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Knocking Out DJ-1 Attenuates Astrocytes Neuroprotection Against 6-Hydroxydopamine Toxicity

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Abstract Astrocytes are the most abundant glial cell type in the brain. Impairment in astrocyte functions can critically influence neuronal survival and leads to neurodegeneration. Parkinson's disease (PD) is a common neurodegenerative disorder, characterized by motor dysfunction that results from progressive neuronal loss. Astrocytic dysfunction was demonstrated in human samples and in experimental models of PD. Mutations in DJ-1 (PARK7) leading to loss of functional protein cause familial PD and enhance sensitivity to oxidative insults. Recently, an increase in DJ-1's expression was found in reactive astrocytes in various neurodegenerative disorders. Here we show that lack of DJ-1 attenuates astrocytes' ability to support neuronal cells, thereby leading to accelerated neuronal damage. DJ-1 knockout mice demonstrated increased vulnerability in vivo to 6-hydroxydopamine (6-OHDA) hemiparkinsonian PD model. Astrocytes isolated from DJ-1 knockout mice showed an inferior ability to protect human neuroblastoma cells against 6-OHDA insult both by co-culture and through their conditioned media, as compared to wild-type astrocytes. DJ-1 knockout astrocytes showed blunted ability to increase the expression of cellular protective mechanisms against oxidative stress mediated via Nrf-2 and HO-1 in response to exposure to 6-OHDA. These experiments demonstrated that lack of DJ-1 impairs astrocyte-mediated neuroprotection.

Keywords Parkinson's disease · DJ-1 · 6-hydroxydopamine

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Introduction

Astrocytes, the most abundant glial cell type in the central nervous system, provide metabolic and trophic support to neurons and modulate synaptic transmission (Przedborski 2001; Yanagida et al. 2009). Accordingly, impairment in astrocyte functions can critically influence neuronal survival. Indeed, data are accumulating on their important role in neurodegenerative diseases. Studies show that astrocyte dysfunction and apoptosis may contribute to pathogenesis of many acute and chronic neurodegenerative diseases, such as, cerebral ischemia, Alzheimer's disease, Pick's disease, amyotrophic lateral sclerosis, and Parkinson's disease (PD) (Rizzu et al. 2004; Mullett et al. 2009; Baulac et al. 2009; Yanagida et al. 2009; Halliday and Stevens 2011; Hauser and Cookson 2011).

PD is the second most common progressive neurodegenerative disorder, affecting more than 1 % of the population above the age of 65. Although most of the patients suffer from sporadic disease, a minor subset of patients has the hereditary form. The identification of disease-associated genes helps unravel basic mechanisms in the pathophysiology of the more common sporadic type of the disease. Several genetic mutations leading to familial PD have been discovered (Corti et al. 2011). Among these, DJ-1 gene deletions and point mutations leading to loss of functional protein were identified as a cause of early onset autosomal recessive PD (Bonifati et al. 2003; Abou-Sleiman et al. 2003). Altered DJ-1 levels and isoforms were found in sporadic PD patients and in PD animal models (Waragai et al. 2006, 2007; Hong et al. 2010; Shi et al. 2011; Akazawa et al. 2010; Devic et al. 2011), implying that DJ-1 has also a role in the sporadic form of the disease. Accumulating evidence suggests that DJ-1 has many functions and plays an important role in the defensive response against oxidative insults (Canet-Aviles et al. 2004; Choi et al. 2006; Lev et al. 2008, 2009, 2012).

In human brains, DJ-1 is highly expressed in astrocytes and is sensitive to oxidative stress conditions (Bandopadhyay et al. 2004; Rizzu et al. 2004). Studies on rodent brains confirmed that DJ-1 is expressed both in neurons and in glial cells, including astrocytes (Bader et al. 2005; Bandopadhyay et al. 2004; Kotaria et al. 2005; Xie et al. 2009). Altered DJ-1 expression in reactive astrocytes in various acute and chronic CNS disorders might imply that astrocytic DJ-1 is important for neuronal–glial interactions in various neurodegenerative diseases. The aim of this study was to investigate the effect of DJ-1 expression on the neuroprotective abilities of astrocytes.

Methods

In Vivo Experimental Models

All animal experiments were carried out according to the National Institute of Health guidelines for the care and use of laboratory animals and approved by the ethical committee of the Tel Aviv University, Israel. Adult male C57bl/6 mice were purchased from Harlan, Israel at the age of 6–8 weeks, weighing 22–28 g. Animals were housed at a standard temperature (22±1 °C), relative humidity (30 %), and in a light-controlled environment (12-h light/12-h dark cycle) with ad libitum access to food and water. Animals were acclimatized for 1 week prior to experimentation, then randomly divided into the experimental groups.

Transgenic DJ-1 Knockout Mice

DJ-1 knockout mice were purchased from Jackson Laboratory. The experimental protocols used were approved by the Insitutional Animal Care and Use Committee at Tel Aviv University. Genetic identification of the transgenic and wild-type (WT) littermates was done using tail cDNA extraction and amplification with the following primers: GCTGAAACTCTGCCATGTGA, ACTTGGAGTCCCCTCGTTTT, TGGATGTGGAATGTG TGCGAG. In the knockout mice, the PCR product is of 280 bp while in WT it is 311 bp.

In Vivo 6-OHDA Hemiparkinsonian Mouse Model

Eight-week-old male C57bl/6 mice (Harlan, Israel; 22–28 g) were used for 6-OHDA hemiparkinsonian mouse model experiments. Mice received a unilateral, right intrastriatal injection of 4 μg 6-OHDA hydrobromide (Sigma) using a stereotaxic surgical procedure. Injections were targeted to the central caudate-putamen using the following coordinates: 0.5 mm anterior to the bregma, 2.0 mm lateral to the bregma, and 2.5-mm deep to the skull surface. Toxin or vehicle was administered in a volume of 2.0 μL at a rate of 0.5 μL/min.

Behavioral Testing: Amphetamine-Induced Rotation Asymmetry

Mice were tested for amphetamine-induced turning behavior 14 and 28 days after intrastriatal 6-OHDA injection. Motor behavior was recorded by video tracking software and systems, EthoVision XT (Noldus), and monitored for 60 min after injection of amphetamine (2.5 mg/kg, s.c.). The net rotation asymmetry score for each test was calculated by subtracting contralateral turns from the turns ipsilateral to the lesion.

HPLC Analysis of Dopamine Content

Each brain hemisphere was subjected to high-performance liquid chromatography (HPLC). Dopamine (DA), 3,4-dihydroxyphenylacetic acid and homovanillic acid concentrations in tissue samples were determined by HPLC method with electrochemical detection. Tissue samples were homogenized on ice (1:10, *w*/*v*) in 0.1 M perchloric acid. Tissue homogenates were centrifuged for 20 min at 15,000×*g* at 4 °C. Supernatants were filtered through polypropylene membrane (Spin-X centrifuge tube filter, Corning, NY, USA). The samples were kept in −80 °C until analyzed by HPLC.

The supernatants of each tissue extract were injected directly to the HPLC system through a reverse phase column (GL-Science, Inertsil ODS-2 5um 4.6×150 mm at room temperature) coupled to an electrochemical detector Coulochem II ESA with a conditioning cell model 5021 and analytical cell model 5011. The working potential was set to 0.35 V on the conditioning cell and 0.1 and −0.35 V on the analytical cell. The mobile phase was 0.05 M monobasic sodium phosphate, with 80 mg/L EDTA, 125 mg/L heptane sulfonic acid, 55 mL of methanol, and 50 mL of acetonitrile pH=2.7. The flow rate was 1.5 mL/min.

Tissue Processing and Immunohistochemistry

Four weeks after 6-OHDA lesioning, mice were deeply anaesthetized by intraperitoneal (i.p.) injection of 7 % chloral hydrate and then underwent cardiac perfusion through the left ventricle with normal saline, followed by 4 % paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4. Brains were removed and fixed by immersion in 4 % paraformaldehyde in 0.1 M phosphate buffer (PB) for 24 h. Following cryoprotection in 30 % sucrose in 0.1 M PB for 48 h and freezing the brains in 2-methylbutane, 10-μm-thick cryosections were prepared with a cryostat. The sections were mounted on slides and stored at −20 °C until use.

Striatal and nigral sections were used for tyrosine hydroxylase (TH) immunohistochemistry. The sections were boiled in 0.01 M citrate buffer, pH 6.0 for antigen unmasking, than permeabilized in PBS containing 0.05 % Triton X-100. Sections were rinsed several times in PBS. Tissue endogenous peroxidase was inactivated by incubation in 0.3 % hydrogen peroxide for 10 min. After three washes in PBS, the sections were pre-incubated in blocking buffer (PBS containing 10 % goat serum) to reduce nonspecific binding and then were incubated overnight at 4 °C in a humidified chamber with anti-TH (1:500, Sigma) or anti- glial fibrillary acidic protein (GFAP) (1:500, Santa Cruz) antibodies. Sections were rinsed in PBS and incubated with secondary antibodies for 30 min at 37 °C following the manufacturer's instructions. The sections were stained with a dimethylaminoazobenzene kit, dehydrated in graded alcohols, cleared with xylene and coverslipped. Control sections were treated with the same protocol but omitting the primary antibody. The images were recorded with an inverted microscope (OLYMPUS) connected to a camera.

In Vitro Experimental Assays

SH-SY5Y Neuroblastoma Cell Line

Human neuroblastoma cells, SH-SY5Y cells, were obtained from ATCC (Rockville, USA) and were grown on tissue culture plates (Greiner, UK) in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10 % fetal calf serum, 1 % L-glutamine and 1 % SPN antibiotics (Biological Industries, Israel). Cells were incubated at 37 °C in a humidified atmosphere with 5 % CO_2 and passaged twice a week.

Astrocytes Primary Cell Culture

Astrocytes were extracted from the cortex of a newborn mouse as we previously described (Fisher-Shoval et al. 2012; Yust-Katz et al. 2012). Briefly, the cortex tissue was dissected from newborn mice brains (p1-p3) and transferred to a Petri dish. All the meninges were pulled off. Pure tissue was transferred to a tube containing cold PBS. The brain was mechanically dissociated and digested by incubation with 1/5*v*/*v* trypsin for 10 min at 37 °C. The reaction was stopped by the addition of complete medium (DMEM, 10 % Serum, SPN, and glutamine) and 50 μL DNAse. The tissue, again, was mechanically digested by pipettation until full dissociation into single cells was accomplished. The cells were washed with complete medium once and centrifuged at 1,100 rpm for 7 min. Supernatant was removed and the pellet was resuspended in a complete medium. The 1×10^6 cells were plated in T75 flask in 10 ml complete medium. The flasks were incubated at 37 °C in 5 % CO_2. The medium was first changed after 24 h and then every 3 days until confluence was achieved, after approximately 8–10 days. Once confluent, the primary culture was placed on a shaker platform horizontally with medium covering the cells, shaken at 250 rpm for 18 h, and the medium that contained microglia was removed. After a few hours, the cells were trypsinized and replated to two new flasks.

The conditioned media of astrocyte cells, grown in serum-free medium (DMEM supplemented with glutamine and antibiotics) for 48 h, was collected and used for the experiments with neuroblastoma SH-SY5Y cells. For co-culture experiments, astrocytes were plated on 12-well Transwells© (BD Falcon™, Becton Dickinson (BD), Franklin Lakes, NJ). The SH-SY5Y cells were plated on 12-well plates and the astrocytes were plated on the upper wells. The transwell© system was subsequently exposed to 6-OHDA (Sigma) for 24 h. The upper wells with the astrocytes were then removed to allow evaluation of SH-SY5Y viability using Alamar blue (Invitrogen).

Alamar Blue Viability Assay

Cells were seeded in 96-well plates at a concentration of 5,000 cells per well and allowed to attach overnight. On the following day, the cells were exposed to increasing doses of 6-OHDA for 24 h. All experiments were done in serum-free medium. Alamar blue is a nontoxic 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 for 2 h. 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 every treatment. The experiments were repeated three times.

Protein Extraction and Western Blotting

Protein extraction and Western blotting were done as previously described (Lev et al. 2008, 2009). The membranes were probed with antibodies for DJ-1 (1:1,000 Santa Cruz), Nrf-2 (1:500, Abcam) and heme oxygenase-1 (HO-1, 1:500, Enzo), and mouse anti-beta-actin (1:10,000, Sigma). Quantification was done by the Odyssey system.

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 post hoc comparison. Differences among groups were considered significant if the probability (P) of error was less than 5 %.

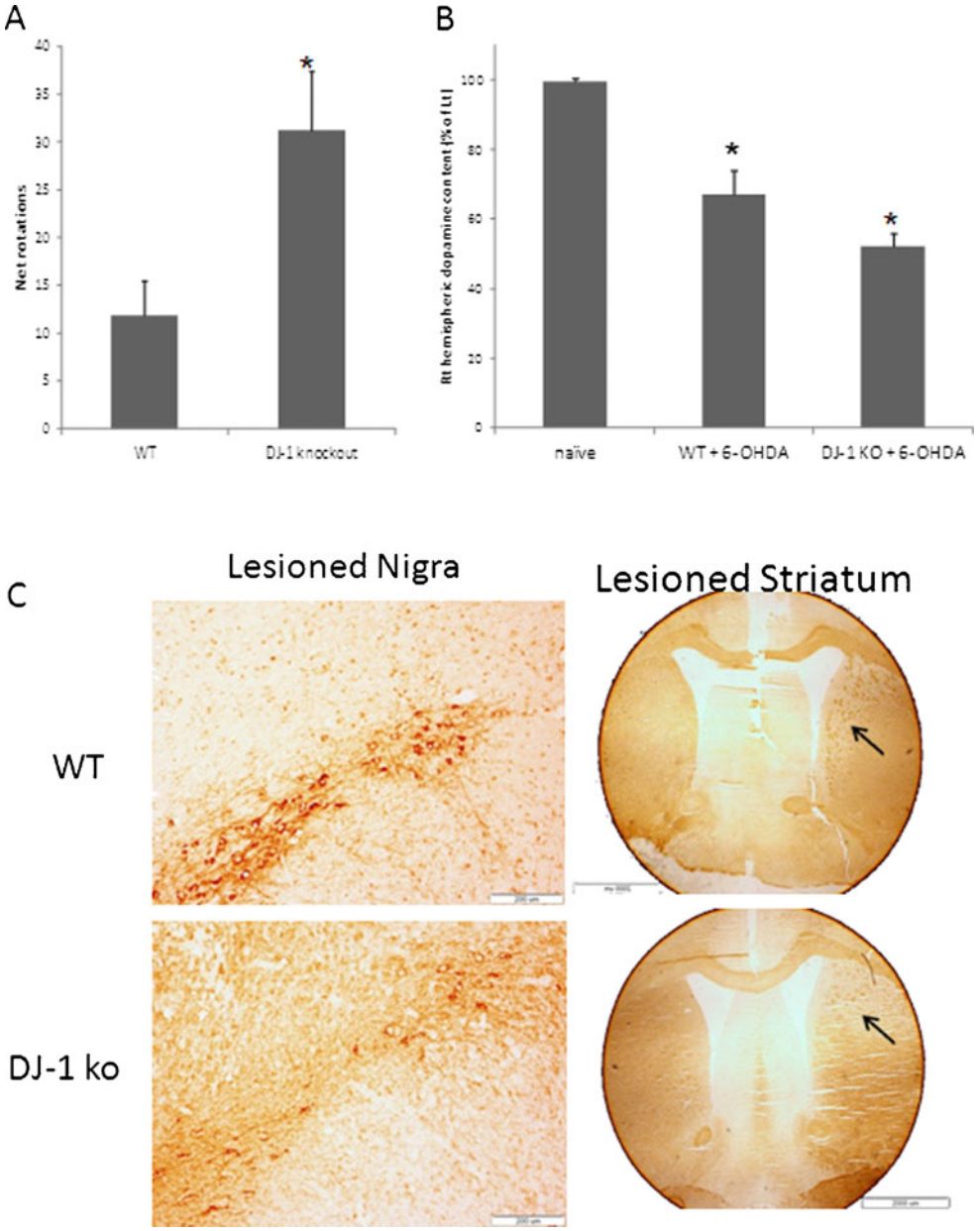

Fig 1 DJ-1 knockout mice are more sensitive to 6-hydroxydopamine toxicity in vivo. A hemiparkinsonian mice model was induced in WT and in DJ-1 knockout mice by stereotactic right intrastriatal injection of 6-OHDA as described in the "Methods" section. Death of dopaminergic neurons resulting in asymmetry between the lesioned (right) and normal (left) hemispheres causes rotational behavior with amphetamine injection. **a** DJ-1 knockout mice showed significantly increased rotational behavior, as compared to WT mice; $n=11$ per group. Data are presented as averages ± s.e.; statistical analysis was done by Student's *t* test, $*p<0.05$. **b** Quantification of hemispheric dopamine content was done by HPLC. 6-OHDA injection into the right hemisphere causes significant reduction in the hemispheric dopamine content. In WT mice, the dopamine content in the lesioned hemisphere decreased to 67 % while in DJ-1 knockout mice to 52 %. Data are presented as averages ± s.e.; $n=3–5$ per group. Statistical analysis was done by ANOVA, $*p<0.05$. **c** Immunohistochemical staining for tyrosine hydroxylase was done on striatal and nigral slices four weeks after 6-OHDA lesioning. Immunohistochemical staining was done on three mice per group, ten slides per mouse. DJ-1 knockout mice showed decreased TH staining indicating enhanced loss of dopaminergic neurons in the right substantia nigra and increased lesion size in the right striatum

Results

DJ-1 Knockout Mice Are More Vulnerable to 6-OHDA Lesioning

Using a stereotaxic surgical procedure, C57bl/6 or DJ-1 knockout transgenic mice were striatally lesioned by 6-OHDA. Behavioral quantification of dopamine depletion, done by amphetamine-induced rotations 2 and 4 weeks after the lesioning, demonstrated that DJ-1 knockout mice are more vulnerable to 6-OHDA lesioning (Fig. 1a). One month after 6-OHDA lesioning, the mice were sacrificed and hemispheric dopamine content was quantified in both hemispheres by HPLC (Fig. 1b). Analysis revealed that intrastriatal 6-OHDA injection resulted in significantly decreased dopamine content in the lesioned hemisphere as compared to the intact hemisphere. Hemispheric dopamine content was similar in unlesioned hemispheres and in naive mice in transgenic DJ-1 knockout mice and in WT (data not shown). Intrastriatal injection of the same toxic dose and at the same volumes of 6-OHDA resulted in decreased dopamine content in the lesioned hemisphere in DJ-1 knockout mice as compared to WT mice. In WT mice, the dopamine content in the lesioned hemisphere decreased to 67 % while in the DJ-1 knockout mice it decreased to 52 % ($p<0.05$, Fig. 1b).

Immunohistological study using anti-thyrosine hydroxylase (TH) antibodies indicated that 6-OHDA lesioning resulted in significant loss of dopaminergic cell bodies in the substantia nigra and dopaminergic cell terminals in the striatum in the lesioned hemisphere in both WT and in DJ-1 knockout mice (Fig. 1c). A significantly increased damage in the dopaminergic system was found in DJ-1 knockout mice as compared to WT mice (Fig. 1c). Immunohistological staining against GFAP of nigral and striatal slices 4 weeks after 6-OHDA lesioning did not reveal significant alterations (data not shown).

DJ-1 Knockout Astrocytes Show Attenuated Ability to Protect Neuronal Cells Against 6-OHDA Toxicity

Primary astrocyte cultures were obtained from newborn DJ-1 knockout and WT mice. Western blotting analysis confirmed that only astrocytes obtained from WT mice, but not DJ-1 knockout mice, express DJ-1 (data not shown). Exposure of cultured astrocytes to increasing doses of 6-OHDA (0–50 μM) demonstrated that astrocytes from both WT and DJ-1 knockout mice are resistant to 6-OHDA toxicity, and cell death was not apparent up to 50 μM (Fig. 2). In contrast, neuronal cells are much more sensitive to 6-OHDA toxicity, as demonstrated by sensitivity of neuroblastoma SH-SY5Y cells to 6-OHDA (Fig. 2).

Next we sought to evaluate the involvement of DJ-1 in the astrocytic neuroprotective abilities. The viability of both WT and DJ-1 knockout astrocytes was not affected by exposure to 6-OHDA (0–50 μM). However, the survival of human neuroblastoma cells, SH-SY5Y, is dramatically affected by increasing doses of 6-OHDA (0–50 μM) (Fig. 2).

First, we examined the ability of astrocytes conditioned media to protect against 6-OHDA-induced neuronal cell death. Conditioned media from both WT and DJ-1 knockout astrocytes demonstrated protective abilities as compared to serum-free medium (Fig. 3a). However, significant differences were present between the abilities of DJ-1 knockout astrocytes and WT astrocytes to preserve neuronal survival with exposure to 6-OHDA (Fig. 3a). Conditioned media obtained from DJ-1 knockout astrocytes showed inferior ability to protect SH-SY5Y neuroblastoma cells against 6-OHDA insult (Fig. 3a) as compared to conditioned media obtained from WT astrocytes. These differences were apparent in all the examined doses of 6-OHDA.

Next, we examined whether the presence of astrocytes during the exposure of the neuroblastoma SH-SY5Y cells to 6-OHDA alters their protective abilities. Again, co-culture with astrocytes significantly protected SH-SY5Y cells from 6-OHDA toxicity (Fig. 3b). Yet, DJ-1 knockout astrocytes showed significantly inferior protective abilities as compared to WT astrocytes (Fig. 3b).

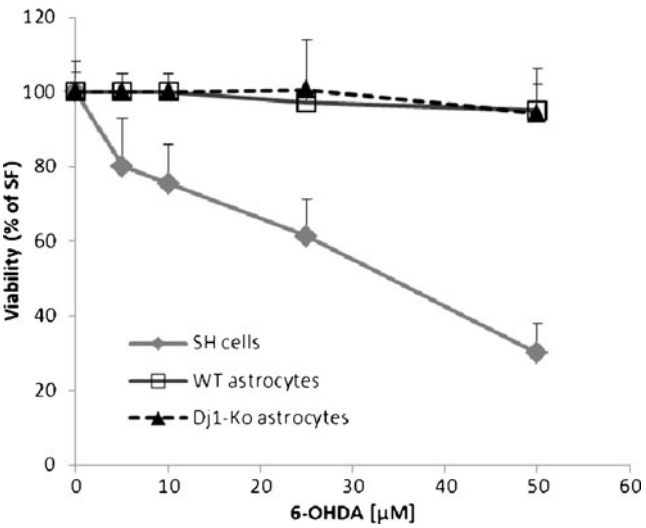

Fig. 2 WT and DJ-1 knockout astrocytes are more resistant to 6-hydroxydopamine toxicity than neuronal cells in vitro. DJ-1 knockout and WT astrocytes and human neuroblastoma cells SH-SY5Y cells were exposed for 24 h to increasing doses of 6-OHDA (0–50 μM). Human neuroblastoma SH-SY5Y cells showed enhanced sensitivity to 6-OHDA toxicity, as compared to WT and DJ-1 knockout astrocytes ($p<0.05$). No significant difference was found between WT and DJ-1 knockout astrocytes Evaluated by Alamar blue assay. Data are shown as averages ± STD. The experiments were done in triplicate for every treatment. The experiments were repeated three times

Reduced Activation of the Nrf2 System in DJ-1 Knockout Astrocytes

The Nrf2 system is an important cellular defense against oxidative insults, including 6-OHDA. Therefore, we examined the ability of DJ-1 knockout versus WT astrocytes to augment Nrf2 expression as well as hemeoxygenase-1 (HO-1), one of its main protective products. The elevation of NRF-2 and HO-1 was measured in the astrocytes following exposure to 6-OHDA at doses that are relevant for astrocytic neuroprotective functions. At these doses, conditioned media and co-culturing of neuronal cells with astrocytes augmented neuronal cell survival, yet DJ-1 knockout astrocytes showed abrogated protective abilities as compared to WT astrocyte (as shown in Fig. 3).

We found that DJ-1 knockout astrocytes have blunted ability to increase the expression of both Nrf2 (Fig. 4) and HO-1 (Fig. 5) in response to exposure to 6-OHDA. The reduced activation of this major defense system may impair the ability of DJ-1 knockout astrocytes to protect neighboring neurons against oxidative insults.

Discussion

Astrocytes are known to harbor a powerful neuroprotective arsenal and to comprise abilities to secrete neurotrophic factors and to activate anti-oxidative stress machinery, thereby support neurons and guard their survival (Przedborski 2001; Takuma et al. 2004; Mullet and Hinkle 2009; Yanagida et al.

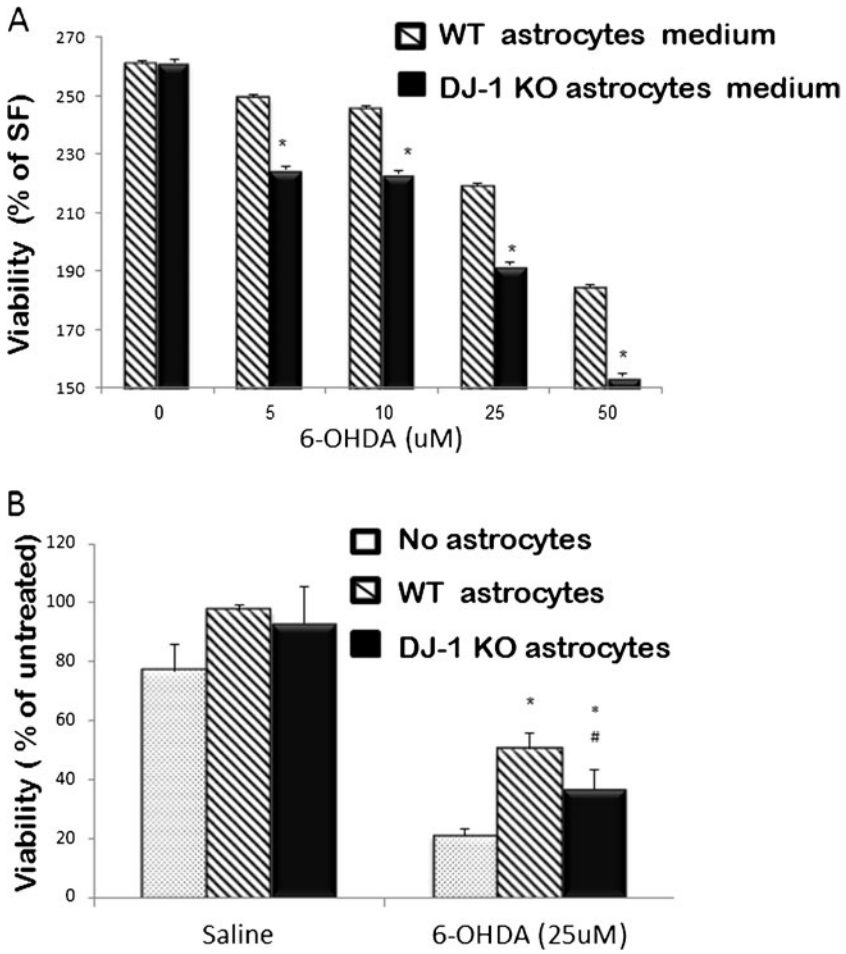

Fig. 3 Lack of DJ-1 impairs astrocytic neuroprotective functions. Human neuroblastoma cells SH-SY5Y were exposed to increasing doses of 6-OHDA, resulting in decreased cell viability. Protective abilities of conditioned media (**a**) or co culture (**b**) of WT and DJ-1 knockout astrocytes were examined. **a** Conditioned media of DJ-1 knockout astrocytes had inferior protective ability on human neuroblastoma cells SH-SY5Y exposed to increasing doses of 6-OHDA as compared to conditioned media of WT astrocytes. **a** Viability was evaluated by Alamar blue. Data are shown as averages ± STD. $^{**}p<0.01$. **b** Dual astrocyte–neuron culture, achieved by co-culturing of DJ-1 knockout astrocytes or WT astrocytes with SH-SY5Y cells demonstrated significantly inferior protective abilities DJ-1 knockout astrocytes as compared to WT astrocytes. Data are shown as averages ± STD. $^{**}p<0.01$ compared neuronal cells alone (without astrocytes); $\#p<0.05$ direct contact with DJ-1 knockout astrocytes as compared to WT astrocytes. Each experiment was done in triplicate for every treatment. The experiments were repeated three times

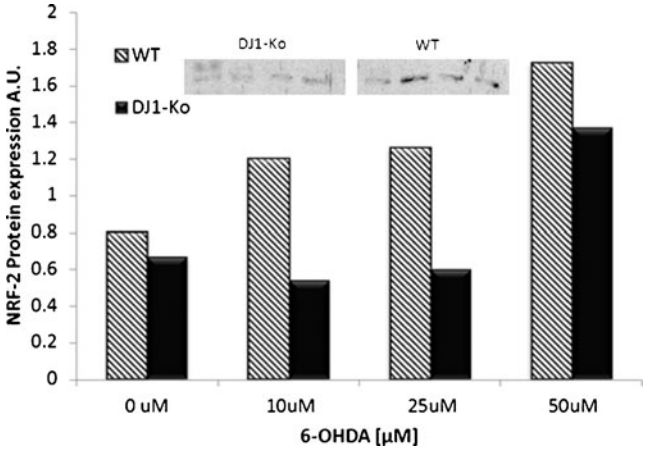

Fig. 4 Attenuated activation of the Nrf2 system in DJ-1 knockout astrocytes. Exposure to 6-OHDA leads to activation of the Nrf2 system and translocation of the Nrf2 protein into the nucleus. Western blots of nuclear fractions obtained from WT and DJ-1 knockout astrocytes were probed with antibodies to Nrf2. DJ-1 knockout astrocytes demonstrated attenuation of Nrf2 activation as shown graphically and in a representative blot

2009). Recent observations suggest that astrocytes may play an important role in neurodegenerative diseases including PD (McGeer and McGeer 2008; Halliday and Stevens 2011; Hauser and Cookson 2011). Postmortem studies found that in sporadic PD brains reactive astrocytes are robustly immunoreactive for DJ-1 (Rizzu et al. 2004; Bandopadhyay et al. 2004). Mutations that eliminate functional DJ-1 cause early onset familial PD (Bonifati et al. 2003; Abou-Sleiman et al. 2003) and extensive research demonstrated DJ-1's abilities to protect neurons against oxidative and toxic insults (Canet-Aviles et al. 2004; Junn et al. 2005; Choi et al. 2006; Lev et al. 2008, 2009, 2012). However, neuronal selective vulnerability to the neurodegenerative process in PD may not reflect only dysfunction of neuronal mechanisms but may also result from altered astrocytic functions and from neuron–astrocyte interactions (Vila et al. 2001; Rizzu et al. 2004; McGeer and McGeer 2008; Mullet and Hinkle 2009; Mythri et al. 2011; Halliday and Stevens 2011; Schmidt et al. 2011; Yokoyama et al. 2011). Since DJ-1 has anti-oxidative stress and anti-apoptotic neuroprotective properties (Junn et al. 2005; Inden et al. 2006; Meulener et al. 2006; Clements et al. 2006; Gorner et al. 2007; Aleyasin et al. 2007, 2010; Fan et al. 2008; Lev et al. 2008, 2009), its massive expression in reactive astrocytes may imply that DJ-1 is needed for astrocyte-mediated neuroprotection. Therefore, we endeavored to explore the function of astrocytic DJ-1 in their abilities to protect neurons against 6-OHDA toxicity.

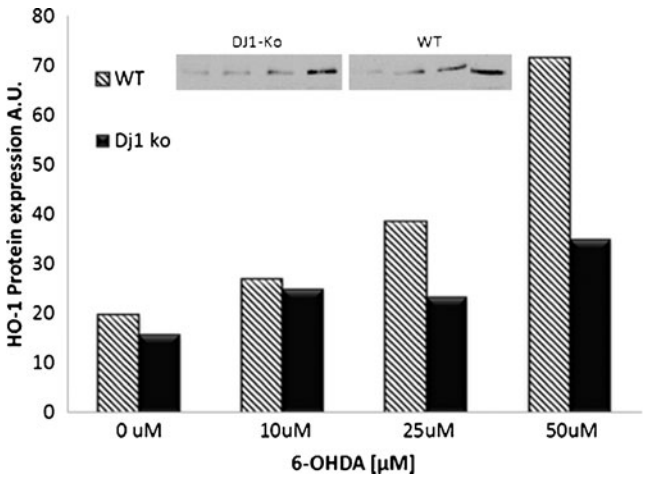

Fig. 5 DJ-1 knockout astrocytes demonstrate attenuated activation of heme oxygenase-1. Exposure to increasing doses of 6-OHDA resulted in increased levels of HO-1 consequential to Nrf2-mediated transcription. DJ-1 knockout astrocytes demonstrated blunted elevation of HO-1 levels as shown by Western blotting (results are shown graphically and by a representative blot)

Using the hemiparkinsonian mice model of PD, we found that DJ-1 knockout mice are more vulnerable than WT mice in vivo to 6-OHDA striatal lesioning, as evident by behavioral, biochemical, and histochemical assays (Fig. 1). In order to delineate whether loss of astrocytic DJ-1 contributes to the augmented vulnerability to 6-OHDA toxicity we employed primary astrocyte cultures obtained from DJ-1 knockout and from WT mice and compared their neuroprotective abilities. Astrocytes obtained from DJ-1 knockout mice did not demonstrate increased sensitivity to 6-OHDA toxicity as compared to WT astrocytes (Fig. 2), yet showed blunted abilities to protect neuronal cells against toxic insults (Figs. 2 and 3).

First, we employed conditioned media obtained from WT or from DJ-1 knockout astrocytes, and examined their ability to protect neuroblastoma cells exposed to increasing doses of 6-OHDA. Conditioned media obtained from DJ-1 knockout astrocytes showed reduced neuroptrotective abilities, as compared to conditioned media of WT astrocytes (Fig. 3). These results imply that soluble, astrocyte-derived factors are involved in the mechanism of astrocyte-mediated protection, and that astrocytes lacking DJ-1 do not secrete these soluble factors as WT astrocytes. These results are in agreement with previous studies that found that DJ-1 knockdown in astrocytes impaired astrocyte-mediated neuroprotection against rotenone and other pesticides (Mullett and Hinkle 2009, 2011).

Next we examined whether DJ-1 knockout affects astrocytes' protective abilities in conditions of indirect contact between astrocytes and neurons, achieved through co-culturing. Co-culture of astrocyte–neuron contact demonstrated also that astrocytes lacking DJ-1 have blunted neuroprotective abilities (Fig. 3). Waak et al. (2009) showed enhanced neurotoxicity of lipopolysaccharide when primary neuron cultures were grown on DJ-$1^{-/-}$ astrocytes as compared to WT astrocytes. They hypothesized that this increased toxicity resulted from increased nitric oxide production by DJ-$1^{-/-}$ astrocytes, thereby demonstrating neurotoxic potential of astrocytic DJ-1 deficiency (Waak et al. 2009). In our experiments, astrocytic DJ-1 deficiency did not cause toxic effects either alone or when

combined with exposure to 6-OHDA (Fig. 3). However, DJ-1 knockout in astrocytes impaired their neuroprotective capacity.

Experiments using astrocyte-conditioned media suggested that astrocyte-released soluble factors were involved in the DJ-1-dependent, astrocyte-mediated neuroprotective mechanism. Furthermore, co-culture experiments supported that direct contact between astrocytes and neurons augment the protective abilities of astrocytes. Yet, even with direct contact, DJ-1 knockout astrocytes demonstrated inferior protective abilities as compared with WT astrocytes. Since DJ-1 is known to protect against oxidative insults, we examined the possible protective system that could mediate this neuroprotection. The transcription factor Nrf2 is currently considered the master regulator of redox homeostasis. Nrf2 regulates the expression of numerous genes involved in xenobiotic and reactive oxygen species detoxification (Clements et al. 2006; Malhotra et al. 2008; Gan et al. 2010; Im et al. 2012). Those genes are collectively termed as phase-II enzymes and share a common promoter enhancer named antioxidant response element that is regulated by Nrf2. HO-1, among many others, is regulated by Nrf2. Recent studies show that DJ-1 activates the Nrf2 system and enhances its activation of the phase II antioxidant genes (Clements et al. 2006; Im et al. 2012). Thus, astrocytic activation of this system could aid in exerting neuroprotection, and lack of DJ-1 could attenuate their protective capacity. Indeed, we found that the Nrf2 system is activated by astrocytes in response to 6-OHDA exposure. Yet, the ability of DJ-1 knockout astrocytes to activate the Nrf2 system and increase HO-1 levels was decreased as compared to WT astrocytes (Figs. 4 and 5). Shin et al. (2012) recently demonstrated that Nrf2 activation and subsequent HO-1 induction (achieved through ethyl-pyruvate treatment) enhances the viability of H_2O_2-treated primary astrocyte cultures, and that ethyl-pyruvate-conditioned astrocyte culture media exert a protective effect on neurons exposed to oxidative insults via paracrine and autocrine effects, including, at least in part, by GDNF and GSH accumulation in media.

Conclusions

Lack of DJ-1 was repeatedly shown to lead to increased vulnerability of neuronal cells in vitro and in vivo to oxidative insults. The results of this study demonstrate that lack of DJ-1 also leads to astrocytic dysfunction, altering their ability to protect neuronal cells by direct contact and altering their secretion of soluble protective factors. We have found reduced activation of the Nrf2 system in DJ-1 knockout astrocytes. Intensive astrocytic DJ-1 response in chronic forms of neurodegenerative disease such as PD, Altzheimer's disease, Pick's disease, and in acute CNS injuries such as stroke, supports the possibility that DJ-1 is important in the physiological protective response of astrocytes in a broad spectrum of disorders. Astrocytes are a major source of antioxidant and trophic support in the brain, therefore DJ-1 overexpression in astrocytes across a broad spectrum of neurodegenerative diseases may reflect an adaptive response against the disease process, attempting to exert protection on surrounding neurons. Neuronal selective vulnerability in PD may therefore reflect not only altered neuronal mechanisms, but also altered astrocytic functions and neuron–astrocyte interactions (Vila et al. 2001; McGeer and McGeer 2008; Ashley et al. 2009; Mythri et al. 2011; Halliday and Stevens 2011; Larsen et al. 2011; Schmidt et al. 2011; Yokoyama et al. 2011). DJ-1 has, therefore, multileveled functions in the preservation and protection on the central nervous system against insults and further studies on its mechanisms of action in astrocytes are warranted.

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