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Molecular Association of Glia Maturation Factor with the Autophagic Machinery in Rat Dopaminergic Neurons: A Role for Endoplasmic Reticulum Stress and MAPK Activation

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Abstract

Parkinson's disease (PD) is one of several neurodegenerative diseases where accumulation of aggregated proteins like α -synuclein occurs. Dysfunction in autophagy leading to this protein build-up and subsequent dopaminergic neurodegeneration may be one of the causes of PD. The mechanisms that impair autophagy remain poorly understood. 1-methyl-4-phenylpyridinium ion (MPP⁺) is a neurotoxin that induces experimental PD *in vitro*. Our studies have shown that glia maturation factor (GMF), a brain-localized inflammatory protein induces dopaminergic neurodegeneration in PD and that suppression of GMF prevents MPP⁺ induced loss of dopaminergic neurons. In the present study, we demonstrate a molecular action of GMF on the autophagic machinery resulting in dopaminergic neuronal loss and propose GMF-mediated autophagic dysfunction as one of the contributing factors in PD progression. Using dopaminergic N27 neurons, primary neurons from wild type (WT) and GMF-deficient (GMF-KO) mice we show that GMF and MPP⁺ enhanced expression of MAPKs, increased the mammalian target of rapamycin (mTOR) activation, endoplasmic reticulum stress markers such as phospho-Eukaryotic translation initiation factor 2 alpha kinase 3 (p-PERK), inositol-requiring enzyme 1 α (IRE1 α). Further, GMF and MPP⁺ reduced Beclin1, focal adhesion kinase (FAK) family interacting protein of 200 kD (FIP200) and autophagy related proteins (ATGs) 3, 5, 7, 16L and 12. The combined results demonstrate that GMF affects autophagy through autophagosome formation with significantly reduced lysosomal-associated membrane protein 1/2, and the number of autophagic acidic vesicles. Using primary neurons we show that MPP⁺ treatment leads to differential expression and localization of p62/sequestosome and in GMF-KO neurons there was a marked increase in p62 staining implying autophagy deficiency with very little colocalization of α -

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Conflict of Interest

The authors declare that there are no conflicts of interest.

Compliance with Ethical Standards Conflict of Interest

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synuclein and p62 as compared with WT neurons. Collectively, this study provides a bidirectional role for GMF in executing dopaminergic neuronal death mediated by autophagy that is relevant to PD.

Keywords

Autophagy dysfunction; Glia maturation factor; Parkinson's disease; Protein aggregation

Introduction

Parkinson's disease (PD) is a neurodegenerative disease, that mainly affects about 3.4% of elderly population over 60–70 years of age worldwide. The onset of PD before the age of 40 is seen in lesser than 5% of the cases in population-based cohorts. The occurrence of PD is generally accepted to range from 100 to 200 per 100,000 people and the annual incidence is thought to be 15 per 100,000. [1–4]. The pathological features of PD are a loss of dopaminergic neurons in the substantia nigra (SN) and the presence of protein deposits-the Lewy bodies. Defects in proteolytic processing of proteins combined with aberrant protein folding and aggregation along with mitochondrial dysfunction contribute to the pathogenesis of PD [5–8]. Lack of clearance of cellular debris by autophagy in dopaminergic neurons has been shown to lead to neuronal cell death in PD [9–12].

By the process of autophagy, cells degrade long-lived cytoplasmic material, oxidatively damaged organelles and misfolded proteins to maintain cellular homeostasis during normal and metabolic stress conditions [13]. Recent studies have shown a dysfunction in the autophagic pathway in the brains of PD patients and in animal models of PD, indicating the importance of autophagy in PD [14,12,15,16]. Several reports indicate that endoplasmic reticulum (ER) stress is closely associated with impaired autophagy in PD progression [17–20]. Accumulation of unfolded proteins in the lumen of ER and the signaling pathways involved in the activation of ER stress response are still unknown [21]. During autophagy progression, the interface between the autophagy and apoptosis is at least partially regulated by beclin 1 [22]. Autophagy gene (ATG)-related proteins coordinate the specific steps involved in autophagy induction, sequestration and execution.

Mitogen activated protein kinases (MAPKs) such as p38 and ERK1/2 respond to inflammatory proteins (cytokines and chemokines) and reactive oxygen species (ROS). Microtubule-associated protein light chain 3 II (LC3-II), which is formed by phosphatidylethanolamine conjugation of LC3-I, translocates to the autophagosome membrane, the process which is essential for the autophagosome formation. In this process, cytoplasmic inclusions are delivered to the lysosome surface where it binds with lysosome associated membrane protein (LAMP) 2 and is rapidly degraded by the hydrolases of the lysosomal acidic vesicles [23].

Glia maturation factor (GMF), a neuroinflammatory protein that is predominantly expressed by astrocytes, microglia and neurons in the brain, was discovered, cloned, and sequenced in our laboratory [24–26]. GMF consists of a 141 amino acid polypeptide chain with a 99% homology between both humans and rodents. It plays a fundamental role in the growth and

survival of brain cells [27–30]. GMF is predominantly expressed in the brain during stress conditions [31,32]. It is also reported that GMF is expressed highly in various other tissues including colon, thymus, and kidney. Complete knockdown of GMF improves various biochemical and behavioral parameters in mice [31,33,34]. Previously, Kaimori et al., [35] reported that GMF is involved in the pathophysiology of renal tubular disease by enhancing the oxidative stress. The deficiency of GMF in renal tubular cells shows resistance against stress of proteinuria. Concurrently, Baldwin et al., [36] showed that transient over expression of GMF, increased the susceptibility of the tumor cells to cytotoxicity of cisplatin, a chemotherapeutic agent via p38 MAPK activation [32]. GMF is one of the major intracellular signal-transducing factor for glial cells and neurons and acts as an intra-neuronal proinflammatory cytokine; increasing its expression under certain stress conditions [30,37,38]. It consists of multiple phosphorylation sites and is phosphorylated by protein kinases A and C, casein kinase and ribosomal S6 kinase [39]. Over expression of GMF in primary astrocyte cultures causes the secretion of neurotrophic factors such as brain derived neurotrophic factors (BDNF) and nerve growth factors (NGF) via the activation of p38 pathway [40]. Earlier findings from our laboratory showed that absence of GMF protects dopaminergic neurodegeneration by reducing neuroinflammatory cascade and significantly improves motor deficits in MPTP intoxicated mice [41]. In addition, deficiency of GMF in primary astrocytes showed a significant tolerance against MPP⁺ induced oxidative stress dependent inflammation [1]. Cyclosporine A, an effective immunophilin/calcineurin inhibitor is an immunosuppressive agent that is implicated in the maturation and folding of native proteins via the autophagic machinery [42]. Cyclosporine A attenuates mitochondrial permeability transition, preserves mitochondrial integrity and improves mitochondrial stability and respiratory function. Since autophagy has been shown to play a protective role in PD and since GMF promotes neuronal degeneration in PD animal model; we decided to study the action of GMF on autophagy. This would help better understand the role of GMF in the pathogenesis of PD and enable better therapeutic interventions in PD. In our present study, we demonstrate that extracellular GMF activates p38 and ERK1/2 MAPKs that mediates mTOR-dependent dysregulation of the autophagy-lysosomal pathway leading to dopaminergic neuronal death.

Materials and Methods

Rat dopaminergic neurons (N27) culture

44Rat mesencephalic dopaminergic (N27) cells were grown in RPMI-1640 (GIBCO, Life Technologies, Grand Island, NY) medium supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO), 1% L-glutamine, 1% penicillin and streptomycin (Life Technologies) as reported previously [43].

Mouse primary neurons culture

Mouse primary neuronal cultures were prepared from wild type (WT) mice as well as GMF-KO mice fetal brains as described previously [38,45,40]. Brain tissues were harvested from the fetuses and the cells were used to grow in to neurons *in vitro*. Briefly, cerebral cortical tissues were obtained from 15 or 16-day fetal mouse brains and cultured in neurobasal medium supplemented with B27, 2 mM L-glutamine and 1% penicillin/streptomycin at

37°C in humidified 5% CO₂ and 95% air. Neurons were grown on poly-D-lysine coated cover glass in six well tissue culture plates for 2 weeks. These cultures represent a nearly pure neuronal population based upon morphological observation under microscope.

Incubation of N27 cells and mouse primary neurons with GMF, MPP⁺, rapamycin and cyclosporine A

N27 cells and mouse primary neurons were grown to ~65% – 85% confluency. N27 cells were incubated for up to 24 h with GMF protein (100 ng/ml), MPP⁺ (300 µM), rapamycin (200 µM) and cyclosporine A (100 nM) (dissolved in Dulbecco's phosphate-buffered saline (DPBS; Life technologies), MPP⁺ is an active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [46]. WT and GMF-KO mice primary neurons were treated with MPP⁺ (20 µM) for 24 h [38]. After GMF and MPP⁺ treatment, cells were fixed with 4% paraformaldehyde for immunostaining. Cell lysates were prepared for the analysis of autophagy markers expression by Western blotting. Protein concentration of the cell lysates was determined using the bicinchoninic acid assay (BCA) protein assay kit (Thermo Scientific, Waltham, MA) as per the manufacturer's instructions.

Western blotting

Briefly, N27 cells were seeded in T25 cm² cell culture flask and allowed to grow about 65–85% confluency. Then the cells were incubated with GMF protein, MPP⁺, rapamycin and cyclosporine A for 24 h. Cells were harvested by trypsinization and washed with PBS. Cells were lysed with RIPA cell lysis buffer containing protease and phosphatase inhibitors. Protein concentration of the cell lysates was determined using the BCA protein assay kit. Lysates were loaded into 4–12% NuPAGE Tris-Glycine gradient gel (Invitrogen, Life technologies, Carlsbad, CA). An equal amount of protein (ranging from 20–35 µg) was loaded in each lane along with a lane containing pre-stained protein markers (Invitrogen). The separated proteins were blotted onto PVDF by wet protein transfer system (Life technologies). After blocking with 5% bovine serum albumin or non-fat milk powder in Tris-buffered saline-Tween20 (TBS-T; TBS+0.05% Tween-20) for 1 h, the membranes were then incubated with the respective antibodies: anti-p38 and p-p38 (Cat No. ab7952 and ab4822), anti-ERK1/2 and p-ERK1/2 (Cat No. ab17942 and 14362) that were purchased from Abcam (Cambridge MA). Anti-mTOR and P-mTOR (Ser2448) (Cat No. CST 2972 and 2971), anti-Beclin 1 (Cat No. CST 3738), anti-IRE1α (Cat No. CST 3294), anti-PERK and p-PERK (Cat No. CST C33E10 and 3179), anti-FIP200 (Cat No. CST 12436), and anti-ATG complexes (Cat No. CST 4445) were purchased from Cell Signaling Technology (Danvers, MA). Anti-β-actin (Cat No. A1978) was purchased from Sigma-Aldrich, (St. Louis, MO). The following dilutions were used for p38 and p-p38, ERK1/2 and p-ERK1/2 (1:700–1:1000), mTOR and p-mTOR, Beclin 1, and LC3-I and LC3-II (1:800), IRE1α and FIP200 (1:500), ATG complexes (1:600–1000), and β-actin (1:2000) in 5% BSA in TBS-T or PBS-T. After incubation with the primary antibodies, the membranes were incubated with appropriate secondary antibodies at a 1:1500–1:3000 dilution. Then, the membranes were washed thrice with TBST for 5 min each. The bands were visualized by treating the membranes with ECL prime western blotting detection reagent (SuperSignal West Pico PLUS Substrate, Cat No. 34580; ThermoScientific). Blots were stripped and re-probed for

β -actin as a loading control. Densitometric quantitation was done using ChemiDoc-It² Imaging System (UVP LLC, Upland, CA).

Enzyme linked immunosorbent assay (ELISA)

Protein levels were quantified by indirect ELISA as previously described [47,48]. Briefly, total cell lysates were prepared and protein concentrations were measured. Protein samples were diluted in PBS coating buffer (Life Technologies) and equal amounts of total protein in a volume of 50 μ l was loaded in each well of a PVC ELISA 96 well plate (Corning), sealed and incubated overnight at 4°C. Unbound protein from samples was discarded and the wells were washed four times with 1X PBST. The remaining protein-binding sites in the protein-coated wells were blocked with blocking buffer containing 1% BSA in PBS, 0.3% H₂O₂ solution for 1 h at room temperature. Afterwards, 50 μ l primary antibodies to beclin or ATG complexes (1:200–400) were loaded to each well and incubated for 2 h at room temperature. Following three washes with PBST, plate wells were incubated with appropriate HRP conjugated secondary antibodies (1:1000–2000) for 1 h at room temperature. Following three washes, wells were loaded with TMB (3, 3', 5, 5'-tetramethylbenzidine, Thermo Scientific) substrate reagent, incubated for 30 min and reaction was stopped with 50 μ l of 2 M sulfuric acid. The optical density was read at 450 nm in a microplate reader (Molecular Devices; Sunnyvale, CA).

Immunocytochemistry

Rat dopaminergic N27 cells and primary neurons were cultivated on glass coverslips pre-coated with poly-D-lysine (Millipore, Billerica, MA). After the treatment the cells were fixed with 4% paraformaldehyde for 1 h and permeabilized by incubation in PBST for 15 min. Then the cells were rinsed three times in PBS, blocked with 5% normal goat serum (NGS) for 30 min and finally incubated for 2 h at room temperature with anti-IRE1 α (1:500 dilution), LAMP1, LC3 II (1:300), and α -synuclein and p62/SQSTM (1:300–400) primary antibodies. The cells were then incubated with goat anti-rabbit IgG conjugated with green fluorescent dye Alexa Fluor 488 (Cat No. A-11008) or red fluorescent dye Alexa Fluor 568 secondary antibody (Cat no. A-11001, ThermoFisher Scientific) for 1 h at room temperature. The coverslips were stained with VECTASHIELD antifade mounting medium with DAPI (Vector laboratories) to stain DNA. Finally, the coverslips were mounted with Vinol (Sigma) onto microscope slides and cells were examined under a Nikon fluorescent microscope.

Confocal microscopy

Confocal imaging was performed on a Leica TCP SP8 laser scanning confocal microscope with a 405-nm diode laser and tunable super continuum white light laser using 63X oil immersion objective (Molecular cytology core facility, University of Missouri). Briefly, after the treatment with GMF protein and MPP⁺, N27 cells and mice primary neurons were incubated with fixative (4% paraformaldehyde) for 1 h at 4°C and permeabilized by incubation in PBST for 15 min. Then the cells were rinsed three times in PBS, blocked with 5% NGS for 30 min and finally incubated for 2 h at room temperature with α -synuclein and p62/SQSTM (1:300) primary antibody. P62/SQSTM and α -synuclein were visualized with goat anti-rabbit IgG conjugated with green (Alexa fluor 488) and red (Alexa Fluor 568) fluorescent dyes. Finally, the cells were counterstained with VECTASHIELD antifade

mounting medium with DAPI. The following excitation/emission band-pass wavelengths were used: 405/420–480 nm (DAPI), 495/505–550 nm (Alexa Fluor 488) and 570/580–630 nm (Alexa Fluor 568).

Autophagy acidic vesicles quantification

To quantitatively estimate autophagy acidic vesicles by intensity of labelling we adopted the Acridine Orange (AO) staining method [49–51] at a concentration of 10 µg/ml of AO dissolved in PBS (pH 7.4). 1 µl AO reagent was added, incubated with treated cells and finally imaged on coverslips. The excitation laser for green fluorescence was 473 nm and for red fluorescence was 559 nm. Emission filters were 520 nm and 572 nm, respectively. We present quantitative measurement of autophagy acidic vesicles as a measure of the intensity of labelling of red fluorescent AO with vesicles in the whole field and scale bars (µm) calculated using ImageJ software.

Statistical analysis

The results were analyzed using GraphPad InStat 3 statistical software. Mean $\pm$ SEM was calculated and analyzed using One-way Analysis of Variance (ANOVA) followed by Tukey-Kramer multiple comparison tests to determine statistically significant differences between the groups. An unpaired t-test was performed when comparing between groups. Results were expressed as mean $\pm$ SEM for four experiments in each group. p values < 0.05 were considered as significant.

Results

GMF activates p38 and ERK1/2 MAPKs expression

The MAPKs are a specific class of serine/threonine kinases, described as stress activated protein kinase and mainly expressed due to extracellular stress and cytokine induction, which mainly respond to extracellular signals in neurodegeneration. There is evidence to support a link between p38 MAPK activation and autophagy [52–54], though the exact mechanism remains elusive. To determine if GMF was involved in the p38 activation in dopaminergic N27 cells, we performed immunoblotting for p-p38 and total p38 (Fig 1A). Results show that GMF exposure significantly increased p38 phosphorylation in N27 cells when compared with control cells (Fig 1A and 1B; * $p < 0.05$). Additionally, N27 cells when incubated with both GMF and MPP⁺ further increased p-p38 expression when compared with other treated groups.

ERK1/2 is a primary factor directing cellular response to pro-inflammatory cytokines and a wide variety of environmental and oxidative stresses. To determine that GMF is involved in the phosphorylation of ERK1/2, we performed ERK1/2 protein expression analysis by immunoblotting method. Exposure of N27 cells to GMF significantly upregulates p-ERK1/2 expression as compared with control cells (Fig. 1C and D; * $p < 0.05$). Incubation of cells simultaneously with both GMF and MPP⁺ further increased p-ERK1/2 protein expression as compared with other groups. Bar graphs showing densitometry quantification of protein bands in immunoblots is obtained as a ratio of the density of p-p38 and p-ERK1/2 over the

density of total p38 and ERK1/2 (B and D) respectively. Values are expressed as arbitrary units and given as mean $\pm$ SEM of four experiments in each group.

GMF activates mammalian target of rapamycin (mTOR) and regulates autophagy

mTOR is a cascade regulator in the central nervous system and is an important kinase in the pathway regulating autophagy in dopaminergic neurons via the extracellular signaling pathway. We determined the action of GMF and MPP⁺ on mTOR activity by immunoblotting analysis. Results showed that GMF significantly upregulates mTOR phosphorylation as compared with untreated control cells (Fig. 2A). Incubation with both GMF and MPP⁺ further increased the phosphorylation of p-mTOR expression relative to either GMF or MPP⁺ treated cells (* p <0.05). Rapamycin, a specific inhibitor of mTOR function has been shown to block mTOR phosphorylation at ser-2448 and to induce autophagy. Our results (shown here Fig. 2B), demonstrate that GMF increases mTOR phosphorylation and through its activation impair autophagy. The effect of rapamycin as an inducer of autophagy is also shown in Fig 2B where p-mTOR level is decreased. Bar graphs showing densitometric quantification of protein bands in immunoblots is obtained as a ratio of the density of p-mTOR over the density of total mTOR. The values are expressed as percentage of control.

GMF reduces the expression of FIP200 and Beclin1

Focal adhesion kinase family interacting protein of 200kD (FIP200) is responsible for autophagosome formation by interacting with ULK protein kinase, a homolog of the yeast autophagy-related protein ATG1. Beclin1, a key player in neurodegeneration, is implicated in the execution of the autophagy-lysosomal pathway. The impairment of autophagy by GMF treatment may be due to the improper complex formation between the interacting proteins. To define whether GMF interferes with the autophagy-lysosomal pathway we performed immunoblotting for FIP200 (Fig. 3A) and determined Beclin1 expression by ELISA (Fig. 3C). Results show that incubation of N27 cells with GMF or MPP⁺ significantly reduced FIP200 (Fig. 3A) and Beclin 1 (Fig. 3C) expression as compared with untreated control cells. Moreover, incubation of cells simultaneously with GMF and MPP⁺ further reduced FIP200 and Beclin 1 expression when compared with control cells (* p <0.05). The results suggest that GMF protein could affect the initiation and nucleation stages of autophagy. Bar graphs show the effect of GMF and provided as a percentage of control (Fig. 3B).

GMF impairs ATG complex of proteins implying a role in autophagosome maturation

We next examined whether GMF interferes with the ATG complex of proteins such as ATG3 (Fig. 4A), ATG5 (Fig. 4B), ATG7 (Fig. 4C), ATG16L (Fig. 4D) and ATG12 (Fig. 4E) by ELISA, which are essential proteins for autophagosome formation and maturation. N27 cells incubated with GMF for 24 h resulted in significant decrease in ATG complex proteins as compared with control cells as shown in Fig 4 (A–E). Incubation with MPP⁺ shows similar effects shown by GMF. These results indicate that GMF closely interferes in the autophagy-lysosomal pathway by downregulating the ATG complex of proteins that leads to dysfunction in autophagy; probably at the elongation step in the autophagosome formation ultimately resulting in dopaminergic cell death. N27 cells treated with GMF and MPP⁺

significantly reduced the expression of ATG complex of proteins as compared with other treated groups. The ATG protein expression levels were normalized to total protein in cells (* $p < 0.05$). Bar graphs show the effect of GMF exposure as a percentage of control.

GMF treatment increases the expression of ER stress markers p-PERK and IRE1 α in N27 cells by activation dependent nuclear translocation

IRE1 α and PERK senses the presence of misfolded proteins in the ER lumen and transduces signals to the cytoplasm and the nucleus in normal brain. Western blot and immunofluorescence results show that exposure of N27 cells to GMF for 24 h significantly increases the ER-stress markers p-PERK (Fig. 5A) and IRE1 α (Fig. 5C and E) expression as compared with untreated control cells. Furthermore, immunocytochemical expression of IRE1 α is reduced in the cytoplasmic region of the cells that were treated with GMF and/or MPP⁺ as compared with control cells (Fig 5E) and also IRE1 α translocates to the nuclei. The results indicate that GMF inhibits autophagy initiation by enhancing ER-stress. Bar graphs showing densitometry quantification of PERK protein band in immunoblots is obtained as a ratio of the density of p-PERK over the density of total PERK (Fig. 5B). IRE1 α protein expressions were normalized with β -actin (Fig. 5D).

Co-expression of LAMP1 and LC3 in N27 cells by Immunofluorescence

Next, we performed immunofluorescence staining of LAMP1 and LC3 to confirm the co-localization of LAMP1 and LC3 in autolysosome formation in N27 cells. The LC3 expression co-localized with the late lysosomal marker LAMP1, indicating the fusion of autophagosome with lysosomes. Exposure of GMF to N27 cells for 24 h qualitatively altered the autolysosome formation and degradation by inhibiting LAMP1 (red fluorescence) and increased LC3 (green fluorescence) expression as compared with control cells (shown in Fig. 6). N27 cells were treated with GMF and MPP⁺ qualitatively reduced immunofluorescence expression and co-localization of these proteins as compared with control cells.

GMF reduces LAMP1 and LAMP2 expression and number of autophagosome acidic vesicles

LAMP1 and LAMP2 are the major heavily glycosylated proteins that maintain the structural integrity of the lysosomal membrane. They are delivered to the phagosomes during the maturation process. N27 cells incubated with GMF for 24 h significantly reduces LAMP1 and LAMP2 expressions as compared with control cells (Fig. 7A and C). Cells incubated simultaneously with GMF and MPP⁺ further reduced LAMP1 and LAMP2 expression as compared with cells incubated either with GMF or MPP⁺ (* $p < 0.05$). Densitometry bar graphs show the effect of GMF exposure on LAMP1 (Fig. 7B) and LAMP2 (Fig. 7D) as a percentage of control. These results along with the results in Fig. 4 (A–E) clearly show that GMF impairs autophagy probably by inhibiting phagolysosome biogenesis and maturation.

Acridine orange (AO), is a lysosomotropic metachromatic fluorochrome that accumulates in acidic vesicular organelles such as autolysosomes, where it emits a red fluorescent signal. Lysosomal membrane permeabilization following GMF or MPP⁺ and rapamycin exposures for 24 h, showed that the red fluorescent signal from AO was markedly reduced, indicating

lysosomal disruption as shown in Fig. 8A. Bar graph shows that the staining intensity of AO labelling within the acidic vesicles was quantitatively reduced in GMF and MPP⁺ treated cells, whereas AO intensity of labelling within vesicles were increased in rapamycin treated cells, when compared with untreated control cells (Fig. 8B). Hence, the red fluorescence intensity that directly correlates with the volume of acidic vesicles; increases with induction of autophagy such as with rapamycin.

Further, we measured phagosome-lysosome fusion using LysoTracker staining along with Hoechst stain. Fig. 9 shows the number and size of positive staining vesicles. GMF treatment restricted the accumulation of LysoTracker stain in the cells. In addition, in the control cells the acidic organelles stained with LysoTracker appeared to be concentrated in the perinuclear region while less intense and more diffused perinuclear staining was detected in the GMF treated cells. Representative confocal images show that incubation of N27 cells with GMF and/or MPP⁺ for 24 h qualitatively reduced the number of LysoTracker stained lysosomes when compared with control cells (Fig. 9).

Involvement of GMF in the expression of α -synuclein and p62/SQSTM in primary neurons

p62/SQSTM is an adaptor protein involved in autophagosome formation with lysosome to degrade protein aggregates like α -synuclein and other cytoplasmic inclusions within the dopaminergic cell. p62 functions as a chaperone for the autophagic removal of protein and organelle debris; hence, p62 expression levels may be correlated with autophagy. When primary neurons from WT and GMF-KO mice were incubated with MPP⁺ for 24 h, WT neurons shows qualitatively reduced p62/SQSTM expression (green color) when compared with untreated cells (Fig. 10). Whereas GMF-KO primary neurons treated with MPP⁺ shows increased p62/SQSTM expression as compared with WT primary neurons. These findings indicate that GMF plays an important role in the dysfunction associated with autophagy that leads to neuronal cell death.

GMF is involved in the aggregation of endogenous α -synuclein in primary neurons

α -synuclein is a toxic cytoplasmic inclusion of the Lewy bodies and over-aggregation and accumulation of it leads to dopaminergic cell death in PD; probably due to dysfunction in autophagy. In the present study we show that primary WT neurons treated with MPP⁺ showed significantly increased α -synuclein aggregation (red color) and reduced p62/SQSTM (green color) expressions as compared with GMF-KO neurons (as shown in Fig. 11). Neurons from GMF-KO mice when incubated with MPP⁺ showed, decreased α -synuclein and increased p62/SQSTM compared with WT primary neurons. The results indicate that GMF could be mediating the clearance of α -synuclein aggregates by regulating autophagy.

Discussion

PD is a chronic neurodegenerative disease that depletes bundle of dopaminergic neurons in the SN and leads to lack of neurotransmission between SN and striatum of the midbrain resulting in bradykinesia, rigidity, rest tremor and postural instability [55,56]. Motor complication appeared when ~50%–60% of dopaminergic neurons degenerate in SN that

leads to a ~70%–80% depletion of dopamine levels in the striatum region. The principle lesion of PD is neuronal loss accompanied by dopaminergic degeneration, but the exact mechanism associated with dopaminergic cell death is still not clearly understood [56–58]. We have previously reported that administration of MPTP significantly increased GMF expression in the SN up to 730 pg/mg of protein (5.3-fold increase) and in the striatum of midbrain up to 559 pg/mg of protein (4.9-fold increase) as compared with untreated control mice brain. These findings clearly indicate that upregulation of GMF expression is mainly associated with MPTP neurotoxicity in the WT mice [41]. Absence of GMF protects nigrostriatal dopaminergic neurons from MPTP induced neurodegeneration by suppressing TNF- α , IL-1 β , GM-CSF, CCL2 and MCP-1 that enhances the motor behavior in mice [41]. In addition, deficiency of GMF protects the astrocytes from MPP⁺ induced oxidative stress and interferes with the inflammatory cascade by reducing the activity of NF- κ B, and reduced levels of proinflammatory TNF- α , IL-1 β , GM-CSF, IL-17, IL-33, and CCL2 protein expressions [1]. These results clearly indicate a key role of GMF in neuroinflammation. GMF is present in both glial cells and neurons as well as in Schwann cells [59, 41]. In this study, we investigated the role played by GMF in the dysfunctions of the autophagy-lysosomal proteolytic system that leads to increased cytoplasmic inclusions resulting in ER stress mediated dopaminergic cell death. Here, we report that GMF mediates dopaminergic neuronal degeneration by activating ERK1/2 pathway and attenuates the autophagy-lysosomal machinery via enhancing ER stress. GMF, a neuroinflammatory protein is highly localized in the brain than other organs in the human body [24,45,61]. GMF has no special secretory leading sequence and acts as an intracellular signal transduction regulator [62,63,26]. In the present study, we found that incubation of N27 cells with GMF activates p38 and ERK1/2 phosphorylation as compared with untreated control cells. Previously, we have reported that GMF activates p38 isoform; and ERK1/2 expression by phosphorylation in C6 glioma cells, suggesting that GMF acts as a stress activated primary responder [61,36]. The action of GMF on ERK1/2 activation in primary astrocytes and microglial cells has been previously studied in this laboratory [40,61]. In astrocytes, GMF overexpression activates p38 activity and the intracellular redox enzyme CuZn-superoxide dismutase (CuZn-SOD) and the transcription factors NF- κ B [64,65]. In the past studies we have shown that overexpression of GMF in PC12 cells activates p38, its downstream MAPKAP-kinase 2, and the final effector tyrosine hydroxylase, accompanied by an increased phosphorylation of these proteins [66,32,24,61].

Autophagy is a tightly regulated intracellular proteolytic system to degrade the cytoplasmic inclusion to maintain intracellular homeostasis [67, 68]. Recent evidence indicates that autophagy dysfunction plays a major role in the pathogenesis of neurodegenerative disorders, especially in PD [20,69]. Deficiency in the elimination or degradation of abnormal cytotoxic protein aggregates, promotes cellular stress, and this ultimately induces dopaminergic cell death [70]. During stress conditions, the kinase mTOR is a critical regulator of autophagy induction. Phosphorylation of mTOR (p-mTOR expression) via ERK1/2 signaling regulates autophagic flux [71–73]. Increasing evidence suggests that phosphorylation of ERK1/2 activates mTOR complexes by phosphorylation of mTOR [74]. Our previous studies demonstrate that GMF activates both p38 and ERK1/2 by phosphorylation in primary neuronal and proximal tubular cells [24–26,61,75]. In this study,

we found that, exposure of N27 cells to GMF and/or MPP⁺ significantly increased the expression of p-mTOR when compared with untreated control cells, indicating that GMF participates in autophagic flux by regulating mTOR expression. During the ERK1/2-mediated mTOR-dependent autophagy-lysosomal pathway, inhibition of mTOR leads to the activation of Beclin1 (a mammalian homolog of yeast ATG6) [76,77]. Our results showed that GMF and MPP⁺ significantly reduced Beclin 1 expression as compared with untreated N27 cells. Previously, we have reported that GMF phosphorylates p38 and NF- κ B in mouse primary astrocytes [25,40,61,78]. Recently, it was implicated that NF- κ B activation negatively regulates autophagy by reducing Beclin 1 expression, which is induced by tumor necrosis factor, ROS and starvation in *in vitro* system [79–81]. Our results clearly demonstrated that GMF exposure attenuates Beclin 1 expression due to p38 and ERK1/2 phosphorylation, as shown in earlier findings [25,40,61,78].

Activated MAPKs such as p38 inhibits autophagy and increases expression of inflammatory mediators through ULK1 phosphorylation [52]. It has been demonstrated that IKK α and IKK β are involved in the activation of the mTOR complex in response to inflammatory molecules such as TNF- α and insulin exposure [82]. Preventing exposure to proinflammatory protein inhibits mTOR expression and further promotes autophagy-lysosomal proteolytic system [83,84], which corroborates our present findings. In the present study, we found that GMF significantly activates mTOR expression by phosphorylation probably due to activation of p38 and ERK1/2. This indicates that GMF interferes with the autophagy pathway by enhancing p-mTOR expression. In addition, expression of ERK1/2 modulates the mTOR expression via phosphorylation of mTOR [74,52,73].

Initiation, execution and degradation of the autophagy-lysosomal depends on ATG complexes of proteins, which include ATG3, ATG5, ATG7, ATG12 and ATG6L. Autophagy-lysosomal degradation consists of multiple steps of autophagosome formation, which include nucleation, expansion, uncoating and completion, and execution [85,14,86,87]. ATG protein complexes are mainly associated with LC3-I and LC3-II that are essential proteins for autophagosome formation. The conjugation between ATG5-ATG12-ATG16 that occurs at the elongation stage of autophagosome formation renders neuroprotective roles of ATG proteins against synucleinopathies [88]. Impairment or decrease in the expression of ATG proteins, results in neurodegeneration and the presence of cytoplasmic inclusions filled with ubiquitinated proteins such as α -synuclein [89,90]. The expression of ATG proteins in brain cells significantly reduced the transcription of α -synuclein and PINK1 expression, which indicates that ATG reduces α -synuclein accumulation via autophagy-lysosomal degradation [91,88]. Our present results demonstrate that exposure to GMF significantly dysregulates ATG protein complex which may enhance the aggregation of α -synuclein implying that GMF may contribute to the aggregation and accumulation of α -synuclein by reducing autophagy markers such as ATG complexes; ultimately leading to dopaminergic cell death.

The lysosomal membrane proteins such as LAMP1 and LAMP2 are mediators of lysosomal action in the autophagy-lysosomal pathway within the neurons. Previously, it was reported that lysosomal markers such as LAMP1 and LAMP2 are deficient in human PD brain as well as in experimental animal models [92]. Extending this observation, we found decreased

expression of LAMP1 and LAMP2 in GMF and/or MPP⁺ treated N27 cells. Immunocytochemical examination in N27 cells revealed that cytosolic LAMP1 expressions was reduced in the rat dopaminergic N27 cells. In addition, GMF treatment reduced the number of autophagic acidic vacuoles. Combined results of the action of GMF on autophagy, presented in this study suggest that though the initial steps in endosome/autolysosome fusion may be unaffected; autophagosome maturation and maturation of the autophagic acidic vesicles leading to degradative clearance of cytoplasmic contents could be the target of GMF and impaired by it.

The ER is the intracellular organelle, mainly involved in the regulation of secretory protein translocation, transportation and quality control of secretory protein folding in the cytoplasmic compartment [93,94]. The intracellular environment and metabolic state can induce ER dysfunction due to accumulation of cytoplasmic debris within the ER lumen, designated as ER stress. Accumulating evidence indicates that ER stress mediated autophagy dysfunction plays an important role in the generic or progressive neurodegenerative disease particularly in PD [95,17,93]. Extensive ER stress induces the activation of p38 phosphorylation that leads to cell death [94,96]. Expression of α -synuclein and its aggregation and accumulation induces ER stress, autophagic flux mediating cell death [97]. In our study, we found that GMF and/or MPP⁺ significantly enhances the phosphorylation of p38 and ERK1/2 that leads to aggregation of α -synuclein and increases ER stress markers such as IRE1 α and phosphorylation of PERK expression as compared with untreated control cells. GMF and/or MPP⁺ induces ER stress mediated autophagy-lysosomal flux via activation and phosphorylation of both p38 and ERK1/2, further leading to dopaminergic neuronal loss. α -synuclein, the intraneuronal inclusion body present in PD has been shown in cell culture systems to be presented to the autophagy-lysosomal pathway after recognition by the adaptor protein p62/SQSTM1. Using primary neurons from WT and GMF-KO mice we determined endogenous p62 levels by immunohistochemistry and we show that GMF strongly associated with impairment of the autophagic machinery in processing intracellular α -synuclein.

Conclusion

Autophagy plays a central role in maintaining cellular homeostasis in the brain and a dysregulation in autophagy is implicated in many neurodegenerative diseases. With non-replicating cells such as neurons, intracellular pathways mediated by proteins like GMF become all the more relevant in the control of the accumulation and degradation of aggregated proteins. Results from the present study indicate that extracellular addition of GMF activates the phosphorylation of p38 and ERK1/2 MAPKs that leads to increased activation of mTOR. Upregulation of mTOR inhibits autophagy markers such as ATG3, ATG5, ATG12 and ATG16L. Furthermore, GMF also reduces lysosomal markers LAMP1 and LAMP2 that consequently leads to impairment of autophagy pathway execution within the lysosome. In addition, GMF enhances the accumulation of α -synuclein due to impairments in the autophagy pathway that leads to ER stress dependent dopaminergic neuron cell death. We show that p62 is required for α -synuclein autophagy and this is regulated by GMF. Future studies aimed at understanding the molecular mechanism of action of GMF to inhibit PD pathogenesis are underway in the laboratory.

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Fig. 1.

GMF activates p38 and ERK1/2 MAPKs expression in dopaminergic N27 cells. N27 cells were seeded in T25 cell culture flasks and incubated with GMF (100 ng/ml) and MPP⁺ (300 μ m) for 24 h under standard conditions. Then the cells were washed with PBS and cell lysates were prepared for western blot, using specific antibodies to p-p38, p-ERK1/2, p38 and ERK1/2. GMF treatment significantly increased p-p38 and p-ERK1/2 expression as compared with control cells (A and C). N27 cells simultaneously incubated with both GMF and MPP⁺ significantly increased p-p38 and p-ERK1/2 expression as compared with other group. Western blot results were quantified by ChemiDoc-It² imaging system analysis software, and the values are expressed as arbitrary units and given as mean $\pm$ SEM (n=4). Bar graphs show the effect of GMF exposure on the relative intensity compared to the control (B and D). *p < 0.05 compared to control. Statistical significance was assessed with one-way ANOVA followed by Tukey's Kramer procedure using GraphPad prism-7 software.

Fig. 2.

GMF increases target of rapamycin (mTOR) expression in dopaminergic N27 cells. N27 cells were seeded in T25 cell culture flask and incubated with GMF (100 ng/ml), MPP⁺ (300 μ M), rapamycin (200 μ M) and cyclosporine A (100 nM) for 24 h under standard conditions. Then the cells were washed with PBS and cell lysates were prepared for western blot analysis, using specific antibodies to total and p-mTOR, expression. GMF, MPP⁺ and cyclosporine A treatment significantly increased expression of p-mTOR when compared with control cells (A). Western blot results were quantified by ImageJ, and the values are

expressed as percentage of control and given as mean $\pm$ SEM (n=4). Bar graphs show the effect of GMF, MPP⁺, rapamycin and cyclosporine A exposure on the relative intensity compared to the control (B). *p< 0.001 and *p< 0.05 compared to control and GMF. *p< 0.001 compared with MPP⁺ and GMF+MPP⁺. Statistical significance was assessed with one-way ANOVA followed by Tukey's Kramer procedure and unpaired t-test was performed when comparing between groups by using GraphPad prism-7 software.

Fig. 3.

Effect of GMF on FIP200 (ULK interacting protein) and Beclin 1 expression in dopaminergic N27 cells. N27 cells were seeded in T25 cell culture flask and incubated with GMF (100 ng/ml) and MPP⁺ (300 μ M) for 24 h under standard conditions. After the incubation period, cells were washed with PBS and cell lysates were prepared from these cells for western blot using specific antibodies to FIP200, then stripped and reprobed for β -actin expression. The concentrations of Beclin 1 were determined by ELISA assay, using cell lysates collected from GMF and MPP⁺ treated cells. GMF and MPP⁺ treatment

significantly reduced expression of FIP200 (A) and Beclin 1 (C) expression as compared with control cells. N27 cells simultaneously treated with both GMF and MPP⁺ significantly reduced FIP200 and Beclin 1 expression as compared with other group. Bar graphs showing the effect of GMF and MPP⁺ exposure on the percentage of control (C). Western blot results were quantified by ChemiDoc-It2 imaging system analysis software, and the values are given as mean $\pm$ SEM (n=4). Bar graphs show the effect of GMF exposure on the relative intensity to the control (B). *p< 0.05 compared to control, GMF, and MPP⁺. *p< 0.001 compared with MPP⁺ and GMF+MPP⁺. Statistical significance was assessed with one-way ANOVA followed by Tukey's Kramer procedure and unpaired t-test was performed when comparing between groups by using GraphPad prism-7 software.

Fig. 4.

GMF reduces ATG protein complexes expression in dopaminergic N27 cells. N27 cells were seeded in T25 cell culture flask and incubated with GMF (100 ng/ml) and MPP⁺ (300 μ m) for 24 h under standard conditions. After the incubation period, cells were washed with PBS, cell lysates were prepared from these cells, and the concentrations of ATG protein complexes were determined by ELISA assay, using specific antibodies to ATG3, ATG5, ATG7, ATG16L and ATG12. GMF and MPP⁺ treatment significantly reduced expression of ATG protein complexes (A–E) as seen by ELISA results and compared with control cells. N27 cells incubated with both GMF and MPP⁺ significantly reduced expression of these proteins as compared with other group. Bar graphs show the effect of GMF and MPP⁺ exposure as a percentage of control (A–E) and the values are expressed as arbitrary units and given as mean $\pm$ SEM of four experiments in each group. * $p < 0.05$ compared to control. Statistical significance was assessed with one-way ANOVA followed by Tukey's Kramer procedure using GraphPad prism-7 software.

Fig. 5.

GMF increases ER stress markers expression in dopaminergic N27 cells. N27 cells were seeded and allowed to grow to confluency and incubated with GMF (100 ng/ml) and MPP⁺ (300 μM) for 24 h under standard conditions. After the incubation period, cells were washed with PBS and these cells prepared for western blot and immunofluorescence study, using specific antibodies such as p-PERK and IRE1α. GMF and MPP⁺ treatment significantly increased expression of p-PERK and IRE1α expression as compared with control cells (A, B, C and D). N27 cells treated simultaneously with both GMF and MPP⁺ significantly

increased p-PERK and IRE1 α expression as compared with other groups. Representative immunocytochemical images show the expression of IRE1 α is reduced in the cytoplasmic region and also IRE1 α translocates to the nuclei in the cells that were treated with GMF and/or MPP⁺ (Fig. 5E). Bar graphs show the effect of GMF and MPP⁺ exposure as a percentage of control (B and D). Western blot bands were quantified by ChemiDoc-It2 imaging system analysis software and the values are expressed as mean $\pm$ SEM (n=3). *p< 0.05 compared to control. Statistical significance was assessed with one-way ANOVA followed by Tukey's Kramer procedure using GraphPad prism-7 software.

Fig. 6.

GMF treatment increases LC3 and decreases LAMP1 immunocytochemical expression in rat dopaminergic N27 cells. N27 cells were incubated in the presence or absence of GMF (100 ng/ml) or MPP⁺ and immunostained for LC3 (green fluorescence) and LAMP1 (red fluorescence). Representative images show that exposure of N27 cells to GMF and MPP⁺ qualitatively increased LC3 and reduced LAMP1 expression as compared to control cells. Incubation of N27 cells with both GMF and MPP⁺ simultaneously qualitatively increased LC3 and reduced LAMP1 expression as compared with other group. Images were taken using Leica TCP SP8 laser scanning confocal microscope with a 405-nm diode laser and tunable super continuum white light laser using 63X oil immersion objective. Scale bar = 100 μ m.

Fig. 7.

GMF reduces lysosomal associated membrane proteins 1 and 2 (LAMP1 and 2) expression in dopaminergic N27 cells. N27 cells were seeded in T25 cell culture flask and incubated with GMF (100 ng/ml) and MPP⁺ (300 μM) for 24 h under standard conditions. After the incubation period, cells were washed with PBS and cell lysates were prepared from these cells for western blot analysis, using specific antibodies for LAMP1 and LAMP2. GMF and MPP⁺ significantly reduced the expression of LAMP1 and LAMP2 (indicated with arrow head) as compared with control cells. Incubation of N27 cells with both GMF and MPP⁺

simultaneously significantly reduced LAMP1 and LAMP 2 expression as compared with other group. Western blot bands were quantified by ChemiDoc-It2 imaging system analysis software and the values are expressed as mean $\pm$ SEM (n=3). *p< 0.05, *p < 0.001 and *p < 0.01 compared to control, GMF, and MPP⁺. *p< 0.001 compared with MPP⁺ and GMF +MPP⁺. Statistical significance was assessed with one-way ANOVA followed by Tukey's Kramer procedure and unpaired t-test was performed when comparing between groups by using GraphPad prism-7 software.

Fig. 8.

GMF decreases autophagic acidic vesicles or autophagosomes in dopaminergic N27 cells. N27 cells were incubated in the presence or absence of GMF (100 ng/ml) or MPP⁺ under standard condition. After incubation period cells were washed twice with PBS and stained with 10 μg/ml of acridine orange (AO) for autophagy lysosomal acidic vesicle (red fluorescence intensity) quantification. Representative images show that exposure of N27 cells to GMF significantly decreased the red fluorescence labelling intensity of AO with acidic vesicles correlates number of autophagy acidic vesicles when compared to control cells. Incubation of N27 cells with both GMF and MPP⁺ simultaneously significantly decreased autophagy acidic vesicles by reducing red fluorescent intensity as compared with other group (A). Images were taken using Leica TCP SP8 laser scanning confocal microscope with a 405-nm diode laser and tunable super continuum white light laser using 63X oil immersion objective. Scale bar = 100 μm. Autophagic acidic vesicle were quantified in the whole field by ImageJ, and the values are expressed as mean ± SEM (n=4). Bar graphs show the AO red fluorescence intensity and correlates with the number of acidic vesicles compared to the control (B). *p< 0.05 compared to control. Statistical significance was assessed with one-way ANOVA followed by Tukey's Kramer procedure using GraphPad prism-7 software.

Fig. 9.

GMF affects intracellular lysosomal morphology as detected by LysoTracker Red DND-99 fluorescent staining in dopaminergic N27 cells. N27 cells were seeded (3×10^6) in 6 well plate with coverslip and incubated with GMF (100 ng/ml) and/or MPP⁺ (300 μ M) for 24 h under standard conditions. After incubation period, the cells were washed with PBS. 1 mM of LysoTracker Red DND-99 (red fluorescence) added and coverslip mounted in a Vectashield [with DAPI for nuclear staining (blue fluorescence)] mounting media and finally visualized under Leica Confocal system. Representative confocal images show that GMF and MPP⁺ affects the cytoplasmic lysosomal mass compared with control cells (n=3). GMF and MPP⁺ qualitatively reduced the LysoTracker Red DND-99 fluorescent stained lysosomal mass as compared with control cells. Images were taken using Leica TCP SP8 laser scanning confocal microscope with a 405-nm diode laser and tunable super continuum white light laser using 63X oil immersion objective. Scale bar = 100 μ m.

Fig. 10.

GMF presence decreases p62/SQSTM immunocytochemical expression in murine primary neuronal cells. Primary WT and GMF-KO mouse neurons were incubated in the presence of MPP⁺ (20 μ M) under standard condition. After incubation period, cells were immunostained for p62/SQSTM (green fluorescence) and counter stained with VectaShield DAPI mounting media for nuclear staining (blue fluorescence). Representative images (n=4) show that exposure of primary WT neurons to MPP⁺ qualitatively reduced p62/SQSTM expression (green color) as compared with control cells. GMF-KO primary neurons treated with MPP⁺ shows qualitatively increased p62/SQSTM expression as compared with WT primary neurons. Images were taken using Leica TCP SP8 laser scanning confocal microscope with a 405-nm diode laser and tunable super continuum white light laser using 63X oil immersion objective. Scale bar = 100 μ m.

Fig. 11.

GMF presence increases α -synuclein and downregulates p62/SQSTM1 immunocytochemical expression in mouse primary neurons. WT and GMF-KO mouse primary neurons were incubated in the presence of MPP⁺ (20 μ M) under standard condition. After incubation period, cells were immunostained for α -synuclein (red fluorescence), p62/SQSTM1 (green fluorescence) and mounted with Vectashield [with DAPI for nuclear staining (blue fluorescent)]. Representative images show that exposure of WT primary neurons to MPP⁺ significantly increased α -synuclein and decreased p62/SQSTM1 expression as compared with control neurons. GMF-KO primary neurons treated with MPP⁺ shows increased p62/SQSTM1 and reduced α -synuclein expression as compared with WT primary neurons (n=3). Images were taken using Leica TCP SP8 laser scanning confocal microscope with a 405-nm diode laser and tunable super continuum white light laser using 63X oil immersion objective. Scale bar = 100 μ m.