Biochimica et Biophysica Acta 1852 (2015) 451–461

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

TNF-α regulates miRNA targeting mitochondrial complex-I and induces cell death in dopaminergic cells

Paresh Prajapati [a,b], Lakshmi Sripada [a,b], Kritarth Singh [a,b], Khyati Bhatelia [a,b], Rochika Singh [a], Rajesh Singh [b,*]

[a] Department of Cell Biology, School of Biological Sciences and Biotechnology, Indian Institute of Advanced Research (IIAR), Koba Institutional Area, Gandhinagar 382007, Gujarat, India
[b] Department of Biochemistry, Faculty of Science, The M. S. University of Baroda, Vadodara 390002, Gujarat, India

ARTICLE INFO

Article history:
Received 1 September 2014
Received in revised form 22 November 2014
Accepted 26 November 2014
Available online 4 December 2014

Keywords:
TNF-α
Inflammation
Parkinson's disease
Mitochondria
miRNA

ABSTRACT

Parkinson's disease (PD) is a complex neurological disorder of the elderly population and majorly shows the selective loss of dopaminergic (DAergic) neurons in the substantia nigra pars compacta (SNpc) region of the brain. The mechanisms leading to increased cell death of DAergic neurons are not well understood. Tumor necrosis factor-alpha (TNF-α), a pro-inflammatory cytokine is elevated in blood, CSF and striatum region of the brain in PD patients. The increased level of TNF-α and its role in pathogenesis of PD are not well understood. In the current study, we investigated the role of TNF-α in the regulation of cell death and miRNA mediated mitochondrial functions using, DAergic cell line, SH-SY5Y (model of dopaminergic neuron degeneration akin to PD). The cells treated with low dose of TNF-α for prolonged period induce cell death which was rescued in the presence of zVAD.fmk, a caspase inhibitor and N-acetyl-cysteine (NAC), an antioxidant. TNF-α alters mitochondrial complex-I activity, decreases adenosine triphosphate (ATP) levels, increases reactive oxygen species levels and mitochondrial turnover through autophagy. TNF-α differentially regulates miRNA expression involved in pathogenesis of PD. Bioinformatics analysis revealed that the putative targets of altered miRNA included both pro/anti apoptotic genes and subunits of mitochondrial complex. The cells treated with TNF-α showed decreased level of nuclear encoded transcript of mitochondrial complexes, the target of miRNA. To our knowledge, the evidences in the current study demonstrated that TNF-α is a potential regulator of miRNAs which may regulate mitochondrial functions and neuronal cell death, having important implication in pathogenesis of PD.

1. Introduction

Parkinson's disease (PD) is the most common neurodegenerative disorder, affecting millions of elderly individuals worldwide [1,2]. The increase in aging population is already showing exponential rise in PD cases. The mechanisms leading to PD had been the focus of research for the last several years; however, there is no effective therapy or any potential marker for monitoring the progression of PD. Neuropathological examination of the post-mortem brain suggests that several regions of the brain are affected, however the loss of dopaminergic (DAergic) neurons in the substantia nigra pars compacta (SNpc) is one of the most prominent features of PD [3]. At the time of clinical presentation approximately 50–70% of DAergic neurons in the nigrostriatal system are already lost [4]. The mechanisms leading to degeneration of DAergic neurons are still not well understood.

Inflammation and its association with neurodegenerative diseases are emerging [5,6]. Several studies provide strong evidences for the association of inflammation with sporadic and familial forms of the PD. The studies of post-mortem human brain obtained from PD patients provided direct evidence of the association with inflammation with PD. HLA-DR-positive reactive microglia were clearly observed within the substantia nigra of PD patients [7]. The increased levels of several pro-inflammatory cytokines (IL1-β, IL-2, IL-6, TNF-α and IFN-γ) were observed in the DAergic nigrostriatal system and the regions outside the SN in PD patients [8–13]. TNF-α is one of the important pleiotropic cytokines and had been implicated in both neuronal survival and death. TNF-α is known to induce ROS (reactive oxygen species) generation in mitochondria [14]. The mitochondrial complex I and complex III are the primary sites of ROS generation. The homeostasis of mitochondria is maintained through selective elimination of defective mitochondria by the process of selective autophagy called as mitophagy [15]. The role of TNF-α in regulation of mitochondrial dysfunction, generation of ROS and implication in mitophagy during PD conditions is not well understood.

The optimal functioning of mitochondria requires more than 1000 proteins. Hence >1000 resident proteins and critical non-coding RNAs (RNaseP, RNA component of MRP and 5S rRNA) are encoded from nuclear genome and are imported into mitochondria for their optimal function [16]. The miRNAs, emerging class of small non-coding RNAs, play important role in the regulation of mRNA copy number and protein level in the narrow physiological range [17]. Recently, our group

* Corresponding author. Tel.: +91 265 2795594.
E-mail address: singhraj1975@gmail.com (R. Singh).

http://dx.doi.org/10.1016/j.bbadis.2014.11.019
0925-4439/

demonstrated the association of non-coding RNAs including miRNAs with mitochondria [18]. The translation of mRNA encoding mitochondrial proteins occurs in close proximity to mitochondria [19]. The association of miRNA to mitochondria suggest its important implication in the regulation of nuclear encoded mitochondrial transcripts. The role of miRNA in the regulation of mitochondrial functions in the presence of TNF-α and its implication in PD progression are not well understood.

In the current study we investigated the role of TNF-α in the regulation of cell death and miRNA mediated mitochondrial functions using DAergic neuronal cells (SH-SY5Y, model of dopaminergic neuron degeneration similar to PD). We observed that increased levels of TNF-α alter the miRNAs targeting nuclear encoded mitochondrial complex-I subunits, induce mitochondrial oxidative stress by dysregulation of complex-I activity and induce cell death.

2. Materials and method

2.1. Cells and reagents

SH-SY5Y cells were grown at 37 °C, 5% CO_2 in Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 (DMEM/F12, Gibco, Invitrogen) supplemented with 15% (v/v) heat-inactivated fetal bovine serum (Gibco, Invitrogen) and 1% penicillin, streptomycin, and neomycin (PSN) antibiotic mixture (Gibco, Invitrogen). GFP-LC3 was provided by Dr. T. Yoshimori (National Institute of Genetics, Shizuoka, Japan) [20]. α-Synuclein-GFP was provided by Dr. Patrik Brundin (Neuronal Survival Unit, Wallenberg Neuroscience Center, Sweden). The primary antibodies used were: mouse monoclonal against caspase-9 (Cell Signaling, USA), rabbit polyclonal against caspase-3 (Cell Signaling, USA), PARP (Cell Signaling, USA), LC3 (Sigma, USA) and β-actin (Abcam, USA). Secondary antibodies: HRP-conjugated anti-rabbit and anti-mouse antibodies (Open Biosystems, USA) were used in the study. Rotenone, H_2O_2, 6-OHDA, N-acetyl-cysteine (NAC), Antimycin A, Sodium azide, Decylubiquinone, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Acridine orange and Propidium Iodide (PI) were purchased from Sigma-Aldrich, USA. TNF-α was purchased from Enzo Life Science (USA), z-VED.fmk from Invivogen (USA), SYBR green from Takara (Japan), first strand cDNA kit, miRCURY miRNA isolation kit from Exiqon (Denmark), Anti-miR-155 from Ambion (USA), Tetramethylrhodamine, methyl ester (TMRM) and ATP assay kit from Invitrogen (USA).

2.2. Cell death assay

The cell viability was analyzed by MTT assay and Acridine/Propidium Iodide staining. SH-SY5Y cells were plated at the density of 1×10^4 cells/per well in 96 well plate. After 24 h of culture, the cells were treated with indicated chemicals. MTT assay was performed at specified time intervals by incubating cells with MTT (0.1 mg/ml) at 37 °C for 1–2 h. The visible blue crystals were solubilized in DMSO and the absorbance was measured at 510 nm using microplate reader μQuant (Biotech Instruments, USA).

For Acridine/Propidium Iodide staining, the cells were plated in 24 well plate at the density of 1.5×10^5 cells/well. The cells were cultured in the presence of TNF-α (20 ng/ml) for 72 h. The Acridine orange/Propidium iodide (0.025 μg/ml and 0.033 μM respectively) solution was added to cells, plates were centrifuged and images were captured using GFP and RFP filter by IX81 fluorescent microscope (Olympus, Japan). Images were captured in monochrome and pseudo-colored and analyzed for green (healthy), orange (apoptotic) and red cells (late apoptotic or necrotic) using Image-Pro Plus (Media Cybernetics, USA). Caspase3/7 activation assay was performed using Caspase-Glo® 3/7 Assay kit (Promega, USA), according to the manufacturer's protocol.

2.3. Western blotting

Cells were treated with TNF-α for indicated time periods. Cells were harvested, washed with ice cold PBS and lysed in NP40 lysis buffer (150 mM NaCl, 50 mM Tris-Cl, 1% NP40, 1 mM PMSF). Protein concentration was determined by Bradford assay and equal protein loaded on 12% SDS-PAGE. Protein was electroblotted on PVDF membrane at 110 V for 1 h. Following the transfer, the membrane was blocked with 5% blocking buffer (5% non-fat dried milk and 0.1% Tween-20 in TBS) for 1 h at room temperature. The membrane was incubated overnight with specific primary antibody. After incubation the membrane was washed three times with TBS-T (TBS containing 0.1% Tween 20) for 10 min and incubated with a secondary antibody at room temperature for 1 h. The membrane was similarly washed three times with TBS-T and protein bands on the membrane were then visualized by using EZ-ECL chemiluminescence detection kit for HRP (Invitrogen, USA) by exposing to X-ray film.

Mitophagy was checked by western blotting of mitochondrial fraction by analyzing the conjugation of LC3 to mitochondria by western blotting using LC3 specific antibody. Mitochondrial fraction was prepared using differential centrifugation method [21] and lysed in Triton X100 lysis buffer. Western blotting was preformed as mentioned above.

2.4. Generation of stable cell line

The stable cell line SH-SY5Y-MTRFP was generated to study the mitophagy and mitochondrial dynamic. To generate SH-SY5Y-MTRFP stable cell line, 1.5×10^5 cells of SH-SY5Y were plated in 24 well plate and transfected with mitochondrial targeted red fluorescent protein vector (pHcRed1-Mito) (Clonetech, USA) using FUGENE HD Transfection Reagent (Roche, Germany) according to the manufacturer's protocol. Media was replaced with DMEM-F12 supplemented with G418 (500 μg/ml) after 48 h of transfection and every alternate day until stable clones were clearly visible. The stable cells were harvested from 24 well plate and transferred to 96 well plate to obtain single clone using serial dilution method. The single clones were transferred and maintained in 6 well plate. After incubation for 15 days, the cells were transferred to 25 cm^2 culture flask and maintained in DMEM-F12 supplemented with 50 μg/ml of G418.

2.5. Analysis of mitophagy by confocal microscopy

SH-SY5Y-MTRFP cells were plated at the density of 1.5×10^5 cells per well in 24 well plate having coverslip and transfected with GFP-LC3 using FuGENE HD Transfection Reagent (Roche, Germany). After 24 h of transfection, the cells were treated with 20 ng/ml of TNF-α for 72 h and co-localization of GFP-LC3 with mitochondria was monitored using Leica TCS-SP5-II confocal microscope (Germany).

2.6. Analysis of reactive oxygen species and mitochondrial membrane potential

ROS was analyzed by microscopy and spectrofluorometry using MitoSOX as mitochondria ROS indicator. SH-SY5Y cells were treated with 20 ng/ml of TNF-α for 48 h. After incubation for indicated time, the cells were stained with MitoSOX Red (1.0 μM) (Invitrogen, USA) in DMEM-F12 for 10 min at 37 °C. Cells were washed three times in fresh DMEM-F12. Images were captured using 10× objective (Olympus IX81 microscope) having same exposure for each well.

The relative quantification of ROS was analyzed by spectrofluorometry. Cells were harvested after treatment as indicated. Cells were suspended in DPBS containing MitoSOX Red (1.0 μM) (Invitrogen, USA) for 10 min. The cells were washed with DPBS and fluorescence was analyzed by fluorescence spectrophotometer (Hitachi, Japan) at Excitation 510 and Emission 580.

Mitochondrial membrane potential ($\Delta\Psi$) was analyzed by spectrofluorometry using TMRM staining. SH-SY5Y cells were loaded with TMRM (200 nM) (Invitrogen, USA) for 15 min after treatment with indicated chemicals. The cells were washed in DPBS and fluorescence was analyzed by fluorescence spectrophotometer (Hitachi, Japan) at Excitation 540 and Emission 570.

2.7. Analysis of complex-I activity

Complex-I activity was determined by BN-PAGE (Blue-Native Page) and Spectro-Photometry. BN-PAGE was performed as described previously [22,23] with minor modifications. Acrylamide (50% w/v)/Bisacrylamide (0.5% w/v) solution was prepared such that it yielded highly porous gels on polymerization, for the separation of higher order protein complexes. The final concentration of the stacking gel was 4% and that of the resolving gel was 10%. Purified mitochondrial fraction was mixed with sample buffer and non-gradient BN-PAGE was performed at room temperature as described previously [22]. The gel was stained with complex-I staining solution (50 mM potassium phosphate buffer (pH 7.0) containing 0.2 mg/ml NBT and 0.1 mg/ml NADH). All gel images were documented using an Epson Perfection 1670 scanner.

The activity of complex-I was determined using decylubiquinone as the electron acceptor and NADH as donor. Briefly, cells were harvested and washed with cold DPBS. Cells were subjected to freeze-thawing two or three times in Freeze–Thaw complete solution (0.25 M Sucrose, 20 mM Tris–HCl (pH 7.4), 40 mM KCl, 2 mM EDTA supplemented with 1 mg/ml fatty acid free BSA, 0.01% Digitonin and 10% Percoll). After completion of the freeze-thawing process, the cells were washed with Freeze–Thaw solution devoid of digitonin to remove the residual digitonin as it interferes with the complex-I enzyme assay. Cells were suspended in complex-I assay buffer (35 mM Potassium Phosphate (pH—7.4), 1 mM EDTA. 2.5 mM NaN_3, 1 mg/ml BSA, 2 μg/ml Antimycin A, 5 mM NADH). Complex-I activity was measured for 3 min by following the decrease in absorbance at 340 nm after the addition of 2.5 mM acceptor decylubiquinone indicating the oxidation of NADH.

2.8. ATP luciferase assay

Total and mitochondrial ATP was performed by using ATP Bioluminescence kit CLS II (Invitrogen, USA). Briefly, SH-SY5Y cells were treated with TNF-α for 72 h. Mitochondrial fraction was isolated by differential centrifugation method [21]. Total cell and mitochondrial fractions were lysed in 0.1% Triton X-100 and ATP levels were analyzed using Luminescent ATP Detection Assay Kit (Invitrogen, USA) according to the manufacturer's instructions. ATP concentration was calculated by plotting standard curve.

2.9. miRNA isolation and analysis by Quantitative Real Time-PCR (qRT-PCR)

SH-SY5Y were plated at density of 5×10^5 cells/well in 6 well plate and treated with 20 ng/ml of TNF-α for 72 h. miRNAs were isolated using miRCURY miRNA isolation kit (Exiqon, Denmark) according to the manufacturer's protocol.

The expression of miRNAs was determined by qRT-PCR. For analysis of miRNA expression, Poly-A tailing of 100 ng miRNA was performed at 42 °C for 1 h using PolyA polymerase (NEB, USA). The cDNA was synthesized using universal degenerate primer and Universal RT mix (Applied Biosystems, USA). qRT-PCR was performed with SYBR Premix Ex Taq II (Takara, Japan) with StepOnePlus™ (Applied Biosystem, USA) for 35 cycles of 95 °C for 15 s and 60 °C for 60 s. 5S rRNA was used as endogenous control.

The target mRNA levels were also analyzed by qRT-PCR. Briefly, total RNA was isolated using Tri Reagent (Life technologies, USA) and was reverse transcribed to synthesize cDNA using cDNA Universal RT mix (Applied Biosystems, USA) according to the manufacturer's instructions. qRT-PCR was performed using SYBR Premix Ex TaqTM (Takara, Japan) as per the manufacturer's instructions for 35 cycles of 95 °C for 5 s and 60 °C for 34 s. GAPDH was used as endogenous control. The relative amount of each gene and miRNA was measured using the $2(-\Delta\Delta CT)$ method [24]. The relative association with standard error was plotted using Graph Pad PRISM 5.0.

2.10. Target analysis

The validated miRNAs were categorized into upregulated and downregulated columns based on the qPCR results. The target pathways of selected miRNA function were determined by selecting all miRNAs of each category, combination of all 5 target prediction tools and ClipSeq with low stringency and corrected p-value < 0.05 [25]. The GO terms and pathways were retrieved for each category and tabulated.

2.11. Statistical analysis

Data are shown as mean ± SEM for no. of observations. Comparisons of groups were performed using ANOVA, Tukey's Multiple Comparison test and Student's t-test for repeated measurements to determine the levels of significance for each group. Each experiment has been repeated minimum two times independently and probability values of $p < 0.05$ were considered as statistically significant.

3. Results

3.1. Prolonged exposure of TNF-α induces cell death in SH-SY5Y

The previously reported clinical studies had provided evidence that TNF-α level is increased in both serum and CSF (cerebral spinal fluid) in PD patients [13,26,27]. This strongly suggests its important role in selective neuronal loss however; its role in pathophysiology of PD is not well understood. In the current study, SH-SY5Y cells (in-vitro model of dopaminergic neuron) were treated with low concentration of TNF-α (20 ng/ml) for different time periods. The soluble TNF-α can bind to TNFR1 and TNFR2 [28], hence the expression pattern of both TNFR1 and TNFR2 in SH-SY5Y was analyzed in the presence/absence of TNF-α using real time PCR. The expression of TNFR1 increased significantly in the presence of TNF-α whereas there was no change in TNFR2 expression (Fig. S1). The binding of TNF-α to TNF-R1 can lead to cell survival and death pathway [29] hence further studies were done to confirm the role of TNF-alpha in neuronal survival/death. SH-SY5Y cells were treated with different doses of TNF-α for prolonged period and cell survival was monitored using MTT assay (Fig. S2A). The cell survival significantly decreased after 72 h of TNF-α treatment as compared to untreated cells (Fig. 1A). The high level of cell death (>30%) was observed after 96 h of TNF-α treatment (Fig. 1A).

To determine the type of cell death on prolonged exposure of TNF-α, Acridine orange/Propidium Iodide staining was performed. The cells stained orange (Fig. 1B) and number increased significantly (Fig. S2B) after 72 h of TNF-α treatment, suggesting that the cell death is mediated by apoptosis. The induction of apoptosis was further confirmed by monitoring the PARP cleavage and caspase activation. PARP is a target of executioner caspases and is cleaved during apoptosis [30,31]. The western blotting showed 110 kDa corresponding to native form of PARP in control as well as TNF-α treated cells. The band of 89 kDa corresponding to cleaved PARP was observed only after 72 h of TNF-α treatment. The cleaved form of PARP was not detected in the untreated cells (Fig. 1C). PARP cleavage strongly suggests the role of caspases in TNF-α induced cell death. Further experiments were performed to confirm the activation of caspases and their role in TNF-α induced cell death. SH-SY5Y cells treated with TNF-α showed the band of 18 kDa corresponding to cleaved subunit of caspase-3 after 72 h whereas it was not detected till 48 h of TNF-α

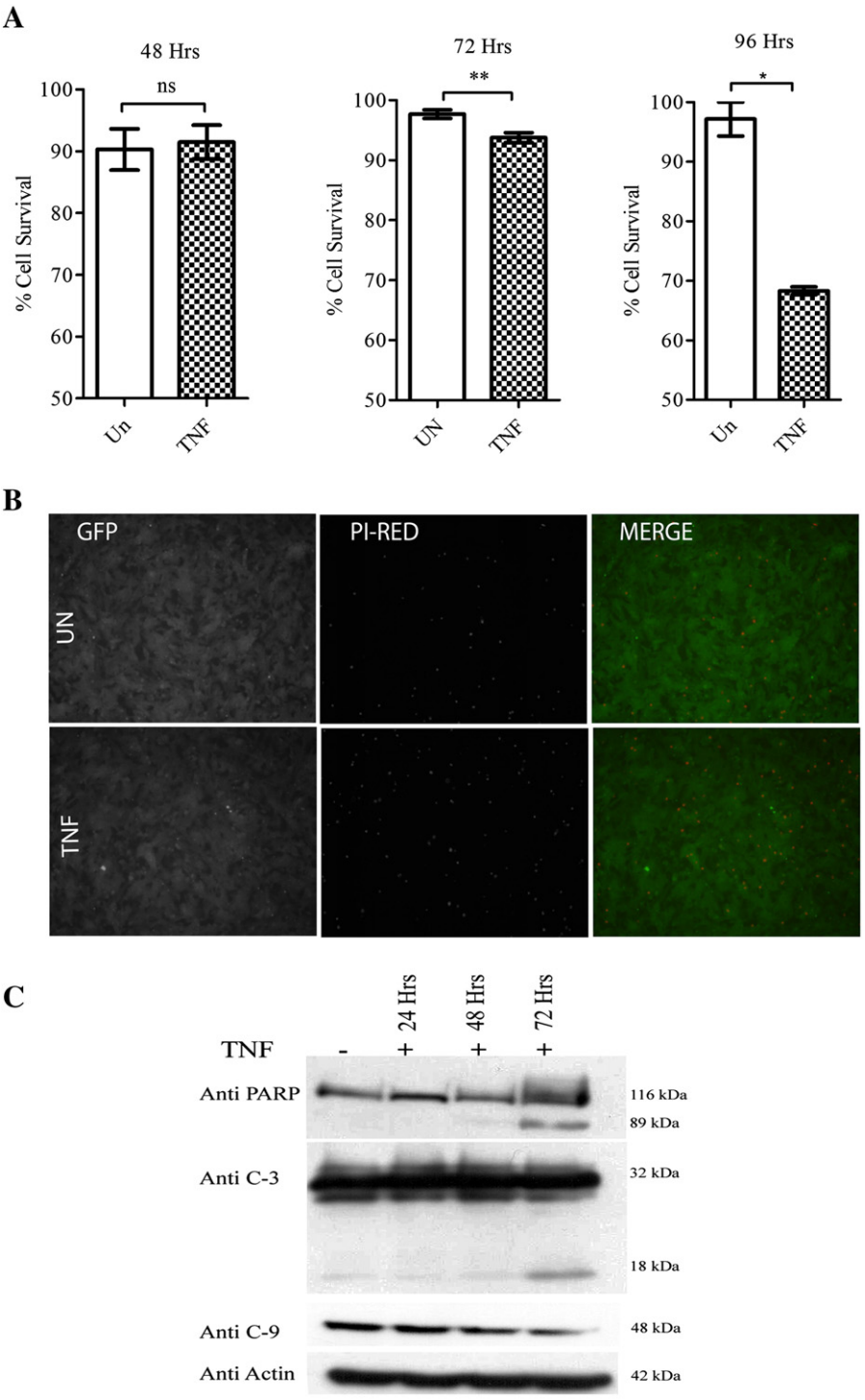

Fig. 1. TNF-α induces apoptotic cell death in SH-SY5Y cells. (A) SH-SY5Y cells were treated with TNF-α (20 ng/ml) for indicated time period and MTT assay was performed for the analysis of cell survival. (B) TNF-α induces apoptosis. SH-SY5Y cells were treated with TNF-α (20 ng/ml) for 72 h. The analysis of the type of cell death was monitored by Acridine orange/Propidium Iodide staining. (C) TNF-α activates caspase-3 and 9 in DAergic cells. SH-SY5Y cells were treated with TNF-α (20 ng/ml) for different time periods. The activation of caspase-3 and 9 was monitored by western blotting using specific antibodies. Asterisk (*) indicates that p value < 0.05, for SEM.

treatment and untreated cells (Fig. 1C). The antibody against caspase-9 used in the study detects only 48 kDa precursor form of caspase-9. The decreased level of 48 kDa band was observed only after 72 h of TNF-α treatment (Fig. 1C). This strongly suggests the activation of caspase-9 in the presence of TNF-α. These observations collectively suggest that prolonged exposure of low doses of TNF-α to dopaminergic neuronal cells, SH-SY5Y induces apoptosis.

3.2. TNF-α sensitizes SH-SY5Y cells to PD stress induced cell death

Neurotoxicants like Rotenone, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or 6-hydroxydopamine (6-OHDA) induce PD like conditions in experimental animal models [32] and induce degeneration of DAergic neurons. SH-SY5Y expresses tyrosine hydroxylase and dopamine transporter and can preferentially uptake 6-OHDA [33]. It

accumulates in the cytosol and induces cell death by generating ROS. Similarly, Rotenone freely enters into the cells and induces neuronal death by inhibiting complex-I activity [34]. We further analyzed if TNF-α sensitizes dopaminergic neurons to cell death in PD stress conditions (Rotenone and 6-OHDA). SH-SY5Y cells were co-treated with sub-toxic doses of 6-OHDA and TNF-α. The cell viability was analyzed by MTT assay. The cell viability significantly decreased in the presence of TNF-α and 6-OHDA as compared to 6-OHDA alone and untreated cells (Fig. 2A). Similar experiments were performed in the presence of rotenone. The presence of TNF-α sensitized rotenone induced cell death as compared to untreated or treated either of them (Fig. 2B). The high levels of α-synuclein expression are known to produce PD-like cellular and axonal pathologies in the nigrostriatal region of both rodents' and primates' brain [35]. Therefore the role of TNF-α was also checked in cell death during α-synuclein mediated in PD stress condition. To confirm this, α-synuclein-GFP was transfected in SH-SY5Y and treated with TNF-α for 72 h and cell death was monitored by PI Staining. The number of PI positive/α-synuclein-GFP transfected cells significantly increased in the presence of TNF-α (Fig. 2C). Collectively, these results suggest that TNF-α sensitizes SH-SY5Y to cell death in the presence of different PD stress conditions.

3.3. TNF-α induces mitochondrial oxidative stress in SH-SY5Y cells

The dysregulation of mitochondria and oxidative stress is one of the primary features of PD stress conditions. The activation of caspase-9 and caspase-3 cascade suggests the dysregulation of mitochondrial functions in the presence of TNF-α, which may be one of the reasons for increased cell death in PD stress conditions. To elucidate the role of TNF-α in oxidative stress, the generation of ROS at mitochondria was monitored by MitoSOX staining. The dye permeates live cells where it is selectively targeted to mitochondria, oxidized by superoxide and generates red fluorescence. The number of MitoSOX positive cell significantly increased in the presence of TNF-α as compared to untreated cells (Fig. 3A) suggesting the induction of mitochondrial ROS. Quantification of MitoSOX fluorescence was performed by spectrofluorometry method

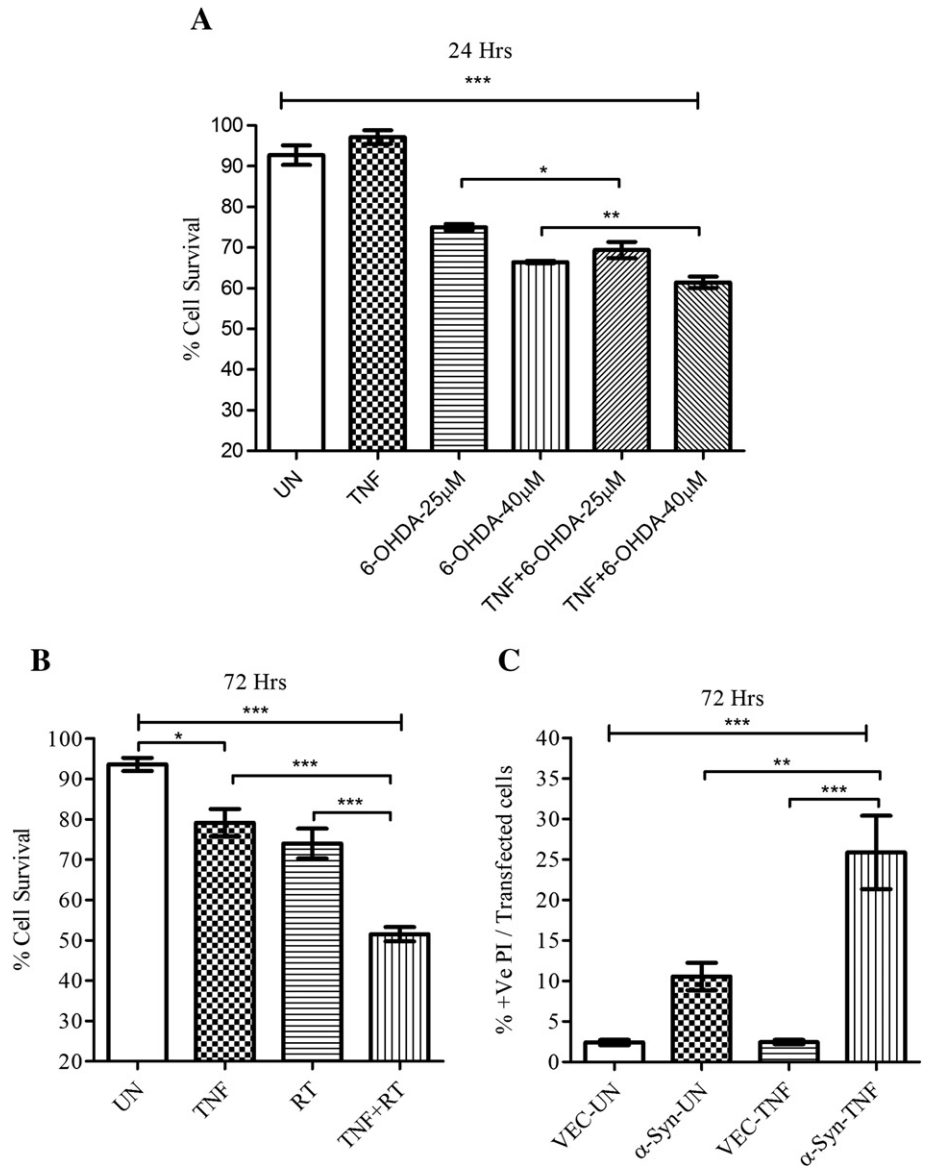

Fig. 2. TNF-α sensitizes SH-SY5Y to cell death in PD stress conditions. (A) SH-SY5Y cells were co-treated with indicated sub-toxic doses of 6-OHDA (25 μM, 40 μM) and TNF-α (20 ng/ml) for 24 h. Cell viability was analyzed by MTT assay. (B) SH-SY5Y cells were treated with Rotenone (25 μM/ml) for 24 h in the presence of TNF-α and MTT assay was performed for cell survival assessment. (C) SH-SY5Y cells were transfected with α-synuclein-GFP and vector control followed by TNF-α (20 ng/ml) treatment. After 72 h of treatment % PI (Propidium Iodide) positive cell/ Transfected was assessed by PI staining. Asterisk (*) indicates that p value < 0.05, for SEM.

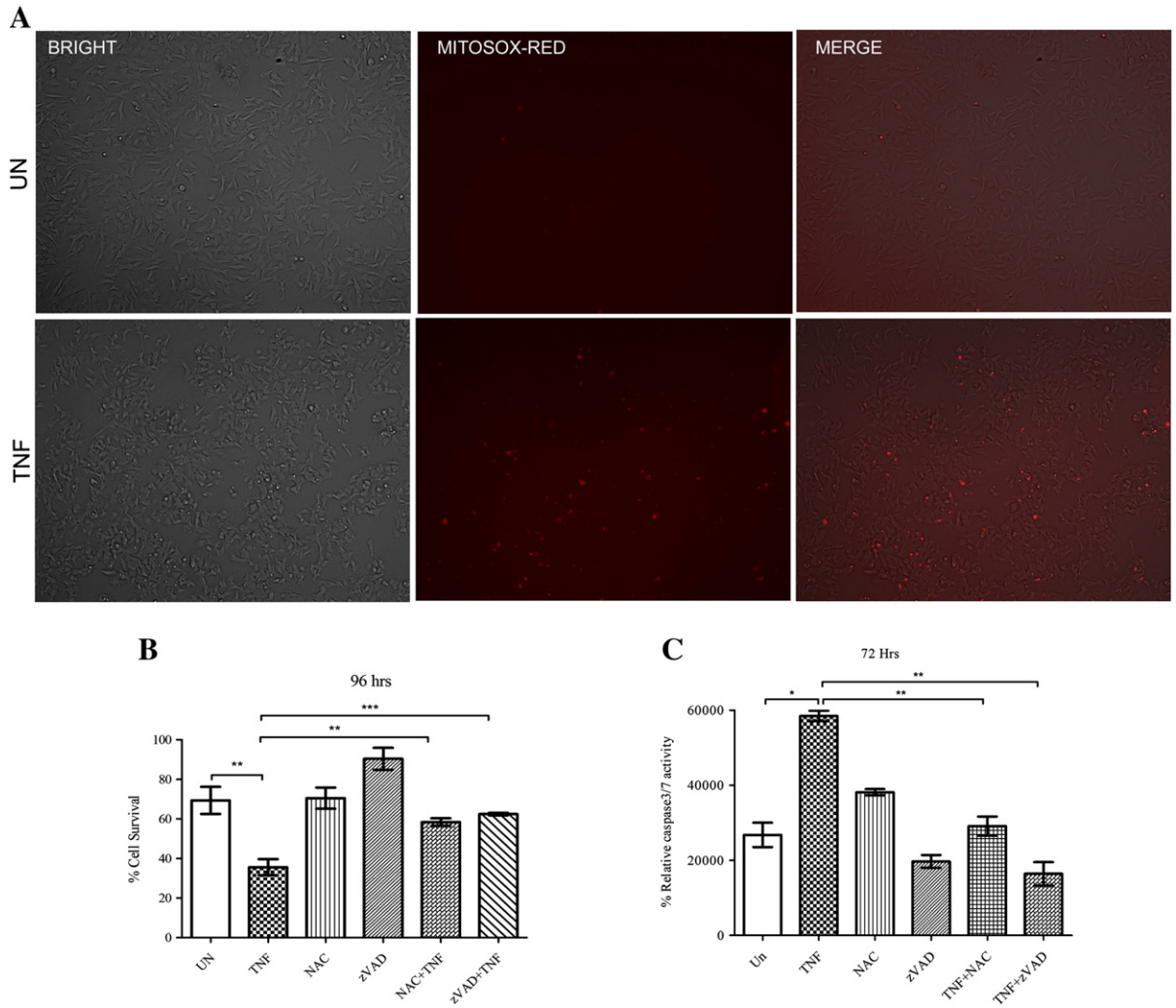

Fig. 3. TNF-α induces mitochondrial oxidative stress and cell death. (A) SH-SYSY cells were treated with TNF-α for 48 h. MitoSOX staining was performed to monitor ROS production using fluorescence microscope. (B and C) SH-SY5Y cells were treated with TNF-α, NAC and z-VAD.fmk either alone or in combination. (B) After 96 h of treatment, cell survival was assessed by MTT assay and (C) caspase 3/7 activity was measured using caspases 3/7 Luciferase assay. Asterisk (*) indicates that p value < 0.05, for SEM.

using rotenone as positive control. The significant increase in fluorescence intensity was observed in the presence of TNF-α (Fig. S3A) suggesting the generation of mitochondrial ROS. The generation of ROS may lead to the loss of mitochondrial membrane potential (ΔΨ), hence TMRM staining was performed in the presence and absence of TNF-α. The fluorescence intensity significantly decreased in the presence of TNF-α (Fig. S3B). To further confirm the role of TNF-α induced oxidative stress in induction of DAergic neuronal death, the cell survival was monitored in the presence of antioxidant (NAC) and caspase inhibitor (z-VED.fmk). TNF-α induced cell death was rescued by NAC and z-VED.fmk (Fig. 3B). The activity of caspase3/7 significantly increased in the presence of TNF-α and decreased in the presence of antioxidant and caspase inhibitor (Fig. 3C). The results here demonstrated that TNF-α induces oxidative stress and subsequently activates caspase dependent cell death pathway in SH-SY5Y cells.

3.4. TNF-α regulates mitochondrial complex-I in dopaminergic cells

The above experiment showed increased oxidative stress in the presence of TNF-α suggesting compromised mitochondrial functions. The mitochondrial complex-I is the entry point of the electrons in the electron transport chain (ETC) [36]. The dysregulation of mitochondrial complex-I results in leakage of electrons and is one of the primary sources of ROS [36]. However, its regulation in the presence of TNF-α and its implication in PD stress are not well understood. Hence, we monitored the mitochondrial complex-I activity by spectrophotometric assay and BN-PAGE. The spectrophotometric assay showed that complex-I activity significantly decreased in the presence of TNF-α. The decrease in complex-I activity was observed as early as 36 h whereas the caspase activation and cell death were evident after 72 h of TNF-α treatment as compared to untreated cells (Fig. 4A). To further confirm this observation, BN-PAGE was performed and in-gel assay for complex-I showed decreased activity in the presence of TNF-α treated cells as compared to untreated cells (Fig. 4B). This suggests that dysregulation of complex-I precedes the cell death in the presence of TNF-α.

TNF-α mediated decrease in mitochondrial complex-I activity may also affect ATP levels in the cells. The total relative cellular and mitochondrial ATP levels were determined in the presence/absence of TNF-α. In TNF-α treated cells, both total cellular and mitochondrial ATP levels significantly decreased as compared to untreated cells (Fig. 4C and D). These evidences strongly suggest that mitochondrial functions may be dysregulated in the presence of TNF-α.

The mitochondrial homeostasis is required for the optimal function of the cell [37]. The defective mitochondria are eliminated by selective process of autophagy called as mitophagy [38,39]. However, TNF-α mediated regulation of mitophagy and its implication in PD disease are not well understood. Mitophagy was monitored by co-localization of autophagy marker (LC3 protein) with mitochondria by confocal

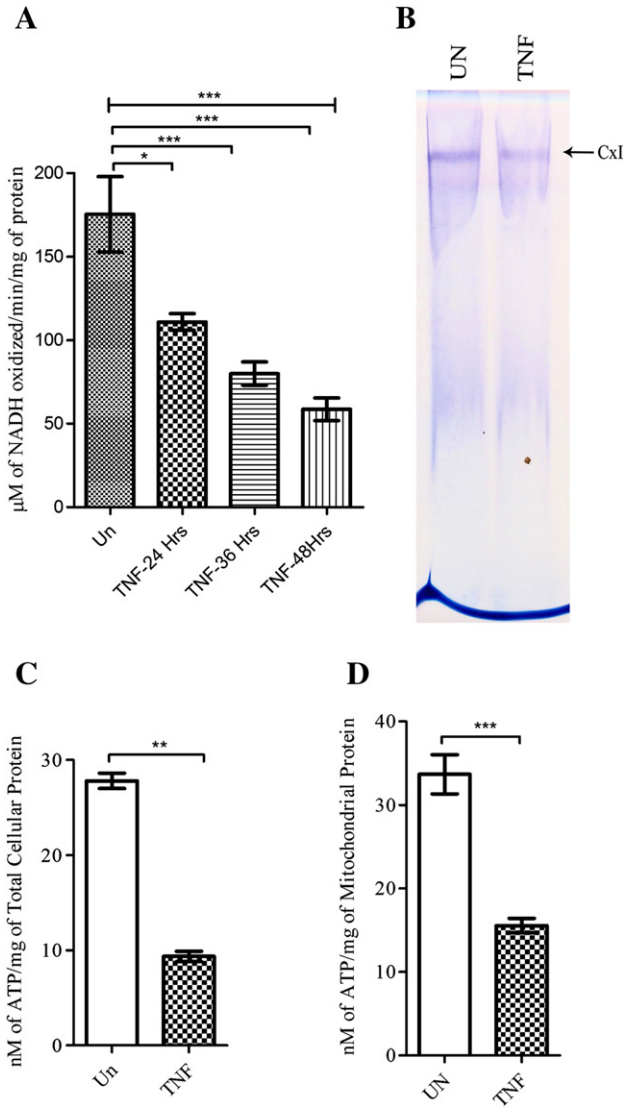

Fig. 4. TNF-α regulates mitochondrial complex-I activity in SH-SY5Y cells. (A) SH-SY5Y cells were treated with TNF-α at different time periods. Complex-I activity was measured at indicated time point of TNF-α treatment by monitoring the decrease in absorbance at 340 nm. (B) Blue Native gel electrophoresis of Triton-X 100 solubilized mitochondrial extracts from TNF-α treated and untreated SH-SY5Y cells was performed. The gel strips were stained with complex-I staining solution. (C and D) After 72 h of TNF-α treatment, ATP levels in untreated and TNF-α treated condition were measured by ATP-dependent luciferase activity. (C) ATP level in whole cell lysates. (D) ATP level in mitochondrial fraction. Asterisk (*) indicates that p value < 0.05, for SEM.

microscopy and western blotting. SH-SY5Y-MTRFP stable cell line was transfected with GFP-LC3 and co-localization was analyzed by confocal microscopy. The co-localization of green LC3 puncta and red mitochondria was clearly observed in the presence of TNF-α as compared to untreated cells (Fig. 5A) and significantly increased in the presence of TNF-α (Fig. S4D). We also hypothesized that mitochondria undergoing mitophagy may be conjugated with LC3 hence we performed western blotting in the presence/absence of TNF-α and rotenone (as positive control for mitophagy inducer). The LC3-II forms, which represent the lipidated form of LC3, increased significantly in the mitochondrial fraction in the presence of rotenone (inhibitor of complex-I, positive control). The lipidated form of LC3 increased in the presence of TNF-α as compared to untreated cells however it was low as compared to rotenone (Fig. 5B). The level of autophagy was also analyzed in the presence of TNF-α by western blotting of LC3. The levels of lipidated form of LC3-II increased in the presence of TNF-α. The turnover increased till 36 h as the levels of LC3-II form increased in the presence of bafilomycin. Interestingly, the level of LC3-II did not change in the presence of bafilomycin (inhibitor of vacuolar H + ATPase (V-ATPase)) after prolonged incubation of 48 and 72 h [40], suggesting the decreased turnover of autophagosome to the lysosome (Fig. S4A). Taken together, all these suggest that the increased levels of TNF-α alter the mitochondrial complex-I activity, induce oxidative stress, and alter mitochondrial turnover through mitophagy which may have important implication in PD conditions.

3.5. TNF-α regulates miRNA expression targeting mitochondrial functions

The evidences in the current study strongly suggest that mitochondrial function is compromised in the presence of TNF-α and leads to cell death. The optimal functioning of mitochondria needs more than 1000 proteins which are nuclear encoded and translocated to the mitochondria. The import/association of non-coding RNA including miRNA to mitochondria is emerging [18,41–43]. The miRNA targets both nuclear encoded and mitochondria encoded mRNA transcripts. Hence, we hypothesized that increased levels of TNF-α observed in PD conditions may alter the levels of miRNA which in turn may regulate mitochondrial functions and cell death. The differentially expressed miRNAs in SH-SY5Y in the presence of TNF-α were selected from previous reports [44]. The expression level of identified miRNAs were analyzed by real time PCR. It was observed that hsa-miR-let-7b, hsa-miR-let-7g, hsa-miR-103, hsa-miR-155, hsa-miR-16-5p, hsa-miR-17, hsa-miR-204, hsa-miR-27 and hsa-miR-98 were up-regulated significantly whereas the levels of hsa-miR-let-7a, hsa-miR-128, hsa-miR-145, hsa-miR-181a, hsa-miR-23a, hsa-miR-23b and hsa-miR-320a significantly decreased in the presence of TNF-α (Fig. S5).

The putative targets of validated miRNAs and their biological significance were determined by combinatorial approach using miRNA function tools of starBase v.2.0 [25,45,46]. The putative targets of up-regulated miRNAs were involved in neuronal differentiation, axonal guidance and nerve projection development (Table A1). The targets of up-regulated miRNAs were also clustered in basic cellular functional like cell cycle, mRNA transport, proteasomal ubiquitin dependent protein catabolism, mRNA transport and stabilization, protein phosphorylation, response to mechanical stress, acetyl CoA synthesis, ubiquitination, ubiquitin proteasome system and mitochondrial import. These processes are important for the mitochondrial homeostasis and functions (Table A2). Similarly, the putative targets of downregulated miRNA regulate mitotic cell cycle, positively regulate protein phosphorylation, negatively regulate ubiquitination, inflammatory genes, neuronal projections, mRNA transport, cAMP response, translation, transcription, protein stabilization and negatively regulate cellular migration (Table A3). The several cellular processes as described above targeted by miRNAs have important implication in the progression of PD.

The starBase analysis also revealed that some of the validated miRNAs may target nuclear encoded subunits of mitochondrial complex-I to complex-V (Table A4). The putative targets of hsa-miR-103, hsa-miR-128, hsa-miR-155, hsa-miR-17, hsa-miR-221, hsa-miR-23a/b, and hsa-miR-27a/b were found to be nuclear encoded mitochondrial complex I subunits: NDUFV1, NDUFS4, NDUFV1, NDUFAB1 and NDUFB5, NDUFA1, NDUFA7 and NDUFC1, NDUSF4 respectively. As observed in the above section, the mitochondrial complex-I was compromised in the presence of TNF-α, hence miRNAs targeting nuclear encoded mitochondrial complex-1 subunit were specifically analyzed by real time PCR. The levels of miR-27a, hsa-miR-17, hsa-miR-103 and hsa-miR-155 significantly increased in the presence of TNF-α (Fig. 6A). The level of NDUFS4 (target of hsa-miR-27a) was decreased significantly in the presence of TNF-α (Fig. 6B). This suggests that TNF-α regulates miRNAs that target mitochondrial complex-I subunits which are critical for neuronal function. Similarly, ATP5G3, a subunit of mitochondrial complex-V is a putative target of miR-155 and miR-27a. It was observed that the levels of both miRNAs (miR-155 and miR-27a) were high and the levels of ATP5G3 (target of miR-155 and

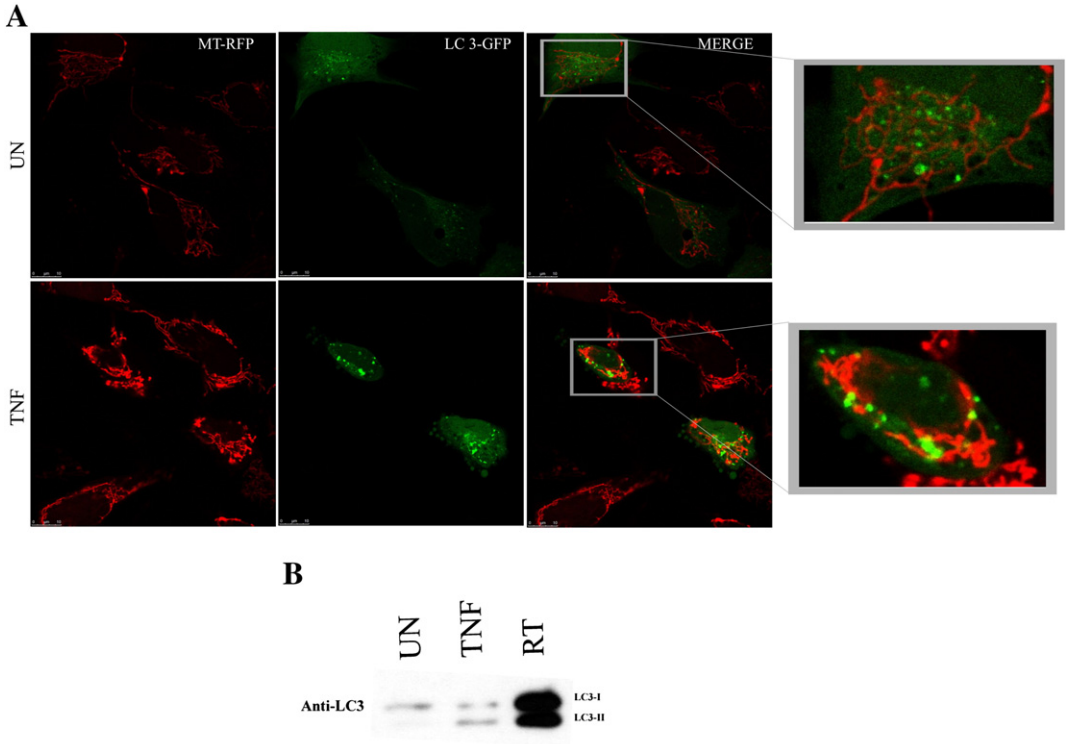

Fig. 5. TNF-α regulates mitophagy in SH-SY5Y cells. (A) SH-SY5Y-MTRFP stable cells were transfected with GFP-LC3. Cells were treated with TNF-α for 72 h and co-localization of GFP-LC3 with red mitochondria was analyzed by confocal microscopy. Scale bars, 10 μm. Graphical representations of the numbers LC3 puncta co-localization with mitochondria per cell in untreated and TNF-α treated condition of representative (Fig. S4B). (B) SH-SY5Y cells were treated with TNF-α for 72 h and mitochondrial fraction was prepared using differential centrifugation method and association of LC3 with mitochondria was analyzed by western blotting using LC3 specific antibody.

miR-27a) transcripts, decreased significantly in the presence of TNF-α (Fig. 6A and B).

The evidence in the current study suggest that hsa-miR-155 target mitochondrial functions and previous reports [47] suggest its implication in cell death hence further experiments were done to confirm its role in TNF-α induced cell death. SH-SY5Y cells were transfected with anti-miR-155 and cell survival was monitored in presence of TNF-α. The cell death significantly decreased in the presence of TNF-α in anti-miR-155 transfected cells as compared to control (Fig. 6C). These evidences suggest that TNF-α alters the expression of miRNAs that target transcripts encoding mitochondrial complex-I and complex-V subunits leading to mitochondrial defects and decline in ATP production during neuronal cell death in PD.

4. Discussion

The epidemiological, GWAS and clinical reports strongly suggest that inflammation is one of the major causes of DAergic neuronal loss and cause of PD. The increased levels of TNF-α, one of the key pro-inflammatory cytokines have been observed in CSF [13,48] and striatum of the PD brain [49]. The role of TNF-α in pathogenesis of PD is not well understood. In the current study we conclude that TNF-α regulates miRNAs that regulate mitochondrial functions, induces oxidative stress and apoptosis.

TNF-α is pleiotropic cytokine and can act as pro-survival or cell death stimuli depending upon the cell type [50–53]. We used DAergic neuronal cell line (SH-SY5Y) to elucidate the molecular mechanisms of TNF-α mediated DAergic loss during PD stress conditions. SH-SY5Y cells were treated with enhanced levels of TNF-α for longer duration mimicking conditions in vivo. The presence of TNF-α specifically up-regulates TNFR1 leading to activation of intracellular cell death pathways. The increased level of TNF-α leads to apoptosis in DAergic neuronal cell line was clearly evident by PARP cleavage, caspase-3 and caspase-9 activation. The increased level of TNF-α showed significantly increased cell death with time. This observation is in consonance with earlier observation of the post-mortem brain of PD patients [54]. This strongly suggests that TNF-α may bind to the cognate receptor and induce the activation of downstream caspase cascade rather than activation of pro-survival pathway of NF-κB activation. TNF-α also sensitized SH-SY5Y to cell death in the presence of rotenone, 6-OHDA and α-synuclein induced PD stress conditions. The activation of caspase-9 in the presence of TNF-α strongly suggests the activation of mitochondrial mediated pathway of apoptosis.

Mitochondrial dysfunction is one of the cardinal features of the PD [55]. TNF-α is known to regulate mitochondrial functions by enhancing ROS, superoxide and H_2O_2 production [56]. These functions are critically regulated by mitochondrial complex-I which is the entry point of the electrons in ETC. Hence, we hypothesized that the prolonged exposure of TNF-α may decrease complex-I activity and mitochondrial ATP production. The prolonged exposure of SH-SY5Y cells to TNF-α showed decrease in mitochondrial complex-I and ATP levels and induces ROS and cell death. The cell death induced by TNF-α was rescued by antioxidants that further emphasized the role of ROS and complex-I. The evidence of the regulation of mitochondrial complex-I activity by TNF-α suggests its important implication in PD stress conditions. The hypothesis is further supported by observation in animal model of ventricular dysfunction where TNF-α regulates ROS production by down regulating the expression of four subunits of the complex-I [57]. These observations should be validated in animal models and post-mortem brain samples obtained from PD patients.

The defective mitochondria are ubiquitinated through PARKIN and degraded through selective process of autophagy called as mitophagy [58]. Mitochondrial homeostasis is critical for neuronal functions hence any alteration in mitochondrial biogenesis or the degradation through mitophagy may lead to neurodegeneration [59]. The evidences here strongly suggest that the turnover of mitochondria is regulated by

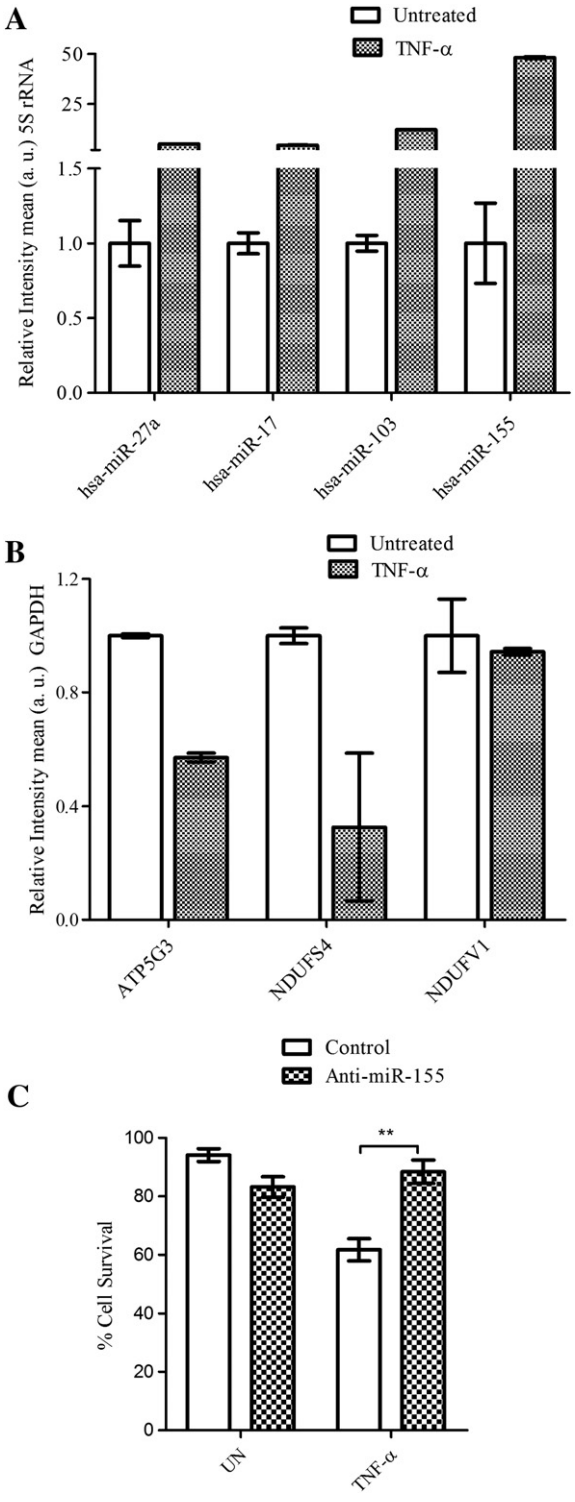

Fig. 6. TNF-α regulates miRNAs targeting mitochondrial complexes. (A) The expression levels of miRNAs which putatively target mitochondrial complex-I subunits were determined using 5S rRNA as endogenous control by qPCR (B) The expression levels of targets comprising nuclear encoded mitochondria complex-I subunits were determined by real time PCR using GAPDH as endogenous control. (C) SH-SY5Y cells were transfected with anti-miR-155 and control. Cell survival was monitored using MTT assay in presence of TNF-α. Asterisk (*) indicates that p value < 0.05, for SEM.

TNF-α. Mitochondria are recognized by the autophagic machinery and conjugated by LC3 in the presence of TNF-α. The induction of mitophagy in the presence of rotenone is in consonance with earlier reports [60,61]. The LC3 conjugation to mitochondria is less prominent in the presence of TNF-α as compared to rotenone. This suggests that defective mitochondria may be accumulated as observed by decreased turnover in the presence of TNF-α leading to generation of ROS and cell death. The levels of mitophagy and its association with TNF-α should be also studied in the clinical samples of PD patient.

The emerging reports strongly suggest that mitochondrial trafficking is crucial for neuronal branching, synaptic plasticity and transmission [62,63]. The mitochondria are immobilized at the neuronal branching point, assist site specific de novo protein synthesis and initiate axonal branching [63,64]. This strongly suggests that mitochondria are important for the neuronal connectivity which is degenerated in different neurodegenerative diseases including PD [65,66]. The optimal functioning of mitochondria requires nuclear encoded genes that are transcribed in the nucleus, translated in the cytoplasm and translocated to mitochondria [16]. The recent reports including ours [18,41–43] suggest that miRNA associates with mitochondria and is important for the regulation of the target in the narrow physiological range. In consonance to it, the current study demonstrated that miRNAs including mitochondria associated miRNA (miR-23a, miR-320a, miR-181, miR-let-7a, miR-let-7 g a) are altered in the presence of TNF-α. Intriguingly, some of these miRNAs: miR-103, miR-17, miR-181a, miR-23a, miR-23b, miR-27b and miR-320 were previously reported to be enriched at synapses [67–69]. The role of miRNAs in the regulation of synaptic functions should be also studied in the presence of TNF-α.

The putative targets of validated miRNA suggested that miRNA may target the genes involved in mRNA stability, transport and transcription, translation, phosphorylation and ubiquitination of proteins, cell cycle and mitochondrial complex subunits. The dysregulation of these processes and targets has been implicated in progression of PD [70–77]. In consonance with the starBase analysis of miRNA and targets, the experimental results showed that TNF-α regulates miRNAs (has-miR-27a and hsa-miR-103) that target the nuclear encoded transcripts of mitochondrial complex-I subunit decreased in the presence of TNF-α. This is further supported by observation where expression of miR-27a impaired mitochondrial biogenesis, structure integrity and complex-I activity which further produced excessive ROS in human cell [78]. Similarly, TNF-α regulates the expression of miR-155 and miR-27a which regulate the transcript levels of ATP5G3, subunit of F1-ATPase. This helps in transport of protons across the mitochondrial inner membrane to the F1-ATPase for synthesis of ATP. This hypothesis is in consonance with recent report demonstrating the import of hsa-miR-181c in mitochondria, which targets complex-IV subunits in myocardial infract [79]. These results strongly suggest that TNF-α may regulate several miRNAs specifically targeting nuclear encoded transcripts encoding mitochondrial targeted proteins.

The data provided here strongly suggest that mitochondrial functions were compromised in the presence of TNF-α leading to bioenergetics crisis and increased cell death in dopaminergic neurons. The study also suggests that TNF-α regulated miRNAs play important role in the regulation of mitochondrial functions suggesting their important implication in PD conditions. The innovation in sequencing technologies for the last few years had shown exponential increase in the number of miRNAs hence it is important to systematically investigate the TNF-α regulated miRNA in dopaminergic neurons. The same needs to be validated in the tissue obtained from the patients having increased levels of TNF-α in serum and CSF and showing the symptoms of Parkinsonism. The studies in this direction will help to better understand the crosstalk of inflammation, miRNA and mitochondria and its implication in PD.

5. Conclusions

Parkinson's disease is a devastating age related neurodegenerative disorder. Pro-inflammatory cytokine like TNF-α is increased in serum, CSF and striatum of PD patients. The current study provided evidence that increased levels of TNF-α decrease mitochondrial complex-I, leading to oxidative stress, activation of caspase cascade leading to mitochondrial dysfunction and cell death. TNF-α regulated miRNAs target

nuclear encoded subunits of mitochondrial ETC. The altered levels of miRNA in the presence of TNF-α reduced the subunit of mitochondrial complex-I and complex-V leading to decreased levels of ATP. This study strongly suggests that TNF-α regulates miRNA targeting mitochondrial functions that may have important implication in dopaminergic neuronal loss in PD.

Acknowledgements

This work was financially supported by the Department of Biotechnology, Government of India (grant number BT/PR14206/MED/30/396/2010 to Rajesh Singh). This work may constitute the PhD thesis of Paresh Prajapati. Lakshmi Sripada and Kritarth Singh received their Senior Research fellowship and Junior Research fellowship respectively from University Grants Commission (UGC), Govt. of India. Khyati Bhatelia received her Senior Research fellowship from Council of Scientific and Industrial Research (CSIR), Govt. of India. Dr. Rochika Singh received her fellowship as DST Young Scientist from Department of Science and Technology (DST), Govt. of India. Authors also acknowledge the facilities developed under DBT Program Support to IIAR.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2014.11.019.

References

[1] V.P. Calabrese, Projected number of people with Parkinson disease in the most populous nations, 2005 through 2030, Neurology 69 (2007) 223–224 (author reply 224).
[2] J.M. Savitt, V.L. Dawson, T.M. Dawson, Diagnosis and treatment of Parkinson disease: molecules to medicine, J. Clin. Invest. 116 (2006) 1744–1754.
[3] H. Braak, E. Ghebremedhin, U. Rub, H. Bratzke, K. Del Tredici, Stages in the development of Parkinson's disease-related pathology, Cell Tissue Res. 318 (2004) 121–134.
[4] M. Orth, A.H. Schapira, Mitochondrial involvement in Parkinson's disease, Neurochem. Int. 40 (2002) 533–541.
[5] S. Amor, F. Puentes, D. Baker, P. van der Valk, Inflammation in neurodegenerative diseases, Immunology 129 (2010) 154–169.
[6] G.C. Brown, J.J. Neher, Microglial phagocytosis of live neurons, Nat. Rev. Neurosci. 15 (2014) 209–216.
[7] P.L. McGeer, S. Itagaki, B.E. Boyes, E.G. McGeer, Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's disease brains, Neurology 38 (1988) 1285–1291.
[8] M. Reale, C. Iarlori, A. Thomas, D. Gambi, B. Perfetti, M. Di Nicola, M. Onofrj, Peripheral cytokines profile in Parkinson's disease, Brain Behav. Immun. 23 (2009) 55–63.
[9] R.J. Dobbs, A. Charlett, A.G. Purkiss, S.M. Dobbs, C. Weller, D.W. Peterson, Association of circulating TNF-alpha and IL-6 with ageing and parkinsonism, Acta Neurol. Scand. 100 (1999) 34–41.
[10] G. Stypula, J. Kunert-Radek, H. Stepien, K. Zylinska, M. Pawlikowski, Evaluation of interleukins, ACTH, cortisol and prolactin concentrations in the blood of patients with Parkinson's disease, Neuroimmunomodulation 3 (1996) 131–134.
[11] D. Blum-Degen, T. Muller, W. Kuhn, M. Gerlach, H. Przuntek, P. Riederer, Interleukin-1 beta and interleukin-6 are elevated in the cerebrospinal fluid of Alzheimer's and de novo Parkinson's disease patients, Neurosci. Lett. 202 (1995) 17–20.
[12] M. Mogi, M. Harada, T. Kondo, P. Riederer, H. Inagaki, M. Minami, T. Nagatsu, Interleukin-1 beta, interleukin-6, epidermal growth factor and transforming growth factor-alpha are elevated in the brain from Parkinsonian patients, Neurosci. Lett. 180 (1994) 147–150.
[13] M. Mogi, M. Harada, P. Riederer, H. Narabayashi, K. Fujita, T. Nagatsu, Tumor necrosis factor-alpha (TNF-alpha) increases both in the brain and in the cerebrospinal fluid from Parkinsonian patients, Neurosci. Lett. 165 (1994) 208–210.
[14] J.J. Kim, S.B. Lee, J.K. Park, Y.D. Yoo, TNF-alpha-induced ROS production triggering apoptosis is directly linked to Romo1 and Bcl-X(L), Cell Death Differ. 17 (2010) 1420–1434.
[15] J.J. Lemasters, Selective mitochondrial autophagy, or mitophagy, as a targeted defense against oxidative stress, mitochondrial dysfunction, and aging, Rejuvenation Res. 8 (2005) 3–5.
[16] S.E. Calvo, V.K. Mootha, The mitochondrial proteome and human disease, Annu. Rev. Genomics Hum. Genet. 11 (2010) 25–44.
[17] N. Tran, G. Hutvagner, Biogenesis and the regulation of the maturation of miRNAs, Essays Biochem. 54 (2013) 17–28.
[18] L. Sripada, D. Tomar, P. Prajapati, R. Singh, A.K. Singh, Systematic analysis of small RNAs associated with human mitochondria by deep sequencing: detailed analysis of mitochondrial associated miRNA, PLoS One 7 (2012) e44873.
[19] M. Spillane, A. Ketschek, T.T. Merianda, J.L. Twiss, G. Gallo, Mitochondria coordinate sites of axon branching through localized intra-axonal protein synthesis, Cell Rep. 5 (2013) 1564–1575.
[20] Y. Kabeya, N. Mizushima, T. Ueno, A. Yamamoto, T. Kirisako, T. Noda, E. Kominami, Y. Ohsumi, T. Yoshimori, LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing, EMBO J. 19 (2000) 5720–5728.
[21] Z.T. Schug, F. Gonzalvez, R.H. Houtkooper, F.M. Vaz, E. Gottlieb, BID is cleaved by caspase-8 within a native complex on the mitochondrial membrane, Cell Death Differ. 18 (2011) 538–548.
[22] L.J. Yan, M.J. Forster, Resolving mitochondrial protein complexes using nongradient blue native polyacrylamide gel electrophoresis, Anal. Biochem. 389 (2009) 143–149.
[23] L.J. Yan, S.H. Yang, H. Shu, L. Prokai, M.J. Forster, Histochemical staining and quantification of dihydrolipoamide dehydrogenase diaphorase activity using blue native PAGE, Electrophoresis 28 (2007) 1036–1045.
[24] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods 25 (2001) 402–408.
[25] J.H. Li, S. Liu, H. Zhou, L.H. Qu, J.H. Yang, starBase v2.0: decoding miRNA-ceRNA, miRNA-ncRNA and protein-RNA interaction networks from large-scale CLIP-Seq data, Nucleic Acids Res. 42 (2014) D92–D97.
[26] M. Mogi, A. Togari, T. Kondo, Y. Mizuno, O. Komure, S. Kuno, H. Ichinose, T. Nagatsu, Caspase activities and tumor necrosis factor receptor R1 (p55) level are elevated in the substantia nigra from Parkinsonian brain, J. Neural Transm. 107 (2000) 335–341.
[27] D. Koziorowski, R. Tomasiuk, S. Szlufik, A. Friedman, Inflammatory cytokines and NT-proCNP in Parkinson's disease patients, Cytokine 60 (2012) 762–766.
[28] A. Caminero, M. Comabella, X. Montalban, Tumor necrosis factor alpha (TNF-alpha), anti-TNF-alpha and demyelination revisited: an ongoing story, J. Neuroimmunol. 234 (2011) 1–6.
[29] D.E. Christofferson, Y. Li, J. Yuan, Control of life-or-death decisions by RIP1 kinase, Annu. Rev. Physiol. 76 (2014) 129–150.
[30] G.V. Chaitanya, A.J. Steven, P.P. Babu, PARP-1 cleavage fragments: signatures of cell-death proteases in neurodegeneration, Cell Commun. Signal 8 (2010) 31.
[31] D. D'Amours, F.R. Sallmann, V.M. Dixit, G.G. Poirier, Gain-of-function of poly(ADP-ribose) polymerase-1 upon cleavage by apoptotic proteases: implications for apoptosis, J. Cell Sci. 114 (2001) 3771–3778.
[32] J. Bove, D. Prou, C. Perier, S. Przedborski, Toxin-induced models of Parkinson's disease, NeuroRx 2 (2005) 484–494.
[33] J. Luthman, A. Fredriksson, E. Sundstrom, G. Jonsson, T. Archer, Selective lesion of central dopamine or noradrenaline neuron systems in the neonatal rat: motor behavior and monoamine alterations at adult stage, Behav. Brain Res. 33 (1989) 267–277.
[34] N. Berndt, H.G. Holzhutter, S. Bulik, Implications of enzyme deficiencies on mitochondrial energy metabolism and reactive oxygen species formation of neurons involved in rotenone-induced Parkinson's disease: a model-based analysis, FEBS J. 280 (2013) 5080–5093.
[35] A. Ulusoy, M. Decressac, D. Kirik, A. Bjorklund, Viral vector-mediated overexpression of alpha-synuclein as a progressive model of Parkinson's disease, Prog. Brain Res. 184 (2010) 89–111.
[36] F.H. Sterky, N.G. Larsson, Complex I: a complex gateway to the powerhouse, Cell Metab. 7 (2008) 278–279.
[37] A.C. Kulkarni, P. Kuppusamy, N. Parinandi, Oxygen, the lead actor in the pathophysiologic drama: enactment of the trinity of normoxia, hypoxia, and hyperoxia in disease and therapy, Antioxid. Redox Signal. 9 (2007) 1717–1730.
[38] M. Redmann, M. Dodson, M. Boyer-Guittaut, V. Darley-Usmar, J. Zhang, Mitophagy mechanisms and role in human diseases, Int. J. Biochem. Cell Biol. 53 (2014) 127–133.
[39] N. Hattori, S. Saiki, Y. Imai, Regulation by mitophagy, Int. J. Biochem. Cell Biol. 53 (2014) 147–150.
[40] T. Yoshimori, A. Yamamoto, Y. Moriyama, M. Futai, Y. Tashiro, Bafilomycin A1, a specific inhibitor of vacuolar-type H(+)-ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells, J. Biol. Chem. 266 (1991) 17707–17712.
[41] T.R. Mercer, S. Neph, M.E. Dinger, J. Crawford, M.A. Smith, A.M. Shearwood, E. Haugen, C.P. Bracken, O. Rackham, J.A. Stamatoyannopoulos, A. Filipovska, J.S. Mattick, The human mitochondrial transcriptome, Cell 146 (2011) 645–658.
[42] S. Bandiera, S. Ruberg, M. Girard, N. Cagnard, S. Hanein, D. Chretien, A. Munnich, S. Lyonnet, A. Henrion-Caude, Nuclear outsourcing of RNA interference components to human mitochondria, PLoS One 6 (2011) e20746.
[43] X. Zhang, X. Zuo, B. Yang, Z. Li, Y. Xue, Y. Zhou, J. Huang, X. Zhao, J. Zhou, Y. Yan, H. Zhang, P. Guo, H. Sun, L. Guo, Y. Zhang, X.D. Fu, MicroRNA directly enhances mitochondrial translation during muscle differentiation, Cell 158 (2014) 607–619.
[44] D. Culpan, P.G. Kehoe, S. Love, Tumour necrosis factor-alpha (TNF-alpha) and miRNA expression in frontal and temporal neocortex in Alzheimer's disease and the effect of TNF-alpha on miRNA expression in vitro, Int. J. Mol. Epidemiol. Genet. 2 (2011) 156–162.
[45] S. Ekimler, K. Sahin, Computational Methods for MicroRNA Target Prediction, Genes (Basel) 5 (2014) 671–683.
[46] J.H. Yang, J.H. Li, P. Shao, H. Zhou, Y.Q. Chen, L.H. Qu, starBase: a database for exploring microRNA-mRNA interaction maps from Argonaute CLIP-Seq and Degradome-Seq data, Nucleic Acids Res. 39 (2011) D202–D209.
[47] R. Nederlof, O. Eerbeek, M.W. Hollmann, R. Southworth, C.J. Zuurbier, Targeting hexokinase II to mitochondria to modulate energy metabolism and reduce ischaemia–reperfusion injury in heart, Br. J. Pharmacol. 171 (2014) 2067–2079.
[48] T. Nagatsu, M. Mogi, H. Ichinose, A. Togari, Changes in cytokines and neurotrophins in Parkinson's disease, J. Neural Transm. Suppl. (2000) 277–290.
[49] M. Chertoff, N. Di Paolo, A. Schoeneberg, A. Depino, C. Ferrari, W. Wurst, K. Pfizenmaier, U. Eisel, F. Pitossi, Neuroprotective and neurodegenerative effects of the chronic expression of tumor necrosis factor alpha in the nigrostriatal dopaminergic circuit of adult mice, Exp. Neurol. 227 (2011) 237–251.

[50] D. Wallach, The TNF cytokine family: one track in a road paved by many, Cytokine 63 (2013) 225–229.
[51] K. Juhasz, K. Buzas, E. Duda, Importance of reverse signaling of the TNF superfamily in immune regulation, Expert. Rev. Clin. Immunol. 9 (2013) 335–348.
[52] M. Shuh, H. Bohorquez, G.E. Loss Jr., A.J. Cohen, Tumor necrosis factor-alpha: life and death of hepatocytes during liver ischemia/reperfusion Injury, Ochsner J. 13 (2013) 119–130.
[53] W.M. Chu, Tumor necrosis factor, Cancer Lett. 328 (2013) 222–225.
[54] T. Nagatsu, M. Sawada, Biochemistry of postmortem brains in Parkinson's disease: historical overview and future prospects, J. Neural Transm. Suppl. (2007) 113–120.
[55] C.A. Gautier, O. Corti, A. Brice, Mitochondrial dysfunctions in Parkinson's disease, Rev. Neurol. (Paris) 170 (2014) 339–343.
[56] N. Mariappan, C.M. Elks, B. Fink, J. Francis, TNF-induced mitochondrial damage: a link between mitochondrial complex I activity and left ventricular dysfunction, Free Radic. Biol. Med. 46 (2009) 462–470.
[57] N. Mariappan, C.M. Elks, M. Haque, J. Francis, Interaction of TNF with angiotensin II contributes to mitochondrial oxidative stress and cardiac damage in rats, PLoS One 7 (2012) e46568.
[58] D. Narendra, A. Tanaka, D.F. Suen, R.J. Youle, Parkin is recruited selectively to impaired mitochondria and promotes their autophagy, J. Cell Biol. 183 (2008) 795–803.
[59] H. Chen, D.C. Chan, Mitochondrial dynamics–fusion, fission, movement, and mitophagy–in neurodegenerative diseases, Hum. Mol. Genet. 18 (2009) R169–R176.
[60] C.T. Chu, J. Ji, R.K. Dagda, J.F. Jiang, Y.Y. Tyurina, A.A. Kapralov, V.A. Tyurin, N. Yanamala, I.H. Shrivastava, D. Mohammadyani, K.Z. Qiang Wang, J. Zhu, J. Klein-Seetharaman, K. Balasubramanian, A.A. Amoscato, G. Borisenko, Z. Huang, A.M. Gusdon, A. Cheikhi, E.K. Steer, R. Wang, C. Baty, S. Watkins, I. Bahar, H. Bayir, V.E. Kagan, Cardiolipin externalization to the outer mitochondrial membrane acts as an elimination signal for mitophagy in neuronal cells, Nat. Cell Biol. 15 (2013) 1197–1205.
[61] M. Frank, S. Duvezin-Caubet, S. Koob, A. Occhipinti, R. Jagasia, A. Petcherski, M.O. Ruonala, M. Priault, B. Salin, A.S. Reichert, Mitophagy is triggered by mild oxidative stress in a mitochondrial fission dependent manner, Biochim. Biophys. Acta 1823 (2012) 2297–2310.
[62] Z.H. Sheng, Mitochondrial trafficking and anchoring in neurons: new insight and implications, J. Cell Biol. 204 (2014) 1087–1098.
[63] Q. Cai, M.L. Davis, Z.H. Sheng, Regulation of axonal mitochondrial transport and its impact on synaptic transmission, Neurosci. Res. 70 (2011) 9–15.
[64] T. Sun, H. Qiao, P.Y. Pan, Y. Chen, Z.H. Sheng, Motile axonal mitochondria contribute to the variability of presynaptic strength, Cell Rep. 4 (2013) 413–419.
[65] E.I. Ginns, S.K. Mak, N. Ko, J. Karlgren, S. Akbarian, V.P. Chou, Y. Guo, A. Lim, S. Samuelsson, M.L. LaMarca, J. Vazquez-DeRose, A.B. Manning-Bog, Neuroinflammation and alpha-synuclein accumulation in response to glucocerebrosidase deficiency are accompanied by synaptic dysfunction, Mol. Genet. Metab. 111 (2014) 152–162.
[66] O. Solis, D.I. Limon, J. Flores-Hernandez, G. Flores, Alterations in dendritic morphology of the prefrontal cortical and striatum neurons in the unilateral 6-OHDA-rat model of Parkinson's disease, Synapse 61 (2007) 450–458.
[67] P. Follert, H. Cremer, C. Beclin, MicroRNAs in brain development and function: a matter of flexibility and stability, Front. Mol. Neurosci. 7 (2014) 5.
[68] R. Saba, P.H. Storchel, A. Aksoy-Aksel, F. Kepura, G. Lippi, T.D. Plant, G.M. Schratt, Dopamine-regulated microRNA MiR-181a controls GluA2 surface expression in hippocampal neurons, Mol. Cell. Biol. 32 (2012) 619–632.
[69] N.R. Smalheiser, G. Lugli, microRNA regulation of synaptic plasticity, Neuromol. Med. 11 (2009) 133–140.
[70] S. Tenreiro, K. Eckermann, T.F. Outeiro, Protein phosphorylation in neurodegeneration: friend or foe? Front. Mol. Neurosci. 7 (2014) 42.
[71] T. Cornelissen, D. Haddad, F. Wauters, C. Van Humbeeck, W. Mandemakers, B. Koentjoro, C. Sue, K. Gevaert, B. De Strooper, P. Verstreken, W. Vandenberghe, The deubiquitinase USP15 antagonizes Parkin-mediated mitochondrial ubiquitination and mitophagy, Hum. Mol. Genet. 23 (2014) 5227–5242.
[72] K.Z. Wang, J. Zhu, R.K. Dagda, G. Uechi, S.J. Cherra III, A.M. Gusdon, M. Balasubramani, C.T. Chu, ERK-mediated phosphorylation of TFAM downregulates mitochondrial transcription: Implications for Parkinson's disease, Mitochondrion 17 (2014) 132–140.
[73] V.A. Morais, D. Haddad, K. Craessaerts, P.J. De Bock, J. Swerts, S. Vilain, L. Aerts, L. Overbergh, A. Grunewald, P. Seibler, C. Klein, K. Gevaert, P. Verstreken, B. De Strooper, PINK1 loss-of-function mutations affect mitochondrial complex I activity via NdufA10 ubiquinone uncoupling, Science 344 (2014) 203–207.
[74] L. Soreq, H. Bergman, Z. Israel, H. Soreq, Deep brain stimulation modulates nonsense-mediated RNA decay in Parkinson's patients leukocytes, BMC Genomics 14 (2013) 478.
[75] K. Fifel, O. Dkhissi-Benyahya, H.M. Cooper, Lack of long-term changes in circadian, locomotor, and cognitive functions in acute and chronic MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) mouse models of Parkinson's disease, Chronobiol. Int. 30 (2013) 741–755.
[76] T. Cali, D. Ottolini, A. Negro, M. Brini, Enhanced parkin levels favor ER-mitochondria crosstalk and guarantee Ca(2+) transfer to sustain cell bioenergetics, Biochim. Biophys. Acta 1832 (2013) 495–508.
[77] S. Ohtsuki, H. Yamaguchi, Y.S. Kang, S. Hori, T. Terasaki, Reduction of L-type amino acid transporter 1 mRNA expression in brain capillaries in a mouse model of Parkinson's disease, Biol. Pharm. Bull. 33 (2010) 1250–1252.
[78] T. Kang, W. Lu, W. Xu, L. Anderson, M. Bacanamwo, W. Thompson, Y.E. Chen, D. Liu, MicroRNA-27 (miR-27) targets prohibitin and impairs adipocyte differentiation and mitochondrial function in human adipose-derived stem cells, J. Biol. Chem. 288 (2013) 34394–34402.
[79] S. Das, D. Bedja, N. Campbell, B. Dunkerly, V. Chenna, A. Maitra, C. Steenbergen, miR-181c regulates the mitochondrial genome, bioenergetics, and propensity for heart failure in vivo, PLoS One 9 (2014) e96820.