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In Vitro Effects of Cognitives and Nootropics on Mitochondrial Respiration and Monoamine Oxidase Activity

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Abstract Impairment of mitochondrial metabolism, particularly the electron transport chain (ETC), as well as increased oxidative stress might play a significant role in pathogenesis of Alzheimer's disease (AD). Some effects of drugs used for symptomatic AD treatment may be related to their direct action on mitochondrial function. In vitro effects of pharmacologically different cognitives (galantamine, donepezil, rivastigmine, 7-MEOTA, memantine) and nootropic drugs (latrepirdine, piracetam) were investigated on selected mitochondrial parameters: activities of ETC complexes I, II + III, and IV, citrate synthase, monoamine oxidase (MAO), oxygen consumption rate, and hydrogen peroxide production of pig brain mitochondria. Complex I activity was decreased by galantamine, donepezil, and memantine; complex II + III activity was increased by galantamine. None of the tested drugs caused significant changes in the rate of mitochondrial oxygen consumption, even at high concentrations. Except galantamine, all tested drugs were selective MAO-A inhibitors. Latrepirdine, donepezil, and 7-MEOTA were found to be the most potent MAO-A inhibitors. Succinate-induced mitochondrial hydrogen peroxide production was not significantly affected by the drugs tested. The direct effect of cognitives and nootropics used in the treatment of AD on mitochondrial respiration is relatively small. The safest drugs in terms of disturbing mitochondrial function appear to be piracetam and rivastigmine. The MAO-A inhibition by cognitives and nootropics may also participate in mitochondrial neuroprotection. The results support the future research aimed at measuring the effects of currently used drugs or newly synthesized drugs on mitochondrial functioning in order to understand their mechanism of action.

Keywords Cognitives · Nootropics · Mitochondrial respiration · Monoamine oxidase · Reactive oxygen species

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder. It is the most common cause of dementia characterized by a progressive impairment of memory and a decline of various cortical functions (e.g., judgment, orientation, behavior). Therapeutic strategies for AD treatment are designed to target cholinergic hypofunctioning, N-methyl-D-aspartate (NMDA) receptor overactivation, amyloid β (Aβ) by interfering with its synthesis, aggregation or degradation, and/or mitochondria and monoamine oxidase (MAO) [1]. Current options for AD treatment are very limited and are based on the administration of cholinesterase (ChE) inhibitors (donepezil, rivastigmine, galantamine) and/or the NMDA receptor antagonist, memantine [2, 3]. Nootropic drugs, such as piracetam and latrepirdine, are also used to treat cognitive impairment in the elderly and for dementia.

Drugs used therapeutically for AD such as donepezil, rivastigmine, and galantamine positively affect cholinergic central transmission, decrease the enzymatic degradation of acetylcholine by the inhibition of acetylcholinesterase (AChE, E.C. 3.1.1.7), and increase both the concentration and persistence of acetylcholine in the synaptic cleft [4]. The first AChE inhibitor licensed for AD therapy was tacrine, which was withdrawn from the market due to its toxicity. 7-methoxytacrine (9-amino-7-methoxy-1,2,3,4-

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tetrahydroacridine, 7-MEOTA), a tacrine derivative, has lower toxicity than tacrine and was described as a candidate molecule for AD treatment [5, 6]. 7-MEOTA derivatives were synthesized and extensively investigated to find less toxic compounds affecting more AD pathological mechanisms [6, 7]. Galantamine, donepezil, and rivastigmine have been shown to possess neuroprotective properties through a mechanism likely unrelated to AChE inhibition [8, 9]. Memantine, a non-cholinergic alternative to AChE inhibitors for AD treatment, preferentially blocks glutamate NMDA receptors without disruption of their normal physiological activity [10]. Latrepirdine (Dimebon™) has been suggested as a neuroprotective agent affecting numerous cellular functions, calcium influx, mitochondrial function, and intracellular metabolic pathways [11] including activation of the energy sensor AMP-activated protein kinase and a reduction of neuronal excitability [12]. Piracetam is described as a metabolic enhancer, which ameliorated the impairment of mitochondrial function induced by β-amyloid (Aβ) [13] or improved mitochondrial dysfunction following oxidative stress [14].

The understanding of the complexity of AD and its underlying mechanisms could be helpful in the search and development of new AD treatments [15]. The brain cells are very vulnerable to oxidative stress due to their high metabolic rate and high production of reactive oxygen species (ROS) as a by-product of oxidative phosphorylation (OXPHOS) in the electron transport chain (ETC) system [16]. The production of ROS by the mitochondria is considered in the pathogenesis of neurodegenerative disorders [17, 18]. Mitochondrion-derived ROS are sufficient to trigger Aβ production, and Aβ itself leads to mitochondrial dysfunction and increased ROS levels [19]. The role of mitochondrial enzyme monoamine oxidase (MAO; EC 1.4.3.4) is important to study in this context because MAO affects both hydrogen peroxide (H_2O_2) production and metabolism of intraneuronal monoamine neurotransmitters. MAO activity is inhibited by many different pharmacological substances [20–22]. Investigating the direct action of ChE inhibitors on components of the monoamine system could be useful for both understanding their therapeutic and/or adverse effects and for the development of new drugs with increased therapeutic efficiency due to dual acetylcholinergic and monoaminergic action. The current study tested the hypothesis that some effects of cognitives and nootropic drugs may be linked to changes in mitochondrial functions. The aim of our study was to detect the direct effects of drugs used in AD treatment plus the effect of 7-MEOTA, a candidate molecule for AD treatment, on activity of ETC complexes, citrate synthase (CS) activity, and the oxygen consumption rate in isolated mitochondria. The effects of 7-MEOTA, memantine, rivastigmine, donepezil, galantamine, latrepirdine, and piracetam were also examined on MAO activity and H_2O_2 production by mitochondria.

Methods and Materials

Media and Chemicals

Buffered sucrose (sucrose 0.32 mol/L, HEPES 4 mmol/L, pH 7.4) was used as isolation medium and preservation medium for isolated mitochondria. Krebs-Henseleit buffer (KH buffer) consisted of 118 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L KH_2PO_4, 1.2 mmol/L $MgSO_4 \cdot 7H_2O$, 25 mmol/L $NaHCO_3$, 11.1 mmol/L glucose (pH 7.4). The mitochondrial respiration medium (MiR05) consisted of sucrose 110 mmol/L, K^+-lactobionate 60 mmol/L, taurine 20 mmol/L, $MgCl_2.6H_2O$ 3 mmol/L, KH_2PO_4 10 mmol/L, EGTA 0.5 mmol/L, BSA essentially fatty acid free 1 g/L and HEPES 20 mmol/L, adjusted to pH 7.1 with KOH (32). Other media were used in enzyme assays. Radiochemicals, 5-hydroxytryptamine [^{3}H] trifluoroacetate ([^{3}H]serotonin) and 2-phenylethylamine [ethyl-1-^{14}C] hydrochloride ([^{14}C]PEA) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA), other chemicals from Sigma-Aldrich Co. (St. Louis, MO, USA).

Isolation of Brain Mitochondria

The mitochondrial fraction and purified mitochondria were isolated and prepared from pig brain cortex as described previously [20, 23]. Samples of mitochondria purified on a sucrose gradient were stored on ice until the assay. The fresh crude mitochondrial fraction (stored on ice) was used for measurement of mitochondrial oxygen consumption rate. Fresh purified mitochondria (stored on ice) were used in experiments with H_2O_2 production. The frozen crude mitochondrial fraction (stored at −70 °C) was used for determination of enzyme activities. The protein concentration was determined by the Lowry method [24], with bovine serum albumin as the standard.

Activity of Citrate Synthase and Respiratory Complexes

Purified mitochondria were resuspended with hypotonic buffer (25 mmol/L potassium phosphate, 5 mmol/L $MgCl_2$, pH 7.2) and ultrasonicated three times to achieve the maximum enzymatic activity. Samples were incubated with selected drugs at a concentration of 50 μmol/L for 30 min at 30 °C. Each independent measurement had a control. Samples were measured in 3 mL of total reaction volume at 30 °C; the final protein concentration was 150 μg/mL. The activity of ETC complexes and CS was measured spectrophotometrically using Uvicon XL spectrophotometer (SECOMAM, Alès, France).

Complex I (NADH dehydrogenase (ubiquinone), EC 1.6.5.3)

The method used has been previously described [25, 26] Complex I activity was measured as the rotenone-sensitive rate of NADH oxidation at 340 nm. The reaction was started by the addition of coenzyme Q_1 (in a final concentration 33 μmol/L) and measured for 10 min.

Complex II + III (Succinate cytochrome *c* oxidoreductase, EC 1.8.3.1)

Mitochondrial complex II + III activity was measured spectrophotometrically as antimycin A sensitive rate of cytochrome *c* reduction at 550 nm using succinate as a substrate [27].

Complex IV (cytochrome *c* oxidase, COX, EC 1.9.3.1).

COX activity was measured as a decrease in absorbance during the oxidation of reduced cytochrome *c* at 550 nm [28].

Citrate synthase (CS, EC 2.3.3.1).

CS activity was measured as a color change of 5,5′-dithiobis-(2-nitrobenzoic) acid (DNTB); the reaction was initiated by the addition of 0.5 mmol/L oxaloacetate and absorbance was measured at 412 nm for 3 min [29].

Mitochondrial Respiration

Mitochondrial oxygen consumption rate was measured at 37 °C using a titration-injection high-resolution oxygraph (Oxygraph-2 k, Oroboros Instruments, Innsbruck, Austria) with Clark-type electrodes. Experimental protocols were adapted from previous studies [30, 31]. The respiration medium MiR05 (oxygen solubility factor: 0.92) was added into chambers to a final volume of 2 mL. The crude mitochondrial fraction was added to a final protein concentration between 0.05–0.20 mg/mL. Plasma membranes were permeabilized by digitonin (573 μg digitonin/mg protein). The respiratory rate of mitochondria in respiratory states related to electron entry through complex I and complex II was assessed using specific mitochondrial substrates: malate + pyruvate for complex I-linked respiration, succinate + rotenone for complex II-linked respiration. Two parallel samples were titrated and measured simultaneously. The final drug concentrations were between 1.25–100 μmol/L, except for piracetam, whose effect was measured up to 10 mmol/L. Oligomycin, rotenone, and antimycin A were used as inhibitors of ATP synthase, complex I and complex III, respectively.

Monoamine Oxidase Activity

MAO-A and MAO-B activities were determined by means of radiochemistry using a previously published method [32, 33]. Briefly, the reaction mixture in KH buffer containing the mitochondria and various drug concentrations was preincubated for 60 min at 37 °C. The reaction was initiated by the addition of a radiolabelled substrate. The final sample volume was 250 μl containing 200 μg of protein, 3.2 μmol/L [^{3}H]serotonin for MAO-A or 10 μmol/L [^{14}C]PEA for MAO-B, and 0.1 μmol/L to 100 mmol/L of drug. MAO activity was measured at 37 °C for 30 min for MAO-A and for 1 min for MAO-B. The reaction was stopped by the addition of 250 μl of 2 N hydrochloric acid. The reaction products were extracted into benzene:ethyl acetate 1:1 (*v*/v), and the radioactivity of extracts in organic phase were measured by liquid scintillation counting (LS 6000IC, Beckman Instruments, Inc., Fullerton, CA, USA).

Hydrogen Peroxide Release

Purified mitochondria in a final concentration of 0.10 mg/mL were incubated in buffer (26.85 mmol/L Hepes, 114.1 mmol/L sucrose, 100 mmol/L KCl, 1.342 mmol/L K_2HPO_4, pH 7.3) with 10 mmol/L succinate, 1.0 mmol/L ADP, and 50 μmol/L of selected cognitives or nootropics for 30 min at 37 °C. The extramitochondrial release of H_2O_2 was determined using 7.5 μmol/L Ampliflu™ Red in the presence of 1.5 units/mL horseradish peroxidase, according to a previously described and slightly modified method [34]. In total, 0.5 μmol/L rotenone and 1.25 μg/mL antimycin A were used to inhibit activities of complex I and complex III, respectively, after 5 min in every measurement. Fluorescence was monitored continuously for 15 min in a fluorometer (Fluoromax-3, Jobin Yvon, Horiba) at an excitation of 571 nm and an emission of 585 nm. The fluorescence intensity was converted to H_2O_2 concentration based on measurement of the fluorescence increase after addition of a known amount of H_2O_2 [35].

Data Analysis and Statistics

Enzyme activities were measured as the slope of time dependence of absorbance using LabPower Junior software (SECOMAM). Relative changes of enzyme activities induced by the drugs were determined assuming that the control sample activity is equal to 100 %. Data from high-resolution respirometry were collected and analyzed using DatLab 4.3 software (Oroboros Instruments, Innsbruck, Austria). Respiratory rates were expressed as mass-specific oxygen flux (pmol O_2 consumed per second/mg of protein in the sample).

Drug-induced inhibition of respiratory rate and MAO activity was analyzed using a four-parameter logistic regression (SigmaPlot, Systat Software Inc., Richmond, CA, USA) to quantify IC_{50} (concentration of a drug that is required for 50 % inhibition) and the residual activity at high drug concentration. Data were expressed as the mean ± standard error.

The results were analyzed by the data analysis software system STATISTICA (version 12.0, StatSoft, Inc., Tulsa, OK, USA). One-sample *t* tests for single means were used to determine whether enzyme activity in the sample with drug added was significantly different from the control. For H_2O_2 production, the effect of inhibitors in the control sample was evaluated by *t*-test for dependent samples. Comparisons

between controls and samples with the drug were performed using ANOVA, and the Dunnett test was utilized for post hoc comparisons. The data are expressed as the mean ± standard deviation.

Results

Activity of CS and Respiratory Chain Complexes

Drug-induced changes in cellular energetics characterized by activity of CS and individual ETC complexes were measured in purified mitochondria. None of the tested drugs significantly affected the CS activity. Significantly decreased complex I activity was caused by donepezil ($p = 0.029$), galantamine ($p = 0.049$), and memantine ($p = 0.010$, Fig. 1a). Significantly increased complex II + III activity was observed after incubation with galantamine ($p = 0.021$, Fig. 1b). Complex IV was insignificantly increased after incubation with latrepirdine ($p = 0.070$, Fig. 1c).

Mitochondrial Respiration

Drug-induced changes in oxygen consumption rate were measured in isolated pig brain mitochondria incubated with substrates for complex I or complex II-linked respiration. A slight decrease in complex I-linked respiration was observed for galantamine and memantine. Increases were induced only by rivastigmine; piracetam, latrepirdine, 7-MEOTA and donepezil did not change the oxygen consumption rate (Fig. 2a). Complex II-linked respiration was insignificantly increased by memantine, slightly increased by latrepirdine, galantamine, and donepezil; piracetam, 7-MEOTA, and rivastigmine did not change the oxygen consumption rate (Fig. 2b). No change was statistically significant. Note the effect of piracetam was measured up to a concentration of 10 mmol/L without any change (data not shown).

The effects of rotenone and antimycin A on complex I-and complex II-linked respiration were measured under the same experimental conditions to confirm the sensitivity of our respiratory assay to known inhibitors of respiration (inset graphs in Fig. 2). We confirmed the full inhibition of both complex I-linked respiration by rotenone and complex II-linked respiration by antimycin A.

Monoamine Oxidase Activity

The dose-dependent effects of pharmacologically different cognitives and nootropic drugs on MAO-A and MAO-B activity were measured in pig brain mitochondria.

MAO-A activity was fully inhibited by all tested drugs; however, higher half maximal inhibitory concentrations (IC_{50}) of cognitive enhancers were observed compared to the known MAO inhibitors, such as iproniazid, pargyline, and clorgyline (Fig. 3a, Table 1). MAO-A was inhibited by the drugs tested in the following order: pargyline > clorgyline > iproniazid > donepezil = latrepirdine =7-MEOTA > memantine = rivastigmine > galantamine > piracetam. Piracetam, galantamine, rivastigmine, and memantine are very weak inhibitors of MAO-A activity. Donepezil, latrepirdine, and 7-MEOTA are relatively strong MAO inhibitors. MAO-B activity was fully inhibited by latrepirdine and memantine; partial inhibition was caused by 7-MEOTA, donepezil, and galantamine. Rivastigmine induced an initial increase in MAO-B activity followed by a rapid decrease (full inhibition) at very high concentrations. Piracetam caused monotonic increase of MAO-B activity, even at very high (millimolar) concentrations (Fig. 3b). IC_{50} values characterizing MAO-B inhibition were higher than MAO-A inhibition, except for galantamine (Table 1).

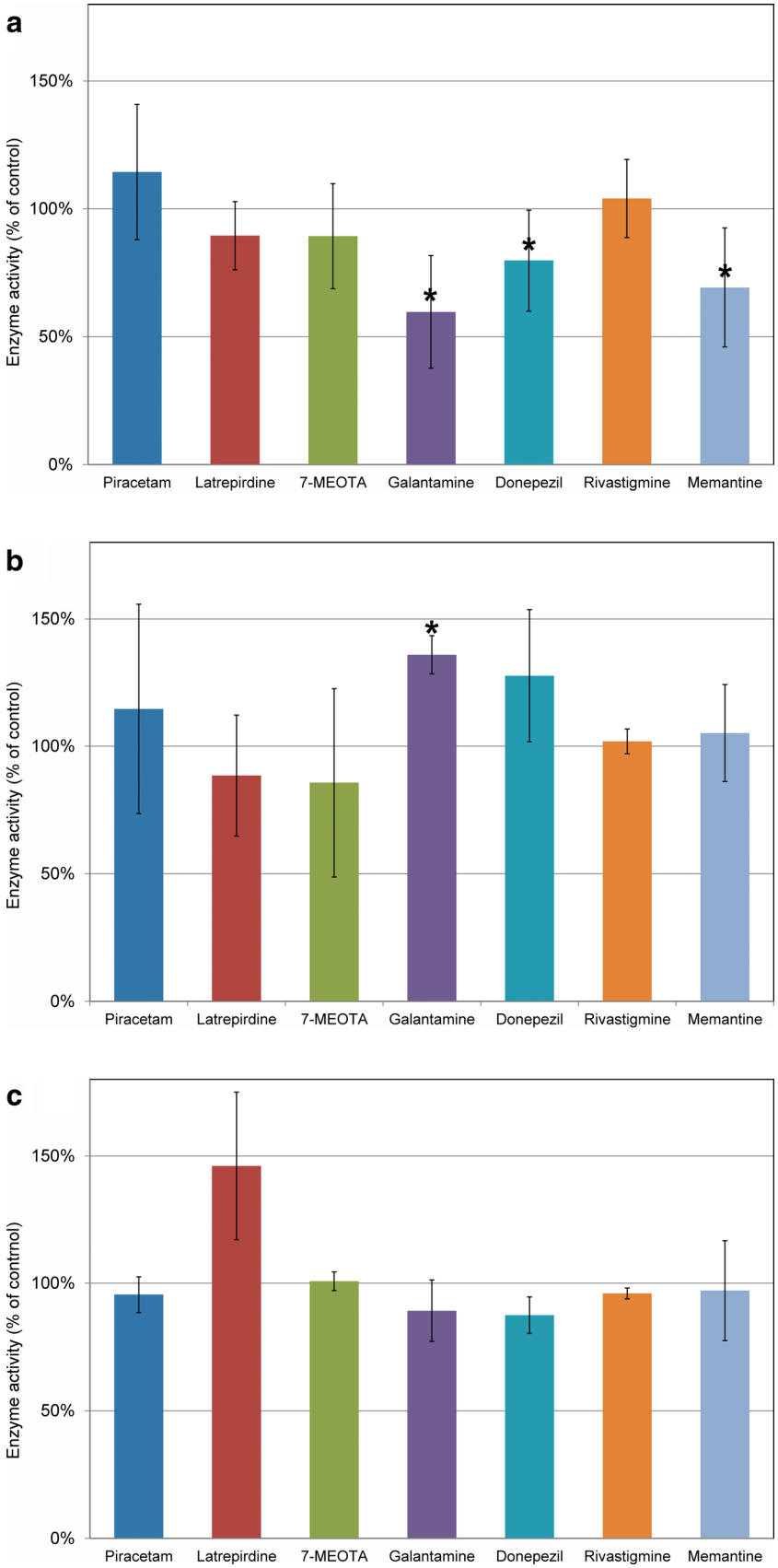
Fig. 1 Drug-induced changes in activity of the **a** complex I, **b** complex II + III, and **c** complex IV in pig brain mitochondria. The sample was incubated with a drug at 30 °C for 30 min, and the enzyme activity was determined relative to control sample with solvent added without the drug. Values are the means ± standard deviation at 3–7 independent measurements. Equality of enzyme activity in sample with drug to enzyme activity in the sample without drug was tested using a *t* test for single means against 100 % (*$p < 0.05$)

Hydrogen Peroxide Release

The effects of cognitives and nootropics on the release of hydrogen peroxide from purified pig brain mitochondria were determined fluorometrically after 30-min incubation with succinate. The addition of rotenone (complex I inhibitor) and antimycin A (complex III inhibitor) followed. The results are summarized in Fig. 4. In the control sample (with added solvent without drug), rotenone had no effect, and antimycin A caused a significant increase in succinate-induced H_2O_2 release ($p < 0.001$). Compared to the control, the succinate + rotenone + antimycin A-induced increase in H_2O_2 release was not significantly affected by any drug tested in this study (Fig. 4).

Discussion

To determine the effect of cognitives and nootropics for AD treatment in mitochondrial energy metabolism, we investigated the in vitro effect of piracetam, latrepirdine, 7-MEOTA, galantamine, donepezil, rivastigmine, and memantine on mitochondrial respiration, enzyme activities of CS and ETC complexes, mitochondrial ROS release, and MAO activity in isolated brain mitochondria.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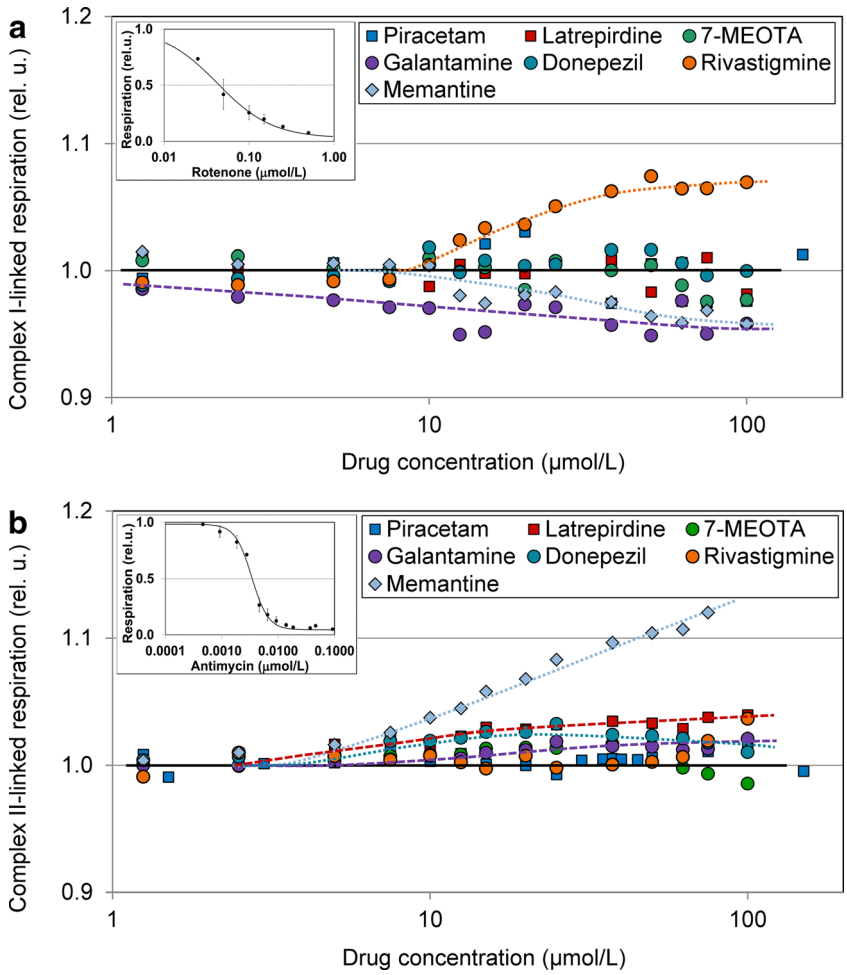

Fig. 2 Drug-induced changes of **a** complex I-linked respiration, **b** complex II-linked respiration in pig brain mitochondria. Dose-response curves are displayed as plots of the relative respiratory rate against the drug concentration, presuming that the relative respiratory rate equals 1 at zero drug concentration. *Lines* show a tendency to decrease, increase or no change in the respiratory rate with increasing drug concentrations. Values are the means of 3–7 independent measurements. *t* tests for single means against the control sample with solvent added without the drug did not confirm significant changes in mitochondrial respiration, even at high drug concentrations. The effect of rotenone and antimycin A is shown in embedded graphs to illustrate their inhibitory effects on complex I-and complex II-linked respiration

The Effects of Cognitives and Nootropics on Mitochondrial Complexes and Respiration

CS is localized in the mitochondrial matrix and commonly used as a quantitative enzyme marker for the presence of intact mitochondria. We did not observe any significant drug-induced changes in CS activity, which confirms CS activity as a proper mitochondrial marker also during pharmacotherapy with cognitive enhancers. Many drugs have been shown to induce a mild and transient inhibition of complex I, which plays a role in their mechanisms of action. Inhibitory effect of tacrine on complex I activity was found in vitro in pig brain mitochondria [36]. We observed mild complex I inhibition by galantamine, donepezil, and memantine, indicating the possible involvement of mitochondrial dysfunction on the adverse effects of high doses of these drugs (Fig. 1a). The activity of complexes II + III and IV was unchanged by all of the drugs examined in the study, except for a significant increase in complex II + III activity by galantamine (Fig. 1b).

The in vitro effects of some cognitives and nootropics on the activity of mitochondrial complexes in isolated mitochondria are apparently linked to high concentrations of these drugs (50 μmol/L). Such concentrations are higher than plasma concentrations at therapeutic doses of the drugs (latrepirdine <0.05 μmol/L, 7-MEOTA <0.2 μmol/L, galantamine 0.2–0.5 μmol/L, donepezil 0.08–0.13 μmol/L, rivastigmine 0.01–0.10 μmol/L, memantine 0.5–1.0 μmol/L), except for piracetam (200–2000 μmol/L). The use of high doses of tested drugs in in vitro experiments can be justified in part by the fact that there is an accumulation of some drugs in the brain and that their intraneuronal concentrations are unknown. Moreover, information on in vitro mitochondrial effects at high drug concentrations can be useful for the design of in vivo experiments, which should confirm or disprove these effects at therapeutic doses.

Galantamine mildly inhibited complex I activity, stimulated complex II + III activity, and did not significantly affect either complex IV activity or the oxygen consumption rate. It can be speculated that the direct inhibitory effects of galantamine on complex I can be compensated by the stimulatory effect of the drug on complex II + III. Memantine partially inhibited complex I activity, did not affect complex II + II or complex IV activity, slightly decreased complex I-linked respiration, and increased complex II-linked respiration. These results are consistent and

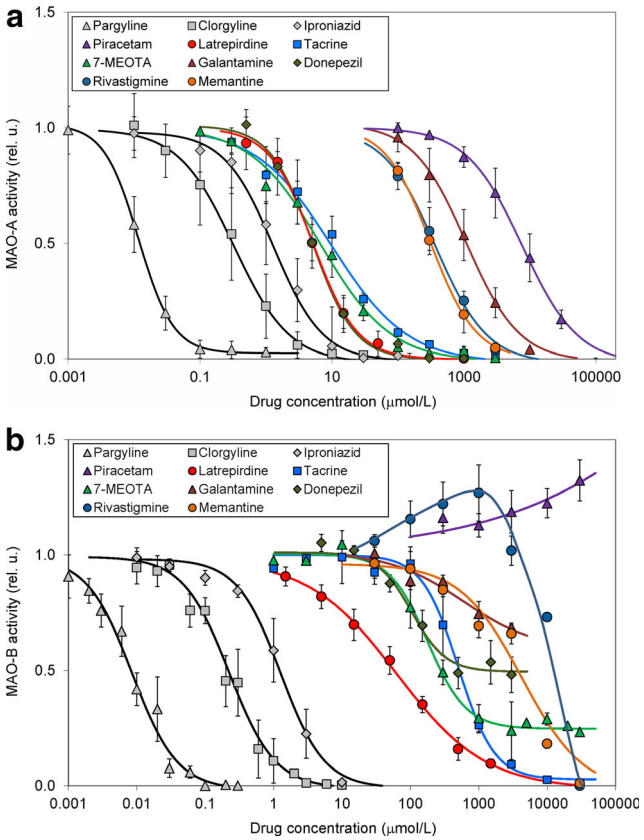

Fig. 3 Changes in basal monoamine oxidase type (MAO) activity caused by cognitive enhancers in the crude mitochondrial fraction of pig brain. Concentration-response curves are displayed as plots of the initial enzyme activity against the drug concentration for **a** MAO-A and **b** MAO-B. The samples were incubated with drugs at 37 °C for 60 min, and the reaction was started by the addition of 3.2 μmol/L [^{3}H]serotonin for MAO-A and by the addition of 10 μmol/L [^{14}C]phenylethylamine for MAO-B. Median effective concentrations (IC_{50}) were calculated using nonlinear regression analysis software. Values are the means calculated from at least five independent measurements. *Lines* represent the best fit curves using the four-parameter logistic function, except for effect of rivastigmine and piracetam on MAO-B activity. Inhibitory effects of pargyline, clorgyline, and iproniazid are displayed for comparison

correspond to previous findings where memantine was protective against the effects of 3-nitropropionic acid (complex II inhibitor) but was ineffective in protection against rotenone-induced toxicity (complex I inhibitor) in spiny striatal neurons. We partially confirmed the results of an earlier in vitro study on the direct effects of acute memantine exposure on isolated mitochondria. In that study when the oxygen consumption rate was increased, complex I activity was increased, and complex IV activity was decreased at high (60 μmol/L) memantine concentrations [37]. However, the differences in complex I- and complex II-linked respiration were not determined in this study. Donepezil was found to gently inhibit complex I (Fig. 1a) with no effect on complex II + III, complex IV, or mitochondrial respiration. It appears that the direct effect of donepezil on ETC activity is not included in the non-cholinergic mechanisms that are associated with its neuroprotective profile [9].

One clinical study found that the cellular respiration supported by complex I and complex II substrates as well as activities of complexes II, III, and IV were increased in AD patients treated with rivastigmine [38]. The mechanisms by which rivastigmine would stimulate ETC are uncertain. Compensatory mechanisms of declines or increases of individual complexes in OXPHOS are also possible. Our data show that there is no significant direct effect of rivastigmine on individual respiratory complexes, and respiration characterizing ECT activity as a whole is indicative of stimulatory effects. Therefore, effects of rivastigmine on mitochondrial respiration may support its neuroprotective action.

7-MEOTA and its derivatives are considered candidate molecules for AD treatment, due to lower toxicity, better antioxidant properties, interaction with muscarinic and nicotinic receptors, and safer metabolization compared to tacrine [5]. In our study, we found that 7-MEOTA did not change the activity of mitochondrial complexes I, II + III, or IV and did not affect the mitochondrial oxygen consumption rate. From this perspective, the 7-MEOTA does not exhibit anti-mitochondrial effects.

Piracetam is supposed to enhance mitochondrial function or at least protect against mitochondrial damage in the aging brain or in people with dementia [39]. Piracetam has been found to improve mitochondrial dysfunction following oxidative stress [14] and also to ameliorate the detrimental effects of Aβ on brain functions [13]. In the current study, piracetam did not induce any change in the activity of complexes I, II + III, or IV. Neither complex I- nor complex II-linked respiration was affected by piracetam at concentrations up to 10 mmol/L. Our results indicate that the piracetam-induced beneficial effect on cognition is not associated with its rapid direct effect on the mitochondrial electron transport system activity. Thus, piracetam seems to be very safe in terms of mitochondrial toxicity even at very high concentrations. This confirmed the results of a previous study, which showed that piracetam did not affect mitochondrial membrane potential and ATP levels in PC12 cells under basal conditions without additional insult [14].

Latrepirdine action is believed to be involved in improving cellular energy balance, stabilizing mitochondrial function by increasing the threshold for nonselective mitochondrial pore opening, increasing the calcium retention capacity of mitochondria and reducing lipid peroxidation [40]. We observed that latrepirdine caused nonsignificant changes in activities of ETC complexes, and in the oxygen consumption rate. A lack of an effect of latrepirdine on mitochondrial respiration and/or activity of the ETC complexes does not indicate an association with its therapeutic effects.

Inhibition of MAO Activity

MAO catalyzes the oxidative deamination of a variety of biogenic and xenobiotic amines with concomitant H_2O_2 production. MAO activity is inhibited by many psychotropic drugs

Table 1 Inhibition of monoamine oxidase (MAO) activity by cognitives and nootropics

Drug	MAO-A		MAO-B		MAO-A/MAO-B
	IC_{50} (μmol/L)	Inhibition	IC_{50} (μmol/L)	Inhibition	
Piracetam	7668 ± 754	Full	increase		nd
Latrepirdine	5.16 ± 0.39	Full	63.3 ± 8.9	Full	0.081
7-MEOTA	6.71 ± 0.72	Full	178 ± 13	Partial	0.038
Tacrine	9.84 ± 1.86	Full	502 ± 44	Full	0.020
Galantamine	1080 ± 137	Full	592 ± 564	Partial	1.826
Donepezil	5.04 ± 0.41	Full	105 ± 17	Partial	0.048
Rivastigmine	370 ± 41	Full	10,085 ± 1034	(Full)	0.037
Memantine	313 ± 22	Full	4261 ± 666	Full	0.073
Pargyline	0.0116 ± 0.0007	Full	0.00853 ± 0.00039	Full	1.361
Clorgyline	0.326 ± 0.039	Full	0.224 ± 0.012	Full	1.454
Iproniazid	1.294 ± 0.098	Full	1.272 ± 0.071	Full	1.017

[20]; and MAO inhibitors are considered potential candidates for AD treatment, due to their ability to inhibit oxidative damage. A series of new multifunctional MAO/ChE inhibitors have been synthesized for AD treatment [41, 42].

While MAO inhibition is not a primary biochemical effect related to therapeutic action of cognitives and nootropics, it can be supposed that changes in MAO activity may be related to some of the effects of these drugs on serotonergic, noradrenergic, and dopaminergic neurotransmission. Moreover, MAO inhibition may participate in antioxidant and neuroprotective effects of cognitives and nootropics. We found that the most potent MAO inhibitors are latrepirdine, 7-MEOTA, and donepezil. The IC_{50} of approximately 5 μmol/L for inhibition of MAO-A indicates that MAO-A activity may be inhibited at therapeutic doses of these drugs. Thus, neuroprotective and antidepressant or mood altering effects can be of added value to the improved cognition by treatment with these drugs. Latrepirdine, 7-MEOTA, donepezil, and memantine were found to be selective MAO-A inhibitors (Table 1), which is in conflict with previous findings that latrepirdine in rat brain homogenate [43] and donepezil in rat liver homogenate [41] preferentially inhibited MAO-B. We suppose that the purification of mitochondria may play a role in this discrepancy because the presence of non-mitochondrial membranes may affect the concentrations of free substances. The lowest potency to inhibit MAO-A was found for piracetam; however, 1 mmol/L piracetam plasma concentration may be reached during therapy, which is sufficient to partially inhibit MAO-A. In contrast, a stimulatory effect on MAO-B was found for piracetam. These findings indicate that the resulting effect of

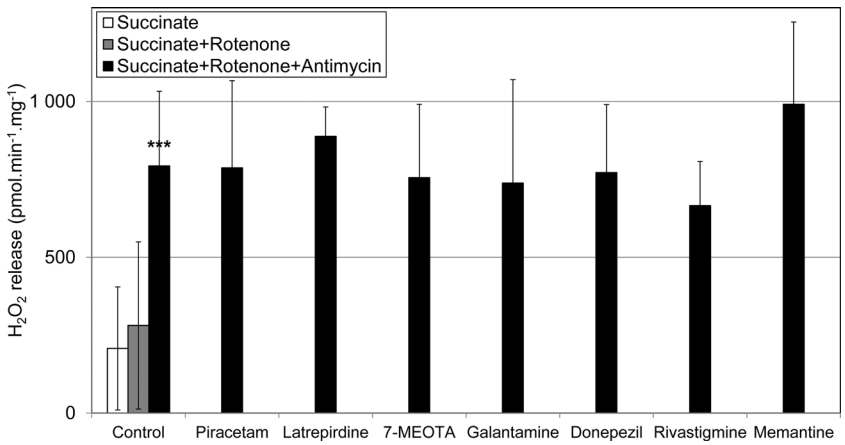

Fig. 4 Drug-induced changes of hydrogen peroxide release from purified brain mitochondria. The samples (0.10 mg protein/mL) were incubated with drugs (50 μmol/L) or with solvent (control) at 30 °C for 30 min in presence of 10 mmol/L succinate. Samples were supplemented with 1.5 units/mL of horseradish peroxidase and 7.5 μmol/L Ampliflu™ Red, and fluorescence of resorufin was monitored. In total, 0.5 μmol/L rotenone and 1.25 μg/mL antimycin A was added to inhibit the activity of complex I and complex III, respectively. Values are the means ± SEM of 3–5 independent measurements. For controls, comparison between hydrogen peroxide release in the presence of succinate, succinate + rotenone, or succinate + rotenone + antimycin A were evaluated by *t*-tests for dependent samples (***$p < 0.001$). Comparisons between control and samples with drug added were performed using ANOVA and post-hoc Dunnett test, which did not find significant changes in the hydrogen peroxide release for any tested cognitive drug

piracetam on MAO activity may be different in various tissues/cells with different representations of MAO-A and MAO-B [44].

Hydrogen Peroxide Production

Respiratory complexes I and III are taken as the source of the most ROS in mitochondria, and ROS generation and release can be regulated by the choice of substrates and inhibitors. During complex I-linked respiration, ROS are produced from complexes I and III, whereas complex II-linked respiration leads to ROS production to some extent by complex III but also through reverse electron flow to complex I. Electron flow through the complexes can be inhibited selectively, e.g., by rotenone (complex I) and antimycin A (complex III), resulting in an alteration in the production of ROS [16].

We measured the drug's effect on the release of mitochondrial H_2O_2 in the presence of succinate + rotenone + antimycin A. Although such ROS production is non-physiological, it can be used to evaluate the protective effect of cognitives and nootropics on mitochondrial ROS production.

In the absence of a tested drug (in the control sample), succinate-dependent H_2O_2 generation was relatively low, and rotenone (complex I inhibitor) had no significant effect. In contrast, antimycin A (complex III inhibitor) significantly stimulated (approximately four-fold) H_2O_2 production in controls (Fig. 4). Because reverse electron transport was inhibited by rotenone and complex I substrates were not added, this antimycin-induced H_2O_2 production could be assigned to complex III.

H_2O_2 production in the presence of succinate + rotenone + antimycin A was not significantly different in control samples and samples with added cognitive enhancers. This is consistent with the finding that the complex II-linked mitochondrial oxygen consumption rate was not significantly altered by the same drugs. Our results confirmed that there are neither protective nor deteriorative effects of cognitive enhancers on succinate + rotenone + antimycin A-stimulated ROS production in mitochondria.

In conclusion, the different in vitro effects of piracetam, latrepirdine, 7-MEOTA, galantamine, donepezil, rivastigmine, and memantine on mitochondrial respiration, the enzymatic activity of CS and ETC complexes, H_2O_2 production, and MAO activity support the presumed hypothesis for the independent mode of the mitochondrial action of various cognitives and nootropics. We confirmed that the cognitives and nootropics utilized in this study have a relatively small direct effect on selected mitochondrial functions, even at high concentrations. The high drug concentration required for this direct mitochondrial effect indicates that non-receptor mechanisms play a role. The inhibitory effects of these drugs on MAO-A may participate in mitochondrial neuroprotection; however, they should be taken into account if there is a co-administration of drugs affecting monoaminergic transmission. Drug-induced changes in mitochondrial functions should be included in a panel of tests for newly synthetized drugs in order to rule out their mitochondrial toxicity and have a better understanding of their mechanisms of action.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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