Review

Melatonin, tunneling nanotubes and anastasis: Cheating cell death

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ABSTRACT

When healthy neurons are exposed to toxins or physiological insults such as ischemia, apoptosis is often initiated. Once underway, this mechanistically-well described process was thought to routinely run its course with the disintegration of the cell and phagocytosis of the debris. Within the last decade, the consistency of this process has been questioned. It is now known that some damaged cells can recover, i.e., they avoid death; this restoration process is referred to as anastasis. The reestablishment of a healthy cell phenotype is highly energy-requiring, so optimally functioning mitochondria are obviously beneficial during the regenerative process. Some healthy mitochondria that end up in regenerating cells are transferred there by adjacent healthier cells through tunneling nanotubes. Tunneling nanotubes generally form under stressful conditions when these micron-size tubules link adjacent cells. These tubules transfer soluble factors and organelles, including mitochondria, between the connected cells. When damaged cells receive high APT-producing mitochondria via this means, they support the ability of the cells to recover. Two recent comprehensive publications show that melatonin aids the transfer of mitochondria through nanotubes that connect neurons thereby likely assisting the recovery of the damaged recipient cell. Thus, melatonin not only protects normal neurons from damage by neutralizing the agents that initiate apoptosis, e.g., free radicals, etc., but also reverses this process once it is underway.

Key words: Apoptosis, intercellular mitochondrial transfer, cell survival, oxidative stress, oxidative phosphorylation, mesenchymal stem cells, ischemia/reperfusion.

1. INTRODUCTION

Not all cells that initiate molecular events that promote proapoptotic protein expression which normally leads to apoptosis implode and die. Some of these severely damaged cells are brought back from the "brink of death" by cellular processes referred to initially as "apoptosis interruptus" (1). Programmed cell death (apoptosis) has been highly conserved throughout evolution and it

plays a central role in a variety of diseases/conditions that compromise the function of tissues. To reduce the subsequent disability that results as a consequence of injury to critical tissues such as the heart and brain, reversal of cell loss and restoration of function could be a major factor in at least partially preserving the physiology of the damaged areas (2). Indeed, Haider *et al.* (3) have referred to apoptosis in failing cardiomyocytes as programmed cell survival which is designed to reverse the disability of cells undergoing molecular insults such as ischemia/reperfusion.

Apoptosis or programmed cell death was initially described 50 years ago (4). It is highly regulated process that is a result of a cascade of reactions in which initiator and executioner caspases, functionally highly precise enzymes directed at mitochondria, lead to morphologically-observable and molecular changes that cause cells to disintegrate. The resulting cellular debris is phagocytized and recycled (Figure 1). Apoptosis is a process of normal cellular turnover including that which occurs during embryological development and various types of induced atrophy (5). Apoptosis also occurs under circumstances that are pathological such as when cells are exposed to toxins (6, 7) or as a result of degenerative diseases (8, 9). Numerous subtypes of apoptosis, e.g., ferroptosis, pyroptosis, etc., have been described which exhibit similar molecular perturbations but have different initiating stimuli (10, 11).

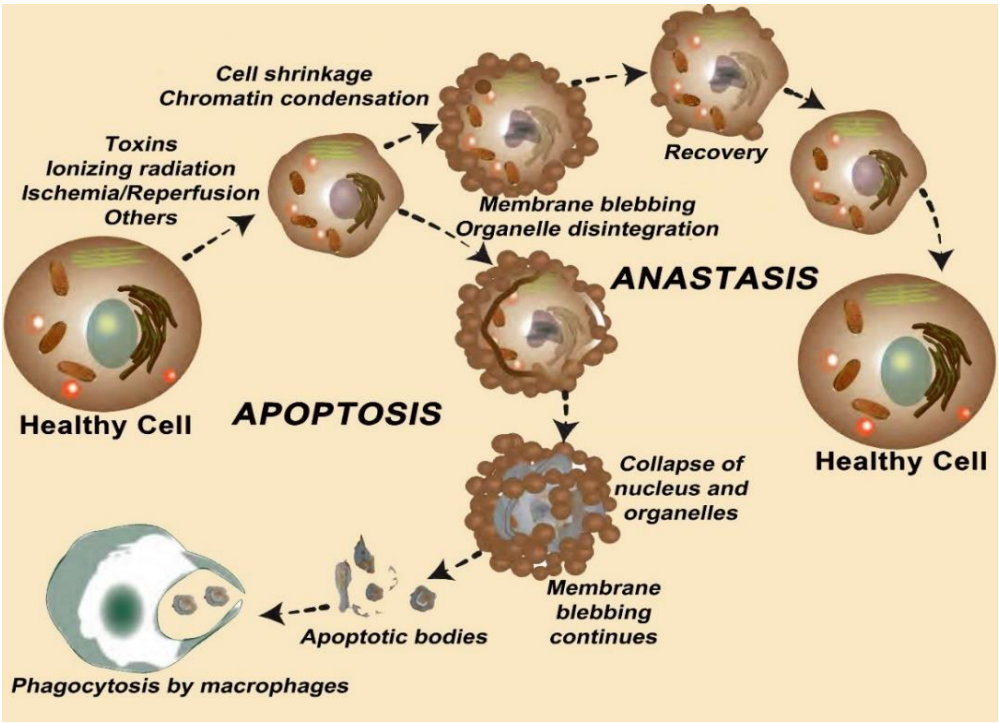

Fig. 1. This figure illustrates the two fates that cells may achieve after apoptosis is initiated.

It has been conventionally thought that with the onset of apoptotic processes, cell fate was determined, i.e., the cell would eventually disintegrate with the remnants being phagocytized and recycled. It is now known that this is not the only possible outcome. Some cells, even when severely damaged, regain normal physiology and are restored to a healthier cell phenotype; this restoration process is referred to as anastasis. While the molecular details of the recovery process are not totally established, since it is a high energy-requiring undertaking the mitochondria are likely intimately involved. Some of the mitochondria arrive in these regenerating cells via tunneling nanotubes which connect them to healthier adjacent cells.

Apoptosis is activated by one of two different pathways, i.e., the mitochondria-mediated or intrinsic pathway or the death receptor-mediated or extrinsic pathway (12). During apoptosis, not all mitochondria in an individual cell are damaged to the same degree. This differential response relates to the cytoplasmic environment surrounding individual mitochondria such as the presence of biomolecular condensates (9) and also to epigenetic influences, post-transcriptional changes of proteins encoded by the mitochondrial genome, etc. (13). The functional preservation of some mitochondria may allow a cell to avoid demolition and rehabilitate its normal physiology (14).

2. ANASTASIS: CELL DEATH AFTER INITIATION OF APOPTOSIS IS NOT INEVITABLE

For many years after its discovery, it was presumed that, once initiated, apoptosis was a non-reversible process with cellular death being unavoidable. About 20 years ago, however, this idea was challenged by observations reported by Haider and colleagues (3), when they noted that the apoptotic cascade does not always run its full course in cardiomyocytes during congestive heart failure. Under these circumstances, the cardiomyocytes display some of the usual characteristics of apoptosis such as cytochrome c release along with the activation of caspase 3, but the nuclei of the cells retain their normal morphology rather than undergoing condensation (15). With the failure of the complete apoptotic cascade, which normally results in cell death, these "wounded" cells survived and proved beneficial in preventing further cardiac deterioration. Thus, as mentioned previously, Haider *et al*. (3) re-classified programmed cell death as programmed cell survival for these particular cells; this was an important phenomenon since it reversed cardiac remodeling which contributes to heart failure and death. The term anastasis (Greek for "returning to life") entered the lexicon as a replacement for programmed cell survival 10 years later when Tang *et al*. (2) noted that Hela cells treated with what was thought to be a lethal dose of ethanol, which clearly induced signs of apoptosis, survived when the damaging agent was removed. While anastasis has been most extensively studied in cultured cells, it has also been seen in vivo (16) (Figure 1).

Cytochrome *c* release from mitochondria and caspase activation, as already mentioned, are essential events leading to cellular suicide (17). Initially, pro-apoptotic proteins translocate to the mitochondria where they damage these organelles; this causes mitochondrial outer membrane permeabilization with the release of a host of factors that contribute to cellular loss (18). Damage to the mitochondria also leads to their functional failure which compromises bioenergetics and ATP production. Since anastasis is a high energy-requiring process, restoration of mitochondrial function is essential for morphological and functional cellular recovery (16). As subsequently discovered, incomplete mitochondrial discharge of cytochrome *c* occurs in some mitochondria with only minor damage so the bioenergetic capability of these organelles is well preserved (19, 20). Thus, the differential damage to mitochondria may determine whether a cell morphologically and functionally disintegrates. In the case of neurons which exhibit extensive arborization of their cell processes, how seriously a given mitochondrion is damaged may relate to its distance from the cell body (21, 22). The minimally-damaged mitochondria, e.g., those in the perikaryon, could provide for the early energy requirements for curtailing cellular death. As anastasis proceeds, mitochondria fuse and regain a more standard morphology with the re-establishment of normal oxidative phosphorylation and ATP production (23). This is likely a critical factor in the return to normal of the functionally impaired cells. While anastasis has been documented in cells previously classified as being healthy, it also occurs if diseased cells, e.g., cancer cells, where it is undesirable since it contributes to the reoccurrence of tumors (24).

The reader is reminded that melatonin synthesis occurs not only in the pineal gland but additionally in the mitochondria of possibly all healthy cells (25, 26) where it supports normal oxidative phosphorylation (miOXPHOS) and ATP production. Conversely, cancer cells presumably do not synthesize this indoleamine in these organelles at least not during the day (27, 28). Indeed, the absence of the usual melatonin production in cancer cell mitochondria is believed to contribute to their oncogenicity (29, 30), given melatonin's well-known anticancer actions (31-33). Many cancer cells also adopt cytosolic aerobic glycolysis in lieu of miOXPHOS. How cells undergoing apoptosis or anastasis handle glucose metabolism has not been investigated according the authors' knowledge. This may be relevant since optimally-functioning mitochondria relative to ample ATP synthesis is an aid to reversing apoptosis and stimulating anastasis with melatonin having a role in determining the ability of mitochondria to maintain miOXPHOS and ATP levels.

3.TUNNELING NANOTUBES, INTERCELLULAR MITOCHONDRIAL TRANSFER AND CELL SURVIVAL

Tunneling nanotubes (TNT) physically connect adjacent cells which allows them to establish cytoplasmic continuity; this cellular modification was first described by Rostow and colleagues in 2004 (34). These micron-sized tubules are composed of filamentous (F)-actin protein and form a network that connects adjacent cells which allow for the transfer of soluble materials and organelles between cells (Figure 2) (35).

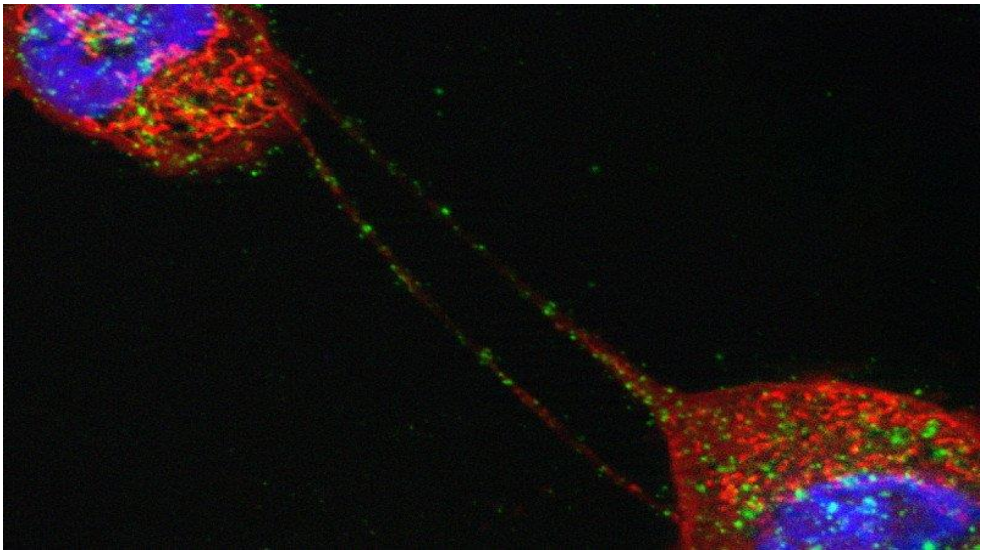

Fig.2. Tunneling nanotubes as visualized utilizing fluorescence microscopy. *The development of nanotubes is initiate under conditions of stress or cellular damage, e.g., as a result of ischemia/reperfusion injury. These micro-sized tubules transfer soluble agents and organelles between the attached cells. The transfer of healthy mitochondria into cells undergoing apoptosis probably assists these cells in recovering their normal morphology and function. Figure provided by Drs. Katia O. Manov-Todorova, Emil Lou and Yevgeniy Romin and reprinted with permission.*

The molecular mechanisms that initiate TNT formation involve the leukocyte specific transcript 1 (LST1), Ras-related protein A (RalA), the cystosolic protein M-sec and the exocyst complex (36, 37). TNT connect cultured cells as well as cells in vivo and have been shown to transport a variety

of different cargoes (38). Microtubules have been identified in what are referred to a thicker TNT whereas the thinner tubules may lack these intrinsic microtubes. It has been speculated that the thicker TNT are designed for long-haul mitochondrial transfer while the thinner tubules are involved in short distance transport (39, 40).

Although the function of TNT has been linked to normal developmental processes and signaling between healthy cells including immune cell activation (41, 42), they have also been implicated in cancer progression since they transport mitochondria and mRNAs from tumor cells to adjacent stromal cells (43). Pathological situations where TNT are reportedly involved include the intracellular transfer of viruses, e.g., HIV-1, and damaged proteins, e.g., β-amyloid, tau, etc., between neurons thereby contributing to neurodegenerative diseases (44, 45).

While TNT have often been observed, there is no consensus on whether there are different subtypes beyond the size differences mentioned above. Some of them are bilaterally open-ended while others are closed and there are other variations as well (46). Many different stimuli initiate the development of TNT; these stimuli are broadly defined as stresses. When a damaged cell establishes a TNT with a healthier cell, the latter cell can transfer cytosolic elements to the target cell to rescue it from apoptosis, i.e., promote anastasis (47).

TNT transfer information from healthy cells to pathological cells as well as vice versa but little is known about what determines the direction of information flow (48, 49). While other mechanisms for intercellular communication also exist (50, 51), it is now accepted that TNT are a major means of communication between damaged cells. The transfer of mitochondria-related products between cells via TNT involve either intact mitochondria or their genes. This process can significantly alter the bioenergetics of the recipient cell and, in the case of cancer cells, it may determine their differentiation state and their resistance to chemotherapies. The evidence shows that the transfer of mitochondrial DNA (miDNA) changes the metabolic phenotype of the target cell for multiple cell generations (52).

Mesenchymal stem cells (MSC) have been extensively investigated in reference to mitochondrial trafficking through TNT (53, 54). MSC are typically added for the purpose of reducing tissue damage and supporting tissue regeneration. The results of numerous preclinical studies have confirmed the therapeutic efficacy of MSC and their secretome in a variety of disease models (55). Importantly, the lack of major histocompatibility complex II (MHCII) in addition to the absence of factors related to T cell activation make MSC highly amenable to their application in clinical trials (56).

Especially for the purposes of this review, the information derived from studies that examined the importance of MSC-related nanotubes in reducing ischemia/reperfusion injury are of special relevance (57, 58). One of the classic examples of ischemia/reperfusion injury occurs when the blood supply to an area of the brain is interrupted such as occurs in a stroke. Mitochondrial dysfunction is a critical contributor to the damage the cells experience during neural hypoxia and vascular re-establishment. In addition to severely compromising miOXPHOS and ATP production, excessive reactive oxygen species generation is a contributing factor to the subsequent death of tissue (59, 60).

In a report by Liu and colleagues (61), MSC proved effective in reducing the size of the infarct area after rats were subjected to middle cerebral artery occlusion when TNT connections were established with the damaged cells. In this study, MSC were injected into the occluded vessel; the transplanted cells interacted with damaged cells at the injured site and transferred healthy mitochondria to the injured cells which aided in their recovery. While the transfer of the heathy mitochondria was likely via TNT, this was difficult to definitely document because of the

complexity of the modeled tissue. This interpretation is, however, consistent with observations which found that astrocytes transport mitochondria, via TNT, into neurons that improved their recovery from stroke (62). Also, the TNT transfer of mitochondria from MSC into neurons under in vitro conditions has been observed (63-65). Regardless of how the healthy mitochondria made their way into the damaged cells of the infarcted tissue, melatonin located in these mitochondria was also transferred, likely contributing to the ability of a larger percentage of the cells to improve their physiology and undergo anastasis (66).

4. MELATONIN, NANOTUBES AND NEURONAL MORPHOLOGICAL AND PHYSIOLOGICAL RECOVERY

Highly metabolically-active tissues such as the brain and the heart rely heavily on a constant supply of oxygen provided by the blood vascular system. Consequently, even a short-term interruption of the arterial supply to these tissues, such as occurs during a stroke or heart attack, has drastic negative effects on these tissues leading to extensive tissue loss, disability or death. Additionally, since the cells are post-mitotic the regenerative capacity of neurons and cardiomyocytes is much less than that seen in some other tissues. For these reasons, the world-wide morbidity and mortality resulting from damage to the brain and heart is vast and there is a persistent search to identify molecules or means to attenuate these impairments.

In the case of both experimentally-induced stroke and the obstruction of the blood supply to portions of the heart, there have been an abundance of studies documenting that melatonin is an effective countermeasure to combat tissue loss and the compromised physiology that is associated with transitory hypoxia followed by reoxygenation (59, 67-71). The damage in the brain is a result of excitotoxicity, the generation of an excess of reactive oxygen species followed shortly by inflammation, all of which involve faulty mitochondrial physiology (72). Some of the mitochondrial dysfunctions that occur during stroke include depolarization of the mitochondrial membrane potential, perturbations of miOXPHOS resulting in increased quantities of ROS being formed and reduced ATP production, accumulation of PTEN-induced putative kinase 1 (PINK1) and Parkin, calcium overload in the matrix, mitochondrial transport pore opening followed by the release of cytochrome c which may initiate the apoptotic cascade.

The use of melatonin to curb the devastating repercussions of neural hypoxia has a long history and is based on the ability of exogenously-administered melatonin to enter the mitochondria (73,74) and to mitigate the associated pathophysiology (75, 76). Moreover, the blood-brain barrier does not limit melatonin's entrance into the CNS (77) where it functions as a versatile direct and indirect antioxidant and as an anti-inflammatory agent (78-80). The protection of the brain from ischemia/reperfusion damage was presumed to be attributed primarily to melatonin's capacity to prevent mitochondrial damage in hypoxic cells which translates into reduced cytochrome c release and fewer apoptotic neurons and glia. The results of recent studies show, however, when apoptotic cells with faltering mitochondria are provided undamaged and highly functional mitochondria from adjacent healthy cells by their transfer through TNT aids in their recovery; i.e., they undergo anastasis (81, 82).

The recent innovative report by Yip and colleagues (81), which includes both in vivo and in vitro documentation, clearly confirms that TNT in the area of infarction transfer mitochondria from healthy to apoptotic cells allowing them to recover. The in vivo experimental model included rats that underwent 50 min common carotid occlusion; 60 min after reperfusion was initiated, purified healthy mitochondria pretreated with melatonin were injected into the infarcted site. The injection

of melatonin-treated mitochondria reduced infarct volume, mitochondrial DNA damage, oxidative stress levels and the number of apoptotic neurons; likewise, lower cytochrome c and CYP1 1A1 release indicated and enhanced number of intact and functional mitochondria in the damaged neurons. Using mitochondrial trackers, Yip and co-workers (81) documented that the injected healthy mitochondria were transferred, via TNT, to the damaged cells and improved cell survival and reduced infarct volume.

For the in vitro studies, Yip *et al*. (81) used neural crest-derived intact and mitochondrial DNA-depleted N2a cells treated with hydrogen peroxide, which initiates cellular apoptosis. In this case, melatonin increased intercellular mitochondrial transfer through TNT from the intact to the mitochondria-depleted cells. Moreover, melatonin enhanced mitochondrial fusion indicative of healthy mitochondria and overcame all the parameters which are related to apoptosis, e.g., reduced expression of mitochondrial Bax and of cleaved caspase 3 and cleaved PARP, among others.

The Nasoni *et al*. (82) report, which was published almost simultaneously with that of Yip and co-workers (81) exclusively used cultured hippocampal neurons (HT22) subjected to oxygen/glucose deprivation, a common in vitro model that simulates the effects of in vivo neural ischemia. The addition of melatonin to the medium in which the cells were grown induced the usual indices of mitochondrial protection including a lower oxidizing environment, elevated expression of PGC1α (peroxisome proliferator-activated receptor-gamma coactivator 1 alpha) and sirtuin 3, maintenance of the expression of TOM20 and TIM23 (mitochondrial membrane translocases) as well as proteins that support mitochondrial biogenesis. As reported by Yip and colleagues (81), using MitoTracker^{TM} Deep Red dye, they also observed that melatonin enhanced mitochondrial transfer between cells via TNT. Neither of these studies noted whether melatonin influenced the development or number of TNT. The findings of both of these comprehensive investigations underpin the importance of melatonin in helping to maintain the function of damaged mitochondria and potentially supporting cellular anastasis.

As mentioned above, it has been routinely assumed that melatonin's protective actions against ischemia and reperfusion was a consequence its ability to maintain redox homeostasis and thereby promote the survival of some cells while others underwent apoptosis. The data presented by both Yip *et al*. (81) and Nasoni *et al*. (82), however, show that melatonin's efficacy extends beyond the mere protection of neurons from degeneration via apoptosis, but also substantiates its capacity to enhance neural regeneration. This information can be leveraged regarding the potential application of melatonin-enriched MSC for restoring brain morphology and function after it has sustained damage. Figure 3 illustrates some of these general phenomena as observed in the two seminal reports reviewed herein (81, 82).

5. CONCLUDING REMARKS

The results of multiple studies from experiments of highly varied designs have proven that melatonin is intimately involved with mitochondrial quality control (9, 23, 83, 84). Considering the critical importance of these organelles in influencing essentially every aspect of cellular physiology, they are also clearly involved in cell durability and perhaps, as discussed herein, with aiding cells to interrupt the apoptotic process and to mediated the recovery of cells to a healthier state.

TNT have attracted the attention of numerous investigators in the last decade. Many of these studies relate to their potential importance of MSC in restoring damaged tissues. While TNT can transport soluble cellular elements as well as cellular organelles including mitochondria, the

transfer of heathy mitochondria to damaged cells that are suffering from an energy shortage likely is indispensable for cells to return to a healthy state, i.e., to undergo anastasis. Anastasis is a high energy-requiring process so the highly functioning mitochondria are a requirement.

Healthy neuronal mitochondria contain uncommonly high levels of melatonin (85) and they have the capability to both synthesize the indoleamine (26) and to extract it from the blood (86). Damaged mitochondria such as those resulting from hypoxia during ischemia change their metabolism markedly due in part to the activation of hypoxia inducible factor 1α (HIF1α), a transcription factor that influences many downstream pathways (87) including glucose metabolism. In the absence of the optimal conversion of pyruvate, a glucose metabolite, to acetyl coenzyme A in the mitochondrial matrix, damaged mitochondria presumably do not synthesize melatonin locally (25, 88). Reduced levels of melatonin in the mitochondria would likely contribute to increased oxidative stress, diminished energy generation and promotion of cellular apoptosis (89, 90). In these situations, the transfer of healthy ATP-producing mitochondria through TNT into the damaged cells may be a required stimulus for the cells to undergo anastasis since cellular restoration is a high energy-requiring process.

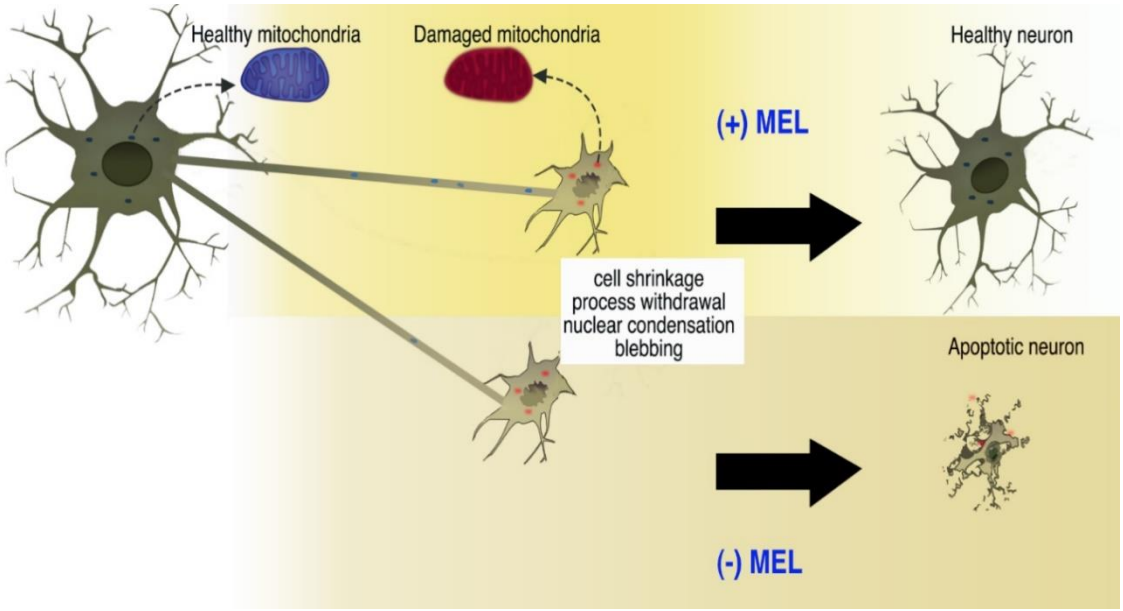

Fig. 3. A schematic representation of the findings uncovered in the reports of Yip et al (81) and Nasoni and colleagues (82).

Some damaged neurons, both in vivo (ischemia/reperfusion) or in vitro (oxygen/glucose deprivation), are restored back to a healthier state even after apoptosis has been initiated likely related to the fact that the addition of melatonin transfers normal mitochondria via tunneling nanotubes into the injured cells. These transferred highly-functioning mitochondria provide the necessary energy to allow the cells to rehabilitate their morphology and function, i.e., to undergo anastasis.

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AUTHORSHIP

All authors contributed to conceptualization, data collection, preparation of the figures, writing and editing the manuscript.

CONFLICT OF INTEREST

The authors report no conflicts of interest.

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