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DJ-1 protects against dopamine toxicity

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Abstract Parkinson's disease (PD) is a slowly progressive neurodegenerative disorder characterized by the loss of dopaminergic neurons. Dopamine is a highly toxic compound leading to generation of reactive oxygen species (ROS). DJ-1 mutations lead to early-onset inherited PD. Here, we show that DJ-1 protects against dopamine toxicity. Dopamine-exposure led to upregulation of DJ-1. Overexpression of DJ-1 increased cell resistance to dopamine toxicity and reduced intracellular ROS. Contrary effects were achieved when DJ-1 levels were reduced by siRNA. Similarly, in vivo striatal administration of 6-hydroxydopamine led to upregulation of DJ-1. Upregulation of DJ-1 was mediated by the MAP kinases pathway through activation of ERK 1, 2 in vitro and in vivo. Hence, oxidative stress, generated by free cytoplasmic dopamine, leads to upregulation of DJ-1 through the MAP kinases pathway. This mechanism elucidates how mutations in DJ-1 prompt PD and imply that modulation of DJ-1 may serve as a novel neuroprotective modality.

Keywords Dopamine · DJ-1 · Parkinson's disease · Oxidative stress · MAP kinases

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Introduction

Parkinson's disease (PD) is a slowly progressive neurodegenerative disorder characterized clinically by bradykinesia, rigidity, tremor, gait dysfunction, and postural instability. The pathological hallmark of the disease is loss of dopaminergic neurons in the substantia nigra pars compacta. The neurotransmitter dopamine is a highly toxic compound (Offen et al. 1996; Jenner and Olanow 1998; Blum et al. 2001; Sulzer 2001; Barzilai et al. 2003; Dawson and Dawson 2003). The enzymatic catabolism of dopamine, via monoamine oxidase, and its non-enzymatic autooxidation, generates cellular damaging reactive oxygen species (ROS) including hydrogen peroxide, hydroxyl radicals and dopamine-quinones (Jenner and Olanow 1998; Sulzer and Zecca 2000). Neurotoxicity due to elevated cytosolic dopamine has long been implicated in the etiology of neurodegeneration in PD (Blum et al. 2001; Sulzer 2001; Barzilai et al. 2003; Dawson and Dawson 2003).

In the past decade genetic causes leading to familial PD have been discovered. DJ-1 deletions and point mutations with loss of functional protein have been shown as a cause of early onset autosomal recessive PD (Bonifati et al. 2003; Abou-Sleiman et al. 2003; Hedrich et al. 2004). Several studies imply that DJ-1 responds to oxidative insults. Upon ROS exposure, DJ-1 undergoes a pI shift from 6.2 to 5.8 (Canet-Aviles et al. 2004; Kinumi et al. 2004; Choi et al. 2006; Lev et al. 2006). Post-mortem studies of brain samples taken from sporadic PD patients showed that the acidic isoforms of DJ-1 are more abundant in PD brains than in controls (Bandopadhyay et al. 2004; Choi et al. 2006). Elevated levels of DJ-1 were also reported in the cerebrospinal fluid of sporadic PD patients (Waragai et al. 2006). These studies imply that DJ-1 has a pathogenic role

not only in inherited cases but also in the more common sporadic form of the disease.

Accumulating evidence suggests that DJ-1 may play a part in the cellular defensive response to oxidative stress, but the mechanism by which mutations in DJ-1 result in early onset PD is still unknown (Martinat et al. 2004; Kim et al. 2005; Betarbet et al. 2006; Inden et al. 2006; Meulener et al. 2006; Lev et al. 2008). Since all known DJ-1 mutations cause decreased protein levels or function and lead to early onset autosomal recessive PD, we hypothesized that DJ-1 might have a special role in protecting dopaminergic neurons against dopamine-induced oxidative stress. Consequently, a deficiency or a malfunction of DJ-1, may inherently lead to increased ROS accumulation and oxidative insults, and consequently to the early demise of dopaminergic neurons. Therefore, the aim of this study was to examine whether DJ-1 has a potential protective effect against dopamine toxicity.

Experimental procedures

Materials

The following reagents were used: Bis-benzimide trihydrochloride (Hoechst 33342; Sigma); Tri-reagent (Sigma, St Louis, MO, USA); pIRES2-acGFP1 plasmid (Chemicon, Temecula, CA, USA); pSilencer2.1-U6 plasmid (Ambion); lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA); rabbit anti-DJ-1 (Chemicon, Temecula, CA, USA); mouse anti-beta actin (Sigma, St Louis, MO, USA); mouse anti-pERK1,2 (Santa-Cruz Biotechnology, CA, USA); mouse anti-tyrosine hydroxylase (Sigma, St Louis, MO, USA); rabbit anti-emerin (Santa-Cruz Biotechnology, CA, USA); Alexa 568-conjugated goat anti-rabbit (Molecular probes, Invitrogen, Eugene, OR, USA); horseradish peroxidase conjugated goat anti-mouse and goat anti-rabbit (Sigma, St Louis, MO, USA); Super Signal West Pico Chemiluminescent substrate (Pierce Biotechnology, Rockford, IL, USA); BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA); random primer (Invitrogen, Carlsbad, CA); Sybr green PCR master mix (Applied Biosystems, Warrington, UK); RNase inhibitor (RNAguard, Amersham Pharmacia biotech); Super Script II RNase H-reverse transcriptase (Invitrogen, Carlsbad, CA, USA); dopamine (Sigma, St Louis, MO, USA), *N*-acetylcysteine (Sigma, St Louis, MO, USA); PD-98059 (Calbiochem, Rosh Haayin, Israel); H_2DCFDA (Sigma, St Louis, MO, USA); Dulbecco's Modified Eagle's Medium (DMEM) (Biological Industries Israel Beit Haemek LTD, Kibbutz Beit Haemek, Israel); fetal calf serum (FCS) (Biological Industries Israel Beit Haemek LTD, Kibbutz Beit Haemek, Israel); diethyl pyrocarbonate (DEPC) water (Biological Industries Israel Beit Haemek LTD, Kibbutz Beit Haemek, Israel); Complete protease inhibitor cocktail tablets (Roche Diagnostics GmbH, Mannheim, Germany); geniticine (G418) (Gibco, Invitrogen); LDH cytotoxicity detection kit (Clontech, Mountain View, CA, USA); Alamar blue (Serotec, Oxford, UK). Other chemicals were purchased from Sigma Chemicals Co., St Louis, MO, USA.

Cellular transfections and treatments

SH-SY5Y human neuroblastoma cells, obtained from the ATCC (Rockville, MD, USA), were stably transfected with pIRES2-acGFP1 plasmid (BD Biosciences, Clontech, Mountain View, CA, USA) containing wild type DJ-1, as reported previously (Lev et al. 2008). We used naïve neuroblastoma cells as well as cells stably transfected with the empty vector as controls. Decreased expression of DJ-1 was achieved by stable transfection with pSilencer2.1-U6 plasmid (Ambion) containing siRNA for DJ-1 (Lev et al. 2008). For targeting human DJ-1 (GGTCATTACACCTACTC TGAGAATCGT), the loop sequence (TTCAAGAGA) flanked by the sense and antisense siRNA sequence, was inserted immediately downstream of U6 promoter in pSilencer2.1-U6 plasmid, according to the instructions of the manufacturer. As negative controls, neuroblastoma cells were transfected with pSilencer2.1-U6 negative control (siRNA-control; Ambion). Negative control plasmids supplied by Ambion express a hairpin siRNA with limited homology to any known sequences in the human, mouse and rat genomes. Transfections were performed using the lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). Stable transfections were achieved by G-418 treatment and were verified by measuring DJ-1 mRNA and protein levels using real-time PCR and Western blotting. Cells were treated by dopamine (0–500 μM; Sigma), *N*-acetylcysteine (NAC 5 mM; Sigma), and PD-98059 (30 μM; Calbiochem, Rosh Haayin, Israel).

Dopamine toxicity assays

Several methods were used in order to determine dopamine toxicity. *Alamar blue:* Cells were seeded in 96-wells plates at the concentration of 5,000 cells per well and allowed to attach over night. On the following day the cells were exposed to increasing doses of dopamine (0–500 μM) for 4 h in serum free medium. Alamar blue is a non toxic reagent which incorporates a redox indicator that changes color in response to metabolic activity. The reduction-induced color change varies proportionately with cell number and time. Solution of alamar blue 10% in serum free medium was added after 4 h of exposure to increasing doses of dopamine, for 2 h. Alamar blue fluorescence was

measured by FLUOstar spectrofluorometer at the excitation wavelength of 544 nm and the emission wavelength of 590 nm. Each experiment was done in triplicate for each treatment. The experiments were repeated three times.

Lactate dehydrogenase (LDH) cytotoxicity

LDH released by damaged cells into the cell culture supernatant was determined using LDH cytotoxicity detection kit (Clontech laboratories, CA, USA), according to the manufacturer's instructions. The amount of LDH activity correlates to the number of damaged cells in the culture. LDH present in the culture supernatant participates in a coupled reaction converting a yellow tetrazolium salt into a red formazan product. The percentage of dead cells was calculated by the following formula of the absorbance values:

$$(\text{triplicate absorbance} - \text{low control})/(\text{maximum absorption} - \text{low control}) \times 100.$$

Maximum absorption was obtained by treating the cells with 1% Triton X-100. The amount of enzyme activity was measured in a microplate reader by absorbance at 490 nm. Each experiment was done in triplicate for each treatment. The experiment was repeated three times.

Hoechst 33342

After the treatment period, the medium was aspired and cells were fixated with 70% ethanol at 4°C for 30 min. Cells were subsequently stained for 40 min with 10 μg/mL of Hoechst 33342 (Sigma). Hoechst 33342 is a cell fluorescent permeable dye with an affinity for DNA. Hoechst 33342 enters cells with intact or damaged membranes and stains DNA in blue, thereby allowing evaluation of cell number in each well. Excitation was done at 346 nm and emission wavelength was determined at 460 nm in FLUOstar spectrofluorometer microplate reader. The experiment was done in triplicate for each treatment. All experiments were repeated at least three times.

Measurement of intracellular reactive oxygen species (ROS)

The generation of ROS, after exposure to increasing dopamine concentrations, was measured using H_2DCFDA (Sigma, Israel), which is incorporated into the cells and cleaved into fluorescent DCF in the presence of ROS. A 10 μM H_2DCFDA was added to the cell suspension, and the cells were incubated in the dark at 37°C for 10 min. DCF fluorescence was measured by FLUOstar spectrofluorometer microplate reader at 520 nm. The generation of ROS was quantitatively assayed by the increase in DCF fluorescence and expressed as percentage of control. Each experiment was repeated at least three times in triplicate.

RNA isolation and real-time quantitative PCR

Total RNA was isolated from cultured neuroblastoma cells using a commercial reagent TriReagent™ (Sigma). The amount and quality of RNA was determined spectrophotometrically using the ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). First-strand cDNA synthesis was carried out from 1 μg of the total RNA using random primer (Invitrogen, Carlsbad, CA, USA) and RT-superscript II (Invitrogen, Carlsbad, CA, USA) reverse transcriptase. Real-time quantitative reverse transcription polymerase chain reaction (PCR) of the desired genes was performed in an ABI Prism 7700 sequence detection system (Applied biosystems, Foster City, CA, USA) using Sybr green PCR master mix (Applied biosystems, Foster City) and the following primers: GAPDH (used as 'housekeeping' gene) sense: CGACAGTCAGCCGCATCTT, GAPDH antisense: CCAATACGACCAAATCCGTTG; DJ-1 sense: CATGAGGCGAGCTGGGATTA, DJ-1 antisense: GCTG GCATCAGGACAAATGAC. Real-time quantitative PCR (qPCR) was performed using AbsoluteTM QPCR SYBR® Green ROX Mix, in triplicates. Quantitative calculations of the gene of interest versus GAPDH were done using the ddCT method.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 and were then incubated in a blocking solution followed by overnight incubation with mouse anti-pERK1,2 (1:50; Santa-Cruz Biotechnology, CA, USA), at 4°C followed by incubation with alexa-568 conjugated goat anti-rabbit antibodies (1:1,000; Molecular probes, Invitrogen, Carlsbad, CA, USA).

In vivo 6-hydroxydopamine hemiparkinsonian mouse model

Eight-week-old male C57BL/6 mice (Harlan, Israel; 22–28 g) were used for 6-hydroxydopamine hemiparkinsonian mouse model experiments. All animals were housed in standard conditions, in a constant temperature (22 ± 1°C), relative humidity (30%), 12-h light: 12-h dark cycle, with free access to food and water. Surgical procedures were performed under the supervision of the Animal Care Committee at the Rabin Medical Center and at Tel Aviv University, Tel Aviv, Israel. Mice received a unilateral, right intrastriatal injection of 4 μg 6-hydroxydopamine hydrobromide (Sigma) using a stereotaxic surgical

procedure. Injections were targeted to the central striatum using the following coordinates: 0.5 mm anterior to bregma, 2.0 mm lateral to bregma, and 2.5 mm deep to the skull surface. Treatments were administered in a volume of 2.0 μL at a rate of 0.5 μL/min. Twenty-four hours after 6-hydroxydopamine lesioning, striatal tissue was collected from both the injected and intact sides for DJ-1 and tyrosine hydroxylase (TH) analysis. For analysis of acute 6-hydroxydopamine effects on phosphokinases striatal tissue was excised after 30 and 45 min of 6-hydroxydopamine injection.

Protein extraction and Western blot analysis

Protein extraction and Western blotting were performed, as previously described (Lev et al. 2006). The membranes were probed with rabbit anti-DJ-1 antibody (1:2,000; Chemicon Laboratories, Yavne, Israel), mouse anti-phospho-ERK 1,2 (1:500; Santa-Cruz Biotechnology, Santa Cruz, CA, USA), and mouse anti-tyrosine hydroxylase (TH, 1:10,000, Sigma) and with mouse anti-beta-actin (1:10,000, Sigma) or rabbit anti-emerin (1:5,000; Santa Cruz), followed by horseradish peroxidase conjugated secondary antibody (1:10,000; Sigma) and developed with the Super Signal West Pico Chemiluminescent substrate (Pierce Biotechnology, Rockford, IL, USA). Densitometry of the specific protein bands was preformed by VersaDoc® imaging system and Quantity One® software (BioRad, Rishon Lezion, Israel).

Statistical analysis

Comparisons of two groups were conducted using a two-tailed Student's *t* test. Statistical analyses among three or more groups were performed using analysis of variance (ANOVA) followed by least-significant difference (LSD) post hoc comparison. Differences among groups were considered significant if the probability (*P*) of error was less than 5%.

Results

Vulnerability to dopamine toxicity and accumulation of intracellular ROS depend on DJ-1 expression levels

Loss-of-function DJ-1 mutations are linked to the degeneration of dopaminergic neurons and PD. Therefore, we hypothesized that decreasing DJ-1 levels by siRNA for DJ-1 may predispose dopaminergic SH-SY5Y neuroblastoma cells to dopamine-induced cell death, while overexpression of DJ-1 may have a protective effect. As an experimental in vitro platform, we generated human neuroblastoma cells overexpressing DJ-1 or expressing siRNA for DJ-1 thereby decreasing DJ-1 levels (Fig. 1).

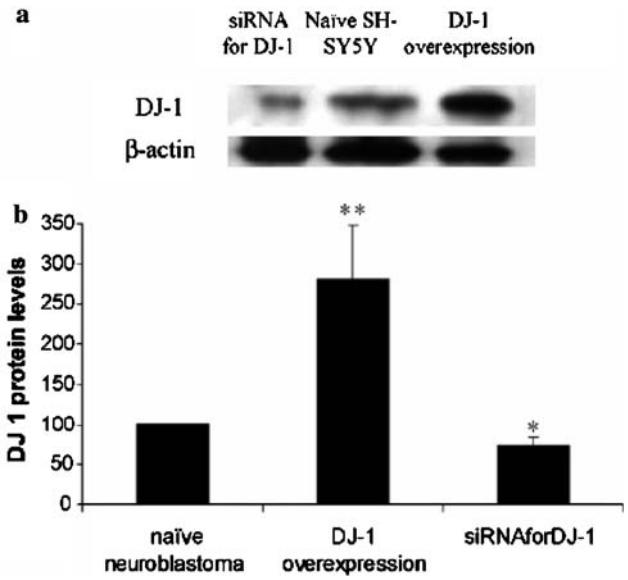
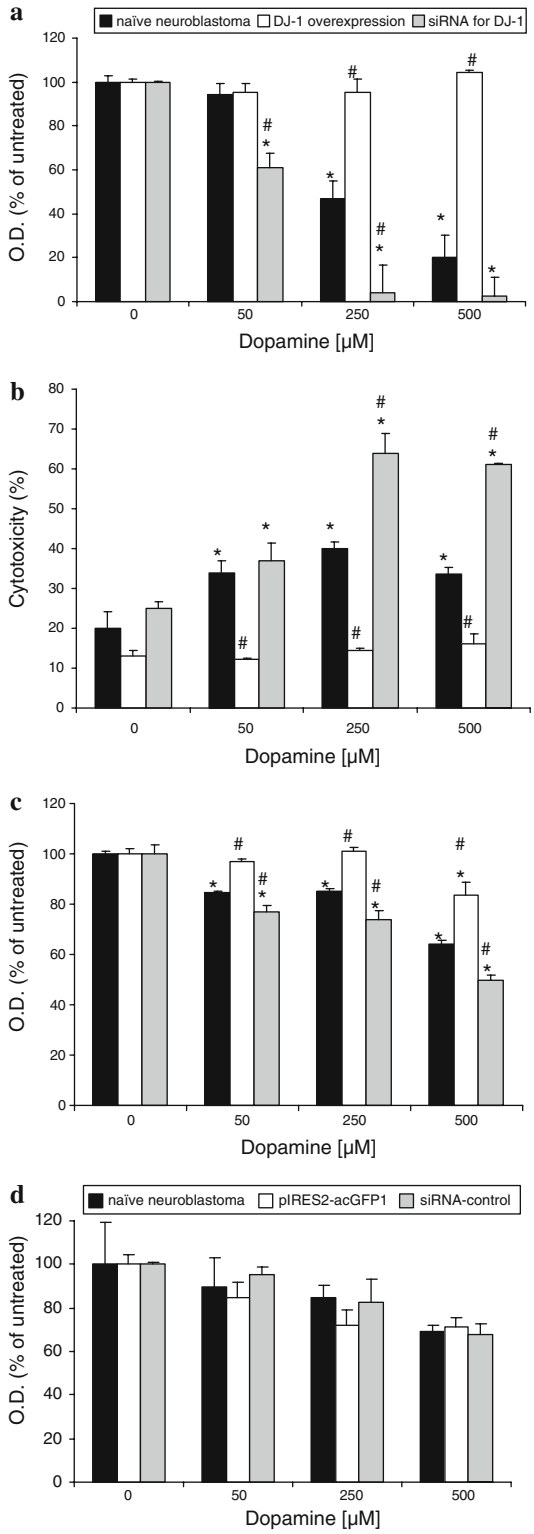


Fig. 1 DJ-1 expression levels in transfected cells. **a** A representative Western blot of DJ-1 protein expression levels in naive SH-SY5Y neuroblastoma cells, cells overexpressing DJ-1 and cells transfected with siRNA for DJ-1. **b** Quantified graph of Western blots analysis of DJ-1 expression levels in control, overexpression and knockdown cells. *Error bars* indicate mean ± SD, * $P < 0.05$ and ** $P < 0.001$ (statistical analysis was done by ANOVA). The analysis was repeated three times

The effects of dopamine exposure were measured using several methods. Alamar blue was used to determine the effects of dopamine exposure on the metabolic activity of the cells. LDH cytotoxicity was used in order to determine dopamine-induced cell death and LDH release into the medium. Hoechst 33342 was used in order to quantitate the number of adherent cells in each well after dopamine exposure. Exposure of neuroblastoma cells to increasing doses of dopamine resulted in decreased metabolic activity (Fig. 2a) and cell death (Fig. 2b, c). Dopamine-induced toxicity was dependent on DJ-1 expression levels; overexpression of DJ-1 protected neuroblastoma cells from the toxic effect of dopamine, while decreasing DJ-1 levels by siRNA resulted in increased vulnerability to dopamine exposure (Fig. 2). The vulnerability to dopamine of cells transfected with the control vectors did not statistically differ from that of naïve neuroblastoma cells (Fig. 2d).

In order to investigate whether dopamine toxicity was mediated through oxidative stress, we measured intracellular ROS. Exposure of cells to dopamine caused a rise in oxidative stress as indicated by increased intracellular ROS (Fig. 3). Overexpression of DJ-1 significantly reduced intracellular ROS accumulation after dopamine exposure, while reducing DJ-1 expression levels by siRNA resulted in elevated intracellular ROS accumulation (Fig. 3).



◀ **Fig. 2** Vulnerability to dopamine toxicity depends on DJ-1 expression levels. Exposure to increasing dopamine concentrations (0–500 μM, for 4 h) caused dose dependent toxicity. Overexpression of DJ-1 conferred resistance to dopamine while decreasing DJ-1 levels by siRNA led to increased vulnerability to dopamine. **a** Alamar blue was used to determine the effects of dopamine exposure on the metabolic activity of the cells. Exposure to increasing doses of dopamine led to inhibition of cell metabolism. Cells overexpressing DJ-1 were more resistant to dopamine while cells expressing siRNA for DJ-1 were more vulnerable to dopamine toxicity. Data are presented as means ± SD. Statistical analysis was done by ANOVA. *Asterisk* indicates OD of treated cells versus no treatment (of the same cells), $P < 0.05$. *Ash* indicates OD of transfected cells versus naïve neuroblastoma treated by the same dopamine concentration, $P < 0.05$. Each experiment was repeated three times in triplicate. **b** LDH cytotoxicity was used in order to determine dopamine-induced cell death and LDH release into the medium. Cytotoxicity is expressed as percentage of dead cells calculated as instructed by the kit manufacturer, as described in the methods section. Exposure to increasing doses of dopamine led to cell death. Cells overexpressing DJ-1 were more resistant to dopamine, while cells expressing siRNA for DJ-1 were more vulnerable to dopamine toxicity. Data are presented as means ± SD. Statistical analysis was done by ANOVA. * Significantly increased cell death of dopamine-treated cells versus no treatment (of the same cells), $P < 0.05$. # Significant change in cell death of transfected cells versus naïve neuroblastoma cells treated by the same dopamine concentration, $P < 0.05$. Each experiment was repeated three times in triplicate. **c** Hoechst 33342 was used in order to quantitate the number of adherent cells in each well after exposure to 0–500 μM dopamine. Dopamine-induced toxicity was dependent on DJ-1 expression levels. Data are presented as means ± SD. Statistical analysis was done by ANOVA. * Significantly increased cell death of dopamine-treated cells versus no treatment (of the same cells), $P < 0.05$. # Significant change in cell death of transfected cells versus naive neuroblastoma cells treated by the same dopamine concentration, $P < 0.05$. Each experiment was repeated three times in triplicate. **d** Hoechst 33342 was used in order to quantitate the number of adherent cells of naïve neuroblastoma cells, cells transfected with pIRES2-acGFP1 empty vector or cell transfected with scrambled siRNA as control for siRNA for DJ-1, after exposure to 0–500 μM dopamine. No significant differences were observed. Data are presented as means ± SD. The experiment was repeated three times in triplicate

Dopamine exposure leads to ROS-mediated upregulation of DJ-1

Since DJ-1 was shown to be protective against dopamine toxicity we hypothesized that exposure to dopamine might lead to upregulation of DJ-1. Indeed, we found that naïve neuroblastoma cells augment DJ-1 expression levels in response to dopamine. Exposure to 50 μM dopamine resulted in a rapid increase in DJ-1 mRNA levels which started within 1 h (Fig. 4a). Pretreatment with the antioxidant *N*-acetyl-cysteine (NAC) abolished the elevation of DJ-1 mRNA induced by dopamine exposure (Fig. 4b), suggesting that the upregulation of DJ-1 is mediated by intracellular ROS generation. In order to verify whether there is upregulation of DJ-1 protein levels we performed Western blotting of neuroblastoma cell extracts that were exposed to dopamine as compared to non treated cells. Significantly increased DJ-1 protein levels were detected after dopamine exposure (Fig. 4c, d). Once more, pretreatment with NAC abolished the upregulation of DJ-1 protein induced by dopamine exposure (Fig. 4c, d).

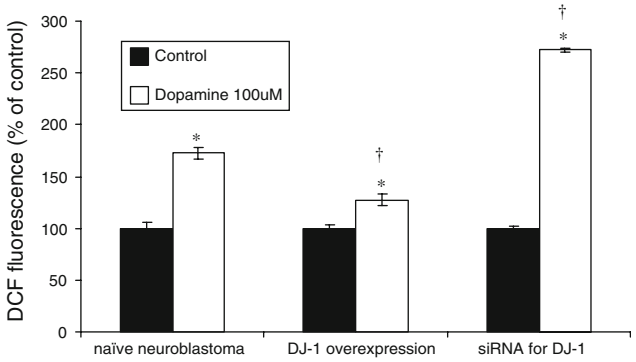


Fig. 3 Dopamine-induced intracellular ROS accumulation depends on DJ-1 expression levels. Dopamine exposure led to increased intracellular ROS, as quantified by the DCF assay. Decreased DJ-1 expression by siRNA for DJ-1 led to an increase in dopamine-induced intracellular ROS. Overexpression of DJ-1 affected a decrease of intracellular ROS. Data are presented as means ± SD, * $P < 0.001$ (*t* test, ROS induced by dopamine versus no treatment). † LSD $P < 0.001$ (ANOVA, dopamine-induced ROS in DJ-1 overexpression or siRNA for DJ-1 as compared to naive neuroblastoma). Each experiment was repeated three times in triplicate

Upregulation of DJ-1 is mediated through activation of extracellular signal-regulated kinase (ERK) 1 and 2

Recent reports indicate that protein kinases, especially the mitogen-activated protein kinases (MAPK) participate in the critical steps of neurotoxic cascades (Leak et al. 2006). Therefore, we investigated the possible involvement of MAPK in the signal transduction pathway that leads to upregulation of DJ-1. Dopamine exposure led to a rapid phosphorylation of extracellular signal-regulated kinase (ERK) 1 and 2 as shown by Western blotting (Fig. 5a, b) and by immunocytochemistry using anti-phospho-ERK antibodies (Fig. 5c). ERK1, 2 activation preceded up-regulation of DJ-1 mRNA. Inhibition of MAPKK by PD-98059 attenuated dopamine-induced DJ-1 upregulation, as shown by real-time PCR (Fig. 5d) and Western blotting (Fig. 5e, f). These results indicate that dopamine exposure leads to rapid activation of ERK 1, 2, leading to DJ-1 upregulation.

In vivo assessment of ROS-induced DJ-1 changes using a 6-hydroxydopamine-induced hemiparkinsonian mouse model

Subsequently, we examined whether ROS also induces upregulation of DJ-1 in vivo. In order to evaluate such in vivo changes, we used a hemiparkinsonian mouse model induced by unilateral intrastriatal 6-hydroxydopamine lesioning. In order to enable the evaluation of the changes in DJ-1 expression levels we used a mild insult of 4 µg 6-hydroxydopamine injection into the right striatum. Twenty-four hours after 6-hydroxydopamine lesioning,

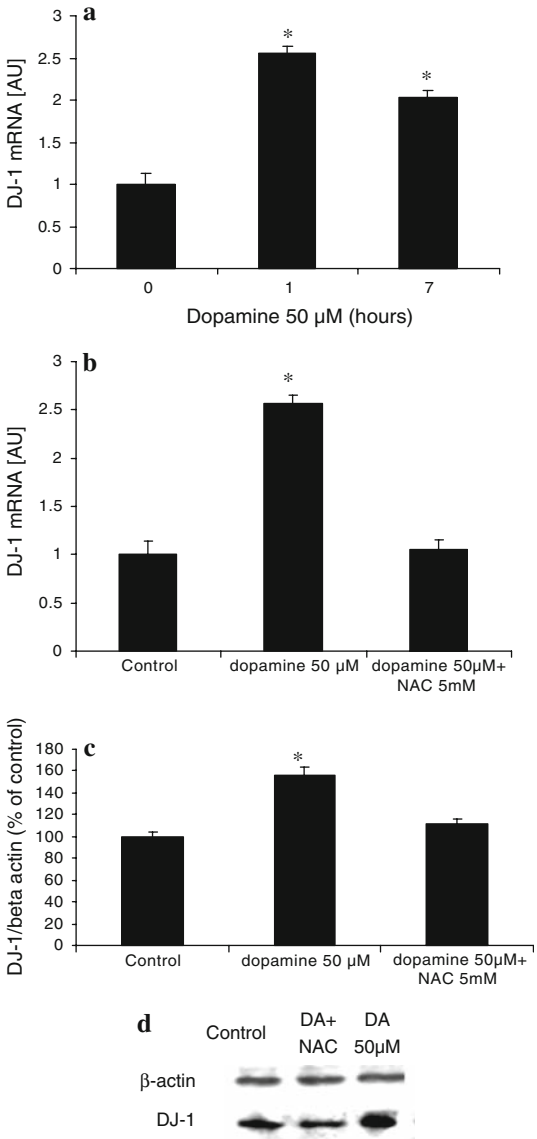


Fig. 4 Dopamine exposure leads to the upregulation of DJ-1 in naïve neuroblastoma SH-SY5Y cells. **a** Exposure of naive neuroblastoma to 50 µM dopamine induced upregulation of DJ-1 mRNA within 1 h. DJ-1 mRNA levels were quantified by real-time PCR, as described in the methods section. GAPDH was used as reference gene. Real-time quantitative PCR was repeated three times, in triplicate. Data are presented as means ± SD, * $P < 0.001$ (ANOVA). **b** DJ-1 mRNA level was quantified after 1 h of exposure to 50 µM dopamine, with and without antioxidant treatment. Significant upregulation of DJ-1 mRNA was noted after exposure to dopamine. Pre-treatment with 5 mM *N*-acetyl cysteine (NAC) abolished DJ-1 mRNA upregulation. DJ-1 mRNA levels were quantified by real-time PCR. GAPDH was used as reference gene. Real-time quantitative PCR was repeated three times, in triplicate. Data are presented as means ± SD, * $P < 0.001$ (ANOVA). **c** Quantization of Western blots of total cell lysates from naïve neuroblastoma cells demonstrates the upregulation of DJ-1 protein levels 24 h after dopamine exposure. Pre-treatment with 5 mM NAC abolished the elevation of DJ-1 protein levels. Data are presented as means ± SD of three independent experiments, * $P < 0.001$ (ANOVA). **d** Representative Western blot of DJ-1 protein levels after exposure to 50 µM dopamine with or without NAC pre-treatment

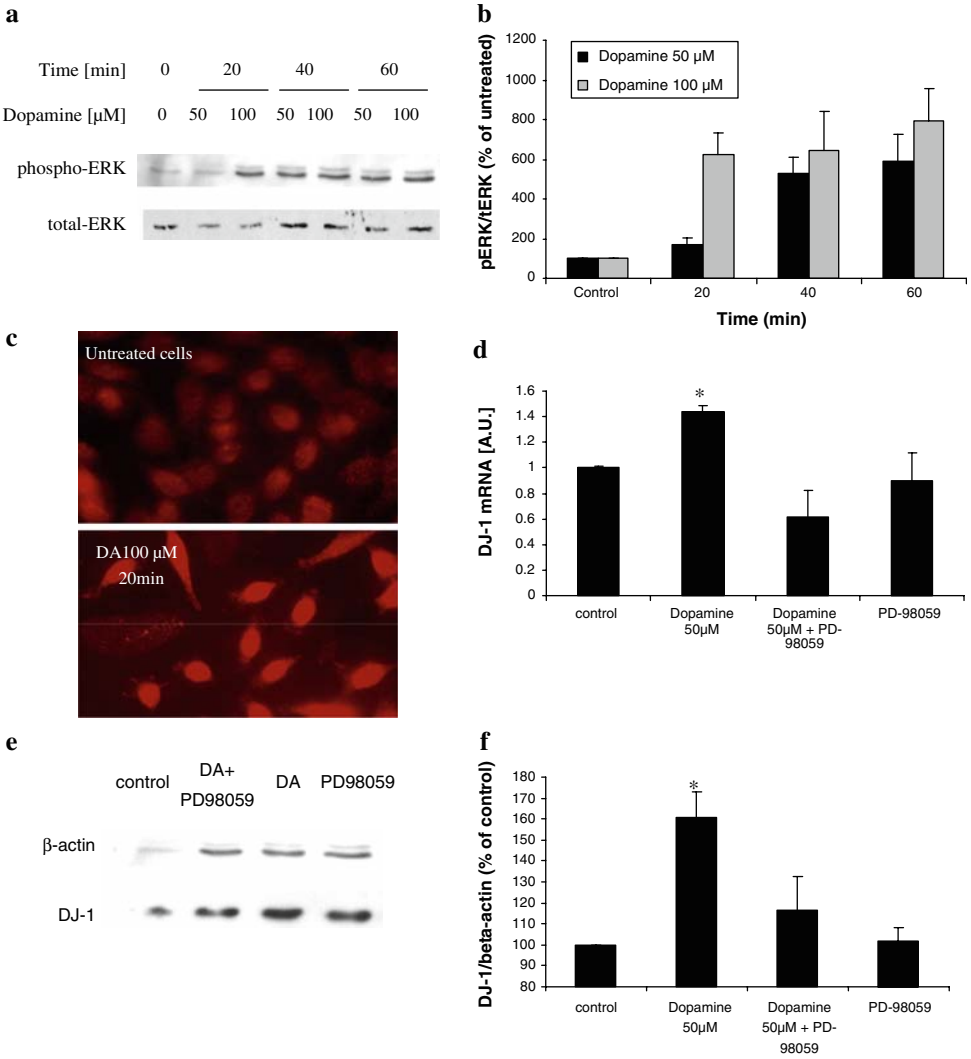


Fig. 5 Upregulation of DJ-1 is mediated by phosphorylation of ERK-1, 2. Exposure of naive neuroblastoma to dopamine induces phosphorylation of ERK-1, 2 within 20–60 min as illustrated by Western blotting. A representative blot is presented in **a** and densitometric quantification is presented in **b**. The experiment was repeated three times. Immunocytochemistry for phosphorylated ERK-1, 2 (**c**) illustrates the increase in ERK-1, 2 phosphorylation 20 min after exposure to dopamine 100 μM. **d** Inhibition of ERK-1, 2 phosphorylation by PD-98059 inhibited DJ-1 mRNA upregulation induced by exposure to 50 μM dopamine for 1 h. Real-time quantitative PCR was repeated three times, in triplicate. Data are presented as means ± SD of three independent experiments, * $P < 0.05$ (ANOVA). **e** A representative Western blot demonstrate that PD-98059 abolished the increase in DJ-1 protein levels induced by exposure to 50 μM dopamine. **f** Quantified graph of Western blots analysis of DJ-1 expression levels in cells treated with 50 μM dopamine or PD-98059. *Error bars* indicate mean ± SD, * $P < 0.05$ (ANOVA). The analysis was repeated three times. Data are presented as means ± SD of three independent experiments

striatal tissue was collected from both the injected and intact sides for DJ-1 and tyrosine hydroxylase (TH) analysis. We found an increased expression of DJ-1 protein 24 h after 6-hydroxydopamine injection in the lesioned striatum as compared to the unlesioned side (Fig. 6a, b). For analysis of acute 6-hydroxydopamine effects on phosphokinases, striatal tissue was excised after 30 and 45 min of 6-hydroxydopamine injection. Consistent with the in vitro results, acute exposure to 6-hydroxydopamine led to the increased phosphorylation of ERK1, 2 (Fig. 6c, d).

Discussion

This study suggests a novel mechanism of neuroprotection against dopamine toxicity. Overexpression of DJ-1 led to increased resistance to dopamine toxicity and reduced intracellular ROS. Reducing DJ-1 levels by siRNA demonstrated contrary effects, increasing intracellular ROS and the susceptibility to dopamine toxicity. Moreover, we found that exposure to dopamine- or 6-hydroxydopamine-induced ROS led in vitro and in vivo to upregulation of DJ-1. Similarly, we previously reported that susceptibility

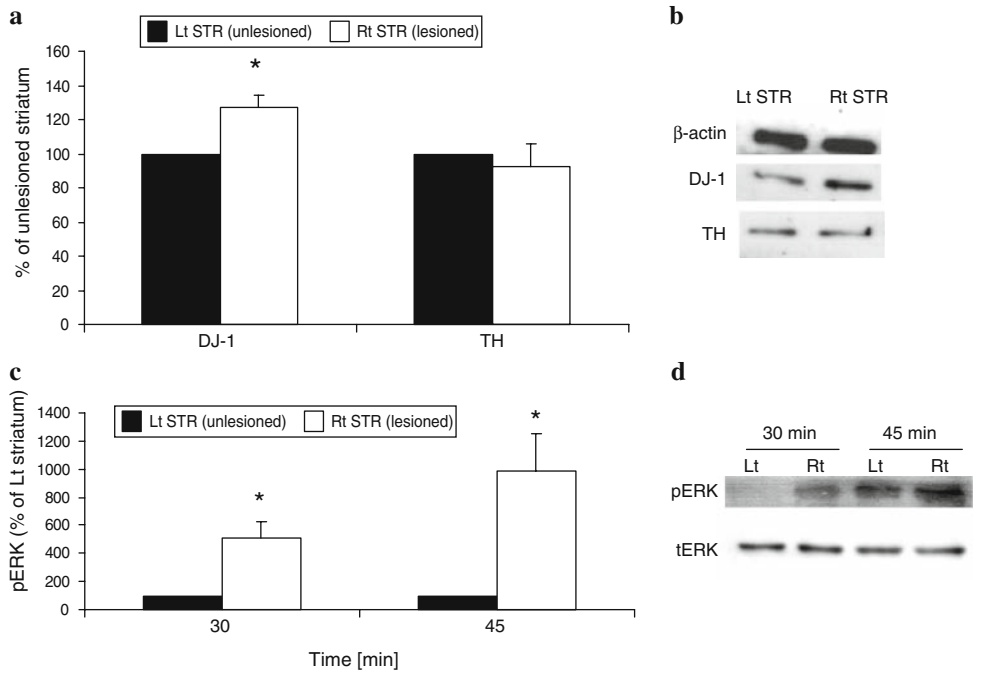


Fig. 6 In vivo intrastriatal 6-hydroxydopamine injection leads to phosphorylation of ERK-1, 2 followed by upregulation of DJ-1. **a** Unilateral (*right*) in vivo 6-hydroxydopamine intrastriatal injection led to the elevation of DJ-1 protein levels, as evaluated by Western blotting. Mildly toxic dose of 6-hydroxydopamine was preferred in order to enable the evaluation of intracellular changes in protein levels prior to cell death, as indicated by the non significant change in tyrosine hydroxylase (*TH*) levels. Data presented as means ± SD. Statistical analysis was done using *t* test. * $P < 0.05$ versus the unlesioned striatum. **b** A representative Western blot of DJ-1 and TH levels in the lesioned (*Rt*) versus the unlesioned (*Lt*) striatum (*STR*). **c** In vivo striatal injection of 6-hydroxydopamine led to rapid phosphorylation of ERK-1, 2 as evaluated by Western blotting. Data presented as means ± SD. Statistical analysis was done using *t* test. * $P < 0.05$ versus the unlesioned striatum. **d** A representative Western blot of phospho-ERK 1, 2 (*pERK*) versus total-ERK (*tERK*) levels in the lesioned (*Rt*) versus the unlesioned (*Lt*) striatum (*STR*) 30 and 45 min after 6-hydroxydopamine injection

of neuroblastoma cells to other dopaminergic neurotoxins such as rotenone and 6-hydroxydopamine was dependent on DJ-1 levels (Lev et al. 2008). Therefore, as an in vivo platform, we used intrastriatal 6-hydroxydopamine lesioning, a commonly used in vivo model for Parkinson's disease. In these mice, upregulation of DJ-1 was detected in the lesioned striatum.

Furthermore, we found that dopamine-induced upregulation of DJ-1 was mediated by the MAP kinases pathway through activation of ERK 1, 2. Inhibition of ERK 1, 2 phosphorylation by the MAPKK inhibitor PD-98059 abolished dopamine or 6-hydroxydopamine-induced DJ-1 upregulation. Isoforms of the mitogen-activated protein kinase ERK have been implicated in both cell survival and cell death. In a recent study ERK 1, 2 were shown to play a role in cell response to oxidative insults (Lin et al. 2008). Exposure of dopaminergic cells to 6-hydroxydopamine was accompanied by a rapid and large increase in phosphorylated ERK1, 2 (Lin et al. 2008). Inhibition of the early phosphorylation of ERK 1, 2 with U0126 increased the generation of ROS by 6-hydroxydopamine as well as 6-hydroxydopamine-induced toxicity (Lin et al. 2008). In contrast, activation of caspase-3 by 6-hydroxydopamine, occurring after 6 h, was increased by inhibition of the early phosphorylation of ERK1, 2. These results suggest that the rapid activation of ERK 1, 2 in dopaminergic cells by oxidative stress serves as a self-protective response, reducing the content of ROS and caspase-3 activation and increasing cell survival. These findings are in agreement with our results. We propose DJ-1 upregulation as the mean of abrogation of the toxicity implicated by the oxidative insult.

DJ-1 is widely distributed and is highly expressed in the brain and extra cerebral tissues (Bandopadhyay et al. 2004; Bader et al. 2005; Olzmann et al. 2007). However, DJ-1 mutations are known to cause early onset autosomal recessive PD (Bonifati et al. 2003; Abou-Sleiman et al. 2003; Hedrich et al. 2004). Although we do not have post mortem studies indicating which neurons are affected by the loss of functional DJ-1 in these patients, since they suffer from parkinsonian symptoms, it is likely that the dopaminergic neurons are affected by their disease. How do dopaminergic neurons become particularly sensitive to mutations that lead to loss of DJ-1 function? Dopamine is inherently unstable and can oxidize to generate ROS. It is synthesized in the cytosol and rapidly sequestered into

synaptic vesicles, where the low vesicular pH and the absence of monoamine oxidase limits dopamine breakdown. Cytosolic dopamine leads to increased oxidative damage and cell death (Blum et al. 2001). Dopamine-producing neurons are specifically susceptible to oxidative stress since neurotransmitters produced by other neurons are less toxic. Furthermore, a recently published study reported that another gene linked to inherited PD, PINK1, phosphorylates the downstream effector TRAP1 to prevent oxidative-stress-induced apoptosis (Pridgeon et al. 2007). This implies that the dysregulation of genes involved in oxidative damage prevention plays a central role in PD pathogenesis.

In our study we have demonstrated that intracellular ROS levels after exposure to dopamine are dependent on DJ-1 expression levels. However, it is unlikely that direct scavenging of free radicals by DJ-1 account for all of its protective effects, since these effects are only modest. Intracellular ROS may only serve as the signal leading to upregulation of DJ-1. Oxidative-induced changes in DJ-1 (Canet-Aviles et al. 2004) imply that DJ-1 may serve as a sensor for increased cytoplasmic levels of ROS, and its rapid upregulation may be a first line defence mechanism of dopaminergic neurons that acts to rapidly counteract dopamine toxicity. Therefore, further research is needed in order to elucidate other mechanisms of cell protection inferred by upregulation of DJ-1.

In conclusion, the findings presented suggest a novel mechanism in which ROS, generated by free cytoplasmic dopamine, lead to the rapid upregulation of DJ-1, which in turn protects dopaminergic neurons against dopamine toxicity and lowers intracellular ROS. This mechanism helps clarify how mutations in DJ-1 trigger early onset PD. Moreover, modulating DJ-1 expression or function might serve as a novel neuroprotective therapy.

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