Chapter 2
TIQ improves impaired mitochondrial morphology in experimental parkinsonism *in vitro* and *in vivo*.

**Introduction:**

Neurons are highly differentiated and unlike other cell types these cells have complex morphology. A typical neuron has a cell body (soma or perikaryon), a long axon and several shorter dendrites, all of which have fairly diverse energy demand that is satisfied by unique mitochondrial subcellular distribution within each neuron. Studies suggest mitochondria as dynamic organelles (Chan, 2006; Okamoto and Shaw, 2005) and to maintain the overall morphology of their population they can drastically change their size and shape from small bodies to long tubular filaments achieved by controlled divide and fuse processes (Detmer and Chan, 2007; Nunnari et al., 1997; Westermann, 2008). Functional gain or loss of PD associated genes such as α-syn (Nakamura et al., 2008), parkin (Greene et al., 2003), DJ-1, phosphatase and PINK1 (Valente et al., 2004), LRRK2 (Niu et al., 2012) and HTRA2 are related with impaired Cx-I activity and atypical mitochondrial dynamics.

Growing evidences from experimental models suggest abnormally fragmented and accumulated mitochondria lead to pathology of sporadic PD. Mitochondrial fission occurs much earlier than apoptosis caused by MPP⁺ (Arnold et al., 2011; Kim-Han et al., 2011; Wang et al., 2011). Chronic exposure of rotenone, a Cx-I inhibitor, also induces mitochondrial fission in neuronal primary culture (Arnold et al., 2011). The metabolite of MPTP, MPP⁺ is shown to inhibit histone deacetylation over Mfn2 promoter, leading to its down regulation culminating in hindered mitochondrial fusion activity (Zhu et al., 2014). 6-OHDA, another neurotoxin that causes PD in experimental animals (Borah and Mohanakumar, 2010; Deumens et al., 2002; Sindhu et al., 2006; Ungerstedt, 1971) causes Drp1-dependent aberrant mitochondrial fragmentation leading to neuronal apoptotic cell death similar to that caused by MPP⁺ (Wang et al., 2011).

Observed beneficial outcome of TIQ on mitochondrial physiology (Chapter 1) under *in vitro* and *in vivo* conditions led us to assess the effect of TIQ on MPTP- or MPP⁺-induced impaired mitochondrial fission-fusion dynamics.
Materials and Methods:

Material and Methods corresponding to this Chapter are provided in the Materials and Methods segment.

Results:

TIQ effects on MPTP-induced nigral mitochondrial fragmentation in mice:

Mitochondrial physiology is heavily dependent on mitochondrial morphology. Following the positive effects of TIQ on mitochondrial function in the striatum, it was thought prudent to investigate the morphological status of mitochondria within TH-immunopositive neurons. Twenty µm mouse brain sections passing through SNpc region were taken and double immunostaining was carried out for Tom-20 (green) that stains mitochondria, and TH (red), an indicator for dopaminergic neurons. TH-immunopositive neurons of MPTP-treated mice had fragmented and swollen mitochondria in contrast to elongated, interconnected and networked mitochondria in controls. TIQ (200 mg/kg, per oral, treated in four doses after MPTP administration) corrected this abnormal mitochondrial morphology to a great extent as seen in Tom-20 staining (Fig. 7).
TIQ normalizes MPTP-induced Drp1 overexpression from mice SN and striatum:

Drp1 assembling and spiraling around the cytoplasmic face of mitochondrial fission site is necessary for mitochondrial division to occur (Bleazard et al., 1999; Smirnova et al., 2001). To investigate the expression level of Drp1, immunoblot was performed on mouse SN and striatal tissue lysates 24 h after the last dose of TIQ. MPTP treatment induced significant Drp1 over-expression in mouse SN (Fig. 8A) and striatum (Fig. 8B) as compared to control at this point of time. TIQ (200 mg/kg, per oral) treatment normalized MPTP-induced Drp1 overexpression in both these regions.

Fig. 7: Effect of TIQ on MPTP-induced mitochondrial fragmentation in DA neurons.
Twenty µm mouse brain sections passing through SNpc region were taken and double immunostaining was carried out for Tom-20 (green) that stains mitochondria, and for tyrosine hydroxylase (TH; red) immunoreactivity. MPTP-induced fragmentation and swelling (arrow head) of mitochondria in TH-immunopositive neurons as compared with control where networked and elongated (arrow) mitochondria are seen. Treatment of TIQ (200 mg/kg; per oral) in MPTP-treated animals attenuated the neurotoxin-mediated aberrant mitochondrial morphology. The images are representative ones from three independent experiments.

Fig. 8: Effect of TIQ on MPTP-induced alteration in dynamin related protein 1 (Drp1) expression. Immunoblots depict MPTP-induced overexpression of Drp1 in mouse striatum (A) and SN (B). Data are represented as ratio of intensities using β-actin as loading control. TIQ treatment significantly attenuated MPTP-induced Drp1 overexpression from both the regions of the brain. TIQ itself did not show any effect on Drp1 expression. The data represented in the bar graphs are Mean ± S.E.M. provided for a minimum of four independent experiments. One way ANOVA followed by Newman Keul’s test; *p ≤ 0.05 as compared to the control group, @p ≤ 0.05 as compared to the MPTP group. n = 4-6 each group.
**TIQ reduces MPTP-induced Drp1 accumulation in mitochondria in mouse striatum:**

To study the mitochondrial localization of Drp1, mitochondrial and cytosolic fractions from the striatum of the mice were isolated as described earlier (Kirsch et al., 1999). Striatal Drp1 levels were found to be significantly increased in the mitochondrial fraction (Fig.9A) and reduced in the cytosolic fraction (Fig. 9B) in MPTP treated mice as compared with respective controls. TIQ (200 mg/kg, per oral, in four doses, and analyzed 24 h later) was able to antagonize these effects of MPTP. Moreover, TIQ alone significantly increased Drp1 level in the cytosolic fraction when compared with control mice (Fig. 9B). Mitochondrial to cytosolic ratio of Drp1 levels (Fig. 9C) revealed MPTP-induced increased Drp1 translocation (>2 fold) to mitochondria. This increase was found to be significantly reduced by TIQ administration both in control and in MPTP-treated mice (Fig.9B).

![Graph showing the effect of TIQ on MPTP-induced increase in Drp1 accumulation in mitochondria](image)

**Fig. 9: Effect of TIQ on MPTP-induced increase in Drp1 accumulation in mitochondria.** Immunoblots corresponding to Drp1 from mitochondrial (A) and cytosolic (B) fractions of mouse striatal tissue show an increased level of Drp1 in mitochondria and a decreased level in the cytosol after MPTP treatment. Data are represented as ratio of intensities normalized with β-actin. MPTP-mediated accumulation of Drp1 in the mitochondria is brought back to a significantly lower level by TIQ treatment with a significant elevation in the cytosol (B). MPTP treatment increased the mitochondrial:cytosolic (mito/cyto) ratio of Drp1 (C), which was significantly reduced by TIQ treatment. TIQ alone also showed an increased level of Drp1 localization in the cytosolic fraction, thereby causing a significant decrease in the mito/cyto ratio (see B, C). The data represented are Mean ± S.E.M. One way ANOVA, followed by Newman Keul’s test; *p ≤ 0.05 as compared to the control group, @p ≤ 0.05 as compared to the MPTP group. n = 3-6 each group.
**TIQ prevents MPTP-induced Drp1-punta formation in mouse SN:**

Observed effect of Drp1 distribution in mitochondria from striatum prompted us to check its distribution within TH-immunopositive neurons. Double immunofluorescent staining of TH and Drp1 in the sections passing though SNpc was undertaken. Punctate Drp1 fluorescence (Fig. 10) was observed in TH-immunopositive neurons of MPTP-treated animals in contrast to diffused Drp1 signal in control suggesting intracellular oligomerization of Drp1, possibly on mitochondria, as shown in Fig. 7 with Tom-20 staining. TIQ treatment abolished the MPTP-induced punctate Drp1 signal in TH-immunopositive neurons of SN. Surprisingly, accumulation of Drp1 signal in the nuclei of all TIQ-treated groups was consistently observed (Fig. 10; see small and bold arrows).
Fig. 10: Effect of TIQ on MPTP-induced Drp1 puncta formation in DA neurons. Twenty µm mice brain sections passing through SNpc region were cut and double immunostained for dynamin related protein 1 (Drp1) (green) and tyrosine hydroxylase (TH) (red). MPTP-induced puncta formation (green) in SN neurons is evident in merged and in the representative inverted images. TIQ treatment in MPTP-treated animals attenuated MPTP-induced puncta formation (see arrows in the third row). TIQ-induced Drp1 translocation to nucleus was also subsided to some extent (see arrows; fourth row; and the diminished intensity of staining). These images are representative photographs from three independent experiments, conducted on different days; n = 3 each group.

**TIQ normalizes MPTP-induced loss in Mfn2 expression from mouse SN:**

MPTP caused a reduction in Mfn2, a mitochondrial fusion protein expression levels in the SN (Fig. 11A), but not in the striatum (Fig. 11 B) of mice when compared with the control. TIQ treatment normalized Mfn2 (Fig. 11 A) expression levels in SN of MPTP-treated animals.
TIQ prevents MPTP-induced cytochrome-c dispersion in mouse nigral dopaminergic neurons:

Dispersed Cyt-c immunostaining in the TH-immunoreactive neurons in the SN of MPTP-treated mice (see second row) in contrast to punctate signal in controls (first row) was very obvious (Fig. 12). Treatment with TIQ was able to attenuate the MPTP-induced cytosolic dispersion of Cyt-c, and the neuronal staining of Cyt-c looked similar to control (Fig. 12, third row). The differences in Cyt-c punctate or diffused staining in the neurons are much clearer in the inverted images on the fifth column).

Fig. 11: Effect of TIQ on MPTP-induced altered mitofusin 2 (Mfn2) expressions. Representative immunoblots from three independent experiments depict MPTP-induced loss in Mfn2 expression from SN, while TIQ treatment significantly attenuated this effect (A). Data are expressed as ratio of intensities normalized by β-actin. MPTP treatment did not alter the expression of Mfn2 in the striatum (B). The data represented are Mean ± S.E.M. One way ANOVA, followed by Newman Keul’s test; *p ≤ 0.05 as compared to the control group; †p ≤ 0.05 as compared to the MPTP-treated group; n = 4.
**Drp1 is overexpressed in human PD postmortem brains:**

Immunoblot analyses carried out on mitochondrial fusion protein Drp1 in the SN of PD patient postmortem brain is provided in Fig 13. A clearly increased expression pattern of Drp1 in PD patients (n = 2) compared to age-matched controls (n = 2) are seen in these samples analyzed (Fig. 13).

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![Fig. 12: Effect of TIQ on MPTP-induced cytochrome-c release in SN dopaminergic neurons.](image-url)

Twenty µm mouse brain sections passing through SN region were cut and immunostained for cytochrome-c (Cyt-c; green) and tyrosine hydroxylase (TH; red). MPTP-induced dispersion of Cyt-c (diffused green signal) in SNpc TH-immunoreactive neuronal cytoplasm as depicted in the merged and inverted microphotographic images are clearly visible. This Cyt-c release is corrected by treatment with TIQ (200 mg/kg; per oral, four injections and analyzed 24 h following the fourth injection of TIQ), which is depicted as green puncta formation, comparable to the punctates seen in control (CON) section. The representative images were obtained from several sections analyzed from each animal from three independent experiments, n = 3 each group.
TIQ normalizes MPP\textsuperscript{+}-induced altered expressions of Drp1 and Mfn2 in SH-SY5Y cells:

Immunoblot was performed to study the effect of TIQ on MPP\textsuperscript{+}-induced alterations, if any in Drp1 and Mfn2 expression levels. In RA-differentiated SHSY5Y cells, MPP\textsuperscript{+} treatment (1 mM) caused a significant elevation of Drp1 (Fig. 14A) and reduction in Mfn2 (Fig. 14B) expression levels. Treatment of TIQ (1 \( \mu \)M) 24 h post MPP\textsuperscript{+} treatment significantly attenuated MPP\textsuperscript{+}-induced alterations in both Drp1 as well as Mfn2 protein levels (Fig. 14A,B). TIQ failed to affect these proteins levels.

Fig. 13: Drp1 is overexpressed in the SN of human PD postmortem brain. Immunoblots performed on SN tissue lysates of human postmortem brains of two control (CON) subjects and two PD patients are provided. An overexpression of Drp1 was evident when compared to CON brain samples. Data are given as ratio of intensities.

Fig. 14: MPP\textsuperscript{+}-induced alterations in Drp1 and Mfn2 are normalized by TIQ treatment. Representative immunoblots from retinoic acid differentiated SH-SY5Y cells evidence MPP\textsuperscript{+}-induced overexpression of Drp1 (A) and down regulation of Mfn2 (B). TIQ treatment significantly attenuated MPP\textsuperscript{+}-induced alteration in Drp1 and Mfn2 levels (A, B). Data presented are as ratio of intensities, Mean ± S.E.M. are provided from three independent experiments; \( n = 3-4 \). One way ANOVA and Newman Keul’s test was followed. \(*p \leq 0.05\) as compared to the control group (CON), \(\oplus p \leq 0.05\) as compared to the MPP\textsuperscript{+} group.
TIQ reduces MPP⁺-induced Drp1 translocation onto mitochondria in SH-SY5Y cells:

In order to study the effect of TIQ on MPP⁺-induced increased Drp1 redistribution on mitochondria, RA-differentiated SH-SY5Y cells were transfected with plasmid for mtRFP to visualize mitochondria and immunostained with Drp1 to study its subcellular distribution. Enlarged inset from merged image clearly demonstrate MPP⁺-induced increase in mitochondrial localization of Drp1 as compared with control cells (Fig. 15A). Treatment of TIQ (1 µM) 24 h post MPP⁺ treatment significantly attenuated MPP⁺-induced increased Drp1 localization on mitochondria. Cells treated with TIQ alone did not display any alteration in mitochondrial localization of Drp1 (Fig. 15A). To confirm our observations we performed immunoblot analysis for Drp1 from mitochondrial fractions of the cells treated with MPP⁺ and/with TIQ. Consistent with immunofluorescence analysis, MPP⁺-induced the overexpression of Drp1 from mitochondrial fraction, while, TIQ treatment significantly attenuated MPP⁺-induced mitochondrial translocation of Drp1 (Fig. 15B).
**TIQ attenuates MPP⁺-induced loss in mitochondrial membrane potential in differentiated SH-SY5Y cells:**

JC-1 staining was carried out to evaluate the effect of TIQ on MPP⁺-induced loss in mitochondrial membrane potential in RA-differentiated SH-SY5Y cells. JC-1 is ratio-metric potential-dependent dye. In mitochondria with high membrane potential JC-1 aggregates and gives red fluorescence as seen in control images (Fig. 16 upper row). JC-1 monomers reside in cytosol and give out green fluorescence as seen in MPP⁺ treated cells (Fig. 16; middle row). There appeared a significant recovery in membrane potential of differentiated cells, when treated with TIQ (note the gain in red fluorescence; Last row). In order to check mitochondrial membrane potential red/green frequency graph is plotted with the help of Image-J software.

Red/green frequency of JC-1 fluorescence revealed MPP⁺-induced loss in mitochondrial membrane potential of differentiated SH-SY5Y cells as compared with control (Fig. 16). A high red:green frequency is observed for the MPP⁺ + TIQ (1 µM) treated cells (Fig. 16).
**TIQ helps to control MPP⁺-induced mitochondrial fragmentation in SH-SY5Y cells:**

MPP⁺-induced overexpression of Drp1 with increased mitochondrial distribution and dip in mitochondrial membrane potential warranted investigation of the effect of TIQ on MPP⁺-induced alteration in mitochondrial morphology. RA differentiated SH-SY5Y cells were transfected with mtRFP to visualize the mitochondria and treated with MPP⁺ (1 mM). Mitochondrial morphology was further analyzed under fluorescent microscope at 2, 4, 8 and 24 h intervals. On the basis of mitochondrial morphology, mitochondria in the cells were categorized under three groups 1; networked and tubular (Fig 17G), 2; intermediate (Fig 17H), and 3; fragmented (Fig 17I) mitochondria. MPP⁺ treatment of SH-SY5Y cells caused significant decrease in percentage cells with networked or tubular in a
time-dependent manner (Fig. 17A). Decrease was also observed for intermediate mitochondria but not in a time-dependent fashion (Fig. 17B). MPP⁺-induced time-dependent increase in cells with mitochondrial fragmentation (Fig 17C) was about 72.78 ± 3.8% of total cells at 24 h after MPP⁺ treatment.

Fig. 17: Effect of TIQ on MPP⁺-induced alterations in mitochondrial morphology of differentiated SH-SY5Y cells. MPP⁺ (1 mM) caused time-dependent alterations in the number of cells with networked or tubular (A), intermediate (B) and fragmented (C) mitochondria when compared to control cells (CON). TIQ treatment (1 µM) significantly reduced the number of cells with fragmented mitochondria (F), and increased the percentage cells with networked mitochondria (D). There observed no significant difference in the number of cells harboring intermediate mitochondria (E). Representative microscopic images (100x) of networked (G), intermediate (H) and fragmented mitochondria (I) are provided. The data represented are Mean ± S.E.M. for three independent experiments conducted on different days; n = 35-40 cells each group per experiment; one way ANOVA followed by Newman Keul’s test was performed; *p ≤ 0.05 as compared to CON, †p ≤ 0.05 as compared to MPP⁺, ‡p ≤ 0.05 as compared to 2 h MPP⁺ and §p ≤ 0.05 as compared to 8 h MPP⁺.
TIQ treatment (1 µM) significantly improved the percentage of cells with networked mitochondria in MPP⁺ treated cells (Fig 17D). Similarly, TIQ helped to reduce the number of cells with fragmented mitochondria (Fig 17F). No significant difference in the number of cells harboring intermediate mitochondria was observed (Fig 17E).

**Discussion:**

The important outcomes elucidated in present Chapter are (i) oral administration of TIQ (200 mg/kg) alleviated MPTP-induced anomalous mitochondrial morphology in nigral dopaminergic neurons in mice, (ii) TIQ treatment antagonized MPTP-induced changed expression levels of mitochondrial dynamic regulatory proteins, increased Drp1 distribution in striatal mitochondria and Drp1 punta formation in TH-immunoreactive neurons in the SNpc region of mice brain, (iii) TIQ led to reduce MPTP-induced Cyt-c diffusion in dopaminergic neurons in the brain of mice and (iv) treatment of TIQ (1 µM) attenuated MPP⁺-induced altered expression levels of Drp1 and Mfn2, increased mitochondrial distribution of Drp1, anomalous mitochondrial morphology and mitochondrial membrane potential loss in RA-differentiated SH-SY5Y cells.

Neuronal cells continually adjust the rate of mitochondrial fission and fusion in response to change in energy demands and to facilitate the distribution of mitochondria (Detmer and Chan, 2007). Imbalances in mitochondrial fusion/fission dynamics could negatively impact neuronal function and neuronal viability. Targeting mitochondrial dynamics as a potential therapeutic for neurodegenerative diseases has been highlighted in recent years (Andreux et al., 2013; Bobela et al., 2015; Youle and van der Bliek, 2012). A group of large GTPases mediating mitochondrial fission and fusion processes have been identified in yeast screens, and most are conserved in mammals, including the fission mediators Drp1 (Dnm1 in yeast) and Fis1, as well as the fusion mediators Mfn1 and 2 (Fzo1 in yeast) and OPA1 (Mgm1 in yeast) (Westermann, 2008). MPTP-, MPP⁺-, 6-OHDA- and rotenone-induced neurotoxicity in cellular and animal models of PD are associated with Drp1-dependent mitochondrial fragmentation (Arnold et al., 2011; Gomez-Lazaro et al., 2008; Rappold et al., 2014; Wang et al., 2011). MPP⁺ inhibits histone deacetylation over Mfn2 promoter reducing Mfn2 expression (Zhu et al., 2014). Moreover, retrograde degeneration of DA neurons was apparent in Mfn2 deficient mice (Pham et al., 2012). In
consistency with previous finding, we report that MPTP-induced mitochondrial fragmentation in SN with concomitant altered Drp1 and Mfn2 expression levels. MPTP caused to increase Drp1 expression in the striatum as well. Interestingly, oral administration of TIQ affords to normalize Drp1 and Mfn2 levels and inhibited mitochondrial fragmentation triggered by MPTP. Consistently, we have also observed MPP⁺-induced altered Drp1 and Mfn2 protein expression levels and associated aberrant mitochondrial morphology in differentiated SH-SY5Y cells, and TIQ (1 µM) could reverse this effect of MPP⁺. It is important to note that our study for the first time revealed an increased Drp1 expression level in SNpc of postmortem PD brains.

Drp1-induced mitochondrial fragmentation requires Drp1 oligomerization on mitochondria and genetic or pharmacological blocking of Drp1 not only restricts neurotoxin effects of parkinsonian toxins but also modifies dopamine release deficit in vivo (Rappold et al., 2014). As expected, our immunoblots depict MPTP-induces increase in Drp1 translocation to mitochondria in the striatum and TIQ treatment redistributed Drp1 back into the cytosol. Immunofluorescent staining for Drp1 within TH-immunopositive neurons depicts MPTP-induced Drp1 puncta formation resembling Drp1 oligomerization. Also, Drp1 translocation into the nucleus of DA neurons was noteworthy in immunofluorescent staining. Though, how Drp1 gets in nucleus and their action therein is not explored here, yet, redistribution of Drp1 into the nucleus to prevent excessive mitochondrial fragmentation offers the prospect of a feedback loop to Drp1 promoter. The report that liver adenocarcinoma cells expressing nuclear Drp1 under hypoxic condition show resistance to chemotherapy might be of some relevance (Chiang et al., 2009). Additionally, we observed that TIQ attenuates MPP⁺-induced Drp1 localization on mitochondria in SH-SY5Y cells.

It is interesting to note that imbalanced mitochondrial fusion/fission dynamics occurs in the initial stages of neurodegenerative diseases including PD (Wang et al., 2011; Xie and Chung, 2012; Zhu et al., 2014). It has been observed that increased Drp1 expression or activity induces mitochondrial fragmentation, which are associated with cytochrome-c mediated apoptosis (Cassidy-Stone et al., 2008; Cereghetti et al., 2010; Estaquier and Arnoult, 2007; Rahimmi et al., 2015; Rappold et al., 2014; Wang et al., 2011). Bax and Bak, pro-apoptotic regulators, interact with Drp1 and Mfn2 during
apoptosis (Estaquier and Arnoult, 2007; Karbowski et al., 2002; Karbowski et al., 2006), supports the functional link between mitochondrial dynamics and apoptosis. Consistent with the findings, we have observed MPTP-induced Cyt-c release is concomitant with mitochondrial fragmentation. Importantly, TIQ treatment blocked the MPTP-induced release of Cyt-c. Altogether findings from this Chapter suggest the possible action of TIQ’s ability to correct MPTP/MPP\(^+\)-induced abnormal mitochondrial morphology.

**Conclusion:**

Results discussed in this Chapter clearly suggest TIQ’s ability to correct MPTP/MPP\(^+\)-induced abnormal mitochondrial fragmentation (*in vivo* and *in vitro*) by normalizing altered Drp1 and Mfn2 protein expression and Drp1 localization on mitochondria. TIQ treatment blocked MPTP-induced Cyt-c efflux from the mitochondria, which suggest TIQ’s ability to inhibit Cyt-c-mediated apoptotic cell death.
References:


Wang, X., Su, B., Liu, W., He, X., Gao, Y., Castellani, R.J., Perry, G., Smith, M.A., Zhu, X., 2011. DLP1-dependent mitochondrial fragmentation mediates 1-methyl-4-