Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 67 (2004) 1965–1974
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Dopamine toxicity involves mitochondrial complex I inhibition: implications to dopamine-related neuropsychiatric disorders

D. Ben-Shachar*, R. Zuk, H. Gazawi, P. Ljubuncic

Research Lab of Psychobiology, Department of Psychiatry, Bruce Rappaport Faculty of Medicine, Rambam Medical Center, Technion ITT, P.O. Box 9649, Haifa, Israel

Received 20 November 2003; accepted 9 February 2004

Abstract

Dopamine, which is suggested as a prominent etiological factor in several neuropsychiatric disorders such as Parkinson's disease and schizophrenia, demonstrates neurotoxic properties. In such dopamine-related diseases mitochondrial dysfunction has been reported. Dopamine oxidized metabolites were shown to inhibit the mitochondrial respiratory system both in vivo and in vitro. In the present study, we suggest an additional mechanism for dopamine toxicity, which involves mitochondrial complex I inhibition by dopamine. In human neuroblastoma SH-SY5Y cells dopamine induced a reduction in ATP concentrations, which was negatively correlated to intracellular dopamine levels ($r = -0.96$, $P = 0.012$), and was already evident at non-toxic dopamine doses. In disrupted mitochondria dopamine inhibited complex I activity with $IC_{50} = 11.87 \pm 1.45$ μM or 8.12 ± 0.75 μM in the presence of CoQ or ferricyanide, respectively, with no effect on complexes IV and V activities. The catechol moiety, but not the amine group, of dopamine is essential for complex I inhibition, as is indicated by comparing the inhibitory potential of functionally and structurally dopamine-related compounds. In line with the latter is the finding that chelatable $FeCl_2$ prevented dopamine-induced inhibition of complex I. Monoamine oxidase A and B inhibitors, as well as the antioxidant butylated hydroxytoluene (BHT), did not prevent dopamine-induced inhibition, suggesting that dopamine oxidation was not involved in this process. The present study suggests that dopamine toxicity involves, or is initiated by, its interaction with the mitochondrial oxidative phosphorylation system. We further hypothesize that this interaction between dopamine and mitochondria is associated with mitochondrial dysfunction observed in dopamine-related neuropsychiatric disorders, such as schizophrenia and Parkinson's disease.

Keywords: Dopamine; Brain mitochondria; Complex I; Neurotoxicity; Parkinson's disease; Schizophrenia

1. Introduction

Dopamine plays a key role in the pathophysiology of several psychiatric and neurological disorders such as schizophrenia, tardive dyskinesia, Parkinson's disease, and Tourette's syndrome. At physiological concentrations dopamine does not induce degenerative processes in brain, and has been shown to activate the expression of cell survival genes and proteins [1]. In contrast, at higher, pathological concentrations numerous studies have shown that dopamine can cause cell death both in vivo and in cell cultures. Thus, administration of dopamine into the striatum [2] results in pre- and postsynaptic damage, while intraventricular injection of dopamine to rats resulted in a dose-dependent death of the animal [3]. Further, toxins which can cause extensive release of dopamine, such as methamphetamine and 1-methyl-4 phenyl-1,2,3,6-tetra hydropyridine (MPTP), also cause degeneration of dopaminergic neurons [4–7]. Dopamine-dependent cell death has been demonstrated in mesencephalic, cerebellar, striatal, and cortical primary neurons cultures [8–12]. It is widely believed that dopamine can induce neurotoxic effects by the formation of highly reactive oxygen species, quinones and semiquinones generated by dopamine auto-oxidation or by its enzymatic metabolism by monoamine oxidase (MAO) leading to a state of oxidative stress [2,10,13–17]. However, elevation of extracellular dopamine concentrations in rat brain in the absence of a neurotoxin did not induce any change in oxidative stress parameters such as lipid peroxidation, protein oxidation, glutathione, and glutathione reductase and peroxidase

* Corresponding author. Tel.: +972-4-8295224; fax: +972-4-8510941.
E-mail address: shachar@tx.technion.ac.il (D. Ben-Shachar).

0006-2952/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2004.02.015

activities [3,18]. An additional mechanism through which dopamine has been suggested to induce neurotoxicity involves apoptotic process [19–23].

Defects in mitochondrial function and inability to maintain cellular ATP levels have been suggested as a possible cause for slow neurodegeneration and aberrations in neuronal diseases such as Parkinson's and Alzheimer's diseases, schizophrenia, bipolar disorder, and aging [24–32]. Accumulating evidence points to a link between dopamine toxicity and the mitochondrial respiratory system. Thus, chronic administration of L-3,4-dihydroxy-phenylalanine (L-DOPA), which was associated with elevated dopamine concentrations in the brain, caused a significant reduction in the activity of complex I of the mitochondrial respiratory chain in rat brain [33]. Additionally, a significant decrease in striatal ATP concentrations was detected after D-methamphetamine or MPTP treatment, which was associated with dopamine-derived neurotoxic effects [34,35]. In intact rat brain mitochondria, dopamine suppressed pyruvate- and succinate-dependent electron transport [36], and in disrupted rat brain mitochondria dopamine inhibited NADH ferricynide reductase activity [3]. Interestingly, both neurotoxins 6-hydroxydopamine and MPP^+ are inhibitors of complex I activity both in vivo and in vitro [37–41]. In addition, dopamine was found to inhibit mitochondrial ATP coupled state 3 respiration, albeit less efficiently than its oxidized species, which led the authors to conclude that dopamine oxidation is a perquisite for the inhibition of mitochondrial respiration [42]. The present paper shows that dopamine-induced cell death is preceded by a reduction of ATP levels and suggests complex I inhibition as a possible additional mechanism by which dopamine impinges on the mitochondrial oxidative phosphorylation system (OXPHOS). The characteristics and specificity of dopamine interaction with mitochondrial complex I is demonstrated as well.

2. Methods

2.1. Cellular ATP and dopamine content

Cellular ATP content was analyzed in human neuroblastoma SH-SY5Y cells (passage 40–43). SH-SY5Y with a neuronal phenotype is a subclone of the SK-N-SH cell line that was established from bone marrow aspirate of a 4-year-old girl with highly malignant tumor. 0.5×10^6 cells were plated in 8 cm Nunc plates and were grown in DMEM with 10% fetal calf serum at 37 °C in a 5% CO_2 humidified incubator. Three days later the medium was replaced with serum-free DMEM for additional 18–20 h. Prior to treatment with dopamine, norepinephrine, or serotonin (2×10^{-7} M to 5×10^{-4} M) for 6 or 18 h, cells were pre-incubated with 0.1 mM pargyline for 30 min. ATP content in cells was measured by a luminometer (Lucy 1, Anthos Labtec, Austria) using the high sensitive Bioluminescent Somatic Cell assay kit from Sigma Chemical Company (St. Louis, MO, USA). Intracellular dopamine content was analyzed following three times wash of cells with an excess of ice-cold PBS. Cells were then dissolved in 100 μl of 0.1 M $HClO_4$ and their intracellular dopamine content was detected by HPLC as described previously [43].

2.2. Preparation of synaptosome-free mitochondria

Brain mitochondria were isolated from male Sprague–Dawley rats (300–350 g) as described previously [3,44]. In short, rats were sacrificed by decapitation, their brains rapidly removed, washed, minced, and homogenized by Dounce homogenizer in ice-cold 10 mM Tris–HCl buffer (pH 7.4) containing 0.25 M sucrose, 1 mM EDTA, and defatted BSA (3% w/v). Single brain homogenates were brought to 25 ml and centrifuged at $2000 \times g$ for 10 min (Sorval RT 6000B centrifuge Sorval, Products L.P. Newtown, Connecticut, USA). The supernatant was collected and centrifuged at $12{,}000 \times g$ for 10 min (Sorval RC5 Plus centrifuge, Sorval, Products L.P. Newtown). The pellet was gently suspended and recentrifuged with the same buffer lacking EDTA and BSA. The pellet was further purified on discontinuous Percoll gradient to remove synaptosomes [3,44]. The mitochondria containing layer was diluted 1:10 in isolation buffer and recentrifuged at $12{,}000 \times g$ for 10 min. The final pellet was stored at −80 °C until use. Mitochondrial protein yields were 8–12 mg per rat brain. Mitochondrial preparation purity was assessed by electron microscopy which showed that 95–98% of the preparation consisted of mitochondria with an intact outer membrane, as well as by cytochrome *c* oxidase activity which was 4.58 ± 0.51 μmol/mg protein/min, similar to its previously reported activity in non-synaptosomal mitochondria isolated from rat brain [44]. All animal procedures were approved by the Technion Animal Care and Use Committee.

2.3. Mitochondrial enzymes activity

NADH CoQ reductase activity was assayed in disrupted mitochondria at 25 °C in 20 mM potassium phosphate buffer (pH 7.2) containing 5 mM $MgCl_2$, 1 mM KCN, 0.14 mM NADH, and 50 μM of CoQ_1 (ubiquinone 5; 2,3-dimethoxy-5-methyl-6-[3-methyl-2-butenyl]-1,4-benzoquinone) in the presence or absence of rotenone as described previously [45–48]. The decrease in NADH absorbance was followed at 340 nm for 1 min with a 3-s interval between successive readings and 1-s initial delay.

NADH ferricyanide reductase activity was determined at V_{max} ferricyanide throughout the study, based on Ref. [49] with several modifications. Kinetic analysis was performed in disrupted mitochondria at 25 °C in

50 mM Tris–HCl buffer (pH 7.4) containing 0.25 M sucrose. The reaction was started by the addition of 0.1 mM potassium ferricyanide and 0.14 mM NADH. The decrease in NADH absorbance was followed at 340 nm for 1 min with a 3-s interval between successive readings and 1-s initial delay.

NADH-cytochrome c reductase (*complexes I–III*) was assayed at 25 °C by following the absorbance of NADH at 340 nm in the presence or absence of rotenone. The assay mixture contained 20 mM potassium phosphate buffer (pH 7.4), 5 mM $MgCl_2$, 1 mM KCN, 0.14 mM NADH, and 10 mg/ml cytochrome *c* (Sigma type III) [46].

Cytochrome c oxidase was assayed by following the decrease in absorbance of reduced cytochrome *c* (final concentration 0.12 mg/ml) at 550 nm as described previously [50].

ATPase activity was assayed in intact mitochondria (3 mg protein/0.5 ml) which were pre-incubated at 37 °C for 5 min in 10 mM Tris–HCl buffer (pH 7.2) containing 125 mM KCl, 5 mM $MgCl_2$, and 5 μM rotenone in the presence or absence of 10 μg/ml oligomycin [51]. The reaction was started by addition of 4 mM ATP and stopped after 5 min by the addition of 0.5 ml 1 M $HClO_4$. ATPase activity was determined by the measurement of P_i released using Fiske–Subbarow reducer [52].

ATP synthase activity was assayed in intact mitochondria (5 mg protein/0.5 ml) which were pre-incubated at 37 °C for 5 min in 10 mM potassium phosphate buffer (pH 7.2) containing 125 mM KCl, 5 mM $MgCl_2$, 5 mM succinate, and 5 μM rotenone in the presence or absence of 10 μg/ml oligomycin [53]. The reaction was initiated by the addition of 4 mM ADP and stopped after 5 min with 0.5 ml 1 M $HClO_4$. ATP was assayed using a commercial kit purchased from Sigma Diagnostic (USA).

Complex I activity using either ferricyanide or CoQ as electron acceptors was determined as described above in the presence or absence of dopamine (10^{-6} to 2×10^{-5} M) and related compounds (10^{-6} to 5×10^{-4} M), which were added exactly 1 min before the start of the reaction to enable 1 min of pre-incubation. At least six experiments were done with each compound in triplicates for each concentration.

2.4. Cell viability and protein concentration

Cell viability was determined by trypan blue. Protein concentration was determined by Bradford reactions.

2.5. Statistical analysis

Analysis of data was performed by SPSS software version 11.0. Multiple comparisons were performed by ANOVA followed by Bonferoni post hoc multiple comparisons test. A two-tailed Student's *t*-test was used when appropriate. Linear regression analysis was used to estimate the relationship between intracellular concentrations of dopamine and ATP. For dose-dependent inhibition, the best fit calculated curve was drawn.

All drugs were purchased from Sigma Chemical Company. Mediums for tissue culture were purchased from Biological Industries Beit Haemek Ltd. (Israel). All other materials were of the highest purity and were purchased from standard commercial sources.

3. Results

3.1. Dopamine and cellular ATP levels

Dopamine effect on mitochondrial function in living cells was studied by measuring intracellular ATP levels in SH-SY5Y cells incubated for 6 or 18 h in the presence of 2×10^{-7} M to 5×10^{-4} M dopamine. SH-SY5Y cells took up dopamine, which was blocked by cocaine. Thus, basal intracellular dopamine levels measured by HPLC were 0.57 ± 0.08 pmol/10^6 cells and increased to 2.40 ± 0.33 pmol/10^6 cells ($P < 0.001$) in the presence of 200 nM dopamine. Cocaine (0.1 mM) had no significant effect on basal dopamine cell content (0.63 ± 0.04 pmol/10^6 cells), but completely blocked dopamine uptake (0.45 ± 0.12 pmol/10^6 cells) in the presence of 200 nM dopamine. Exposure of SH-SY5Y cells to various concentrations of dopamine (2×10^{-7} M to 5×10^{-4} M) for 18 h, but not for 6 h, in the presence of 0.1 mM pargyline resulted in a dose-dependent decrease in intracellular ATP levels in viable cells (Fig. 1A). Moreover, a significant ($r = -0.96$, $P = 0.012$) inverse correlation was found between intracellular dopamine levels, as detected by HPLC, and ATP content (Fig. 1B). Extracellular dopamine caused cell death only at concentrations $\geq 2 \times 10^{-4}$ M (Fig. 2), whereas a significant ($P < 0.05$) reduction in ATP intracellular levels was already observed at 5×10^{-5} M of dopamine. Norepinephrine and serotonin at the same concentrations had no effect on cell viability nor did they affect intracellular ATP concentrations in viable cells (Fig. 1A).

3.2. Effects of dopamine on mitochondrial NADH dehydrogenase activity

Dopamine inhibited NADH oxidation in the presence of the electron acceptor CoQ with an IC_{50} of 11.87 ± 1.45 μM (Fig. 3). NADH CoQ reductase activity was highly sensitive to rotenone inhibition, with a residual activity of less than 5%. In the presence of the artificial electron acceptor ferricyanide, dopamine inhibited the oxidation of NADH with an IC_{50} of 8.12 ± 0.75 μM (Fig. 4) in accordance with our previous findings [3]. Dopamine had no effect on NADH ferricyanide reductase activity when the reaction began with the addition of the enzyme without a pre-incubation period. In addition, dopamine inhibited the rotenone sensitive NADH-cytochrome *c*

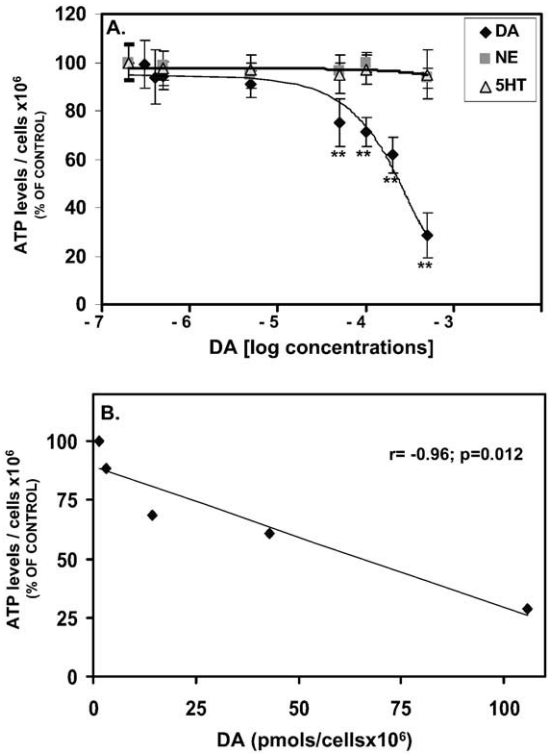

Fig. 1. Dopamine-induced dose-dependent decrease in ATP levels in viable human neuroblastoma SH-SY5Y cells. (A) Cellular ATP levels. (B) The correlation curve between intracellular concentrations of dopamine analyzed by HPLC, and ATP analyzed by luminometer, in viable SH-SY5Y cells exposed for 18 h to different concentrations of dopamine (2×10^{-7} to 5×10^{-4}). ATP intracellular data are means ± S.E.M. of duplicates from five experiments. $^{*}P < 0.05$, $^{**}P < 0.001$ vs. control. Correlation was determined by linear regression of the means of five experiments measured in duplicates of dopamine and ATP intracellular levels.

reductase (complexes I–III) activity in the presence of cytochrome *c* as the electron acceptor, in a dose-dependent manner (Table 1). Complex I activity in mitochondria isolated from rat liver or heart was inhibited by dopamine to the same extent as in brain mitochondria (data not shown).

Table 1
Dopamine-induced inhibition of NADH-cytochrome *c* reductase activity

Inhibition (%)	Dopamine (μM)
37.5 ± 3.5	10
75.1 ± 6.4	20
87.6 ± 13.1	30

NADH-cytochrome *c* reductase activity was measured in the presence and absence of various concentrations of dopamine. Dopamine significantly ($P < 0.001$) inhibited complexes I–III activity in all used concentrations. The control activity (100%) was 10.47 ± 0.38 nmol/mg protein/min. Data are means ± S.E.M. values of six determinations.

To study the possibility that inhibition of complex I activity by dopamine results from lack of substrate, electron acceptor, or from product inhibition, 0.14 mM NADH and/or 0.1 mM ferricyanide, as well as of 0.14 mM NAD^{+}, were added at different time intervals after the beginning of the reaction. None of these drugs could prevent dopamine-induced inhibition of complex I activity. MAO inhibitors such as tranylcypromine, clorgyline, and pargyline also had no effect on dopamine-induced inhibition of the enzyme. Throughout the entire process pH was stable.

Dopamine is an active catechol, which can be auto-oxidized to toxic oxygen species including semiquinones and quinones [13,15,17], which can cause an inhibition of NADH oxidation and reduction in mitochondrial membrane potential. Thus, we examined the effects of the strong antioxidant butylated hydroxytoluene (BHT) on NADH oxidation in the presence and absence of dopamine. BHT at concentrations of 10^{-5} to 10^{-3} M had no effect on NADH dehydrogenase basal activity, nor did it affect dopamine-induced inhibition of the enzyme activity. In

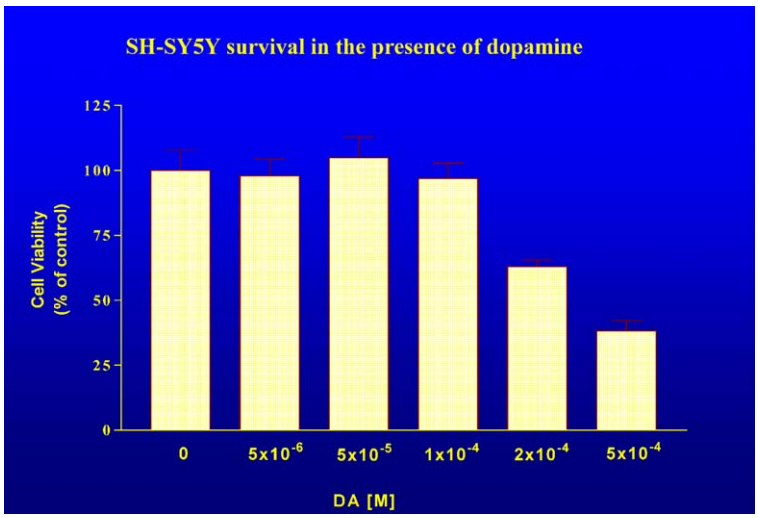

Fig. 2. Dopamine-induced dose-dependent cell death in human neuroblastoma SH-SY5Y cells. Cells were pre-incubated for 30 min with 0.1 mM pargyline before the addition of dopamine at different concentrations (5×10^{-6} to 5×10^{-4}). After 18 h their viability was determined using trypan blue. Data are means ± S.E.M. of six experiments. The significance of the difference ($P < 0.0001$) was analyzed by one-way ANOVA followed by pot hoc Bonferroni test. $^{*}P < 0.001$ vs. control.

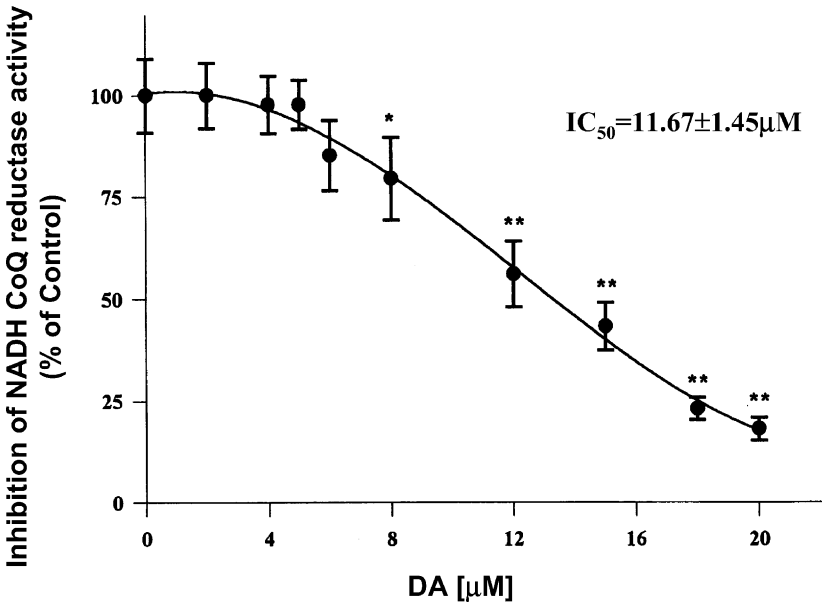

Fig. 3. Dopamine-dependent inhibition of NADH CoQ reductase activity in mitochondrial preparation. Disrupted mitochondrial preparation (0.5 mg protein/ml) were pre-incubated with various concentrations of dopamine for 1 min before the start of the reaction. The best fitted calculated trend line is depicted. Each point represents the means ± S.E.M. of six experiments. $^{*}P < 0.05$, $^{**}P < 0.001$ vs. control.

line with the latter is the finding that $FeCl_2$ prevented dopamine-induced inhibition in a dose-dependent manner (Fig. 5).

3.3. Effects of dopamine-related compounds on mitochondrial NADH ferricyanide reductase activity

The interaction between NADH ferricyanide reductase and other biogenic amines, dopamine precursors and its metabolite, a bi-catechol and ethylamine (10^{-6} M to 5×10^{-4} M) was studied. The calculated IC_{50} values of each compound are represented in Table 2. Inhibition of complex I activity was not specific for dopamine, since noradrenaline, a less reactive catecholamine [54], the dopamine precursor and metabolite L-3,4-dihydroxyphenylalanine (L-DOPA) and 3,4-dihydroxyphenylacetic acid (DOPAC), respectively, as well as the specific toxin for catecholaminergic neurons, 6-hydroxydopamine (6-OHDA), all inhibited the enzyme, although not as efficiently as dopamine. Interestingly, the bi-catechol nordihydroguaiaretic acid (NDGA) also inhibited NADH dehydrogenase activity, while triethylamine and ethylamine had no effect. Serotonin (5-HT) as well as L-tyrosine did not inhibit NADH dehydrogenase activity.

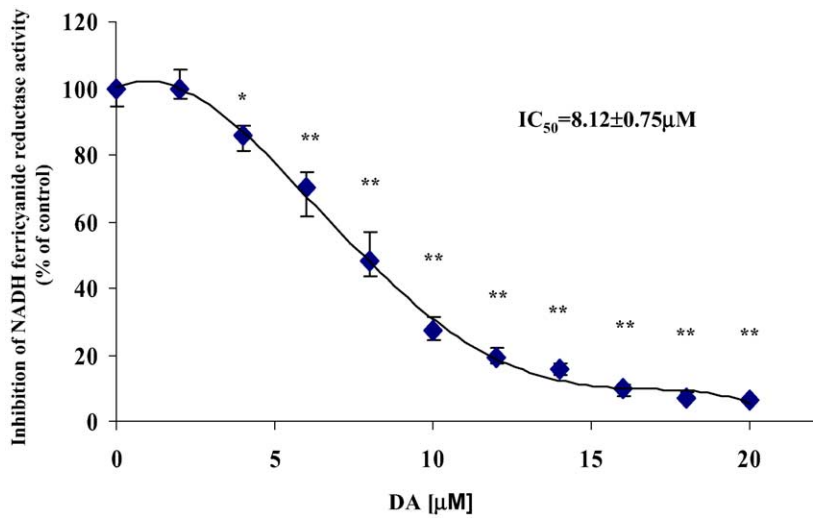

Fig. 4. Dopamine-dependent inhibition of NADH ferricyanide reductase activity in mitochondrial preparation. Disrupted mitochondrial preparation (0.25 mg protein/ml) were pre-incubated with various concentrations of dopamine for 1 min before the start of the reaction and enzyme activity was determined at V_{max} ferricyanide as described in Section 2. The best fitted calculated trend line is depicted. Each point represents the means ± S.E.M. of five experiments. $^{*}P < 0.01$, $^{**}P < 0.001$ vs. control.

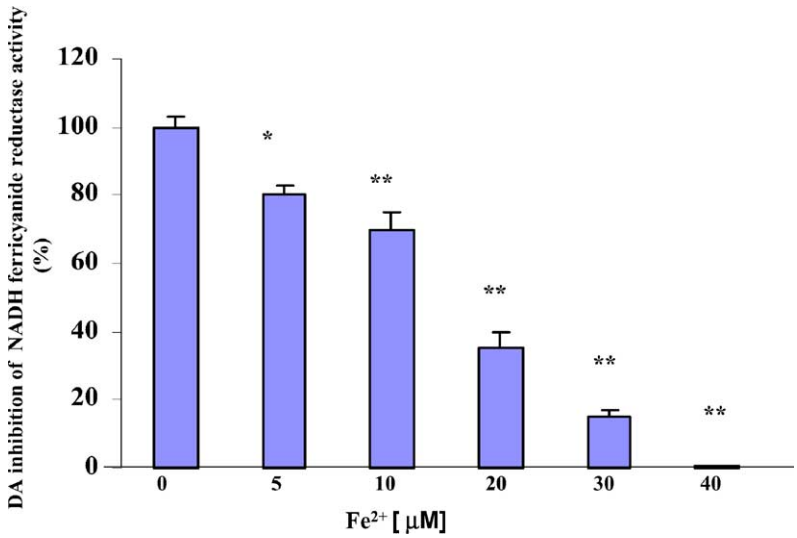

Fig. 5. Prevention of dopamine-dependent inhibition of NADH ferricyanide reductase activity by $FeCl_2$. Dopamine 5 μM and Fe^{2+} (5–40 μM) were added 1 min before the start of the reaction and enzyme activity was determined at V_{max} ferricyanide as described in Section 2. Five micromoles of dopamine inhibited complex I activity by about 30 ± 5.5%. Each point represents the mean ± S.E.M. of four experiments. $^{*}P < 0.02$, $^{**}P < 0.001$ vs. control.

Table 2
Inhibition NADH dehydrogenase activity in mitochondrial preparation by dopamine and related compounds

IC_{50} (μM)	Drug
8.12 ± 0.75	Dopamine
15.56 ± 1.40	Noradrenaline
10.42 ± 0.80	L-DOPA
24.64 ± 3.10	6-OHDA
61.20 ± 8.08	DOPAC
50.60 ± 2.50	NDGA
>200	5-HT
>200	L-Tyrosine
>200	Triethylamine
>200	Ethylamine

NADH ferricyanide reductase activity was followed at V_{max} ferricyanide as described in the Section 2. IC_{50} was calculated from the enzyme activity in the presence of 10 various doses (10^{-6} M to 5×10^{-4} M) of the various drugs. Data are means ± S.E.M. of five to seven experiments.

3.4. Effects of dopamine on ATPase, ATP synthase, and cytochrome c oxidase activities

The activities of these enzymes were studied in order to verify whether dopamine inhibitory effect is specific for complex I or is a rather non-specific effect on the mitochondrial respiratory system. None of these enzymes were affected even at relatively high concentrations of dopamine (2×10^{-6} M to 10^{-4} M), or noradrenaline (8×10^{-6} M to 4×10^{-4} M).

4. Discussion

The key role dopamine plays in the pathophysiology of neuronal as well as psychiatric disorders, suggests that dopamine is associated with neuronal injury. Indeed, numerous studies have demonstrated its neurodegenerative and apoptotic properties both in vivo and in tissue cultures [1,2,8,10–12,13,15,23,54,55]. Dopamine neurotoxicity is commonly attributed to the accumulation of toxic oxygen species following its metabolism by MAO or auto-oxidation. In addition, several studies have suggested that dopamine or its oxidized metabolites can induce an increase in the expression of mitochondrial pro-apoptotic genes, as well as interfere with mitochondrial respiration [1,3,33,36,42]. Concomitant with the latter, the results of the present study demonstrate a dose-dependent decrease in ATP production negatively correlated with an intracellular increase in dopamine levels in SH-SY5Y neuroblastoma cells ($r = -0.96$, $P = 0.012$). Norepinephrine and serotonin had no effect on ATP levels or cell viability. ATP reduction was observed in the presence of MAO inhibitor thus excluding the involvement of dopamine oxidized enzymatic metabolites, but not of its auto-oxidation derived metabolites. Still, the high negative correlation between intracellular intact dopamine, as detected by HPLC, and ATP levels, suggests that dopamine per se can interfere with mitochondrial function. Moreover, the reduction in intracellular ATP levels was already observed at non-toxic dopamine doses, suggesting that impairment of mitochondrial main function, ATP production, precedes dopamine-induced cell death. Indeed, it has been shown that a decrease in ATP production can lead to neuronal depolarization followed by activation of excitatory amino acids receptors, as well as other potentially detrimental effects, including impaired intracellular calcium buffering, generation of reactive oxygen species and apoptosis [56–59]. It was previously shown that dopamine at 100 μM affects cell viability after 4 h of exposure [60], while at the present study cell death was observed following exposure of cells to 200 μM for 18 h. However, in the latter study, unlike the

present study, cells were treated with dopamine in the presence of fetal calf serum and in the absence of pargyline, both inducers of dopamine derived oxidative species. This, together with the ability of antioxidants such as ascorbic acid, to prevent dopamine toxicity, imply that oxidative stress-related mechanisms were involved in their study. In addition, the discrepancy between both studies further suggests that different dopamine-related mechanisms are involved in ATP reduction observed in the present study. In a previous study [12], it was reported that in striatal cultures exposed to 250 μM dopamine for 6 h, ATP/ADP ratios were unchanged. Similar results were obtained in the present study following a 6-h exposure period of cells to dopamine. In addition, striatal cells in the former study were unable to take up dopamine, as indicated by their insensitivity to nomifensine, suggesting the involvement of an extracellular mechanism of dopamine-induced toxicity. Finally, the results of both studies support the previous notion that dopamine may exert its toxicity via either an extraneuronal (transport-independent) or an intraneuronal (transport-dependent) process [12,21,61,62].

To elucidate the mechanism by which dopamine affects mitochondrial ATP levels, we studied its direct interaction with the oxidative phosphorylation system. Dopamine inhibited complex I activity in the presence of CoQ, ferricyanide, or cytochrome *c* (complexes I–III) as an electron acceptor in a dose-dependent manner. Dopamine did not alter ATPase, ATP synthase, and cytochrome *c* oxidase activities, which points to the specificity of its interaction with complex I.

Previously, it has been reported that dopamine and its metabolites showed either no inhibition or only a weak inhibition of NADH-linked mitochondrial respiration and complex I activity [42,63]. Indeed, numerous studies have suggested that dopamine oxidative products, are involved in dopamine neurotoxicity as well as in MPP^+-induced toxicity [2,7,8,10–13,15,17,42,54,55,64]. In the present study, the dopamine inhibitory effect was contingent on pre-incubation, in as much as no inhibition of complex I was observed upon addition of the enzyme as the last component of the reaction. This could suggest that dopamine is either metabolized or oxidized before being able to inhibit complex I. However, MAO inhibitors as well as the strong antioxidant BHT, which in itself has no effect on complex I activity, did not prevent dopamine inhibition of complex I, indicating that hydrogen peroxide, superoxide, and lipid-derived radicals do not participate in complex I inactivation. Interestingly, it was previously reported that following 1-h pre-incubation, dopamine suppressed pyruvate- and succinate-dependent electron transport in intact rat brain mitochondria [36]. The latter, together with the in vivo findings demonstrating a significant inhibition of complex I by dopamine and L-DOPA [33], which was not associated with altered oxidative stress parameters [3,18], as well as the finding that the highly reactive 6-OHDA-induced inhibition of complex I in vivo and in mitochondrial preparations [40,41], are in accordance with the present data.

An alternative explanation to the indirect effect of dopamine on complex I is a direct interaction between both. As previously mentioned, a dopamine inhibitory effect was detected in the presence of ferricyanide, which accepts electrons from Fe–S cluster 1 [65,66]. This suggests that dopamine might interact with the enzyme at a site that lies between the binding site for NADH and the iron–sulphur cluster N1, before the more hydrophobic part of the complex, which includes the sites for rotenone, piericidin A and MPP^+ [67–69]. The hydrophilic characteristics of all compounds which were tested in the present study and altered complex I activity, supports the suggestion that the interaction site is located on the peripheral part of the enzyme. Thus, an interaction between dopamine and the low potential Fe–S cluster could explain the inhibition of NADH oxidation. Indeed, it was reported that NADH dehydrogenase activity was inhibited by iron chelators probably by displacing the labile sulfur of the Fe–S cluster and chelating the iron [70]. Catechols are known iron chelators. Thus, being a catechol, dopamine could chelate iron and thereby inhibit the enzyme. In this connection, it is notable that addition of Fe^{2+} prevented the $30 \pm 5.5\%$ inhibition of complex I activity induced by 5 μM dopamine in a dose-dependent manner, with complete inhibition at 40 μM of Fe^{2+}. The effect of other compounds, which relate to dopamine either by structure or by function, further supports the aforementioned hypothesis, in as much as the catechol, and not the ethylamine, moiety of dopamine is essential for affecting complex I. Thus, noradrenaline, L-DOPA, DOPAC, 6-OHDA, and nordihydroguaiaretic acid, the latter having two catechol rings combined by dimethylbutane, inhibited the enzyme, while ethylamine and triethylamine as well as serotonin, which has an indole group, and the dopamine precursor L-tyrosine, had no effect on NADH dehydrogenase. Although conclusive evidence for a direct interaction between dopamine and Fe–S cluster calls for studies using methods such as electron spin resonance (ESR), the possibility of a direct interaction between dopamine and complex I is in line with the protective effect of Fe^{2+}, the brief duration of the pre-incubation period, as well as the lack of effect of the antioxidant and MAO inhibitors.

MPP^+ neurotoxicity was shown to be a multi-component process involving both mitochondrial dysfunction by inhibition of complex I activity, and ROS generated by vesicular dopamine displacement [7]. The result of the present study together with previous reports which implicate the involvement of highly reactive oxygen species in dopamine inhibition of mitochondrial respiration, suggest that parallel processes, i.e. ROS production alongside inhibition of complex I activity, can be implied for dopamine-induced neurotoxicity. Furthermore, the findings that ATP production preceded DA-induced cell death, which

was previously associated with ROS formation and apoptosis, as well as the important role of mitochondria in the latter processes, may suggest that impairment of mitochondrial OXPHOS precedes DA-induced ROS formation and apoptosis.

The present study shows that exposure of SH-SY5Y cells to both serotonin and norepinephrine had no effect on intracellular ATP levels. While serotonin has not been shown to be taken up by these cells and did not inhibit mitochondrial complex I activity, norepinephrine was shown to be taken up by these cells [71], and to inhibit complex I activity, although less efficient than dopamine. The latter is in line with norepinephrine less potent chelation capability as compare to dopamine, and could be associated with its inability to reduce intracellular ATP levels. Notably, however, is the recently reported finding that the noradrenergic locus coeruleus is the major site of subcortical neuronal loss in both Parkinson and Alzheimer diseases [72], suggesting that both catechols are involved in neurodegenerative processes. However, the lack of effect of norepinephrine on intracellular ATP levels in cell cultures, as well as the Parkinson disease specific dopaminergic cell loss in the substantia nigra par compacta, in contrary to the disease non-specific cell loss in the locus coeruleus, may suggest that despite sharing some similarities, dopamine and norepinephrine neurotoxic effects are induced via different mechanisms.

Finally, the results of the present study suggest that an interaction between dopamine and mitochondrial complex I may be an early step in the process involved in dopamine toxicity and thus may be of relevance to the pathophysiology of dopamine-related neurological and psychiatric disorders such as Parkinson's disease and schizophrenia. In line with the latter are the findings of altered complex I activity both in periphery and in brain of patients with Parkinson's disease and schizophrenia, in which dopamine abnormal transmission is a prominent etiological factor [28,32,73–77]. Thus, complex I deficiency has been described in the substantia nigra, as well as in platelets, in sporadic Parkinson's disease. Further, evidence for the imperative role complex I plays in Parkinson's disease is the ability of its inhibitor MPTP to cause parkinsonism in human and in primates, which is remarkably similar, both clinically and pathologically, to idiopathic Parkinson's disease [37–39,78]. Likewise, the common pesticide rotenone, which is an inhibitor of complex I, induces anatomical, neurochemical, behavioral, and neuropathological features of Parkinson's disease in rodents [79]. The mechanism that underlies complex I deficient activity in Parkinson's disease is still unknown. Many studies have claimed that the increased turnover of dopamine reported in Parkinson's disease [80] leads to an extensive free radical generation from dopamine metabolism and/or auto-oxidation, with consequent complex I dysfunction. However, treatment of patients with MAO inhibitors such as deprenyl, or antioxidants, such as vitamin E, has not yet been proved as neuroprotective [81]. Conceivably, a direct inhibition of complex I activity by dopamine may be an additional alternative mechanism. Interestingly, in schizophrenia, which is associated with increased dopamine transmission, with no evidence for neurodegeneration due to free radicals formation, several independent lines of evidence indicate mitochondrial dysfunction in general, and complex I aberrations in particular, in brain and in platelets or lymphocytes (for review, see [29]). Complex I activity alterations in the periphery were shown to be disease state dependent, while in brain a decrease was observed in the prefrontal cortex, which is a brain area fundamental for the pathophysiology of schizophrenia, and is extensively innervated by the dopaminergic system [32,75,76]. In addition, the relevance of dopamine complex I interaction to the pathology of schizophrenia was exhibited by significantly higher susceptibility of complex I activity in mitochondria isolated from platelets of schizophrenic patients to dopamine inhibition as compared with the control subjects.

In conclusion, our findings suggests mitochondrial complex I as a potential target for dopamine, further highlighting the complexity and multitude of processes involved in dopamine neurotoxicity.

Acknowledgments

This study was supported by the Israel Ministry of Health Grant, the Rambam Medical Center Research and Development Fund, and the Center for Absorption in Science, Ministry of Immigrant Absorption, State of Israel.

References

[1] Weinreb O, Mandel S, Youdim MBH. cDNA gene expression profile homology of antioxidants and their antiapoptotic and proapoptotic activities in human neuroblastoma cells. FASEB J 2003;17:935–7.
[2] Fillox F, Townsend JJ. Pre- and postsynaptic neurotoxic effects of dopamine demonstrated by intrastriatal injection. Exp Neurol 1993; 119:79–88.
[3] Ben-Shachar D, Zuk R, Glinka Y. Dopamine neurotoxicity: inhibition of mitochondrial respiration. J Neurochem 1995;64:718–23.
[4] Sirinathsinghji DJ, Heavens RP, McBribde CS. Dopamine-releasing action of 1-methyl-4-phenyl-1,2,3,6-tetra-hydropyridine (MPTP) and 1-methyl-4-phenylpyridine (MPP+) in the neostriatum of the rat. Brain Res 1988;443:101–16.
[5] Chiueh CC, Krishna G, Tulsi P, Obata T, Lang K, Huang SJ, et al. Intracranial microdialysis of salicylic acid to detect hydroxyl radical generation through dopamine autooxidation in the caudate nucleus: effects of MPP+. Free Radic Biol Med 1992;13:581–3.
[6] O' Dell SJ, Weihhmuller FB, Marshall JF. Methamphetamine-induced dopamine terminals alteration by dopamine D1 and D2 antagonists. J Neurochem 1993;60:1792–9.
[7] Lotharius J, O'Malley KL. The parkinsonism-inducing drug 1-methyl-4-phenylpyridinium triggers intracellular dopamine oxidation. A novel mechanism of toxicity. J Biol Chem 2000;275:38581–8.
[8] Rosenberg PA. Catecholamine toxicity in cerebral cortex of dissociated cell culture. J Neurosci 1988;8:2887–94.

[9] Michell PP, Hefti F. Toxicity of 6-hydroxydopamine and dopamine for dopaminergic neurons in cell culture. J Neurosci Res 1990;26: 428–35.
[10] Tanaka M, Sotomatsu A, Kanai H, Hirai S. DOPA and dopamine cause cultured neuronal death in the presence of iron. J Neurochem 1991; 101:198–203.
[11] Mytilineou C, Han SK, Cohen G. Toxic and protective effects of L-DOPA on mesencephalic cell cultures. J Neurochem 1993;61: 1470–8.
[12] McLaughlin BA, Nelson D, Erecinska M, Chesselet MF. Toxicity of dopamine to striatal neurons in vitro and potentiation of cell death by a mitochondrial inhibitor. J Neurochem 1998;70:2406–15.
[13] Cohen G. Oxidative stress in the nervous system. In: Sies H, editor. Oxidative stress. Academic Press: London; 1985. p. 383–401.
[14] Founstedt B. Role of catechol autoxidation in the degeneration of dopamine neurons. Acta Neurol Scand 1990;129:12–4.
[15] Chiueh CC, Miyake H, Peng MT. Role of dopamine autoxidation, hydroxyl radical generation and calcium overload in underlying mechanisms involved in MPTP-induced parkinsonism. Adv Neurol 1993;60:251–8.
[16] Hastings TG, Zigmond MJ. Identification of catechol-protein conjugates in neostriatal slices incubated with [3H] dopamine: impact of ascorbic acid and glutathione. J Neurochem 1994;63:1126–32.
[17] Lee CS, Song EH, Park SY, Han ES. Combined effect of dopamine and MPP+ on membrane permeability in mitochondria and cell viability in PC12 cells. Neurochem Int 2003;43:147–54.
[18] Loeffler DA, DeMaggio AJ, Juneau PL, Havaich MK, LeWitt PA. Effects of enhanced dopamine turnover in vivo on glutathione oxidation. Clin Neuropharmacol 1994;17:370–9.
[19] Ziv I, Melamed E, Nardi N, Luria D, Achiron A, Offen D, et al. Dopamine induced apoptosis-like cell death in cultured chick sympathetic neurons—a possible novel pathogenetic mechanism in Parkinson's disease. Neurosci Lett 1994;170:136–40.
[20] Gabbay M, Tauber M, Porat S, Simantov R. Selective role of glutathione in protecting human neuronal cells from dopamine-induced apoptosis. Neuropharmacology 1996;35:571–8.
[21] Simantov R, Blinder E, Ratovitski T, Tauber M, Gabbay M, Porat S. Dopamine induced apoptosis in human neural cells: inhibition by nucleic acid antisense to the dopamine transporter. J Neurosci 1996; 74:39–50.
[22] Massenaro JM, Gong L, Kuage H, Baker I, Wyatt RJ. Dopamine induces apoptotic cell death of cathecolaninergic cell line derived from the central nervous system. Mol Pharmacol 1996;50:1309–15.
[23] Offen D, Ziv I, Panet H, Wasserman L, Stein R, Melamed E, et al. Dopamine-induced apoptosis is inhibited in PC12 cells expressing bcl-2. Cell Mol Neurobiol 1996;17:289–304.
[24] Beal MF. Does impairment of energy metabolism result in cytotoxic neuronal death in neurodegenerative illnesses? Ann Neurol 1992;31: 119–30.
[25] Beal MF. Aging energy and oxidative stress in neurodegenerative diseases. Ann Neurol 1995;38:357–66.
[26] Cavalier L, Jazin E, Eriksson I, Prince J, Bave B, Oreland L, et al. Decreased cytochrome *c* oxidase activity and lack age related accumulation of mtDNA in brain of schizophrenics. Genomics 1995;29: 217–28.
[27] Whatley SA, Curi D, Marchbanks RM. Mitochondrial involvement in schizophrenia and other functional psychoses. Neurochem Res 1996;21:995–1004.
[28] Ben-Shachar D, Zuk R, Gazawi H, Reshef A, Sheinkman A, Klein E. Increased mitochondrial complex I activity in platelets of schizophrenic patients. Int J Neuropsychopharmacol 1999;2:245–53.
[29] Ben-Shachar D. Mitochondrial dysfunction in schizophrenia: a possible linkage to dopamine. J Neurochem 2002;83:1241–51.
[30] Kingsbury AE, Cooper M, Schapira AH, Foster OJ. Metabolic enzyme expression in dopaminergic neurons in Parkinson's disease: an in situ hybridization study. Ann Neurol 2000;50:142–9.
[31] Kato T, Kato N. Mitochondrial dysfunction in bipolar disorder. Bipolar Disord 2000;2:180–90.
[32] Dror N, Karry R, Mazor M, Sheinkman A, Klein E, Ben-Shachar D. State dependent alterations in mitochondrial complex I activity in platelets: a potential peripheral marker for schizophrenia. Mol Psychiatry 2002;7:995–1001.
[33] Przedborski S, Jackson-Lewis V, Muthane U, Jiang H, Ferreria M, Naini AB, et al. Chronic levodopa administration alters cerebral mitochondrial respiratory chain activity. Ann Neurol 1993;34: 715–23.
[34] Chan P, Di Monte DA, Luo JJ, DeLanney LE, Irwin I, Langston JW. Rapid ATP loss caused by methamphetamine in the mouse striatum: relationship between energy impairment and dopaminergic neurotoxicity. J Neurochem 1994;62:2484–7.
[35] Royland JE, Delfani K, Langston JW, Janson AM, Di Monte DA. 7-Nitroindazole prevents 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced ATP loss in the mouse striatum. Brain Res 1999;839: 41–8.
[36] Cohen G, Farooqui R, Kesler N. Parkinson's Disease: a new link between monoamine oxidase and mitochondrial electron flow. Proc Natl Acad Sci USA 1997;94:4890–4.
[37] Ramsay RR, Salach JI, Singer TP. Uptake of the neurotoxin 1-methyl-4-phenylpyridine (MPP+) by mitochondria and its relation to the inhibition of the mitochondrial oxidation of NAD+-linked substrates by MPP+. Biochem Biophys Res Commun 1986;134:743–8.
[38] Nicklas WJ, Vyas I, Heikkila RE. Inhibition of NADH-linked oxidation in brain mitochondria by 1-methyl-4-phenyl-pyridine, a metabolite of the neurotoxin, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. Life Sci 1985;36:2503–8.
[39] Mizuno Y, Saitoh T, Sone N. Inhibition of mitochondrial NADH-ubiquinone oxidoreductase activity by 1-methyl-4-phenylpyridinium ion. Biochem Biophys Res Commun 1987;143:294–9.
[40] Glinka Y, Youdim MBH. Inhibition of mitochondrial complex I and IV by 6-hydroxydopamine. Eur J Pharmacol 1995;292:329–32.
[41] Dabbeni-Sala F, Di Santo S, Franceschini D, Skaper SD, Giusti P. Melatonin protects against 6-OHDA-induced neurotoxicity in rats: a role for mitochondrial complex I activity. FASEB J 2001;15:164–70.
[42] Berman SB, Hatings TG. Dopamine oxidation alters mitochondrial respiration and induces transition in brain mitochondria: Implications for Parkinson's disease. J Neurochem 1999;73:1127–37.
[43] Ben-Shachar D, Klein E, Tabak A, Finberg JPM. Effect of single and repeated ministration of fluvoxamine on noradrenaline release in rat brain. Eur J Pharmacol 1997;332:237–42.
[44] Sims NR. Rapid isolation of metabolically active mitochondria from rat brain and subregions using percoll density gradient centrifugation. J Neurochem 1990;55:698–707.
[45] Ragan CI, Wilson MT, Darley-Usmar VM, Lowe PN. Subfractionation of mitochondria and isolation of the proteins of oxidative phosphorylation. In: Darely-Usmar VM, Rickwood D, Wilson M, editors. Mitochondria, a practical approach. London: IRL Press; 1987. p. 79–112.
[46] Hatefi Y. Preparation and properties of the enzyme complexes of the mitochondrial oxidative phosphorylation system. Methods Enzymol 1978;53:3–10.
[47] Krige D, Carroll MT, Cooper JM, Maesden CD, Schapira AHV. Platelet mitochondrial function in Parkinson's disease. Ann Neurol 1992;32:782–8.
[48] Estornell E, Fato R, Pallotti F, Lenaz G. Assay conditions for the mitochondrial NADH:coenzyme Q oxidoreductase. FEBS 1993;332: 127–30.
[49] Singer TP. Determination of the activity of succinate, NADH, choline and glycerophosphate dehydrogenases. In: Glick E, editor. Methods of biochemical analysis, vol. 22. NY: Inter. Science; 1974. p. 123–175.
[50] Laclau MJ, Rigoulet M, Averet N, Leverve X, Dubourg L, Carbonneau A, et al. Relationship between age-dependent changes in the effects of

almitrine on H^+-ATPase/ATPsynthase and the pattern of membrane fatty acid composition. Biochim Biophys Acta 1993;1141:90–4.
[51] Storrie B, Madden EA. Isolation of subcellular organelles. Methods Enzymol 1990;182:203–25.
[52] Fiske CH, Subbarow YJ. The calorimetric determination of phosphorus. Biol Chem 1925;66:375–81.
[53] Nagamune H, Fukushima Y, Takada J, Yoshida K, Unami A, Shimooka T, et al. The lipophilic weak base Au-1421 is a potent protonophore type cationic uncoupler of oxidative phosphorylation in mitochondria. Biochim Biophys Acta 1993;1141:231–7.
[54] Graham DG. Catecholamine toxicity: a proposal for molecular pathogenesis of manganese neurotoxicity in Parkinson's disease. Neurotoxicology 1984;5:38–96.
[55] Ben-Shachar D, Riederer P, Youdim MBH. Iron melanin interaction and lipid peroxidation: implication for Parkinson's disease. J Neurochem 1991;57:1609–14.
[56] Simpson PB, Russell JT. Role of mitochondrial Ca2+ regulation in neuronal and glial cell signaling. Brain Res Rev 1997;26:72–81.
[57] Babcock DF, Hill B. Mitochondrial oversight of cellular Ca2+ signaling. Curr Opin Neurobiol 1998;8:398–404.
[58] Green DR, Reed JC. Mitochondria and apoptosis. Science 1998; 281:1309–12.
[59] Wallace DC. Mitochondrial diseases in man and mouse. Science 1999;283:1482–8.
[60] Gomez-Santos C, Ferrer I, Santidrian AF, Barrachina M, Gil J, Ambrosio S. Dopamine induces autophagic cell death and alpha-synuclein increase in human neuroblastoma SH-SY5Y cells. J Neurosci Res 2003;73:41–50.
[61] Bloom FE, Algeria S, Groppetti A, Revuelta A, Costa E. Lesions of central norepinephrine terminals with 6-OH-dopamine: biochemistry and fine structure. Science 1969;166(910):1284–6.
[62] Javitch JA, D'Amato RJ, Strittmatter SM, Snyder SH. Parkinsonism-inducing neurotoxin, *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine: uptake of the metabolite *N*-methyl-4-phenylpyridine by dopamine neurons explains selective toxicity. Proc Natl Acad Sci USA 1985; 82:2173–7.
[63] Morikawa N, Nakagawa-Hattori Y, Mizuno Y. Effect of dopamine, dimethoxyphenylethylamine, papaverine, and related compounds on mitochondrial respiration and complex I activity. J Neurochem 1996;66:1174–81.
[64] Jenner P. Oxidative stress in Parkinson's disease. Ann Neurol 2003; 53:S26–38.
[65] Lusty CJ, Machinist JM, Singer TP. Studies on the respiratory chain-linked nicotinamide adenine dinucleotide dehydrogenase. VII. Labile sulfide group in the dehydrogenase and related proteins. J Biol Chem 1965;240:1804–10.
[66] Gutman M, Singer TP. EPR studies on the iron sulfur centers of DPNH dehydrogenase during redox cycle of the enzyme. Biochem Biophys Res Commun 1973;44:1572–8.
[67] Singer TP. Mitochondrial electron transport inhibitors. Methods Enzymol 1979;55:454–62.
[68] Singer TP, Ramsay RR. The reaction site of rotenone and ubiquinone with mitochondrial NADH dehydrogenase. Biochim Biophys Acta 1994;1187:198–202.
[69] Gluck MR, Krueger MJ, Ramsay RR, Sablin SO, Singer TP, Nicklas WJ. Characterization of the inhibitory mechanism of 1-methyl-4-phenylpyridinium and 4-phenylpyridine analogs in inner membrane preparations. J Biol Chem 1994;269:3167–74.
[70] Harmon HJ, Crane FL. Inhibition of mitochondrial electron transport by hydrophilic metal chelators. Determination of dehydrogenase topography. Biochim Biophys Acta 1976;440:45–8.
[71] Joyce PI, Atcheson R, Marcus RJ, Heffernan AM, Rowbotham DJ, Lambert DG. Interaction of local anaesthetic agents with the endogenous norepinephrine transporter in SH-SY5Y human neuroblastoma cells. Neurosci Lett 2001;305:161–4.
[72] Zarow C, Lyness SA, Mortimer JA, Chui HC. Neuronal loss is greater in the locus coeruleus than nucleus basalis and substantia nigra in Alzheimer and Parkinson diseases. Arch Neurol 2003;60:337–41.
[73] Schapira AHV, Cooper JM, Dexter D. Mitochondrial complex I deficiency in Parkinson's disease. J Neurochem 1990;54:823–7.
[74] Singer TP, Ramsay RR, Ackrell BA. Deficiencies of NADH and succinate dehydrogenases in degenerative diseases and myopathies. Biochim Biophys Acta 1995;1271:211–9.
[75] Burkhardt C, Kelly JP, Lim YH, Filley CM, Parker WD. Neuroleptic Medications inhibit complex I of the electron transport chain. Ann Neurol 1993;33:512–7.
[76] Maurer I, Moller HJ. Inhibition of complex I by neuroleptics in normal human brain cortex paralles the extrapyramidal toxicity of neuroleptics. Mol Cell Biochem 1997;174:255–9.
[77] Greenamyre JT, Sherer TB, Betarbet R, Panov AV. Complex I and Parkinson's disease. IUBMB Life 2001;52:135–41.
[78] Langston JW, Ballard P, Tetrud JW, Irwin I. Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. Science 1983;219:979–80.
[79] Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV, Greenamyre JT. Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nat Neurosci 2000;3:1301–6.
[80] Hornykiewicz O, Kish SJ. Biochemical pathophysiology of Parkinson's disease. Adv Neurol 1987;45:19–34.
[81] Parkinson's Disease Study Group. Effects of tocopherol and deprenyl on the progression of disability in early Parkinson's disease. N Engl J Med 1993;328:176–83.