NEURAL REGENERATION RESEARCH
February 2016, Volume 11, Issue 2
www.nrronline.org

PERSPECTIVE

The inherent high vulnerability of dopaminergic neurons toward mitochondrial toxins may contribute to the etiology of Parkinson's disease

Although the exact mechanism(s) of the degeneration of dopaminergic neurons in Parkinson's disease (PD) is not well understood, mitochondrial dysfunction is proposed to play a central role. This proposal is strongly strengthened by the findings that compromised mitochondrial functions and/or exposure to mitochondrial toxins such as rotenone, paraquat, or MPTP causes degeneration of the midbrain dopaminergic system and manifest symptoms similar to Parkinson's disease in primates and rodents (Goldman, 2014). In fact, the specific dopaminergic toxin MPTP is one of the most commonly used models in the mechanistic studies of environmental factors associated with the etiology of PD, particularly due to the availability of direct and unequivocal clinical and biochemical evidence from human and primate subjects. Several decades of intense studies in many laboratories have led to the proposition of a general mechanism for the specific dopaminergic toxicity of MPTP (Przedborski and Jackson-Lewis, 1998). The salient features of this mechanism are (a) lipophilic pro-toxin, MPTP, freely crosses the blood-brain barrier and enters the brain; (b) in glial cells monoamine oxidase-B converts it to the toxic metabolite MPP^+; (c) the polar MPP^+ is extruded into the extracellular space through organic cation transporter-3; (d) presynaptic dopamine transporter (DAT) takes it up specifically into dopaminergic neurons; (e) in dopaminergic neurons, MPP^+ accumulates in the synaptic vesicles and/or mitochondria; (f) mitochondrial MPP^+ inhibits the mitochondrial complex-I of the electron transport chain, leading to cellular ATP depletion and excessive reactive oxygen species (ROS) production causing apoptotic cell death (Lotharius and O'Malley, 2000; Storch et al., 2004). Although this mechanism is generally well accepted, numerous recent studies seriously challenge the central dogma of this proposal that the specific dopaminergic toxicity of MPP^+ is primarily due to the specific uptake into dopaminergic neurons through presynaptic DAT.

Recent studies clearly show that MPP^+ is taken up not only into dopaminergic cells, but also into other neuronal and non-neuronal cells with varying efficiencies through a number of diverse transporters, including organic cation transporters (Müller et al., 2005), non-specific plasma membrane amine transporters (Engel and Wang, 2005) and through other less characterized pathways. Our recent studies show that MPP^+ uptake even into dopaminergic cells occurs through multiple pathways including DAT [Kadigamuwa et al., 2015; Le and Wimalasena (manuscript in preparation)]. In addition, MPP^+ effectively accumulates in the mitochondria of most cells without significant specificity. However, almost all *in vivo* as well as *in vitro* studies to date unequivocally demonstrate that MPP^+ is specifically and highly toxic to dopaminergic cells in comparison to other cell types (Lotharius and O'Malley, 2000; Storch et al., 2004). Therefore, in addition to efficient uptake through membrane transporters, some intrinsic characteristics of dopaminergic cells must also be responsible for the specific high vulnerability to MPP^+ and similar mitochondrial toxins.

Recently, we have carried out a detailed *in vitro* study (Kadigamuwa et al., 2015) to test this possibility using two simple cyanine dyes[1,1′-diethyl-2,2′-cyanine and 1,1′-diethyl-4,4′-cyanine (**Figure 1**)], based on their unique properties and structural similarities to MPP^+. Similar to MPP^+, they are chemically inert aromatic quaternary ammonium salts with no functional groups, but possess highly conjugated extended π electron systems. On the other hand, they are relatively more lipophilic in comparison to MPP^+ due to the presence of non-polar steric bulk, suggesting that they may accumulate nonspecifically in all cell types through simple diffusion and do not require a specific transporter, in contrast to MPP^+. Therefore, they are good candidates for a comprehensive comparative study to test the above proposals experimentally.

Both cyanines non-specifically and freely accumulate in mouse dopaminergic (MN9D), human dopaminergic (SH-SY5Y) and human liver (HepG2) cells in large quantities with similar efficiencies, as expected. They also effectively accumulate in the mitochondria of both cell types at high concentrations leading to strong mitochondrial membrane depolarization. While cellular and mitochondrial accumulations of cyanines and their mitochondrial membrane potential depolarization effects are similar in both cell types, increased ROS production and effective cell death were observed only in dopaminergic cells, similar to MPP^+. Remarkably, both cyanines are about 1,000 fold more potent dopaminergic toxins in comparison to MPP^+ under similar experimental conditions (IC50s for MPP^+ and cyanines are in the ranges of 100 μM and 100 nM, respectively). The excessive and specific ROS production in response to cyanine and MPP^+ treatments correlated well with their respective dopaminergic toxicities. In agreement with this proposal, the water soluble anti-oxidant, ascorbate, reduces the ROS production and effectively protects cells from the toxicity of cyanines as well as MPP^+ (Kadigamuwa et al., 2015). Therefore, the specific dopaminergic toxicities of these toxins are primarily due to their abilities to induce the excessive ROS production, specifically in those cells and not due to the specific uptake, as previously proposed.

Both cyanines and MPP^+ accumulate in the mitochondria and depolarize the mitochondrial membrane potentials of both cell types to a similar extent, but the excessive ROS production and toxicities are limited only to dopaminergic cells. These results suggest that some key physiological differences between these cell types must be responsible for their sharply contrasting behaviors. One obvious difference between these two cell types is that only dopaminergic cells contain and maintain high levels of DA which is oxidatively sensitive and known to amplify the intracellular ROS production, especially under the conditions where cellular ATP levels are depleted and/or synaptic DA storage is disrupted (Hauser and Hastings, 2013). We found that the depletion of intracellular DA in MN9D cells through the inhibition of tyrosine hydroxylase (the rate limiting enzyme in the catecholamine biosynthetic pathway) by L-α-methyl-*p*-tyrosine, prior to cyanine or MPP^+ treatments reduces the ROS production and cell death induced by both cyanine and MPP^+ (Kadigamuwa et al., 2015). This supports the possibility that intracellular DA in dopaminergic cells exacerbates the toxin mediated ROS production and toxicity. However, even drastic DA depletion causes the reduction of cyanines or MPP^+ mediated ROS production and toxicity only by about 30–35%, which suggests that there may be other key cellular differences that may also contribute to the increased ROS production and specific dopaminergic toxicity of these toxins.

The expression of antioxidant enzymes superoxide dismutase, catalase, and glutathione peroxidase in animal brain are known to be significantly low in comparison to the liver (Grankvist et al., 1981). We found that dopaminergic MN9D cells which are the most sensitive to these toxins contain substantially lower levels of all three enzymes in comparison to PC12 and HepG2 cells (Kadigamuwa et al., 2015), parallel to the reported results for rat and mouse brain, relative to their liver and adrenal gland, respectively (Grankvist et al., 1981). Therefore, in addition to the presence of high levels of DA, the overall low expression levels of the antioxidant enzymes in dopaminergic cells may also contribute to the specific excessive ROS production in these cells in response to cyanine or MPP^+ treatments. In contrast, other cell types may possess the capacity to effectively cope with the relatively moderate levels of ROS production, most likely due to the absence of DA and presence of high levels of antioxidant enzymes. Thus, we propose that the presence of high levels of oxidatively sensitive DA together with the expression of low levels of antioxidant enzymes [and other inherent characteristics of dopaminergic cells such as greater demand for energy (for example see Bolam and Pissadaki, 2012)] may contribute to the specific dopaminergic toxicity of these cyanines as well as MPP^+ (**Figure 2**).

The inhibition of complex I or III of the mitochondrial electron transport chain is known to increase the ROS production in isolated mitochondria under aerobic conditions (Chen et al., 2003). Accordingly, dopaminergic toxicity of MPP^+ could be due to the inhibition of complex I leading to the over-production of ROS (Lotharius and O'Malley, 2000). However, the origin of the ROS production with cyanines is not clear currently, since they are not known to inhibit the mitochondrial complex I or III. However, several previous studies have shown that hydrophobic

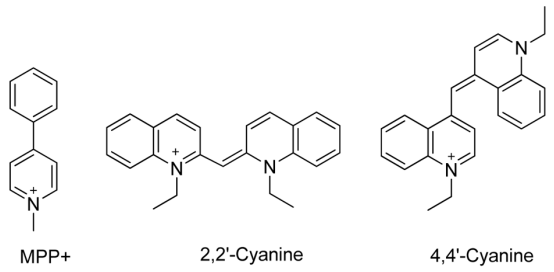

Figure 1 Structures of 1-methyl-4-phenylpyridinium (MPP^+), 1,1′-diethyl-2,2′-cyanine (2,2′ Cyanine) and 1,1′-diethyl-4,4′-cyanine (4,4′-Cyanine).

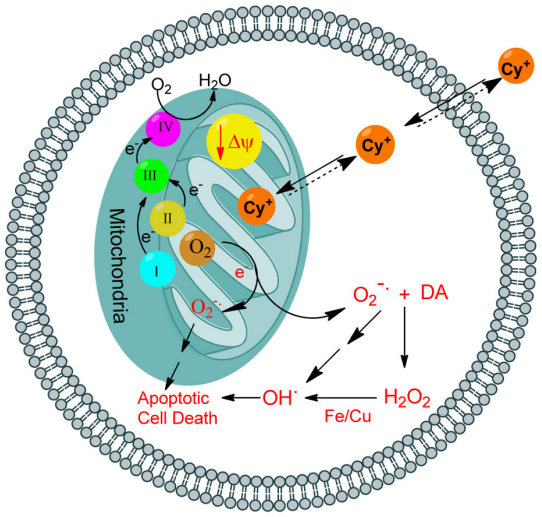

Figure 2 Proposed mechanism of the dopaminergic toxicity of cyanines.
Cationic lipophilic cyanines (Cy^+) freely accumulate in the mitochondria of all cell types causing non-specific mitochondrial membrane depolarization. They increase the reactive oxygen species (ROS) levels specifically in dopaminergic cells leading to the apoptotic cell death, similar to MPP^+. The exact mechanism of ROS production by these cyanines has not been identified at present (for further details, see the text). The specific ROS production and cell death are amplified by the presence of high levels of dopamine (DA) and low levels of antioxidant enzymes in these cells.

MPP^+ derivatives are better complex I inhibitors than MPP^+ itself and they are shown to bind to the hydrophobic rotenone/piericidine binding site of the complex I (Gluck et al., 1994). Therefore, as mentioned above, since these cyanines are structurally similar to MPP^+ and highly hydrophobic in comparison to MPP^+, they may also effectively inhibit the complex I in a similar fashion leading to excessive ROS production. However, additional studies are certainly necessary to determine the exact mechanism of increased ROS production in dopaminergic cells by these cyanines.

The two cyanines, 1,1′-diethyl-2,2′-cyanine and 1,1′-diethyl-4,4′-cyanine, employed in our study are prototypical of a family of lipophilic cationic dyes that are commonly used in industry and scientific research. They are widely used in the production of photographic films, computer storage and video recording media, solar cells, and in photochemical research. They are also frequently used as inhibitors of extra-neuronal noradrenaline, plasma membrane monoamine, and organic cation transporters (Schomig et al., 1993; Engel and Wang 2005), and even as precursors in the development of chemotherapeutics (Chen et al., 2005) and neuro-protective agents (Ohta et al., 2011; Uchida et al., 2012). In spite of their structural resemblance to MPP^+ (**Figure 1**), wide usage, and ability to freely accumulate in the cytosol and in the mitochondria of most cell types, their physiological or neurotoxicological properties have not been studied or reported. Based on our findings, especially the similarities of cell specificities and the mechanistic characteristics of toxicities between MPP^+ and cyanines, it is tempting to speculate that cationic lipophilic cyanine dyes could be stronger *in vivo* dopaminergic toxins than MPP^+.

Taken together, the high vulnerability of dopaminergic cells to toxins such as MPP^+ and cyanines is not due to the specific uptake, but could be due to their inherent predisposition to mitochondrial toxin mediated ROS production (**Figure 2**). Therefore, presence of these types of mitochondrial toxins in the environment could contribute to the etiology of PD and minimizing the environmental exposure to them could help to decrease the occurrence of PD. However, obviously the above *in vitro* findings must be confirmed with appropriate *in vivo* models to further advance these highly critical proposals.

The studies cited from the author's laboratory were carried out by his lab members Chamila Kadigamuwa, Viet Le and Sumudu Mapa. The author thanks Nivanthika Wimalasena and Shyamali Wimalasena for critical reading of the paper.

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***Accepted:** 2015-12-08*
***doi:** 10.4103/1673-5374.177730* ***http**://www.nrronline.org/*
***How to cite this article:** Wimalasena K (2016) The inherent high vulnerability of dopaminergic neurons toward mitochondrial toxins may contribute to the etiology of Parkinson's disease. Neural Regen Res 11(2):246-247.*

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