



Overexpression of the Neuron-Specific Drp1 Isoform that Includes Exon 3 (ISO1) Increases Mitochondrial Respiratory Capacity in Neurons

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BACKGROUND

The physiological relevance of the mitochondrial fission protein, Drp1, and thus mitochondrial fission to brain development and neuronal function is becoming increasingly clear. The absence of Drp1 function severely affects brain development (Wakabayashi et al., 2009) and a dominant-negative mutation (A395D) in the Drp1 gene was found in a newborn child with abnormal brain development and optic atrophy (Waterham et al., 2007). Drp1 has eight different splicing isoforms attributed to inclusion/exclusion of three alternative exons (E3, 16 and 17). Our previous *in vitro* studies demonstrated that cultured cortical neurons express several alternatively spliced Drp1 transcripts with or without exon 3 and that Drp1 is required for neuronal survival. Inclusion of exon 3 (E3+) is specific to postmitotic neurons, while exon 3 exclusion (E3-) was always observed in non-neuronal cells. The importance of Ex3 to Drp1 function and hence mitochondrial function has not been investigated. In these studies, we have found that inclusion of Ex3+ in Drp1 ISO1 confers a unique neuron-specific function in regulating mitochondrial bioenergetics. We also found that the Drp1 ISO1 – mediated increase in mitochondrial functions is dependent on the Bcl-xL protein.

HYPOTHESES

1. Expression of the neuron-specific Drp1 isoform (ISO1: Ex3+/Ex16+/Ex17+) increases mitochondrial respiratory capacity and mitochondrial fission in mouse postnatal cortical neurons when compared with the non-neuronal isoform of Drp1 (ISO3: Ex3-/16-/17-).
2. Expression of Drp1 ISO1 in non-neuronal cells (3T3 mouse fibroblasts) impairs mitochondrial respiratory capacity.
3. The Drp1 ISO1-mediated increase in OCR is dependent on Bcl-xL protein.

METHODS

Plasmid Construction and lentivirus production:

Human pcDNA3-Drp1 for transient expression of wild-type Drp1 (Smirnova et al., 1998) was kindly provided by Dr Richard Youle (NIH). It is important to note that this cDNA lacks exon 3, 16 and 17. Expression constructs for FLAG-tagged wild-type (Drp1^{WT}) and dominant-negative mutant Drp1 (Drp1^{K38A}) were created as follows. Using pcDNA3-Drp1 as a template, a cDNA fragment encoding N-terminally FLAG-tagged partial Drp1 protein was prepared by PCR with forward primer: GGA TCC GCC ACC ATG GAT TAC AAG GAT GAC GAC GAT AAG GAG GCG CTA ATT CCT GTC ATA AAC and reverse primer: AAA TCC ACA AGT GTC AAA TTG ACA ACG (BamHI site is singly underlined and the sequence encoding FLAG tag was dashed). The amplified fragment contains the HindIII site and this was digested with BamHI and HindIII. Subsequently, the resultant fragment and the HindIII-XhoI fragment of pcDNA3-Drp1 were introduced together into the BamHI-XhoI site of the pcDNA3 vector (Invitrogen) to produce pcDNA3 FLAG-Drp1^{WT}. An expression construct for the dominant negative mutant of Drp1, pcDNA3 FLAG-Drp1^{K38A}, was created using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene). For lentivirus-mediated expression of Drp1^{WT} and Drp1^{K38A}, DNA fragments were liberated from the respective pcDNA3 FLAG-Drp1 vectors by digestion with NdeI and XhoI. These fragments were inserted into the NdeI-XhoI site of the pSL6 IRES-GFP vector (Uo et al., 2007) to produce lentiviral transfer vectors co-expressing EGFP and either FLAG-tagged Drp1^{WT} or Drp1^{K38A} from the same transcripts.

Each cDNA encoding a variable splicing variant of human Drp1 was created using pcDNA3 FLAG-Drp1^{WT} as a parental plasmid. Human Drp1 cDNA encoding transcript variant 1 (NM012062.2) which lacks exon 3 but contains exon 16 and 17 was purchased from Origene (Rockville, MD) and used as a template for PCR in some cases. Primers with DNA sequence corresponding to exon 3 were used for PCR to introduce the exon 3 sequence into the human Drp1 cDNA. Further information about the cloning methods and primers is available upon request.

Seahorse XF24 Metabolic Flux Analysis:

An XF24 Analyzer (Seahorse Bioscience) was used to measure oxygen consumption (OCR) and extracellular acidification rate (ECAR). Before running the assay, the XF24 Sensor cartridge was loaded with 75µl pre-warmed (37°C) injection CCCP and KCN compounds into sensor ports and biosensors were calibrated for approximately 25 min in a calibration reagent. Oxygen Consumption Rate (OCR) is expressed as nanomoles per min and Extracellular Acidification Rate (ECAR) in milli-pH (mPH) units per minute. Primary cultures of postnatal cortical neurons were seeded in XF 24-well microplates at a density of 8 x 10⁴ cells per well in 250µl Neurobasal Medium + B27 supplement for 4 days before the experiment. 3T3 mouse fibroblasts were seeded at a density of 7 x 10⁴ cells per well in 250µl DMEM +10% CBS medium and then incubated at 37°C / 5% CO₂ for one day before the experiment. On the day of metabolic flux analysis, cells were changed to unbuffered DMEM (DMEM base medium (Sigma D5030) supplemented with 17 mM glucose (Sigma G8270), 10mM sodium pyruvate (Sigma S8636), 2mM GlutaMax (Gibco 35050-061) and 32mM NaCl (Sigma S3014), adjusted to pH7.4) and incubated at 37°C in a CO₂ free incubator for 1 h. All injection reagents were adjusted to pH 7.4 on the day of assay. Four baseline and three CCCP and KCN response rates were measured. For relative measurements comparing metabolic rates after the addition of CCCP and KCN to the baseline, we expressed the data as a percentage of OCR or ECAR change over baseline. Percentage of change was calculated as the value of change (Response rate – Basal Rate) divided by the value of baseline readings (Basal Rate) multiplied x 100. Upon completion of an XF assay a cell count measurement was performed for each well and the data was normalized by the number of cells per well. Assay medium was removed, followed by addition of 100µl 0.01% SDS and 100µl of a solution of 4 µg/ml Hoechst 33342 in buffer containing 10 mM Tris HCl, 1 mM EDTA, 1 M NaCl at pH 7.4. After 1h incubation at 37°C with shaking, the cells were mixed and Hoechst fluorescence intensity was measured at a wavelength of 355nm excitation and 460 nm emission using a Fusion™ plate reader.

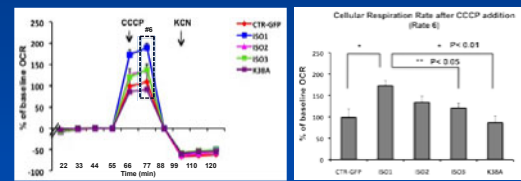
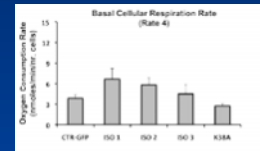
Statistical Analysis:

Data analysis was carried out using ANOVA followed by Holm-Sidak *posthoc* test Test for multiple comparisons or Student's *t*-test for two groups' comparison. Means ± SEM values for n = 3 – 14 separate experiments are presented. Differences were considered significant when p<0.05.

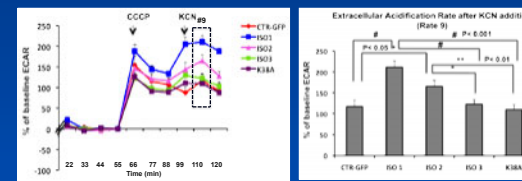
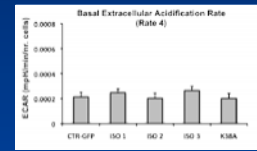
RESULTS

THE NEURON-SPECIFIC DRP1 ISOFORM (ISO1) INCREASES MITOCHONDRIAL RESPIRATORY CAPACITY AND MITOCHONDRIAL FISSION IN NEURONS

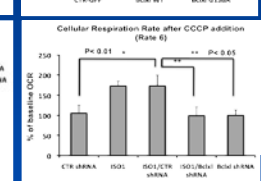
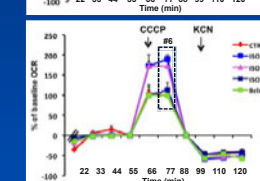
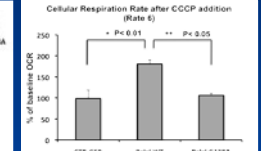
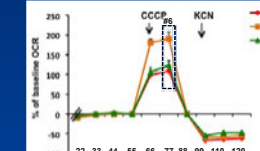
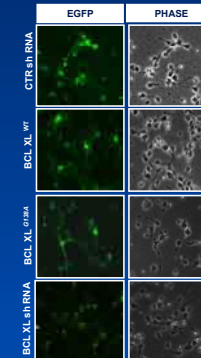
ELEVATION OF OCR IN ISO1-INFECTED NEURONS



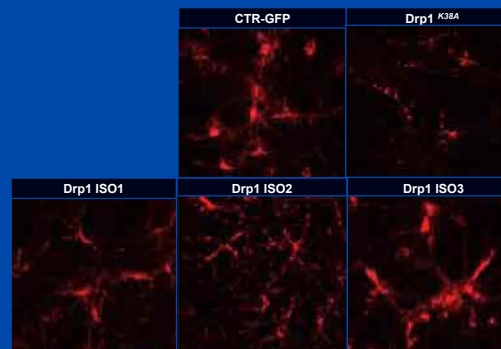
ELEVATION OF ECAR IN ISO1-INFECTED NEURONS



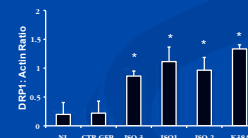
DRP1 ISO1 – MEDIATED INCREASE IN OCR IS DEPENDENT ON BCL-XL



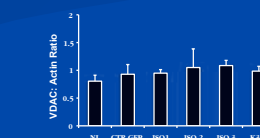
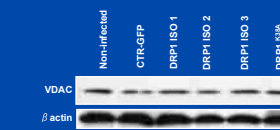
TMRM FLUORESCENCE IN POSTNATAL CORTICAL NEURONS OVEREXPRESSING DRP1 ISO1, ISO2, ISO3 AND DRP1 K38A



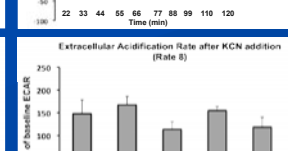
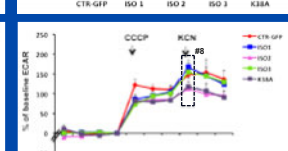
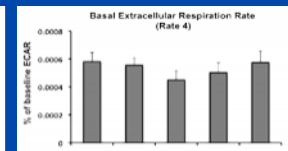
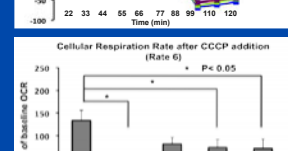
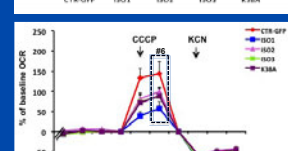
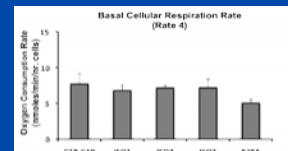
OVEREXPRESSION OF THE DRP1 PROTEIN



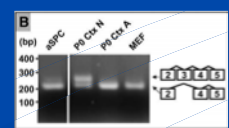
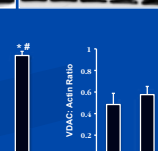
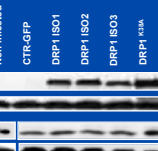
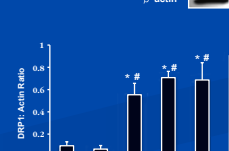
DRP1 EXPRESSION DOES NOT CHANGE MITOCHONDRIAL MASS



OVEREXPRESSION OF DRP1 ISO1 DECREASES MITOCHONDRIAL RESPIRATORY CAPACITY IN NON-NEURONAL CELLS (3T3 MOUSE FIBROBLASTS)



OVEREXPRESSION OF DRP1 ISOFORMS IN 3T3 CELLS DOES NOT CHANGE MITOCHONDRIAL MASS



CONCLUSIONS

- Neuron-specific Drp1 ISO1(Ex3+) increases basal respiration and mitochondrial respiratory capacity in postnatal cortical neurons compared to non-neuronal Drp1 isoforms.
- In response to inhibition of mitochondrial respiration neurons expressing Drp1 ISO1 show a robust increase in ECAR - a compensatory response in which neurons up-regulate glycolysis to compensate for mitochondrial respiratory inhibition.
- Drp1 ISO1 exhibits a unique function in neurons; inclusion of Ex3+ in non-neuronal cells (3T3 mouse fibroblasts) resulted in a decrease in OCR. In addition, the inhibition of Drp1 function in 3T3 cells does not affect mitochondrial respiration suggesting that mitochondrial function in non-neuronal cells is not as dependent on Drp1.
- Drp1 ISO1-mediated increase in OCR is dependent on Bcl-xL expression.

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