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Developmental changes in trak-mediated mitochondrial transport in neurons

Omar Loss, F. Anne Stephenson *

School of Pharmacy University College London, 29/39 Brunswick Square, London WC1N 1AX, United Kingdom

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ABSTRACT

Previous studies established that the kinesin adaptor proteins, TRAK1 and TRAK2, play an important role in mitochondrial transport in neurons. They link mitochondria to kinesin motor proteins via a TRAK acceptor protein in the mitochondrial outer membrane, the Rho GTPase, Miro. TRAKs also associate with enzyme, O-linked *N*-acetylglucosamine transferase (OGT), to form a quaternary, mitochondrial trafficking complex. A recent report suggested that TRAK1 preferentially controls mitochondrial transport in axons of hippocampal neurons whereas TRAK2 controls mitochondrial transport in dendrites. However, it is not clear whether the function of any of these proteins is exclusive to axons or dendrites and if their mechanisms of action are conserved between different neuronal populations and also, during maturation. Here, a comparative study was carried out into TRAK-mediated mitochondrial mobility in axons and dendrites of hippocampal and cortical neurons during maturation in vitro using a shRNA gene knockdown approach. It was found that in mature hippocampal and cortical neurons, TRAK1 predominantly mediates axonal mitochondrial transport whereas dendritic transport is mediated via TRAK2. In young, maturing neurons, TRAK1 and TRAK2 contribute similarly in mitochondrial transport in both axons and dendrites in both neuronal types. These findings demonstrate maturation regulation of mitochondrial transport which is conserved between at least two distinct neuronal subtypes.

1. Introduction

Despite the brain being only 2% of the body weight, it consumes 20% of the body's resting energy. A typical neuron consumes ~4.7 million ATP molecules per second to power various brain functions such as neuronal survival, axonal growth and branching, generation of action potentials and synaptic transmission (Attwell & Laughlin, 2001; Zhu et al., 2012). Mitochondria are the cellular organelles that generate the ATP. For efficiency, they must be located adjacent to the sites requiring energy. It is known that mitochondria travel from their site of synthesis, the soma, along the axons and dendrites of neurons (anterograde movement) or vice versa (retrograde movement) to satisfy the energy demand essential for neuronal function. Indeed, defective mitochondrial trafficking and impaired mitochondrial function are increasingly implicated in neurological diseases (reviewed in (Chan, 2006; Mattson et al., 2008).

At any given time ~20–50% mitochondria in neurons are mobile (Brickley and Stephenson, 2011; Bros et al., 2015; Cai et al., 2005; Chang and Reynolds, 2006; Chang et al., 2006; Chen and Sheng, 2013; Jimenez-Mateos et al., 2006; Ligon and Steward, 2000; MacAskill et al., 2009a; Morris and Hollenbeck, 1993; Nguyen et al., 2014; Overly et al., 1996; Pekkurnaz et al., 2014; Ruthel and Hollenbeck, 2003; Trushina et al., 2012; van Spronsen et al., 2013; Wang and Schwarz, 2009). Mitochondrial transport in neurons has been shown to undergo maturation changes. For example in early studies, their movement in axons of growing neurons showed changes in the percentages of moving mitochondria with differences in anterograde versus retrograde transport. These observed changes were dependent on the length of the axons (Morris and Hollenbeck, 1993; Ruthel and Hollenbeck, 2003). More recently, Chang and Reynolds (2006) found that mitochondrial mobility in cortical neurons is greater in young neurons (5 days in vitro, DIV) compared to mature neurons (14 DIV) despite them having mitochondria with similar functionality properties i.e. their ability to buffer Ca^{2+} and their membrane potential. Mitochondrial transport has been reported to differ between axons and dendrites of maturing hippocampal neurons. In axons, mitochondrial velocity was higher compared to in dendrites (Ligon and Steward, 2000; Overly et al., 1996).

Anterograde and retrograde movement of mitochondria in axons and dendrites is microtubule based. It is now generally accepted that the majority of transport is mediated by a quaternary complex of

Abbreviations: DIV, days in vitro; PSD-95, postsynaptic density protein 95; MAP, microtubule-associated protein 2; shRNA, short hairpin RNA; scrRNA, scrambled RNA; FITC, fluorescein isothiocyanate; EGFP, enhanced green fluorescent protein; DsRed, *discosoma* red fluorescent protein; DAPI, 4′,6-diamidino-2-phenylindole; PBS, phosphate-buffered saline.

* Corresponding author.
E-mail address: anne.stephenson@ucl.ac.uk (F.A. Stephenson).

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proteins. This complex is composed of the motor proteins, kinesin or dynein; the trafficking kinesin adaptor proteins (TRAKs); the TRAK acceptor protein, the outer mitochondrial membrane Rho GTPase, Miro, and the post-translational modification enzyme, *N*-acetylglucosamine transferase (OGT) (reviewed in (Schwarz, 2013; Stephenson and Brickley, 2011).

The family of TRAK proteins was initially identified in humans as the homologues of the *Drosophila melanogaster* gene product, Milton (Brickley et al., 2005; Stowers et al., 2002). Whereas *Drosophila* carries one Milton gene, mammals have two encoding TRAK1 and TRAK2. Decreased TRAK1 and TRAK2 expression and also the use of a TRAK2 dominant negative to inhibit the formation of the quaternary complex, leads to a decrease in mitochondrial mobility in hippocampal neurons (Brickley and Stephenson, 2011). The TRAK mitochondrial trafficking complex is also regulated by Miro and OGT. Both over-expression and down-regulation of Miro affect the transport of mitochondria in dendrites of hippocampal neurons (Macaskill et al., 2009b). Further, increases in Ca^{2+} concentration alter the protein-protein binding properties of Miro and kinesin resulting in the inhibition of mitochondrial transport via dissociation of the trafficking complex (MacAskill et al., 2009a, 2009b). Increased levels of extracellular glucose decrease mitochondrial movement in axons of hippocampal neurons as a consequence of activation of OGT (Pekkurnaz et al., 2014).

A recent report suggested that TRAK1 and TRAK2 have potentially distinct roles in mitochondrial transport in different neuronal subcellular compartments since immunocytochemical studies revealed that TRAK1 was prevalently localized in axons whereas TRAK2 was more abundant in dendrites (van Spronsen et al., 2013). More support for this premise was that TRAK1-shRNA gene knockdown resulted in a decrease in mitochondrial mobility in axons (Brickley and Stephenson, 2011; van Spronsen et al., 2013) but in contrast, TRAK2-shRNA gene knockdown had no effect on axonal mitochondrial transport (Brickley and Stephenson, 2011) but van Spronsen et al. (2013) found that it impaired dendritic mitochondrial mobility. A subsequent investigation into TRAK1/2 subcellular distribution found a similar predominantly axonal distribution for TRAK1 and a dendritic distribution for TRAK2 (Loss and Stephenson, 2015). However, the demarcation between axonal versus dendritic distribution was not as evident as described by van Spronsen et al. (van Spronsen et al., 2013). A key difference between these two reports was that the study of van Spronsen et al. (2013) used 14 DIV hippocampal neurons whereas that of Loss and Stephenson (2015) used 6 DIV hippocampal neurons. A direct comparison of the findings between the two groups is therefore not tenable since there may be important maturation differences in mitochondrial transport at distinct stages of maturation. To address this, we have performed a systematic, comparative study in which the properties of TRAK-mediated mitochondrial transport were investigated in two different types of cultured primary neurons during maturation. The results are reported herein.

2. Materials and methods

2.1. Constructs and antibodies

The plasmids pDsRed1-Mito, pGreenTRAK1scrRNA (TRAK1-scrRNA), pGreenTRAK1shRNA (TRAK1-shRNA), pGreenTRAK2scrRNA (TRAK2-scrRNA) and pGreenTRAK2shRNA (TRAK2-shRNA) were as described previously (Brickley and Stephenson, 2011; Loss and Stephenson, 2015).

The following antibodies were used: rabbit polyclonal anti-TRAK1 antibodies (973–988), generated as described by Loss and Stephenson (2015); sheep anti-TRAK2 (874–889) antibodies, generated as described by Brickley et al. (2005); mouse monoclonal anti-tau (TAU-5), (RRID:AB_1603723, Abcam, Cambridge, UK, Cat. N. ab80579); mouse monoclonal anti-microtubule associate protein-2 (MAP-2), (RRID:AB_297885, Abcam, ab11267); mouse monoclonal anti-β-actin (RRID:AB_722536, Abcam ab40864); rabbit polyclonal anti-PSD95 (RRID:AB_444362, Abcam, ab18258), mouse monoclonal anti-synaptophysin [SY38] (RRID:AB_2198854, Abcam, ab8049); goat polyclonal anti-mouse IgG1 secondary antibody, Alexa Fluor 633 conjugate (RRID:AB_2535768, Thermo Fisher Scientific, Waltham, MA USA, A-21126); goat polyclonal anti-mouse IgG (H + L) secondary antibody, Alexa Fluor 594 conjugate (RRID:AB_2534073, Thermo Fisher Scientific, A-11005); goat polyclonal anti-rabbit IgG (H + L) secondary antibody, Alexa Fluor 594 conjugate (RRID:AB_2534095, Thermo Fisher Scientific, A-11037); goat polyclonal anti-mouse IgG (H + L) secondary antibody, Alexa Fluor 488 conjugate (RRID:AB_2534069, Thermo Fisher Scientific, A-11001).

2.2. Culturing and transfection of hippocampal and cortical neurons

Cultures of rat hippocampal and cortical neurons were prepared at a density of ~30,000 cells/cm^2 on poly-D-lysine- (1 μg/ml) and laminin (2 μg/ml)-coated glass bottom culture dishes (P35G-1.5-14-C, Mattek Corporation, US) from hippocampi or cerebral cortices dissected from E18 rat embryos by standard methods (Goslin et al., 1998). Cultures were grown for 4–12 days in complete neurobasal media which was neurobasal media (Life Technologies) containing a 1 in 50 dilution of B27 (Life Technologies), 0.5 mM GlutaMax (Life Technologies), 0.4% (w/v) glucose and 1× penicillin/streptomycin. Transfection of neurons was performed at 3–4, 7–8 or 11–12 days in vitro (DIV) using the calcium phosphate method. In brief, 48 h prior to transfection, neurobasal complete medium with antibiotics was removed and stored at 37 °C in 5% CO_2 and replaced with fresh neurobasal complete medium. The transfection reaction was performed by addition of 6 μg EndoFree plasmid DNA at a ratio of 1:1 (pDsRed-Mito + pGreenTRAK1scrRNA, pGreenTRAK1shRNA, pGreenTRAK2scrRNA or pGreenTRAK2shRNA) to a solution containing 0.25 M $CaCl_2$. This was added to an equal volume of 2× HEPES-buffered saline, pH 7.14, and incubated at room temperature for 25 min. The transfection mixture was added to the neurons dropwise and incubated at 37 °C in 5% CO_2 for 30 min. The media was aspirated and the neurons were washed twice with fresh neurobasal media prior to addition of the previously removed media. Live imaging and subsequent fixation of neurons with 4% (w/v) paraformaldehyde was carried out 48–72 h after transfection.

Cultures were analyzed from 3 different maturation stages, i.e. 6 DIV, 10 DIV and 14 DIV to encompass the full range of previous reports on TRAK-mediated mitochondrial trafficking (Brickley and Stephenson, 2011; Loss and Stephenson, 2015; van Spronsen et al., 2013).

2.3. Immunoblotting

Cell lysates were prepared using 1× RIPA solubilisation buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.1% [v/v] SDS, 0.5% [v/v] sodium deoxycholate, 1% [v/v] Triton X-100, 1 mM phenylmethylsulphonyl fluoride, 0.5 mg/ml protease inhibitor cocktai. Immunoblotting was carried out exactly as described by Brickley et al. (2005) using final antibody concentrations of 1 μg/ml anti-TRAK1 (973–988) and 2 μg/ml anti-TRAK2 (874–889).

2.4. Immunocytochemistry

Neurons fixed with 4% (w/v) paraformaldehyde in glass bottom culture dishes were permeabilized and blocked with a solution containing 0.2% (v/v) Triton X-100 (Sigma, UK) and 10% (v/v) foetal bovine serum in PBS for 1 h at room temperature. Primary antibodies were diluted in the above solution, added to the cells and incubated overnight at 4 °C. Cells were washed three times with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) containing 0.1% (v/v) Tween 20 (Sigma, UK) for 10 min with gentle rotation. Secondary antibodies were added at a dilution 5 times the concentration of the