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Piracetam improves mitochondrial dysfunction following oxidative stress

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1 Mitochondrial dysfunction including decrease of mitochondrial membrane potential and reduced ATP production represents a common final pathway of many conditions associated with oxidative stress, for example, hypoxia, hypoglycemia, and aging.

2 Since the cognition-improving effects of the standard nootropic piracetam are usually more pronounced under such pathological conditions and young healthy animals usually benefit little by piracetam, the effect of piracetam on mitochondrial dysfunction following oxidative stress was investigated using PC12 cells and dissociated brain cells of animals treated with piracetam.

3 Piracetam treatment at concentrations between 100 and 1000 μM improved mitochondrial membrane potential and ATP production of PC12 cells following oxidative stress induced by sodium nitroprusside (SNP) and serum deprivation. Under conditions of mild serum deprivation, piracetam (500 μM) induced a nearly complete recovery of mitochondrial membrane potential and ATP levels. Piracetam also reduced caspase 9 activity after SNP treatment.

4 Piracetam treatment (100–500 mg kg^{-1} daily) of mice was also associated with improved mitochondrial function in dissociated brain cells. Significant improvement was mainly seen in aged animals and only less in young animals. Moreover, the same treatment reduced antioxidant enzyme activities (superoxide dismutase, glutathione peroxidase, and glutathione reductase) in aged mouse brain only, which are elevated as an adaptive response to the increased oxidative stress with aging.

5 In conclusion, therapeutically relevant *in vitro* and *in vivo* concentrations of piracetam are able to improve mitochondrial dysfunction associated with oxidative stress and/or aging. Mitochondrial stabilization and protection might be an important mechanism to explain many of piracetam's beneficial effects in elderly patients.
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Abbreviations: AD, Alzheimer's disease; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid; ANOVA, analysis of variance; CNS, central nervous system; GPx, glutathione peroxidase; GR, glutathione reductase; H_2O_2, hydrogen peroxide; NaN_3, sodium azide; PC12 cells, rat pheochromocytoma cells; R123, rhodamine 123; ROS, reactive oxygen species; SNP, sodium nitroprusside; SOD, superoxide dismutase; TMRE, tetramethylrhodamineethyl-ester; TTFA, thenoyltrifluoroacetone

Introduction

Piracetam, the prototype of the so-called 'nootropic' drugs (Giurgea, 1982), is used in many countries to treat cognitive impairment in aging, brain injuries, as well as dementia (Croisile *et al*., 1993; Waegemans *et al*., 2002). Although its clinical efficacy is still a matter of dispute, a recent meta-analysis of all available (published and not published) clinical studies provided compelling evidence for the global efficacy of piracetam in a diverse group of older subjects with cognitive impairment (Waegemans *et al*., 2002).

Similar to the situation in man, piracetam has also been shown to improve cognitive function in animals, but its mode of action is not yet been finally known (Giurgea, 1982; Muller *et al*., 1999). Findings that piracetam's efficacy is usually associated with conditions of disturbed brain function like aging, that is, young healthy animals usually benefit little or nothing from piracetam treatment (Valzelli *et al*., 1980; Muller *et al*., 1997), has led to the speculation that piracetam's mechanism of action is associated with biochemical deficits of the aged brain. This assumption was later supported by observations that piracetam specifically enhances membrane fluidity in aged brain material, showing no effect at all in membranes from young brains (Muller *et al*., 1997). Since piracetam's effects at the membrane level were observed at concentrations also needed in pharmacological experiments to improve cognition (Muller *et al*., 1997), we proposed that by restoring age-related membrane alterations piracetam improves brain function and finally cognition (Scheuer *et al*., 1999). At the subcellular level, piracetam's effects on membrane fluidity could be demonstrated for synaptosomal plasma membranes of aged mouse brains and also for mitochondrial membranes (Muller *et al*., 1999).

Piracetam's improving effects on the fluidity of aged synaptosomal membranes could easily explain the beneficial

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effects of piracetam on age-related deficits of several mechanisms of signal transduction (receptor density and function, transmitter release) (Stoll *et al*., 1992; Muller *et al*., 1999), since these mechanisms are disturbed in the aging brain probably due to a decrease of membrane fluidity. Piracetam's recently reported effects on impaired glucose uptake might also be a consequence of its effects on membrane fluidity (Naftalin *et al*., 2004). On the other hand, evidences that piracetam's beneficial effects on the fluidity of aged mitochondrial membranes might contribute to its therapeutic efficacy are rather indirect and orginate from observations that piracetam might improve glucose uptake and utilization as well as ATP production (Domanska-Janik & Zaleska, 1977; Benzi *et al*., 1985; Heiss *et al*., 1988). Even if these effects led to the term 'metabolic enhancer', sometimes used to characterise piracetam and related nootropics, the mechanism of this effect and its possible relationship to mitochondrial function remained obscure.

As already mentioned, piracetam's beneficial effects are usually associated with impaired brain function such as aging, hypoxia, glucose deprivation, injuries, or neurodegeneration (Giurgea, 1982; Muller *et al*., 1999). It is quite remarkable that all those situations, even if other deficits are also present, are associated with mitochondrial dysfunction. Accordingly, together with our initial observations of improved mitochondrial membrane properties by piracetam, we speculated that piracetam might enhance mitochondrial function or at least protect mitochondria in situations of enhanced damage. Using specific fluorescence dyes to monitor mitochondrial membrane potential but also using other means to characterize mitochondrial function, the present communication reports a series of *in vitro* and *in vivo* experiments strongly indicating improvements of mitochondrial function as a specific property of piracetam.

Methods

Chemicals

Rhodamine 123 (R123) and tetramethylrhodamineethylester (TMRE) were purchased from Invitrogen, Karlsruhe (Germany). ViaLight HT kit was purchased from Cambrex, Verviers (Brussels). Hydrogen peroxide (H_2O_2), sodium nitroprusside (SNP), rotenone, thenoyltrifluoroacetone (TTFA), antimycine, sodium azide (NaN_3), and oligomycine were obtained from Sigma, Munich (Germany). Piracetam was a gift from UCB (Brussels).

Cell culture

PC12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum and 5% heat-inactivated horse serum, 50 U ml^{-1} penicillin, 50 μg ml^{-1} streptomycin, and 400 μg ml^{-1} G418 at 37°C in a humidified incubator containing 5% CO_2.

Animal brain tissue

Young (2–3 months) and old (22–24 months) female Naval Medical Research Institute (NMRI) mice used in this study were from Harlan-Winkelmann GmbH (Borchen, Germany). The latter were obtained at an age of 12 months and maintained at the Biocenter's animal care facility until use. All animals were housed in plastic cages with water and food *ad libitum* and were maintained on a 12-h light/dark cycle. Animals were handled according to the German guidelines for animal care.

Mice were killed by decapitation and brains were quickly dissected on ice (method modified after Stoll *et al*., 1992). After removing the cerebellum, the tissue was minced into 2 ml of medium I (NaCl 138, KCl 5.4, Na_2HPO_4 0.17, K_2PO_4 0.22, glucose 5.5 and sucrose 58.4 all in mmol l^{-1}, pH 7.35) with a scalpel and further dissociated by trituration through a nylon mesh (pore diameter 1 mm) with a pasteur pipette. The resulting suspension was filtered by gravity through a fresh nylon mesh with a pore diameter of 102 μM and the dissociated cell aggregates were washed twice with medium II (NaCl 110, KCl 5.3, $CaCl_2 \cdot H_2O$ 1.8, $MgCl_2 \cdot 6\ H_2O$ 1, glucose 25, sucrose 70, and HEPES (4-2-hydroxyethyl)-1-piperazineethanesufonic acid) 20 all in mmol l^{-1}, pH 7.4) by centrifugation ($400 \times g$ for 3 min at 4°C). In all, 100 μl of the suspension was used for protein determination. After centrifugation, cells were resuspended in 6 ml DMEM and distributed on a 48 well plate for the measurement of mitochondrial membrane potential. The data were expressed as fluoroscence unit per mg ml^{-1} protein.

Measurement of mitochondrial membrane potential

PC12 cells were plated the day before at a density of 2×10^5 cells well^{-1} in a 24-well plate. The mitochondrial membrane potential was measured using the fluorescence dye R123 (Baracca *et al*., 2003). Transmembrane distribution of the dye depends on the mitochondrial membrane potential. The dye was added to the cell culture medium at a concentration of 0.4 μM for 15 min. The cells were washed twice with Hanks' balanced salt solution and the fluorescence was determined with a fluorescence reader (Victor® multilabel counter) at 490/535 nm.

The mitochondrial membrane potential of dissociated brain cells was also measured with R123 at a concentration of 0.4 μM for 15 min. For detailed information about the preparation, see under Animal brain tissue.

To test acute and fast changes in mitochondrial membrane potential, the fluorescence dye TMRE (Collins *et al*., 2002) was used at a concentration of 0.4 μM for 15 min. TMRE exhibits a characteristic increase in fluorescence at 490/590 nm after challenging mitochondria with membrane potential-decreasing drugs (Krohn *et al*., 1999). The mitochondrial membrane potential was recorded and then the complex inhibitors (rotenone 2 μM, TTFA 10 μM, antimycine 2 μM, oligomycine 10 μM, and NaN_3 10 mM) were added.

Determination of ATP levels with a bioluminescence assay (ViaLight™ HT)

PC12 cells were plated the day before at a density of 2×10^4 cells well^{-1} in a white 96-well plate. The kit is based upon the bioluminescent measurement of ATP (Crouch *et al*., 1993). The bioluminescent method utilizes an enzyme, luciferase, which catalyzes the formation of light from ATP and luciferin. The emitted light is linearly related to the ATP concentration and is measured using a luminometer.

Piracetam treatment

PC12 cells were treated in three different ways. First, the protective effect of piracetam on recovery after oxidative stress was tested. Thus, PC12 cells were treated for 24 h with SNP (0.5 mM), piracetam was added 30 min after the onset of SNP exposure. Second, PC12 cells were pretreated for 30 min with SNP, then the medium was exchanged and piracetam was added for 23 h. Third, PC12 cells were pretreated for 6 h with piracetam, after that the mitochondrial membrane potential was recorded, and then the complex inhibitors were added.

Dissociated brain cells were treated for 6 h with SNP (2 mM), and piracetam was added 1 h after the onset of SNP exposure.

Treatment of animals

The treated animals received 100, 250, or 500 mg kg^{-1} piracetam in 0.9% NaCl solution p.o. once daily for 2 weeks. Control animals were treated with 0.9% NaCl alone. The tests were implemented 24 h after the last feeding. The dissociated brain cells of untreated and treated mice were incubated for 4 or 6 h with H_2O_2 (2 mM) or SNP (2 mM).

Measurement of caspase 9 activity

PC12 cells were plated the day before at a density of 5×10^6 cells per culture dish (10 cm diameter). After pretreatment for 22 h with piracetam, SNP was added for 2 h. The cells were harvested, and, after a centrifugation step, the culture medium was aspirated. The cell-containing pellet was washed with PBS and lysed in 100 μl of lysis buffer (10 mM HEPES, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM EDTA, 1 mM dithiothreitol, 1 μg ml^{-1} pepstatin A, 1 μg ml^{-1} leupeptin, 0.1% CHAPS, pH 7.4). Lysates were centrifuged at 20,000 $\times$ g for 10 min at 4°C, and the supernatant was used for caspase assay.

Caspase 9 activity was measured by cleavage of the colorimetric substrate Ac-LEHD-pNA for caspase 9. The production of pNA was monitored over 30 min in a photometer at 405 nm. The caspase activity is expressed as change in absorption units. One unit was defined as the amount of enzyme required to cleave 1 pmol of NA per min of incubation per 5×10^6 cells. For details, see Marques *et al.* (2003).

Cu/Zn-superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR) activity assays

For determination of antioxidative enzyme activities, we prepared frozen brain hemispheres. Tissue samples without cerebellum were washed in 1 ml EDTA and 1 ml Tris buffer (20 mM, pH 7.4) and homogenized with a Potter homogenizer in 20 mM Tris buffer. The resulting homogenates were centrifuged at 8500 $\times$ g for 10 min at 4°C. The clear supernatants were used to determine the activity of SOD, GPx and GR according to Leutner *et al.* (2001) and Schuessel *et al.* (2004).

SOD activity

The assay of Cu/Zn-SOD is based on the method of Nebot *et al.* (1993), utilizing the commercially available SOD Assay Kit from Merck Biosciences, Bad Soden (Germany). For elimination of interfering substances and Mn-SOD activity, Cu/Zn-SOD enzyme activity was assayed after the extraction procedure with chloroform and ethanol according to the supplier's manual. SOD activity was determined from the V_s/V_c ratio of the autoxidation rates of the chromophore BXT-01050 at 37°C measured in the presence (V_s) and absence (V_c) of sample.

One SOD activity unit is defined as the activity that doubles the autoxidation background ($V_s/V_c = 2$).

GPx activity

The assay of GPx (cytosolic GPx) is based on the reaction described by Paglia & Valentine (1967) using the commercially available Cellular GPx Assay Kit from Merck Biosciences, Bad Soden (Germany). Tert-butylhydroperoxide was used as substrate.

One unit of GPx is defined as the activity that converts 1 mmol of reduced glutathione per litre per minute at 25°C.

GR activity

The GR-activity assay is based on the method of Mizuno & Ohta (1986). Enzymatic activity was assayed photometrically by measuring NADPH consumption during the enzymatic reaction: in the presence of GSSG and NADPH, GR reduces GSSG and oxidizes NADPH to yield NADP, resulting in a decrease of absorbance at 340 nm. We used the commercially available GR Assay Kit from Merck Biosciences, Bad Soden (Germany).

One unit of GR is defined as the activity that converts 1 mmol of NADPH per litre per minute at 25°C.

Statistics

Data are given as mean $\pm$ s.e.m. n is the number of independent experiments usually performed in triplicates. In animal studies it is the number of animals. For statistical comparison, Student's t-test, one-way ANOVA followed by Tukeys *post hoc* test or repeated-measures ANOVA followed by Tukeys *post hoc* test were used. P-values less than 0.05 were considered statistically significant.

Results

Protection against NO-induced oxidative stress in PC12 cells

Oxidative stress accumulates with aging and plays an important role in the pathogenesis of neurodegenerative disorders including Alzheimer's disease (AD) (Butterfield *et al.*, 1999; Christen, 2000; Wei & Lee, 2002; Sastre *et al.*, 2003; Emerit *et al.*, 2004). For our initial experiments using PC12 cells in tissue culture, we experimentally induced acute oxidative stress using different approaches to mirror acutely damages, which are usually seen in the aged brain only after years (Marques *et al.*, 2003; Keil *et al.*, 2004).

Nitric oxide (NO) and its derivative peroxynitrite radical induce oxidative stress. NO inhibits cytochrome c oxidase activity (Brunori *et al.*, 1999; Sarti *et al.*, 2000; Cooper, 2002). Inhibition of mitochondrial respiratory chain by NO may

cause apoptosis (Brown & Borutaite, 2002; Vieira & Kroemer, 2003). In PC12 cells, SNP, a NO donor, led to a reduction of mitochondrial membrane potential and ATP levels (Figure 1).

Under basal conditions without additional damage, piracetam did not affect mitochondrial membrane potential and ATP levels in PC12 cells even after 24 h incubation and at rather high concentrations (data not shown). However, piracetam was able to reduce mitochondrial membrane potential changes and to enhance ATP levels, when added 30 min after the onset of SNP exposure (Figure 1a). Additionally, piracetam improved the recovery of mitochondrial membrane potential and ATP levels after exchanging the medium following SNP exposure (Figure 1b).

Protection against mitochondrial damage due to reduction of neurotrophic support in PC12 cells

Cells require serum to maintain growth *in vitro*. Serum provides growth and survival factors and its removal causes cellular stress. Serum deprivation leads to a rise of ROS and a decrease of ATP levels in neurons (Atabay *et al*., 1996).

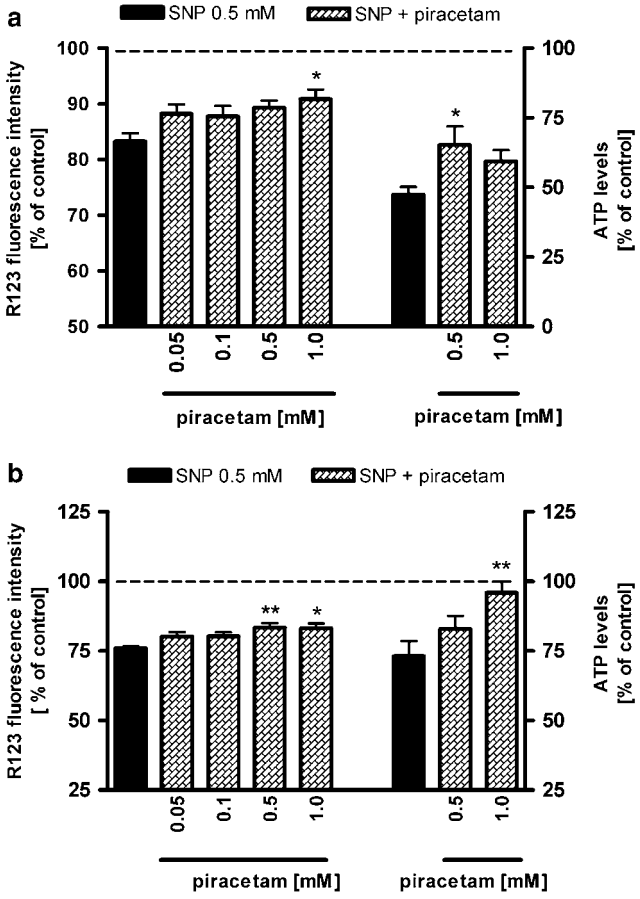

Figure 1 (a) Treatment with piracetam after NO (SNP) insult improves the reduction of mitochondrial membrane potential and enhances ATP levels. PC12 cells were incubated for 24 h with 0.5 mM SNP, piracetam was added 30 min after insult. (b) Improvement of recovery of mitochondrial membrane potential and ATP levels by piracetam following NO exposure. PC12 cells were incubated for 30 min with 0.5 mM SNP; after washing, piracetam was added for 23 h. Data are expressed as means ± s.e.m. ($n = 6$–12. $^{**}P < 0.01$, $^{*}P < 0.05$ vs SNP control, one-way ANOVA followed by Tukey's *post hoc* test.

Additionally, serum and glucose deprivation in PC12 cells cause peroxidation of their cell membrane lipids and decrease intracellular SOD activity (Ochiai *et al*., 2004). We have previously shown that serum deprivation enhances apoptosis in PC12 cells (Leutz *et al*., 2002). Here, we could demonstrate that serum deprivation leads to a decrease of mitochondrial membrane potential (Figure 2a). Interestingly, piracetam was able to protect mitochondria against cellular stress following serum deprivation. The decrease of mitochondrial membrane potential after serum deprivation was significantly reduced (Figure 2a).

Under conditions of mild serum deprivation, when serum concentrations not lower than 2% were used, piracetam (500 μM) induced a nearly complete recovery of mitochondrial membrane potential (Figure 2b). ATP levels are more sensitive to serum deprivation (Figure 2c). Reduced ATP levels were already seen at 10% serum. Under this condition, piracetam completely restored ATP levels, while at lower serum concentrations only a partial restoration was seen.

Piracetam protects PC12 cells against damage induced by inhibitors of the respiratory chain

Biochemical analysis of CNS tissue from patients with AD has yielded evidence for abnormalities of components of the electron transport chain (Parker Jr *et al*., 1994b). In many AD patients, cytochrome *c* oxidase activity is impaired in the CNS (Kish *et al*., 1992) and even in other tissues, including platelets (Parker Jr *et al*., 1994a; Bosetti *et al*., 2002; Cardoso *et al*., 2004).

Thus, we investigated the possible efficacy of piracetam to protect individual complexes of the mitochondrial respiratory chain after treatment with different respiratory chain complex inhibitors. Complexes I, II, and III were protected at concentrations as low as 500 μM piracetam (Figure 3, Table 1). A significant protection of complexes IV and V was observed at a concentration of 1000 μM piracetam (Table 1).

Other antidementiva and antipsychotic drugs had no effect on mitochondrial membrane potential and ATP levels

We investigated the putative protective effects of other antidementiva and psychotropic drugs like memantine, galantamine, haloperidol, imipramine, fluoxetine, or trolox on mitochondrial function by measuring mitochondrial membrane potential and ATP levels under similar conditions. PC12 cells were incubated with SNP for 24 h, 30 min after insult the drugs were added. Interestingly, none of the investigated drugs was able to diminish the decrease of mitochondrial membrane potential and the reduction of ATP levels (Figure 4a and b). Thus, the protective effect of piracetam under similar conditions shows a substantial specificity.

Piracetam reduces caspase 9 activity after SNP exposure

Caspase 9 represents the last specific step of the intrinsic pathway of apoptosis, which is triggered from mitochondria following oxidative stress (Kroemer & Reed, 2000). The activation of caspase 9 leads to activation of the executor caspase 3, which is common also for the extrinsic apoptotic pathway, and finally leads to apoptotic cell death. Caspase 9 is activated in PC12 cells after SNP exposure with a maximum

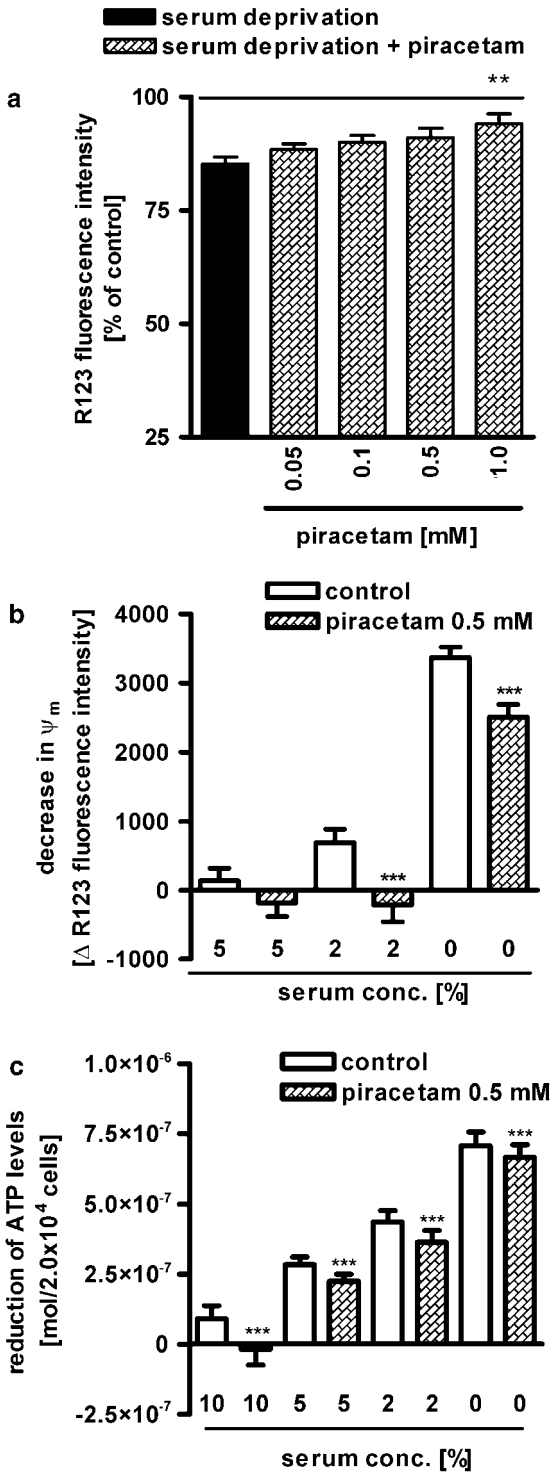

Figure 2 (a) Treatment with piracetam improves the reduction of mitochondrial membrane potential induced by 24 h serum deprivation. Piracetam was added 30 min after insult. Data are expressed as means ± s.e.m. ($n = 6$). $^{**}P < 0.01$ vs serum deprivation control, one-way ANOVA followed by Tukey's *post hoc* test. (b) Treatment with piracetam ameliorates the decrease of mitochondrial membrane potential induced by 24 h stepwise serum deprivation. Piracetam was added 30 min after insult. Data are expressed as means ± s.e.m. ($n = 10$–15). $^{***}P < 0.001$ vs % control, Student's paired *t*-test. (c) Treatment with piracetam ameliorates the reduction of ATP levels induced by 24 h stepwise serum deprivation. Piracetam was added 30 min after insult. Data are expressed as means ± s.e.m. ($n = 10$–15). $^{***}P < 0.001$ vs % control, Student's paired *t*-test.

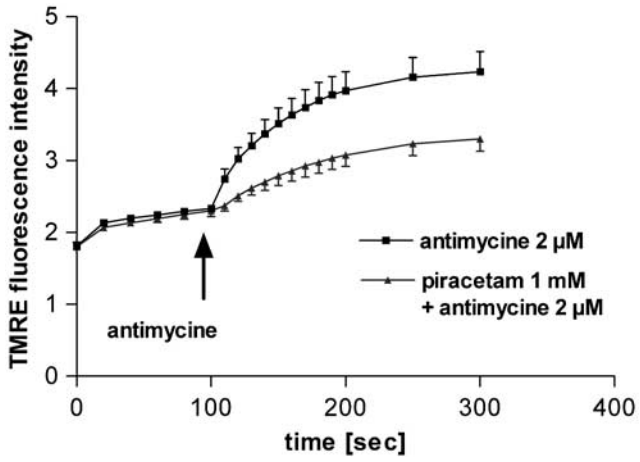

Figure 3 Pretreatment with piracetam reduces antimycine-induced changes in mitochondrial membrane potential evoked by inhibition of complex III. PC12 cells were pretreated for 6 h with piracetam. Mitochondrial membrane potential was recorded with the dye TMRE and antimycine was added after 100 s. Data are expressed as means ± s.e.m. ($n = 4$).

Table 1 Protective efficacy of piracetam on the individual complexes of the mitochondrial respiratory chain

		Protection by piracetam (mmol l^{-1})		
Complex	*Damage*	*0.50*	*1.00*	*10.00*
Complex I	Rotenone	+	+	+
Complex II	Thenoyltrifluoroacetone	+	+	+
Complex III	Antimycine	+	+	++
Complex IV	Natriumazide		+	++
Complex V	Oligomycine		+	++

Pretreatment with piracetam diminished mitochondrial membrane potential changes evoked by different complex inhibitors. Unpaired student's *t*-test, $^{++}P < 0.01$, $^{+}P < 0.05$ vs control cells ($n = 4$–10).

after 2 h of SNP treatment (data not shown). Pretreatment of PC12 cells with piracetam for 22 h, following SNP for 2 h, reduced the caspase 9 activity significantly (Figure 5).

Protection against NO-induced oxidative stress in dissociated brain cells from young and aged mice in vitro

Consistent with our findings in PC12 cells, piracetam is not protective by itself in dissociated brain cells *in vitro*, since the mitochondrial membrane potential of dissociated brain cells did not differ between *in vitro* untreated control cells and cells treated with piracetam (data not shown). However, piracetam was protective against SNP-induced mitochondrial dysfunction in cells of aged (Figure 6b) but not of young mice (Figure 6a). This effect was already seen at the rather low piracetam concentration of 500 μM.

Piracetam normalizes the basal level of mitochondrial membrane potential in vivo

Brain cells of aged mice show a decreased mitochondrial membrane potential compared to young mice (Figure 6c). Contrary to our *in vitro* data, piracetam treatment of young

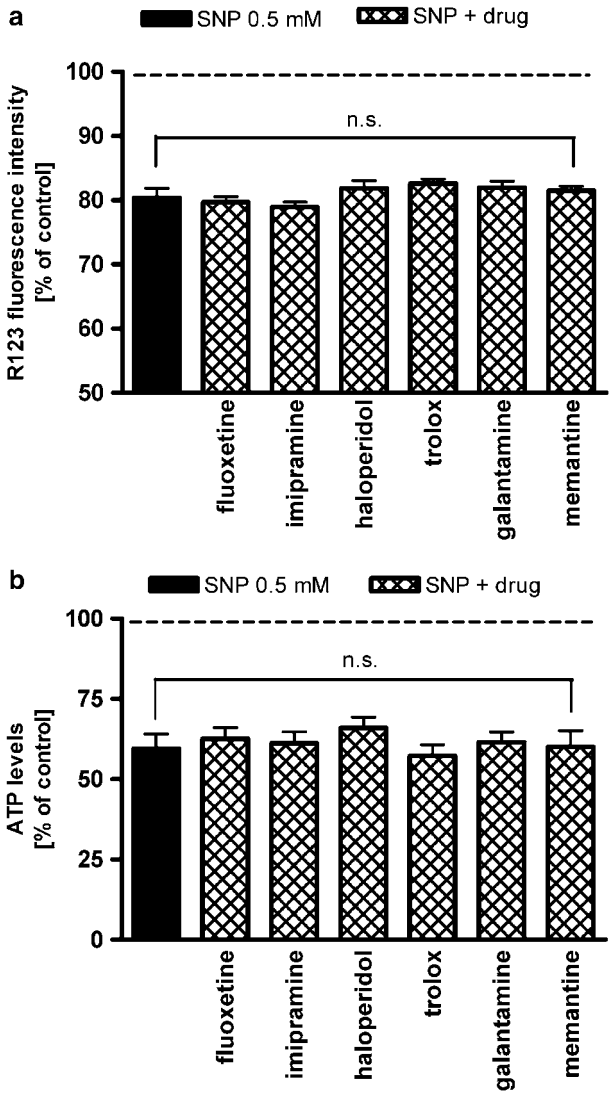

Figure 4 (a) No protective effect of other drugs on mitochondrial membrane potential. The concentration of all drugs was 10 μM. (b) No protective effect of other drugs on ATP levels.

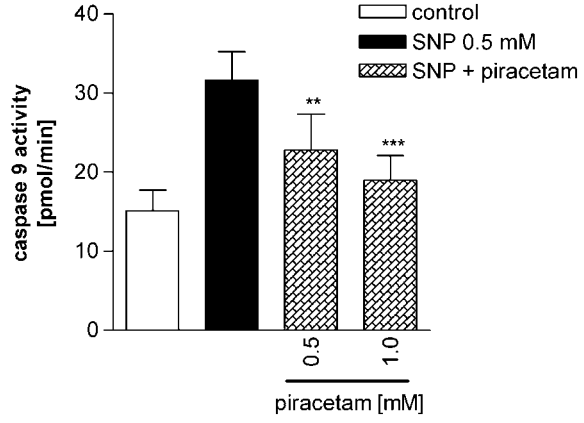

Figure 5 Treatment with piracetam reduces caspase 9 activity after SNP exposure. PC12 cells were pretreated for 22 h with piracetam, then SNP 0.5 mM was added for 2 h. Data are expressed as means ± s.e.m. ($n = 5$). ***$P < 0.001$, **$P < 0.01$ vs SNP control, Repeated-measures ANOVA followed by Tukey's *post hoc* test.

and aged mice for 14 days normalized mitochondrial membrane potential in aged mice without showing an effect in young animals (Figure 6c).

Protection against H_2O_2- and NO-induced oxidative stress in dissociated brain cells from young and aged mice treated in vivo

When piracetam was given orally for 2 weeks, decrease of mitochondrial membrane potential by H_2O_2 was significantly diminished in young and old treated mice (data not shown). Piracetam was protective against NO-induced mitochondrial dysfunction in aged mice only (Figure 6d). The protection in aged mice was always more pronounced than in young animals. Therefore, the dose dependence of piracetam's protective effects was investigated in aged mice only.

Consistent with our first study, piracetam normalized basal levels of mitochondrial membrane potential and protected against H_2O_2- and SNP-induced mitochondrial dysfunction, with first effects already seen at concentrations of 250 mg kg^{-1} (Figure 7a–c).

Effects of aging and treatment with piracetam on antioxidative enzyme activities in the mouse brain

Antioxidative enzymes are the primary defense mechanism to protect biological macromolecules from oxidative damage, and are upregulated in aged mouse brain as an adaptive response to oxidative stress (Leutner *et al*., 2001; Schuessel *et al*., 2005). Therefore, we investigated the effect of piracetam treatment on the activities of SOD, GPx, and GR in young mice (2–3 months old) and old mice (22–24 months old).

We confirmed a significant increase in GPx and GR activity in aged mice compared to young mice (Figure 8b and c). The activity of SOD had also a tendency to increase with age (Figure 8a). Piracetam treatment decreased the activities of all three enzymes in aged mice nearly to the level of young animals (Figure 8a–c). In young mice, a only small and not significant decrease of antioxidative enzymes could be observed (Figure 8).

Discussion

The data presented clearly show that piracetam protects mitochondria against different conditions associated with oxidative stress including aging. Piracetam's protecting effects on mitochondrial damage induced *in vitro* are small, but reproducible and highly significant. This is not surprising, since the conditions used to induce oxidative stress *in vitro* are not physiological and rather massive, in contrast to the small and slowly occurring changes induced by aging, which however were sometimes completely reversed by piracetam treatment. This was also the case for the adaptive elevation of antioxidant enzyme activities. When mild conditions were uesd *in vitro* (e.g. partial serum deprivation), a complete protection of mitochondria was seen by piracetam treatment in PC12 cells. Moreover, piracetam was highly effective *in vivo* in pathophysiologically relevant situations of brain dysfunction.

Piracetam does not possess radical scavenging properties (Bentue-Ferrer *et al*., 1989; Horvath *et al*., 2002). This observation was recently confirmed by us since piracetam up

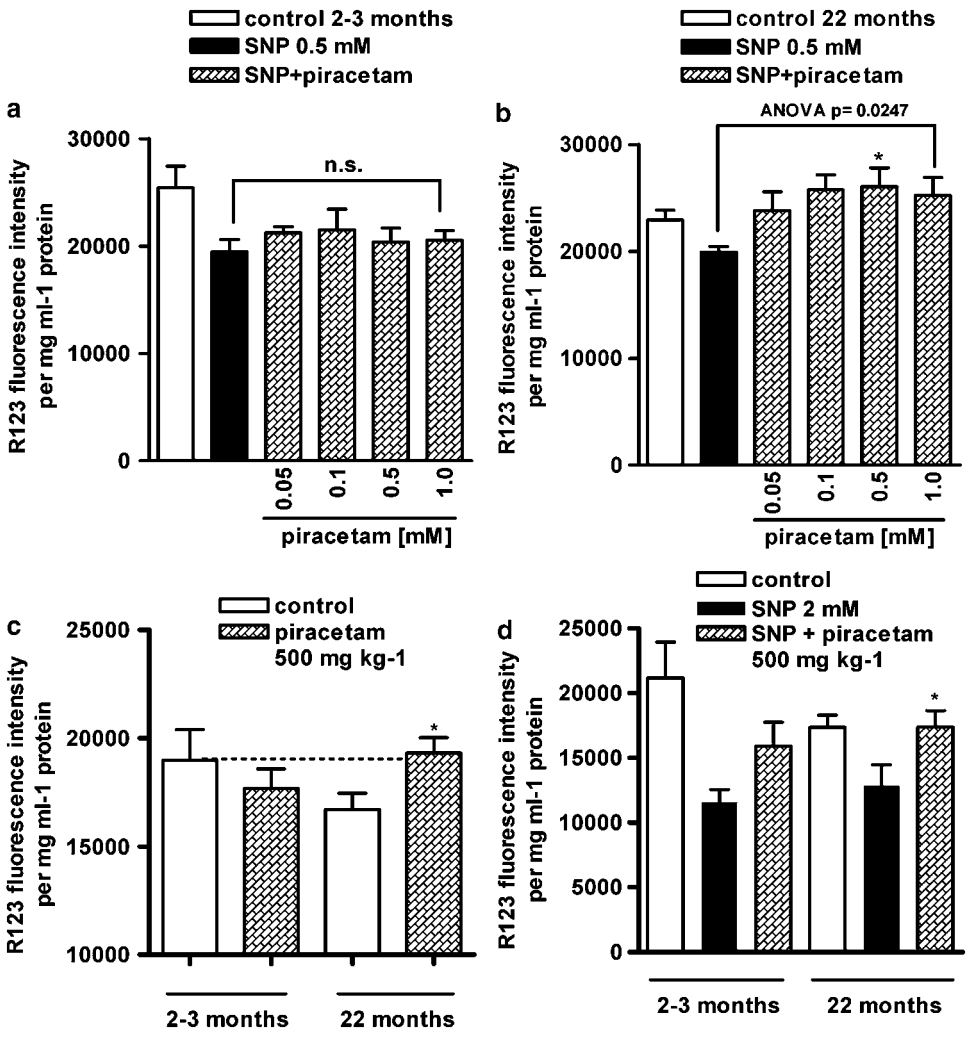

Figure 6 (a) Treatment with piracetam after NO (SNP) insult improves the reduction of mitochondrial membrane potential. Membrane potential was measured after 1 h preincubation with 0.5 mM SNP and 6 h incubation with piracetam in dissociated brain cells of young mice. (b) Dissociated brain cells of aged mice. Data are expressed as means ± s.e.m. ($n = 4$–7). *$P < 0.05$ vs SNP control, one-way ANOVA followed by Tukey's *post hoc* test. (c) Treatment with piracetam stabilizes basal levels of mitochondrial membrane potential in aged mice. The treated animals received 500 mg piracetam kg^{-1} in 0.9% NaCl solution p.o. once daily for 2 weeks. Control animals were treated with 0.9% NaCl solution alone. Data are expressed as means ± s.e.m. ($n = 6$–8). *$P < 0.05$ vs control, Student's unpaired *t*-test. (d) Piracetam treatment protects against NO induced mitochondrial damage. Young (2–3 months) and aged mice (22 months) were treated with 500 mg piracetam kg^{-1} in 0.9% NaCl solution p.o. once daily for 2 weeks. Control animals were treated with 0.9% NaCl solution alone. Membrane potential of dissociated brain cells was measured after 4 h incubation with 2 mM SNP. Data are expressed as means ± s.e.m. ($n = 6$–8). *$P < 0.05$ vs SNP control, Student's unpaired *t*-test.

to 5 mM had no activity at all in two typical *in vitro* antioxidant assays (guajacol and oxyhemoglobin assay) (unpublished observations). Moreover, the classical radical scavenger trolox was not able to protect mitochondria under similar conditions (Figure 4). Thus, it seems quite likely that piracetam acts directly at the mitochondrial level, presumably by improving mitochondrial membrane properties (Muller *et al*., 1999). This is also supported by our observation that the protective effects were also seen in the recovery phase, when the oxidative stressor was already removed. Moreover, initial experiments from our lab also indicate similar effects of piracetam on isolated mouse brain mitochondria of animals treated with piracetam (unpublished observations). The concentrations of piracetam effective *in vitro* (100–1000 μM) and the doses used in the *in vivo* experiments (100–500 mg kg^{-1}) are quite well within the plasma concentrations seen in patients treated with the standard dose of about 5 g daily, which range between 200 and 2000 μM (Saletu *et al*., 1986; 1995). Thus, it is quite likely that similar effects are also taking place in the brain of piracetam treated patients (Heiss *et al*., 1988; Eckert *et al*., 1999).

At such therapeutically relevant concentrations, piracetam seems to interact rather unspecifically with the polar head groups of the lipid bilayer of cellular membranes (Peuvot *et al*., 1995; Muller *et al*., 1997). Its structural analogue aniracetam binds to specific recognition sites associated with AMPA-sensitive glutamate receptors (Fallarino *et al*., 1995; O'Neill *et al*., 2004). However, piracetam does not interact with these sites up to 2 mM (Fallarino *et al*., 1995).

While the interaction of piracetam with membranes shows little further changes of membrane properties under normal conditions, it significantly enhances reduced membrane fluidity, for example, in the aging or even Alzheimer brain (Muller *et al*., 1997; Eckert *et al*., 1999). All the conditions

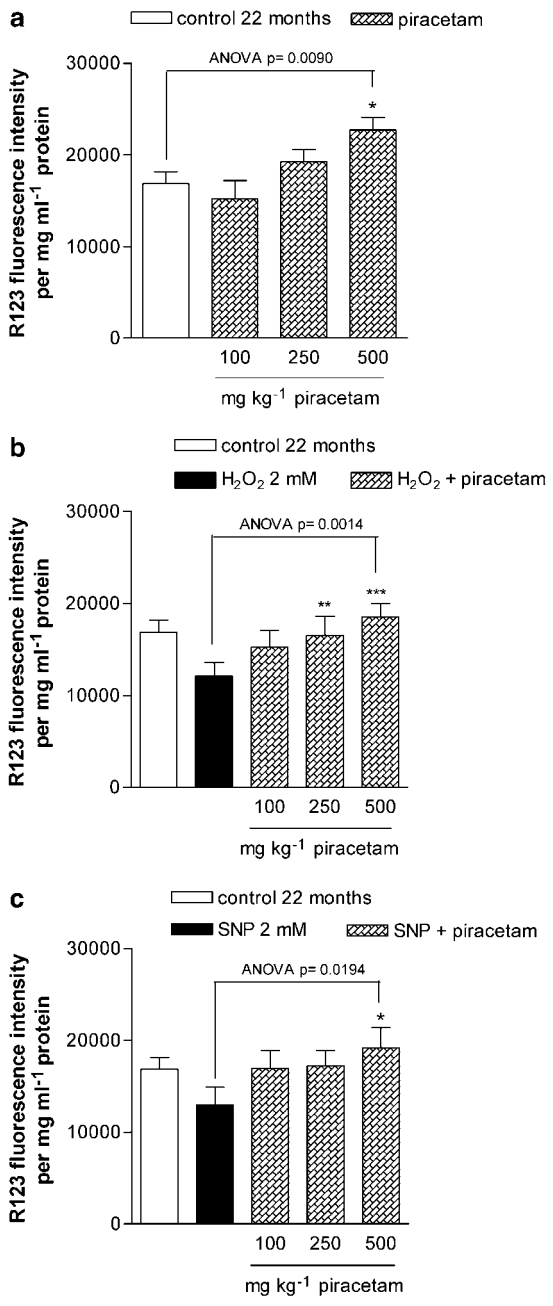

Figure 7 (a) Treatment with piracetam stabilizes concentration dependently the basal levels of mitochondrial membrane potential in aged mice. (b) Treatment with piracetam protects against H_2O_2-induced mitochondrial damage in a concentration dependent manner. (c) Treatment with piracetam protects against SNP induced mitochondrial damage concentration dependently. The treated animals received 100, 250 and 500 mg kg^{-1} piracetam in 0.9% NaCl solution p.o. once daily for 2 weeks. Control animals were treated with 0.9% NaCl solution alone. Membrane potential of dissociated brain cells was measured after 4 h incubation with H_2O_2 or SNP. Data are expressed as means ± s.e.m. ($n=8$). ***$P<0.001$, *$P<0.01$, *$P<0.05$ vs control, repeated-measures ANOVA followed by Tukey's multiple comparion test.

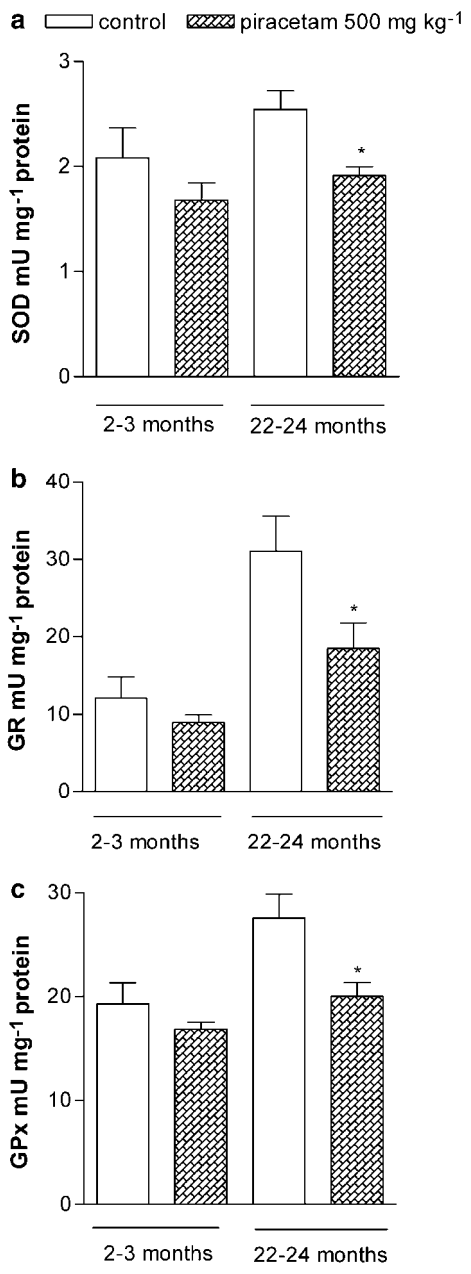

Figure 8 Effects of aging and treatment with piracetam on SOD (a), GPx (b) and GR activity (c) in young and aged mice. The treated animals received 500 mg kg^{-1} piracetam in 0.9% NaCl solution p.o. once daily for 2 weeks. Control animals were treated with 0.9% NaCl solution alone. Activities of antioxidant enzymes are increased in aged mice. Treatment with piracetam reduces the enzyme activities significantly in old mice. Data are expressed as means ± s.e.m. ($n=7$). *$P<0.05$ vs control, Student's unpaired t-test.

associated with positive effects of piracetam on mitochondrial function in the present report may also be associated with decreased membrane fluidity due to enhanced lipid peroxidation and other damages. Thus, it seems quite plausible that piracetam improves mitochondrial function by enhancing membrane fluidity. However, direct proof for this mechanism still needs to be given. Piracetam also improved mitochondrial membrane potential after stimulation with several inhibitors of the mitochondrial respiratory chain, where complexes I, II, and III were specifically sensitive. Since the complexes are located in the inner mitochondrial membrane, this structure might benefit specifically by piracetam.

Impaired mitochondrial function and decreased activity of the respiratory chain will lead to reduced ATP levels, decreased glucose uptake, and finally to neuronal dysfunction,

situations which all can be improved by piracetam (Benzi *et al*., 1985; Saletu *et al*., 1986; 1995; Heiss *et al*., 1988; Szelies *et al*., 2001). Impaired mitochondrial function will finally also lead to activation of caspases and apoptotic cell death. Observations with piracetam of Gabryel *et al*. (2002), about improved MTT reduction and enhanced ATP levels in astrozytes after hypoxia and reoxygenation, and by Pelsman *et al*. (2003), about reduced apoptosis after H_2O_2 treatment of human cortical neurons, together with our observation of reduced caspase 9 activity following oxidative stress, fit perfectly into the findings of a direct mitochondrial stabilization by piracetam. This might lead to improved respiratory chain function and ATP production on the one hand and reduced apoptosis signalling on the other hand.

Piracetam also markedly protects against mitochondrial dysfunction induced by serum deprivation and even seems to enhance or normalize mitochondrial function in situations of mildly reduced trophic support.

Serum deprivation leads to a decrease of cellular ATP/ADP ratio (Gottlieb *et al*., 2002) and to an energy deficiency. Piracetam is known to enhance cerebral glucose metabolism (Heiss *et al*., 1988). Moreover, Piracetam antagonizes inhibition of human erythrocyte D-glucose transport by barbiturates, diazepam, melatonin, and galanin (Naftalin *et al*., 2004). Thus, enhanced glucose transport in the cells might contribute to the stabilization of mitochondrial function during serum deprivation. In addition, serum deprivation induces opening of the permeability transition pore (Furuno *et al*., 2001). Due to the membrane-stabilizing properties, piracetam might inhibit the opening of the permeability transition pore and so protect mitochondria under serum-deprivated conditions. Furthermore, piracetam has been shown to be specifically active in preventing hypoxia-induced sensitization of animals for convulsive drugs (Rauca *et al*., 2000). Interestingly, this kindling-like phenomenon has been associated with reduced levels of trophic factors. The assumption that piracetam also compensates for cellular (mitochondrial) dysfunction associated with reduced neurotrophic support and might even improve neurotrophic mechanisms could be an explanation that piracetam might enhance the restitution and reorganization of neuronal circuits after periods of brain damage in experimental animals or patients following stroke (Coq & Xerri, 1999; Szelies *et al*., 2001).

In summary, protection against mitochondrial dysfunction, improved ATP production, and prevention of apoptotic signals may be important features of piracetam's beneficial effects in aging and neurodegeneration. Since mitochondrial dysfunction and reduced energy metabolism seem to be very early events during the course of AD (Mielke *et al*., 1994; Santens *et al*., 2001; Eckert *et al*., 2003; Keil *et al*., 2004), the present findings can easily explain piracetam's beneficial effects on glucose utilization in AD patients (Domanska-Janik & Zaleska, 1977; Benzi *et al*., 1985; Heiss *et al*., 1988) together with its effects on glucose uptake.

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