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Dopamine modulates mitochondrial function in viable SH-SY5Y cells possibly via its interaction with complex I: Relevance to dopamine pathology in schizophrenia

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Abstract

Deleterious effects of dopamine (DA) involving mitochondrial dysfunction have an important role in DA-associated neuronal disorders, including schizophrenia and Parkinson's disease. DA detrimental effects have been attributed to its ability to be auto-oxidized to toxic reactive oxygen species. Since, unlike Parkinson's disease, schizophrenia does not involve neurodegenerative processes, we suggest a novel mechanism by which DA impairs mitochondrial function without affecting cell viability. DA significantly dissipated mitochondrial membrane potential ($\Delta\psi_m$) in SH-SY5Y cells. Bypassing complex I prevented the DA-induced depolarization. Moreover, DA inhibited complex I but not complex II activity in disrupted mitochondria, suggesting complex I participation in DA-induced mitochondrial dysfunction. We further demonstrated that intact mitochondria can accumulate DA in a saturated manner, with an apparent $K_m = 122.1 \pm 28.6$ nM and $V_{max} = 1.41 \pm 0.15$ pmol/mg protein/min, thereby enabling the interaction between DA and complex I. DA accumulation was an energy and Na^+-dependent process. The pharmacological profile of mitochondrial DA uptake differed from that of other characterized DA transporters. Finally, relevance to schizophrenia is demonstrated by an abnormal interaction between DA and complex I in schizophrenic patients. These results suggest a non-lethal interaction between DA and mitochondria possibly via complex I, which can better explain DA-related pathological processes observed in non-degenerative disorders, such as schizophrenia.

Keywords: Dopamine; Mitochondria; Mitochondrial membrane potential; Complex I; Mitochondrial dopamine uptake; Schizophrenia

1. Introduction

Dysregulation of dopamine (DA) homeostasis has been suggested to play an important role in the pathophysiology of several psychiatric and neurological disorders such as schizophrenia and Parkinson's disease. In neurodegenerative disorder the degeneration of catecholaminergic neuron has been attributed to increased cytosolic levels of DA and its oxidized metabolites [1–5]. In schizophrenia, however, DA increased level [6,7], is not associated with neuronal death. The mechanism by which DA exerts its toxic effects *in vivo* is still unclear, yet several processes have been suggested, including the induction of a state of oxidative stress [8,9], apoptosis [10–12] as well as interference with mitochondrial respiration.

Mitochondria are responsible for many essential processes including energy production, intracellular calcium buffering, neurotransmitter transmission, apoptosis and ROS production, all leading either to cell death or playing a role in synaptic plasticity such as structural remodeling and long term functional changes [13,14]. Indeed, impaired mitochondrial function and the inability to maintain cellular ATP levels have been suggested as a possible cause for pathological processes in DA-related non-degenerative disorders such as schizophrenia as well as in neurodegenerative diseases such as Parkinson's [15–21].

Accumulating experimental evidence suggests that DA can inhibit the mitochondrial respiratory system. Thus, elevated rat

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brain DA concentrations following chronic administration of L-DOPA or D-methamphetamine resulted in a reduction of the activity of the first complex of the respiratory system (complex I) and ATP levels in the striatum [22,23]. In a neuronal cell line DA induced a reduction in cellular ATP levels without affecting cell viability [24]. In isolated intact rat brain mitochondria DA inhibited ATP coupled state III respiration [25] and suppressed pyruvate- and succinate-dependent electron transport [26]. In disrupted mitochondria from both rat brain and human platelets DA reversibly inhibited complex I activity but not that of complexes IV of the respiratory system and of ATPase/ATP synthase (complex V) [27,28]. Two mechanisms have been suggested for DA interference with mitochondrial respiration. The first, involves DA enzymatic catabolism or autooxidation to highly reactive oxygen species (ROS), and has been suggested to underlie cell death in Parkinson's disease. The second mechanism, involves a direct reversible inhibition of complex I activity, which can disrupt mitochondrial activity leading to abnormal neuronal transmission, rather than cell death, as is observed in schizophrenia [24]. Indeed, studies focusing on the mitochondrial respiratory system in schizophrenia have revealed brain region specific alterations in the activities of complexes I–III, II and IV [19,29,30]. Complex I activity was also altered in platelets and lymphocytes of schizophrenic patients in a disease specific and state dependent manner [16]. In addition, abnormal expression of mitochondrial genes in general, and of nuclear encoded genes of complex I, in particular, was also observed both in brain and in the periphery of schizophrenic patients [15–18,20,31].

The aforementioned findings suggest that the interaction between DA and complex I can play an important role in the pathophysiology of non-degenerative disorders such as schizophrenia. In order to verify such an interaction between DA and mitochondria, we investigated the ability of DA to enter the mitochondria and disrupt mitochondrial function via its interaction with complex I in viable cells. The results of the present study demonstrate that in intact cells DA causes dissipation of mitochondrial membrane potential ($\Delta\psi_m$) possibly via its interaction with complex I. Moreover, the results demonstrate that intact mitochondria can accumulate DA through their highly selective inner membrane, enabling its interaction with complex I. The kinetics and pharmacological profile of this process were characterized. Finally, a schizophrenia-specific pathological interaction between DA and complex I is demonstrated in mitochondria isolated from platelets of patients and healthy controls suggesting that this interaction may be of relevance for schizophrenia.

2. Materials and methods

2.1. Cell treatment

1×10^6 human neuroblastoma SH-SY5Y cells were plated in DMEM supplemented with 10% fetal calf serum, L-glutamine and antibiotics at 37 °C in a 5% CO_2 humidified incubator. Following 24 h, the medium was replaced with serum free DMEM for an additional 18–20 h. Prior to treatment, cells were preincubated with 0.1 mM tranylcypromine (MAO A and B inhibitor) for 30 min.

Cell viability was determined by Trypan Blue. Protein concentration was determined by Bradford reagent.

2.2. Imaging mitochondrial membrane potential

2.2.1. Confocal microscopy

Mitochondrial membrane potential ($\Delta\psi_m$) was assessed with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1) [32,33]. The positively charged JC-1 is specific for mitochondrial membrane potential and its color alters reversibly from green (J-monomer, at low JC-1 concentration) to red (J-aggregates, at high JC-1 concentration) with increased mitochondrial membrane potential. For confocal microscopy cells were loaded for 15 min with 5 μM JC-1 in HBSS (HEPES buffered salt solution containing: 137 mM NaCl, 5 mM KCl, 0.9 mM $MgSO_4$, 1.4 mM $CaCl_2$, 10 mM $NaHCO_3$, 0.6 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 5.6 mM glucose and 20 mM HEPES pH 7.4) at 37 °C. Cells were washed twice with HBSS and incubated for additional 30 min. Images were acquired, before and 1–10 min after the addition of 10^{-4} M DA, by a Radiance 2001 laser scanning confocal imaging system (Nikon Eclipse E600). A 488-nm excitation from an argon laser was used in conjunction with a 510 nm dichroic mirror. Emitted light was further separated by a 575 nm dichroic mirror before being filtered at 530±10 nm and 590±17 nm, the wavelengths corresponding to the peak fluorescence from the monomer and aggregate signals, respectively. The ratio of fluorescence emission 590:530 nm was used as an indicator of $\Delta\psi_m$ [32,33]. Data analysis was performed using Image Pro Plus MediaCybernetics software.

2.2.2. Flow cytometry

Suspended cells (10^6 cells/ml of HBSS or PBS) were pretreated with different drugs before the incubation with JC-1 at 37 °C as follows: 5 μM of the uncoupler carbonyl cyanide m-chlorophenylhydrazine (CCCP), 0.001–10 μM of complex I inhibitor, rotenone or 5×10^{-5} to 10^{-4} M DA for 5 min. Since a significant dissipation of $\Delta\psi_m$ was already observed with 1 μM rotenone this concentration was used throughout the study. For succinate induced respiration, 5 mM succinate was added to cells immediately before treatment. Cells were washed twice at room temperature and analyzed by FACS Calibur (BECTON DICKINSON, USA) equipped with a single argon ion laser. Samples were excited at 488 nm and the ratio of fluorescence emission at 590:530 (red: green) was determined as an indicator of mitochondrial membrane potential [34]. A minimum of 10,000 events per sample were analyzed, using the "Cell Quest" software.

2.3. Intracellular DA

Intracellular DA was measured by HPLC with an electrochemical detector (ESA Inc. MA, USA). Cells were incubated as specified for the JC-1 reaction in the presence or absence of three different concentrations of DA (5×10^{-5} to 5×10^{-4}) and of 5 mM succinate. The reaction was terminated by centrifugation at 280×*g* at room temperature for 15 min. The pellet was washed three times with 100 times excess of buffer and centrifuged between each wash. The final pellet was placed on ice and dissolved in 100 μl of 0.1 M $HClO_4$. After freezing and thawing the homogenate was centrifuged at 12,000×*g* at 4 °C, and aliquots of the supernatant were analyzed with HPLC. The electrodetection was performed at +0.3 V. The mobile phase contained 0.1 M NaH_2PO_4, 0.27 mM Na_2EDTA, 1 mM octanesulfuric acid, 4.5% acetonitrile, 2.5% methanol (pH=2.75). Data were analyzed using Borwin software [35].

2.4. Reactive oxygen species (ROS) production

The formation of intracellular ROS was measured by incubating SH-SY5Y cells (2×10^5 cells) suspended in HBSS in the presence of 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA, Molecular Probes) (final concentration 25 μM) for 30 min at 37 °C [36]. H_2DCFDA is a stable non-fluorescent molecule that passively diffuses into cells, where the acetate can be cleaved by intracellular esterases to produce a polar diol that is well retained within the cells. The diol can then be oxidized by ROS to a fluorescent form. Redox state of cells was determined by exciting H_2DCFDA-loaded cells at 485 nm and detection of emitted fluorescence was performed at 520 nm using FLUOstar OPTIMA (BMG Labtech, Germany). After loading, DA (10^{-6} and 10^{-4} M) was added to the cells at different concentrations and measurements were obtained every 5 min for up to 30 min. H_2O_2 (10^{-4} M) was used as a positive control.

Superoxide formation was measured by incubating SH-SY5Y cells (2×10^5 cells) suspended in HBSS using Red mitochondrial superoxide indicator

(MitoSOX, Molecular Probes) (final concentration 5 μM) for 10 min at 37 °C [37]. Superoxides were determined by exciting MitoSOX-loaded cells at 485 nm and detection of emitted fluorescence was performed at 580 nm using FLUOstar OPTIMA (BMG Labtech, Germany). After loading, DA (10^{-6}, 10^{-4} M) were added to the cells at different concentrations and measurements were obtained every 5 min for up to 30 min. Rotenone (10 μM) was used as a positive control.

2.5. Preparation of synaptosome-free mitochondria from rat brain

Brain mitochondria were isolated from male Sprague–Dawley rats (300–350 g) as previously described [38]. 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 1000×*g* for 10 min. The supernatant was collected and centrifuged at 12,000×*g* for 10 min. 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 [38]. The mitochondria containing layer was diluted 1:10 in isolation buffer and centrifuged at 12000×*g* for 10 min. The final pellet was immediately used for uptake experiments. Mitochondrial protein yields were 8–12 mg per rat brain. All animal procedures were approved by the Technion Animal Care and Use Committee.

Purity and integrity of mitochondria, isolated by Percoll and thoroughly washed, were studied by electron microscopy as described previously [38] and by evaluating respiratory control rates in the presence of ADP (state 3 respiration rates) that was determined polarographically with a thermostatically controlled Clark oxygen electrode (Strathkelvin Instruments LTD Glasgow, Scotland) [39].

2.6. Mitochondrial swelling

Ca^{2+} induced mitochondrial swelling was determined spectrophotometrically (Milton Roy Spectronic Genesis 5, Rochester, NY, USA) at 25 °C as previously described [40,41]. The decrease in absorbance at 540 nm was monitored in a standard swelling buffer (215 mM mannitol, 50 mM sucrose, 10 mM KCl, 3 mM KH_2PO_4, 0.5 mM $MgCl_2$, 10 mM HEPES pH 7.4, 5 mM succinate, 2 μM rotenone and 1 μg/ml oligomycin) and in the buffer used for DA accumulation experiments. Ca^{2+} (100 μM) and DA (2×10^{-7} to 5×10^{-5} M) were added 2 and 5 min after the addition of intact mitochondria, respectively.

2.7. Mitochondrial enzyme activities

NADH CoQ reductase (complex I) activity was assayed in disrupted mitochondrial (0.2 mg protein/ml) at 25 °C in 20 mM potassium-phosphate buffer pH 7.2. Containing 5 mM $MgCl_2$ and 1 mM KCN. The reaction was started by the addition of 0.14 mM NADH and 50 μM of ubiquinone-5;2,3-dimethoxy-5-methyl-6-[3-methyl-2-butenyl]-1,4-benzoquinone (CoQ_1) in the presence or absence of rotenone (10 μM). The decrease in NADH absorbance was followed at 340 nm for 1 min with a 3-s interval between successive readings and 1 sec initial delay [42,43]. The residual activity of complex I was less than 2–4% in rat brain mitochondria and 10–15% in platelet mitochondria.

Succinate CoQ oxidoreductase (complex II) activity was assayed in disrupted mitochondria (0.1 mg protein/ml) at 25 °C, in 50 mM potassium-phosphate buffer pH 7.4 containing 0.05 mM K EDTA and 1 mM KCN. The reaction was started by the addition of 50 μM CoQ_1 and 0.06 mM 2,6-Dichloroindophenol sodium salt hydrate (DCIP) with or without 20 mM K-succinate. Activity was assayed by following the decrease in the absorbance of DCIP at 600 nm as previously described [44].

Cytochrome c oxidase (complex IV) activity was assayed in mitochondrial preparation (0.1 mg protein/ml) at 25 °C in 40 mM potassium-phosphate buffer pH 6.2. The reaction was started by the addition of reduced cytochrome *c* (final concentration 0.12 mg/ml). Activity was assayed by following the decrease in absorbance of reduced cytochrome *c* at 550 nm [45].

2.8. Dopamine accumulation in intact mitochondria

Intact mitochondria (1 mg protein) were preincubated at 30 °C for 5 min in 10 mM HEPES pH 7.4 buffer containing 195 mM mannitol, 65 mM sucrose, 5 mM KCl, 1 mM $MgCl_2$, 1 mM KH_2PO_4, 5–100 mM NaCl, 0.2 mM tranylcypromine and 0.2 mM ATP or 5 mM potassium succinate. 10–400 nM [^{3}H]-dopamine (24.01 ci/mmol NEN, Boston, MA) was added in the presence or absence of 10^{-4} M of cold DA and incubated for an additional 2 min at the initial, linear rate of the reaction. Incubation was terminated by rapid filtration through GF/B filters followed by three washes with ice-cold buffer. Dried filters were counted for tritium content in liquid β-scintillation counter (1409 WALLAC, Finland). Pharmacological specification of the accumulation of 50 nM ^{3}[H]-dopamine was studied in the presence of 11 different concentrations of various drugs (10^{-7} to 10^{-3} M) which were pre-incubated with intact mitochondria for 5 min. The solutions, which were used to dissolve the drugs, were added to control mitochondria.

The accumulation of DA into intact mitochondria was also measured by HPLC. The reaction was terminated by centrifugation at 12,000×*g* for 10 min at 4 °C and processed as described above for the measurement of intracellular DA.

2.9. Subjects

A total of 77 inpatients and 24 control subjects participated in the study. All patients met DSM-IV criteria for schizophrenia (acute exacerbation), bipolar disorder (BD) depressed type or recurrent major depression (MDD). Consensus diagnosis by two senior psychiatrists was based on extended clinical interview and patients' chart review. Patients with schizoaffective illness were excluded. Twenty-four subjects without prior psychiatric history served as a control group. This group was age and sex matched to the schizophrenic group. Table 1 displays some of the demographic characteristics of the study groups. All schizophrenic patients ($n=50$) were in a state of acute psychotic exacerbation. At the time of blood sampling 25 were medication-free for at least 1 month and most patients had not received medication for significantly longer periods. Upon admission some short acting benzodiazepine was used when necessary to control agitation and restlessness until blood was collected after which antipsychotic treatment was started. The remaining 25 schizophrenic patients were receiving at the time of the study various antipsychotic medications at conventional doses including haloperidol ($n=7$), chlorpromazine ($n=6$), perphenazine ($n=4$), clozapine ($n=6$) and resperidone ($n=2$). Some received additional anticholinergic medications and benzodiazepines. MDD patients ($n=17$) received an antidepressant treatment, mostly selective serotonin reuptake inhibitors, or tricyclic antidepressants. BD patients ($n=10$), all depressed at the time of the study, were on a combination of a mood stabilizer and antidepressants. Patients who needed additional medications for other medical conditions were excluded. All patients were given an explanation about the purpose of the study and provided a written informed consent. The study was approved by the Institutional Review Board.

Table 1
Clinical data for the different groups

	Controls	Schizophrenic patients		Depressed patients	
		Medicated	Unmedicated	MDD	BD
No. of patients	24	25	25	17	10
Gender (F/M)	13 / 11	10 / 15	14 / 11	11/6	6/4
Age±SD	35.0±9.8	25.9±7.0	32.0±13.0	53.0±14.0[a]	50.0±13.5[b]
(range)	(22–50)	(18–43)	(18–60)	(33–75)	(27–75)
Duration of illness		5.2±5.5	4.2±10.0	10.3±10.3	18.0±11.1[c]
(range)		(0.5–20)	(0.5–20)	(0.5–34)	(2–41)

A significant difference for age and duration of illness was demonstrated in depressed patients as compared with the schizophrenic or control groups, which did not differ from each other. The significance of the difference ($df=100$ $F=19.291$ $p<0.0001$ for age of subjects; $df=76$ $F=6.720$ $p=0.0005$ for duration of illness) was analyzed by one way ANOVA followed by Bonferroni. [a]$p<0.001$($t=5.033$); [b]$p<0.01$ ($t=3.533$)vs. control, [c]$p<0.01$($t=5.367$) vs. schizophrenic patients.

2.10. Isolation of mitochondria from platelets

Blood (50–80 ml) was collected from the cubital vein without tourniquet between 8 and 10 AM and mixed 9:1 with 3.8% (w/v) tri-sodium citrate. Platelets were isolated (1–3 h after blood sampling) by centrifugation at 1000×*g* for 30 min from platelet-rich plasma. Isolated platelets were immediately washed with Tyrode's buffer pH 7.4 containing 1 mM EDTA as previously described [46]. The platelet pellet was gently re-suspended in ice cold 10 mM Tris buffer pH 7.4 containing 250 mM sucrose and 1 mM EDTA and disrupted by a Dounce-A homogenizer (20–30 strokes). The breakdown of the platelets was verified by light microscopy. The homogenate was centrifuged at 1000×*g* at 4 °C for 20 min to remove unbroken cells. The supernatant, containing the cytoplasmic extract was centrifuged at 12,000×*g* at 4 °C for 15 min and an enriched mitochondrial fraction was isolated on Percoll as previously described [17,27,47]. Electron microscopy, showed a sediment of intact mitochondria with few dense granules and α-granules. The final preparation was immediately stored at −70 °C until use.

2.11. Data analysis

Statistical significance was determined by one way ANOVA followed by Bonferroni post hoc multiple comparisons test. For the effect of succinate effects paired *t*-test was used. SPSS 14.0 program was used for all analyses.

2.12. Chemicals

All drugs were purchased from Sigma Chemical Company St. Louis, MO, USA. JC-1 was purchased from Molecular Probes (Eugene, USA). Media for tissue culture was 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. DA mitochondria interaction in intact cells

3.1.1. DA dissipation of $\Delta\psi_m$

Previously, we have shown that DA inhibits complex I activity in disrupted brain mitochondria. In the present study we investigated whether this inhibition influences mitochondrial functionality in intact viable cells. DA effect on mitochondrial function was studied by measuring changes in mitochondrial membrane potential ($\Delta\psi_m$) as a marker for the oxidative phosphorylation system (OXPHOS) activity in living SH-SY5Y cells. Confocal microscopy demonstrated that DA (10^{-4} M) caused a significant depolarization of $\Delta\psi_m$ in cultured SH-SY5Y loaded with JC-1. 5 μM CCCP applied for 5 min was used as a positive control (Fig. 1A). Quantification of 8–10 experiments, 3–4 scans in each, demonstrated a significant depolarization of $\Delta\psi_m$ by DA (30%; $p<0.032$). Similar effect was observed in the presence of rotenone (32%; $p<0.025$) and a more extensive depolarization was caused by CCCP (50%; $p<0.001$) (Fig. 1B).

Previously it was shown that confocal microscopy should be used with caution for measurements of $\Delta\psi_m$ kinetics due to an uncoupling effect of the laser illumination itself [48], therefore, DA effects on mitochondrial function in living cells were further studied by measuring changes of $\Delta\psi_m$ using the flow cytometry technique. Treatment of SH-SY5Y cells with DA or rotenone, for 5–10 min, resulted in a significant dissipation of mitochondrial membrane potential (20–30%; $p<0.012$, 70–80%; $p<0.01$, respectively). CCCP almost completely abolished mitochondrial membrane potential (95%; $p<0.01$) (Fig. 2). Addition of 10^{-4} M cocaine, which prevents DA uptake into cells [24], abolished DA dissipation of $\Delta\psi_m$, suggesting that intracellular DA is involved in this process.

DA had no effect on cell viability under our various assay conditions.

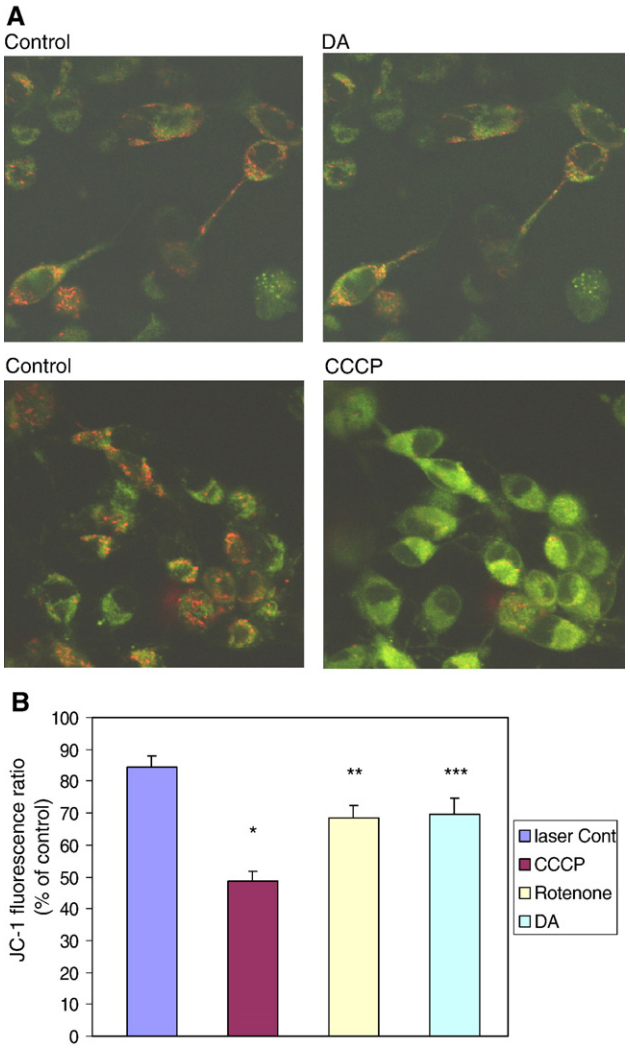

Fig. 1. DA dissipation of $\Delta\psi_m$ in SH-SY5Y analyzed by confocal microscopy. Cultured SH-SY5Y cells in HBSS were loaded for 15 min with 5 μM of the fluorescent probe- JC-1 at 37 °C. Scans were taken after cells were treated with: 5 μM CCCP, 1 μM rotenone and 10^{-4} M DA for 5 min. (A) Representative scans of cells control cultures and cells treated with DA or CCCP (B) Quantification of the results of 8–10 different experiments. Data analysis was performed using Image Pro Plus MediaCybernetics software. Results are means ± SEM. The significant difference between treatments was analyzed by ANOVA followed by Bonferroni post-hoc test. *$p=0.0013$, **$p=0.025$, ***$p=0.032$ vs. control.

3.1.2. DA, complex I, and dissipation of $\Delta\psi_m$

In order to study whether DA effect on $\Delta\psi_m$ in intact cells is mediated through complex I inhibition, we bypassed complex I by adding succinate, which donates electrons through succinate CoQ oxidoreductase (complex II) to CoQ of the OXPHOS. Succinate can be taken up by the Na+-coupled carboxylate transporters 2 and 3, which are expressed in brain neuronal and

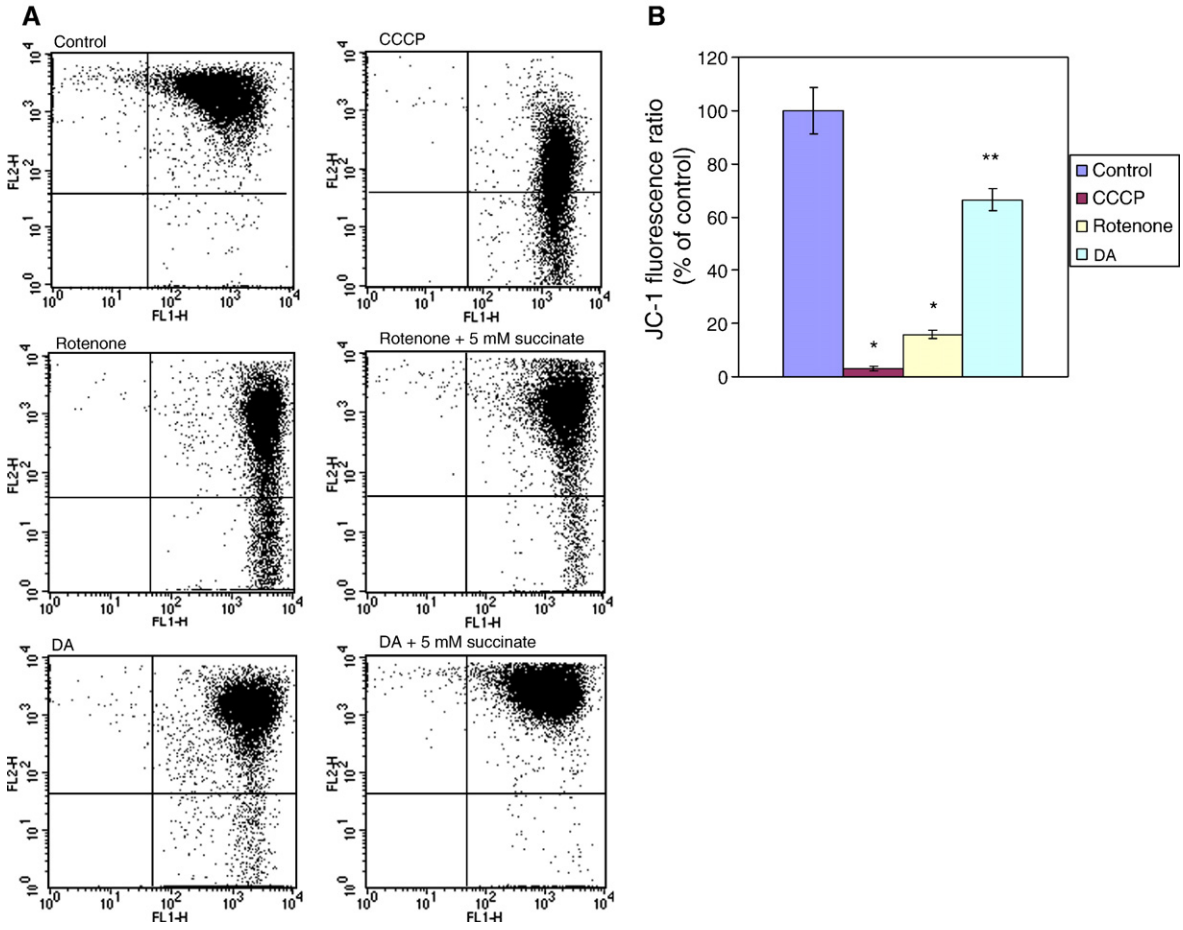

Fig. 2. DA dissipation of $\Delta\psi_m$ in SH-SY5Y analyzed by FACS. Suspended SH-SY5Y cells (10^6 cells/ml HBSS or PBS) were pretreated with 5 μM of CCCP, 1 μM of rotenone or 10^{-4} M of DA for 5 min, before the incubation with JC-1 at 37 °C. For succinate induced respiration, 5 mM succinate was added to cells immediately before treatment. (A) Representative FACS scans of cells treated with DA, rotenone and CCCP with or without succinate. FL-1-H — red fluorescent aggregates, F-2-H — green fluorescent monomers. (B) Quantification of FACS results. Quantification of the succinate FACS appears in Fig 3. Data are means ± SEM of 10 experiments. The significant difference between treatments was analyzed by ANOVA followed by Bonferroni post-hoc test *$p<0.001$; **$p<0.012$ vs. control.

glia cells [49]. In the presence of succinate, the ability of DA (5×10^{-4} M) to dissipate $\Delta\psi$m was completely abolished. Rotenone (1 μM) induced dissipation of $\Delta\psi_m$ was partially prevented by succinate (60% depolarization as compared to 80% without succinate). Notably, succinate ability to reverse rotenone-induced dissipation of $\Delta\psi_m$ was attenuated in the presence of DA in a dose dependent manner (Fig. 3). Such DA induced attenuation was not observed in the presence of 10 μM rotenone. Further support for the inability of DA to inhibit complex II was demonstrated by the lack of effect of 5×10^{-4} M DA on complex II activity in disrupted mitochondrial preparation (Table 2), as opposed to its ability to inhibit complex I activity (data not shown, [24]).

3.1.3. ROS Formation in the presence of DA

To determine whether ROS formation is involved in DA induced dissipation of $\Delta\psi_m$ we analyzed superoxide and H_2O_2 production using 5 μM MitoSOX and 25 μM H_2DCFDA, respectively, in SH-SY5Y cells treated with 10^{-4} and 10^{-6} M DA for up to 30 min. During this time interval no significant increase in ROS production was observed in the presence of DA. On the contrary, a significant reduction in ROS formation as detected by MitoSOX and H_2DCFDA was observed in cells treated with 10^{-4} M DA with no change in the presence of 10^{-6} M DA. The latter unexpected results call for further studies. An increase in ROS production was observed in the presence of 10 μM rotenone or 10^{-4} M H_2O_2 serving as positive controls for superoxide and H_2O_2 production (Table 3).

3.1.4. Intracellular DA concentration

To ensure that intracellular concentrations of DA are increased under our various assay conditions, DA was analyzed by HPLC. Basal levels of DA, in cells suspended in 1 ml serum free medium and treated with 0.1 mM tranylcypromine was 0.13 ± 0.04 pmol/10^6 cells. Following incubation with 10^{-4} M DA for 10 min, intracellular DA levels increased to 15.6 ± 0.7 pmol/10^6 cells ($p<0.001$), indicating that about 0.1% of the extracellular dopamine was taken-up by 10^6 cells. Succinate had no effect on basal as well as DA induced intracellular DA levels. Although tranylcypromine was used to inhibit MAO, a small amount of DOPAC was still formed in the presence of 10^{-4} M DA and was about 12% of DOPAC formed in the absence of the inhibitor (1.2 ± 0.2 vs. 10.1 ± 1.1 pmol/10^6 cells, respectively).

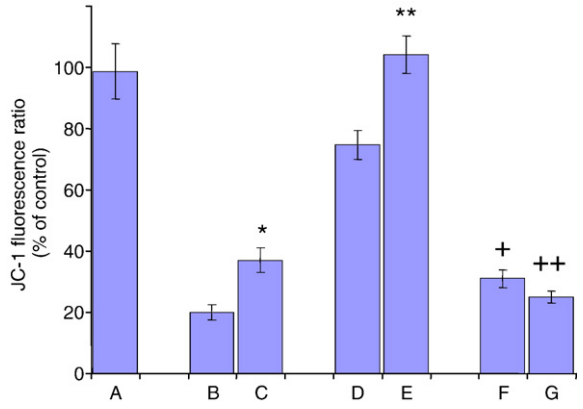

Fig. 3. Succinate overcomes DA induced dissipation of $\Delta\psi_m$ in the presence or absence of rotenone analyzed by FACS. In order to bypass the electron transport via complex I, suspended SH-SY5Y cells (10^6 cells/ml) were treated with succinate (5 mM), the complex II substrate, in the presence or absence of 10^{-4} M and 5×10^{-4} M of DA and/or 1 μM of rotenone for 5 min, before the incubation with JC-1 at 37 °C. (A) Succinate. (B) Rotenone. (C) Succinate+rotenone. (D) DA 10^{-4} M; (E) Succinate+DA 10^{-4} M. (F) Rotenone+succinate+DA 10^{-4} M. (G) Rotenone+succinate+DA 5×10^{-4} M. Data are means±SEM of 12 experiments. The significant difference induced by succinate was analyzed by paired *t*-test *p<0.0069 (C) vs. (B); **p<0.003 (E) vs. (D); $^{+}$p<0.043 (F) vs. (C); $^{++}$p<0.029 (G) vs. (C).

3.2. DA uptake by mitochondria

3.2.1. Integrity and purity of isolated mitochondria

Integrity and purity of the isolated mitochondria were evaluated by electron microscopy, which showed that 95–98% of the preparation consisted of mitochondria with an intact outer membrane. No synaptic vesicles/synaptosomes were observed. Membrane integrity of mitochondria was further demonstrated by their inability to metabolize NADH in the presence of CoQ or ferricyanide. Purity of preparation was further established by the determination of cytochrome *c* oxidase and NADH CoQ_1 reductase activities. Cytochrome c oxidase activity in our preparation was 4.58±0.51 μmol/mg protein/min, which is similar to its previously reported activity in non-synaptosomal mitochondria isolated from rat brain [38]. NADH CoQ reductase activity in disrupted mitochondrial preparation was highly sensitive to rotenone inhibition with a residual activity of less than 3%. Functional metabolic integrity of the mitochondrial preparation was assessed by measurement of its respiratory activity, which resulted in respiratory control ratio of 6.5±0.5 (mean±SEM; $n=5$) in standard respiration buffer and of 6.9±0.4 (mean±SEM; $n=5$) in the buffer used for DA uptake experiments. Moreover, swelling of mitochondria in a standard swelling buffer and in the uptake buffer demonstrated similar results. Thus, the decrease in absorbance at 540 nM during 10 min without any additions was negligible and similar in both buffers. The addition of Ca^{2+} (100 μM) induced a significant decrease in optical density of 0.125±0.011 and 0.133±0.017 (mean±SEM $n=7$) during 8 min for standard swelling buffer and uptake buffer, respectively. DA (2×10^{-7} to

Table 2
DA does not inhibit succinate CoQ oxidoreductase activity

	Succinate CoQ oxidoreductase (μmol/mg protein/min)
Control (without succinate)	0.0053±0.0023
Control (with succinate)	0.0202±0.0014
DA (with succinate)	0.0211±0.0005

Effects of 5×10^{-4} M DA on succinate CoQ oxidoreductase activity were measured in the presence of 20 mM succinate in disrupted rat brain mitochondria. Data are means±SEM of 3 experiments.

Table 3
DA does not induce ROS formation

Drugs	Superoxides production (% of Control)	H_2O_2 production (% of Control)
Control	100.0±1.1	100.0±1.8
DA 10^{-4} M	85.0±1.1[c]	89.6±3.3[b]
DA 10^{-6} M	108.2±1.3	94.1±1.5
Rotenone (10 μM)	110.7±1.6[a]	–
H_2O_2 10^{-4} M	–	112.0±1.3[c]

The effect of DA on the formation of superoxides and ROS (specifically H_2O_2) was measured loading cell with MitoSOX and H_2DCFDA, respectively as described in Materials and methods. Data represent results of loaded cells incubated with the different drugs for 30 min. Data are means±SEM of 4–6 experiments. The significance of the difference ($df=3$ $F=31.1$ $p<0.0001$ for superoxide; $df=3$ $F=23.3$ $p=0.0001$ for ROS) was analyzed by one-way ANOVA followed by Bonferroni post-hoc test. [a]$p<0.031$; [b]$p<0.0094$; [c]$p<0.003$ vs. the control.

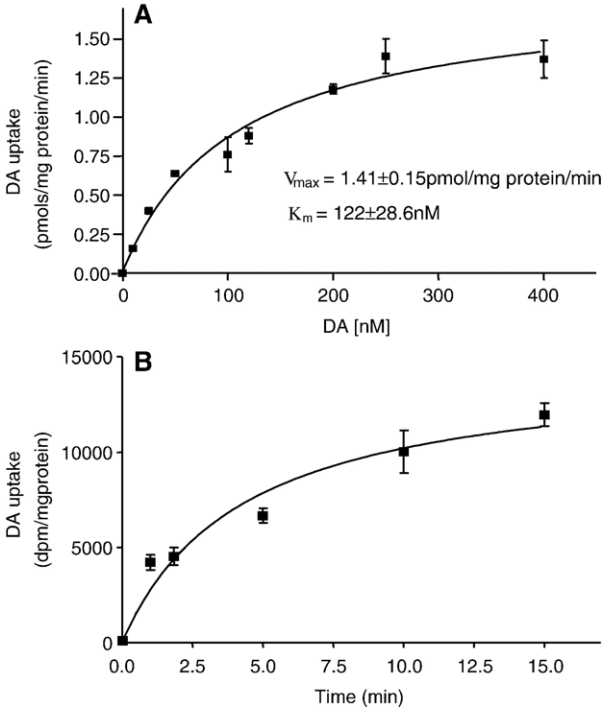

Fig. 4. The saturation and time dependent curves of dopamine accumulation in intact mitochondria. (A). The accumulation of ^{3}[H]-dopamine (10–400 nM) during 2 min of incubation at 30 °C by intact mitochondria (1 mg protein) was determined in the presence or absence of unlabelled dopamine (10^{-4} M). Data are means±SEM of triplicates of the specific accumulation from 6 experiments in which succinate was used as a respiratory substrate. (B). Time dependent accumulation of dopamine in intact mitochondria. Following 5 min of pre-incubation, intact mitochondria were incubated for 1–15 min at 30 °C and the accumulation of 50 nM ^{3}[H]-dopamine in the presence or absence of cold dopamine (10^{-4} M) was determined. Dopamine accumulation reached 50% of its maximum within 4 min. Data are means±SEM of triplicates of the specific accumulation from 3 experiments.

5×10^{-5} M) did not cause mitochondrial swelling and did not affect Ca^{2+} induced swelling in both buffers.

3.2.2. Characteristics of dopamine accumulation by mitochondria

^{3}[H]-dopamine was taken up by intact mitochondria in a saturable (Fig. 4A) and time dependent (Fig. 4B) manner with kinetic constants derived from Lineweaver–Burk plots of apparent $K_m=122.0\pm28.6$ nM and $V_{max}=1.41\pm0.15$ pmol/mg protein/min. Assuming 1 μl matrix volume/mg mitochondrial protein [50], the calculated internal DA concentration for a typical uptake experiment (50 nM DA; 2 min incubation) was 2.56±0.04 μM, thus representing a concentration gradient of about 50-fold.

In order to ensure that DA, rather than one of its metabolites, was taken up by mitochondria, DA accumulation was measured by HPLC. Fig. 5 shows that dopamine *per se* is taken up by intact mitochondria. Moreover, in the absence of tranylcypromine, a non-selective MAO A and B inhibitor, DA accumulation is significantly reduced ($p<0.001$), probably due to its enzymatic metabolism (Fig. 5 column B), further supporting that DA, and not its MAO metabolites, was taken up by the mitochondria.

DA accumulation by intact mitochondria was Na^+ dependent, and substitution of Na^+ with Li^+ almost abolished DA uptake (Fig. 5). Studying three additional concentrations of Na^+ indicated a bell shape dependency. Thus, at low 5 mM and high 100 mM concentrations of Na^+, no accumulation of DA was observed, and at 20 mM of Na^+, DA accumulation was 45± 7.5% (mean±SEM $n=4$) of that observed at 50 mM Na^+. Na^+ dependent DA accumulation by intact mitochondria took place following the addition of either ATP or succinate. In the absence of both external energy sources, DA accumulation was almost completely abolished. Finally, disruption of mitochondrial membrane potential by the addition of 5 μM of the uncoupler carbonyl cyanide m-chlorophenylhydrazine (CCCP) also prevented the accumulation of DA (Fig. 5).

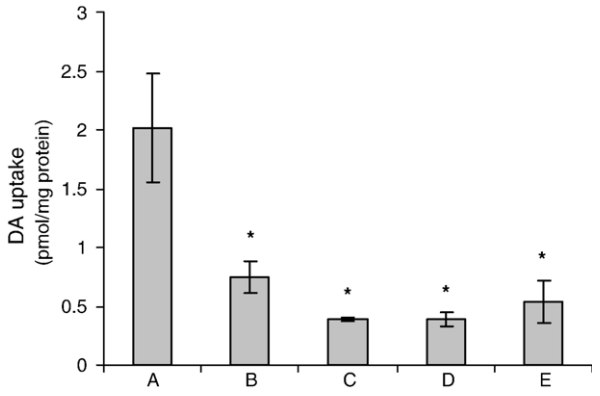

Fig. 5. The dependence of dopamine accumulation in intact mitochondria on Na^+, energy and membrane potential. Accumulation of dopamine (50 nM) in the presence of different drugs by intact mitochondria was determined by HPLC with an electrochemical detector. Incubation was performed as described for the accumulation of ^{3}[H]-dopamine, but the reaction was terminated by centrifugation at 15,000×*g* at 4 °C for 15 min. The pellet was washed three times with an excess of ice-cold buffer with in-between centrifugation. The mitochondria in the final pellets were disrupted by 0.1 M $HClO_4$ and by freezing and thawing. The supernatant was separated by centrifugation and was analyzed by HPLC. (A) Uptake under control conditions (B) in the absence of the MAO inhibitor tranylcypromine (C) in the presence of 50 mM LiCl instead of 50 mM Na^+ (D) in the absence of succinate and (E) in the presence of 5 μM of the uncoupler carbonylcyanide m-chlorophenyl-hydrazine (CCCP). Data are means±SEM of triplicates from 4 experiments. The significant difference between treatments was analyzed by ANOVA followed by Bonferroni post-hoc test. *$p<0.001$ for all treatments vs. control.

Table 4
The effect of various drugs on dopamine accumulation by intact rat brain mitochondria

Compound	IC_{50} (M)
NE	1.1×10^{-4}
MPP^+	1.5×10^{-5}
5HT	$>10^{-2}$
Spermine	$>10^{-2}$
Nomifensine	$>2.5\times10^{-3}$
GBR-12909	$>10^{-3}$
Cocaine	$>2\times10^{-3}$
Desipramine	$>2\times10^{-3}$
Reserpine	$>10^{-2}$
SCH-23390	$>10^{-2}$
Sulpiride	$>10^{-2}$
SKF-38393	$>10^{-2}$
Quinpirole	$>10^{-2}$

The specific uptake of ^{3}[H]-dopamine (50 nM) by intact mitochondria was determined in the presence of eleven different concentrations (10^{-3} to 10^{-7} M) of the various drugs. Data are the results of triplicate determinations from 4 experiments for each drug.

3.3. DA accumulation in the presence of various drugs

The effect of various DA-related drugs on DA accumulation by mitochondria is shown in Table 4. 1-methyl-4-phenylpridinum (MPP+) and norepinephrine (NE) inhibited DA accumulation with IC_{50} of 15.3±0.4 μM and 112±5 μM, respectively (Fig. 6A and B). Inhibition by MPP+ and NE showed a simple noncompetitive inhibition pattern (Fig. 6C and D). Other amines such as serotonin (5-HT) and spermine did not affect DA uptake (Table 3). Moreover, ^{3}[H]-5HT, which has an indol group instead of a catechol, was not accumulated by intact mitochondria (data not shown). In addition, DA accumulation was not inhibited by GBR-12909 and nomifensin (inhibitors of DA plasma membrane transporter-DAT) or by reserpine (inhibitor of the vesicular monoamine transporter-VMAT). Nor was DA accumulation inhibited by cocaine or desipramine (inhibitors of monoamines and NE plasma membrane transporters, respectively). DA D1 and D2 receptors agonists (SKF-38393 and quinipirole) or antagonists (SCH-23390 and sulpiride) did not inhibit DA accumulation, suggesting that DA does not bind to a known DA-binding site.

3.4. Increased susceptibility of complex I activity to DA inhibition in platelets of schizophrenic patients

The relevance of DA complex I interaction to schizophrenia was verified in mitochondria isolated from platelets of schizophrenic patients and control subjects. Similar to previous reports,

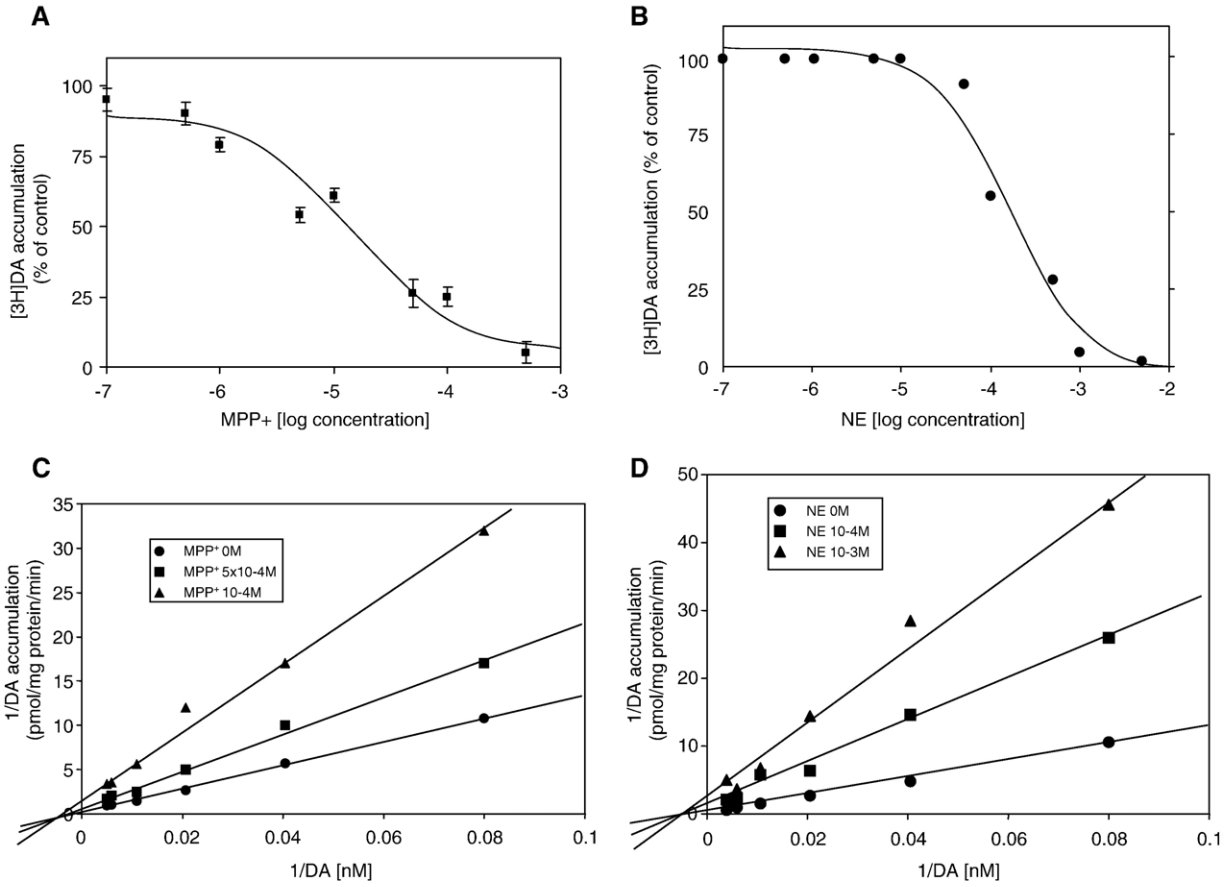

Fig. 6. Inhibition of ^{3}[H]-dopamine accumulation in intact mitochondria by MPP^{+} and norepinephrine. Intact mitochondria were pre-incubated with the 10^{-7} to 10^{-3} M MPP^{+} (A) and norepinephrine (B) for 5 min before the addition of 50 nM ^{3}[H]-dopamine. Data are means ± SEM of triplicates from 6 experiments. Double reciprocal Lineweaver–Burk analysis was performed on the inhibition curves for MPP^{+} (C) and Norepinephrine (D). ^{3}[H]-dopamine (10–250 nM) accumulation experiments in the presence of different concentrations of the inhibitors were performed. Data are means of triplicates from a representative experiment replicated 4 times.

complex I activity was significantly increased in schizophrenic patients at acute psychosis regardless whether they were medicated or not, as compared with control subjects [16]. Addition of 10^{-5} M DA resulted in a significant inhibition of complex I activity both in patients and in normal subjects. However, in schizophrenic patients inhibition of mitochondrial complex I was twice as much as in control subjects (69.7% vs. 38.6%). Patients with affective disorders did not differ from the controls (Table 5).

Table 5
The degree of dopamine inhibition of mitochondrial complex I activity in platelets of schizophrenic patients

Subjects	Complex I activity (% of control)	Dopamine inhibition (%)
Controls (n=24)	100.0±40.2	38.6±13.4
Medicated schizophrenics (n=25)	242.3±64.4*	69.1±19.9*
Unmedicated schizophrenics (n=25)	225.1±41.5*	72.6±15.0*
Recurrent major depression (n=17)	103.3±34.1	31.7±16.5
Bipolar disorder (depressed type, n=10)	104.50±44.7	34.5±13.6

Complex I activity (NADH CoQ reductase) was measured as described in Materials and methods. In control subjects rotenone sensitive complex I activity is 131.87±53.1 nmol/mg protein/min. Values are means±SD. Results were analyzed by ANOVA followed by Bonferroni post hoc test. *p<0.001 vs. controls and vs. both groups of patients with affective disorder.

4. Discussion

The present study provides evidence for DA ability to affect mitochondrial function, possibly through its interactions with complex I. This DA-mitochondria interaction was not associated with cell death, suggesting it as a preferable mechanism for DA deleterious effects in non-degenerative DA-disorders such as schizophrenia. Mitochondrial membrane potential ($\Delta\psi_m$) was used as a marker for the OXPHOS activity in human neuroblastoma SH-SY5Y cells. Both confocal microscopy and FACS results show a significant dissipation (25–30%) of $\Delta\psi_m$ by DA already after 5 min of treatment with no effect on cell survival. These findings are in line with a previous report on DA induced reduction of mitochondrial membrane potential in EBV transformed B lymphocytes [51]. DA induced dissipation of $\Delta\psi_m$ was prevented in the presence of cocaine, which inhibits the uptake of DA both by DA and NE plasma membrane transporters, suggesting that DA-induced mitochondrial dysfunction depends on DA uptake into the cells.

Microdyalisis studies report that extracellular DA concentrations can range between 3 and 750 nM depending on neuronal activation and brain region[52,53]. Synaptic concentrations of DA can range between 30 and 100 mM following activation of DA cell bodies [54]. In SH-SY5Y cells as well as in isolated

mitochondria the concentration generally used for DA is at the range used in this study (5×10^{-5} to 10^{-4} M). At such concentrations exposure of cells to DA for 12–18 h does not affect cell viability or causes apoptosis [24,39,55]. Under pathological condition, which are associated with massive release of DA, as in schizophrenia [7], mitochondria enriched nerve terminals are exposed to increased DA levels due to its increased re-uptake and synthesis [56]. Therefore, to investigate DA effects on mitochondria, cells were exposed to 100 μM DA for up to 10 min. In addition, to prevent DA metabolism to DOPAC and the production of H_2O_2, which can lead to a state of oxidative stress, MAO A and B inhibitor was used throughout the study.

To study the involvement of complex I in DA-induced alterations of $\Delta\psi_m$ the OXPHOS was activated through complex II, thereby bypassing complex I. Succinate, a complex II substrate, completely abolished DA-induced dissipation of $\Delta\psi_m$, suggesting that DA affect $\Delta\psi_m$ by inhibiting the OXPHOS prior to complex II, namely complex I. One may argue that succinate successfully reversed DA inhibition of complex II. If so, it is anticipated that the ability of succinate to reverse rotenone induced dissipation of $\Delta\psi_m$ through complex I inhibition, will not be affected by DA. However, in the present study DA-attenuated succinate ability to reverse rotenone (1 μM) induced dissipation of $\Delta\psi_m$, suggesting that DA inhibits complex I. Our previous findings suggest that DA inhibits complex I at a site located between the binding site for NADH and the iron–sulphour cluster N1 [24], while rotenone inhibits electron efflux from complex I through the ubiquinone docking site(s) on the downstream portion of the complex [57]. Further support for the involvement of complex I in DA induced inhibition of $\Delta\psi_m$, is the inability of DA to inhibit complex II activity, shown in the present study, as well as that of complexes IV and V [24], as opposed to its inhibitory effect on complex I [24] in disrupted mitochondrial preparation. Although other sites of interaction between DA and mitochondria, prior to complex I, cannot be ruled-out, based on our previous and the present results, it is tempting to hypothesis that DA ability to inhibit complex I, which is a major determinant of mitochondrial respiration [58], can lead to mitochondrial dysfunction. Interestingly, our previous findings in SH-SY5Y demonstrated a dose-dependent decrease in ATP production, which negatively correlated with intracellular increase in DA levels [24].

Numerous studies have claimed that DA deleterious effects on mitochondrial function are primarily due to its catabolism by MAO to H_2O_2 and oxygen free radicals, or due to its auto-oxidation to reactive quinones [59,60]. In this study however, exposure of cells for up to 30 min was not associated with ROS formation including H_2O_2 and superoxides. These results are not totally unexpected as cells were pretreated with the MAO A and B inhibitor, tranylcypromine significantly reducing the formation of DOPAC and therefore also the formation of H_2O_2. In addition, HPLC results show that under our assay conditions 0.3 μM DOPAC was formed in cells exposed to the highest DA concentration, indicating that intracellular levels of H_2O_2 were elevated by 0.3 μM at most. It was previously reported that physiological concentrations of H_2O_2 are in the range of 25–60 μM and that exposing cells to 100 μM H_2O_2 for at least 1.5 h is needed in order to induce alterations in $\Delta\psi_m$ [61]. Therefore, the observed alterations in $\Delta\psi_m$ induced by the brief exposure of cells to DA in our experiments cannot be linked to H_2O_2 formation. It is well established that inhibition of complex I can induce the production of superoxides. It was previously suggested that the site of rapid superoxide generation by complex I is in the region of the ubisemiquinone-binding sites and not upstream at the flavin or low potential Fe–S centers [62]. Our previous results suggest that DA inhibits complex I between the binding site for NADH and cluster N1 [24] and therefore a detectable production of superoxide, is not likely to occur. In line with the latter are the findings that Ca^{2+}, which inactivates complex I and inhibits electron transport upstream of the primary site(s) of free radical production, limits the production of superoxides in submitochondrial particles [63]. However, other studies suggest that superoxide generation is increased in the inactive slowly oxido-reduction activated D form of the enzyme and therefore do not support the aforementioned study [64]. In addition, recent reports have shown that superoxides can be generated at the tightly bound FMN [65,66], which maybe located upstream to the proposed DA binding site. Nevertheless, under our experimental conditions we could not detect superoxide formation in the presence of DA, suggesting that either superoxides were not generated in association with DA inhibition, or that in the presence of DA superoxides were detoxified by mitochondrial or cellular protective mechanisms. To overcome the formation of reactive quinones cells were suspended in HBSS solution pH 7.4 that limits oxidation. In addition, cells were treated with DA for 10–15 min, while previous findings demonstrated that more than 2 h were needed for a sufficient build-up of the damaging quinones to produce inactivation of OXPHOS in cells [28]. These findings are in favor of a direct interaction between DA and complex I, leading to the dissipation of $\Delta\psi_m$.

In order to be able to inhibit complex I activity in intact mitochondria, DA has to cross the mitochondrial highly selective inner membrane. Indeed, HPLC studies demonstrate that DA can be accumulated by synaptosomal-free, intact, coupled and respiring mitochondria. The accumulation of exogenous ^{3}[H]-DA by mitochondria fulfilled several criteria for a transporter mediated process. DA accumulation by mitochondria was rapid and reached 50% of its maximum within 4 min. It showed saturability with an apparent K_m of 122.0±28.6 nM, similar to that of striatal DAT [67] and V_{max} of 1.41±0.15 pmol/mg protein/min, similar to that of DAT in cerebral cortex, hypothalamus, midbrain, medulla oblongata and cerebellum [68]. A concentration gradient for DA of about 50-fold was formed, assuming 1 μl matrix volume per mg mitochondrial protein [50]. Accordingly, 2.56±0.04 μM DA can be accumulated in the mitochondria following their exposure to 50 nM of DA for 2 min, which is far less than the concentration range at nerve endings following release and reuptake of DA, and therefore in the range of the concentration of DA needed for complex I inhibition [24].

DA accumulation was an energy-dependent process. The kinetics of DA accumulation was similar when energy for the process was available either by substrate (succinate) oxidation

via the electron transport system, or from ATP hydrolysis in the absence of succinate (data not shown). In the presence of an uncoupler CCCP DA was not taken up, further supporting its uptake dependence on coupled functioning mitochondria. DA accumulation by mitochondria was dependent on sodium concentrations in a bell shape dependent manner. A possible explanation for such a curve is that at high intracellular Na^+ concentrations mitochondrial Ca^{+2} efflux can be increased, leading to inhibition of mitochondrial respiration and to a reduction in ATP production [69] and consequently to a decrease in DA uptake. The mechanism by which intracellular Na^+ enables DA accumulation in the mitochondria is still unclear. We suggest that mitochondrial Na^+ gradient, which is kept by the mitochondrial Na^+/H^+ exchanger, can serve as a driving force for this process [70–73]. The involvement of directly or indirectly Na^+ dependent mitochondrial ion transporters such as the Na^+/H^+, Na^+/Ca^{+2} and H^+/Ca^{+2} exchangers and H^+ symporter [74,75] in DA uptake is currently under investigation. It is well documented that the resting cytosol concentration of Na^+ is 5–10 mM, whereas DA accumulation by the mitochondria was considerable at 20 mM Na^+ and increased at 50 mM Na^+. However, during synaptic activation, powerful fluorescent indicators sensitive to cytosolic concentrations of Na^+, revealed measurable changes in $[Na^+]i$ (5–10 folds). Such changes in $[Na^+]i$ were observed during synaptic activation of glutamate receptors in midbrain DA neurons and Purkinje cells [76–79] as well as during hyperpolarization of hippocampal pyramidal cells and DA cells in the substantia nigra pars compacta [50,80]. These observations suggest that mitochondria can be exposed to significant fluctuations in intracellular $[Na^+]$, which under certain conditions, such as synaptic activation, can induce Na^+ dependent mitochondrial DA accumulation. In our cellular model DA induced 20–30% dissipation in $\Delta\psi_m$ possibly via interaction with complex I. An attractive possibility is that physiological fluctuations in intracellular $[Na^+]$ enable DA accumulation in mitochondria. Further studies on the role intracellular $[Na^+]$ plays in DA induced alteration of mitochondrial function are currently underway.

The characteristics of mitochondrial DA accumulation described above is not compatible with the idea of DA binding to a mitochondrial membrane-binding site, as was previously suggested [81]. This notion is further supported by the inability of DA D1 and D2 agonists and antagonist to inhibit mitochondrial DA accumulation. DA uptake is unlikely due to its positive charge, since two other amines, 5-HT and spermine were not taken-up by mitochondria and did not inhibit DA uptake, respectively. Mitochondrial DA accumulation is also probably not due to its interaction with MAO, since in the absence of a MAO inhibitor DA accumulation was attenuated, and since 5-HT was unable to compete with DA.

The pharmacological profile of DA accumulation by mitochondria differed from that of DAT (Na^+ gradient linked transporter) and that of VMAT (H^+ gradient linked transporter). Neither DAT specific inhibitors (GBR-12909 and nomifensine), nor a VMAT specific inhibitor (reserpine), affected mitochondrial DA accumulation. Interestingly, MPP^+, which is accumulated by mitochondria and inhibits complex I activity with an $IC_{50}=700$ μM [50,57], inhibited DA accumulation by mitochondria with a K_i of 15.3 ± 0.4 μM in a non-competitive manner. The noncompetitive inhibition of DA uptake by MPP^+ suggests that DA uptake depends on mitochondrial membrane potential, similar to previous findings on mitochondrial uptake of other positively charged molecules such as polyamines [82]. Norepinephrine, which efficiently competes with DA on the VMAT [16,83,84] and less so on the DAT [17,19,85–87], also inhibited mitochondrial DA accumulation in a noncompetitive manner but was 10 times less efficient than MPP^+. These results suggest that under certain conditions, such as increased intracellular sodium concentrations or extensive DA transmission, an active process of DA uptake by mitochondria, which is distinct from that of both DAT and VMAT, can take place.

The results hitherto suggest that under pathological states associated with increased $[Na^+]_i$ due to synaptic activation, abnormal DA transmission and/or dysfunction of MAO, an interaction between DA and mitochondrial complex I could be part of the pathogenesis. Such abnormalities have been reported in schizophrenia [88–93]. In line with the latter, in the present study we showed a pathological interaction between DA and complex I in schizophrenia. The data show that 10^{-5} M DA inhibited complex I activity in schizophrenic patients twice as much as in control subjects, in disrupted mitochondria isolated from platelets. This finding further suggests that DA modulation of complex I activity is abnormal in this DA-related pathological condition. The interaction between DA and complex I and whether it contributes to the pathology of schizophrenia is still unclear, since complex I activity in schizophrenic patients is disease state dependent, increased in patients with positive symptomatology and decreased in those with residual schizophrenia [16,94]. However, one cannot exclude the possibility that the increased inhibition of complex I by DA could be an endogenous means to compensate for the increase in complex I activity in schizophrenia. Alternatively, the higher exposure to DA in patients, may lead to the increased complex I activity to balance the enhanced DA inhibitory effect (for a more detailed discussion on the interaction between DA and complex I see Ben-Shachar 2002). Interestingly, in a previous study it was demonstrated that the extent of DA induced reduction in $\Delta\psi_m$ was similar in EBV transformed lymphocytes of schizophrenic patient and controls [51]. One explanation for these findings can be the higher susceptibility of complex I to DA in patient in whom an increased complex I activity was reported.

The validity of peripheral abnormalities as proxies for measuring brain dysfunction has been questioned. Mitochondria are partly independent organelles, contain their own DNA and their OXPHOS is highly preserved along evolution and in different tissues. Biochemical and pharmacological similarities have been observed between blood platelets and 5HT or DA containing neurons of the CNS [95]. Numerous studies have shown that platelets from schizophrenic patients behave differently than those isolated from healthy controls in dopamine uptake, 5-HT content arachidonic acid metabolism, inositol phosphate levels and disturbance of calcium homeostasis [95–97]. In addition, a reduction in imipramine binding was found

both in platelets and in postmortem brains of deceased depressed patients and patients who committed suicide [98]. In patients with Parkinson's or Alzheimer's diseases, a correlation has been demonstrated between platelet and postmortem brains for the reduction in mitochondrial complex I and cytochrome *c* oxidase activities, respectively [99,100]. The aforementioned substantiate the supposition that alterations in peripheral mitochondrial complex I abnormalities may be reflected in brain. Indeed, abnormalities in the OXPHOS were observed both in blood cells and in postmortem brain specimens [29,88,101,102].

Together with the previously mentioned inhibitory effect of DA on complex I activity, the findings in schizophrenic patients may indicate that in addition to DA receptors, complex I is a potential target for DA. In this connection it is notable that mitochondria are target organelles for DA by the virtue of the fact that MAO, the main metabolizing enzyme of DA, is located on their outer membrane. Under normal conditions MAO is probably protecting the mitochondria from the vicious effects of DA.

In view of the ability of DA to impair cell energy metabolism, or to cause cell death by forming highly reactive oxygen species, it is tempting to hypothesize that DA may affect mitochondria in a dual manner depending on the intracellular state. Consequently, we suggest that DA induced oxidative mechanism is more likely associated with neurodegenerative DA disorders such as Parkinson's disease, while a mechanism involving an interaction between DA and complex I, can better explain the pathology of non-degenerative DA disorders such as schizophrenia. Interference with mitochondrial function can effect normal neuronal transmission, which may impair optimal functioning of critical circuits necessary for normal cognitive, emotional and behavioral functions.

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