HHS Public Access

Author manuscript

Ageing Res Rev. Author manuscript; available in PMC 2021 September 01.

Published in final edited form as:

Ageing Res Rev. 2020 September ; 62: 101128. doi:10.1016/j.arr.2020.101128.

Inter and Intracellular mitochondrial trafficking in health and disease

Santhanam Shanmughapriya[a], **Dianne Langford**[b], **Kalimuthusamy Natarajaseenivasan**[b,c,*]

[a]Heart and Vascular Institute, Department of Medicine, Department of Cellular and Molecular Physiology, Pennsylvania State University, College of Medicine, Hershey, PA, USA

[b]Department of Neuroscience, Lewis Katz School of Medicine at Temple University, Philadelphia, PA, USA

[c]Department of Microbiology, Bharathidasan University, Tiruchirapalli, Tamil Nadu, India

Abstract

Neurons and glia maintain central nervous system (CNS) homeostasis through diverse mechanisms of intra- and intercellular signaling. Some of these interactions include the exchange of soluble factors between cells via direct cell-to-cell contact for both short and long-distance transfer of biological materials. Transcellular transfer of mitochondria has emerged as a key example of this communication. This transcellular transfer of mitochondria are dynamically involved in the cellular and tissue response to CNS injury and play beneficial roles in recovery. This review highlights recent research addressing the cause and effect of intra- and intercellular mitochondrial transfer with a specific focus on the future of mitochondrial transplantation therapy. We believe that mitochondrial transfer plays a crucial role during bioenergetic crisis/deficit, but the quality, quantity and mode of mitochondrial transfer determines the protective capacity for the receiving cells. Mitochondrial transplantation is a new treatment paradigm and will overcome the major bottleneck of traditional approach of correcting mitochondria-related disorders.

Keywords

TRAK; Miro; Kinesin; mitochondrial transplantation; extracellular vesicles; tunneling nanotubes; mitochondrial extrusion

1. Introduction

Mitochondria are one of the major ancient double membrane systems that arose around two billion years ago (Friedman and Nunnari, 2014) from engulfment of α-proteobacterium by a

*Corresponding Author: Kalimuthusamy Natarajaseenivasan, PhD, Department of Neuroscience, Lewis Katz School of Medicine at Temple University, Philadelphia, PA, USA. tuf29518@temple.edu, 215 707-5487.

Conflict of Interest

No potential conflict of interest was reported by the authors

precursor of the modern eukaryotic cell. Mitochondria have an inert ability to produce ATP through respiration leading them to become a driving force in evolution. Although mitochondria maintained the double membrane structure and retained the core ability to produce ATP, the overall form and composition can be drastically altered in response to the microenvironment. Apart from its traditional role as a cell powerhouse, mitochondria also possess a myriad of additional functions including calcium (Ca^{2+}) buffering capacity (Granatiero et al., 2017; Pallafacchina et al., 2018; Szymanski et al., 2017), generation of reactive oxygen species (ROS) (Demine et al., 2019; Sena and Chandel, 2012; Singh et al., 2019; Stefanatos and Sanz, 2018), regulation of apoptosis (Bock and Tait, 2020; Galluzzi et al., 2018; Gong et al., 2019; Marchi et al., 2018), activation of endoplasmic reticulum (ER) stress response (Csordas et al., 2018; Marchi et al., 2018; Szymanski et al., 2017), as well as in the broad range sequelae of mitochondrial dysfunction. Mitochondria are also implicated in many common diseases including but not restricted to cardiovascular disease, Alzheimer's disease (AD) and Parkinson's disease (PD), metabolic disorders, muscular dystrophy, and in the process of normal aging (Diaz-Vegas et al., 2020).

The causes of these mitochondrial diseases are literally a collection of changes in thousands of genes that influence mitochondrial function. It has been accepted that mitochondria are comprised of over 1,000 proteins that vary within and between species in response to cellular and tissue-specific organismal needs. However, what exists in cellular mitochondria is a small, approximately 16 kbp, circular mitochondrial genome present in a vast excess of copies relative to nuclear chromosomes. Thus, mitochondrial composition is plastic in nature and is under the control of dual genomes (mitochondrial and nuclear) that add a layer of complexity to the genomic and proteomic composition of this organelle. Most of the mitochondrial proteins are encoded by nuclear genes and 13 proteins which are the core constituents of the mitochondrial electron transport chain (ETC) complexes are encoded by the mitochondrial DNA (mtDNA). Additionally, the mitochondrial genome encodes 22 transfer RNAs (tRNAs) and 2 mitochondrial ribosomal RNA (rRNA), which are essential components for the mitochondrial translational apparatus (Kopinski et al., 2019; Wallace, 2018). The nuclear-encoded mitochondrial proteins are translated on cytosolic ribosomes and actively imported and sorted into mitochondrial sub-compartments by outer and inner membrane translocase machinery. The ETC complex generates an electrochemical gradient, mitochondrial membrane potential ($\Delta\Psi_m$) through the coupled transfer of electrons to oxygen (O_2) and the transport of protons (H^+) from the matrix across the inner mitochondrial membrane (IMM) into the intermembrane space (IMS). The $\Delta\Psi_m$ powers the terminal complex V of the ETC, the ATP synthase, which is likened to an ancient rotary turbine machine that catalyzes the synthesis of most cellular ATP. Additionally, the generated $\Delta\Psi_m$ is linked to a critical mitochondrial function, the mitochondrial Ca^{2+} uptake through the mitochondrial calcium uniporter (MCU) (Baughman et al., 2011; De Stefani et al., 2011). The functional status of mitochondria depends on the $\Delta\Psi_m$ and any perturbation of $\Delta\Psi_m$ activates a cascade of signaling events that either activate the repair mechanism or eliminate defective mitochondria (Friedman and Nunnari, 2014).

Having established mitochondria as multivariate signal processors in nearly all eukaryotes, in recent years the diverse functionality of mitochondria has been linked to their morphological complexity. An observation made over seven decades ago revealed the

plasticity of mitochondria (Porter et al., 1945), eg: fibroblast mitochondria are usually long and filamentous (1 to 10 μm in length with a constant diameter of ~700 nm), whereas, mitochondria in hepatocyte are more uniformly spheres or ovoids (Youle and van der Bliek, 2012). Additionally, mitochondria in many cell types form a complex reticulum, whereas axonal mitochondria are separated from these reticular structures and exist as discrete organelles of typically 1–3 μm in length (Chang and Reynolds, 2006). The phenotypic state of mitochondria has been linked to their capacity to produce energy, cell viability, and death (Mishra and Chan, 2014; Youle and van der Bliek, 2012). Though the link between phenotypic and functional state of mitochondria is not always observed in every context, it is true for a plethora of scenario that actively respiring mitochondria are long and filamentous, while short circular fragmented mitochondria are often associated with pathological conditions (Mishra and Chan, 2014). Thus, the pleomorphic nature of mitochondria makes it apparent that these organelles are far from static.

Mitochondria are highly dynamic organelles that constantly undergo shape change through fusion and fission to form a continuous network. Constant cycles of fission and fusion promote the intermixing and the homogenization of mitochondrial proteins, lipids, and DNA among different mitochondria within the same cell (Mishra and Chan, 2014). Also, mitochondrial dynamics allow selective elimination of mitochondria through mitophagy and mitochondrial biogenesis replenishes the mitochondrial pool, thus maintaining a healthy mitochondrial repertoire within the cell (Glancy et al., 2017). Specifically, in neurons the mitochondria are transported anterograde from the cell body to the processes of the cell, or retrograde back to cell body, enabling the removal of damaged mitochondria or replenishing healthy mitochondria (Dai et al., 2014; Suen et al., 2010; Valenci et al., 2015). Mitochondrial transport in neurons is facilitated by the microtubule-based adaptor or motor proteins trafficking system, consisting of kinesin/dynein (motor), Milton/Trak (adaptor) and Miro (mitochondrial receptor protein) (Mandal and Drerup, 2019; Schwarz, 2013).

Apart from these intracellular mitochondrial dynamics, horizontal transfer of mitochondria between mammalian cells was recently shown, thereby challenging current concepts of mitochondria and mtDNA segregation and inheritance (Torralba et al., 2016). This transcellular transfer of mitochondria promotes the incorporation of the transferred mitochondria into the endogenous network of the recipient cells, contributing to changes in the bioenergetic profile and other functional properties of the recipient cell. In addition, the intercellular transfer of mitochondria also involves the horizontal transfer of mitochondrial genes, which has important implications in physiopathology of mitochondrial dysfunction. The diverse mechanisms like formation of tunneling nanotubes, extracellular vesicles, and formation of gap junctions, naked mitochondrial ejection, or cytoplasmic fusion mediate intercellular mitochondrial transfer (Chang et al., 2019; Nakamura et al., 2020). This review focuses on both intracellular and intercellular mitochondrial movement, the contributing molecular mechanisms, molecular machinery and the impact on cell survival with a special focus on brain cells.

2. Neuronal energy demand and supply

Neuronal functioning requires high energy and a constant supply of ATP is critical for neuron growth, survival and function (Nicholls and Budd, 2000). The brain occupies only 2% of the body weight, but consumes 20% of the body's resting energy production (Tomasi et al., 2013). Within the brain, neurons utilize 70–80% of the energy and neighboring glial cells utilize the remainder. For example, a cortical neuron in the human brain utilizes up to ~4.7 billion ATP molecules/second to power various biological processes (Zhu et al., 2012). In the brain, synapses are the primary sites of ATP consumption, where mitochondria supply ~93% of the ATP demand, with the remaining ~7% provided through glycolysis (Harris et al., 2012). Due to such a high energy demand, neurons require specialized mechanisms to maintain energy homeostasis throughout the cell, particularly in distal synapses and in axons (Saxton and Hollenbeck, 2012; Sheng, 2014). The larger size of neurons precludes the rapid diffusion of ATP from one end of the cell to the other (Hubley et al., 1996; Sun et al., 2013). This implies that the energy production must be spatially matched to local energy usage. Thus, it is necessary for the mitochondria to be located close to the sites of high energy demands, including pre- and post-synaptic domains, the axon initial segment, nodes of Ranvier and growth cones (Hollenbeck, 1996, 2005; Hollenbeck and Saxton, 2005). Axons and synapses are highly plastic and undergo spontaneous and activity-dependent remodeling, thereby changing mitochondrial distribution. Therefore, the position of mitochondria in neurons must be controlled on rapid timescales to match changes in synaptic input. Live cell imaging using mitochondrial targeted fluorescent dyes or genetically encoded mitochondrial proteins reveal that in neurons, the mitochondrial network is very dynamic, undergoing fission and fusion events with individual mitochondria exhibiting bi-directional transport, stopping, starting, persistent docking in certain regions, and changing direction (Schwarz, 2013). The mean neuronal mitochondrial transport velocities in invertebrates and vertebrates lie within the range of ~0.1–1.4 μm/s (Melkov and Abdu, 2018; Schwarz, 2013). In addition to the fast transport, a lower velocity (~50 μm/h or even slower) has been identified in developing neurons (Miller and Sheetz, 2004). In mature neurons, about 20–30% of axonal mitochondria are motile (Chen and Sheng, 2013; Kang et al., 2008); while ~15% either briefly pause or dock at synapses; and ~14% of motile mitochondria dynamically pass through presynaptic terminals. An anchored mitochondrion within the presynaptic terminals provides a stable and continuous ATP supply. Conversely, a motile axonal mitochondrion temporally supplies ATP thus, changing synaptic energy levels and synaptic activities. Mitochondria are transported between the soma and distal axon and dendritic terminals by microtubule (MT)-based motor proteins dependent transport mechanism: Kinesin-1 (KIF5) and dynein motors drive anterograde and retrograde movement, respectively (Fig. 1).

3. Intracellular mitochondrial transport

3.1. Motor proteins for mitochondrial movement

3.1.1. Kinesin: Kinesin superfamily proteins (KIFs) and cytoplasmic dynein mediate long-distance transport of mitochondria and other membranous organelles or cargoes through mechanisms that depend on the polarity and organization of neuronal MTs and

require ATP hydrolysis (Hirokawa et al., 2010; Martin et al., 1999). Axonal MT are uniformly arranged with their plus-end directed distally and minus end towards the soma. Of the 45 kinesin motor genes identified, the Kinesin-1 (KIF5) family drives plus-end directed anterograde transport of neuronal mitochondria (Cai et al., 2005; Gorska-Andrzejak et al., 2003; Hurd and Saxton, 1996; Pilling et al., 2006). Kinesin-1 contains two heavy chains (KHC) and two light chains (KLC). The N-terminal domain of kinesin-1 is the motor domain with ATPase activity and binds directly to the MTs and the C-terminal is the cargo-binding domain (Fig. 1A). There are three isoforms of KIF5 (KIF5A, 5B, and 5C). KIF5A is ubiquitously present; whereas, KIF5B and 5C are specific for neurons (Kanai et al., 2000). Disruption of KIF5-mitochondrial coupling impairs mitochondrial transport, thus reducing the number of mitochondria in the distal axons (Hurd and Saxton, 1996; Tanaka et al., 1998).

3.1.2. Dynein: Cytoplasmic dynein guides MT-based retrograde transport in axons. Dynein contains multiple subunits including two catalytic heavy chains (DHC), several intermediate (DIC), light-intermediate (DLIC) and light chains (DLC). The C-terminus of the DHC is the motor domain required for the dynein movement. Dynactin is a large complex protein that binds directly to dynein and MTs through its $p150^{Glued}$ subunit (Waterman-Storer et al., 1995). Both dynein and dynactin associate with mitochondria and drive its retrograde transport (Fig. 1B) (Karki and Holzbaur, 1999; King and Schroer, 2000; Waterman-Storer et al., 1995). Disruption of the dynactin complex does not disrupt the binding of motor proteins to the mitochondria, but interrupts both the anterograde and retrograde transport, suggesting that dynactin is probably involved in the regulation of both KIF5 and dynein driven bi-directional mitochondrial transport (Haghnia et al., 2007).

3.1.3. Myosin: While the MT-based motor proteins quickly transport cargoes and organelles through lengthy axons, myosin drives short distance trafficking along with actin filaments at the presynaptic terminals and growth cones (Morris and Hollenbeck, 1993). Myosins I, II, V, and VI were reported to facilitate mitochondrial transport in axons. Myosin V is a two-headed motor containing unique globular tail domain and can interact directly with either a kinesin motor or with the light-chain of dynein (Fig. 1C and 1D) (Langford, 2002), raising the possibility that the dual-motor complex may facilitate co-ordination of mitochondrial long-range transport along microtubules and short-range movement on actin filaments. For example, Myo19 was identified as a mitochondria-associated myosin and its overexpression in neuronal cell lines increased mitochondrial motility (Quintero et al., 2009). Later with the help of proximity labeling, Miro was identified as the potential binding partner of Myo19. The interaction studies showed Miro1 to bind directly to a C-terminal fragment of the Myo19 tail region. These results show Miro to coordinate microtubule and actin-based mitochondrial movement (Fig.1E) (Oeding et al., 2018)

3.2. Motor-adaptor complex for mitochondrial movement

Motor proteins recruit mitochondria by associating with their respective motor adaptor proteins and mitochondrial membrane receptors (Fig. 1). This motor/adaptor/receptor complex ensures targeted mitochondrial trafficking and precise regulation of their distribution in response to changes in neuronal activity (Brickley et al., 2005; Brickley and

Stephenson, 2011; Fransson et al., 2003; Fransson et al., 2006; Glater et al., 2006; Guo et al., 2005; Stowers et al., 2002). The *Drosophila* protein Milton was the first identified motor adaptor protein that links mitochondrial OMM protein Miro to the KIF5 cargo binding domain (Glater et al., 2006). There are two mammalian orthologues: TRAK1 and TRAK2, sometimes referred to as GRIF1 and OIP106 (Beck et al., 2002; Brickley et al., 2005). TRAK1/2 lacks an obvious domain structure other than an extensive coiled-coil region, which includes a significant region of homology with the Huntingtin-associated protein 1 (HAP-1) (Stowers et al., 2002). The interaction of TRAK with KIF5 is direct, as TRAK contains one N-terminal KIF5 binding site and two dynein/dynactin binding sites; one at the N-terminal and the other at the C-terminus (van Spronsen et al., 2013). Thus, TRAK can mediate both KIF5 and dynein driven bi-directional transport of axonal mitochondria. Mammalian TRAK proteins are also recruited to endosomal compartments and associate with γ-aminobutyric acid ($GABA_A$) receptors, K+ channels, and Hrs protein highlighting their role in neuronal cargo transport (Grishin et al., 2006; Kirk et al., 2006; Webber et al., 2008). Depleting TRAK1 or overexpressing a dominant negative mutant impairs axonal mitochondrial motility (Brickley and Stephenson, 2011; van Spronsen et al., 2013). Conversely, overexpression of TRAK2 robustly enhances mitochondrial motility (Chen and Sheng, 2013).

3.3. Motor-adaptor-receptor complex for mitochondrial movement

Miro, the mitochondrial outer membrane protein functions as the receptor of the motor/adaptor/receptor complexes by binding to the motor adaptor, Milton (or TRAK1/2) thus, recruiting KIF5 to the mitochondrial surface (Glater et al., 2006) (Fig. 1). Miro is a member of the Rho-GTPase family with two homologous domains with small GTPases that are separated by two Ca^{2+} binding EF-hand motifs. At the carboxyl terminus of Miro there is a trans-membrane domain that tethers it to the surface of the OMM (Fransson et al., 2006). *Drosophila* has a single Miro (Guo et al., 2005); whereas, mammals have two Miro orthologs, Miro-1 (RhoT1) and Miro-2 (RhoT2) and they share 60% sequence identity (Aspenstrom et al., 2007). Biochemical data originally linked Milton and Miro only to KIF5, but *Drosophila* phenotypes indicated that they may also be needed for dynein-mediated transport (Stowers et al., 2002).

3.4. Regulation of mitochondrial movement

3.4.1. Reversible arrest by cytosolic Ca^{2+}—Cytosolic Ca^{2+} (cCa^{2+}) is one of the best studied regulators of mitochondrial movement. Elevation of cCa^{2+} stops both the anterograde and the retrograde movement of mitochondria (Chang et al., 2006; Rintoul et al., 2003; Szabadkai et al., 2006). Such elevation of cCa^{2+} occurs during physiological stimuli including the activation of glutamate receptors in dendrites, action potential in axons, and by neuromodulators that trigger Ca^{2+} release from the stores (Ohno et al., 2011; Rintoul et al., 2003; Saotome et al., 2008; Yi et al., 2004). Studies have shown ~0.4 μM of cCa^{2+} is needed to cause 50% reduction in mitochondrial movement and complete arrest occurs at low micromolar concentrations (Saotome et al., 2008; Yi et al., 2004). The docking of mitochondria in the region of high Ca^{2+} maintains cCa^{2+} homeostasis by (i) buffering the cCa^{2+} and (ii) also providing ATP for the active efflux of Ca^{2+} from the cell. In addition, subcellular regions with inadequate ATP are likely to have elevated cCa^{2+} and the arrest of

mitochondria in such regions will provide ATP for biological processes (Wang and Schwarz, 2009; Yang and Steele, 2000). Detailed examination of the mechanism underlying this regulation suggested that the EF hand of Miro plays a critical role in the Ca^{2+}-mediated regulation of mitochondrial movement. Two mechanisms are proposed for the molecular mechanism downstream to Ca^{2+} binding: (i) Ca^{2+} binding to Miro triggers the dissociation of either Miro/TRAK or TRAK/KIF5 interaction, thereby uncoupling the KIF5 from the mitochondrial surface (Macaskill et al., 2009) (Fig. 2A); (ii) KIF5 switches from the active state to an inactive state by binding to the Ca^{2+} bound Miro through the head domain of kinesin, thus, preventing it from binding to microtubules (Wang and Schwarz, 2009) (Fig. 2B). These two proposed mechanisms are noteworthy in the context of health and disease. The first is readily reversible and will allow mitochondrial movement as soon as the cCa^{2+} is removed. The second explains the selective arrest of mitochondrial motility.

3.4.2. Irreversible arrest by PINK/Parkin pathway—An additional mechanism of mitochondrial arrest by PINK1 (PTEN-induced putative kinase 1)/Parkin-mediated pathway is very different from the readily reversible Ca^{2+}-mediated mitochondrial docking. PINK1 is a protein kinase and Parkin is an E3 ubiquitin ligase, and together they initiate a pathway for the autophagic clearance of mitochondria. Physiologically, PINK1 is recruited and imported into the mitochondria and then inactivated by proteolytic cleavage. Conversely, in a damaged mitochondria with compromised mitochondrial membrane potential ($\Delta\Psi m$) PINK1 accumulates on the surface of the mitochondria and recruits Parkin to the site. The potential involvement of a PINK/Parkin mechanism for the mitochondrial movement was confirmed by the observation of a biochemical association between PINK1 and Miro/Milton complex (Weihofen et al., 2009). Subsequently, Parkin was also shown to bind this complex, and this interaction was greatly increased in depolarized mitochondria (Wang et al., 2011). Binding of PINK1 phosphorylates Miro at various sites of which S156 is indispensable for the activity of Parkin. The consequence of activating the PINK/Parkin pathway is the proteasome-dependent degradation of Miro and consequent release of KIF5/Milton complex from the mitochondrial surface (Chan et al., 2011; Wang et al., 2011) (Fig. 2C). This proteolytic and irreversible mechanism of mitochondrial docking will facilitate the removal of quarantined mitochondria by mitophagy and prevent them from fusing with other healthy mitochondria.

3.5. Mitochondrial anchoring

One of the most intriguing anchoring mechanisms involves the association of mitochondria with microtubules through the protein syntaphilin (SPH) (Fig. 2D). SPH acts as a static anchor specific for axonal mitochondria (Kang et al., 2008). SPH specifically targets the outer membrane of the axonal mitochondria through its C-terminal mitochondria-targeting domain and axon-sorting sequence and arrests motile mitochondria by anchoring them to the MTs. Dynein light chain LC8 also appears to be involved with the SPH-based docking (Chen et al., 2009). Deleting SPH results in a robust increase in axonal mitochondrial motility and conversely overexpression of SPH abolishes mitochondrial motility (Kang et al., 2008). However, SPH is absent from the genome of *Drosophila,* although flies have the stationary pool of mitochondria like mammals in their neurons.

These observations clearly show that mitochondria are not just static organelles afloat in the cytosolic milieu, but that they are dynamic organelles that traffic, fuse and divide (Jakobs, 2006). In axons, motile mitochondria can become stationary and stationary mitochondria can be remobilized in response to changes in bioenergetic status and synaptic activity. Thus, axonal mitochondria deploy an anchoring mechanism in addition to the motor-driven transport. These anchored/docked mitochondria serve as the power plants for stable and continuous ATP supply at the synapses. Thus, SPH can serve as an attractive molecular target for investigations into mechanisms recruiting motile mitochondria into activated synapses.

4. Intercellular mitochondrial transfer

Organelle exchange represents a special form of intercellular communication that allows unidirectional or bidirectional transfer, not only of signals, small molecules, or ions but also defined intracellular structures such as mitochondria, lysosomes, endosomal vesicles and plasma membrane components (Rogers and Bhattacharya, 2013). Transport of mitochondria from one cell to another has gained importance in recent years as a significant mediator of cellular health and fitness. Mitochondrial transfer supports the exogenous replacement of damaged mitochondria, thereby rescuing mitochondrial defects (Hayakawa et al., 2016; Patananan et al., 2016). Most studies used stem cells as mitochondria donors, but some utilized immortalized or primary cells from the same or different species to serve as mitochondrial donors (Berridge et al., 2016). It was previously unknown whether mitochondrial transfer was a physiologically relevant process or was observed only in the *in vitro* environment. But in recent years, several reports provide evidence for transfer of mitochondria to occur *in vivo* and to be involved in diverse pathophysiological conditions such as tissue injury and cancer progression (Ahmad et al., 2014; Hayakawa et al., 2016; Islam et al., 2012; Moschoi et al., 2016; Tan et al., 2015). The molecular and signaling mechanism(s) by which cells containing dysfunctional mitochondria acquire functional mitochondria from other cells and how this process is regulated remains unknown. But it is hypothesized that cells likely possess mechanisms to trigger organelle exchange in response to injury signals originating from distressed recipient cells. However, the molecular cues that initiate this process remain unidentified. The first evidence of functional mitochondrial transfer was observed in intrinsic mitochondria-depleted recipient cells from mesenchymal stem cells/fibroblasts (Spees et al., 2006). Careful examination of mitochondrial and nuclear DNA polymorphisms in these rescued clones excluded cell fusion as the mechanism of mitochondrial transfer. Also, the transfer did not occur through the passive uptake of mitochondrial fragments or isolated organelles but involved an active process such as the formation of tunneling nanotubes (TNTs) or vesicular transfer of mitochondrial fragments (Spees et al., 2006). TNTs are so far described as the major cellular structure that mediates intercellular mitochondrial transfer. Other mechanisms have been proposed including, extracellular vesicles, naked mitochondria ejection, and gap junctions.

4.1 Tunneling nanotubes

TNTs are nanotubular structures produced by the outgrowth of filopodia-like cell membrane protrusions that connect with the target cell. The membrane from each cell extends to fuse

together, thereby forming a tightly connected bridge that is not tethered to the substrate, but rather hovering in the extracellular space (Gerdes et al., 2007). The discovery of TNTs in 2004 emerged as a novel mechanism of cell-to-cell communication, demonstrating the ability of mammalian cells to donate or receive organelles from other cells (Rustom et al., 2004). TNTs are different from other cellular protrusions, as TNTs are straight with a small diameter (20–500nm) and a length up to 100 μm. They are dynamic structures formed *de novo* within minutes and display lifetimes ranging from minutes to several hours (Bukoreshtliev et al., 2009; Rustom et al., 2004). TNTs contain a skeleton mainly composed of F-actin and transport proteins that facilitate active transport of cargo and mitochondria along these structures (Ahmad et al., 2014). TNTs were first described in cultured pheochromocytoma PC12 cells (Rustom et al., 2004) and subsequent studies have shown that they connect a variety of cell types including epithelial, fibroblastic, immune, neuronal and glial cells (Abounit and Zurzolo, 2012; Gousset et al., 2009; Rustom et al., 2004; Wang and Gerdes, 2011) and an evidence confirm their existence *in vivo* (Chinnery et al., 2008). Studies have shown the involvement of TNTs in mitochondrial transport, the repair of cell damage, the activation of enhanced immune responses, and cell metabolic reprogramming (Bukoreshtliev et al., 2009; Gousset et al., 2009; Islam et al., 2012; Jackson et al., 2016; Sinha et al., 2016).

Though the transport of mitochondria through TNTs is defined (Fig. 3A), the directionality of mitochondrial transfer and other indispensable factors essential to initiate and promote TNT formation are not fully understood. A study identified the involvement of S100A4, an extracellular protein and its receptor RAGE (Receptor for Advanced Glycation End Product) in guiding TNTs growth direction. During stress, hippocampal neurons and astrocytes initiated the formation of TNT's after p53-mediated activation of caspase 3. Activated caspase 3 cleaves S100A4 creating a gradient of low levels of S100A4 in initiating cells (injured cells) towards a higher concentration in astrocyte target cells (Sun et al., 2012). The results from this study reveal that damaged cells (neurons) transfer cellular contents to astrocytes to spread the danger signals to initiate mitochondrial transfer. But no information regarding mitochondrial participation was provided by this study. These studies on TNT formation and mitochondrial transfer raise intriguing questions (i) on the degree of cellular damage required to initiate intercellular mitochondrial transfer and (ii) the mechanism(s) by which healthy cells detects the degree of metabolic shutdown of the stressed cells and makes a judgment to restore the functionality of the stressed cells, instead of permitting apoptosis. This line of investigation is of great interest in deciding the fate of terminally differentiated cells, including adult neurons and cardiomyocytes.

Spees *et al.* described the transfer of mitochondria from mesenchymal stem cells (MSCs) to respiration-deficient cancer cells through TNTs (Spees et al., 2006), but the direction of transfer or whether the MSCs received any signal from cancer cells to facilitate mitochondrial transfer was not described. Koyanagi *et al.*, showed the transfer of mitochondria exclusively through TNTs from human endothelial progenitor cells (EPCs) to neonatal undifferentiated cardiomyocytes in a process intending to sustain their maturation (Koyanagi et al., 2005). Yang *et al.*, while using microfluidic channels to track TNT formation and exchange of biological materials, observed MSCs forming TNTs and transferring mitochondria to cardiomyocytes, as opposed to fibroblasts (Yang et al., 2016).

But why MSCs have a greater propensity to form TNTs compared to other cells remain unanswered. Similarly, MSC improve the survival of endothelial cells and cardiomyocytes after ischemic-reperfusion injury (in an *in vitro* model system deprived of glucose and oxygen and then reoxygenated) by transferring its mitochondria independent of cellular transdifferentiation or the release of paracrine factors (Han et al., 2016; Liu et al., 2014b). Mitochondrial transfer from MSCs to lung epithelium attenuated cigarette-smoke induced lung damage (Li et al., 2014), while transfer between MSC and innate immune cells via TNTs enhanced the capacity of alveolar macrophages to engulf invading bacteria in a pneumonia model (Jackson et al., 2016). A follow-up study reported that Miro1 regulated the efficiency of intercellular movement of mitochondria. Overexpression of Miro1 in MSC increased the transfer of mitochondria from stem cells to stressed alveolar epithelial cells via intercellular TNTs, thus attenuating epithelial cell apoptosis, and reducing inflammatory cell infiltration, collagen deposition, and mucus hypersecretion in the lungs (Ahmad et al., 2014). Cancer cells also acquire mitochondria from the surrounding healthy cells to optimize and repair damaged metabolic machinery and promote survival. One such critical donor of healthy mitochondria to cancer cells are stromal cells. Cancer and stromal cells communicate through TNTs and mitochondrial transfer via TNTs is shown to enhance the chemoresistance to doxorubicin and survival (Pasquier et al., 2013). In addition, mitochondrial transfer from bone marrow mesenchymal cells to acute myelogenous leukemia cells *in vivo* confers chemoresistance and survival (Moschoi et al., 2016).

4.2. Gap Junctions

Gap junctions have evolved into specialized cell to cell structures between eukaryotic cells and allow direct metabolic and electrical communication between almost all cell types. Generally, gap junction channels allow the passive diffusion of molecules of up to 1,000 Daltons and they transport nutrients, metabolites, second messengers, cations, anions and mitochondria (Evans and Martin, 2002; Islam et al., 2012; Mistry et al., 2019; Otsu et al., 2009; Yao et al., 2018). Nearly 20 connexin genes and 3 pannexin genes contribute to gap junction proteins in mice and humans. Connexin oligomerizes and forms channel-like structures or gap junctions between cells. Live optical imaging showed gap-junction-mediated intercellular mitochondrial transfer between bone marrow derived stromal cells (BMSC) and alveolar-epithelial cells in a lipopolysaccharide (LPS)-injured mouse model. BMSC infused to the trachea of LPS-treated mice attached to the alveolar epithelial cells by a mechanism involving connexin, particularly connexin 43. BMSC established Cx43-containing gap junctional channels (GJCs) with the alveolar epithelia releasing mitochondria-containing microvesicles that were engulfed by the epithelial cells (Fig. 3B). The presence of BMSC-derived mitochondria in the epithelia was confirmed microscopically (Islam et al., 2012). Mitochondrial acquisition by the alveolar cells restored ATP concentration and increased the secretion of pulmonary surfactant. Additionally, silencing of Rieske protein (RISP), a subunit of the mitochondrial complex III in BMSCs reduced their capacity to restore ATP levels in alveolar cells, preventing protection from LPS-injury. This implicated mitochondrial transfer as one of the plausible mechanisms of MSC-mediated therapy in models of acute lung injury and other inflammatory diseases. Recent study demonstrated that overexpression of Cx43 in iPSC derived MSCs facilitated mitochondrial transfer between iPSC-MSCs and epithelial cells in both *in vitro* and *in vivo*

model of asthma inflammation (Yao et al., 2018). Cx43-based intercellular gap junctional communication also occurred in co-culture of MSCs and endothelial cells (Otsu et al., 2009). Recently, it was shown that the transfer of mitochondria from BMSCs to hematopoietic stem cells (HSCs) is an early physiological event in the mammalian response to acute bacterial infection (Mistry et al., 2019) . Mechanistically, it was observed that ROS-induced oxidative stress regulates the opening of connexin channels in a system mediated by phosphoinositide 3-kinase (PI3K) activation, which allowed the transfer of mitochondria from BMSCs to HSCs (Mistry et al., 2019).

4.3. Extracellular Vesicles

Every cell type releases a heterogenous population of vesicles to the extracellular environment. These vesicles range from 40 to 1000 nm and are referred to as extracellular vesicles (EVs). EVs can be divided into exosomes (30 to 100 nm in diameter), microvesicles (MVs) (100 nm to 1 μm diameter), and apoptotic bodies (1 to 2 μm) depending on their origin and molecular composition (Zappulli et al., 2016). Apoptotic bodies have been less studied due to their rapid elimination by phagocytic cells (Pitt et al., 2016). Exosomes and microvesicles were initially described as vesicles meant to remove archaic proteins in immune cells and reticulocytes (Paolicelli et al., 2019). More recently, they have been shown to originate from almost all cell types and are reported to mediate cell-to-cell communication in a growing number of physiological and pathological situations (Paolicelli et al., 2019). Growing evidence indicates that EVs containing lipids, proteins, RNAs and mitochondria represent an efficient way to transfer functional cargoes from one cell to another. In this line, the discovery of EVs have brought a new format of interactions to an already multifaceted communication network and a new mechanism of signal transmission (Hervera et al., 2018; Valadi et al., 2007).

Mitochondrial components have been detected in EVs (Fig. 3C), although the mechanisms by which mitochondrial proteins or mtDNA are loaded in EVs are still unknown. Smaller EVs like exosomes can transport mostly small RNAs (Valadi et al., 2007), but genomic and mtDNA have also been detected (Guescini et al., 2010; Sansone et al., 2017). Larger EVs such as MVs can contain entire mitochondria (Berridge and Neuzil, 2017). MVs carrying mitochondria can be secreted by different cell types, as observed in astrocytes, neurons and mesenchymal stem cells (Davis et al., 2014; Guescini et al., 2010; Hayakawa et al., 2016; Sinha et al., 2016) and received by epithelial cells, immune cells, astrocytes, and neurons (Torralba et al., 2016). The process of mitochondrial transfer does not always protect damaged cells but also functions to recycle organelles in other cells by a transcellular degradation process (Davis et al., 2014; Hayakawa et al., 2016). Additionally, MSC-derived EVs containing mitochondria have been shown to modulate macrophage function by enhancing their respiration and phagocytic activity (Morrison et al., 2017). In a recent study, mitochondria was observed in EVs derived from the pro-inflammatory HLA-DR+ subsets of airway myeloid derived regulatory cells (MDRCs), which are known regulators of T cell responses in asthma. Importantly, these exosomally transferred mitochondria co-localize with the mitochondrial network and generate reactive oxygen species within recipient T cells (Morrison et al., 2017).

Apart from mitochondria being expelled in EVs, mitochondria possess the capacity to produce their own vesicles in order to transport mitochondrial proteins and lipids to other organelles within the cell (Bozi et al., 2016; Bragoszewski et al., 2017; Braschi et al., 2010; Cadete et al., 2016; McLelland et al., 2014; Neuspiel et al., 2008; Soubannier et al., 2012a; Soubannier et al., 2012b; Sugiura et al., 2014). These mitochondria-derived vesicles are an alternative means of quality control alongside the action of mitochondrial proteases, ubiquitin-mediated proteosomal degradation, and mitophagy (Bragoszewski et al., 2017). As one of mitochondria's first line of defense, MDVs expel damaged proteins in order to prevent complete mitophagy. These MDVs are formed by selective incorporation of protein cargoes which can include outer/inner membrane and matrix components (McLelland et al., 2014; Neuspiel et al., 2008; Soubannier et al., 2012a; Soubannier et al., 2012b; Sugiura et al., 2014). The mechanisms that dictate cargo selection are still unclear, however proteins are known to be selected based on their target destination and the nature of mitochondrial stress. MDVs destined for lysosomal degradation contain protein kinase PINK1 and cytosolic ubiquitin E3 ligase Parkin (McLelland et al., 2014; Soubannier et al., 2012a; Soubannier et al., 2012b), and those following the peroxisome pathway carry the retromer complex and MAPL protein (Braschi et al., 2010; Neuspiel et al., 2008).

4.4. Mitochondrial extrusion

Mitochondrial extrusion is another possible mechanism for mitochondria to transfer from one cell to another. Extrusion allows the release of mitochondria or mitochondrial components from cells under specific conditions during which mitochondria become unfit to remain in cells. Retaining damaged mitochondria can produce large quantities of ROS (Lyamzaev et al., 2008) and under such circumstances, cells tend to dispose of their mitochondria into the intercellular space. During TNFα-induced cell death, cytoplasmic vacuoles engulf mitochondria, which subsequently fuse with the plasma membrane, releasing free mitochondria to the extracellular medium (Maeda and Fadeel, 2014). Likewise, LPS stimulation promotes the fusion of autophagolysosomes with the plasma membrane and releases mitochondrial components into extracellular space (Unuma et al., 2015). Neutrophils can extrude mitochondrial components including specifically oxidized mitochondrial nucleoids (Caielli et al., 2016). Under high concentrations of ROS, HeLa cells can extrude fragments of mitochondria through selective elimination or mitoptosis (Lyamzaev et al., 2008). Basophils release mtDNA in an ROS-dependent manner to create extracellular DNA traps to fight bacteria (Morshed et al., 2014). Likewise, eosinophils rapidly release mtDNA in the extracellular space to bind to and kill infectious bacteria (Yousefi et al., 2008). Mitochondrial extrusion not only occurs *in vitro* but also *in vivo*. A handful of studies have shown the release of naked or encapsulated mitochondria into the extracellular milieu. Nakajima *et al.*, in 2008 used a mouse model to confirm the release of naked mitochondria into the intercellular space after an anti-Fas antibody injection. In response to this treatment, cytoplasmic vacuoles engulfed fragmented mitochondria and extrude them from apoptotic hepatocytes (Nakajima et al., 2008). Likewise, activated platelets released respiration-competent mitochondria, both as free organelles or mitochondrial encapsulated within microparticles (Boudreau et al., 2014). A number artificial techniques that is independent of biological interaction between the isolated organelle and the recipient cells were developed for the transfer of naked mitochondria.

Examples include mitochondrial transformation (Kesner et al., 2016), magnetomitotransfer (Macheiner et al., 2016), MitoCeption (Caicedo et al., 2015), and mitochondrial invasion (Popkov et al., 2017). In 2016, transfer of naked mitochondria into somatic mammalian cells using photothermal nanoblade was devised (Wu et al., 2016). The use of photothermal nanoblade bypasses endocytosis and cell fusion and showed to rescue the pyrimidine auxotroph phenotype and respiration of ρ0 cells that lack mtDNA.

5. Mitochondrial transfer in the central nervous system

Astrocytes have broad and diverse roles in the central nervous system (CNS), and are involved in the regulation of neurodevelopment, neurotransmission, metabolism and blood flow (Attwell et al., 2010; Iadecola and Nedergaard, 2007; Khakh and Sofroniew, 2015). Under normal conditions, astrocytes protect neurons against oxidative stress and excitotoxicity and provide neurotrophic support (Ouyang et al., 2013; Wang and Cynader, 2001). By contrast, astrocytes under stressful conditions may withdraw this protection or release deleterious factors such as inflammatory cytokines that damage neurons (Khakh et al., 2017; Liddelow et al., 2017; Trias et al., 2018; Yun et al., 2018). Recent proof-of-concept studies demonstrate that the neuroprotective functions of astrocytes may include transfer of mitochondria to damaged neurons (Hayakawa et al., 2016; Huang et al., 2019; Lippert and Borlongan, 2019; Volobouева et al., 2007). Not just astrocytes transfer mitochondria, but recent emerging data suggest the entire nervous system to benefit from cell to cell transfer of mitochondria for different purposes and under different physiological conditions. For example, (i) the transfer of mitochondria to astrocytes from neurons allowed cells to breakdown nonfunctional mitochondria (transmitophagy) (Davis et al., 2014); (ii) the transfer of healthy mitochondria from astrocytes to stressed neurons promoted neuroprotection and neurorepair (Hayakawa et al., 2016; Huang et al., 2019; Lippert and Borlongan, 2019); (iii) the transfer of functional mitochondria from endothelial progenitor cells (EPCs) to brain endothelial cells improved cell viability and barrier function (Hayakawa et al., 2018a; Hayakawa et al., 2018b); (iv) transfer of hematopoietic stem and progenitor cells' (HSPCs) mitochondria to neurons restored mitochondrial function along with frataxin expression in a mouse model of Friedreich's ataxia (Rocca et al., 2017).

5.1. Transmitophagy in the CNS

It is generally accepted that healthy cells degrade their own mitochondria. But recently, recycling of these organelles by other cells in a process called transcellular degradation of mitochondria or transmitophagy has been reported. The transmitophagy process is mediated by the cellular evulsions containing mitochondria from neurons, in which these structures are taken up by astrocytes and then recycled (Davis et al., 2014). The reason for transmitophagy is not known, but it has been hypothesized that focal axon damage stimulates the process. Another theory posits that transporting the damaged mitochondria back to the neuronal soma is energy demanding and thus the CNS makes use of astrocytes to perform the quality control process of removing the unfunctional mitochondria (Davis et al., 2014). It was observed that the retinal ganglion cell axons of wild type (WT) mice shed mitochondria at the optic nerve head (ONH). Based on the frequency of axonal protrusions and evulsions, within the ONH of adult WT mice, it was observed that most axons contain at

least one such structure filled with mitochondria at various stages of degeneration. Further, considering the number of mitochondria per protrusion/evulsion, ~1 million retinal ganglion cell mitochondria were estimated to be degraded at the ONH of mice at any one time. But this study left several questions unanswered: (i) it is unknown whether the axonal mitochondria removed at the ONH are only resident mitochondria within axons at this location or whether other axonal mitochondria fated to undergo degradation are actively transported to the ONH for disposal; (ii) why axonal mitochondria are degraded selectively at the ONH. But morphologically similar structures filled with degrading mitochondria were also found in the cortex at a 10-fold lower density, suggesting transmitophagy of axonal mitochondria by astrocytes is a widespread phenomenon in the CNS (Davis et al., 2014).

Additionally, astrocytes were shown to phagocytose whole synapses elsewhere in the visual pathway (Chung et al., 2013). Because synapses usually contain mitochondria, it is quite possible that the molecular machinery used by astrocytes in the phagocytosis of synapses may also be used in the phagocytosis of axonal mitochondrial evulsions in the ONH and elsewhere. Although this description of a transmitophagy process is new for the nervous system, transcellular degradation of mitochondria is also seen upon fertilization. Because mitochondrial genome is believed to be maternally inherited, immediately after fertilization sperm-derived paternal mitochondria trigger localized autophagy and active lysosomal degradation in the oocyte cytoplasm (Sato and Sato, 2011). Similarly, autophagy-assisted phagocytosis is also involved in the turnover of photoreceptor outer segments by retinal pigment epithelial cells (Kim et al., 2013), and thus may be a common mechanism used in the nervous system.

5.2. Transfer of healthy mitochondria to stressed neurons

Mitochondria comprise the intracellular cores for energetics and viability, but under some conditions mitochondria might also be released into the extracellular space and taken up by recipient cells (Falchi et al., 2013). The ability to exchange mitochondria represents a tangible mode of cell-to-cell signaling in the CNS. Considering the new concept of intercellular organelle transfer, in 2016, Hayakawa et al., (Hayakawa et al., 2016) showed CD38 signaling mediated release of functional mitochondria from activated astrocytes. The released mitochondria were then taken up by damaged cortical neurons where ATP levels were restored after oxygen-glucose deprivation/reperfusion (OGD/R) injury. Additionally, treatment with extracellular mitochondria containing particles, released from cultured astrocytes in a mouse model of focal cerebral ischemia provided neuroprotection (Hayakawa et al., 2016). Thus, *in vitro* astrocyte-to-neuron mitochondrial delivery and *in vivo* astrocyte-derived mitochondrial transfer promoted neuronal survival, plasticity, as well as improved energetic capacity and neurological functioning. Suppression of CD38 signaling by short interfering RNA reduced extracellular mitochondria transfer and worsened neurological outcomes. These findings suggest a new mechanism of neuro-glial crosstalk that may contribute to endogenous neuroprotective and neurorecovery mechanisms after stroke (Hayakawa et al., 2016).

If astrocyte-derived mitochondria can be shown to be inside damaged neurons, it is possible that bioenergetic support to the damaged neuron could be provided. Mitochondria could

serve as a direct therapeutic agent in numerous metabolic degenerative diseases. By simply increasing the number of functional mitochondria with intact motility in injured neurons may provide better regenerative capacity both in the peripheral and central nervous systems. However, mechanistic explanations of how these mitochondria are released into the cytoplasm or used to provide bioenergetic and metabolic support for damaged neurons needs to be addressed. One possibility is that the mitochondria are independent from receptor-mediated or protein coupled signaling to induce their effects. Cytokines, miRNA, transcription factors and other cell effectors usually require the activation of specific signaling pathways to induce a response in the target cell (Godlewski et al., 2015). We can speculate that the exogenous mitochondria once inside the cell can begin functioning and possibly fuse with resident mitochondria to build a pool of intact mitochondria to sustain bioenergetic demands during recovery. It is possible that transfer of replication competent functional mitochondria into recipient cells with damaged mitochondria *in vivo* requires membrane tunneling nanotube connections or intercellular bridges with cytoskeletal structures to direct movement, as observed in cell culture systems (Rustom et al., 2004). On the other hand, transfer of mitochondria via discrete membrane-bound particles is likely to result in transcellular mitochondrial degradation or recycling.

6. Mitochondrial transplantation: a new paradigm of therapeutic strategy

Without doubt, mitochondria are the master organelle of cellular energetics, fueling multiple process like proliferation, migration, differentiation and stress resistance. Thus, the concept of “mitochondrial medicine”, medical intervention targeting mitochondria, launches a new line of biomedical exploration. So far, more than 400 clinical trials for mitochondrial-targeted medical intervention including completed trials have been registered at ClinicalTrials.gov. Mitochondrial therapy aims to restore mitochondrial functions, however there is currently no treatment to cure mitochondria-related diseases caused by bioenergetic crisis or by accumulation of mitochondrial ROS. Therefore, a new paradigm of mitochondrial therapy based on either direct or systemic delivery of mitochondria from autologous sources was established (Birsa et al., 2013; Gollihue et al., 2018a). Such supplementation of exogenous healthy mitochondria to replace damaged mitochondria improved cellular bioenergetics, reversed excessive ROS production, and restored mitochondrial function (Gollihue and Rabchevsky, 2017; Liu et al., 2014a). In fact, growing body of evidence demonstrate promising outcome for mitochondrial transplantation strategies under many experimental conditions such as cardiac ischemia, ischemia reperfusion of liver, and neurodegenerative models (Nakamura et al., 2020).

Notably in a pioneering clinical study, mitochondrial transplantation was performed on five pediatric patients dependent upon extracorporeal membrane oxygenation (ECMO) support due to myocardial dysfunction related to ischemia and reperfusion (IR) (Emani et al., 2017). Autologous mitochondria isolated from the abdominal muscles were injected intracardially and all patients showed marked improvement in their myocardial function. Additionally, in a recent safety and efficacy study by the same group of researchers reported that intracorononary injection of mitochondria resulted in rapid uptake and specific biodistribution of exogenous mitochondria throughout the healthy swine heart. They also showed the mitochondrial administration to be safe with no effect on coronary patency.

Moreover, they demonstrated enhanced myocardial function following IR injury (Shin et al., 2019).

However, a view point article raised several concerns regarding their findings (Bertero et al., 2018) questioning: (i) how mitochondria could survive the extracellular environment with high Ca^{2+}, (ii) whether the mitochondria remain viable and efficiently produce ATP, and (iii) did the mitochondria pass through cell membranes to produce sufficient ATP to boost cardiac contractile function. In direct response, the authors (McCully et al., 2020) affirmed (i) the presence of cell-free mitochondria in the blood containing physiological Ca^{2+} and Na^{+} concentrations (Al Amir Dache et al., 2020), (ii) several studies have demonstrated functional integration of exogenous mitochondria into recipient cells in media containing 1.8 mM Ca^{2+}, representing physiological concentration (Cowan et al., 2017; Kesner et al., 2016; Pacak et al., 2015), (iii) mitochondria transplanted by either direct or intracoronary injection increased total tissue ATP content, oxygen uptake, contractile function, and upregulated proteins associated with mitochondrial function (Masuzawa et al., 2013; Shin et al., 2019), and (iv) transplanted mitochondria produced no systemic inflammatory responses (Emani et al., 2017; Masuzawa et al., 2013; Ramirez-Barbieri et al., 2019; Shin et al., 2019). As the first successful clinical trial for mitochondrial transplantation and based on similar cell death pathways as cardiac IR injury, it is logical to foster the emerging concept of mitochondrial transplantation after CNS injury to support oxidative phosphorylation in recipient cells (Gollihue et al., 2018b; Hayakawa et al., 2018a; Hayakawa et al., 2016).

6.1. Therapeutic use of exogenous mitochondria for CNS injury or disease.

6.1.1 Parkinson's disease.—Mitochondrial dysfunction aggravates the progression of PD, manifested by increased oxidative stress dysregulated bioenergetic homeostasis, and reduced viability of affected SN dopaminergic neurons. Though mitochondria targeting antioxidants proved to have great potential and promising outcomes in animal models and preclinical tests (Jin et al., 2014; Park et al., 2018), existing agents have limited effect in preventing neuronal deterioration. In this regard, instead of targeting a specific aspect of mitochondrial function, supplementing exogenous mitochondria to damaged regions may potentially be an innovative therapeutic strategy. Chang *et al.* demonstrated that cell-penetrating peptide-based (Pep-1) mitochondrial delivery in 6-hydroxydopamine (OHDA)-treated PC12 cells rescued mitochondrial respiratory function, improved cell viability and promoted neurite growth in the presence of nerve growth factor (Chang et al., 2016). Additionally, xenogenic/allogenic injection of mitochondria into medical forebrain bundle (MFB) of 6-OHDA-unilaterally infused PD rats enhanced the survival of dopaminergic neurons and effectively sustained mitochondrial functions by restoring normal levels of mitochondrial complex I–IV and relieving mitochondrial oxidative stress *in vivo* (Chang et al., 2016). In another study by Shi *et al.*, MPP (1-methyl-4-phenyl-pyridinium)-treated SH-SY5Y neuroblastoma neuron-like cells incubated with intact mitochondria increased cell viability in a dose-dependent manner (Shi et al., 2017). ATP production, mitochondrial complex I activity and cell survival were rescued after mitochondrial supplementation, while levels of ROS significantly decreased. Also, the initial report by Shi *et al.*, showed that systemic intravenous mitochondrial administration to the MPTP (1-methyl-4-phenyl-1,2,3,6-tertrahydropyridine)-induced PD mouse model prevented disease progression (Shi et al.,

2017). *In vivo* distribution of intravenously-injected mitochondria was found in multiple organs, including brain within 2h of injection.

6.1.2 Ischemic stroke.—Current intervention for stroke is very limited due to narrow therapeutic time window after the occurrence of ischemic stroke. After ischemic stroke, lack of glucose and oxygen supply disturbs ATP synthesis in mitochondria, results in energy imbalances, dysregulation of cellular homeostasis and finally neuronal cell death. Therefore targeting mitochondria is a promising approach for neuroprotection after stroke (Russo et al., 2018). In light of intercellular mitochondrial transfer in stroke, exogenously injected mitochondria was observed to be distributed in neuron, astrocytes, and microglia in the peri-infarct area at 4 weeks after transient focal ischemia. Concomitantly, mitochondrial transplantation significantly improved motor function and decreased the infarct area, and TUNEL-positive cells (Huang et al., 2016). More recently, mitochondria allografted from skeletal muscle decreased the infarct volume, and attenuated neurological deficits, cellular oxidative stress, and apoptosis at 28 days after stroke in a rat model of focal ischemia (Zhang et al., 2019).

Studies implicate that stem cells may have the ability to transfer mitochondria into injured cells through tunneling nanotubes, microvesicles, gap junctions, cell fusion or direct uptake (Heyck et al., 2019; Liu et al., 2018; Paliwal et al., 2018) and stem cell mediated mitochondrial transfer may be a key mechanism for neuroprotection and neurorepair. It has been reported that mitochondria are transferred from mesenchymal multipotent stromal cells to co-cultured neurons (Babenko et al., 2015). In this context, studies showed that intravenous injection of mesenchymal multipotent stromal cells to middle cerebral artery occlusion (MCAO) rats reduced the infarction area and improved post-stroke neurological indexes. Treatment of primed stem cells which had been previously co-cultured with neurons caused a more pronounced beneficial outcome in rats after stroke (Babenko et al., 2015). Very recently, Liu and colleagues demonstrated the transfer of mitochondria from transplanted mesenchymal stem cells (MSCs) to damaged brain endothelial cells after transient focal cerebral ischemia in rats (Liu et al., 2019).

6.1.3 Traumatic brain injury and spinal cord injury.—Traumatic brain injury (TBI) and spinal cord injury (SCI) have been some of the most important medical issues worldwide given the lack of effective treatment. The initial study investigating the feasibility of mitochondrial transplantation in SCI reported that supplementation of a pool of healthy mitochondria into L1/L2 contusion SCI rat model acutely sustained cellular bioenergetics in the injured spinal cord and improved locomotor activity, whereas, long-term effects on neuroprotection and tissue sparing were not observed (Gollihue et al., 2018a). However, transplanted mitochondria were found to distributed and localized within various cell types including microglia/brain macrophages, endothelial cells, pericytes, astrocytes, and oligodendrocytes and no evidence of uptake in neurons (Gollihue et al., 2018a). In a TBI rat model, supplement of freshly isolated mitochondria derived from rat cortical neurons into injured hippocampal neurons promoted neurite re-growth and restored membrane potential of the injured neurons (Chien et al., 2018).

6.1.4 Schizophrenia.—Effectiveness of mitochondrial transplantation for schizophrenia has recently been described. Bilateral injection of freshly isolated mitochondria into the intra-prefrontal cortex in SZ model rats, prevented attention-deficit characterized cognitive impairment in SZ along with an improvement in mitochondrial membrane potential (Robicsek et al., 2018). However, fundamental mechanisms of how transplanted mitochondria influence brain function in a SZ rat model remain to be fully understood.

6.2. Clinical application of mitochondrial transplantation techniques.

Previous proposals for treating mitochondrial dysfunction have been targeting specific mitochondrial residents and fusion and fission regulators (El-Hattab et al., 2017; Wang et al., 2016). The outcome of these approaches has not been satisfactory and the emerging line of approach is to supplement freshly isolated mitochondria, ie., mitochondrial transplantation to injury sites. A number of *in vivo* studies documented feasible approaches of mitochondrial transplantation, including microinjection directly into affected sites in SCI, stroke and PD models (Chang et al., 2016; Gollihue et al., 2018a; Kaza et al., 2017; Masuzawa et al., 2013), and intravenous administration in PD and fatty liver models (Fu et al., 2017; Shi et al., 2017) (Table 1). Nonetheless, it is conceivable that clinical translation of mitochondrial transplantation would face great challenge. The effectiveness of mitochondrial therapy is expected to be variable among patients due to the heterogeneity of pathogenesis and efficiency of mitochondrial internalization into affected tissues. Also, the therapeutic outcome of mitochondrial transplantation largely depends upon the isolation protocol, numbers and quality of isolated mitochondria, the proper route of organelle delivery and tissue-specific differential uptake. The major pros and cons that has to be addressed before the implementation of mitochondrial transplantation in clinical settings are:

6.2.1 Source and quality of mitochondrial isolation.—Functional homogeneity of isolated mitochondria is critical for achieving therapeutic efficiency. By comparing the mitochondria enriched fractions from various tissues for mitochondrial number, purity, and membrane potential, it was identified that muscle-derived mitochondria may be a good candidate for isolation and transplantation. In protein adjusted mitochondrial suspension, muscle had the highest mitochondrial number and the MitoTracker/JC1 values (Zhang et al., 2019). Future studies are warranted to define mitochondrial dosages with a common unit ie., number of mitochondria or protein concentration. Additionally, MitoTracker dependent mitochondrial labeling has been commonly used to assess mitochondrial quality and number. But membrane-potential dependent labeling with MitoTracker dyes may cause background signals and non-specific detections when used at higher concentrations. So, mitochondrial labeling with fluorescent proteins can be an alternative approach. Molecular probes that drive expression of fluorescent proteins in cell-specific mitochondria may be useful to quantify the mitochondrial dose to be used for transplantation.

6.2.2 Storage of isolated mitochondria.—Mitochondrial storage is one of the key aspect of clinical translation. It is known that the function of isolated mitochondria could be influenced by freeze-thaw cycles and also by storage temperature (Araki, 1977). Thus, freshly isolated mitochondria are the best option for mitochondrial transplantation (Roushandeh et al., 2019). The isolation and preparation of autogenic mitochondria is rapid

and purified mitochondria are viable within 30 min, a time frame within the clinical time-frame for use in surgery (Kaza et al., 2017; McCully et al., 2016). But is it impossible to store mitochondria? Back in 2006, it was shown that ~50% of normal respiratory function was maintained in brain-derived mitochondria that was cryopreserved in 10% (v/v) dimethylsulfoxide at −80°C (Nukala et al., 2006). Another study showed trehalose-frozen mitochondria to preserve mitochondrial ultrastructure along with retaining ability to produce ATP and to import proteins (Yamaguchi et al., 2007). If we can freeze and thaw isolated mitochondria without functional disruption, mitochondrial transplantation therapy will be potentially applicable at the patient bedside. Further studies are warranted to investigate this idea and assess the feasibility of storing mitochondria until transplantation.

6.2.3 Cellular uptake of transplanted mitochondria.—We do not yet understand how transplanted mitochondria maintain oxygen consumption rate (OCR), rescue impaired mitochondrial function, attenuate oxidative damage, and sustain neuronal survival. It is also not known whether mitochondria must be internalized into cells in order to improve the disease condition or whether they can function as extracellular mitochondrial vesicles/ particles (Hayakawa et al., 2018a). If mitochondria are maintained extracellular, it is unlikely that mitochondria can survive in high extracellular Ca^{2+} milieu. In that case, to maintain the mitochondrial integrity, Acetyl-l-carnitine (ALC)/ N-acetylcysteine amide (NACA) can be co-administered to maintain both host and grafted mitochondria. But if mitochondria are internalized, delivering mitochondria within specialized hydrogels that control mitochondrial release or understanding the mechanisms by which mitochondria enter cells will be intriguing to determine optimal therapeutic time windows for mitochondrial transplantation after injury.

More importantly, in CNS injury, in addition to maintenance of cell survival, regenerative outcome characterized by neurite re-growth, de novo synaptogenesis and the restoration of neuronal activity should be inclusively evaluated. Therefore, increased understanding of the mitochondrial quality, dose, mechanisms underlying mitochondrial delivery and cellular uptake will facilitate the translation of mitochondrial transplantation in clinic.

6.3 Clinical trials on mitochondrial transplantation.

The burgeoning of mitochondrial therapy opened a new era of reversing mitochondria function in human diseases. Thus far registered clinical trials for treating diseases based on mitochondrial delivery technique have been launched. Till date there is only one completed clinical trial which aimed to treat infertility by autologous mitochondrial injection into oocytes (NCT #02586298). An ongoing trial trues to demonstrate the feasibility of mitochondrial transplantation, using autologous mitochondrial injection (NCT #02851758), for rehabilitating myocardial ischemia/reperfusion injury and is currently recruiting participants. The usefulness of mitochondria within translational medicine can not only be exemplified by artificial mitochondrial transplantation therapy, but can also be evidenced in other areas such as the use of the mitochondria or the mtDNA as a biomarker of the severity of the disease. In a study, the quantity and activity of extracellular mitochondria in the cerebrospinal fluid (CSF) was measured by the fluorescent probe JC1, which showed to be an effective prognostic factor after subarachnoid hemorrhage (SAH) in mice and patients

(Chou et al., 2017). By measuring the red and green fluorescence it was determined that SAH patients with better prognosis at 3 months had more active mitochondria (red/green) in the CSF. Additionally, the use of MitoTracker Red CMXRos defined the origin of the mitochondrial flow showing that patients with the best prognoses had mitochondria of astrocytic origin when compared with those of microglial, endothelial or platelet origin (Chou et al., 2017).

7. Concluding Remarks

Considering the complex impact of mitochondrial motility within and between cells, it is important to emphasize that mitochondria are highly dynamic organelles especially in neurons. They travel the length of the axons from the cell body to supply energy at distance processes and travel back to the cell body. Motile mitochondria target to the place of increased energy demand, provide energy and then re-mobilize. This pattern of mitochondrial motility (anterograde and retrograde) was considered the classical mechanism of mitochondrial movement. But recently, transcellular transfer of mitochondria has been identified as a new mechanism for mitochondrial movement. In this case, mitochondria are transferred to supply energy to the recipient cells or to undergo transmitophagy. The type of mitochondrial transfer, aimed to rescue the recipient cellular functions needs to be considered with caution, since whether transferred mitochondria remain functional remains a point of contention. Thus, while donor cells can transfer and establish functional mitochondria into recipient cells with damaged mitochondrial DNA, deliberation will continue over the mode of delivery, the cellular source, and the mechanism(s) of transfer and cellular internalization of these mitochondria (Berridge et al., 2016). Since mitochondria originated from bacteria, the mitochondrial DNA and their components are highly immunogenic and care must be taken to determine the nature and reactivity of the mitochondria that is being used for mitochondrial transfer/transplant. Depending on the nature of mitochondria, inter- and intracellular signals vary, resulting in either survival or death of the recipient cells. Because of the prominent role of mitochondria in both neuronal physiology and pathology, priority should be to determine whether the transcellular transfer of mitochondria contributes to health or disease in various research and clinical settings.

Acknowledgements

This work was supported by NIH R01 MH107340, NIH CNAC pilot grant, and NIH K99/R00 4R00HL138268-03.

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Highlights

- This review highlights recent advances and provided comprehensive summary on the pathophysiological implications of inter and intracellular mitochondrial transfer.
- Different regulatory mechanisms and the molecular machinery involved in intracellular mitochondrial movement are discussed.
- Several promising results on artificial mitochondrial supplementation therapy are reviewed.
- A new paradigm for mitochondrial therapy based on organelle delivery as a strategy to treat neurological disorders is described.

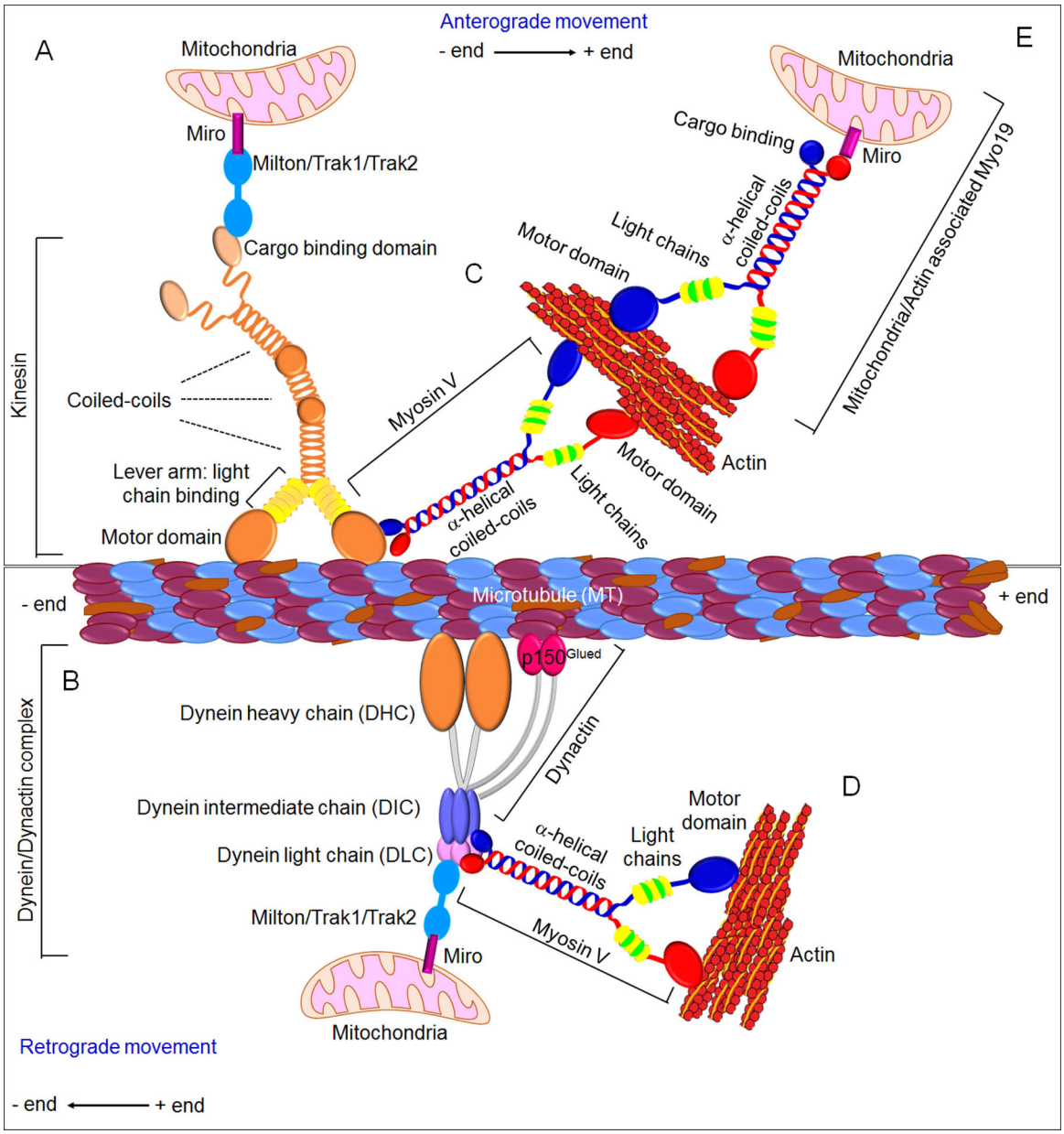

Fig. 1. Key components of the Motor-adaptor-receptor complex for intracellular mitochondrial movement.

Axonal microtubule (MT) are uniformly arranged with their plus end (+end) directed distally and minus end (−end) towards soma. (**A**) Plus-end directed anterograde transport (−end to +end) of mitochondria is mediated by the Kinesin motor. The N-terminal domain the motor domain with ATPase activity and binds directly the MT. The C-terminal is the cargo binding domain. Milton (or TRAK1/2) is the motor adaptor protein that links Miro (present in the outer mitochondrial membrane) to the cargo binding domain of Kinesin. (**B**) Minus-end directed retrograde transport (+end to −end) of mitochondria is mediated by the cytoplasmic MT-based dynein motor. Dynein contains multiple subunits including two catalytic heavy chains (DHC), several intermediate chains (DIC) and light chains (DLC). DHC is the motor domain required for dynein movement. Dynactin is a large protein

complex that interacts with dynein and MT through the $p150^{Glued}$ subunit. Both dynein and dynactin together drive the retrograde mitochondrial movement. (**C and D**) Actin filament based short distance mitochondrial transport is mediated by myosin. Myosin is a two-headed motor protein with a unique globular tail domain that can interact directly with kinesin motor (**C**), or with DLC (**D**) raising the possibility of this motor to facilitate both long-range transport along MT and short-distance transport along actin filaments. (**E**) Myo19 was identified as the mitochondria associated myosin. Myo19 directly interacts with Miro through its C-terminal fragment of the tail region.

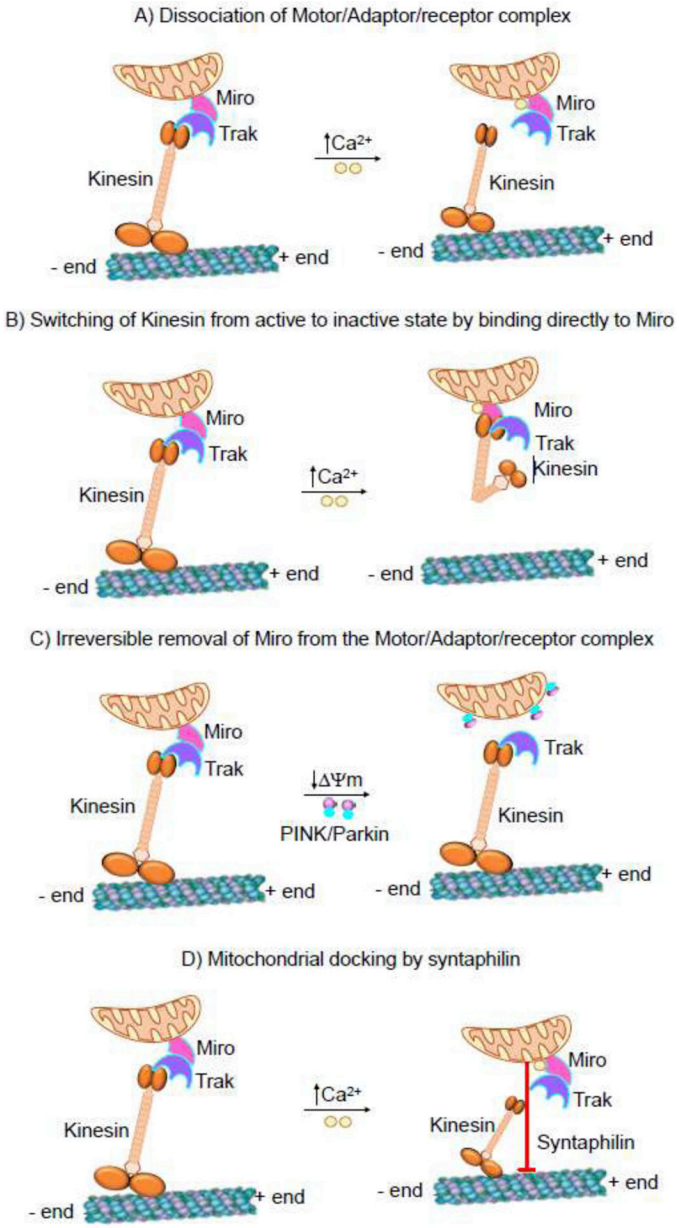

Fig. 2. Molecular regulation of mitochondrial movement.

(**A**) *Dissociation of motor/adaptor/receptor complex*. Binding of Ca^{2+} to the Miro EF hands facilitate the dissociation of either Miro/TRAK or KIF5/TRAK interaction, thereby uncoupling the KIF5 from the mitochondrial surface. (**B**) *Switching of kinesin from active to inactive state by directly binding to Miro*. KIF5 switches from the active state to an inactive state by binding to the Ca^{2+} bound Miro through the head domain of kinesin, thus preventing it from binding to microtubules. (**C**) *Irreversible removal of Miro from the complex.* PINK1 binds to and phosphorylates Miro to signal Parkin. The consequence of activating the PINK/Parkin pathway is the proteasome-dependent degradation of Miro and consequent release of KIF5/TRAK complex from the mitochondrial surface. (**D**) *Mitochondrial docking by syntaphilin.* Syntaphilin (SPH) acts as a static anchor specific for axonal mitochondria.

Depending on the energy requirement mitochondria binds the microtubules through the protein syntaphilin and docks at the site of high energy demand.

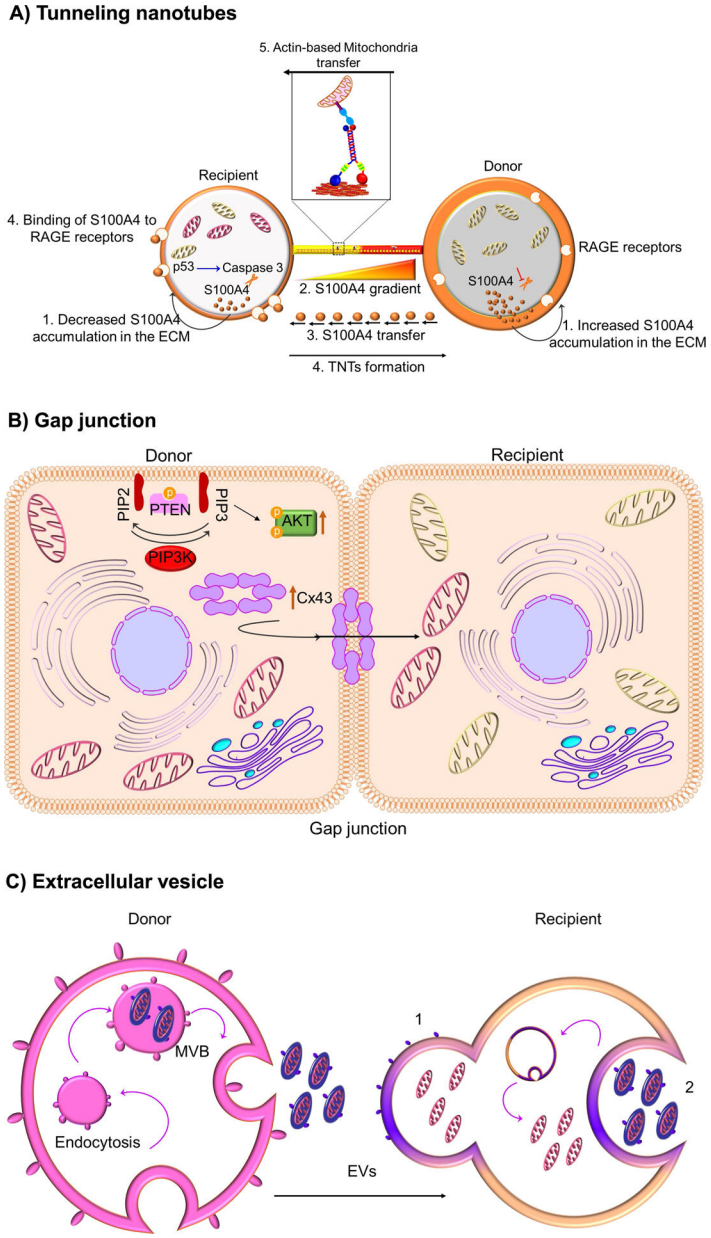

Fig. 3. Mode of intercellular mitochondrial transfer.

(**A**) *Tunneling nanotubes.*P53-mediated activation of caspase 3 cleaves S100A4, creating a gradient of low levels of S100A4 in initiating or recipient cells (injured cells) towards a higher concentration in target or donor cells (healthy cells). It is suggested that RAGE in recipient cells act as the putative receptor for the high concentration of S100A4 in donor cells and this co-ordination of S100A4 and RAGE guides TNT direction. Mitochondria transfer through nanotunnels is believed to be facilitated by Actin/Miro-based transport machinery. (**B**) *Gap junction*. Connexin, specifically connexin 43 oligomerizes to form a channel at the gap junction which facilitates the transfer of mitochondria. Mechanistically, it was shown that ROS-induced oxidative stress regulates the opening of connexin channels in a system mediated by phosphoinositide 3-kinase (PI3K). (**C**) *Extracellular vesicle.* The

multivesicular bodies (MVB) release extracellular vesicles (EVs) from the donor cell. These released EVs can fuse with recipient cells (1) or engulfed (2) to release the EVs' content

Table 1:

Exogenous mitochondria transplantation in CNS disease and injury

No	Disease/injury model	Source of mitochondria	Recipient cells	Mode/Route of delivery	Outcome	Reference
1	*In vitro* ischemia	Mesenchymal multipotent stromal cells	Cortical neurons and astrocytes	Co-culture	Improved cell viability	(Babenko et al., 2015)
2	Traumatic brain injury (TBI mouse)	Cortical neurons	Hippocampal neurons	Add in medium	Enhanced neuroregeneration	(Chien et al., 2018)
3	Transient focal cerebral ischemia (mouse)	Mouse cortical astrocytes	Peri-infarct cortex	Direction injection or autologous secretion	Promoted adjacent neuronal survival and plasticity after injury transfer	(Hayakawa et al., 2016)
4	Parkinson's disease (PD rats)	PC12: human osteosarcoma cybrids	Brain neurons	Intracerebroventricular	Improved motor function and attenuated the deterioration of dopaminergic neurons	(Chang et al., 2016)
5	Parkinson's disease (PD mouse)	HepG2	Multiple tissues including brain	Intravenous injection	Improved behavior test, increased ETC activity, ATP, decreased ROS, apoptosis and necrosis	(Shi et al., 2017)
6	Spinal cord injury L1/L2 contusion (SCI rats)	PC12/rat skeletal muscle (allograft)	Brain macrophages, pericytes, endothelial cells, glia	Microinjection at mediolateral grey matter	Maintenance of acute mitochondrial bioenergetics, and enhanced behavioral recovery	(Gollihue et al., 2018a)
7	Schizophrenia (SZ rats)	Human lymphocyte/rat brain (heterograft/ allograft)	Prefrontal cortex	Direct injection	Prevented dissipation of mitochondrial membrane potential and attentional deficit	(Robicsek et al., 2018)
8	Ischemic Reperfusion brain injury (IR rats)	BHK cells	Neuron, astrocytes, microglia in peri-infarct area of the ischemic hemisphere	Intrafemoral artery injection	Improve motor function, decrease infarct area and cell death	(Huang et al., 2016)
9	Ischemic Reperfusion brain injury (IR rats)	Pectoralis major Muscle (autologous)	Neurons around the ischemic penumbra	Intracerebroventricular	Decrease infarct volume, neurological deficits, cellular oxidative stress, apoptosis, and gliosis, promote neurogenesis	(Zhang et al., 2019)
10	Ischemic Reperfusion brain injury (IR rats)	Mesenchymal stromal cells	Per-infarct area	Intra-arterial	Improve mitochondrial function in peri- infarct area and functional recovery	(Liu et al., 2019)