

DJ-1 knock-down impairs astrocyte mitochondrial function



N.J. Larsen, G. Ambrosi, S.J. Mullett, S.B. Berman, D.A. Hinkle
 Department of Neurology and Pittsburgh Institute for Neurodegenerative Diseases (PIND)
 University of Pittsburgh School of Medicine, Pittsburgh, PA, USA.



345.2

Background

Experimental stress induces DJ-1 translocation from the cytosol to the mitochondria. This may stabilize mitochondrial function.

DJ-1 is over-expressed in reactive sporadic Parkinson's disease (PD) astrocytes, but is unchanged in PD neurons. This may represent an attempt by astrocytes to protect themselves, and neighboring neurons, against disease progression.

Mutations in the DJ-1 gene (*PARK7*) which eliminate its expression cause early-onset forms of genetic PD. The loss of astrocytic DJ-1 may be critical.

DJ-1 knock-down astrocytes are impaired in their capacity to protect co-cultured neurons against rotenone (a mitochondrial respiratory chain complex I inhibitor that causes experimental parkinsonism).

DJ-1 knock-down may impair astrocyte mitochondrial functioning as a mechanism through which DJ-1 deficiency impairs astrocyte-mediated neuroprotection.

Methods

Cell cultures: Astrocytes were prepared from P1 CD1 mouse cerebral cortex.

siRNA transfections (DJ-1 knock-down): Anti-mouse DJ-1 siRNA was diluted in serum-free media and incubated with Hi-Perfect transfection reagent (Qiagen) to form transfection complexes. Sequence AGG CGC GGC TGC AGT CTT TAA (siDJ#2, targets 5' end of ORF) was used to effect DJ-1 knock-down to ~5% endogenous levels. Sequence AAT TCT CCG AAC GTG TCA CGT (siNS, non-silencing) was used as a transfection control. Each was used at 5 ng RNA: 0.5 µl transfection reagent and incubated with the astrocytes for 72 hr.

Treatments: Treatments were performed at 20 days *in vitro* (DIV) in Neurobasal/1X B27 (anti-oxidant free)/1X ABAM. Fresh rotenone powder or p-trifluoromethoxy carbonyl cyanide phenyl hydrazine (FCCP) was diluted in DMSO and then in media. The final concentrations used in the experiments are shown in the figures.

Mitochondrial isolation: Astrocytes were collected, washed, and homogenized using a Dounce glass tissue homogenizer at 4 °C using a mitochondrial isolation buffer containing 225 mM mannitol, 75mM sucrose, 5mM HEPES, 1mg/ml fatty acid-free bovine serum albumin, and 1mM EGTA at pH 7.4. Astrocyte mitochondrial fractions were then isolated away from the cytosolic fractions using differential centrifugation.

Western blots: Protein lysates or mitochondrial/cytosol fractions were electrophoresed, transferred to membranes, and bands detected by an Odyssey infrared imager (LiCor). DJ-1 was detected using a polyclonal rabbit primary antibody (NeuroMics, 1:5000) followed by an Alexa 680-conjugated secondary (Invitrogen, 1:20,000). β-actin was detected using a monoclonal primary antibody (Sigma, 1:2000) followed by a IRdye-800-conjugated secondary (LiCor, 1:20,000). Cyclooxygenase IV (COX IV) was detected using a monoclonal primary antibody (AbCam) at 1:5000.

Bioenergetics: Astrocytes were cultured in 24-well plates to be used with an XF24 Extracellular Flux Analyzer (Seahorse Bioscience). Mitochondrial respiration was measured by oxygen consumption rates (OCR) and glycolytic flux was measured by extracellular acidification rates (ECAR) relative to unbuffered DMEM. Data were normalized to total astrocytes by glial fibrillary acidic protein (GFAP) in-cell Western blot bioassay. To do this, the cells were lightly fixed in 4% paraformaldehyde and permeabilized with 0.2% v/v Triton X-100 in PBS. GFAP signals were quantified using an Odyssey infrared imager. Polyclonal rabbit anti-GFAP (Dako, 1:5000) primary antibody was detected using an Alexa 680-conjugated (1:5000) secondary. Infrared signal was quantified in each well with background removed.

Assessments of mitochondrial membrane potential (ΔΨm): Astrocytes were cultured in 35mm glass-bottom dishes and incubated with 1µM JC-1 dye (5',6'-tetrachloro-1',1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide, Molecular Probes) at 37 °C for 30 min prior to toxin treatments and image analysis. Entire fields were selected at random and imaged live every 30s for 2h using a confocal microscope with Fluoview FV10-ASW 2.0 software. Fields were excited at 488 nm. Red emission (590 nm, JC-1 aggregate form) and green emission (525 nm, JC-1 monomeric form) intensities were recorded, and red-to-green shift was assessed as a measure of ΔΨm depolarization.

Assessments of mitochondrial dynamics

Plasmid transfections: Plasmids containing mitochondria-targeted red fluorescence protein (mtRFP, provided by R. Youle, NINDS) were used to identify mitochondria. Plasmids containing mitochondria-targeted, photoactivatable green fluorescence protein (mtPA-GFP, also from RY) were used to track mitochondria from a region of interest. Transfections were performed 48h prior to experiments using 2 µg DNA/3 µL Transfectin Lipid Reagent (BioRad) in media for 4h.

Whole-cell mitochondrial motility: mtRFP-transfected astrocytes were treated with rotenone then imaged live using a fluorescence microscope (Olympus IX71) and SPOT Advanced software version 4.6. Three astrocytes from each treatment group were individually excited at 543 nm and sequential images were taken every 10s for 5m at room temperature. Motility was assessed using ImageJ software by identifying all individual mitochondria on the initial image and tracking the number of times each crossed a pre-set radius threshold (a "trajectory") through subsequent images.

Mitochondrial egress, fusion, and fission: Astrocytes were co-transfected with mtRFP and mtPA-GFP and visualized via live image capture using a confocal microscope (Olympus IX81) and ImageJ software. Astrocytes were put in fresh, highly buffered media at 37 °C and placed into a non-CO₂ incubation chamber at 37 °C. Circular regions of interest (ROIs) were produced at (i) proximal processes and (ii) the cell body of each studied cell using a 405 nm laser pulse to photoactivate the mitochondria (PA-GFP+). Rotenone was then applied and sequential images were collected every 10s over 15m from each ROI and the surrounding cell. Each event was assessed by manual counting, as described below: An "egress event" occurred each time a mitochondrion (PA-GFP+ or RFP+) exited or entered a ROI. The number of egress events was normalized to the total number of starting PA-GFP+ mitochondria in the ROI under study.

A "fusion event" was defined as the mixing of contents between a PA-GFP+ mitochondrion and a closely-approximated RFP+ mitochondrion anywhere in the cell. To account for motility, fusion counts were normalized to egress events. A "fission event" was defined as a single PA-GFP+ mitochondrion that split into two or more components. Fission events were normalized to the total number of starting PA-GFP+ mitochondria in the ROI under study.

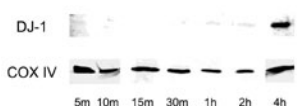


Fig. 1: Rotenone stimulates DJ-1 translocation to astrocyte mitochondria.

Wild-type astrocytes were treated with 40 nM rotenone for the times shown. By Western blots, DJ-1 was first detectable in the mitochondrial fractions of rotenone-treated astrocytes after 4h of treatment. This suggests that rotenone stimulates translocation of DJ-1 from the cytosol to the mitochondria in astrocytes, as it does for many other types of cells.

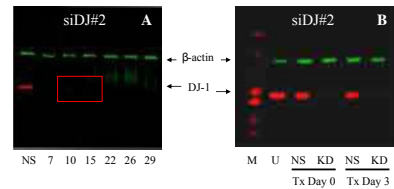


Fig. 2: DJ-1 knock-down is present throughout the entire experimental period.

(A) Western blot showing the time course of siRNA-mediated DJ-1 knock-down in cultured astrocytes (in days post-siDJ#2 transfection, siNS transfection shown at 7 days). All toxin experiments were performed between days 10 and 13 post-transfection (red box). (B) Western blot showing siDJ#2-mediated DJ-1 knock-down (KD) at times corresponding to day 0 and day 3 of toxin treatments, relative to siNS-transfected (NS) and untransfected (U) astrocytes.

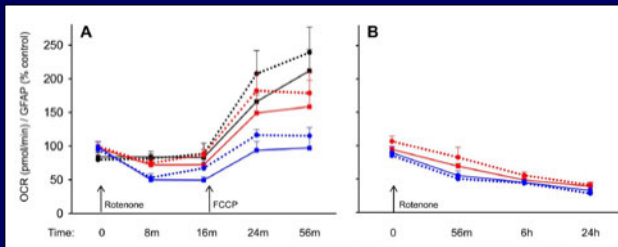


Fig. 3: DJ-1 knock-down does not reduce astrocyte oxygen consumption rates.

Mitochondrial respiration was analyzed by measuring oxygen consumption rates (OCRs) in living astrocytes using a Seahorse XF24 Analyzer. Wild-type (non-silencing siRNA-transfected) and DJ-1 knock-down astrocytes (siDJ#2 siRNA-transfected) were compared. We hypothesized that DJ-1 knock-down would reduce OCR, relative to wild-type, at baseline and after treatment with both rotenone (a complex I inhibitor that should reduce OCR) and FCCP (an uncoupler that should increase OCR).

(A) Baseline OCR measurements were collected. Rotenone was then added at 0, 20, or 40 nM and two further measurements were collected. 500 nM FCCP was then added to each rotenone treatment group. The graph represents OCR data normalized to same-well GFAP levels expressed relative to OCR/GFAP from same-time no rotenone/no FCCP control wells. Mean ± S.E. from 5 replicate experiments is shown. As expected, rotenone treatment reduced the OCR, and this effect persisted after 24h treatment. However, there was no difference between wild-type and DJ-1 knock-down astrocyte OCRs at baseline or after treatment with rotenone at any time point.

(B) Baseline OCR measurements were collected. Rotenone was then added at 20 or 40 nM. The graph represents OCR data normalized to same-well GFAP levels expressed relative to OCR/GFAP from same-time no rotenone control wells. Mean ± S.E. from 5 replicate experiments is shown. As expected, rotenone treatment reduced the OCR, and this effect persisted after 24h treatment. However, there was no difference between wild-type and DJ-1 knock-down astrocyte OCRs at baseline or after treatment with rotenone at any time point.

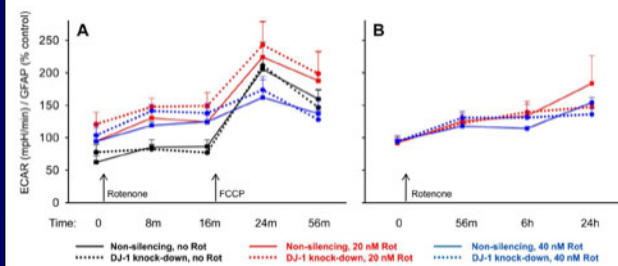


Fig. 4: DJ-1 knock-down does not increase astrocyte extracellular acidification rates.

Glycolytic flux was analyzed by measuring extracellular acidification rates (ECARs) in living astrocytes using a Seahorse XF24 Analyzer. Wild-type (non-silencing siRNA-transfected) and DJ-1 knock-down astrocytes (siDJ#2 siRNA-transfected) were compared. We hypothesized that DJ-1 knock-down would increase ECAR, relative to wild-type, at baseline and after treatment with both rotenone (a complex I inhibitor that should increase ECAR) and FCCP (an uncoupler that should increase ECAR).

(A) Baseline ECAR measurements were collected. Rotenone was then added at 0, 20, or 40 nM and two further measurements were collected. 500 nM FCCP was then added to each rotenone treatment group. The graph represents ECAR data normalized to same-well GFAP levels expressed relative to ECAR/GFAP from same-time no rotenone/no FCCP control wells. Mean ± S.E. from 5 replicate experiments is shown. As expected, rotenone and FCCP treatments stimulated the ECAR. There was no difference, however, between wild-type and DJ-1 knock-down astrocyte ECARs at baseline or after treatment with either rotenone or FCCP at any time point.

(B) Baseline ECAR measurements were collected. Rotenone was then added at 20 or 40 nM. The graph represents ECAR data normalized to same-well GFAP levels expressed relative to ECAR/GFAP from same-time no rotenone control wells. Mean ± S.E. from 5 replicate experiments is shown. As expected, rotenone treatment stimulated the ECAR, and this effect persisted after 24h treatment. However, there was no difference between wild-type and DJ-1 knock-down astrocyte ECARs at baseline or after treatment with rotenone at any time point.

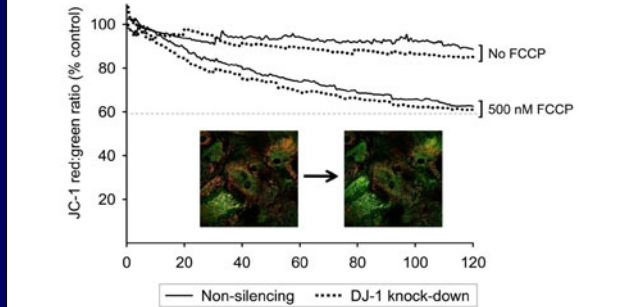


Fig. 5: DJ-1 knock-down in astrocytes does not (i) alter mitochondrial membrane potential (Ψm) at baseline or (ii) enhance FCCP-induced Ψm depolarization.

Astrocytes were imaged every 30s over 2h to determine the JC-1 dye red:green emission ratio. A red → green shift, or reduced ratio, represents a depolarization in Ψm. Untreated astrocytes displayed a small reduction in red:green fluorescence as a function of time, but there was no significant difference between wild-type (non-silencing siRNA-transfected) and DJ-1 knock-down (siDJ#2 siRNA-transfected) cells. As expected, 500nM FCCP (an uncoupler) reduced red:green more robustly. Maximal Ψm depolarization occurred by ~110m (the 60% control asymptote line). However, there was again no significant difference in Ψm between the wild-type and DJ-1 knock-down astrocytes.

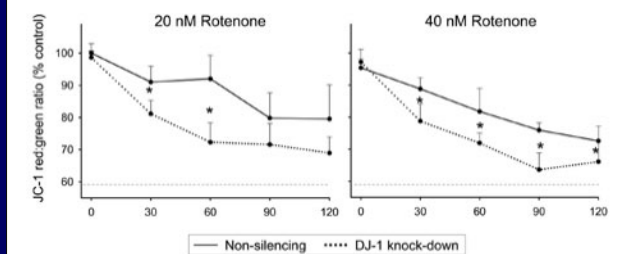


Fig. 6: DJ-1 knock-down in astrocytes enhances rotenone-induced mitochondrial membrane (Ψm) depolarization.

Astrocytes were treated with 20 or 40 nM rotenone and imaged every 30s for 2h to determine the JC-1 dye red:green emission ratio. A red → green shift, or reduced ratio, represents a depolarization in Ψm. As expected, rotenone treatment reduced red:green. However, rotenone treatment reduced Ψm less robustly than FCCP (Fig. 5). Under these conditions we were able to detect a significantly enhanced Ψm depolarization in DJ-1 knock-down (siDJ#2 siRNA-transfected) astrocytes relative to wild-type (non-silencing siRNA-transfected) astrocytes. This suggests that DJ-1 knock-down astrocytes are impaired in their capacity to maintain Ψm in the presence of complex I inhibition. Mean ± S.E. shown, n=6.

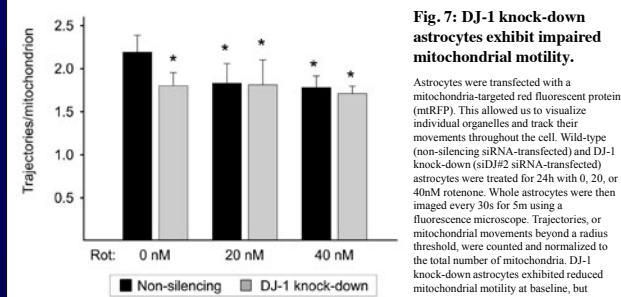


Fig. 7: DJ-1 knock-down astrocytes exhibit impaired mitochondrial motility.

Astrocytes were transfected with a mitochondria-targeted red fluorescent protein (mtRFP). This allowed us to visualize individual organelles and track their movements throughout the cell. Wild-type (non-silencing siRNA-transfected) and DJ-1 knock-down (siDJ#2 siRNA-transfected) astrocytes were treated for 24h with 0, 20, or 40nM rotenone. Whole astrocytes were then imaged every 30s for 5m using a fluorescence microscope. Trajectories, or mitochondrial movements beyond a radius threshold, were counted and normalized to the total number of mitochondria. DJ-1 knock-down astrocytes exhibited reduced mitochondrial motility at baseline, but rotenone treatment did not reduce motility further. Wild-type astrocytes responded to rotenone treatment with the same level of reduced mitochondrial motility. These findings suggest that astrocyte mitochondrial motility is impaired to a similar extent by DJ-1 deficiency and rotenone treatment, but that the combination is neither additive nor synergistic. Mean ± S.E. shown, n=6, asterisks (*) refer to comparisons with wild-type/0 nM rotenone.

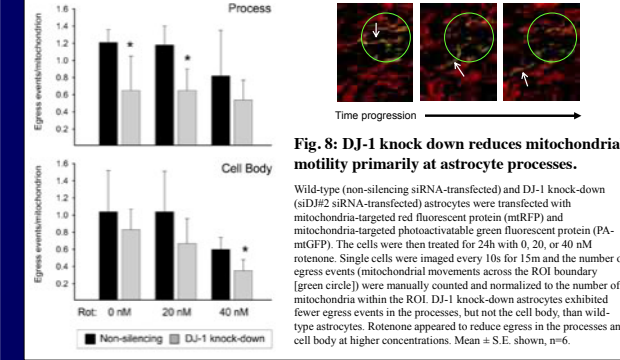


Fig. 8: DJ-1 knock down reduces mitochondrial motility primarily at astrocyte processes.

Wild-type (non-silencing siRNA-transfected) and DJ-1 knock-down (siDJ#2 siRNA-transfected) astrocytes were transfected with mitochondria-targeted red fluorescent protein (mtRFP) and mitochondria-targeted photoactivatable green fluorescent protein (PA-mtGFP). The cells were then treated for 24h with 0, 20, or 40 nM rotenone. Single cells were imaged every 10s for 15m and the number of egress events (mitochondrial movements across the ROI boundary [green circle]) were manually counted and normalized to the number of mitochondria within the ROI. DJ-1 knock-down astrocytes exhibited fewer egress events in the processes, but not the cell body, than wild-type astrocytes. Rotenone appeared to reduce egress in the processes and cell body at higher concentrations. Mean ± S.E. shown, n=6.

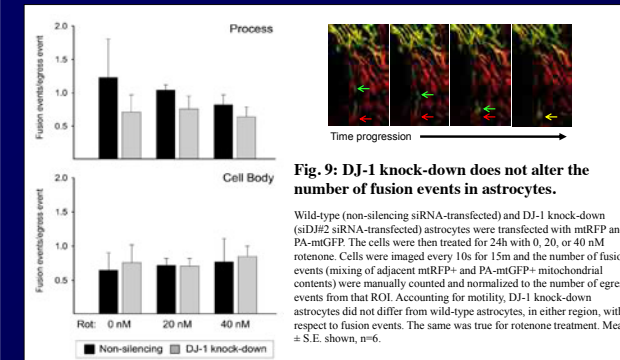


Fig. 9: DJ-1 knock-down does not alter the number of fusion events in astrocytes.

Wild-type (non-silencing siRNA-transfected) and DJ-1 knock-down (siDJ#2 siRNA-transfected) astrocytes were transfected with mtRFP and PA-mtGFP. The cells were then treated for 24h with 0, 20, or 40 nM rotenone. Cells were imaged every 10s for 15m and the number of fusion events (mixing of adjacent mtRFP+ and PA-mtGFP+ mitochondrial contents) were manually counted and normalized to the number of egress events from that ROI. Accounting for motility, DJ-1 knock-down astrocytes did not differ from wild-type astrocytes, in either region, with respect to fusion events. The same was true for rotenone treatment. Mean ± S.E. shown, n=6.

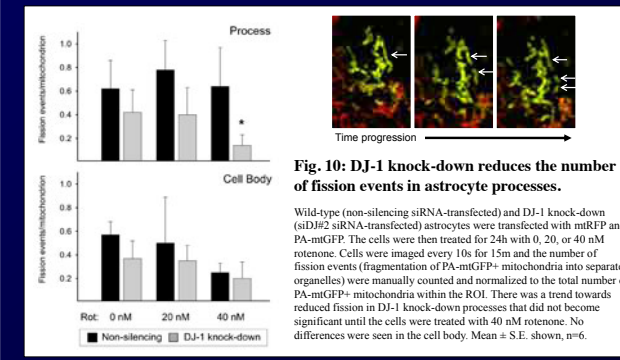


Fig. 10: DJ-1 knock-down reduces the number of fission events in astrocyte processes.

Wild-type (non-silencing siRNA-transfected) and DJ-1 knock-down (siDJ#2 siRNA-transfected) astrocytes were transfected with mtRFP and PA-mtGFP. The cells were then treated for 24h with 0, 20, or 40 nM rotenone. Cells were imaged every 10s for 15m and the number of fission events (fragmentation of PA-mtGFP+ mitochondria into separate organelles) were manually counted and normalized to the total number of PA-mtGFP+ mitochondria within the ROI. There was a trend towards reduced fission in DJ-1 knock-down processes that did not become significant until the cells were treated with 40 nM rotenone. No differences were seen in the cell body. Mean ± S.E. shown, n=6.

Conclusions

DJ-1 deficiency did not alter mitochondrial respiratory function or glycolytic flux in astrocytes.

DJ-1 deficiency enhanced rotenone-induced depolarization of the mitochondrial membrane potential in astrocytes.

DJ-1 deficiency reduced mitochondrial motility in astrocytes. This was particularly prominent in cellular processes.

DJ-1 deficiency reduced mitochondrial fission rates in astrocytes. This was particularly prominent with rotenone treatment.

Support: University of Pittsburgh Department of Neurology