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Organophosphate Pesticide Chlorpyrifos Impairs STAT1 Signaling to Induce Dopaminergic Neurotoxicity: Implications for mitochondria mediated oxidative stress signaling events

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Abstract

The organophosphate (OP) pesticide chlorpyrifos (CPF), used in agricultural settings, induces developmental and neurological impairments. Recent studies using *in vitro* cell culture models have reported CPF exposure to have a positive association with mitochondria-mediated oxidative stress response and dopaminergic cell death; however, the mechanism by which mitochondrial reactive oxygen species (ROS) contribute to dopaminergic cell death remains unclear. Therefore, we hypothesized that STAT1, a transcription factor, causes apoptotic dopaminergic cell death via mitochondria-mediated oxidative stress mechanisms. Here we show that exposure of dopaminergic neuronal cells such as N27 cells (immortalized murine mesencephalic dopaminergic cells) to CPF resulted in a dose-dependent increase in apoptotic cell death as measured by MTS assay and DNA fragmentation. Similar effects were observed in CPF-treated human dopaminergic neuronal cells (LUHMES cells), with an associated increase in mitochondrial dysfunction. Moreover, CPF (10 μ M) induced time-dependent increase in STAT1 activation coincided with the collapse of mitochondrial transmembrane potential, increase in ROS generation, proteolytic cleavage of protein kinase C delta (PKC δ), inhibition of the mitochondrial basal oxygen consumption rate (OCR), with a concomitant reduction in ATP-linked OCR and reserve capacity, increase in Bax/Bcl-2 ratio and enhancement of autophagy. Additionally, by chromatin immunoprecipitation (ChIP), we demonstrated that STAT1 bound to a putative regulatory sequence in the NOX1 and Bax promoter regions in response to CPF in N27 cells. Interestingly, overexpression of non-phosphorylatable STAT1 mutants (STAT1Y701F and STAT1S727A) but not STAT1 WT construct

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Conflict of interest

The authors declare that they have no conflict of interest.

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attenuated the cleavage of PKC δ and ultimately cell death in CPF-treated cells. Furthermore, small interfering RNA knockdown demonstrated STAT1 to be a critical regulator of autophagy and mitochondria-mediated proapoptotic cell signaling events after CPF treatment in N27 cells. Finally, oral administration of CPF (5mg/kg) in postnatal rats (PNDs 27–61) induced motor deficits, and nigrostriatal dopaminergic neurodegeneration with a concomitant induction of STAT1-dependent proapoptotic cell signaling events. Conversely, co-treatment with mitoapocynin (a mitochondrially-targeted antioxidant) and CPF rescued motor deficits, and restored dopaminergic neuronal survival via abrogation of STAT1-dependent proapoptotic cell signaling events. Taken together, our study identifies a novel mechanism by which STAT1 regulates mitochondria-mediated oxidative stress response, PKC δ activation and autophagy. In this context, the phosphorylation of Tyrosine 701 and Serine 727 in STAT1 was found to be essential for PKC δ cleavage. By attenuating mitochondrial-derived ROS, mitoapocynin may have therapeutic applications for reversing CPF-induced dopaminergic neurotoxicity and associated neurobehavioral deficits as well as neurodegenerative diseases.

Keywords

Chlorpyrifos; mito-apocynin; neurotoxicity; oxidative stress, STAT1; mitochondrial bioenergetics; autophagy

INTRODUCTION

Organophosphates (OPs), such as chlorpyrifos (CPF), are among the most commonly used broad spectrum pesticides worldwide. CPF is extensively used for pest control in agricultural settings, flea treatments and in residential settings. It has been placed under increased restriction in the United States due to increased risk of developmental neurotoxicity (Slotkin et al., 2005; U.S.EPA, 2002). In animals and humans, the primary mode of action is related to the inhibition of the enzyme acetylcholinesterase (AChE), resulting in high levels of acetylcholine in the peripheral and central nervous system, thereby leading to cholinergic neurotoxicity via overstimulation of postsynaptic nicotinic and muscarinic receptors (Slotkin et al., 2005). Epidemiological studies alone have failed to provide conclusive evidence regarding whether chronic low-level exposures to OPs result in persistent neurological deficits in occupationally exposed humans (Banks and Lein, 2012; Kamel and Hoppin, 2004). Therefore, the limitations of epidemiological studies highlight the need to implement improved cell culture and animal models that predict the potential toxic effects of low-dose exposure for prolonged periods of time (Samsam et al., 2005). Emerging evidence suggests a positive association between OP pesticide exposure and symptoms of Parkinsonism (Gorell et al., 1998; Wang et al., 2014). Animal studies have shown selective effects on the nigrostriatal dopaminergic system with other OP pesticides, including dichlorvos (Ali et al., 1980) and heptachlor (Miller et al., 1999). These data suggest that the nigral dopaminergic system could serve as a potential target for pesticides, given that a marked reduction in striatal dopamine metabolism was evidenced (Moreno et al., 2008).

Parkinson's disease (PD) is a neurodegenerative disorder, characterized by a progressive loss of dopaminergic neurons, that is often manifested by tremor, rigidity, motor dysfunction and

cognitive impairment. Though the etiopathology of PD is unknown, numerous reports implicate pesticide-induced oxidative stress in the pathogenesis of Parkinson's disease progression. Several studies had reported that CPF induces excessive free radical production by disrupting mitochondrial electron transport chain (ETC) complex 1 activity while depleting the antioxidant defenses necessary for scavenging free radicals (Hroudova et al., 2011; Khambay and Jewess, 2000; Lee et al., 2012b). For example, in a recent study, exposure of rats to CPF was found to be associated with elevated levels of various markers of free-radical-associated oxidative damage including TBARS (a lipid peroxidation product), DNA damage, and depletion of oxidative-stress-susceptible TH⁺ neurons (Abolaji et al., 2017; Lee et al., 2012b). Interestingly, CPF-induced ROS generation also modulates various signaling pathways including MAP Kinase and NF- κ B, which further regulate downstream signaling pathways associated with cell death (Zhang et al., 2015). Consistent with a role for ROS in the mechanism of dopaminergic neurodegeneration, N-acetyl cysteine (NAC) (an antioxidant inhibitor) attenuated CPF-induced dopaminergic neuronal death (Lee et al., 2012b). Taken together, excessive free radical generation and subsequent activation of oxidative stress-induced signaling events might regulate CPF-induced dopaminergic neuronal cell death.

JAK-STAT signaling plays a major role in both neuronal survival and cell death. STAT1 activation has been reported in various neurological pathological events that lead to cell death (Dedoni et al., 2010; Takagi et al., 2002a). Treatment with IFN- β , Paraquat, MPP⁺ activated STAT1 either directly via JAK1/2-dependent or indirectly via ROS and p38 MAPK kinase-dependent mechanism in neuronal cells (Dedoni et al., 2010; Ihle, 2001; Junyent et al., 2010; Kim and Lee, 2005; Mangano et al., 2012; Ramsauer et al., 2002; Simon et al., 1998). Upon activation, STAT1 is phosphorylated on Tyrosine 701 and Serine 727 residue, thereby inducing its dimerization and subsequent translocation to the nucleus, where STAT1 binds to GAS/ISRE elements present on the promoter region of genes that regulate pro-inflammatory cytokines, NOX (an NADPH oxidase subunit), apoptosis and cell cycle arrest regulators, namely caspases, Fas and Bax (Kim and Lee, 2007; Manea et al., 2010; McBride and Reich, 2003; Soond et al., 2007). STAT1 has been shown to regulate cell death by both the transcriptional-dependent expression of proapoptotic genes and via the non-transcriptional signaling pathway (Kim and Lee, 2007). However, the role of STAT1 and its downstream signaling pathways in regulating CPF-induced cell death in neuronal cells remains poorly understood.

Protein kinase C delta (PKC δ), a redox-sensitive serine threonine kinase and a member of the novel PKC isoform family, is highly expressed in various brain cell types. Previously, our lab has demonstrated the role of PKC δ in modulating the effects of various neurotoxicants, including pesticide-induced dopaminergic neuronal cell death (Harischandra et al., 2014; Jin et al., 2014; Kanthasamy et al., 2006; Kanthasamy et al., 2003b; Latchoumycandane et al., 2011; Latchoumycandane et al., 2005). Oxidative stress-induced activation of caspases and subsequent caspase-3-dependent cleavage and activation of PKC δ kinase activity has been associated with DNA fragmentation and dopaminergic neuronal cell death (Kanthasamy et al., 2010; Kanthasamy et al., 2003a; Kanthasamy et al., 2003b; Kitazawa et al., 2003). CPF has been reported to induce ROS-dependent mitochondrial depolarization, followed by cytochrome c release and caspase 9 and 3 activation (Lee et al.,

2012b); however, it is unclear whether PKC δ plays a major role as a downstream signaling molecule during CPF-induced dopaminergic neuronal cell apoptosis.

Despite a wealth of evidence supporting a positive association between pesticide exposure and the etiology of PD the consequence of specific pesticide exposure and risk of developing PD has not been well studied. Here we explore the mechanism(s) by which CPF-induce dopaminergic neurotoxicity and to what extent antioxidant treatment modulate drug-induced neurotoxicity. In addition, we also assessed whether repeated exposure of young rats to CPF-induce dopaminergic neurotoxicity and whether mitoapocynin, a mitochondria targeted antioxidant alters the vulnerability of dopaminergic neurons to STAT1-mediated proapoptotic cell signaling events and resultant neurobehavioral deficits.

METHODS

Chemicals and Reagents

Chlorpyrifos (CPF, C₉H₁₁Cl₃NO₃PS) was received from Chem Service, Inc., West Chester, PA (cat #S-11459A1). DMSO, glutathione S-transferase and, BHT (3,5-di-tert-butyl-4-hydroxytoluene) were purchased from Sigma (St. Louis, MO, USA). z-VAD-FMK, Z-DEVD-FMK, and Ac-LEHD-AMC were obtained from Cayman chemicals (Ann Arbor, Michigan). Chloromethyl-2',7'-dichlorofluorescein diacetate (CM-H2DCFDA), JC-1 dye, RPMI 1640, minimal essential medium (MEM), fetal bovine serum, L-glutamine, penicillin and streptomycin were purchased from Invitrogen (Gaithersburg, MD). Antibodies against p-Y701 STAT1, p-S727 STAT1, STAT1, PARP, caspase-3, LC3B, p62 and beclin1 were from Cell Signaling Technology (Danvers, MA); anti-NOX-1 and anti-alpha-synuclein (phospho S129) antibodies were purchased from Abcam (Cambridge, MA); and anti-PKC δ , anti-tubulin, anti-Bcl-2, and anti-Bax antibodies were purchased from Santa Cruz Biotech (Dallas, TX). Rat/Mouse Cytochrome c Quantikine ELISA kit was purchased for R&D Systems (Minneapolis, MN). Halt protease and phosphatase inhibitor cocktail (100 x) and SYTOX green dye were purchased from Thermo Fisher Scientific (Waltham, MA). The Cell Death Detection ELISA^{plus} kit was purchased from Roche Molecular Biochemicals (Indianapolis, IN). N-acetyl cysteine (NAC) and TH antibody were purchased from Calbiochem/EMD Biosciences (Gibbstown, NJ).

Cell culture

The immortalized rat mesencephalic dopaminergic neuronal cell line (N27) was provided by Dr. K. N. Prasad, University of Colorado Health Sciences Center (Denver, CO). N27 cells were grown in RPMI 1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine, 50 units penicillin, and 50 μ g/ml streptomycin and maintained at 37°C in a humidified atmosphere consisting of 5% CO₂/95% air. The media was replaced at least every two days.

Undifferentiated LUHMES cells were cultured as described in our previous publication (Sarkar et al., 2017). Briefly, cells were propagated in Advanced DMEM/F-12 supplemented with 1 x N-2 supplement (Invitrogen), 2 mM L-glutamine, and 40 ng/ml recombinant basic FGF (Invitrogen) on plastic flasks or multi-well plates pre-coated with 50 μ g/ml poly-L-ornithine and 1 μ g/ml fibronectin. Differentiation of LUHMES cells was initiated by the

addition of differentiation medium containing Advanced DMEM/F-12, 1 x N-2 supplement, 2 mM L- glutamine, 1 mM dibutyl cAMP (Invitrogen), 1 µg/ml tetracycline (Invitrogen), and 2 ng/ml recombinant human glial cell line-derived neurotrophic factor (Invitrogen). After 2 days, cells were trypsinized and seeded onto multi-well plates at a cell density of 1.5×10^5 cells/cm². LUHMES cells differentiate into a dopaminergic phenotype after an additional 3-day culture in differentiation medium. Post differentiation, cells were treated with CPF for the desired time period.

Western blotting

The cells were collected and washed once with ice-cold PBS and resuspended in modified RIPA lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 100 mM NaCl, 0.1% SDS, 1 x Halt protease and phosphatase inhibitor cocktail, 1 mM PMSF, 1 mM sodium orthovanadate, 1 µg/ml leupeptin, 1 µg/ml pepstatin, and 10 µg/ml aprotinin, for 10 minutes. The lysates were further sonicated in ice-cold water for 4 minutes and then centrifuged at 16,000×g for 15 minutes at 4°C. Supernatant was collected and protein concentration was quantified using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific) or Bradford reagent (Bio-Rad). Equal amount of sample was resuspended in 1x SDS loading dye, boiled at 100°C for 5 minutes, loaded on a 10, 12.5 or 15% SDS-PAGE gel, and resolved electrophoretically at 100V for 1.5 h. After electrophoresis, proteins were transferred to a nitrocellulose membrane at 100V for 2 h. Non-specific binding sites on the membrane were blocked by incubating the membrane in 5% BSA solution for 1 h at room temperature. The membranes were probed with the indicated primary antibody (1: 1000), washed three times for 5 minutes each, followed by incubation with a secondary fluorescence labeled antibody (1:7500). The antibody-bound proteins were detected by LI-COR Odyssey classic imaging system. Band intensity was analyzed using ImageJ software.

Immunocytochemistry

N27 or LUHMES cells were plated on poly-D-lysine (20 µg/ml)-coated glass cover slips at initial seeding density of 40×10^3 and 200,000 cells, respectively. After treatment, the cells were gently washed with PBS and fixed in 4% paraformaldehyde for 10 minutes. The cells were washed three times with PBS for 5 min each time and blocked with blocking reagent (0.4% BSA, 5% donkey serum, and 0.2% Triton-X 100 in PBS) for 30 min. Cells were incubated with the primary antibodies against TH (1:2500), p-STAT1 Y701 (1:300), p-STAT1 S727 (1:300) or LC3B (1:500) overnight at 4°C and then washed 4 times for 5 minutes each with PBS. Cells were then incubated with Alexafluor-conjugated donkey anti-rabbit/mouse secondary antibody (1:1000) at RT for 60–90 min. After another four 8-min washes in PBS, the cells were counterstained with Hoechst 33342 (final concentration of 10 µg/ml in PBS) at RT for 4 min. Finally, cells were washed once in PBS and mounted on a glass slide with Fluoroguard antifade mounting medium (Sigma). The cells were observed under a Nikon inverted fluorescence microscope (Model TE-2000U) and pictures were captured with a SPOT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI) with 60× magnification lens. Confocal imaging was performed at the Iowa State University Microscopy Facility using a Leica DMEIR2 confocal microscope with 63X oil objective.

Transfection/Electroporation

Electroporation of small interfering RNAs (siRNAs) was conducted as per manufacturer's instruction by using a Nucleofector device and the Cell line Nucleofector kit (Cat # VCA-1003, Lonza, Walkersville, MD). STAT1-, Map1LC3- and PKC δ -specific siRNAs (Silencer Select ID #sl29043, #sl34506, and #s71696, respectively) as well as the scrambled negative control siRNA (Cat #AM4611) were purchased from Ambion/ThermoFisher Scientific (Austin, TX). Plasmids including eGFP empty vector, eGFP-WT STAT1, eGFP-STAT1^{Y701F} and eGFP-STAT1^{S727A} were obtained from Addgene (Cambridge, MA). Three million of N27 cells were resuspended in 100 μ l of the electroporation buffer supplied with the kit, along with 1.5 μ g of gene-specific siRNA or scrambled negative siRNA. The cells were then electroporated using the nucleofector program #A23. Following electroporation, the cells were immediately transferred to pre-warmed culture medium and subsequently plated for 48 h.

Caspase-3 activity assay

Caspase-3 activity was determined as previously described (Kaul et al., 2003). Briefly, N27 cells (400,000 per well) were seeded on 6-well plates. After treatment, cells were lysed in a buffer containing 50mM Tris-HCl (pH 7.4), 1 mM EDTA, and 10 mM EGTA, 10 μ M digitonin and 1x protease-phosphatase inhibitor for 15 min at 37°C. Cell lysates were centrifuged at 900 $\times$ g for 5 min and the supernatants were incubated with a caspase-3 specific fluorogenic substrate (Ac-DEVD-AMC) at 37°C for 1 h. Caspase activity (cleaved substrate) was measured fluorometrically using the SpectramaxTM microplate reader (Molecular Devices Corp., Sunnyvale, CA) with excitation and emission wavelengths of 380 nm of 460 nm, respectively. Caspase-3 activity was expressed as percentage of control (fluorescence units per milligram of protein per hour).

ROS generation assay

As described in our previous publications (Latchoumycandane et al., 2011; Lawana et al., 2017), intracellular ROS generations were measured by using chloromethyl-2',7'-dichlorofluorescein diacetate (CM-H2DCFDA). Briefly, cells were plated in 96-well plates at the seeding density of 40,000 cells/well. After treatment, cells were washed with PBS and further incubated with 10 μ M CM-H2DCFDA dye at 37°C for 45 minutes in the dark. The cells were washed twice with PBS to remove extracellular dye. The formation of the fluorescent product dichlorofluorescein (DCF) was analyzed fluorometrically using a SpectramaxTM microplate reader at excitation and emission wavelengths of 488 nm and 525 nm, respectively.

GSH assay

N27 cells were plated at the initial density of 1×10^6 cells/T-25 flask. After treatment, the cells were suspended in the cell lysis buffer containing 50 mM Tris, pH 7.4, 5 mM EDTA and 0.001% BHT and indirectly sonicated in the ice-cold water. Cell lysate were centrifuged at 15,000 $\times$ g for 10 minutes at 4°C. The reaction was started at RT by adding 1 mM of monochlorobimane and 10U/mL of glutathione S-transferase to the cell lysate supernatant.

After 30 minutes, the change in the fluorescence of samples was measured using a Spectramax™ microplate reader with excitation and emission wavelengths of 480 nm and 645 nm, respectively. The results were presented as percentage of control.

MTS assay

Cell viability was determined using the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega) as per manufacturer's instructions. N27 cells were plated in a 96-well plate at an initial seeding density of approximately 20,000 cells per well. The cells were treated with CPF for another 24 h. After treatment, cells were incubated with MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) for 1 h at 37°C. The amount of soluble formazan, an indicator of cell viability, was quantified spectrophotometrically at 490 nm.

DNA fragmentation assay

Twenty-four hour post treatment with CPF, N27 cells were collected and lysed in 300 µl of cell lysis buffer for 30 min at RT and subsequently spun down at 200×g for 10 min. The supernatant was then used to measure DNA fragmentation using the Cell Death Detection ELISA^{plus} kit as per the manufacture's protocol or as previously described (Anantharam et al., 2002). The extent of DNA fragmentation was measured at 405 nm using a Spectramax™ microplate spectrophotometer.

Mitochondrial membrane potential measurement

The changes in mitochondrial membrane potential were monitored by 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolcarbocyanineiodide (JC-1) staining. The green fluorescence emitted by monomeric form of JC-1 dye in the cytosol was detected fluorometrically by excitation/emission wavelengths 485/535 nm and the red fluorescence emitted by concentrated aggregates in healthy mitochondria was detected at excitation/emission wavelengths of 550/600 nm using a Spectramax™ microplate reader. The changes in mitochondrial membrane potential were calculated as the red/green ratio and expressed as percentage of control.

Chromatin immunoprecipitation (ChIP)

The ChIP assays were performed using a CFQP-TT Express Enzymatic kit (Active Motif) as per the manufacturer's recommendations. Briefly, $\sim 1 \times 10^7$ N27 cells were treated with or without CPF (50 µM) for 4 and 6 h followed by cross-linking with 1% formaldehyde in complete media for 15 min with gentle rocking at RT. Cells were then washed twice with ice-cold PBS and treated with glycine solution for 5 min to stop the cross-linking reaction. Cells were lysed in buffer containing SDS and protease inhibitor mixture. Chromatin was enzymatically digested to 200–1500 bp fragments (verified through running on a 1% agarose gel) by incubation with the enzymatic shearing cocktail for 12 min at 37°C. The sheared chromatin (7 µg) was used to set up immunoprecipitation reactions containing protein G magnetic beads along with 2 µg STAT1 antibody or mouse IgG. Equal amount of chromatin was set aside to be used as an input control. After extensive washing of the beads, reversal of cross-links, and proteinase K digestion, the eluted DNA in the immunoprecipitated samples

was analyzed by PCR using the primers spanning the binding sites of the specified transcription factors on the murine *NOX-1* and Bax promoter region. The primer sequences used to amplify the Bax promoter region between –322 to –21 relative to the transcription initiation site as predicted by MatInspector software were: forward 5'-TCCTGGCTGGCTTTGAGTTT-3' and reverse 5'-GCCCCGCAGAACTAGTCACS'. The primer sequences used to amplify the NOX-1 promoter region between –254 to –90 relative to the transcription initiation site (Decressac et al.) as predicted by MatInspector software were: forward 5'-TCTCAGCCTAATTACGTGGTCT-3' and reverse 5'-AAGCCCTGCCTATGACAACC-3'. Conditions of linear amplification were determined empirically for the primers. PCR conditions are as follows: 94°C 3 min; 94°C 30 sec, 59°C 30 sec, and 72°C 30 sec for 35 cycles, 72°C for 5 min. The PCR products were resolved by electrophoresis in a 1.0% agarose gel and visualized after ethidium bromide staining.

ATP production measurement

Intracellular ATP levels were measured using the CellTiter Glo® Luminescent Cell Viability assay kit, according to the manufacturer's instructions (Promega). Briefly, N27 cells (50×10^3 cells/well) were seeded in a 96-well cell culture plate. At the end of the incubation period, the media was removed and equal volume of CellTiter Glo® Reagent was added to the individual wells. The plate was put on the orbital shaker for 2 minutes to ensure cell lysis and further incubated for another 10 minutes to stabilize the luminescence signal. The luminescence was recorded by setting the integration time to around 0.25–1 s per well using a BioTek synergy 2 multimode plate reader. The luminescence intensities were normalized to the total protein content and expressed as percentage of control group.

Measurement of oxygen consumption rate

Mitochondrial bioenergetics profile via OCR measurements were performed using the Seahorse XF24 Extracellular Flux analyzer (Agilent, Santa Clara, CA), as described previously (Pike Winer and Wu, 2014). Briefly, N27 cells (20,000/well) were plated into XF24 polystyrene cell culture plates (Seahorse Bioscience, North Billerica) and incubated for another 24 h in an incubator with 5% CO₂, humidified at 37°C. Sensor cartridges were hydrated overnight in the non-CO₂ incubator. After treating with 10 µM CPF for 12 h, the cells were washed with prewarmed bicarbonate-free, XF cell mitostress assay medium containing 10 mM glucose, 1 mM sodium pyruvate and 2 mM L-glutamine. Cell plates were incubated at 37°C in a non-CO₂ incubator for 60 minutes prior to the start of the assay. Meanwhile, sensor cartridge was loaded with oligomycin (1 µM), FCCP (1 µM), rotenone (0.5 µM) and antimycin A (0.5 µM) in the respective ports and kept in the instrument for calibration. Prior to the start of the experiment, the cell culture lid was exchanged with sensor cartridge. The instrument took 3 baseline measurements, referred to as baseline OCR, followed by sequential addition of mitostressors as indicated above. OCR was expressed as pmoles/min and normalized by per mg protein.

Animals and Treatments

Male and female SD rats were purchased from Charles River Laboratories, the pups weaned from mothers on postnatal day (PND) 22 were acclimatized 1–2 rats per cage under 12h light/dark standard conditions with constant temperature ($22 \pm 1^\circ\text{C}$) and humidity (relative,

30%), with free access to food, water and enrichments as approved by IACUC at Iowa State University (Ames, IA). Both male and female rats were randomly grouped into four groups with 6–8 rats per group (equal male to female ratio). CPF was dissolved in corn oil and administered daily by oral gavage to rats at a dose of 5 mg/kg/day beginning at PND 27 through PND 61. We chose the dosage of CPF (5 mg/kg) because it has been used in other developmental studies (Aldridge et al., 2005; Auman et al., 2000; Carr et al., 2011; Dam et al., 2000; Meyer et al., 2005; Slotkin et al., 2005). The assigned groups included control (corn oil), mitoapocynin (mito-apo, three times per week for 5 weeks), CPF, and mito-apo + CPF. The dose of mitoapocynin used in the present study was similar to our previous publication (Langley et al., 2017). At the end of the treatment period (24 h after last dose), rats were subjected to behavioral, neurochemical and biochemical studies.

Behavior analysis

Animals were subjected to locomotor behavioral analysis using an automated device (Model RXYZCM-16; Accuscan Columbus, OH, USA). Rats were kept in activity chamber (size- 40 X 40 X 30.5 cm) with Plexiglas lid with holes for ventilation for 10 min test sessions. Data were collected and analyzed by a VersaMax analyzer (Model CDA-8, AccuScan) and were processed using statistical tools listed below.

HPLC analysis

Brains were harvested and different brain regions were collected and stored as described previously (Ghosh et al., 2012; Ghosh et al., 2016). Striatal tissues were lysed for extraction of neurotransmitters using an antioxidant lysis buffer (100 μ M Perchloric acid, 0.05% EDTA and 0.1% $\text{Na}_2\text{S}_2\text{O}_5$) and isopropanol (as an internal standard). Using a reversed-phase column high-performance liquid chromatography, dopamine (DA) and 3,4-dihydroxyphenyl acetic acid (DOPAC) were separated at the flow rate of 0.6 ml/min. A semi-automatic system equipped with temperature control refrigerator (ESA Inc, Bedford, MA, USA) was used to perform the analysis. Data were collected and were normalized to tissue weight for each sample.

Statistical analysis

Results were expressed as mean $\pm$ standard deviation (SD) or mean $\pm$ standard error or mean (SEM). The statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA). *p*-values were determined by one way ANOVA followed by post hoc Tukey test. *p*-values < 0.05 were considered statistically significant.

RESULTS

Chlorpyrifos induces a dose-dependent increase in cell death in both N27 dopaminergic neuronal cell line and human mesencephalic (LUHMES) cells

We first investigated the neurotoxic effects of CPF in N27 cells, a well-characterized dopaminergic neuronal cell culture model. N27 cells are an immortalized dopaminergic cell line isolated from fetal rat mesencephalic cultures that express dopamine transporter (DAT) and tyrosine hydroxylase (TH) as well as DA generation (Adams et al., 1996). We and others have routinely used this cell line as a model system to study mitochondrial oxidative stress-

mediated cell death signaling events in response to numerous stressors including dieldrin, manganese (Mn), and paraquat (Anantharam et al., 2002; Kitazawa et al., 2001; Kitazawa et al., 2003; Latchoumycandane et al., 2005; Peng et al., 2005). In the initial set of experiments, we determined the optimal concentration of CPF that causes dopaminergic cell death. N27 dopaminergic neuronal cells were exposed to increasing concentrations of CPF (100 nM-100 μ M) for 24 h. Assessment of cell viability using the MTS assay revealed a dose-dependent reduction in cell viability (Fig. 1A), falling by approximately 50% and 70% of control levels at 50 and 100 μ M, respectively. To further assess CPF-induced apoptotic cell death, we performed DNA fragmentation analysis. For this purpose, N27 cells were exposed to increasing concentrations (300 nM-300 μ M) of CPF for either 24 h (Fig. 1B) or 48 h (Sup. Fig 1 A) and cell death was assessed using DNA fragmentation analysis. CPF induced a dose-dependent increase in DNA fragmentation, confirming other reports that CPF induces neuronal cell death (Caughlan et al., 2004a; Park et al., 2013b). We found that exposure of N27 cells to 100 and 300 μ M CPF-induced maximal cell death. In the present study we implemented 10 μ M of CPF for our mechanistic studies owing to the fact that this is the lowest concentration that induces a significant increase in apoptotic cell death in N27 dopaminergic cells. Furthermore, this concentration has been shown to mimic the *in vivo* concentration reached following organophosphate exposure (Buratti et al., 2007). These results are consistent with previous reports showing CPF causes abnormalities in mitochondrial transport and organellar movement at 10 μ M in cortical neurons in culture (Caughlan et al., 2004b; Middlemore-Risher et al., 2011). Thus, our studies suggest that exposure of N27 dopaminergic neuronal cells to low levels of CPF may perturb mitochondrial function via an oxidative stress mechanism. In the published literature, 50–150 μ M CPF has been routinely used to evaluate the mechanisms underlying CPF-induced neurotoxicity (Crompton et al., 2000; Dam et al., 1999; Lee et al., 2014; Park et al., 2013a; Roy et al., 1998). In this context, micromolar CPF levels have been detected in the blood of human newborn babies living in an agricultural community (Huen et al., 2012). Therefore, studies utilizing micromolar concentrations of CPF *in vitro* could provide novel insights into the biological basis of neurodegenerative effects in a living system.

Given the limited availability of mesencephalic primary dopaminergic neurons in culture, we further established CPF-induced dopaminergic neurotoxicity using Lund human mesencephalic (LUHMES) cells. LUHMES cells exhibit extensive neurites and become postmitotic neuron-like cells upon differentiation. These cells express the three dopaminergic neuronal markers TH, DAT, and Nurr 1 (Zhang et al., 2014). As shown in Fig. 1C, incubation of LUHMES cells with increasing concentration (10 nM-100 μ M) of CPF for 24 h resulted in a dose-dependent increase in apoptotic cell death as assessed via DNA fragmentation analysis. The IC₅₀ for inducing DNA fragmentation was deduced to be 1.34 μ M for LUHMES cells. Moreover, CPF at 300 nM not only caused neuritic shortening (Fig. 1D) but also induced markers of cell death including ROS generation, loss of MMP, and caspase-3 activation in LUHMES cells (Sup. Fig. 1). To our knowledge, this is the first evidence that CPF causes apoptotic cell death of LUHMES cells at such a low concentration (300 nM). Consistent with our report a recent study (Tong et al., 2017) demonstrated that of the three cell lines tested namely LUHMES, NSCs, SH-SY5Y cells, LUHMES cells were found to exhibit greater cytotoxicity sensitivity given that they express low levels of Bcl-2

and survivin, while SH-SY5Y cells exhibited resistance to apoptosis owing to increased expression of Bcl-2. Thus, our studies raise the possibility that LUHMES cells are well suited for neurotoxicity-cytotoxicity screening and that low levels of expression of Bcl-2 may explain the increased vulnerability of LUHMES cells to apoptotic cell death at lower concentrations. Therefore, based on the cell death analysis data, we used a concentration of 10 μ M and 300 nM CPF for N27 cells and LUHMES cells, respectively for the remainder of the studies. Furthermore, these concentrations are within the toxicologically relevant range that has been shown to elicit neurodegenerative effects.

It has been postulated that pesticides contribute to death of dopaminergic neurons via upregulation of various caspases including caspase-3 (Lee et al., 2012b). Therefore, we examined caspase-3 activation, which serves a key role in apoptosis execution (Vaughan et al., 2002), and the cleavage of poly (ADP-ribose) polymerase (PARP), which is a well-established substrate of activated caspase-3 (Decker and Muller, 2002). N27 cells were exposed to CPF (10 μ M) for the indicated time periods, and at the end of the incubation period caspase-3 activation and PARP cleavage were determined using fluorometric plate reader analysis and WB analysis, respectively. As shown in Fig. 1E, CPF exposure resulted in a time-dependent increase in caspase-3 activation with significant activation evidenced as early as 6 h. Similarly, CPF increased the proteolytic cleavage of PARP in a time-dependent manner, with maximal activation evidenced at 24 h (Fig. 1F). To further clarify the role of caspase in CPF-induced cytotoxicity, N27 cells were pre-treated with pan caspase inhibitor z-VAD- FMK (100 μ M) as well as caspase-3 inhibitor z-DEVD- FMK (50 μ M) for 1 h followed by treatment with 10 μ M CPF for another 24 h (Fig. 1G). Assessment of cell viability revealed that caspase inhibition conferred significant protection against CPF-induced cytotoxicity. In particular, caspase-3 inhibition reduced apoptosis by ~45%. Our results confirm the susceptibility of dopaminergic neurons to apoptotic cell death following CPF exposure. Taken together, these results indicate that CPF induced apoptotic cell death at least in part via caspase-3 activation and proteolytic cleavage of PARP.

CPF contributes to oxidative stress by upregulating ROS generation and downregulating GSH levels in N27 cells

Reactive oxygen species (ROS) mediated oxidative stress has been identified as an initiator event in the inducing of neuronal cell death in several pesticides (Lee et al., 2012b). For example, in a previous study, we demonstrated that dieldrin induces free radical generation and that pretreatment with antioxidants such as superoxide dismutase (SOD) attenuated oxidative stress-associated dopaminergic neuronal cell death (Kitazawa et al., 2001). To examine the involvement of ROS generation in CPF-induced cytotoxicity, N27 cells were treated with CPF for the indicated time period and ROS generation was examined using the redox-sensitive CM- H2DCFDA dye. CPF treatment induced significant ($p < 0.05$) ROS generation as early as 12 h post-treatment, with a persistent increase in ROS generation evidenced thereafter (Fig. 2A). Conversely, an early increase (6 h) in mitochondria-derived superoxides, which remained elevated for the remainder of the treatment duration, was evidenced in CPF-treated N27 cells (Fig. 2B). Oxidative stress arises when free radical generation exceeds anti-oxidant defense in the cell (Mittler, 2002). Glutathione (GSH), a major intracellular antioxidant, protects the cells against oxidative stress-induced

cytotoxicity (Mittler, 2002; Schulz et al., 2000). Pesticides have been reported to induce apoptotic cell death by depleting intracellular GSH (Coleman et al., 2012). To examine the effect of CPF on GSH levels, N27 cells were treated with 10 μ M CPF for various amounts of time and GSH levels were determined. CPF induced a significant ($p < 0.05$) reduction in GSH levels at 18 h post-CPF treatment (Fig. 2C). To further investigate the functional significance of ROS induction in CPF-induced cytotoxicity, N27 cells were pretreated with 5 mM N-acetyl cysteine (NAC), a free radical scavenger and a precursor of GSH, for 12 h followed by treatment with CPF for another 24 h. Evaluation of the extent of the oxidative stress response revealed that NAC pretreatment significantly attenuated both ROS generation ($p < 0.01$) and CPF-induced cytotoxicity ($p < 0.001$) (Fig. 2C, D). Thus, our data indicate that CPF treatment induces ROS generation with a concomitant decline in GSH levels, hence highlighting the pivotal role of redox mechanisms in initiating dopaminergic cell death.

CPF-induced mitochondrial dysfunction is associated with Bax upregulation and down regulation of Bcl-2 expression

Increased oxidative stress is associated with mitochondrial depolarization. Mitochondrial damage serves as an initial trigger for the induction of intrinsic apoptotic pathways in a variety of neuronal cell types (Kitazawa et al., 2005; Moon et al., 2005). To examine the effect of CPF on mitochondrial membrane potential, we examined the time dependent changes in mitochondrial membrane potential using JC-1 dye. As shown in Fig. 3A, quantification of mitochondrial membrane potential (MMP) using spectrometric plate reader analysis revealed a time-dependent reduction in MMP, whereby CPF induced a significant time-dependent reduction in mitochondrial membrane potential starting at 12 h post-treatment, with maximal reduction (~50%) evidenced at 24 h (Fig. 3A). Thus, these data demonstrate that CPF exposure induces mitochondrial dysfunction via dissipation of MMP.

Bcl-2, a member of the anti-apoptotic Bcl family, plays an important regulatory role in preserving mitochondrial function by blocking activation of the intrinsic, mitochondria-mediated apoptotic pathway by preventing release of proapoptotic mitochondrial proteins, such as cytochrome *c*, into the cytosol. Following exposure to cellular stressors, including DNA-damaging agents and oxidative stressors, proapoptotic proteins such as Bax translocate to the mitochondria, leading to the disruption of the integrity of the mitochondrial outer membrane (MOM), subsequently inducing downstream apoptotic cell death signaling events (Bedner et al., 2000; Park et al., 2013b). To further define the neurotoxic mechanism of CPF, we analyzed the expression of key apoptotic proteins by immunoblotting (Fig. 3B). A time-dependent upregulation of Bax protein levels with a concomitant down regulation of the antiapoptotic protein, Bcl-2, was evidenced in CPF-treated cells (Fig. 3B). Our results are consistent with previous studies demonstrating that dieldrin, an organochlorine pesticide, induced dopaminergic toxicity via downregulation of Bcl-2 expression in PC-12 cells (Kitazawa et al., 2005). Taken together, these results indicate that CPF-induced apoptosis might be mediated at least in part via alteration of the expression levels of apoptosis-related factors, namely Bax and Bcl-2, which might be attributable to mitochondrial dysfunction.

Proteolytic cleavage of PKC δ regulates CPF-induced apoptotic cell death in N27 dopaminergic cells

Protein kinase C delta (PKC δ), a redox-sensitive serine threonine kinase and member of the novel PKC isoform family, is highly expressed in various types of brain cells including dopaminergic neuronal cells (Leibersperger et al., 1991). PKC δ -dependent cell signaling events have been shown to play a neurotoxic role under oxidative stress conditions. Furthermore, caspase-3-dependent proteolytic cleavage and activation of PKC δ kinase activity has been shown to mediate apoptotic cell death induced by a variety of neurotoxicants including dieldrin, Mn, and MPP⁺ in N27 cells, indicating a positive association between PKC δ activation and apoptotic cell death (Kanthasamy et al., 2010; Kanthasamy et al., 2003a; Kanthasamy et al., 2003b; Kitazawa et al., 2003; Latchoumycandane et al., 2005). Therefore, we next investigated whether PKC δ activation could regulate CPF-induced cell death in N27 dopaminergic cells by determining the time-dependent proteolytic activation of PKC δ in N27 cells. Our results demonstrated that CPF induced a significant time-dependent increase in the proteolytic cleavage of PKC δ (41–38 kDa fragments) (Fig. 3C). Given that prolonged exposure (18h) to CPF resulted in a marked increase (~66%) in the magnitude of cleavage fragments of PKC δ , we evaluated whether this activation contributes to cytotoxicity in CPF-treated N27 cells. For this purpose, N27 cells were transfected with scrambled or PKC δ siRNA and exposed to either CPF (10 μ M) or vehicle for 24 h and cytotoxicity was determined via DNA fragmentation assay. PKC δ knockdown inhibited CPF-accentuated DNA fragmentation by ~50% as compared to scrambled-transfected N27 cells, further confirming the proapoptotic role of PKC δ in regulating CPF-induced dopaminergic neurotoxicity (Fig. 3D). Next, to further examine whether caspase-3-dependent proteolytic cleavage of PKC δ mediates CPF-induced neuronal toxicity in N27 cells, we used cells either stably expressing PKC δ mutant (PKC δ^{D327A}) (which is insensitive to proteolytic cleavage by caspase-3) or LacZ (Lu et al., 2007). As anticipated, CPF-induced apoptotic cell death was attenuated in N27 cells stably overexpressing PKC δ^{D327A} mutant as compared with LacZ-expressing cells (Fig. 3E). Taken together, these results indicate that caspase-3-dependent proteolytic cleavage of PKC δ was partially responsible for CPF-induced dopaminergic cell death.

CPF induces STAT1 phosphorylation-dependent apoptotic cell death in N27 cells

During the course of investigating the mechanisms underlying CPF-induced dopaminergic cell death, we hypothesized that STAT1, whose activation is influenced by PKC δ , might play a role in CPF-induced apoptotic cell death. STAT proteins belong to a family of transcription factors that plays a major role in the regulation of growth, differentiation, and cell death. In particular, STAT1 activation has been evidenced in the ischemic brain (Aneja et al., 2004; Takagi et al., 2002a). In addition, STAT1 has been shown to play a critical role in the generation of ROS, regulation of interferon- γ -induced cytokine signaling, and cellular response (Moriwaki et al., 2006; Takagi et al., 2002a). Upon activation, STAT1 is phosphorylated at either Y701 or S727 (Bromberg and Darnell, 2000). Y701 phosphorylation plays a pivotal role in STAT1 dimerization, nuclear translocation, and DNA binding. On the other hand, S727 phosphorylation potentiates the transcriptional activity of tyrosine phosphorylated STAT1. Alternatively, in other cases it has been shown to be involved in signaling events even in the absence of phosphorylation at the Y701 site

(Bromberg and Darnell, 2000; Decker and Kovarik, 2000; Varinou et al., 2003). To examine STAT1 activation, we determined the temporal profile of STAT1 activation post-CPF treatment. As shown in Fig. 4A, exposure of N27 cells to CPF resulted in STAT1 phosphorylation at both serine 727 and tyrosine 701. Because close parallelism has been demonstrated between phosphorylation of STAT1 and nuclear translocation (McBride and Reich, 2003), p-STAT1 localization following CPF treatment was visualized using fluorescence microscopy. Immunocytochemical (ICC) studies revealed that at 12 h post-CPF treatment, pSTAT1 S727 was localized in the nucleus (red fluorescence) (Fig. 4B), suggesting its role in the transcriptional regulation of proteins linked to oxidative stress and apoptosis, at least in dopaminergic neurons. Taken together, this pattern of increased phosphorylation of STAT1 and nuclear translocation preceding CPF-induced dopaminergic cell death suggests that STAT1 transcriptional activity might at least in part contribute to CPF-induced dopaminergic neurotoxicity. To examine whether CPF treatment induces STAT1 binding to DNA/GAS element, N27 cells were transfected with the STAT1-responsive luciferase construct. Dual luciferase reporter assay demonstrates that CPF treatment as early as 4 h induces a two-fold increase in STAT1 transcription factor binding to the consensus GAS element (Fig. 4C), indicating that STAT1-dependent genes might play a critical role in CPF-induced toxicity.

STAT1 regulates CPF- induced dopaminergic cell death via caspase-3 activation and PARP cleavage

Previous studies have indicated that STAT1 signaling might contribute to neuronal toxicity (Dedoni et al., 2010; Hsu et al., 2014; Kim and Lee, 2007; Schlatterer et al., 2012; Takagi et al., 2002a; West et al., 2004). Therefore, we sought to investigate the functional impact of siRNA-mediated STAT1 knockdown (STAT1 KD) on CPF-induced cytotoxicity in N27 cells. Transfection of N27 cells with STAT1 siRNA for 48 h reduced the basal levels of STAT1 by over 90%, highlighting effective knock down of STAT1 (Fig. 5A). N27 cell were subsequently treated with or without CPF (10 μ M) for 24 h. The results showed that CPF induced apoptotic cell death and that STAT1 KD inhibited CPF-induced DNA fragmentation by 80% as compared to WT scramble-transfected N27 cells treated with CPF, further confirming the role of STAT1 in regulating CPF-induced apoptotic cell death (Fig. 5C). Furthermore, morphological analysis using an inverted microscope revealed that N27 cells displayed a shrunken appearance with a concomitant marked reduction in cell density in CPF-treated cells that were transfected with the scrambled siRNA. In contrast, STAT1 KD reduced CPF-induced cellular damage with a concurrent improvement in the cell density (Fig. 5D). Likewise, CPF- mediated reduction in N27 cell viability and DNA fragmentation was also abrogated by STAT1 KD (Fig. 5B and C). Interestingly, STAT1 has been shown to regulate various caspases, including caspase-3 expression, in response to variety of stimuli in various cell types (Kumar et al., 1997; Sironi and Ouchi, 2004). Because CPF activates the intrinsic apoptotic pathway by upregulating caspase-3, we examined whether STAT1 modulates CPF-induced neuronal cell death by regulating caspase-3 activity. Scrambled and STAT1 siRNA-transfected N27 cells were treated with CPF for 18 h, and caspase-3 activity was determined spectrophotometrically. Exposure of scrambled siRNA transfected N27 cells to CPF (10 μ M) resulted in a marked increase in caspase-3 activity; conversely, STAT1 KD resulted in a near complete blockade of caspase-3 activation (Fig. 5E). Next, we examined

whether STAT1 regulates CPF-induced PARP cleavage. N27 cells transfected with scrambled or STAT1 siRNA were exposed to CPF or vehicle for another 24 h. As anticipated, CPF-induced PARP cleavage decreased markedly upon STAT1 knockdown as compared with scrambled siRNA transfected cells (Fig. 5F). Taken together, our results suggest that STAT1 may at least in part contribute to apoptotic cell death via a caspase-3 mediated PARP cleavage.

STAT1 regulates CPF-induced oxidative stress by regulating NOX-1 and ROS generation

Oxidative stress plays a major role in inducing CPF-mediated apoptotic cell death (Ki et al., 2013; Lee et al., 2012b; Park et al., 2013b). Therefore, we examined whether STAT1 regulates CPF-induced ROS production. To determine whether STAT1 is involved in CPF-induced ROS production, we measured the intracellular levels of ROS in both scrambled and STAT1 siRNA transfected N27 cells, 24 h post-CPF treatment (10 μ M), using ROS-sensitive fluorescent dye CM-H₂DCFDA. The fluorescent intensities, reflecting the levels of intracellular ROS, were measured with a fluorescent plate reader. As shown in Fig. 6A, CPF treatment (10 μ M) induced a 66% reduction in ROS levels in STAT1 KD N27 cells as compared with scrambled siRNA transfected cells exposed to the same drug. NOX-1, an NADPH oxidase subunit, is a major regulator of ROS generation in N27 dopaminergic neuronal cells (Choi et al., 2012). Various neurotoxicants have been reported to induce oxidative stress by upregulating NOX-1 protein levels (Cristovao et al., 2009). Interestingly, STAT1 signaling has been shown to upregulate NOX-1 expression (Kumatori et al., 2002; Manea et al., 2010). Our study indicates that STAT1 KD ablates the induction of ROS following CPF treatment, so we tested the hypothesis that STAT1 may be required for CPF-induced expression of NOX-1. Scrambled and STAT1 siRNA transfected N27 cells were treated with CPF (10 μ M) for 24 h and protein lysates were harvested and immunoblotted for NOX-1 protein expression. As shown in Fig. 6B, STAT1 siRNA inhibited CPF-induced NOX-1 expression and provided further evidence for the role of STAT1 in CPF-induced oxidative stress.

Next, we examined whether STAT1 bound to the NOX-1 promoter region upon CPF stimulation of N27 cells. To address this question, we performed chromatin immunoprecipitation (CHIP) assay in N27 cells exposed to CPF for varying periods of time. For the CHIP assays, we used CPF at a concentration of 50 μ M in order to demonstrate an upper limit of concentration whereby STAT1 exhibited profound transcriptional effects on proapoptotic markers. Using the MatInspector promoter analysis search program, we screened the rat NOX-1 promoter for potential STAT1 binding sites. Using primers spanning the predicted STAT1 binding site on NOX-1 promoter, we performed CHIP on the DNA extracted from N27 cells treated with or without CPF for 4 and 6 h, respectively. As shown in Fig. 6C, CPF treatment increased the recruitment of STAT1 to the endogenous NOX-1 promoter at both 4 and 6 h, respectively, in N27 cells, indicating that NOX-1 is subject to transcriptional regulation by STAT1. Excessive free radical generation often induces oxidative stress by downregulating GSH levels (Schmuck et al., 2002). Because a decrease in GSH levels indicates cellular oxidative stress, we examined the GSH levels in scrambled siRNA transfected and STAT1 KD cells treated with or without CPF for 24 h (Fig. 6D). CPF treatment resulted in 48% reduction in GSH levels in scrambled transfected cells while only

a 22% reduction in GSH levels was evidenced in STAT1 KD N27 cells. Taken together, these results indicate that STAT1 plays a major role in regulating antioxidant GSH levels and ROS generation in a NOX-1 dependent manner in CPF-treated cells.

STAT1 regulates CPF-induced bioenergetics deficits in CPF-treated N27 cells

To further evaluate the role of STAT1 in regulating CPF-induced mitochondrial dysfunction, we used a Seahorse XF24 Analyzer to assess the bioenergetics profile of mitochondria in both scrambled and STAT1 siRNA transfected cells treated with or without CPF for 12 h. By exposing N27 cells to the various mitochondrial toxicants and stressors, namely oligomycin, an ATP-synthase inhibitor, followed by FCCP, a mitochondrial uncoupler, and rotenone/antimycin A, mitochondrial ETC inhibitor, we systematically measured basal oxygen consumption rate (OCR), maximal respiratory capacity, ATP coupled respiration, and spare reserve capacity. As shown in Fig. 7A, the real-time change in OCR levels in WT and STAT1 KD N27 cells following sequential addition of mitochondrial stressors was determined. Interestingly, before addition of oligomycin, we observed slightly elevated levels of basal respiration, indicative of oxidative phosphorylation (OXPHOS) (Huijing and Slater, 1961), in STAT1 KD N27 cells as compared to scrambled siRNA transfected control cells, suggesting that STAT1 might have an inhibitory effect on mitochondrial OXPHOS functioning. At 12 h post-CPF (10 μ M) treatment, CPF induced a significant reduction (~45%) in basal respiration rate in scrambled siRNA transfected N27 cells as compared to a marginal (~10%) reduction in STAT1 KD cells, highlighting the detrimental effect of STAT1 on the mitochondrial bioenergetics profile (Fig. 7B). To determine maximal respiration capacity that cells can attain, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), an uncoupler of mitochondrial oxidative phosphorylation/ETC, was added to stimulate the oxygen consumption in cells necessary for maintaining proton gradient across inter-mitochondrial membranes independent of ATP synthase activity or production. As shown in Fig. 7C, maximal respiration was suppressed by 86% in CPF-treated scrambled siRNA transfected N27 cells; however, STAT1 KD cells showed a near complete restoration of maximal respiration. To further measure the cells' ability to cope with increasing energetic demands, cells were treated with rotenone/antimycin A and spare reserve capacity was quantified. As shown in Fig. 7D, CPF treatment led to a dramatic reduction (~75%) in spare respiratory capacity in scrambled siRNA transfected cells, while only marginal reduction in spare reserved capacity (15%) was observed in drug-treated STAT1 KD cells, indicating that STAT1 is linked to CPF-induced mitochondrial bioenergetic deficits in N27 dopaminergic cells. Because change in oxygen consumption is directly linked to ATP production, we quantified ATP-linked respiration in both scrambled siRNA transfected and STAT1 KD N27 cells. As depicted in the bioenergetics profile (Fig. 7E), post-CPF (10 μ M) treatment, we observed a 56% reduction in ATP production in WT N27 cells, while only a marginal reduction in ATP levels was observed in STAT1 KD cells. To further confirm changes in ATP levels post-CPF treatment, we independently determined ATP levels using the highly sensitive luminescence-based CellTiter Glo® assay (Fig. 7F). Concordant with our Seahorse bioanalyzer experimental results, post-oligomycin treatment, we observed about 60% reduction in ATP levels in scrambled siRNA transfected N27 cells treated with CPF, but only a partial (30%) decline in ATP levels in drug-treated STAT1 KD N27 cells, further confirming that STAT1 is a critical determinant of CPF-induced mitochondrial dysfunction.

STAT1 mediates upregulation of Bax/Bcl-2 ratio and mitochondrial release of cytochrome c in CPF-treated N27 cells

STAT1 has been recently reported to alter mitochondrial biogenesis (Boengler et al., 2010; Sisler et al., 2015); however, whether STAT1 regulates CPF-induced mitochondrial dysfunction remains to be investigated. The role of STAT1 in regulating CPF-induced impairment in mitochondrial function was examined in scrambled transfected and STAT1 siRNA transfected N27 cells exposed to 10 μ M CPF for 24 h. Post-CPF treatment, we observed mitochondrial dysfunction as evidenced by significant depolarization of mitochondrial membrane potential (reduction of 56%) in WT siRNA transfected N27 cells. On the contrary, knock down of STAT1 preserved the mitochondrial membrane potential (Fig. 8A). Given that STAT1 has been reported to modulate proapoptotic Bax levels while downregulating anti-apoptotic Bcl-2 and Bcl-xL levels (Stephanou et al., 2000), we examined the impact of STAT1 KD on CPF-induced upregulation of Bax and Bcl-2 protein levels in N27 cells. As expected, treatment with CPF markedly downregulated Bcl-2 protein levels in scrambled siRNA transfected cells, while Bcl-2 protein levels were minimally altered in STAT1 knockdown N27 cells, suggesting that activated STAT1 negatively regulates Bcl-2 expression levels following treatment with CPF in N27 cells (Fig. 8B). Conversely, CPF treatment (10 μ M) elevated Bax/Bcl-2 ratio by ~ 4 fold in scrambled transfected N27 cells, while STAT1 KD markedly inhibited (~2 fold) CPF-induced Bax/Bcl-2 ratio, indicating a proapoptotic role of STAT1 in CPF-induced dopaminergic neuronal toxicity (Fig. 8B). Additionally, STAT1 has also been reported to induce Bax expression by binding to the putative regulatory sequence in the Bax promoter region. To examine whether STAT1 plays a major role in upregulating CPF-induced Bax expression in N27 cells, we performed chromatin immunoprecipitation (ChIP) analysis on the DNA extracted from N27 cells treated with CPF for 4 and 6 h, respectively. As shown in Fig. 8C, CPF treatment induced marked recruitment of STAT1 to the Bax promoter at both 4 h and 6 h in N27 dopaminergic cells, indicating that STAT1 might regulate the transcriptional activation of Bax.

In response to pesticide exposure, Bax translocates to the mitochondria and initiates mitochondrial depolarization by increasing the permeability of the outer membranes of mitochondria, thereby leading to the release of cytochrome c into the cytosol and subsequent activation of downstream caspase-mediated intrinsic apoptotic pathways (Bedner et al., 2000; Park et al., 2013b). To examine whether STAT1 regulates release of cytochrome c, scrambled and STAT1 siRNA transfected N27 cells were treated with CPF for 18 h and the extent of cytochrome c release from mitochondria to the cytosol was determined via an ELISA-based cytochrome c assay kit. While CPF induced a dramatic increase (4 fold) in the release of cytochrome c from the mitochondria of scrambled siRNA transfected cells, the effect was partially inhibited (3 fold) in the mitochondrial fraction isolated from STAT1 KD cells (Fig. 8D). Taken together, our results indicate that STAT1 plays a critical role in regulating CPF-induced mitochondrial dysfunction via modulation of Bax and Bcl-2 protein levels as well as via mitochondrial release of cytochrome c.

STAT1 regulates CPF-induced proteolytic cleavage of PKC δ

In a previous report, it was demonstrated that induction of PKC δ by DNA-damaging agents contributes to apoptotic cell death via a STAT1-dependent mechanism (DeVries et al., 2004). Therefore, we next investigated whether STAT1 could regulate PKC δ cleavage in N27 cells. Here we determined the magnitude of expression of cleavage products of PKC δ in CPF-stimulated cells that were previously transfected with either the STAT1 siRNA or scrambled siRNA (Fig. 9A). STAT1 knockdown completely blocked CPF-induced proteolytic cleavage of PKC δ , highlighting the pivotal role of STAT1 in the regulation of PKC δ activation. Next, to further determine whether STAT1 phosphorylation could control PKC δ cleavage, we overexpressed constitutively active STAT1 as well as nonphosphorylatable STAT1 mutant such as STAT1Y701F and STAT1Ser727A with subsequent exposure to CPF for 12 h. As shown in Fig. 9B, overexpression of STAT1 resulted in a marked increase in the proteolytic cleavage of PKC δ ; however, overexpression of STAT1 mutated at tyrosine 701 or serine 727 markedly attenuated CPF-induced PKC δ cleavage, by ~47% and ~60% respectively (Fig. 9B). Taken together, these results indicate that PKC δ is a downstream target of STAT1 and that phosphorylation at tyrosine 701 or serine 727 might be a critical determinant of proteolytic cleavage of PKC δ in response to CPF in dopaminergic neuronal cells.

Relationship between apoptotic cell death and autophagy in CPF-treated cells: Role of STAT1 during CPF-induced autophagy in N27 cells

Emerging evidence supports a role for autophagy and apoptosis crosstalk during CPF-induced cell death (Dai et al., 2015; Park et al., 2013b). Given that oxidative stress has been implicated in the regulation of autophagy, we sought to examine whether CPF induces autophagy and to what extent STAT1 governs the autophagic mechanism. N27 cells were treated with CPF for various durations and the expression levels of LC3B (microtubule-associated protein 1 light chain 3B), a marker of autophagy, were evaluated by immunoblotting and immunocytochemistry (ICC). As shown in Fig. 10A, CPF treatment induced a marked upregulation of LC3B II levels at 18 h and 24 h, respectively, in line with previous reports that CPF induces autophagy as a protective response during CPF-induced dopaminergic cell death (Park et al., 2013b). To further confirm immunoblot data, ICC studies were performed at 6 and 18 h, respectively, post-CPF treatment. We observed a time-dependent increase in the accumulation of LC3-positive punctate structures in CPF-treated cells, whereas a diffused pattern of staining was evidenced in vehicle-treated control cells, further confirming the induction of autophagy following CPF treatment (Fig. 10B). Similarly, beclin-1, an autophagy-related gene 6 (Atg6), was also upregulated post-CPF treatment in a time-dependent manner (Fig. 10A). Another well-characterized marker of autophagy, p62, also called sequestosome (SQSTM1), was also upregulated in CPF-treated N27 cells (Fig. 10A). The marker p62 is itself degraded by autophagy and therefore serves as a marker for autophagic flux (Komatsu et al., 2007; Pankiv et al., 2007). Inhibition of autophagy in HeLa cells has been shown to cause the accumulation of p62 (Jiang and Mizushima, 2015). In line with this finding, CPF-induced LC3B II accumulation positively correlated with p62, indicating inhibition of autophagic flux, which might in part be related to impaired lysosomal clearance machinery. The relationship between apoptosis and autophagy is rather complex and inhibition of autophagy may either trigger or attenuate apoptotic cell death in a manner specific to cell type and context (Boya et al., 2005;

Djavaheri-Mergny et al., 2006; Pattingre and Levine, 2006). Therefore, we evaluated the functional significance of autophagy during CPF-induced cell death. Accordingly, we examined whether pretreatment with pharmacological inhibitor of autophagy, bafilomycin A, a vacuolar H⁺-ATPase inhibitor, can modulate CPF-induced toxicity. For this purpose, N27 cells were pretreated with bafilomycin A (10 nM) for 1 h followed by treatment with CPF for another 24 h. In addition, in a separate set of experiments, N27 cells were pretreated with an mTOR inhibitor and an autophagy inducer, rapamycin (10 nM, 1 h), prior to CPF treatment. At the end of the treatment period, cell viability was assessed via DNA fragmentation assay. As shown in Fig. 10C, pretreatment with bafilomycin A significantly exacerbated CPF-induced cytotoxicity ($p < 0.05$), whereas rapamycin significantly ($p < 0.05$) reduced CPF toxicity as compared with CPF-treated cells, indicating a neuroprotective role for autophagy during CPF-induced cytotoxicity. Finally, to verify the importance of autophagy in CPF-induced apoptotic cell death we sought to investigate the role of LC3 during CPF-induced cell death. To this end, we knocked down LC3 using siRNA. As shown in Fig. 10D, siRNA-mediated knockdown of LC3 significantly ($p < 0.01$) increased the magnitude of apoptotic cell death as compared with scrambled transfected cells treated with CPF, suggesting that induction of autophagy may serve as a protective response to limit the magnitude of apoptotic cell death. Taken together, these results indicate a neuroprotective role for autophagy during CPF-induced dopaminergic cell death. STAT1 has been shown to be involved in promoting apoptotic cell death in response to cardiac ischemia reperfusion via negative regulation of mitophagy (Bourke et al., 2013). By inhibiting mitophagy, the clearance of damaged mitochondria is also inhibited, thereby promoting cell death. Next, we investigated the effect of STAT1 on CPF-induced autophagy. Knockdown of STAT1 was performed in N27 cells by transfecting siRNA specific for STAT1. N27 cells were transfected with STAT1 or scrambled siRNA for 48 h and then subsequently exposed to CPF (10 μ M) for another 24 h. As shown in Fig. 10E, STAT1 knockdown in CPF-treated cells resulted in marked reduction in autophagosomal markers, suggesting that absence of STAT1 activation promotes the effective clearance of autophagosomes, thereby limiting cell death. Indeed, in a previous study increased numbers of mitochondria were found within autophagosomes of STAT1^{-/-} hearts, suggesting an increased rate of autophagy and resultant clearance of damaged mitochondria, thereby limiting the deleterious effects of mitochondria-derived ROS-mediated cell signaling events (Bourke et al., 2013). Likewise, p62 levels were also reduced upon STAT1 knockdown in CPF-treated cells (Fig. 10E), further supporting the restoration of autophagic flux and enhanced lysosomal clearance. Given that STAT1 knockdown ameliorated CPF-induced mitochondrial superoxide levels, our studies suggest that improvement in the rate of lysosomal clearance may facilitate the elimination of ROS-generating damaged mitochondria, thereby limiting cell death response. Moreover, antioxidants have been shown to reduce autophagy via reduction in the cellular oxidative stress response upon exposure to dopaminergic neurotoxins in dopaminergic neuronal cells, further supporting an important role for the oxidative stress response in the induction of autophagy (Chandramani Shivalingappa et al., 2012). Taken together, our studies demonstrate an anti-apoptotic role for autophagy and unravel a unique role for STAT1 in this process.

Activation of STAT1 and induction of autophagosomal markers are partially mediated via mitochondrial ROS mechanism

Previous studies from our lab have shown that mitoapocynin (mitoapo), a mitochondrially targeted and orally bioavailable derivative apocynin, protects against oxidative stress, glial mediated inflammation, and nigrostriatal neurodegeneration in cellular and animal models of PD (Ghosh et al., 2016; Langley et al., 2017). To determine the effect of mitoapocynin on CPF-induced apoptotic cell death, we performed DNA fragmentation analysis in N27 cells that were pretreated with mitoapo prior to CPF stimulation in accordance with a previous study (Ghosh et al., 2016). As demonstrated in Fig. 11A, CPF significantly ($p < 0.001$) enhanced DNA fragmentation; intriguingly, mitoapo (10 and 30 μM , 1 h) pretreatment partially (40–54%) attenuated CPF-induced DNA fragmentation. To further explore the mechanism by which mitochondrial ROS is involved in cell death and autophagy regulation (Azad et al., 2009; Chen et al., 2007; Scherz-Shouval et al., 2007) upon CPF stimulation, we investigated the levels of STAT1 and autophagosomal markers in CPF-treated cells in the presence or absence of mitoapo (30 μM). We found that CPF-treatment induced marked increase in ROS (~5 fold, Fig. 11B) and superoxide (~2 fold, Fig. 11C) generation as well as activation of STAT1 (~1 fold, Fig. 11D) and induction of autophagosomal markers such as LC3 and beclin-1 levels, respectively by 1 fold (Fig. 11E) and that mitoapo applied 60 min before CPF resulted in a significant ($p < 0.05$) abrogation of the aforementioned markers (Fig. 11B-E). These results indicate that the mitochondrial oxidative stress mechanism is important in the regulation of CPF-induced cytotoxicity partly via regulation of STAT1 activation and autophagy function.

Mitoapocynin abrogates CPF-induced nigral dopaminergic neurotoxicity and associated neurobehavioral deficits via attenuation of mitochondria-mediated proapoptotic cell signaling events in postnatal rats

After establishing that CPF induces dopaminergic neurotoxicity in both mouse and human neurons in culture, we investigated the influence of repeated oral administration of CPF on dopaminergic neuronal survival in postnatal rats. To date there is a paucity of information on the impact of repeated exposure of CPF in adolescent rats (Terry et al., 2003). Based on reports that CPF induces dopaminergic neurodegeneration in neonatal rats (Zhang et al., 2015), we hypothesized that postnatal CPF exposure would be associated with dopaminergic neurodegeneration and resultant neurobehavioral deficits via mitochondrial oxidative stress mechanisms and STAT1 activation. We therefore investigated whether exposure of postnatal rats to CPF may cause motor deficits and dopaminergic neurotoxicity concomitant with the induction of mitochondria-mediated oxidative stress mechanisms. For this purpose, postnatal rats were orally administered CPF (5.0 mg/kg) from PND 27 to PND 61 or 10 mg/mg mitoapocynin or in combination, as described in the Methods section. At the end of the last dosing period, neurobehavioral deficits were determined. Representative activity maps (Fig. 12A) of open-field movements revealed a marked decrease in locomotor movements in CPF-treated mice as compared with CPF/mitoapocynin co-treated mice, which displayed improved locomotion. In this context, CPF decreased horizontal and vertical activity and total movement time by 58%, 50%, and 72%, respectively (Fig. 12B, C, D). A similar trend was evidenced in previous studies demonstrating persistent hyposensitivity of the monoaminergic system (Moreno et al., 2008). Additionally, consistent with previous reports

from our lab and others (Ghosh et al., 2010; Ghosh et al., 2016; Kalyanaraman et al., 2008; Kavitha et al., 2013), we observed a close resemblance of locomotor deficits to the Parkinsonian phenotype. Our results indicate that CPF causes persistent motor deficits. Surprisingly, motor deficits were rescued by mitoapo co-treatment, highlighting the role of mitochondria-derived oxidative stress in the mechanism of CPF-induced motor deficits. Next, we also examined whether mitoapo by itself alters locomotor activity. As shown in Fig. 12A-D, oral administration of mitoapocynin alone failed to alter behavioral parameters. Additionally, neurochemical analysis revealed a marked decrease of striatal dopamine (~34%) and DOPAC (64%) levels in CPF-treated rats (Fig. 13A). Furthermore, reduction in DA levels were found to parallel reduced expression of TH (Fig. 12E and 13B) indicative of dopaminergic neurodegeneration. To further evaluate the mechanistic basis of CPF-induced dopaminergic neurotoxicity, we elucidated the role of STAT1-dependent proapoptotic cell signaling events in CPF-induced dopaminergic neurotoxicity. As anticipated, loss of TH levels positively correlated with upregulation of proapoptotic signaling events, including STAT1, Bax, caspase-3 cleavage, and PKC δ in both substantia nigra (Fig. 12F, G, H, I) and striata (Fig. 13C, D, E, F), further affirming our *in vitro* data indicating STAT1-dependent proapoptotic cell signaling events in CPF-induced dopaminergic neurodegeneration. Conversely, we found that mitoapocynin-induced restoration of TH expression levels coincided with a significant ($p < 0.05$) repression of STAT1-mediated proapoptotic cell signaling events in the substantia nigra and striata of CPF-treated rats, further supporting a role for mitochondria-mediated STAT1-dependent oxidative stress signaling events in the mechanism of dopaminergic cell death.

Next, using WB analysis we quantified the expression levels of S129 phosphorylation of alpha-synuclein (α -Syn) in CPF treated rats that were pretreated with either vehicle or mitoapocynin. A growing body of evidence suggests that alpha synuclein is a major component of Lewy bodies, where S129 phosphorylation has been implicated in the aggregation and dopaminergic neurotoxicity in PD (Dzamko et al., 2014; Karampetsou et al., 2017). CPF treatment significantly ($p < 0.001$) increased nigral and striatal S129 phosphorylation of α -Syn as compared with control animals (Sup. Fig. 2). Intriguingly, pretreatment with mitoapocynin significantly inhibited CPF-induced S129 phosphorylation of α -Syn which coincided with reduced apoptotic cell death, indicating a deleterious role for phosphorylated α -Syn (S129) during CPF-induced dopaminergic neuronal loss. To examine the brain region specific vulnerability to CPF-induced neurotoxicity, we assessed caspase-3 and STAT1 activation in the cortex of CPF treated rats. As expected, there was no significant difference in the expression levels of the afore mentioned proapoptotic markers in animals treated with CPF as compared with control rats (Sup. Fig. 3). Taken together our studies confirm the selective vulnerability of nigrostriatal dopaminergic neurons to CPF-induced neurotoxicity.

Next, we assessed whether autophagy in adolescent rats is altered upon CPF treatment. As anticipated, autophagy was significantly ($p < 0.05$) upregulated in the nigra and striata of CPF-treated rats (Fig. 12J and 13G). Intriguingly, autophagy, which is partly regulated by mitochondria-derived ROS (Azad et al., 2009; Chen et al., 2007; Scherz-Shouval et al., 2007), was found to be significantly ($P < 0.05$) downregulated in rats that received a combination of mitoapo and CPF, further suggesting that autophagy may represent an

adaptive response to limit the deleterious effects of oxidative stress-induced neuronal injury (Cherra and Chu, 2008; Choi et al., 2010; Lee et al., 2012a; Navarro-Yepes et al., 2014). Furthermore, our study raises the possibility that in the presence of mitoapocynin, the absence of oxidative stress response obviates the need for induction of autophagy to limit neurotoxicity. Overall, these results demonstrate that exposure of postnatal rats to low-dose CPF can cause dopaminergic neurotoxicity and associated neurobehavioral deficits via mitochondrial oxidative stress-mediated cell signaling events.

DISCUSSION

Recent studies have implicated mitochondria-mediated oxidative stress mechanisms in CPF-induced cell death, suggesting that some of the neurotoxic effects of CPF might be attributed to mitochondria-dependent proapoptotic cell death signaling events. In the present study, we demonstrate that CPF exposure induces mitochondria-mediated apoptotic cell death of dopaminergic neuronal cells both *in vitro* and *in vivo*, which is in line with previously published reports (Abolaji et al., 2017; Dai et al., 2015; Lee et al., 2012b). Furthermore, we, for the first time, demonstrate that STAT1 plays an essential role in CPF-induced dopaminergic neuronal apoptosis and that PKC δ and autophagy are critical regulators of CPF-induced apoptotic cell death. In this context, STAT1 siRNA-mediated knockdown-associated dopaminergic neuroprotection is due, in part, to: (1) suppression of mitochondrial dysfunction, (2) inhibition of PKC δ cleavage, (3) prevention of mitochondria-dependent proapoptotic cell signaling events, and (4) restoration of autophagic clearance machinery. In addition, we found that mitoapocynin, a mitochondrially-targeted antioxidant, significantly inhibited dopaminergic neurotoxicity and rescued motor deficits via downregulation of STAT1-dependent proapoptotic cell signaling events upon postnatal exposure to CPF. These findings highlight the pivotal role of mitochondria-mediated STAT1-dependent oxidative stress mechanisms in CPF-induced dopaminergic neurotoxicity.

Elevated STAT1 levels in neuronal cells undergoing cell death has been demonstrated previously. For example, neurotoxicants such as paraquat and MPP $^{+}$, which also inhibit mitochondrial complex I, have been reported to induce STAT1 activation in both neuronal cell culture and in dying dopaminergic neurons in the substantia nigra (Junyent et al., 2010; Mangano et al., 2012). Despite these studies, the molecular mechanisms underlying STAT1-mediated neurodegeneration remain poorly understood. Oxidative stress plays a major role in the initiation and propagation of pesticide-induced neuronal toxicity (Andersen, 2004; Franco et al., 2010). Furthermore, it is well established that oxidative stress may be a causative factor in nigral dopaminergic cell death (Wang and Michaelis, 2010). Interestingly, the role of STAT1 in regulating generation of ROS by modulating NOX expression has been recently reported (Kim and Lee, 2005). Following interferon- γ treatment, ROS generation was associated with the STAT1-mediated transcriptional upregulation of NOX-1 and NOX-4 in smooth muscle cells (Manea et al., 2010). In agreement with that study, our results, for the first time, demonstrate that STAT1 regulates CPF-induced NOX-1 protein expression and ROS generation in N27 cells via binding to the NOX-1 promoter region. Furthermore, phosphorylation of STAT1 at serine 727 and tyrosine 701 was found to induce proapoptotic Bax expression by binding to the promoter region of Bax, while downregulating anti-apoptotic Bcl-2 and Bcl-xl (Chang et al., 2009; Stephanou et al., 2000). In our study, CPF

induced a significant increase in the Bax/Bcl-2 ratio in a time-dependent manner, which coincided with mitochondrial depolarization and activation of caspase-3 activity and subsequent PARP cleavage. Together, our results highlight the pivotal role of neuronal STAT1 in mediating the upregulation of mitochondria-mediated proapoptotic factors such as upregulation of Bax protein expression while downregulating Bcl-2 levels (Fig. 8B).

One common mechanism by which pesticides induce neuronal cell death is by altering the mitochondrial bioenergetics profile and depleting the ATP production necessary for neuronal cell survival (Charli et al., 2016). The difference between basal and maximal OCR, termed spare receptor capacity, can be used to support oxidative stress that is dependent on increased energy demands. Our study, for the first time using the high-throughput Seahorse XF24 Analyzer, demonstrates the ability of CPF to induce mitochondrial bioenergetic deficits. An interesting finding of our study is that CPF decreased maximal respiration and reserve capacity. In fact, the response evidenced in CPF-treated cells is consistent with that hypothesized for complex-I inhibitor (Giordano et al., 2012). Indeed, a dose-dependent reduction in mitochondrial complex I activity was found in CPF-treated PC12 cells, further confirming that mitochondrial dysfunction via complex-I inhibitory activity of ETC may mediate ROS generation (Lee et al., 2012b). To date, the role STAT1 in the regulation of mitochondrial bioenergetics remains poorly defined. Our results revealed that siRNA-mediated knockdown of STAT1 reversed CPF-induced mitochondrial bioenergetics deficits in N27 cells. It therefore appears that STAT1 exerts negative modulatory effects on cellular bioenergetics effects in CPF-treated cells. In agreement, STAT1 has been demonstrated to exhibit negative inhibitory effects on the transcription of genes that are involved in oxidative phosphorylation and mitochondrial biogenesis, both of which are implicated in neurodegenerative diseases, including PD (Meier and Larner, 2014; Pitroda et al., 2009). Further studies will investigate the functional crosstalk between STAT1 signaling and major mitochondrial metabolic mechanisms in cells subjected to oxidative stress.

Pesticides have been well documented to induce caspase-dependent activation of both intrinsic and extrinsic apoptotic pathways (Franco et al., 2010). A recent study in dopaminergic PC-12 cells signified the role of CPF-induced ROS in mediating mitochondrial dysfunction and activation of intrinsic apoptotic pathways (Lee et al., 2012b). In accordance with this study, CPF induced mitochondrial dysfunction and initiated intrinsic apoptotic pathways in N27 cells, while inhibition of caspase-3 activity rescued N27 cells from CPF-induced cell death, indicating that caspase-3 might at least in part contribute to CPF-induced cell death. Interestingly, caspase-3 has been reported to modulate neurotoxicant-induced cell death via proteolytic cleavage of PKC δ (Anantharam et al., 2002; Kanthasamy et al., 2006; Kanthasamy et al., 2008; Yang et al., 2004). Furthermore, we have previously demonstrated that suppressing PKC δ activity by pretreatment with Z-DEVD, a caspase-3 inhibitor, inhibited or attenuated pesticide-induced dopaminergic neuronal cell death (Kitazawa et al., 2005; Latchoumycandane et al., 2005; Yang et al., 2004; Zhang et al., 2007), suggesting a positive feedback loop between PKC δ cleavage/activation and caspase-3 activity in the mechanism of cell death. Indeed, cells overexpressing the PKC δ cleavage-resistant mutant demonstrated increased resistance to the dopaminergic neurotoxic effects of CPF, further highlighting the significance of proteolytic cleavage of PKC δ in CPF-induced dopaminergic neurotoxicity. Likewise, in our studies inhibition of caspase-3 activity

coincided with the inhibition of PKC δ cleavage and resultant cell death in STAT1 KD N27 cells treated with CPF, suggesting that STAT1 might regulate PKC δ cleavage at least in part via caspase-3 activation in dopaminergic neuronal cells. Consistently, in CPF-treated cells, enhancement of PKC δ cleavage and resultant cell death was evidenced in cells overexpressing STAT1 WT, while a marked reduction of PKC δ cleavage and cell death was evidenced in cells overexpressing kinase- defective STAT1 mutants (STAT1Y701F and STAT1S727A). Together, our studies indicate that STAT1 promotes CPF-induced cell death partly via enhancement of proteolytic cleavage of PKC δ .

Autophagy and ubiquitin proteasomal system (UPS) represent the two major protein degradative systems in the cell. The exact role of autophagy in cell survival is unclear; however, accumulating evidence favors a neuroprotective role for autophagy. Little is known about the mechanisms regulating autophagy. During CPF-induced dopaminergic neurotoxicity, autophagy appears to participate in protecting the neurons against oxidative stress-induced neuronal injury. For example, Park et al. demonstrated that pretreatment with rapamycin, an autophagy inducer, protected SH-SY5Y cells from CPF-induced apoptotic cell death (Park et al., 2013b), while inhibition of autophagy via 3-methyladenine (3-MA) exacerbated CPF-induced cell death in SH-SY5Y cells, suggesting a neuroprotective role for autophagy in the mechanism of CPF-induced dopaminergic neurotoxicity (Gonzalez-Polo et al., 2007; Park et al., 2013b). Consistent with these reports, our functional studies revealed a putative crosstalk between autophagy and apoptosis in CPF-treated cells, whereby pretreatment with rapamycin protected N27 cells against CPF-induced cytotoxicity, while bafilomycin A exacerbated the neurotoxic effects of the drug. Additionally, siRNA-mediated knockdown of LC3-potentiated CPF-induced cytotoxicity, further confirming the role of autophagy as an anti-apoptotic mechanism. Intriguingly, we found that STAT1 activation coincided with increased ROS generation and upregulation of autophagy, consistent with the hypothesis that ROS generation is required for the induction of autophagy in CPF-treated cells (Dai et al., 2015). This finding agrees with previous work in which IFN- α - induced autophagy was linked to the activation of the JAK-STAT1 signaling pathway in B-cells (Dong et al., 2015). Alternatively, STAT1-mediated impairment in autophagy may have led to the generation of excessive ROS and subsequent buildup of proapoptotic proteins, thereby leading to the induction of deleterious cell death signaling events. Indeed, Bourke et al. demonstrated that STAT1 may be involved in negatively regulating the autophagic mechanism, thereby leading to cardiac infarct (Bourke et al., 2013). Furthermore, the temporal pattern of p62 accumulation coincided with upregulation of LC3B-II in CPF-treated cells, indicative of impaired autophagic flux. In this context, accumulation of the autophagic substrate p62 alongside increased LC3B II levels have been implicated in impaired autophagic flux owing to impaired lysosomal clearance machinery (Zhang et al., 2016). Increased generation of ROS has been implicated in CPF-induced neuronal cell death (Lee et al., 2012b; Saulsbury et al., 2009). Thus, initial blockade of ROS may represent a major mechanism of neuroprotection associated with mitoapocynin. In this study, we, for the first time, demonstrated the mechanism of mitoapocynin- induced neuroprotection by showing that it affords protection against CPF-induced dopaminergic cell death via improved clearance of autophagic vacuoles in a STAT1- and mitochondrial ROS-dependent manner. Our studies raise the possibility that CPF-induced oxidative stress upregulates

autophagic mechanisms to facilitate the clearance of proapoptotic proteins; however, impairment of lysosomal clearance mechanisms and the resulting impairment of autophagic flux further accentuates the progression of apoptotic cell death signaling events, ultimately resulting in dopaminergic neurodegeneration. Further studies aimed at investigating autophagosomal lysosomal clearance mechanisms would improve our understanding of the exact contribution of mitochondrial ROS mechanisms to CPF-induced autophagy and associated dopaminergic neurotoxicity.

Adolescence has been increasingly recognized as a period of brain remodeling with profound changes in brain function (Giedd and Rapoport, 2010). For example, the developing brain has been shown to be more vulnerable to the injurious effects of environmental toxins (Grandjean and Landrigan, 2014). In this context, adolescents are more vulnerable to OP toxicity than adults (Furlong et al., 2006). The exact mechanisms underlying the increased vulnerability of developing brains to CPF remain poorly understood; however, a study by Aldridge et al. indicated that CPF targets neurotransmitter systems such as the cholinergic, noradrenergic, dopaminergic, and serotonergic systems (Aldridge et al., 2005). Other studies have demonstrated that dopamine levels may be unaltered or else elevated following exposure to a high dose of CPF (Karen et al., 2001; Pung et al., 2006), which is attributed to downregulation of monoamine oxidase levels (Xu et al., 2012). In the present study, we observed a reduction in dopamine levels indicating that the exposure level is a critical determinant of alteration of neurotransmitter levels. Indeed, Savy et al. demonstrated that low-level CPF exposure results in a reduction in the DA levels in the caudate putamen (Savy et al., 2015). Although the mechanism of action of CPF was not investigated in this study, it is likely that impairment in dopaminergic neuronal integrity is a likely cause of the reduction in DA levels in CPF-treated rats.

Tyrosine hydroxylase, a rate limiting enzyme in the DA biosynthesis pathway is used as a surrogate marker for the dopaminergic neurons (Daubner et al., 2011). The reduction of TH expression within the striata of CPF-treated rats positively correlates with decreased motor function and is consistent with the loss of TH positive neurons and motor deficits in PD patients (Gelb et al., 1999). In this context, a prominent biochemical feature in the striata of PD patients and MPTP injected mice include decreased levels of DA (Chung et al., 2011; Jackson-Lewis and Przedborski, 2007). In fact, behavioral deficits have been shown to be closely associated with the degree of neuronal dysfunction (Schwartz and Huston, 1996). Furthermore, previous studies have shown that striatal DA levels mirror the number of TH neurons. In this context, reduction in DA levels induced by unilateral injection of S129A α -Syn in the substantia nigra pars compacta was found to closely parallel the level of TH+ cells in the striatum (Gorbatyuk et al., 2008). Likewise, other studies using an MPTP (a Parkinsonian neurotoxicant) mouse model of PD (Ghosh et al., 2007; Ghosh et al., 2009) have demonstrated that a reduction in TH positive neurons and fibers in the midbrain and striatum respectively, positively correlates with loss of DA levels and locomotor deficits indicative of loss of TH immunoreactive neurons. Furthermore, to confirm the dopaminergic neurotoxicant effects of CPF, recently Zhang et al. using stereological analysis quantified the number of TH immunoreactive neurons in the SN of CPF (5 mg/kg-identical to the dose used in the present study) and control rats. Their studies revealed a time-dependent reduction in the number of TH-immunoreactive (TH-ir) neurons in the substantia nigra pars compacta

(SNpc) of rats at 46 days as compared with 16 days following CPF exposure (Zhang et al., 2015). Accordingly, loss of nigrostriatal TH expression levels and accompanying depletion of DA and DOPAC levels and locomotor deficits in the current study support a role for altered dopaminergic neuronal integrity during CPF-induced neurotoxicity.

Aggregation of α -Syn has been identified as a key factor in both sporadic and familial PD (Goedert et al., 2013; Lashuel et al., 2013; Virgone-Carlotta et al., 2013). Under normal physiological conditions α -Syn exists in their non-phosphorylated form; however, oxidative stress response and impairment in proteasomal function is believed to alter α -Syn oligomerization and aggregation leading to the toxic effects evidenced in synucleinopathies (Fujiwara et al., 2002; Lashuel et al., 2013; Visanji et al., 2011). Furthermore, phosphorylation of α -Syn has been shown to impart aggregation capacity to the α -Syn assemblies and may in turn promote the onset of neuronal dysfunction (Karampetsou et al., 2017). Moreover, in both fly and rodent genetic models of PD, G protein-coupled receptor kinases (GRKs) overexpression was associated with an increase of pS129 levels and elevated cellular loss (Ribas et al., 2007; Sato et al., 2011). In line with these findings, our studies showed that reduced TH levels positively correlated with increased phosphorylated α -Syn at the residue S129 (pS129), reduced DA levels and elevated apoptotic cell death indicative of loss of nigrostriatal dopaminergic neuronal integrity. Indeed previously we have demonstrated that increased expression of α -Syn predisposes dopaminergic neurons to dieldrin (pesticide)-induced apoptotic cell death (Sun et al., 2005). Conversely, mitoapocynin, mitochondria targeted antioxidant associated attenuation of apoptotic cell death in the midbrain and striatum coincided with the attenuation of pS129 α -Syn expression levels, restoration of DA levels and improvement in neurobehavioral deficits suggesting that increased expression of pS129 α -Syn may be linked to oxidative stress-induced dopaminergic neuronal loss in response to CPF. Given that oxidative stress is implicated in CPF-induced dopaminergic neurotoxicity and that the present findings bear close resemblance to the pathogenesis of idiopathic PD, it is plausible that it might at least in part contribute to the positive association between OP exposure and higher prevalence of neurodegenerative diseases. Indeed, a recent population-based case control study conducted in the Central valley of California found exposure to a wide range of OPs to be associated with increased odds of developing PD (Wang et al., 2014). The current data indicate that the nigrostriatal dopaminergic system could serve as a potential target for pesticides such as OPs based on the fact that a close parallelism was evidenced between reductions in DA levels, loss of nigrostriatal dopaminergic neuronal integrity, and resultant locomotor deficits in CPF-treated rats.

To explore the potential signaling events underlying CPF-induced dopaminergic cell death, we investigated the expression of STAT1-mediated proapoptotic cell signaling events, including PKC δ activation. Our studies indicated that exposure to CPF during the postnatal stages induced robust STAT1 activation and PKC δ cleavage, concomitant with increased Bax/Bcl-2 ratio in the substantia nigra and striatum upon repeated CPF dosing. For example, STAT1 activation has been evidenced in degenerating neurons in an experimental model of cerebral ischemia and spinal cord injury (Osuka et al., 2011; Takagi et al., 2002b). Based on these reports, it is tempting to speculate that STAT1 activation in nigral dopaminergic neurons may serve as a critical determinant of CPF-induced apoptotic cell death.

Alternatively, STAT1 has been shown to be activated in LPS-stimulated microglial cells (Przanowski et al., 2014) and microglial activation has been shown to precede CPF-induced dopaminergic neurotoxicity (Zhang et al., 2015). Thus, it is plausible that CPF-induced microglial activation might have contributed to STAT1 activation in the substantia nigra. Nevertheless, our data from *in vitro* studies provide strong evidence that STAT1 activation may be a novel factor that increases the vulnerability of dopaminergic neurons to apoptotic cell death. In contrast, both STAT1 activation and caspase-3 activation were absent in the cortical region of CPF treated rats further highlighting the brain region specific vulnerability to CPF-induced apoptotic cell death. Our studies are consistent with previous studies demonstrating that CPF exposure is associated with structural changes in the developing brain (Rauh et al., 2012), presumably via disruption of cellular machinery controlling neuronal replication and differentiation, apoptosis, and neural circuit formation (Roy et al., 2004; Roy et al., 2005; Slotkin and Seidler, 2009). Akin to our *in vitro* data, mitoapocynin, a mitochondrial-targeted antioxidant also ameliorated the induction of STAT1-dependent oxidative stress-mediated proapoptotic signaling events (striatum and substantia nigra) and restored dopaminergic neuronal function as well as improved locomotor deficits. Consistent with our finding, it has been demonstrated that STAT1 activation is ameliorated by antioxidants such as NAC and diphenyl iodinium, an NADPH oxidase inhibitor, in response to pro-oxidant stimulus (Simon et al., 1998). Thus, it is plausible that the inhibition of mitochondrial-derived ROS could have been sufficient to attenuate proapoptotic signaling events, thus leading to the preservation of dopaminergic neurons and rescue of neurobehavioral deficits. Furthermore, mitoapocynin also attenuated CPF-induced upregulation of autophagy, further confirming the pivotal role of oxidative stress response in the induction of autophagy (Azad et al., 2009; Cherra and Chu, 2008; Choi et al., 2010; Scherz-Shouval et al., 2007). Concordant with our findings, Park et al. demonstrated that NAC, an antioxidant, inhibited LC3 upregulation in CPF-treated SH-SY5Y cells, indicating that ROS mediates CPF-induced autophagy (Park et al., 2017). Collectively, our results demonstrate a positive association between STAT1 activation and mitochondria-mediated proapoptotic cell signaling events in nigrostriatal dopaminergic neuronal loss and associated motor deficits following CPF exposure during the developmental period. Further research will be required to elucidate whether activation of STAT1-dependent proapoptotic cell death signaling proteins remain elevated in the substantia nigra upon discontinuation of CSF for prolonged periods of time.

In summary, in our present study we demonstrated that CPF induced the activation of STAT1, resulting in the induction of mitochondria-mediated proapoptotic signaling events and subsequent dopaminergic cell death via the modulation of autophagic functions both *in vitro* and *in vivo*. The data presented herein support a role for mitochondria-mediated oxidative stress response in enhanced STAT1 phosphorylation and downstream PKC δ cleavage as well as autophagy regulation during CPF-induced dopaminergic neurotoxicity and neurobehavioral deficits. Our findings, together with previously demonstrated altered redox mechanisms, stress the relevance of mitochondria-mediated proapoptotic cell signaling events as critical regulators of CPF-induced dopaminergic neurotoxicity. Given the pivotal role of STAT1 in the regulation of proapoptotic cell signaling events during brain injury, their enhanced activation could partly explain the detrimental effects of CPF on

dopaminergic neuronal integrity. Our data also highlights that mitoapocynin might be a promising interventional strategy for the reversal of CPF-induced dopaminergic neurotoxicity and associated behavioral deficits.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

CPF	chlorpyrifos
STAT1	Signal transducer and activator of transcription 1
ROS	reactive oxygen species
MMP	mitochondrial membrane potential
GSH	reduced glutathione
PKCδA	Protein kinase C δ
PAPR	Poly (ADP-ribose) polymerase
NOX1	NADPH oxidase 1
LC3B	Microtubule-associated proteins 1A/1B light chain 3B
TH	tyrosine hydroxylase
DA	dopamine
DOPAC	3,4-dihydroxyphenyl acetic acid

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Highlights

- Organophosphate chlorpyrifos (CPF) induces dopaminergic neurotoxicity in dose-dependent manner.
- STAT1 is crucial for CPF-mediated oxidative stress, mitochondrial damage, apoptosis and DNA damage causing dopaminergic neurotoxicity.
- Mitoapocynin, a mitochondrial antioxidant, attenuates CPF-mediated dopaminergic neuronal loss both *in vitro* and *in vivo*.

Figure 1. CPF-induced dopaminergic cell death via caspase mediated mechanism in N27 dopaminergic neuronal cells.

CPF reduces cell viability in a dose-dependent manner in N27 and differentiated LUHMES dopaminergic neurons. N27 cells treated with increasing concentrations of CPF (100nM-300nM) for 24h and cell death was evaluated using both (A) MTS assay and (B) DNA fragmentation assay. (A) The effects of CPF on dopaminergic neuronal cell viability evaluated using the MTS assay. N27 dopaminergic neuronal cells were exposed to increasing concentrations of CPF and IC50 was determined at the end of 24h using the MTS

assay. Results are expressed as percentage of vehicle treated cells. **(B)** Quantification of CPF-induced apoptosis of N27 cells using DNA fragmentation analysis. The relative concentration of nucleosomes in cells treated with or without CPF was determined by Elisa based cell death detection ELISA^{plus} assay. Results are representative of at least 3 independent experiments and expressed as Mean $\pm$ SEM, N=6. * p <0.05, ** p <0.01 and *** p <0.001 indicates level of significant difference between CPF versus Control group. **(C)** Differentiated LUHMES cells were treated with increasing concentrations of CPF (10nM –100 μ M) for 24h and cell viability was determined using the DNA fragmentation assay. The relative concentration of nucleosomes in cells treated with or without CPF was determined by Elisa based cell death detection ELISA^{plus} assay. Results are representative of at least 3 independent experiments and expressed as Mean $\pm$ SEM, N=4. * p <0.05, ** p <0.01 and *** p <0.001 indicates level of significant difference between CPF versus Control group. **(D)** CPF treatment reduces neuritic length in differentiated LUHMES human dopaminergic neurons. ICC analysis of differentiated LUHMES cells exposed to 300nM CPF for 24. Scale bar: 100 μ m. The bar graph represents the neurite length, measured using imageJ software. Results are representative of at least 3 independent experiments and expressed as Mean $\pm$ SEM, (N=6). *** p <0.001 indicate significant difference between Control versus CPF treated group. **(E)** CPF-induces a time dependent increase in caspase 3 activity. N27 cells were treated with or without CPF (10 μ M) for various time periods (6–24h). Cell lysates were prepared and incubated with Ac-DEVD-AMC, a caspase 3 specific substrate, at 37°C for 1h. Caspase 3 activity was determined spectrophotometrically using Ac-DEVD-AMC substrate and outcomes are expressed as of FU/mg protein. Data calculated as percentage of control and are representative of at least 3 independent experiments. Data expressed as Mean $\pm$ SEM. Each time point was performed in triplicates (N=3). * p <0.05, ** p <0.01 and *** p <0.001 vs control. **(F)** CPF induce time-dependent increase in PARP cleavage in N27 cells. Post treatment with CPF, cell lysates were prepared and membranes were probed with anti-PARP antibody, which recognize both native (116kDa) and cleaved (89kDa) PARP fragment. Cleaved PARP band density was normalized to β -actin. The bar graph represents the mean $\pm$ SEM of atleast 4 independent experiments. * p <0.05 and ** p <0.01 vs Control group. **(G)** Effect of pan caspase and caspase-3 inhibitor on CPF induced neuronal cell death. N27 cells were pretreated with Z-VAD-FMK (pan caspase inhibitor; 100 μ M) or Z-DEVD-FMK (Caspase-3 inhibitor; 50 μ M) for 1h followed by incubation with CPF 10 μ M for another 24h. Post treatment cytotoxicity was assessed via DNA fragmentation analysis and results were expressed as percentage of control. Data expressed as mean $\pm$ SEM, N=6. * p <0.05, ** p <0.01 and *** p <0.001 indicates significant change from Control group, while ### p <0.001 indicate significant difference between CPF and CPF+ caspase inhibitor (s) treated group.

Fig. 2. Oxidative stress response is a critical determinant of CPF-induced cell death.

Effect of CPF on ROS production. (A) N27 cells were treated with or without CPF (10µM) for the indicated time period. Post treatment cells were incubated with ROS sensitive H2DCFDA dye and DCF fluorescence was determined 60min later. Change in the fluorescence reading, was determined via spectrophotometer. Each data point was converted to percentage of control. Data expressed as mean $\pm$ SEM and representative of at least 3 independent experiments performed in six replicates. (B) Time dependent increase in mitochondria derived superoxide levels, as assessed via MitoSOX™ Red dye. Each data

point was converted to percentage of control. Data expressed as mean $\pm$ SEM and representative of at least 2 independent experiments performed in six replicates per group. **(C)** CPF-induces time-dependent reduction in GSH levels in N27 cells. N27 dopaminergic cells were treated with 10 μ M CPF for the indicated time period. Post treatment, CPF-induced changes in glutathione levels were measured spectrophotometrically using monochlorobimane based fluorometric assay. Data expressed as mean $\pm$ SEM and representative of at least 2 independent experiments with N=6 per group. **(D, E)** N-acetyl cysteine (NAC) attenuated CPF-induced ROS generation and cytotoxicity in N27 cells. N27 cells were pretreated with NAC (5mM) for 12h followed by treatment with and without CPF (10 μ M) for another 24h and the levels of CPF-induced ROS generation **(D)** and cell viability **(E)** was assessed spectrophotometrically by redox sensitive CM-H2DCFDA dye and DNA fragmentation analysis, respectively. Data expressed as mean $\pm$ SEM (N=6) and representative of at least 2 independent experiments performed in six replicates per group per group. * p <0.05, ** p <0.01 and *** p <0.001 vs Control group; ## p <0.01 and ### p <0.001 indicates significant differences between CPF and CPF + NAC treated cells.

Figure 3. CPF-induced mitochondrial depolarization is accompanied by mitochondria mediated proapoptotic cell signaling events.

(A) Effect of CPF on mitochondrial depolarization. N27 cells were treated with and without 10 μ M CPF for the indicated time periods. CPF-induced mitochondrial depolarization cells were quantified fluorometrically using JC-1 dye. Representative data from three independent experiments are shown and the numerical values represents Mean $\pm$ S.E.M. (N= 6). (B) Effect of CPF on Bax/Bcl-2 ratio. N27 cells were treated with or without 10 μ M CPF for the indicated time periods. Cell lysates were prepared and immunoblotted for Bcl-2 and Bax and tubulin was used as the loading control. All blots shown are representative of at least 3

independent experiments. Panel on the right hand side shows the densitometric scanning analysis for Bcl2 and Bax was performed, and the Bax/Bcl2 ratio was determined. The results represent mean $\pm$ S.E.M. from at least 3 independent experiments. * p <0.05, ** p <0.01, *** p <0.001 indicates significant difference between CPF treated and control group. (C) CPF-induced time dependent increase in PKC δ cleavage. N27 cells were exposed to the indicated time period and cell lysates were immunoblotted for PKC δ using an antibody, which recognize both native and cleaved bands. β -actin was used as the loading control. All blots shown are representative of at least 4 independent experiments. Right panel, quantification of relative band intensity of cleaved PKC δ and normalized to β -actin. (D) PKC δ genetic knockdown attenuates CPF-induced dopaminergic cell death. N27 were transfected with PKC δ specific siRNA or scrambled siRNA for 48h and CPF (10 μ M) was added for another 24h. At the end of the incubation period, the extent of apoptotic cell death was determined spectrophotometrically using ELISA-based cell death assay kit. The results are expressed as Mean $\pm$ S.E.M. performed in replicates of six and performed at least three independent experiments. Data are expressed as percentage of control. * p <0.05, ** p <0.01 and *** p <0.001 indicates level of significant difference from Control group, whereas ### p <0,001 indicates significant differences between CPF-treated N27 were transfected with PKC δ siRNA or scrambled siRNA. (E) Over expression of caspase-3 cleavage resistant mutant of PKC δ attenuates CPF-induced cell death. N27 cells stably expressing the Lac Z vector or caspase-3 cleavage resistant PKC δ (CRM; PKC δ ^{D327A}) were treated with and without CPF. The extent of DNA fragmentation measured spectrophotometrically as described above. Results shown are Mean $\pm$ S.E.M. from at least three independent experiments and expressed as % of control. *** p <0,001 represent significant difference between CPF treated LacZ empty vector transfected cells vs CPF-treated PKC δ -CRM-V5 mutant group.

Figure 4. CPF-induces STAT1 activation in N27 dopaminergic neuronal cells.

Time-dependent increase in phosphorylation of STAT1 at both Tyr⁷⁰¹ and Ser⁷²⁷ and its nuclear import. (A) N27 cells were treated with CPF (10 μ M) for the indicated time period and the cell lysates were immunoblotted for phospho-Tyr701 (pY701), phospho-Ser727 (pS727) and STAT1. Bottom panel represents quantification of the relative band intensity normalized to total STAT1 levels. Results are normalized to control values and are shown as Mean $\pm$ S.E.M. from at least three independent experiments. * p < 0.05, ** p < 0.01 and *** p < 0.001 vs Control group. (B) CPF induced intracellular translocation of phosphorylated

STATE Representative immunofluorescent images obtained from LUHMES cells that were treated with or without CPF (300nM) for 12h. Phosphorylated STAT1 levels as visualized using an antibody against STAT1 (p-Ser727) (red fluorescence) and subsequently counterstained with Hoechst (blue). Experiments were performed in triplicates and representative of at least 3 independent experiments. Scale bar in C=100 μ M. (C) STAT1 activation determined by luminescence assay in N27 cells stimulated with or without CPF. To measure STAT1 activation, N27 cells were transfected with the STAT1-responsive GAS reporter/ luciferase construct. The cells were subsequently treated with and without CPF(50 μ M) for 4 hours and the cells were harvested at the end of the incubation period for the determination of STAT1 activation using luminescence based dual luciferase assay. The firefly luciferase results were normalized using the internal Renilla luciferase activity. The assays were performed in duplicate, N=8. The results were expressed as relative luciferase unit, Mean $\pm$ SD. **p<0.01 represent significant difference between control and CPF treated group.

Figure 5. STAT1 mediates CPF-induced cytotoxicity in N27 cells.

(A) N27 cells were transfected with scrambled or STAT1 siRNA, 48h post transfection, STAT1 knockdown efficiency was evaluated by immunoblotting for STAT1. Right panel represents densitometric scanning analysis for STAT1. Results are normalized to control values and are shown as Mean $\pm$ S.E.M. from at least three independent experiments. $*p < 0.05$, and $***p < 0.001$ vs Control group (Scrambled siRNA transfected cells exposed to vehicle). (B, C) STAT1 KD confers resistance against CPF-induced cell death. Scrambled and STAT1 siRNA transfected N27 cells were treated with CPF (10 μ M) for another 24h and

cell viability was determined using the DNA fragmentation assay **(B)** or MTS assay **(C)**. Results are expressed as percentage of control and each value represent Mean $\pm$ SEM from atleast 3 independent experiments. * $p < 0.05$; *** $p < 0.001$ vs scrambled siRNA transfected vehicle treated N27 cells, while ## $p < 0.01$ and ### $p < 0.001$ vs scrambled siRNA transfected CPF-treated N27 cells. **(D)** STAT1 KD markedly reduces CPF induced cell shrinkage in N27 cells. Phase contrast light microscopy images reveal that STAT1 KD attenuated CPF-induced reduction in cell density and cell death associated morphological changes. Representative image (20x) from 3 independent experiments. **(E)** Effect of STAT1 KD on CPF-induced caspase-3 activity. N27 cells were transfected with scrambled or STAT1 siRNA as detailed in methods and subsequently were treated with CPF (10 μ M) for 18h. CPF-induced caspase 3 activity was measured fluorometrically by detecting free cleaved product from the caspase 3 specific peptide substrate, DEVD-AFC. Results are expressed as FU/mg protein. Results are expressed as percentage of control and each value represent Mean $\pm$ SEM (N=6). *** $p < 0.001$ vs scrambled transfected siRNA transfected vehicle treated N27 cells, # $p < 0.05$ vs scrambled siRNA transfected CPF-treated N27 cells. **(F)** STAT1 regulates CPF-induced PARP cleavage. N27 cells were transfected with scrambled or STAT1 siRNA, followed by treatment with CPF (10 μ M) for 24h, cell lysates were prepared and immunoblotted for PARP with antibody recognizing parental and cleaved PARP (89kDa). (Right panel) Densitometric scanning analysis revealing attenuation of CPF-induced PARP cleavage in STAT1 KD cells. Data shown are mean $\pm$ S.E.M, and represented as % control. Immunoblot representative of atleast three independent experiments. ** $p < 0.01$ vs scrambled siRNA transfected cells exposed to vehicle, # $p < 0.05$ vs scrambled siRNA transfected cells exposed to CPF.

Figure 6. STAT1 regulates NOX-1-mediated oxidative stress and GSH levels in CPF- treated cells. (A, B) Effect of STAT1 knock down on CPF-induced ROS production and NOX-1 protein expression. N27 cells were transfected with either scrambled siRNA or STAT1-specific siRNA as described above, subsequently, N27 cells were exposed to CPF (10µM) for 24h, followed by incubation with CM-H2DCFDA for 60min and intracellular ROS levels were measured fluorometrically using Spectramax microplate reader (A). The data represent Mean $\pm$ S.E.M. of at least 3 independent experiments. Each treatment group was performed in replicates of six. (B) STAT1 KD attenuates CPF-induced NOX-1 upregulation.

Representative immunoblot for NOX-1 from N27 cells transfected with scrambled siRNA or STAT1 siRNA followed by vehicle or CPF (10 μ M, 24h) treatment. Scrambled and STAT 1 siRNA transfected N27 cells were treated with CPF (10 μ M) for 24h. Cell lysates were prepared & immunoblotted for NOX-1. Histogram representing NOX-1 band intensity normalized to β -actin. Data represented as mean $\pm$ S.E.M. The blots are representative of at least 3 independent experiments. (C) Chromatin immunoprecipitation analysis of GAS/ISRE element within NOX-1 promoter region. Time-dependent binding of STAT1 transcription factors to the proximal promoter region of NOX-1 in N27 cell line treated with CPF. The N27 cell were treated with CPF 50 μ M for 4h and 6h, post incubation cells were fixed and DNA was extracted and subjected to chromatin immunoprecipitation (CHIP) assay using the STAT1 antibodies and primers specific to the proximal promoter of NOX-1. Representative image of amplified PCR product resolved on 1% agarose gel is shown in figure. Input lanes confirms that equal amount of DNA was used for the initial immunoprecipitation assay. Data is representative of 3 independent experiments. (D) STAT1 KD restores CPF-induced GSH depletion. N27 cells were transfected with either scrambled siRNA or STAT1-specific siRNA and exposed to either vehicle or CPF. 24h post CPF treatment, the intracellular GSH levels were determined spectrophotometrically. The data is presented as percentage of control. Data represented as mean $\pm$ S.E.M (N=6). *** p <0.001 vs vehicle treated scrambled siRNA transfected cells, # p <0.05 and ## p <0,01 vs scrambled siRNA transfected cells exposed to CPF.

Figure 7. Effects of STAT1 knock down on CPF-induced mitochondrial bioenergetics deficits in N27 cells.

STAT-1 KD preserves mitochondrial bioenergetic profile following CPF treatment in N27 dopaminergic cells. (A) Schematic representation of the mitochondrial bioenergetics profile in relation to OCR levels, in both scrambled and STAT1 KD N27 cells treated with or without CPF (10 μ M) for 12h. Post treatment, the change in the Oxygen consumption rate (OCR) levels were determined using sea horse XF-24 analyzer. Basal respiration (B), maximal respiration (C), spare respiratory capacity (D) and ATP-linked respiration/ATP

levels (**E**) were calculated by measuring change in OCR levels post sequential addition of oligomycin (1 μ M), FCCP (1 μ M), and Rotenone/Antimycin A (0.5 μ M), respectively, as indicated by arrow in the figure 7A. (**F**) ATP levels, measured at 24h post CPF (10 μ M) treatment via CellTiter-Glo® Luminescent assay. The data is presented as percentage of control. Data represented as mean $\pm$ S.E.M (N=6). * p <0.05 and *** p <0.001 vs Control group, # p <0.05 and ## p <0.01 Vs CPF-treated scrambled siRNA transfected N27 cells. Results are representative of atleast 3 independent experiment performed in replicates of three. Data expressed as mean $\pm$ S.E.M. * p <0.05, *** p <0.001 vs vehicle treated scrambled siRNA transfected cells, whereas, # p <0.05 and ## p <0.001 vs CPF treated scrambled siRNA transfected cells.

Figure 8. Effects of STAT-1 knockdown on CPF-induced modulation of Bax/Bcl2 ratio and mitochondrial release of cytochrome C

(A) STAT1 KD attenuates CPF-induced collapse of mitochondrial membrane potential. N27 cells were transfected with scrambled or STAT1 siRNA as detailed above and subsequently treated with 10 μ M CPF for 24h. Post treatment, CPF- induced mitochondrial depolarization was quantified fluorometrically using JC-1 dye as described in methods section. (B) STAT1 knockdown preserves mitochondrial function by upregulating Bcl-2 and down regulating Bax expression. N27 cells were transfected with scrambled siRNA or STAT1 siRNA and

treated with 10 μ M CPF for 24h, cell lysates were prepared and immunoblotted for Bcl2 and Bax. β -actin was used as an internal control. Panel on the right hand side shows the densitometric scanning analysis for Bcl2 and Bax was performed, and the Bax/Bcl2 ratio was determined. The results represent mean $\pm$ S.E.M. from at least 3 independent experiments. * p <0.05, *** p <0.001 vs vehicle treated scrambled siRNA transfected cells; ^{##} p <0.01 vs CPF treated scrambled siRNA transfected cells. (C) STAT1 binds to Bax promoter post CPF treatment. N27 cells were treated with CPF 50 μ M for 4h and 6h, respectively. Post treatment, cells were harvested and processed for chromatin extraction as described in material method section. The same experimental conditions for CHIP related experiments as detailed above, was implemented here. The immunoblot is representative of at least 3 independent experiments. (D) Effect of STAT1 KD on CPF-induced cytosolic translocation of cytochrome c. Scrambled siRNA or STAT1 siRNA transfected N27 cells were treated with CPF 10 μ M for another 18h, the cytosolic fraction and mitochondrial fraction were extracted and cytochrome c release was determined using a spectrophotometer. The results are expressed as percentage of control and expressed as Mean $\pm$ SEM, N=4. * p <0.05, *** p <0.001 vs vehicle treated scrambled siRNA transfected cells; ^{##} p <0.01 vs CPF treated scrambled siRNA transfected cells.

Figure 9. Effects of STAT1 knockdown and overexpression of STAT1 on CPF-induced PKC δ cleavage.

(A) STAT1 KD inhibited the proteolytic cleavage of PKC δ in CPF-treated cells. N27 cells were transfected with either scrambled or STAT1 siRNA as detailed above. Post treatment with CPF (10 μ M) for 24h, PKC δ cleavage was quantified using Western blotting analysis. The results are expressed as percentage of control and expressed as Mean $\pm$ S.E.M. The blots are representative of at least three independent experiments. *** p <0.001 vs vehicle treated scrambled siRNA transfected cells; ## p <0.01 vs CPF treated scrambled siRNA

transfected cells. **(B)** Overexpression of STAT1 exacerbated proteolytic cleavage of PKC δ in CPF-treated N27 cells. N27 cells were transfected with either eGFP empty vector, eGFP WT STAT1, eGFPSTAT1Y701F or eGFP STAT1S727 mutant. Upon treatment completion (12h), cell lysates were prepared and immunoblotted for PKC δ and tubulin. (RHS panel) Blots were quantified using densitometric scanning analysis. Blot shown are mean ratio of cleaved PKC δ to tubulin. Data shown as Mean $\pm$ SD of at least three independent experiments.

* $p < 0.05$, *** $p < 0.001$, ### $p < 0.001$ vs CPF treated eGFP empty vector transfected N27 cells.

Figure 10. Analysis of autophagy and its regulation by STAT1 during CPF-induced dopaminergic cell death.

(A) Effect of CPF on autophagic markers expression in N27 cells. N27 cells were treated with CPF (10 μM) for the indicated time period. Cell lysates were prepared and immunoblotted for autophagy markers. Equal protein loading was ensured by using β-actin as loading control. Representative blot from 4 independent experiments is presented. Quantification of blots using densitometric scanning analysis (right panel). (B) Immunofluorescence images depicting the extent of autophagic vacuole formation in CPF

treated N27 cells. N27 cells were treated with 10 μ M of CPF for 6h and 18h followed by immunostaining for LC3. The cells were counterstained with Hoechst stain for nuclear labelling. CPF-induced significant time dependent increase in LC3 positive punctate structures, while a diffused pattern of distribution was evidenced in vehicle treated controls cells. **(C)** Effect of pharmacological interference of autophagy during CPF-induced cytotoxicity. N27 cells were pretreated with either Bafilomycin (10nM) or rapamycin (10nM) for 1h followed by treatment with or without CPF (10 μ M) for another 24h and the levels of CPF-mediated cytotoxicity was assessed by the DNA fragmentation assay. The data represent mean $\pm$ SEM, N=6. *** p <0.001 vs vehicle treated control group; # p <0.05 vs CPF treated group. **(D)** LC3B siRNA knockdown sensitizes cells to CPF-induced cytotoxicity. N27 cells were transfected with scrambled or LC3B siRNA for 48h, subsequently treated with CPF (10 μ M) for another 24h and cell viability was determined using DNA fragmentation analysis. Results are expressed as percentage of control and presented as Mean $\pm$ SEM, N=6–8. *** p <0.001 versus scramble control cells exposed to vehicle, ## p <0.01 vs scrambled siRNA transfected CPF treated group. **(E)** STAT1 knockdown attenuates CPF-induced upregulation of autophagic markers. N27 cells were transfected with scrambled or STAT1 siRNA as described above and incubated with CPF (10 μ M) for another 24h. LC3B, beclin and p62 protein expressions were compared between whole cell lysates of scrambled transfected control cells and STAT1 siRNA transfected cells that were treated with or without CPF. β -actin is used as a loading control. The normalized densitometric band intensity analysis was presented as Mean $\pm$ S.E.M. Data representative of at least 4 independent experiments** p <0.01 and *** p <0.001 versus scrambled control cells exposed to vehicle, # p <0.05 vs scrambled siRNA transfected CPF exposed group.

Figure 11. Mito apocynin (Mito-apo) protects against CPF induced neurotoxicity in N27 cells via amelioration of ROS generation and inhibition of STAT1 phosphorylation.

Dopaminergic N27 neuronal cells were pretreated with either the vehicle or the indicated concentration of mito-apo for 1h followed by CPF (10μM) for another 24h. The cultures were assayed for cell death, oxidative species generation, STAT1 activation and autophagic markers (A) Mitoapocynin attenuate CPF-induced DNA fragmentation in N27 cells. Estimation of DNA fragmentation in N27 cells treated with CPF in the presence or absence of mitoapocynin. Cell death was assessed via Roche cell death ELISA kit and the data

expressed as percentage of the control group. Data represented as mean $\pm$ SEM, (N=6). **(B)** Mitoapocynin attenuates CPF-induced ROS generation. Fluorometric analysis of cellular ROS measured using CM-H2DCFDA in dopaminergic neuronal cells. DCF fluorescence was normalized to the control group. Data presented as Mean $\pm$ SEM (N=6). Mitoapocynin attenuate CPF-induced Mitosox generation in N27 cells. **(C)** Mitochondria-derived superoxide was determined using Mito-Sox Red® fluorogenic dye in N27 cells exposed to CPF in the presence or absence of mitoapocynin. Change in fluorescence intensity was calculated as percentage of control (vehicle treated group) and represented as Mean $\pm$ SEM (N=6). **(D, E)** Mitoapocynin attenuates STAT1 activation and upregulation of autophagic markers. **(D)** Immunoblotting analysis of lysates prepared from N27 cells treated with CPF in the presence or absence of mitoapocynin. Densitometric scanning analysis revealed that mitoapocynin attenuated STAT1 phosphorylation in CPF treated cells. Membranes were probed with STAT1 and p-STAT1 (pS727) antibodies and were normalized to β -actin (used as a loading control). Data shown are Mean $\pm$ SEM from at least 3 independent experiments. **(E)** Quantification of bands revealed a reduction in the levels of autophagic markers in cells that received a combination of mitoapocynin and CPF. Data represented as percentage of control group. Each value represents the Mean $\pm$ SEM from at least 3 independent experiments. * p <0.001, ** p <0.01 and *** p <0.001 indicates significant difference from Control group, while # p <0.001, ## p <0.01 and ### p <0.001 indicates significant differences between CPF and CPF + mito-apo treated cells.

Figure 12. Mito-apo attenuated the CPF-mediated locomotor deficit and induction of STAT1 dependent proapoptotic cell signaling events in the rat substantia nigra (SNpc).

Neurobehavioral impairments in CPF (5mg/kg) (35-day repeated dose regimen) treated rats. Both male and female SD rats were subjected to daily oral administrations of CPF (5 mg/kg) or corn oil or CPF/mitoapocynin combination for 35 consecutive days (from PND 27 to 61) as described in methods section. 24h after the last dose, rats were subjected to locomotor analysis using VersaMax open field apparatus. (A-D) Versa plot showing movement track of rats collected over 10 min and various locomotor activity parameters were measured concurrently. Quantification of locomotor activity using the VersaMax Analyzer.

Representative (B) horizontal activity; (C) vertical activity and (D) total distance travelled (cm). Mitoapocynin attenuated CPF-induced TH depletion and upregulation of STAT-1 and other proapoptotic markers in the substantia nigra. Mice were sacrificed at the end of behavioral analysis and SN was extracted and lysates were prepared for WB analysis. Representative blots showing the expression of TH (E), STAT1 and phosphorylated STAT1 (Y701 and S727 residue) (F); Bax and Bcl-2 (G); caspase-3 (H); PKC δ (I) and LC3 (J). Data are represented as means $\pm$ SEM of 6–8 rats per group. * $p < 0.001$, ** $p < 0.01$ and *** $p < 0.001$ versus the Control group, while # $p < 0.001$ and ## $p < 0.01$ indicates significant differences between CPF and CPF + mito-apo treated group.

Figure 13. Mito apo confers resistance against dopaminergic neurotoxicity in the striata of CPF treated rats.

Rats subjected to the same treatment conditions as described in Fig. 12. 24h after the last CPF dose rats were sacrificed and the striata was harvested for biochemical analysis. (A) Mitoapocynin confers resistance against CPF-induced depletion of DA and its metabolite DOPAC levels. Quantification of striatal dopamine and its metabolite DOPAC levels by HPLC analysis in the striatum of CPF treated rats treated with or without mitoapocynin. DA and dihydrophenyactic acid (DOPAC) levels were determined in extracts prepared from striata of CPF treated rats with or without mitoapocynin using HPLC with coulometric

detection. Results are represented as mean $\pm$ SEM with 6–8 rats per group. **(B)** Mitoapocynin attenuate CPF-induced modulation of TH and STAT-1 associated proapoptotic markers in the striata. Western blotting analysis of lysates prepared from striatal tissues. Representative immunoblots depicting expression of TH **(B)**; STAT1, pSTAT1 Y701 and pSTAT1 S727 **(C)**; Bax and Bcl-2 **(D)**; caspase-3 **(E)**; PKC δ **(F)**; and LC3 **(F)**. β -actin was used as a loading control. The densitometric scanning analysis indicate the normalized band intensity and are represented as mean $\pm$ SEM, N=6 rats per group. * p <0.001, ** p <0.01 and *** p <0.001 vs Control group, while # p <0.05 and ## p <0.01 indicates significant differences between CPF and CPF + mito-apo treated group.