Biochimica et Biophysica Acta 1777 (2008) 1289–1300

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

A peptide containing residues 26–44 of tau protein impairs mitochondrial oxidative phosphorylation acting at the level of the adenine nucleotide translocator

A. Atlante [a], G. Amadoro [b], A. Bobba [a], L. de Bari [a], V. Corsetti [b,c], G. Pappalardo [d], E. Marra [a], P. Calissano [b,c], S. Passarella [e,*]

[a] Institute of Biomembranes and Bioenergetics, CNR, Via G. Amendola 165/A, 70126 Bari, Italy
[b] Institute of Neurobiology and Molecular Medicine, CNR, Via del Fosso di Fiorano 64-65, 00143 Rome, Italy
[c] European Brain Research Institute (EBRI), Via del Fosso di Fiorano 64-65, 00143 Rome, Italy
[d] Institute of Biostructures and Bioimaging, CNR, Viale A. Doria 6, 95125 Catania. Italy
[e] Department of Health Sciences, University of Molise, Via F. De Sanctis, 86100 Campobasso, Italy

ARTICLE INFO

Article history:
Received 3 April 2008
Received in revised form 27 July 2008
Accepted 31 July 2008
Available online 6 August 2008

Keywords:
Mitochondria
Tau fragment
Cerebellar granule cells
Oxidative phosphorylation
ATP synthesis
Neurotoxicity

ABSTRACT

Having confirmed that adenovirus-mediated overexpression of NH_2-tau fragment lacking the first 25 aminoacids evokes a potent neurotoxic effect, sustained by protracted stimulation of NMDA receptors, in primary neuronal cultures we investigated whether and how chemically synthesized NH_2-derived tau peptides, *i.e.* NH_2-26–44 and NH_2-1–25 fragments, affect mitochondrial function. We tested both fragments on each step of the processes leading to ATP synthesis via oxidative phosphorylation: *i)* electron flow via the respiratory chain from physiological substrates to oxygen with the activity of each individual complex of the respiratory chain investigated in some detail, *ii)* membrane potential generation arising from externally added succinate and *iii)* the activity of both the adenine nucleotide translocator and *iv)* ATP synthase. Oxidative phosphorylation is not affected by NH_2-1–25 tau fragment, but dramatically impaired by NH_2-26–44 tau fragment. Both cytochrome *c* oxidase and the adenine nucleotide translocator are targets of NH_2-26–44 tau fragment, but adenine nucleotide translocator is the unique mitochondrial target responsible for impairment of oxidative phosphorylation by the NH_2-26–44 tau fragment, which then exerts deleterious effects on cellular availability of ATP synthesized into mitochondria.

1. Introduction

It is a widely accepted notion that tau protein, which is abundant in neurons and less common elsewhere, modulates the stability of microtubules. In Alzheimer's disease (AD), as well as in other human dementias, tau undergoes a series of post-translational changes including abnormal phosphorylation, glycosylation, glycation, and truncation [for refs see [1]], which may render tau more prone to form aggregated structures, the neurofibrillary tangles, which constitute a major hallmark of AD. Following such aggregation the microtubules disintegrate, collapsing the neuron's transport system, with consequent altered communication between neurons and eventually ending in cell death.

Inappropriate neuronal apoptosis is present in AD, as well as in other neurodegenerative diseases. Tau is a substrate for the apoptotic protease(s), *i.e.* caspase(s) and calpain, *in vivo* and an effector of apoptosis itself *in vitro* in established cell lines [1–4], suggesting that an incorrect proteolysis may generate one or more tau fragments that further induce cell death, so contributing to the progression of neurodegeneration by an "autocatalytic process" [3,4].

With the aim of decoding the functional role of the N-terminal domain of tau, in distinction to the C-terminal tail which is reported in the literature to contain the microtubule binding domains [see [5]], Amadoro et al. [6] overexpressed, using adenovirus-mediated infection in primary neuronal cultures, some N-derived fragments located around different protease(s)-cleavage consensus sites in the tau NH_2 domain. The authors showed that high intracellular levels of tau N-terminal fragments lacking the first 25 amino acids evoke a potent neurotoxic effect and induce a necrotic type of cell death, as sustained by protracted stimulation of *N*-methyl-D-aspartate (NMDA) extrasynaptic receptors. The NH_2-26–44 tau fragment was the minimal

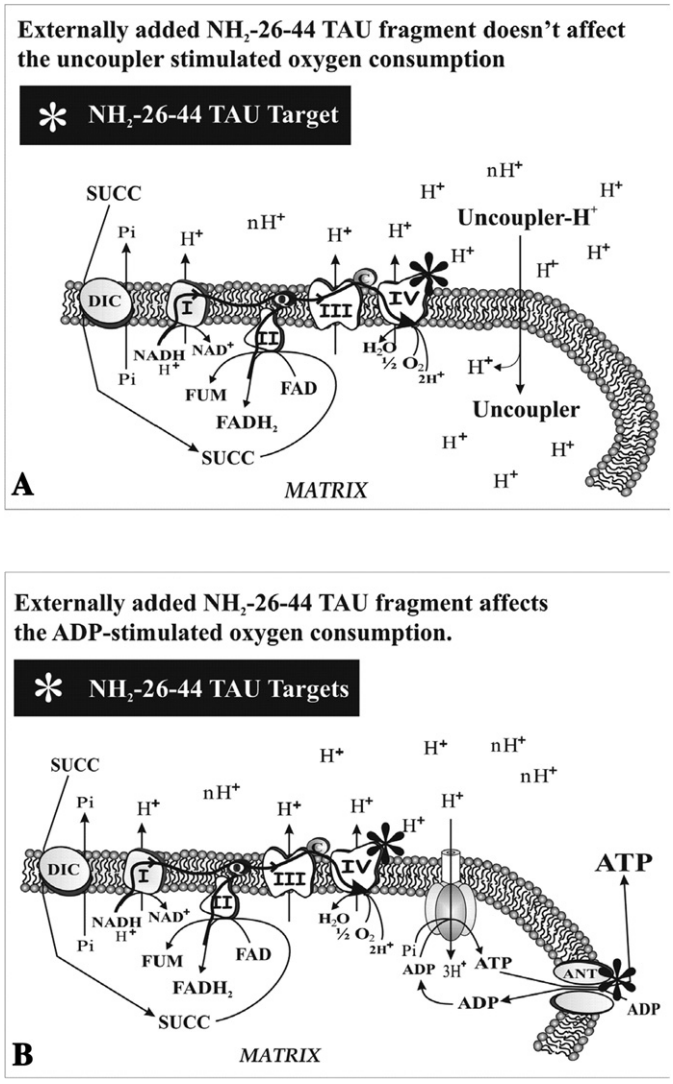
Abbreviations: AA, antimycin A; AD, Alzheimer Disease; ANT, adenine nucleotide translocator; Ap5A, P_1,P_5-Di(adenosine-5′)penta-phosphate; ATP D.S., ATP detecting system; ATR, atractyloside; BME, basal medium Eagle; β-*OH*,, β-hydroxybutyrate; CGC, cerebellar granule cells; CN^-, potassium cyanide; *Control*, trace in the absence of NH_2-tau fragments; COX, cytochrome oxidase; MERS, mersalyl; NMDA, *N*-methyl-D-aspartate; OLIGO, oligomycin; PBS, phosphate buffer saline medium; RAM, rotenone plus antimycin A plus mixothiazole; RCI, respiratory control index; ROT, rotenone; S.D., standard deviation; SUCC, succinate

* Corresponding author. Tel.: +39 (0874) 404243, +39 (080) 5443364; fax: +39 080 5443317.
E-mail addresses: a.atlante@ibbe.cnr.it (A. Atlante), passarel@unimol.it (S. Passarella).

0005-2728/$ – see front matter © 2008 Published by Elsevier B.V.
doi:10.1016/j.bbabio.2008.07.004

active moiety, which retained a marked necrotic effect, while the NH_2-1–25 tau fragment was inactive [1,6].

However, the mechanism underlying the NH_2-26–44 tau fragment-dependent necrosis remains to be established. Here we compare NH_2-26–44 tau and NH_2-1–25 tau fragments with respect to their effects on mitochondrial function. In particular, we investigate each step of the processes leading to ATP synthesis via oxidative phosphorylation: electron flow via the respiratory chain from certain substrates to oxygen with the activity of each individual complex of the respiratory chain investigated in some detail, membrane potential generation and the activity of both the adenine nucleotide translocator (ANT) and ATP synthase (see Scheme 1). Oxidative phosphorylation is not affected by NH_2-1–25 tau fragment, but dramatically impaired by NH_2-26–44 fragment. We show that both cytochrome *c* oxidase (COX) and the ANT are targets of NH_2-26–44 tau fragment, but ANT is the unique mitochondrial target responsible for impairment of oxidative phosphorylation.



Scheme 1. Effect of NH_2-26–44-tau fragment on the oxygen consumption rate under Uncoupling (A) and ATP synthesis (B) conditions. NH_2-26–44-tau fragment has no effect on the rate of succinate-dependent oxygen consumption stimulated by an uncoupler (A), in spite of the capability to impair cytochrome *c* oxidase. Contrarily, in the presence of ADP (B), inhibition takes place due to NH_2-26–44-tau fragment effect on the Adenine nucleotide translocator (ANT). For details see the text. Main abbreviations: DIC, dicarboxylate carrier; FUM, fumarate; Pi, inorganic phosphate; SUCC; succinate. * indicate the NH_2-26–44-tau fragment targets.

2. Materials and methods

2.1. Reagents

Tissue culture medium and fetal calf serum were purchased from GIBCO (Grand Island, NY) and tissue culture dishes were from NUNC (Taastrup, Denmark). All enzymes and biochemicals were from Sigma Chemicals Co. (St Louis, MO, USA). Synthetic peptides: NH_2-tau peptides were synthesized by Sigma Genosys, (Sigma Genosys, Haverhill, UK) and purified to >95% homogeneity by reversed-phase high pressure liquid chromatography on C-18 silica columns with monitoring of A_{214} (peptide bonds). Authenticity and purity of peptides were verified by MALDI mass spectrometry analysis.

2.2. Cell culture and treatments

Primary cultures of cerebellar granule cells (CGC) were obtained from dissociated cerebellar of 7-day-old Wistar rats as described by Levi et al. [7]. Cells were plated in basal medium Eagle (BME, Invitrogen, Gibco) supplemented with 10% fetal calf serum, 25 mM KCl, 2 mM glutamine and 100 μg/ml gentamycin on dishes coated with poly-L-Lysine. Arabinofuranosylcytosine (10 μM) was added to the culture medium 18–22 h after plating to prevent proliferation of non-neuronal cells.

2.3. Preparations of cell homogenate and mitochondria

Culture medium was removed and the plated CGC were washed with phosphate buffer saline medium (PBS), containing 138 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 15 mM KH_2PO_4, pH 7.4. Cell integrity was quantitatively assessed by the inability of cells to oxidize externally added succinate, and by the ability of ouabain to block glucose transport. Homogenates were prepared from a cell suspension by 10 strokes with a Dounce homogeniser at room temperature. Cytosolic lactate dehydrogenase was released and subsequent treatment with Triton X-100 did not cause further release. Mitochondria were isolated from cell homogenates [8]. Briefly, the cellular homogenate was centrifuged at 1500 ×*g* for 10 min. The pellet was resuspended and centrifuged at 1500 ×*g* for 10 min. The combined supernatants were centrifuged again at 1500 ×*g* for 10 min. The supernatant obtained was centrifuged at 17000 ×*g* for 11 min. The pellet represents the mitochondrial fraction. All the operations were carried out on ice and the centrifugations at 4 °C. Purified mitochondria, incubated in PBS, were checked both for their coupling efficiency by measuring the respiratory control index, *i.e.* (oxygen uptake rate after ADP addition)/(oxygen uptake rate before ADP addition), which reflects the ability of mitochondria to produce ATP, and for their intactness by measuring in the post-mitochondrial supernatant the activities of adenylate kinase (ADK, E.C.2.7.4.3) and glutamate dehydrogenase (GDH, E.C.1.4.1.3), which are marker enzymes of the mitochondrial intermembrane space and matrix, respectively. Protein content was determined according to Waddel and Hill [9] with bovine serum albumin used as a standard.

2.4. Polarographic measurements

O_2 consumption was measured polarographically by means of a Gilson 5/6 oxygraph using a Clark electrode [10]. The cell homogenate in PBS (about 0.2 mg protein) was incubated in a thermostated (25 °C) water-jacketed glass vessel (final volume equal to 1.5 ml), and polarographic measurements were performed as substrate-inhibitor analyses in the presence of 1 mM ADP or 0.12 μM FCCP. The following substrates and inhibitors were used: 5 mM β-hydroxybutyrate (β-OH), 3 μM rotenone (ROT), 5 mM succinate (SUCC), 0.8 μM antimycin A (AA). Instrument sensitivity was such as to allow rates of O_2 uptake as low as 0.5 natom min^{-1} mg^{-1} protein to be followed.

2.5. Photometric measurement of mitochondrial respiratory chain complexes I–IV

Enzymatic activities were assayed photometrically at 25 °C. Each assay was performed at least in duplicate with two different volumes of CGC (0.2 mg protein) solubilised with 0.5% Triton X-100 and incubated in 2.5 ml of a medium containing Phosphate-Tris 50 mM and EDTA 2 mM. *Complex I+III* and *Complex II+III* were measured at 548–540 nm by following the reduction of ferricytochrome *c* (10–50 μM) after the addition of 0.5 mM NADH or 10 mM succinate, respectively. The extinction coefficient for cytochrome *c* is 21.1 μM^{-1} cm^{-1} [for refs see [11]]. *Complex IV*, *i.e.* cytochrome *c* oxidase, was measured by recording the decrease in absorbance during the cyanide-sensitive oxidation of 40 μM ferrocytochrome *c* (reduced with substoichiometric concentrations of potassium ascorbate) at 548–540 nm [12]. Time-dependent absorbance changes were recorded with a Jasco double-beam/double-wavelength spectrophotometer UV-550. The rates of ferricytochrome *c* reduction or ferrocytochrome *c* oxidation were obtained as tangents to the initial parts of the progress curves.

2.6. Safranine O response assay

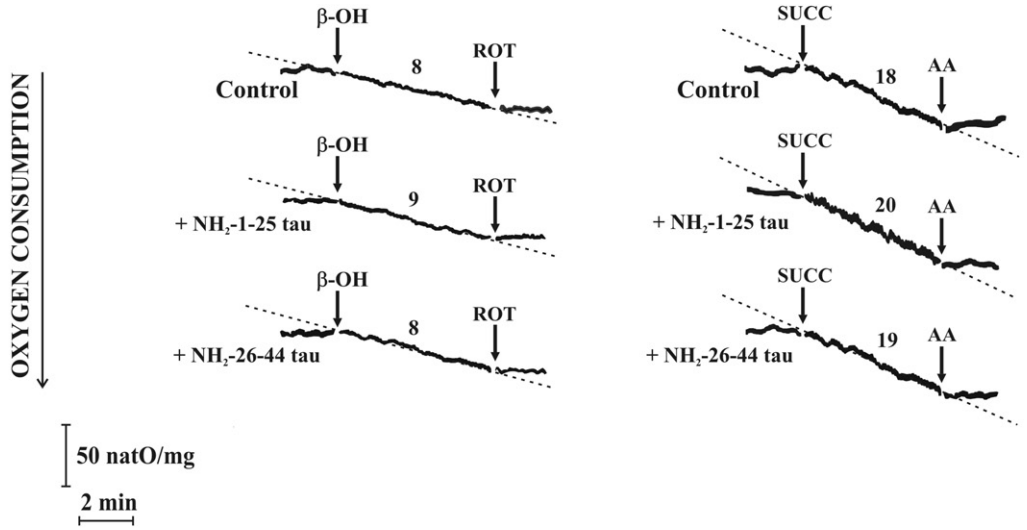
The safranine O response was monitored as in Atlante et al. [13]. Time-dependent absorbance changes were recorded with a Jasco double-beam/double-wavelength spectrophotometer UV-550 with wavelengths of 520–554 nm. Measurements were carried out at 25 °C in 2 ml of standard medium consisting of 200 mM sucrose, 10 mM KCl, 1 mM $MgCl_2$, 20 mM HEPES–Tris, pH 7.2, containing 1 mM safranine O and 0.1 mg protein.

2.7. ADP/ATP carrier measurements

Cell homogenates (0.1 mg protein), containing mitochondria, were incubated at 25 °C in 2 ml of standard medium consisting of 200 mM sucrose, 10 mM KCl, 1 mM $MgCl_2$, and 20 mM HEPES–Tris pH 7.2. The appearance of ATP in the extramitochondrial phase, due to externally added ADP, was assayed, as in Atlante et al. [10], by using the ATP detecting system (ATP D.S.) consisting of glucose (2.5 mM), hexokinase (HK, 0.5 e.u.), glucose-6-phosphate dehydrogenase (G-6-PDH, 0.5 e.u.) and $NADP^+$ (0.2 mM) in the presence of P_1,P_5-Di(adenosine-5′) penta-phosphate (Ap5A), a specific inhibitor of adenylate kinase. The rate of $NADP^+$ reduction in the extramitochondrial phase was determined following the absorbance increase at 334 nm, measured as the tangent to the initial part of the progress curve and expressed as nmol $NADP^+$ reduced/min mg cell protein. Control experiments were carried out in the presence of atractyloside (ATR) to ensure that the ADP/ATP exchange was mediated exclusively by the ANT [10].

2.8. Measurement of ATPase activity

ATPase activity was assayed, as reported in [14], by measuring mitochondrial swelling, which occurred when ATP hydrolysis was elicited in mersalyl-treated mitochondria incubated with ATP. One minute after addition of mersalyl (20 nmol mg^{-1} protein) to the



Respiratory substrate	Oxygen consumption rate (V_{O_2}) (natoms/min x mg cell protein)																		
	Control						+ NH_2-1-25 tau							+ NH_2-26-44 tau					
	Exp.1	*Exp.2*	*Exp.3*	*Exp.4*	*Exp.5*	$\bar{V}_{O_2} \pm$ s.d.	*Exp.1*	*Exp.2*	*Exp.3*	*Exp.4*	*Exp.5*	$\bar{V}_{O_2} \pm$ s.d.	*p*	*Exp.1*	*Exp.2*	*Exp.3*	*Exp.4*	*Exp.5*	$\bar{V}_{O_2} \pm$ s.d.	*p*
β-OH-butyrate	7	8	8	7	9	7.8 ± 0.84	7	8	7	10	8	8.0 ± 1.22	*p>0.05*	9	10	7	7	9	8.4 ± 1.34	*p>0.05*
succinate	17	25	18	24	22	21.2 ± 3.56	21	24	18	24	23	22.0 ± 2.55	*p>0.05*	17	25	20	24	19	21.0 ± 3.39	*p>0.05*

Fig. 1. Effect of NH_2-tau fragments on mitochondrial oxygen consumption (*state 4*) by CGC homogenates due to physiological respiratory substrates, β-hydroxybutyrate and succinate. CGC homogenates (0.2 mg cell protein) were incubated at 25 °C in a water-jacketed glass vessel and consumption of O_2 was monitored polarographically. β-hydroxybutyrate (β-OH, 5 mM), succinate (SUCC, 5 mM), rotenone (ROT, 3 μM) and antimycin A (AA, 0.8 μM) were added at the indicated times. *Control* indicates the traces in the absence of NH_2-tau fragments. NH_2-tau fragments (1–25 and 26–44, each 1 μM) were added 2 min before the addition of the respiratory substrates. Rates of oxygen uptake were expressed as natom O_2/min mg cell protein. In the table the means+S.D. of the oxygen consumption rates are reported. The S.D. was obtained from five experiments, carried out by using different cell preparations. ANOVA and Bonferroni test: No statistically significantly differences when $p>0.05$.

mitochondrial suspension, 2 mM ATP was added and, after 1 min, ATP hydrolysis was induced by adding 0.12 μM FCCP.

2.9. Statistical analysis

Experiments were carried out in triplicates and repeated at least three times. Data were expressed as means ± Standard Deviation (S.D.) ($n=3$). Statistical significance of the data was evaluated using the one-way analysis of variance (ANOVA) followed by post-hoc Bonferroni test. $p<0.05$ was considered as significant for all analyses.

3. Results

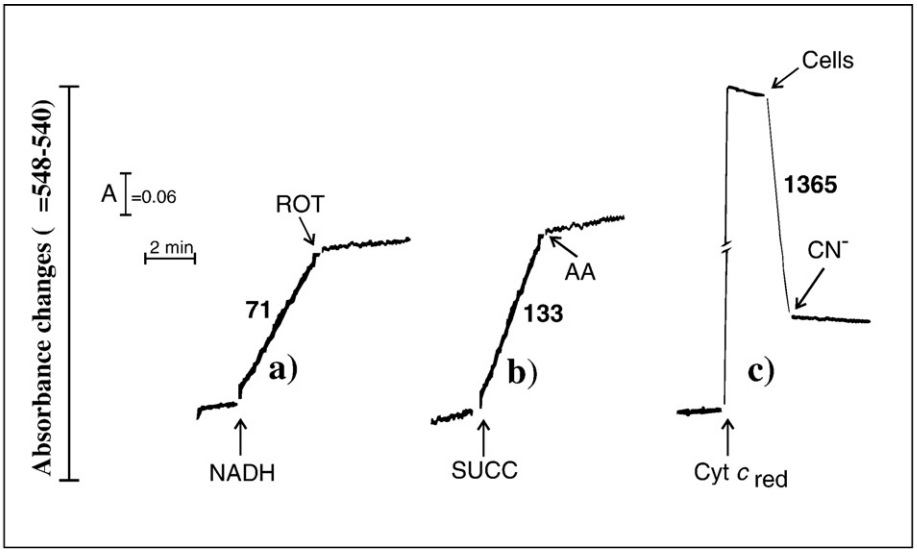
To ascertain whether and how NH_2-1–25 and NH_2-26–44 tau fragments affect mitochondrial oxidative phosphorylation in CGC homogenate, we examined separately all the steps leading to ATP synthesis and export from the mitochondria. These are: *a)* Respiratory substrate transport across the inner mitochondrial membrane; *b)* Substrate oxidation by specific dehydrogenases; *c)* electron flow along the respiratory chain; *d)* $\Delta\mu_{H+}$ generation; *e)* ADP/ATP exchange across the inner mitochondrial membrane via ANT; *f)* $\Delta\mu_{H+}$ driven ATP synthesis by ATP synthase.

Since oxidative phosphorylation occurs only in intact mitochondria, we used either cell homogenate containing mitochondria or isolated mitochondria for these experiments, as in [10]. In either case mitochondrial coupling was previously checked.

3.1. Effect of NH_2-tau fragments on oxygen consumption by CGC homogenate

First oxygen consumption by CGC homogenate was monitored in *State 4*, *i.e.* in the absence of either ADP or uncoupler, arising from different substrates whose oxidation is physiologically linked to *Complex I* (β-hydroxybutyrate) and *Complex II* (succinate).

In a typical experiment (Fig. 1), neither NH_2-1–25 nor NH_2-26–44 tau fragments had any significant effect on oxygen consumption rate, with respect to the control ($v=8$ natom O_2/min/mg cell protein), in the case of rotenone-sensitive oxidation of β-hydroxybutyrate (β-OH, 5 m*M*) ($v=9$ and 8 natom O_2/min/mg cell protein, respectively) (Fig. 1) or on mitochondrial respiration due to externally added succinate (SUCC, 5 m*M*) ($v=18$, 20 and 19 natom O_2/min/mg cell protein in the



RESPIRATORY COMPLEX ACTIVITY (nmoles/min mg ± s.d.)					
Complexes	**Control**	**+ NH_2-1-25 tau**	***p***	**+ NH_2-26-44 tau**	***p***
NADH:ferricytochrome *c* oxidoreductase	71 ± 12	69 ± 8	*p>0.05*	73 ± 7	*p>0.05*
Succinic:ferricytochrome *c* oxidoreductase	137 ± 19	123 ± 14	*p>0.05*	133 ± 13	*p>0.05*
Cytochrome *c* oxidase	1324 ± 141	1297 ± 168	*p>0.05*	657 ± 48	*p<0.001*

Fig. 2. Effect of NH_2-tau fragments on respiratory chain complex activities. CGC were collected and solubilised (see Materials and methods section). Then respiratory chain complexes were assayed as reported in the Materials and methods section. (a) *Complex I+III*: 0.1 mg protein of CGC were incubated at 25 °C in 2.5 ml of standard medium, containing 10 mM Pi, 2 mM EDTA and 2 mg/ml BSA, in the presence of 50 μM ferricytochrome *c*, 1 mM KCN and 0.1% potassium deoxycholate. Further additions: 0.5 mM NADH and 3 μM rotenone (ROT). (b) *Complex II+III*: 0.2 mg protein of CGC were incubated at 25 °C in 2.5 ml of standard medium in the presence of 10 μM ferricytochrome *c*, 0.1% potassium deoxycholate and 3 μM rotenone. At the times indicated by the arrows, the following were added: 10 mM succinate (SUCC) and 0.8 μM Antimycin A (AA). (c) *Complex IV*: 40 μM ferrocytochrome *c* was added to a spectrophotometric cuvette containing in 2.5 ml 3 μM rotenone and 0.8 μM antimycin A and the absorbance was recorded. The reaction was started with the addition of 7.5 μg protein of CGC, and blocked with 1 mM cyanide (CN^-). Numbers along the traces give the rate of ferricytochrome *c* reduction by either NADH or succinate (traces a and b), respectively) and ferrocytochrome *c* oxidation, (trace c)), measured as tangent to the initial part of the progress curve and expressed as nmoles min^{-1} mg^{-1} cell protein. In the table the means+S.D. of the respiratory complex activities in the absence or presence of NH2-tau fragments (1–25 and 26–44, each 1 μM) are reported. S.D. was obtained from six experiments, carried out by using different cell preparations from different groups of animals and the variations ranged within 10%. ANOVA and Bonferroni test: No statistically significantly differences when $p>0.05$; Statistically significantly differences when $p<0.001$.

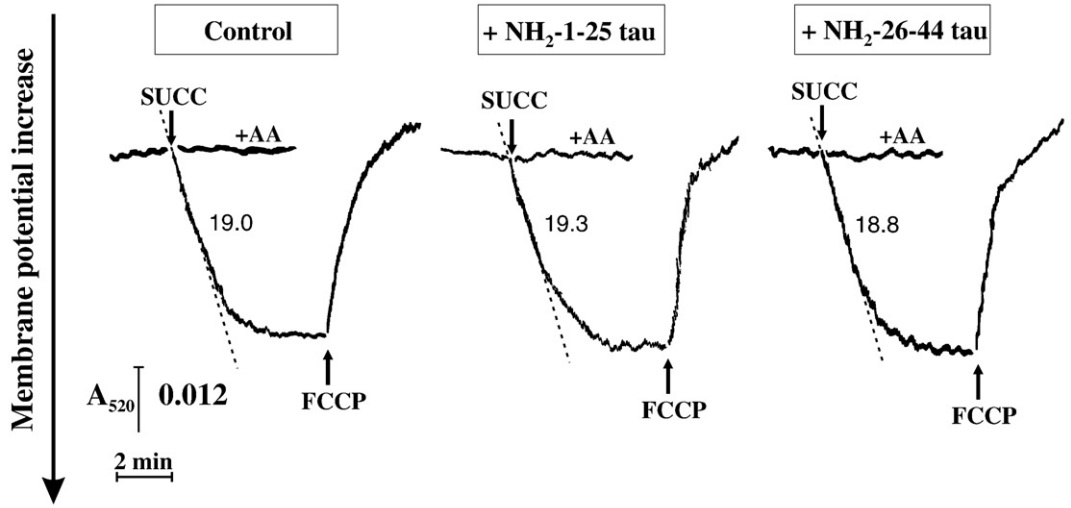
control and following treatment with NH_2-1–25 and NH_2-26–44 tau fragments, respectively) (Fig. 1).

A statistical analysis according to ANOVA followed by the Bonferroni test (five experiments carried out with 5 different cell homogenates) (see Table to Fig. 1) confirms that addition of the NH_2-1–25 fragment treatment did not change the rate of oxygen uptake with any of the substrates investigated ($p>0.05$).

The results in Fig. 1 do not show unambiguously that tau fragments are without effect on oxygen uptake by *complexes I* and *II* because oxygen uptake requires transport of the externally added substrate (either via diffusion in the case of β-hydroxybutyrate or in a carrier mediated manner in the case of succinate), and so the possibility arises that the transport step is rate limiting. This would mask any possible effects of the tau fragments. To rule out this possibility, a parallel experiment was carried out in which the activities of the respiratory chain complexes, *i.e. Complex I-III*: NADH-ferricytochrome *c* oxidoreductase and *Complex II–III*: Succinic:ferricytochrome *c* oxidoreductase were assayed. Moreover *Complex IV* was also monitored. This was made photometrically (for details see the Materials and methods) with the results shown in Fig. 2. The NH_2-1–25 tau fragment was without effect on the respiratory complex activities in all cases. On the other hand the presence of NH_2-26–44 tau fragment led to about 50% of inhibition of the activity of *Complex IV*, monitored as cyanide-sensitive oxidation of reduced cytochrome *c* (Fig. 2c and Table, *line 3*), as in [12]. No effect of NH_2-26–44 tau fragment was found when either 0.5 mM NADH (used to measure *Complex I+III*) or 10 mM succinate (used to measure *Complex II+III*) were added to solubilised CGC to reduce the added ferricytochrome *c* (50 μM) (Table 1, *lines 1 and 2*). In the same experiment we confirmed that these reactions were almost completely inhibited by rotenone and antimycin, respectively (see Fig. 2a and b).

3.2. Effect of NH_2-tau fragments on generation of mitochondrial membrane potential in isolated CGC mitochondria

Isolated mitochondria were supplemented with succinate (5 mM), the only respiratory substrate which donates reducing equivalents directly to the respiratory chain, and the generation of membrane potential ($\Delta\psi$) was monitored by the decrease of safranine O absorbance at 520 nm (Fig. 3). Neither the rate nor the extent of $\Delta\psi$ generation was significantly affected by the addition of NH_2-tau fragments ($p>0.05$). Generation of $\Delta\psi$ was completely prevented by antimycin A (AA, 0.8 μM), an inhibitor of *Complex III* of the respiratory chain, and, as expected, the membrane potential was abolished by FCCP (0.12 μM). It should also be noted that the NH_2-1–25 and NH_2-26–44 tau fragments neither generate $\Delta\psi$ nor abolish endogenous $\Delta\psi$.



Samples	generation									
	Rate (A_{520}/min x 10^3)					Extent (A_{520} x 10^3)				
	Exp.1	*Exp.2*	*Exp.3*	*Exp.4*	$\bar{M} \pm$ s.d.	*Exp.1*	*Exp.2*	*Exp.3*	*Exp.4*	$\bar{M} \pm$ s.d.
control	19.5	19.0	20.1	17.5	19.0 ± 1.08	37	38	40	33	37.0 ± 2.9
+ NH_2-1-25 tau	17.7	19.3	17.9	18.5	18.3 ± 0.72	34	39	41	35	37.2 ± 3.3
+ NH_2-26-44 tau	19.0	18.8	17.5	19.2	18.6 ± 0.77	38	36	32	42	37.0 ± 4.1

Fig. 3. Effect of NH_2-tau fragments on generation of mitochondrial membrane potential. Mitochondria (0.1 mg protein) isolated from CGC were suspended at 25 °C in 1.5 ml of PBS containing safranine O (1 μM). Arrows denote the following additions: succinate (SUCC, 5 mM), FCCP (0.12 μM). NH_2-tau fragments (1–25 and 26–44) were added 2 min before succinate addition at a final concentration of 1 μM. *Control* indicates the trace in the absence of NH_2-tau fragments. Where indicated antymicin A (AA, 0.2 μg) was added 2 min before addition of the substrate. The safranine O response was monitored at 520 nm as described in the Materials and methods section. In the table, both the $\Delta\psi$ generation rate and the extent values (±S.D.), in the absence or presence of NH_2-tau fragments (1–25 and 26–44, each 1 μM), expressed as ΔA_{520}/min mg cell protein and ΔA_{520}=(A_{520} value before succinate addition)−(A_{520} value after $\Delta\psi$ generation due to succinate addition) respectively, are reported.

3.3. Effect of NH_2-tau fragments on mitochondrial ANT

Having ascertained that neither of the NH_2-tau fragments has any effect on *Complex I* and *II*-dependent oxygen uptake and that they do not abolish the mitochondrial membrane potential, we investigated their effect on *Uncoupled/State 3* respiration in which oxygen uptake is stimulated either by using an uncoupler or by addition of ADP, respectively. The difference is that in the former case the stimulation of oxygen uptake derives from collapse of the electrochemical proton gradient without ATP synthesis, whereas in the latter case ADP is used to drive ATP synthesis.

In the first set of experiments, the uncoupler FCCP was used to stimulate succinate oxidation. Mitochondria from cell homogenate in *State 4*, *i.e.* in the presence of the succinate alone, took up oxygen at a rate of 19 natom O_2/min mg cell protein. The addition of FCCP increased the rate of oxygen uptake up to 116 natom O_2/min mg cell protein (Fig 4a) with an Uncoupling Control Ratio (the ratio of the rate of oxygen uptake in uncoupled respiration and *State 4*) equal to 6. No change in the rate of oxygen uptake occurred when NH_2-tau fragments were added separately either before (Fig. 4a'–a") or after the respiratory substrate (Fig. 4b'–b"), further confirming that the electron flow along the respiratory chain is not affected by either fragment. These data also confirmed that neither of the NH_2-tau fragments functions as an uncoupler.

On the other hand, when ADP was used to stimulate the oxygen uptake arising from externally added succinate (Fig. 4b), the rate of oxygen uptake increased (to 67 natom O_2/min mg cell protein) with RCI value equal to 3.5. In the presence of the NH_2-26–44 tau fragment (1 μM), added either before (Fig. 4c') or after ADP (Fig. 4d'), the rate enhancement due to ADP phosphorylation was essentially abolished. The NH_2-1–25 tau fragment (1 μM) was without effect (Fig. 4c"–d").

Given that generation of $\Delta\mu_{H+}$ is not affected by either NH_2-tau fragment (see Fig. 3), it was necessary to examine other possible causes for the NH_2-26–44 fragment-dependent inhibition of ADP-stimulated oxygen uptake. To this end, we investigated both ADP/ATP exchange via ANT and ATP synthase activity [see [10]].

In the case of ANT, we resorted to a procedure that allows for the continuous monitoring of ATP efflux from CGC homogenate incubated with ADP [10]. In a typical experiment, the homogenates, treated with AP_5A to inhibit adenylate kinase and thus preventing mitochondrial ATP synthesis in a manner not dependent on oxidative phosphorylation [see also [10]], were incubated in the presence of an ATP detecting system (ATP D.S.) (see Materials and methods section). The ATP concentration in the extramitochondrial phase of the homogenates was negligible as shown by the fact that no increase in the absorbance measured at 334 nm was found in the presence of glucose, hexokinase, glucose-6-phosphate dehydrogenase and $NADP^+$. As a result of addition of ADP (0.04 mM), an increase in the NADPH absorbance was observed indicating the appearance of ATP in the extramitochondrial phase (Fig. 5A). As shown in Fig. 5B, NADPH formation derives from *i)* ADP uptake into mitochondria in exchange for endogenous ATP, *ii)* ATP synthesis from imported ADP via ATP synthase and *iii)* efflux of the newly synthesized ATP from the mitochondria in exchange for further ADP via ANT (it should be noted that, under the reported experimental conditions, the energy for ATP synthesis is apparently given by the oxidation of endogenous substrates).

As expected, NADPH formation was completely inhibited by the presence of oligomycin (OLIGO, 10 μM), an inhibitor of ATP synthase, further confirming that no ATP could be synthesized via substrate-level

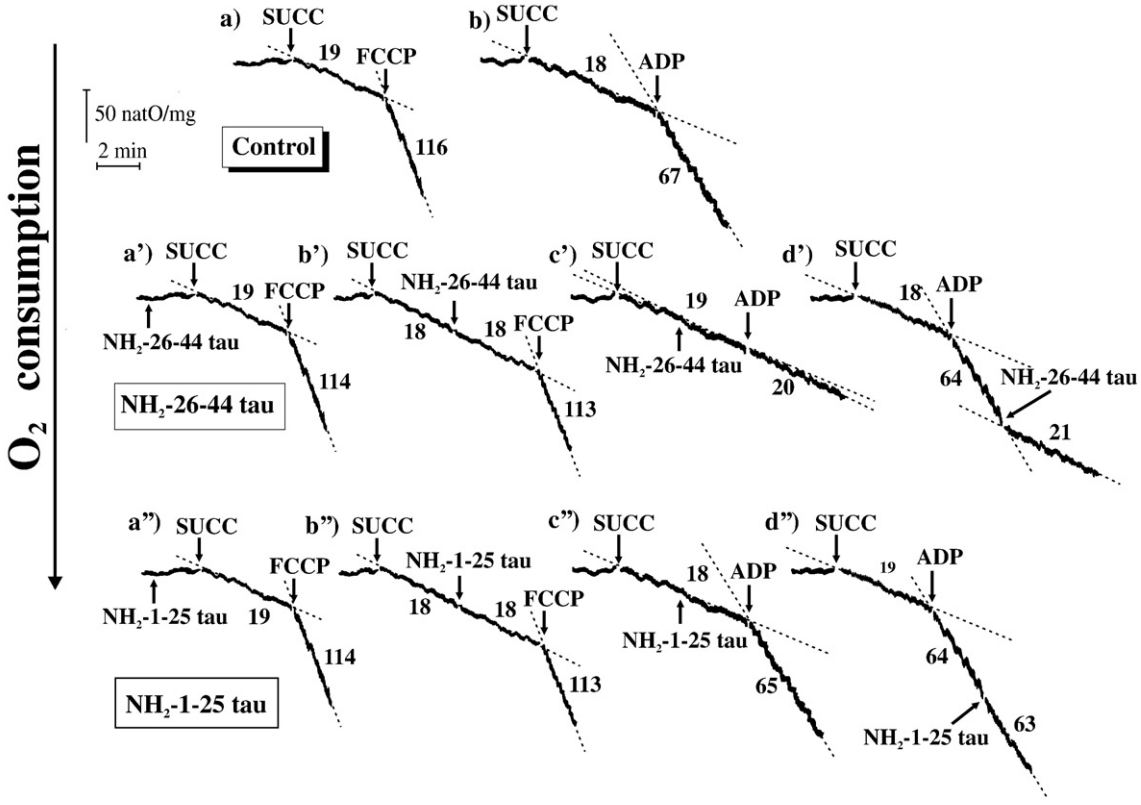


Fig. 4. Effect of NH_2-tau fragments on mitochondrial oxygen consumption (*state 3*) by CGC homogenates due to succinate plus either FCCP or ADP. CGC homogenates (0.2 mg cell protein) were incubated at 25 °C in a water-jacketed glass vessel and consumption of O_2 was monitored polarographically. Succinate (SUCC, 5 mM), FCCP (0.12 μM), ADP (1 mM) and NH_2-tau fragments (1–25 and 26–44, each 1 μM) were added at the indicated times. *Control* indicates the traces in the absence of NH_2-tau fragments. The respiratory control index (RCI), *i.e.* oxygen uptake rate after FCCP/ADP addition/oxygen uptake rate before FCCP/ADP addition, reflects the ability of mitochondria either to respond to the abolishment of the electrochemical proton gradient or to produce ATP. Rates of oxygen uptake were expressed as natom O_2/min mg cell protein.

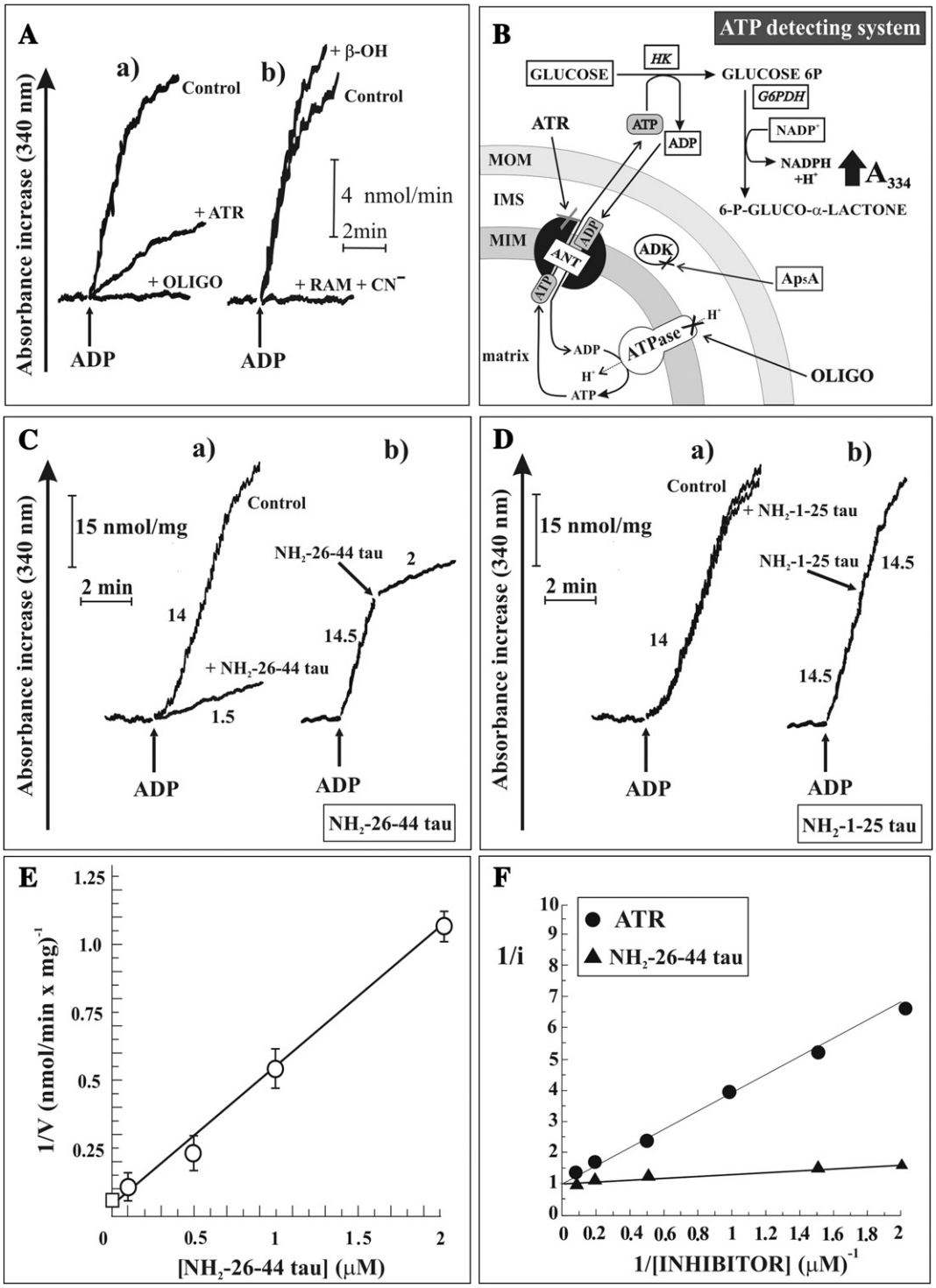


Fig. 5. Effect of NH_2-tau fragments on the ADP/ATP exchange via ANT in CGC. Appearance of ATP due to ADP addition to CGC homogenates (0.1 mg protein) was monitored as described in the Materials and methods. NH_2-tau fragments (1–25 and 26–44) were added, 2 min before ADP, at a final concentration of 1 μM. *Control* depicts the traces in the absence of NH_2-tau fragments.(A) Atractyloside (ATR, 10 μM), oligomycin (OLIGO, 10 μM), β-OH-butyrate (5 mM) and a cocktail of respiratory chain inhibitors, *i.e.* rotenone (3 μM), antimycin A (0.8 μM) and mixothiazole (6 μM) (RAM) plus cyanide (CN^-, 1 mM), were added to the reaction mixtures. ADP concentration was equal to 0.04 mM. (B) Scheme representing the ATP Detecting System. For details see the text. Main abbreviations: Ap5A, P1,P5-di(adenosine-5′)penta-phosphate; ANT, adenine nucleotide translocator; ATR, atractyloside; HK, hexokinase; G6PDH, glucose-6-Pi dehydrogenase; OLIGO, oligomycin; IMS, inner mitochondrial space; MIM, mitochondrial inner membrane; MOM, mitochondrial outer membrane. (C and D) Appearance of ATP due to ADP (0.5 mM) addition to CGC homogenates in the absence (*Control*) or presence of either NH_2-1–25 (C) or NH_2-26–44-tau fragment (D) (1 μM each). (E and F) The plot of 1/absorbance increase rate against NH_2-26–44 tau fragment concentration and the plot of 1/i against 1/[inhibitor], where $i = 1 - v_i/v_o$; v_i and v_o are the rates of ADP/ATP exchange in the presence and in the absence of inhibitor, *i.e.* either atractyloside (ATR, ○) or NH_2-26–44 tau fragment (▲), respectively, are reported.

phosphorylation in the homogenates (Fig. 5Aa). The addition of atractyloside (ATR, 10 μM), an ANT inhibitor, strongly inhibited NADPH formation (Fig. 5Aa), as expected in light of the K_i value (about 3 μM) [see [10]] and of the ADP and atractyloside concentrations used. In a parallel experiment, to ascertain that mitochondria themselves can drive ATP synthesis without any limitation due to energy deficit, we energized cell homogenates by adding β-OH (5 mM), thus increasing membrane potential (Fig. 5Ab). Since the rate of ATP

production was not significantly increased under these conditions, we concluded that the mitochondria themselves can generate the electrochemical proton gradient needed for ATP synthesis. Conversely, prevention of $\Delta\mu_{H+}$ formation from endogenous substrates by the addition of a cocktail of the electron flow inhibitors rotenone, antimycin A, mixothiazole (RAM) and cyanide (CN^-) resulted in blocking of ATP production (Fig. 5Ab).

The rate of NADPH formation found in these experiments was approximately 14 nmol $NADP^+$ reduced/min mg cell protein, in good agreement with values obtained by Atlante et al. [10]. When NH_2-26–44 tau was added, this rate was strongly decreased to 1.5 and 2 nmol $NADP^+$ reduced/min mg cell protein with the fragment added either before (Fig. 5Ca) or after ADP (Fig. 5Cb) respectively. NH_2-1–25 tau fragment was without effect (Fig. 5Da, b).

Since ANT activity measured in mitochondria could depend on a number of events including electron flow across the mitochondrial membrane, electrochemical proton gradient generation, ATP synthase activity and the adenine nucleotide content of the mitochondria, we investigated the rate limiting step of the process leading to ATP efflux from the mitochondria.

That the rate of NADPH formation mirrored the rate of ADP/ATP exchange was verified by measuring the rate of ATP appearance, due to externally added ADP, in the absence or presence of increasing concentrations of either atractyloside or NH_2-26–44 tau fragment (Fig. 5E, F). Data obtained have been plotted both as a Dixon plot and as 1/i against 1/[Inhibitor], where the fractional inhibition $i = 1 - v_i/v_o$ (v_i and v_o were the rates of ATP efflux in the presence or absence of the inhibitor, respectively). In the former case the intercepts of the line obtained by fitting the experimental points obtained in the presence of either NH_2-26–44 tau fragment (Fig. 5E) or atractyloside (not shown) coincide with that obtained in the absence of inhibitor. This allows the conclusion that ANT itself is the target of NH_2-26–44 tau fragment. K_i is about 0.5 μM. In the latter case, the intercept on the *y*-axis was 1 showing that atractyloside, as well as NH_2-26–44 tau fragment, completely prevented ADP/ATP exchange (Fig. 5F).

The conclusion that neither the electron flow across the mitochondrial membrane, nor the electrochemical proton gradient, nor the ATP synthase (see below) limited the rate of NADPH formation is also suggested by the observation that NH_2-26–44 did not impair the electron flow along the respiratory chain, $\Delta\psi$ generation (see above), or also ATP synthase activity (see below). This conclusion is also confirmed by the finding that the coupled enzymatic system used to detect ATP outside mitochondria was not rate limiting *per se* by showing that addition of 5 μM ATP resulted in an increase of the rate of change of absorbance, whereas no rate increase occurred when the concentrations of either the substrate or enzyme components of the ATP D.S. were increased. Taken together, these studies indicate that the major action of the NH_2-26–44 tau fragment on mitochondrial oxidative phosphorylation is at the level *only* of the ANT.

3.4. Effect of NH_2-tau fragments on mitochondrial ATP synthase

The sensitivity of mitochondrial ATPase to NH_2-tau fragments was investigated by checking whether these fragments could prevent the FCCP-stimulated swelling of mersalyl-treated mitochondria incubated with 2 mM ATP. This swelling has been reported to occur when phosphate and ADP formation (caused by ATP hydrolysis via ATPase stimulated by FCCP) takes place in the matrix of mitochondria in which the phosphate carrier is inhibited by mersalyl [see [14]]. As expected, mitochondrial swelling was strongly inhibited in the presence of oligomycin (OLIGO, 10 μM), which inhibits ATP synthase (Fig. 6Aa), as well as partially inhibited in the presence of atractyloside (ATR, 10 μM) (Fig. 6Ab). As a control, it was verified that no swelling occurred when mersalyl was absent (Fig. 6Ac). No change in the rate of mitochondrial swelling, with respect to the control, was found when either NH_2-1–25 or NH_2-26–44 tau fragment was added after ATP addition and accumulation into mitochondria (Fig. 6Ba, Ca), thus showing that there was no inhibition of mitochondrial ATPase. Nonetheless mitochondria showed swelling with a rate and to an extent lower than that found in controls (Fig. 6Cb) when they were previously incubated with NH_2-26–44 tau fragment, but not with NH_2-1–25 fragment (Fig. 6Bb). This finding suggests that the observed impairment is likely due to the effect of NH_2-26–44 tau fragment on ATP accumulation into mitochondria. On the other hand, these data besides demonstrating that both NH_2-tau fragments tested were ineffective on ATP synthase, confirm the inhibitory effect of NH_2-26–44 tau fragment on ANT, and suggest that the inhibition is likely non-competitive.

4. Discussion

We show here that externally added NH_2-26–44 tau fragment can impair oxidative phosphorylation as investigated *in vitro* with CGC homogenates containing intact coupled mitochondria and that this occurs due to the impairment of the mitochondrial ANT (Figs. 4 and 5). Such an impairment could account for the reduced availability of ATP already proposed as the cause of the excitotoxic NMDAR activity-mediated death in Alzheimer disease. In this case the reduced ATP availability in the cytosol could in turn cause glutamate release from the cell and then excitotoxicity [1,6] (see Scheme 2).

The conclusion that NH_2-26–44 tau fragment inhibits ADP/ATP exchange as measured in intact coupled mitochondria rests on the confirmation, as in [10], that the increase of the measured absorbance reflects the rate of ANT mediated ADP/ATP exchange. This is shown by the experiment in which the reciprocal of absorbance increase rate was plotted as a function of either NH_2-26–44 tau fragment (Fig. 5E) or atractyloside concentration (not shown). In both cases the intercepts of the line obtained by fitting the experimental points obtained in the presence of NH_2-26–44 tau fragment or of atractyloside coincide with that obtained in the absence of inhibitor.

The implication of this is that ANT itself is the target of NH_2-26–44 tau fragment. Consistently we confirmed that the decrease in ANT activity does not depend on the failure of mitochondria to generate membrane potential, which is used as driving force for ATP efflux, by showing that the addition to mitochondria of β-hydroxybutyrate, a respiratory substrate which enters mitochondria via diffusion with an increase in membrane potential, is not accompanied by stimulation of ANT transport activity (Fig. 5Ab). Consistently, we have found that the activity of the ATP synthase, assayed as in [14], was not affected by NH_2-26–44 tau fragment (Fig. 6). Interestingly, since inhibition of ATPase-dependent swelling is found only when NH_2-26–44 tau fragment was added before ATP used at 2 mM concentration and was not removed by subsequent addition of ATP at up to 10 mM (not shown) we might suggest that the inhibition is non-competitive. It should be noted that in late apoptosis a non-competitive-like inhibition of ANT was found, probably due to caspase(s) activity [10], but not dependent on a direct caspase–ANT interaction. However since NH_2-26–44 tau fragment is likely to be generated during apoptosis given that the N-terminal domain of tau contains consensus sequences suitable for cleavage by caspase(s) [2,15], which are activated in apoptotic degenerating neurons in AD [16–20], the possibility exists that caspase(s) gradually inhibit/s ANT as a result of NH_2-tau cleavage and generation of toxic NH_2-26–44 tau fragment. In this case NH_2-26–44 tau fragment should bind ANT.

Possible effects of tau fragments on other steps of the process leading to ATP synthesis, via oxidative phosphorylation, in energized mitochondria have been investigated by measuring *State 3* and *4* respiration: NH_2-tau fragments failed to impair the electron flow in the mitochondrial respiratory chain (Figs. 1, 2), $\Delta\mu_{H+}$ generation (Fig. 3) and $\Delta\mu_{H+}$-driven ATP synthase (Fig. 6). These conclusions are not unique: Cardoso et al. [21] found no differences in NADH-ubiquinone oxidoreductase (*Complex I*) or succinate dehydrogenase-cytochrome *c*

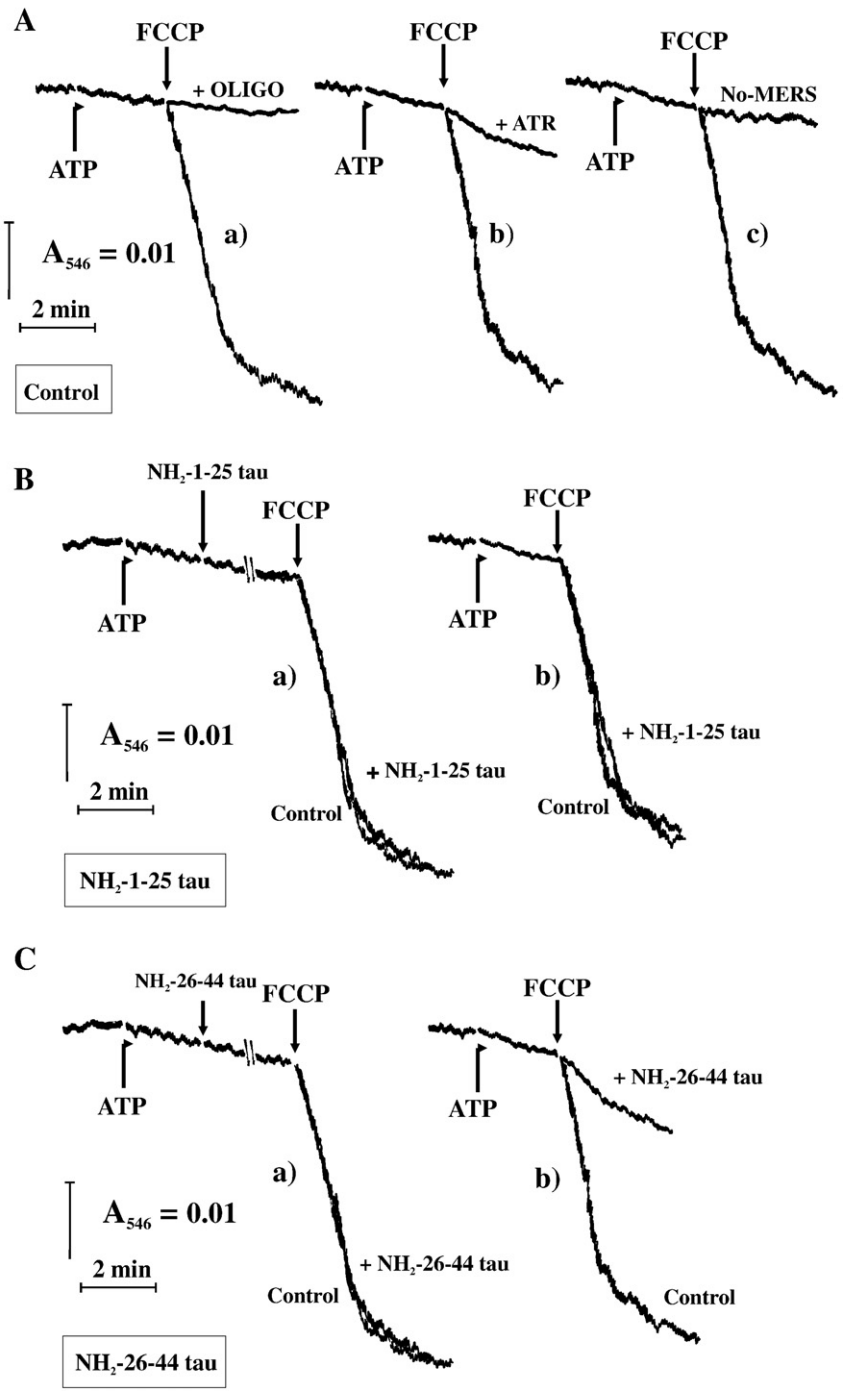
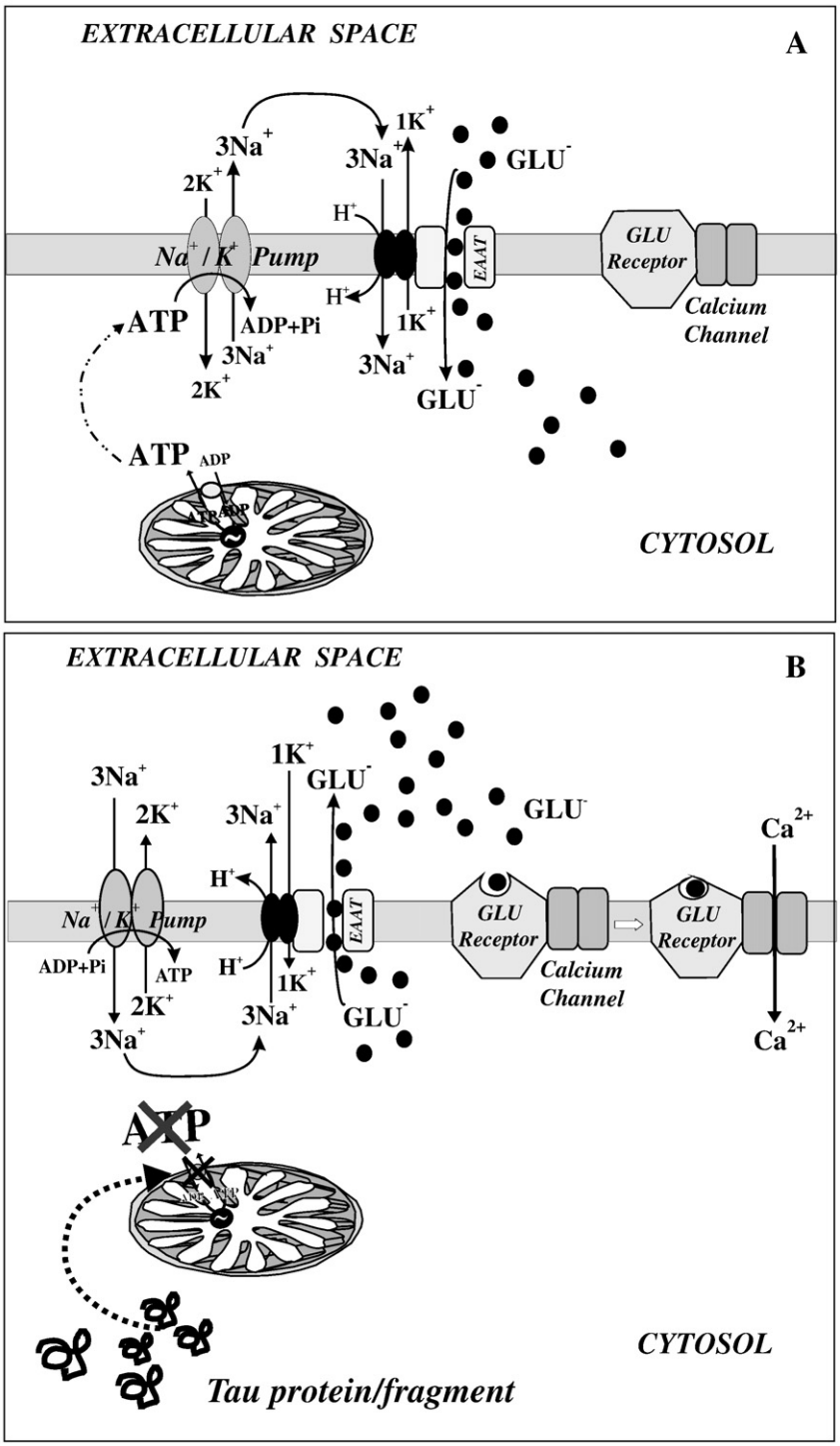


Fig. 6. Effect of NH_2-tau fragments on mitochondrial ATP synthase in CGC. ATP hydrolysis, induced by externally added FCCP to mersalyl-treated-mitochondria (0.2 mg protein), was followed as reported in the Materials and methods. Arrows indicate where additions were made: 2 mM ATP, 0.12 μM FCCP, 1 μM NH_2-1–25 or-26–44 tau fragment. *Control* indicates the traces in the absence of NH_2-tau fragments. Oligomycin (OLIGO, 2 μM) and atractyloside (ATR, 10 μM) (in A,a and b) were added 2 min before ATP addition; No-MERS (in A,c) indicates that mersalyl (20 nmol/mg mitochondrial protein) is not present in the mitochondrial suspension. NH_2-tau fragments (1–25 or 26–44) were added where indicated (B,a and C,a) and 2 min before ATP addition (in B,b and C,b). Absorbance changes at 546 nm were continuously recorded with a Perkin-Elmer Lambda 5 spectrophotometer.

reductase (*Complex II/III*) activities between mitochondria isolated from AD platelets in which the ATP levels proved to decrease and control mitochondria. In another paper Mancuso et al. [22] showed no decrease in activity of F_1F_0-ATPase in hippocampus and platelets of AD cases.

Our results also call into question some previous observations about the possible role of cytochrome oxidase in AD. It was proposed that a decrease in COX activity and/or in the levels of COX polypeptides, responsible for mitochondrial dysfunction, may be the fundamental cause of AD [23–26]. However, we show that the inhibition of COX activity by NH_2-26–44 fragment is of secondary importance since COX is not limiting the electron flow along the respiratory chain due to both β-OH and succinate (Fig. 1). The COX activity assay, measured as an isolated step of the respiratory chain, as well as the failure to impair oxygen uptake when either β-hydroxybutyrate or succinate was used as respiratory substrate, strongly suggests that COX capacity exceeds that required to support respiration, *i.e.* COX is not the limiting step in oxygen uptake.



Scheme 2. (A) Normal conditions: *i)* Mitochondria produce ATP; *ii)* Na^+/K^+ pump utilizes cell ATP to transport Na^+ and K^+ across plasma membrane thus maintaining membrane potential; *iii)* Extracellular glutamate level is kept below toxic level by its reuptake through high-affinity Na^+-dependent excitatory amino acid transporter (EAAT) [63]. (B)TAU fragment production conditions : *i)* Tau fragment affects mitochondrial ATP production, *ii)* the transmembrane gradients of Na^+ collapse due to a reversal of the Na^+/K^+ pump; *iii)* electrogenic Na^+-coupled transporter (EAAT) reverses, then releasing glutamate into the extracellular space; *iv)* high extracellular glutamate level causes excitotoxic neuronal death, which involves activation of ionotropic glutamate receptors and excessive calcium influx in neurons [64] and induce excitotoxic death. Main abbreviations: EAAT, excitatory amino acid transporter; GLU, glutamate.

On the other hand, in distinction with [27], in which an elevated rate of respiration in the absence of ADP in neocortical homogenate derived from Alzheimer's patients was found, we show that NH_2-tau fragments do not cause mitochondrial uncoupling (Fig. 4). We suggest these effects could derive from the action of compounds accumulated in cells during AD, for instance oxidized lipids leading to mitochondrial uncoupling.

Since the same fragment does not impair FCCP-stimulated oxygen consumption, but only ADP-stimulated oxygen consumption (Fig. 4), we can exclude the steps which are shared by the two processes (see Scheme 1) as being responsible for the impairment of ATP production. Notice that if the dicarboxylate carrier or the succinate dehydrogenase were sensitive to NH_2-26–44 tau fragment, thus becoming the rate limiting step of the process leading to oxygen uptake, its rate in the

presence of tau fragment under both ATP and uncoupler conditions would remain the same. This is not the case.

The experimental approach used to address the role of NH_2-26–44 fragment in CGC energy metabolism merits some discussion. Since mitochondrial ATP synthesis and efflux outside mitochondria is a multistep process requiring intact coupled mitochondria and occurring in a manner regulated by the rate of the limiting step, any study dealing with isolated enzyme/proteins or with artificial systems is of limited use. Thus we have used CGC homogenates to study the different steps resulting in the availability of ATP outside mitochondria both in their entirety and as partial or individual processes. In the first case we have found that ATP appearance outside mitochondria caused by ADP addition decreases in the presence of NH_2-26–44 tau fragment. In agreement with [28], this fragment was used in 0.5–1 μM range: under physiological conditions, the tau concentration in a neurons is about 2 μM [28,29] and essentially all of the tau protein (>95%) is tightly bound to microtubules. However, under pathological conditions, as in the case of tau truncation, modified tau detaches from microtubules and accumulates in free form at least up to 0.5–1 μM. The mechanism by which NH_2-26–44 tau fragment impairs ANT are at present a matter of speculation. Since Yang et al. [30] showed that as a consequence of glycation, PHF-tau from AD and AGE-tau generate oxygen free radicals and since Cente et al. [31] reported that expression of a human truncated variant form of tau protein leads to the accumulation of ROS, the possibility that ANT impairment derived from ROS–protein interaction has been considered. However, in a control experiment, not reported here, we have found that in our model system no ROS production occurs under condition in which ANT (itself a target of ROS [10]) is impaired.

The possibility that ANT impairment derives from protein–protein interaction should be carefully considered. Several studies exist in which different aspects of putative interaction sites, such as hydrophobicity, residue propensities, size, shape, solvent accessibility, and residue pairing preferences, are examined [32–43]. Although each of these parameters provides some information indicative of protein interaction sites, none of them perfectly differentiates interaction sites from the rest of the protein surfaces. As far as the interaction of tau peptides and ANT is concerned, a simple comparison of the peptides and the ANT isoform sequences, based on the six-transmembrane model (three-repeat domain structure) proposed by Klingenberg [44], does not reveal any element which could be predictive of a direct interaction such as cysteine [45] or proline [46] residues. Nonetheless it must also be considered that since NH_2-26–44 tau fragment is a non-competitive inhibitor, it does not interact with the catalytic but with some other site of the enzyme which could distort the enzyme's structure thus effecting also the catalytic binding site. In particular, to characterize the mechanism(s) regulating ANT function may be important not only in the elucidation of pathways contributing to necrosis but also in understanding the pathogenesis of various taupathies which may result from the altered function of ANT. We intend therefore to investigate the tau peptide-ANT interaction further by using specific antibodies against the two proteins and a co-immunoprecipitation approach which can be considered as one of the most useful techniques for revealing protein–protein interactions.

The data reported here confirm a clear association between mitochondrial dysfunction and neuronal damage/death in the physiopathology of Alzheimer's disease characterized by the altered function and/or structure of tau protein. Previous studies have demonstrated enhanced susceptibility to oxidative insults caused by mitochondrial dysfunction following overexpression of wild-type or mutant [47-51], or truncated variants of human tau protein [31,52]. Our results indicate that it is a short NH_2-tau fragment that impairs mitochondrial function, providing a possible cellular link between NH_2-tau truncation, mitochondrial dysfunction and excitotoxic neuronal death. Therefore, it is tempting to speculate that whenever an improper apoptotic event is triggered in certain neuronal populations, NH_2-derived tau fragment is generated and binds directly to mitochondria, thus inhibiting their functions similarly to the deleterious effect of SOD 1 (superoxide dismutase 1) in amyotrophic lateral sclerosis [53,54] and of mutant Htt (polyQ expanded-Huntington) in Hungtington disease [55–57]. In addition, it has been reported that microtubules and microtubule-associated proteins (MAPs) intracellularly interact with rat brain mitochondria [58] and that β-amyloid binds *in vivo* with ABAD (Abeta-binding alcohol dehydrogenase) in the mitochondria of AD patients and transgenic mice [59]. Probably at the level of mitochondria, both Alzheimer proteins, *i.e.* β-amyloid and tau proteins, exhibit synergistic effects finally leading to accelerating neurodegenerative mechanisms.

Moreover it has been shown that *en route* to apoptosis, alteration of the ANT occurs resulting in opening of the permeability transition pore, this depending on caspase(s) action on pore component/s other than ADP/ATP translocator [10]. Our results might suggest the possibility that in apoptosis caspase(s) cause/s also tau truncation [6] finally inducing a further ANT impairment.

All together the results reported here and in [1,6] prove that caspase(s) play/s a dual pivotal role in apoptosis not only by degradation of endogenous tau thereby reducing the intracellular endogenous pool of full length tau available for binding to microtubules, but also by generating the production of the neurotoxic NH_2-26–44 tau fragment that impairs the mitochondrial ANT and then ATP cellular availability.

The picture emerging from this and previous papers to account for AD progression is that caspase(s) activity in the late phase of neuron apoptosis leads to tau truncation producing the toxic tau fragment [60,61]. This results in neurodegeneration arising from glutamate excitotoxicity in the surrounding tissue [62] (see Scheme 2). This is in contrast with the conclusions of Chung et al. [4] who proposed a pro-apoptotic effect of tau cleavage product generated by caspase-3. The knowledge that mitochondrial dysfunction, with a key role played by ANT impairment, is of central importance in Alzheimer's disease opens a window for new therapeutic strategies aimed to preserve/ameliorate mitochondrial function, and represents an exciting challenge for biochemists.

Acknowledgements

The authors thank Prof Shawn Doonan for his critical reading of the manuscript and Mr Vito Petragallo for his skilful technical assistance with tissue culture.

This work was partially financed by MIUR— Contributi straordinari di ricerca/aree obiettivo 1 (to E.M.) and by Fondi di Ricerca di Ateneo del Molise and FIRB RBNE03B8KK_003 (to S.P.).

References

[1] G. Amadoro, A.L. Serafino, C. Barbato, M.T. Ciotti, A. Sacco, P. Calissano, N. Canu, Role of N-terminal tau domain integrity on the survival of cerebellar granule neurons, Cell Death Differ. 11 (2004) 217–230.
[2] T.T. Rohn, R.A. Rissman, M.C. Davis, Y.E. Kim, C.W. Cotman, E. Head, Caspase-9 activation and caspase cleavage of tau in the Alzheimer's disease brain, Neurobiol. Dis. 11 (2002) 341–354.
[3] L. Fasulo, G. Ugolini, M. Visentin, A. Bradbury, C. Brancolini, V. Verzillo, M. Novak, A. Cattaneo, The neuronal microtubule-associated protein tau is substrate of caspase-3 and an effector of apoptosis, J. Neurochem. 75 (2000) 624–633.
[4] C.W. Chung, Y.H. Song, I.K. Kim, W.J. Yoon, B.R. Ryu, D.G. Jo, H.N. Woo, Y.K. Kwon, H.H. Kim, B.J. Gwag, I.H. Mook-Jung, Y.K. Jung, Proapoptotic effects of tau cleavage product generated by caspase-3, Neurobiol. Dis. 8 (2001) 162–172.
[5] J. Avila, J.J. Lucas, M. Perez, F. Hernandez, Role of tau protein in both physiological and pathological conditions, Physiol. Rev. 84 (2004) 361–384 (Review).
[6] G. Amadoro, M.T. Ciotti, M. Costanzi, V. Cestari, P. Calissano, N. Canu, NMDA receptor mediates tau-induced neurotoxicity by calpain and ERK/MAPK activation, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 2892–2897.
[7] G. Levi, F. Aloisi, M.T. Ciotti, V. Gallo, Autoradiographic localization and depolarization-induced release of acidic amino acids in differentiating cerebellar granule cell cultures, Brain Res. 290 (1984) 77–86.

[8] A. Almeida, J.M. Medina, A rapid method for the isolation of metabolically mitochondria form rat neurons and astrocytes in primary culture, Brain Res. Brain Res. Protoc. 3 (1998) 209–214.
[9] W.J. Waddel, C. Hill, A simple ultraviolet spectrophotometer method for the determination of protein, J. Lab. Clin. Med. 48 (1956) 311–314.
[10] A. Atlante, A. Bobba, L. de Bari, F. Fontana, P. Calissano, E. Marra, S. Passarella, Caspase-dependent alteration of the ADP/ATP translocator triggers the mitochondrial permeability transition which is not required for the low-potassium-dependent apoptosis of cerebellar granule cells, J. Neurochem. 97 (2006) 1166–1181.
[11] S. Trumbeckaite, J.R. Opalka, C. Neuhof, S. Zierz, F.N. Gellerich, Different sensitivity of rabbit heart and skeletal muscle to endotoxin-induced impairment of mitochondrial function, Eur. J. Biochem. 268 (2001) 1422–1429.
[12] D.C. Wharton, A. Tzagaloff, Cytochrome c oxidase from beef heart mitochondria, Methods Enzymol. 10 (1967) 245–253.
[13] A. Atlante, L. de Bari, A. Bobba, E. Marra, P. Calissano, S. Passarella, Cytochrome *c*, released from cerebellar granule cells undergoing apoptosis or excytotoxic death, can generate protonmotive force and drive ATP synthesis in isolated mitochondria, J. Neurochem. 86 (2003) 591–604.
[14] A. Atlante, S. Passarella, E. Quagliariello, G. Moreno, C. Salet, Haematoporphyrin derivative (Photofrin II) photosensitization of isolated mitochondria: inhibition of ADP/ATP translocator, J. Photochem. Photobiol. B 4 (1989) 35–46.
[15] N. Canu, L. Dus, C. Barbato, M.T. Ciotti, C. Brancolini, A.M. Rinaldi, M. Novak, A. Cattaneo, A. Bradbury, P. Calissano, Tau cleavage and dephosphorylation in cerebellar granule neurons undergoing apoptosis, J. Neurosci. 18 (1998) 7061–7074.
[16] T.T. Rohn, E. Head, J.H. Su, A.J. Anderson, B.A. Bahr, C.W. Cotman, D.H. Cribbs, Correlation between caspase activation and neurofibrillary tangle formation in Alzheimer's disease, Am. J. Pathol. 158 (2001) 189–198.
[17] G. Ugolini, A. Cattaneo, M. Novak, Co-localization of truncated tau and DNA fragmentation in Alzheimer's diseases neurons, Neuroreport 8 (1997) 3709–3712.
[18] M.C. Gastard, J.C. Troncoso, V.E. Koliatsos, Caspase activation in the limbic cortex of subjects with early Alzheimer's disease, Ann. Neurol. 54 (2003) 393–398.
[19] K. Saito, J.S. Elce, J.E. Hamos, R.A. Nixon, Widespread activation of calcium-activated neutral proteinase (calpain) in the brain in Alzheimer disease: a potential molecular basis for neuronal degeneration, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 26–28.
[20] Veeranna, T. Kaji, B. Boland, T. Odrljin, P. Mohan, B.S. Basavarajappa, C. Peterhoff, A. Cataldo, A. Rudnicki, N. Amin, B.S. Li, H.C. Pant, B.L. Hungund, O. Arancio, R.A. Nixon, Calpain mediates calcium-induced activation of the ERK1/2, MAPK pathway and cytoskeletal phosphorylation in neurons: relevance to Alzheimer's disease, Am. J. Pathol. 165 (2004) 795–805.
[21] S.M. Cardoso, I. Santana, R.H. Swerdlow, C.R. Oliveira, Mitochondria dysfunction of Alzheimer's disease cybrids enhances Abeta toxicity, J. Neurochem. 89 (2004) 1417–1426.
[22] M. Mancuso, F. Coppedè, L. Murri, G. Siciliano, Mitochondrial cascade hypothesis of Alzheimer's disease: myth or reality? Antioxid. Redox Signal. 9 (2007) 1631–1646.
[23] S.J. Kish, C. Bergeron, A. Rajput, S. Dozic, F. Mastrogiacomo, L.J. Chang, J.M. Wilson, L.M. DiStefano, J.N. Nobrega, Brain cytochrome oxidase in Alzheimer's disease, J. Neurochem. 59 (1992) 776–779.
[24] R.E. Davis, S. Miller, C. Herrnstadt, S.S. Ghosh, E. Fahy, L.A. Shinobu, D. Galasko, L.J. Thal, M.F. Beal, N. Howell, W.D. Jr Parker, Mutations in mitochondrial cytochrome *c* oxidase genes segregate with late-onset Alzheimer disease, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 4526–4531.
[25] M.F. Beal, Oxidative metabolism, Ann. N. Y. Acad. Sci. 924 (2000) 164–169.
[26] E.M. Mutisya, A.C. Bowling, M.F. Beal, Cortical cytochrome oxidase activity is reduced in Alzheimer's disease, J. Neurochem. 63 (1994) 2179–2184.
[27] N.R. Sims, J.M. Finegan, J.P. Blass, D.M. Bowen, D. Neary, Mitochondrial function in brain tissue in primary degenerative dementia, Brain Res. 436 (1987) 30–38.
[28] M.R. Reynolds, R.W. Berry, L.I. Binder, Site-specific nitration differentially influences tau assembly in vitro, Biochemistry 44 (2005) 13997–14009.
[29] T.C. Gamblin, R.W. Berry, L.I. Binder, Modeling tau polymerization in vitro. A review and synthesis, Biochemistry 42 (2003) 15009–15017.
[30] L.S. Yang, H. Ksiezak-Reding, Calpain-induced proteolysis of normal human tau and tau associated with paired helical filaments, Eur. J. Biochem. 233 (1995) 9–17.
[31] M. Cente, P. Filipcik, M. Pevalova, M. Novak, Expression of a truncated tau protein induces oxidative stress in a rodent model of tauopathy, Eur. J. Neurosci. 24 (2006) 1085–1090.
[32] C. Chothia, J. Janin, Principles of protein–protein recognition, Nature 256 (1975) 705–708.
[33] C.J. Tsai, S.L. Lin, H.J. Wolfson, R. Nussinov, Studies of protein–protein interfaces: a statistical analysis of the hydrophobic effect, Protein Sci. 6 (1997) 53–64.
[34] J. Janin, C. Chothia, The structure of protein–protein recognition sites, J. Biol. Chem. 265 (1990) 16027–16030.
[35] A.P. Korn, R.M. Burnett, Distribution and complementarity of hydropathy in multisubunit proteins, Proteins 9 (1991) 37–55.
[36] I.A. Vakser, C. Aflalo, Hydrophobic docking: a proposed enhancement to molecular recognition techniques, Proteins 20 (1994) 320–329.
[37] L. Young, R.L. Jernigan, D.G. Covell, A role for surface hydrophobicity in protein-protein recognition, Protein Sci. 3 (1994) 717–729.
[38] S. Jones, J.M. Thornton, Protein–protein interaction: a review of protein dimer structures, Prog. Biophys. Mol. Biol. 63 (1995) 131–165.
[39] S. Jones, J.M. Thornton, Principles of protein–protein interactions, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 13–20.
[40] T.A. Larsen, A.J. Olson, D.S. Goodsell, Morphology of protein–protein interfaces, Structure 6 (1998) 421–427.
[41] L. Lo Conte, C. Chothia, J. Janin, The atomic structure of protein–protein recognition sites, J. Mol. Biol. 285 (1999) 2177–2198.
[42] F. Glaser, D.M. Steinberg, A. Vakser, N. Ben-Tal, Residue frequencies and pairing preferences at protein–protein interfaces, Proteins 43 (2001) 89–102.
[43] P. Chakrabarti, J. Janin, Dissecting protein–protein recognition sites, Proteins 47 (2002) 334–343.
[44] M. Klingenberg, Molecular aspects of the adenine nucleotide carrier from mitochondria, Arch. Biochem. Biophys. 270 (1989) 1–14.
[45] E. Majima, N. Yamaguchi, H. Chuman, Y. Shinohara, M. Ishida, S. Goto, H. Terada, Binding of the fluorescein derivative eosin Y to the mitochondrial ADP/ATP carrier: characterization of the adenine nucleotide binding site, Biochemistry 37 (1998) 424–432.
[46] R.M. Kini, H.J. Evans, Prediction of potential protein-protein interaction sites from amino acid sequence identification of a fibrin polymerization site, FEBS Lett. 385 (1996) 81–86.
[47] K. Stamer, R. Vogel, E. Thies, E. Mandelkow, E.M. Mandelkow, Tau blocks traffic of organelles, neurofilaments, and APP vesicles in neurons and enhance oxidative stress, J. Cell Biol. 156 (2002) 1051–1063.
[48] A. Ebneth, R. Godemann, K. Stamer, S. Illenberger, B. Trinczek, E. Mandelkow, Overexpression of tau protein inhibits kinesin-dependent trafficking of vesicles, mitochondria, and endoplasmic reticulum: implication for Alzheimer's disease, J. Cell Biol. 143 (1998) 777–794.
[49] D.D. Santagata, T. Fulga, A. Duttaroy, M.B. Feany, Oxidative stress mediates tau-induced neurodegeneration in *Drosophila*, J. Clin. Inv. 117 (2007) 236–245.
[50] E. Thies, E.M. Mandelkow, Missorting of tau in neurons causes degeneration of synapses that can be rescued by the kinase MARK2/Par-1, J. Neurosci. 27 (2007) 2896–2907.
[51] D.C. David, S. Hauptmann, I. Scherping, K. Schuessel, U. Keil, P. Rizzu, R. Ravid, S. Drose, U. Brandt, W.E. Muller, A. Eckert, J. Gotz, Proteomic and functional analyses reveal a mitochondrial dysfunction in P3201L tau transgenic mice, J. Biol. Chem. 280 (2005) 23802–23814.
[52] N. Zilka, P. Filipcik, P. Koson, L. Fialova, R. Skrabana, M. Zilkova, G. Rolkova, E. Kontsekova, M. Novak, Truncated tau form sporadic Alzheimer's disease suffices to drive neurofibrillary degeneration in vivo, FEBS Lett. 580 (2006) 3582–3588.
[53] R. Liu, B. Yuan, S. Emadi, A. Zameer, P. Schulz, C. McAllister, Y. Lyubchenko, G. Goud, M.R. Sierks, Single chain variable fragments against beta-amyloid (Abeta) can inhibit Abeta aggregation and prevent abeta-induced neurotoxicity, Biochemistry 43 (2004) 6959–6967.
[54] A. Ferri, M. Cozzolino, C. Crosio, M. Nencini, A. Casciati, E.B.V. Gralla, G. Rotilio, J. Selverstone Valentine, M.T. Carrì, familial ALS-superoxide dismutases associate with mitochondria and shift their redox potentials, Proc. Natl. Acad. Sci. 103 (2006) 13860–13865.
[55] A.V. Panov, C.A. Gutekunst, B.R. Leavitt, M.R. Hayden, J.R. Burke, W.J. Strittmatter, J.T. Greenamyre, Early mitochondrial calcium defects in Hungtington's disease are direct effect of polyglutamines, N. Neurosci. 5 (2002) 731–736.
[56] A.V. Panov, J.R. Burke, W.J. Strittmatter, J.T. Greenamyre, In vitro effects pf polyglutamine tracts on Ca2+-dependent depolarization of rat and human mitochondria: relevance to Hungtington's disease, Arch. Biochem. Biophys. 410 (2003) 1–6.
[57] I.S. Seong, E. Ivanova, J.M. Lee, Y.S. Choo, E. Fossale, M.A. Anderson, J.F. Gusella, J.M. Laramie, R.H. Myers, M. Lesort, M.E. MacDonald, HD CAG repeat implicates a dominant property of huntingtin in mitochondrial energy metabolism, Hum. Mol. Genet. 14 (2005) 2871–2880.
[58] A. Rendon, D. Jung, V. Jancsik, Interaction of microtubules and microtubule-associated proteins (MAPs) with rat brain mitochondria, Biochem. J. 269 (1990) 555–556.
[59] J.W. Lustbader, M. Cirilli, C. Lin, H.W. Xu, K. Takuma, N. Wang, C. Caspersen, X. Chen, S. Pollak, M. Chaney, F. Trinchese, S. Liu, F. Gunn-Moore, L.F. Lue, D.G. Walker, P. Kuppusamy, Z.L. Zewier, O. Arancio, D. Stern, S.S. Yan, H. Wu, ABAD directly links Abeta to mitochondrial toxicity in Alzheimer's disease, Science 304 (2004) 448–452.
[60] P. Lipton, Ischemic cell death in brain neurons, Physiol. Rev. 79 (1999) 1431–1568 (Review).
[61] B. Pettmann, C.E. Henderson, Neuronal cell death, Neuron 20 (1998) 633–647 (Review).
[62] S.M. Rothman, J.W. Olney, Excitotoxicity and the NMDA receptor-still lethal after eight years, Trends Neurosci. 18 (1995) 57–58 (Review).
[63] N.C. Danbolt, Glutamate uptake, Prog. Neurobiol. 65 (2001) 1–105.
[64] D.W. Choi, Glutamate receptors and the induction of excitotoxic neuronal death, Prog. Brain Res. 100 (1994) 47–51.