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Suppression of glia maturation factor expression prevents 1-methyl-4-phenylpyridinium (MPP+)-induced loss of mesencephalic dopaminergic neurons

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Abstract

Inflammation mediated by glial activation appears to play critical role in the pathogenesis of Parkinson disease (PD). Glia maturation factor (GMF), a proinflammatory protein predominantly localized in the central nervous system was isolated, sequenced and cloned in our laboratory. We have previously demonstrated an immunomodulatory and proinflammatory functions for GMF, but its involvement in 1-methyl-4-phenylpyridinium (MPP^+), active metabolite of classical parkinsonian toxin MPTP, induced loss of dopaminergic neurons have not been studied. Here we show that altered expression of GMF has direct consequences on the production of reactive oxygen species (ROS) and NF-κB mediated production of inflammatory mediators by MPP^+. We examined MPP^+- induced dopaminergic neuronal loss in primary cultures of mouse mesencephalic neuron/glia obtained from GMF-deficient (GMF-KO) and GMF-containing wild type (Wt) mice. We demonstrate that deficiency of GMF in GMF-KO neuron/glia led to decrease production of ROS and downregulation of NF-κB mediated production of TNF-α and IL-1β as compare to Wt neuron/glia. Additionally, overexpression of GMF induced dopaminergic neurodegeneration, whereas GMF downregulation by GMF-specific shRNA protected dopaminergic neurons from MPP-induced toxicity. Subsequently, GMF deficiency ameliorates antioxidant balance, as evidenced by the decreased level of lipid peroxidation, less ROS production along with increased level of glutathione; and attenuated the dopaminergic neuronal loss via the downregulation of NF-κB mediated inflammatory responses. In conclusion, our overall data indicate that GMF modulates oxidative stress and release of deleterious agents by MPP^+ leading to loss of dopaminergic neurons. Our study provides new insights into the potential role of GMF and identifies targets for therapeutic interventions in neurodegenerative diseases.

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Keywords

Glia maturation factor; Reactive oxygen species; Oxidative stress; NF-κB; Cytokines; Neurodegeneration

Introduction

Parkinson's disease (PD), a neurodegenerative disorder, is characterized by progressive loss of dopaminergic neurons in substantia nigra pars compacta (SNc) resulting in debilitating motor symptoms. Postmortem human brain tissues and experimental studies in PD suggested that oxidative stress, mitochondrial dysfunction, apoptosis and inflammation contribute to the dopaminergic degenerative process, primarily due to the higher vulnerability of nigral neurons to free radicals (Dexter et al., 1994; Lastres-Becker et al., 2012; Dexter and Jenner, 2013). There is mounting evidences that both oxidative stress and inflammation play a pivotal role in the neurodegeneration associated with PD (Lin and Beal, 2006; Choi et al., 2011; Hirsch et al., 2012; Taylor et al., 2013). Oxidative stress is known to threat lipids, proteins, and DNA; this, along with decreased antioxidant enzymes, has been observed in the postmortem PD human brains and experimental models of PD (Henchcliffe and Beal, 2008; Khan et al., 2013). In neuro-degenerative disorders, glial activation is in close proximity to damaged or dying dopaminergic neurons. Additionally, patients with PD show persistent and high level of glial activation at the site of degeneration of dopaminergic neurons (Hirsch et al., 2003; Kim and Joh, 2006). Additionally, *in vitro* and *in vivo* investigations have shown that ROS is a potent trigger of microglial activation, stimulating the production of proinflammatory mediators and the up-regulation of NF-kB and p38 mitogen activated protein kinase (MAPK) signaling pathways leading to cellular degeneration and death (Castro-Caldas et al., 2012; Roy et al., 2012).

1-methyl-4-phenylpyridinium (MPP^+), oxidized form of the classical parkinsonian toxin MPTP, causes oxidative stress, mitochondrial damage and ultimately cell death. MPP^+ is highly selective and toxic for nigrostriatal dopaminergic neurons both *in vivo* and *in vitro* (Noelker et al., 2013; Zhai et al., 2013). The mechanisms underlying the dopaminergic degenerative process observed in PD are not well understood, which has hampered development of successful neuroprotective strategy. Glia Maturation Factor (GMF), a highly conserved brain protein, was previously isolated, sequenced and cloned in our laboratory (Lim et al., 1990; Kaplan et al., 1991; Zaheer et al., 1993). Recently it has been shown that GMF deficiency reduces neuronal loss in a murine model of Multiple sclerosis and Alzheimer's disease. It has been previously documented that GMF is an intracellular regulator of stress-activated signal transduction and activates p38 MAP kinase and transcription factor NF-κB in astrocytes (Lim and Zaheer, 1996; Zaheer and Lim, 1996; Lim et al., 2000; Zaheer et al., 2001). GMF has an apparently high rate of oxidase activity causing the formation of ROS, which can initiate lipid peroxidation and damage cell membranes (Kaimori et al., 2003; Zaheer et al., 2004). A study by Kaimori et al has demonstrated an important role for GMF in the pathophysiology of disease through increased oxidative stress (Kaimori et al., 2003). However, the role of GMF in MPP^+-induced dopaminergic neurodegeneration was not investigated. In this study, we measured

the markers of oxidative stress, as well as the expression of proinflammatory mediators and tyrosine hydroxylase, to investigate the hypothesis that deficiency of GMF may regulate cellular antioxidant defenses, and protect dopaminergic neurons from MPP^+-induced oxidative and inflammatory responses.

Experimental Procedures

Reagents

Enzyme-linked immunosorbent assay (ELISA) kits for tumor necrosis factor- alpha (TNF-α), and IL-1 β were purchased from R&D Systems (Minneapolis, MN). Assay kits for ROS and NF- κB were purchased from Cell Biolabs, Inc (San Diego, CA) and Abcam (Cambridge, MA). Assay kits for reduced glutathione (GSH) and Lipid peroxidation were from Cayman Chemical Company (Ann Arbor, MI) and OxisReseach (Burlingame, CA). Antibody for tyrosine hydroxylase was purchased from Millipore (Temecula, CA). MPP^+ iodide was purchased from Sigma-Aldrich (St. Louis, MO). Pregnant C57BL/6 mice were from Charles River (Wilmington, MA). GMF-deficient (GMF-KO) mice were maintained by backcross breeding to C57BL/6 for 10–12 generations. These mice were bred and maintained in the animal colony at The University of Iowa according to the guidelines of Institutional Animal Care and Use Committee (IACUC).

Cell culture

Primary mesencephalic glia/neuron cultures were prepared from the ventral mesencephalic tissues of embryonic day 12–13 mice essentially as described (Gao et al., 2002; Gao et al., 2003; Zhang et al., 2005). Immunocytochemical analysis indicated that the glia/neuron cultures were made up of approximately15% Iba1-positive microglia, 50% GFAP-positive astrocytes, and 40% NeuN-positive neurons, of which approximately 5% were TH+ neurons. There was no significant difference in the composition of the glia/neuron cultures between GMF-KO and Wt mice. Transient transfection to overexpress GMF was carried out essentially as described earlier (Zaheer et al., 2001; Zaheer et al., 2002; Zaheer et al., 2007). Replication-defective human adenovirus vector containing a full length GMF cDNA (GMF-V) or cytoplasmic lacZ cDNA (LacZ) at 10 MOI (multiplicity of infectivity) were added to cells in serum-free and antibiotic free DMEM/F12 medium for 4 h. At the end of infection period, cells were gently rinsed once and then neuron-glia culture media was added. The efficiency of infection was estimated to be over 95% as determined by X-gal staining. Mixed cultures were grown for 3–5 days before indicated treatments. Immunostaining for dopaminergic (DA) neurons was performed following fixation of the cultured cells by using TH-antibody (Millipore) (Takahashi et al., 1997; Rommelfanger et al., 2004)).

MPP^+ Treatments

For MPP^+ treatments, primary cultures of mouse glia-neuron obtained from Wt and GMF-KO mice were seeded into 24 well plates or cell culture flasks (25 cm^2) at 1X 10^6 cells /ml in complete medium for overnight at 37°C. Then the cells were incubated with various concentrations of MPP^+ (5, 10 and 20 μM) for 24 and 48 h in serum free medium. After the termination of incubation, cells were collected for the assays of LPO, GSH, NO, ROS, and

the culture supernatant were collected for estimation of proinflammatory cytokines. Protein was assayed in culture supernatant by BCA method.

Rotenone treatments

Primary cultures of mouse glia-neuron obtained from Wt and GMF-KO mice were seeded into cell culture flasks (25 cm^2) in complete medium for overnight at 37°C. Then the cells were incubated with 100 nM concentration of rotenone for 24 and 48 h in serum free medium as described (Sherer et al., 2003). After the termination of incubation, cells were collected for the assays of LPO.

Lipid Peroxidation and Nitric Oxide (NO) Assays

Measurement of malonaldehyde (MDA) and 4-hydroxyalkenals as an indicator of lipid peroxidation, and NO were detected according to the kit manufacturer's instructions. The MDA results were expressed as μmol MDA/mg protein. The NO results were expressed as μmol NO release/mg protein.

Glutathione (GSH)

GSH level was estimated according to the kit manufacturer's instructions. The GSH results were expressed as in terms μmol GSH formed/mg protein using a molar extinction coefficient of 13.6 $X10^3$ M^{-1} cm^{-1}

Measurement of ROS Generation

ROS formation was detected by a commercially available kit according to the manufacturer's instruction using a compound, DCFH-DA. In brief, cells were seeded into 96 well ELISA plate with 100 ul of DCFH-DA/media for 1 h at 37°C followed by MPP^+ treatment. Plate was subjected to fluorescence analysis at 485 nm excitation and 535 nm emissions using a fluorescence plate reader (Turner biosystems, Madison, WI).

NF-κB Assay

NF-κB activity was determined by mouse phospho-Rel A/NF-κB p65 immunoassay kit (Abcam, Cambridge, MA) according to the manufacturer's instruction. Briefly, cells were seeded in 96 well plate and incubated with 5, 10 and 20 μM concentration of MPP for 24, and 48 h at 37 °C. After pre incubation in blocking buffer, cells were incubated overnight with primary antibody at 4 °C followed by appropriate secondary antibody. Cells were then exposed to substrate solution for 20–40 minutes and read the plate using a fluorescence plate reader.

Estimation of cytokines by ELISA

TNF-α, and IL-1β, protein concentrations in the culture supernatants were estimated by ELISA as specified in the manufacturer's protocol. The lower detection limits of these ELISA are in the range of 8–12 pg/ml.

Tyrosine hydroxylase (TH) immunocytochemistry

Cultures were washed with phosphate buffer saline (PBS), fixed for 30 min at room temperature with 4% paraformaldehyde in PBS. Following three rinses in PBS, cultures were pre-incubated for 30 min with 3% normal goat serum (NGS). Cells were then incubated overnight at 4°C with anti-TH antibody (1/500, Millipore) in 1% NGS, followed by biotin-conjugated goat anti-rat Ig, avidin-biotin complex (Vectastain kit, Vector Laboratories, Burlingame, CA, USA), and 3,3′-diaminobenzidine as substrate. TH-positive cells in these cultures could be identified as dopaminergic neurones. NIH Image J software was used to quantify the TH positive neurons. For quantification purposes, at least five randomly selected fields were examined and averaged. Numbers of TH positive cells are represented as percentage of control.

Statistical Analysis

Results are expressed as mean ±SEM. Statistical analysis of the data was done by applying the analysis of variance (ANOVA), followed by Tukey's Kramer analysis using GraphPad InStat 3. The p value less than 0.05 were considered statistically significant.

Results

Dopaminergic neuron loss significantly enhanced by GMF-overexpression

To evaluate the role of GMF in selective dopaminergic cell loss, we infected primary cultures of mouse mesencephalic neuron/glia with a replication- defective adenovirus containing a full length GMF cDNA (GMF-V, 10 MOI) or vehicle control phosphate buffer saline (PBS), GMF-V plus GMF- shRNA or control shRNA. Dopaminergic neurons were immunostained with anti-TH antibody after seven days. The results demonstrated that the overexpression of GMF induced dopaminergic neurodegeneration, whereas GMF-downregulation by GMF-specific shRNA protected dopaminergic neurons in culture. Control shRNA treatments had no effect (Figure 1A). The results also revealed significantly reduced numbers of TH-positive neurons in GMF overexpressing neuron/glia cells. However, GMF-specific shRNA rescued the loss of TH positive nuerons in culture as compared to control shRNA (Figure 1B). GMF levels in cells were determined by ELISA following vehicle control PBS, GMF-V, GMF-V plus GMF- shRNA or control shRNA. Results (Figure 1C) show significant overexpression of GMF by GMF-V administration and efficient down-regulation of GMF expression by GMF-shRNA treatment. Control shRNA treatments had no effect.

Significant suppression of MPP+-induced dopaminergic neuron toxicity in neuron/glia cultures derived from GMF-deficient mice

The overreaching hypothesis of this study is that GMF plays a pivotal role in regulation of the glial mediated neuroinflammatory cascade that results in degeneration of dopaminergic neurons. To test our hypothesis, primary neuronal/glial cells were prepared from day-14 embryos obtained from GMF-deficient mice and wild type littermates. Mixed neuronal/glial cells were grown on coated plates for 4–6 days. Twenty four hours following parkinsonian toxin MPP+ (5 μM) treatment, the extent and selectivity of neurodegeneration were

determined by tyrosine hydroxylase (TH)-immunostaining of neurons. The results have shown selective degeneration of DA neurons by MPP+ in neuron-glia derived from wild type mice as compare to barely detectable degeneration in cells derived from GMF-deficient mice (Figure 2A). Moreover, our quantitative data indicates significant decrease in number of TH-positive neurons in neuron-glia derived from wild type mice as compared to cell derived from GMF-deficient mice (Figure 2B). DA neurons from GMF-deficient mice were comparatively resistance to MPP+ toxicity. These results show that GMF-deficient dopaminergic cell are more resistant to MPP-induced toxicity than the GMF-containing Wt cells.

GMF-downregulation protects dopaminergic neurons from MPP-induced toxicity

To validate our data on downregulation of GMF to protect the dopaminergic neuronal loss, we used GMF-shRNA to inhibit GMF expression in mixed neuron/glia cultures. Neuron/glia cells were exposed to GMF-shRNA for 48 h prior to MPP^+ treatment. The cultures were then immunostained for TH-positive neurons (**A**). We found significant loss of TH-positive cell in MPP+ treated cells (**B**) as compared to controls (**A**), and treatment with GMF-shRNA (**C**) reduced the loss of dopaminergic neurons as compared to their control shRNA (D). These results demonstrate that GMF-shRNA treatment attenuated not only the loss of dopaminergic neurons numbers but also the observed shrunken neurite processes due to MPP^+ treatment (Figure 3A & 3B).

GMF deficiency protects primary neuron-glia from MPP^+-induced oxidative damage

Reactive oxygen (ROS) and nitrogen (RNS) species are considered as key mediators in inflammation and play an important role in the pathogenesis of PD. Increased levels of reactive oxygen and nitrogen species are found in the inflammatory microenvironment, which can overwhelm the inherent antioxidant defense mechanisms causing cell death. Therefore, we studied the levels of ROS and NO following MPP+ treatment in relation to GMF levels by using different genotypes (wild type and GMF-deficient) of neuron/glia cells. Primary neuron/glia cells were treated with MPP+ (5, 10 and 20 μM) for 24 and 48 h. Lipid peroxidation in terms of MDA concentration following MPP^+ treatment in the absence of GMF (GMF-deficient neuron- glia) or presence of GMF (Wt neuron-glia) was estimated in the culture supernatant to demonstrate the extent of GMF-dependent oxidative damage to lipids. We found significantly ($p< 0.05$) decreased level of MDA (1.74± 0.12; 2.39±0.09 and 2.98± 0.03 μmol MDA/mg protein) in GMF-deficient neuron-glia cultures as compared to control Wt astrocytes (4.09± 0.37; 5.95±0.23 and 8.29±0.94 μmol MDA/mg protein) at 24 h with 5, 10 and 20 μM MPP^+ treatments (Figure 4A). We found almost similar effect at 48 h of MPP^+ treatments. We demonstrate that GMF-deficient neuron-glia cells showed significant and marked reduction of MDA level as compared to Wt neuron-glia cells followed by MPP^+ treatment. Thus, this culture system allowed us to examine specific effects of GMF-deficient in MPP^+-induced oxidative damage to lipids in primary neuron-glia cultures.

GSH is the major antioxidant in the brain cells which buffers free radicals. The GSH content following MPP^+ treatment in the primary neuron-glia cells derived from GMF-deficient and Wt mice was estimated to demonstrate the role of GMF in MPP^+-induced free radicals

imbalance in neuron-glia culture system (Figure 4B). A significant increase ($p<0.05$) in GSH content was detected in the cells lacking GMF when compared to cells expressing GMF following MPP^+ treatment for 24 h in a dose -dependent (5, 10 and 20 μM) fashion. Similarly, we found significant ($p<0.05$) increase in GSH content in the GMF-deficient derived neuron-glia as compared to Wt neuron-glia following MPP^+ treatment at 48 h in a dose-dependent manner. There was no significant difference at the basal level of MDA and GSH in neuron/glia cell of either genotype. Moreover, we estimated the lipid peroxidation following rotenone treatment (as a positive control) to assess how the lack of GMF affects the regulation of oxidative stress in rotenone-induced mixed culture (Figure 4C). We found almost similar effect following rotenone treatment in the cells of both genotypes. We demonstrate that GMF-KO neuron-glia cells showed significant and marked reduction of MDA level as compared to Wt neuron-glia cells followed by MPP^+ and rotenone treatments.

Pathologic studies in postmortem PD brains and in MPTP-treated mice suggest that NO plays a critical role in PD. Therefore, in the present study, we have estimated the production of total NO (Figure 5A) and we found significantly decreased level of total NO in GMF-KO neuron-glia cells (6.54± 0.17; 8.17±0.85 and 11.31± 0.19 μmol/L) when compared to Wt neuron-glia cells (15.82± 0.53; 30.92±1.06 and 46.21± 5.88μmol/mg protein) following MPP^+ toxicity in dose-dependent fashion at 24 h. Similarly, we found significant ($p<0.05$) decrease in NO level in the GMF-KO neuron-glia cells as compared to Wt neuron-glia cells following MPP^+ treatment at 48 h. To determine whether deficiency of GMF suppress the MPP^+-induced ROS production in neuron-glia cells, we measured total ROS production in Wt neuron-glia cells and GMF-deficient neuron-glia cells following MPP^+ treatment at two different time points. The production of ROS within the cells was determined by monitoring a conversion of $DCFH_2$-DA to DCF. ROS level was significantly ($p<0.05$) decreased in GMF-deficient neuron-glia cells (50.98± 7.42; 60.78±9.80 and 76.17±15.30 DCF) when compared to Wt neuron-glia cells (168.62± 23.52; 230.39±8.82 and 295.09±34.31 DCF) at 5, 10 and 20 μM respectively, at 24 h after MPP^+ treatment. Similarly, we found significant ($p<0.05$) decrease in ROS in the GMF-deficient neuron-glia cells as compared to Wt neuron-glia cells following MPP^+ treatment at 48 h (Figure 5B). There was no significant difference at the basal level of NO and ROS of neuron/glia cell of each genotype. Thus, on the basis of these data we report that GMF-deficient neuron-glia cells are resistant to MPP^+-induced oxidative damage due to decrease lipid peroxidation, ROS, and NO production.

GMF-Dependent Down-regulation of NF-κB Activity in primary neuron-glia cultures

Among many transcription factors involved in the intracellular signaling pathway, nuclear factor-kappa B (NF-κB) is the most important factor known to be exquisitely sensitive to cellular oxidative status. There is mounting evidence that endogenous ROS promote NF-κB activation and NF-κB regulates the expression of several proinflammatory cytokines. Our results (Figure 6) show significant decrease ($p< 0.05$) in NF-κB activity in GMF-deficient neuron-glia cells when compared to Wt neuron-glia cells following 5, 10, 20 μM MPP^+ treatments for 24, and 48 h. There is no significant difference at the basal level of NF-κB activity in neuron/glia cell of either genotype.

Diminished level of cytokines in primary neuron-glia cultures following MPP^+-treatment

ROS has been identified as second messengers in cells and play a role in response to a number of inflammatory signaling mediators, including tumor necrosis factor-a (TNF- α), interleukin-1β (IL-1β), and post-translation modification of signaling molecules (Wang et al., 2007). ROS is required for the downstream signaling effects. Moreover, TNF-α, and IL-1β are reported be over-expressed under neurotoxin insults. We found significantly ($p<0.05$) decreased release of TNF-α (120.22±20.56; 196.61 ±33.61 and 287.72±43.61 pg/ml) and IL-1β (63.31±10.42; 84.84±7.02 and 190.16±27.52 pg/ml), at 5, 10 and 20 μM respectively, in GMF-deficient neuron-glia cells when compared to the release from Wt neuron-glia cells TNF-α (205.22±31.11; 383.52±38.02 and 501.01±31.11) and IL-1 β (254.97±10.97; 273.01±13.27 and 256.54±25.51 pg/ml) with concentration 5, 10 and 20 μM MPP^+ respectively following MPP^+ treatment at 24 h (Figure 7). Similarly, we found the significant ($p<0.05$) decrease in proinflammatory cytokines in the GMF-deficient neuron-glia cells as compared to Wt neuron-glia cells following MPP^+ treatment at 48 h in a dose-dependent manner. These results indicate that MPP^+-induced release of proinflammatory cytokines were significantly inhibited in GMF-deficient neuron-glia cells. There is no significant difference at the basal level of cytokines in neuron/glia cell of either genotype.

Discussion

In the current study we examined whether GMF is required in the production of proinflammatory mediators such as reactive oxygen species (ROS), reactive nitrogen species (RNS), NF-κB, and cytokines by parkinsonian toxin MPP+, the active metabolite of MPTP, in *in-vitro* studies. For this purpose we conducted experiments on primary cultures of mouse mesencephalic neuron/glia cells derived from wild type (GMF containing) mice, GMF-deficient (GMF-KO) mice, and GMF-overexpressing cells. Overexpression of GMF in cultured cells was achieved by transfection with a highly efficient GMF-adenovirus construct (Zaheer et al., 2001; Zaheer et al., 2002; Zaheer et al., 2007). Thus, by studying side-by-side signal transudations in the total absence of endogenous GMF and in the overwhelming presence of GMF (along with those in the normal amount of GMF), a stark contrast was obtained that helped delineate the functions of GMF at cellular level in the setting of PD.

The overreaching hypothesis of this study is that GMF plays a pivotal role in regulation of the glial mediated neuroinflammatory cascade that results in degeneration of DA neurons. To test our hypothesis, primary neuron-glia cells derived from GMF-deficient and wild type mice were treated with MPP+ and the extent and selectivity of neurodegeneration were determined by TH-immunostaining of neurons. The results have shown selective degeneration of DA neurons by MPP+ in neuron-glia derived from wild type mice as compare to cells derived from GMF-KO mice. DA neurons from GMF-deficient mice were comparatively resistance to MPP+ toxicity. These results have shown less loss of DA neurons in absence of GMF, whereas overexpression of GMF via adenoviral delivery aggravated neuronal loss. Reactive oxygen (ROS) and nitrogen (RNS) species are considered as key mediators in inflammation and play an important role in the pathogenesis of PD. Evaluation of production of ROS, NO, NF-κB activation, and inflammatory

cytokines changes by MPP+ treatment in cells of different genotypes (derived from wild type and GMF-deficient) demonstrated that down regulation of GMF function in primary mouse neuron-glia cells confers protection against the neurotoxin MPP^+. The GMF knockdown resulted in decreased oxidative stress, decreased production of inflammatory mediators and ultimately less dopaminergic neuronal death. We also demonstrate that overexpression of GMF in primary neuron-glia culture enhanced the loss of dopaminergic neurons and suppression of endogenous GMF by using GMF-shRNA protected the dopaminergic neurons from MPP^+-induced toxicity. To the best of our knowledge, this study is the first to document a significant effect of GMF on MPP^+ toxicity in primary mouse neuron-glia.

Our results showed that GMF-deficient neuron-glia are capable of fighting against MPP+-induced oxidative stress and a variety of proinflammatory mediators such as TNF-α and IL-1β through the suppression of NF-κB, which suggests that deficiency of GMF could effectively reduce MPP^+-induced toxicity. We and others have previously reported that GMF deficiency decrease the oxidative loads and limits the inflammatory response via the downregulation of NF-κB and its responsive genes (Kaimori et al., 2003; Zaheer et al., 2007; Zaheer et al., 2008). Oxidative stress is a central event and one of the major factors leading to cell death in PD (Jenner, 1998; Hwang, 2013; Subramaniam and Chesselet, 2013). MPP+ is thought to selectively kill dopaminergic neurons and to elicit severe parkinsonism-like symptoms in humans, primates and culture systems (Langston and Irwin, 1986; Spittau et al., 2012; Khan et al., 2013). ROS serving as a key mediator are responsible for MPP+-induced oxidative damage in dopaminergic neuronal cells. This contribution of ROS may be related to translocation of NF-κB and activation of proinflammatory mediators, which together contribute to cell death. Glutathione, a major endogenous antioxidant, decreases the toxic effects of ROS. Altered cellular redox status can activate redox-sensitive transcription factors such as NF-κB. In the present study, we found increased lipid peroxidation product MDA level, nitric oxide and overproduction of ROS which, in turn, caused oxidative damages to membrane lipid and proteins, and ultimately led to a decrease in GSH content. Our results are consistent with previous reports where MPP^+-induced toxicity leads to oxidative damage and dopaminergic neurodegeneration (Zhang et al., 2007; Xiao et al., 2011). We also found that GMF-deficient neuron/glia cells are resistant to rotenone induced oxidative stress in term of lipid peroxidation as compare to cells derived from Wt mice. These results demonstrate that deficiency of GMF decreases oxidative stress induced by MPP^+ and rotenone as well.

Several lines of evidence suggest an important relationship between neuroinflammation and neuronal loss in PD (Hirsch et al., 2012; Gonzalez et al., 2013; More et al., 2013). NF-κB is a major regulator of a number of physiological processes in neurons, astrocytes, and microglia, including defense against oxidative stress and neuroinflammation (O'Neill and Kaltschmidt, 1997). Altered cellular redox status can activate redox-sensitive transcription factors such as NF- κB (Janssen-Heininger et al., 2000; Acquier et al., 2013; Yang et al., 2013). It has been previously reported that over expression of GMF increased IL-1β and TNF-α cytokine production by upregulation of NF-κB nuclear translocation in primary mouse astrocytes (Zaheer et al., 2007) (Zaheer et al., 2001). These previous findings and those of the present study show that GMF deficiency prevents MPP+-induced nuclear

translocation of NF-κB and inhibits production of proinflammatory cytokines TNF-α and IL-1β in primary neuron-glia. These results suggest a mechanistic basis for the attenuation of neuroinflammation in neuron-glia by GMF-shRNA.

Conclusion

The overall results have clearly demonstrated that enhanced expression of GMF is associated with dopaminergic neurotoxicity whereas deficiency of GMF might retard the progression of MPP^+-induced toxicity in neuron-glia by resistant to oxidative stress and downregulation of NF-κB mediated inflammatory responses. Understanding the signal transduction pathways that facilitate the biological function of GMF may improve our knowledge of the events leading to loss of dopaminergic neurons and may help identify targets for therapeutic interventions.

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Glossary

ANOVA	analysis of variance
DA	dopamine
DCFDA	2',7'–dichlorofluorescein diacetate
ELISA	enzyme-linked immunosorbent assay
GMF	glia maturation factor
GFAP	glial fibrillary acidic protein
GMF-KO	GMF knockout
GSH	glutathione
Iba1	ionized calcium binding adaptor molecule 1
IL-1β	interleukin-1beta
MDA	malonaldehyde
MAPK	mitogen activated protein kinase
MPP	1-methyl-4-phenyl-pyridine
MPTP	1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine
NF-κB	nuclear transcription factor-κB
NGS	normal goat serum
NO	nitric oxide
PBS	phosphate buffer saline

PD	Parkinson’s disease
ROS	reactive oxygen species
RNS	reactive nitrogen species
SNc	Substantia nigra pars compacta
TH	tyrosine hydroxylase
TNF-α	tumor necrosis factor-alpha
Wt	wild type

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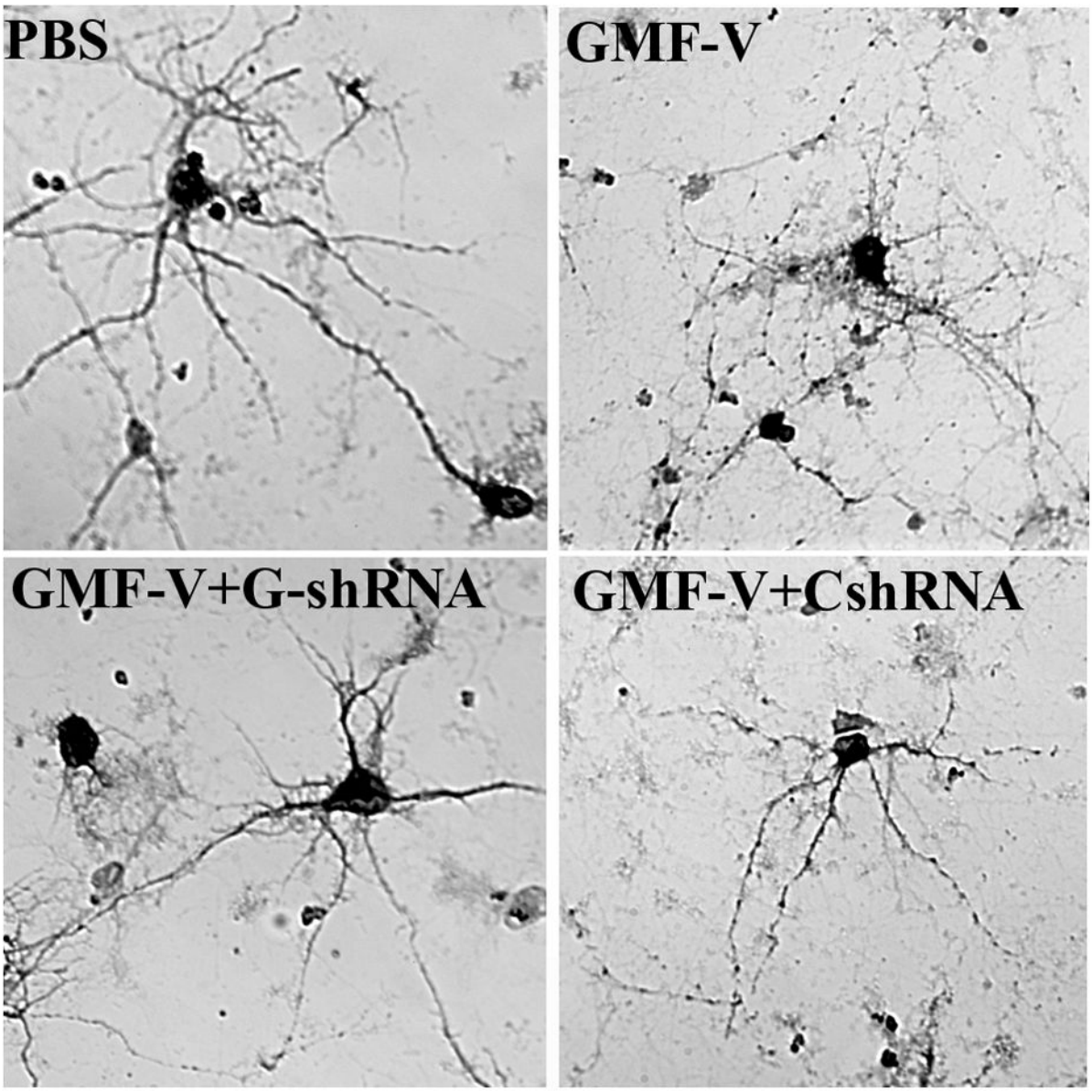
Highlights

- Overexpression of GMF exacerbates dopaminergic neuron loss
- Selective inhibition of GMF expression prevents loss of dopaminergic neurons
- GMF-deficient neuron/glia are resistant to oxidative stress



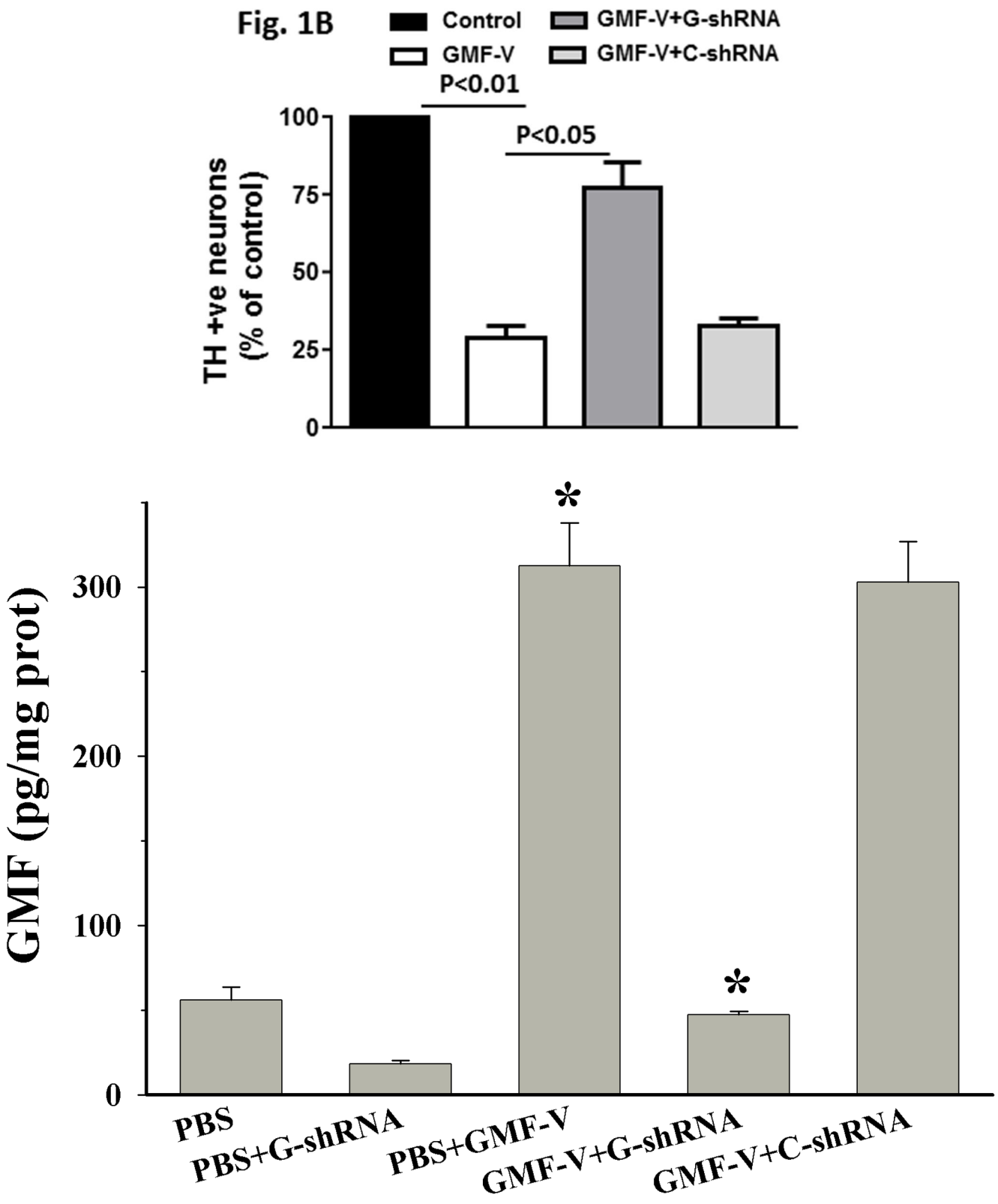
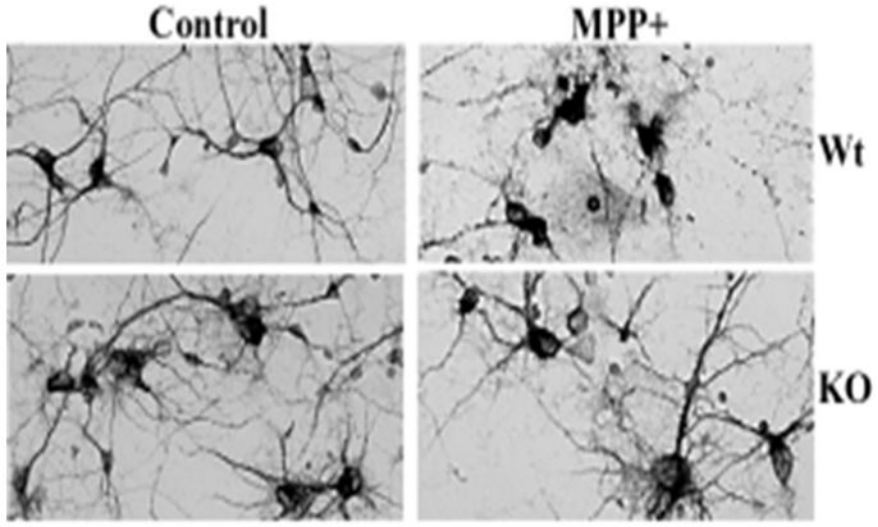


Figure 1.

Dopaminergic neuron loss significantly enhanced by GMF-overexpression. Primary glia/neuron cells were prepared from the ventral mesencephalic tissues of embryonic day 12–13 wild type mice essentially as described in Methods. Transient transfection to overexpress GMF was carried out by the addition of a replication-defective adenovirus containing a full length GMF cDNA (GMF-V, 10 MOI) or vehicle control (None), GMF-V plus GMF-shRNA (G-shRNA) or control shRNA (CshRNA) in serum-free and antibiotic free DMEM/F12 medium for 4 h. At the end of infection period, cells were gently rinsed once and then neuron-glia culture media was added. Immunostaining for dopaminergic (DA) neurons was performed using TH-antibody. Figure 1A shows representative images stained for DA neurons (TH-positive cells). Figure 1B shows quantification of TH-positive cells. The data shown are representative of at least three separate experiments. Figure 1C shows GMF levels following various treatments in primary neuronal cells as determined by ELISA. Note efficient down-regulation of GMF expression by GMF-shRNA administration in cells.



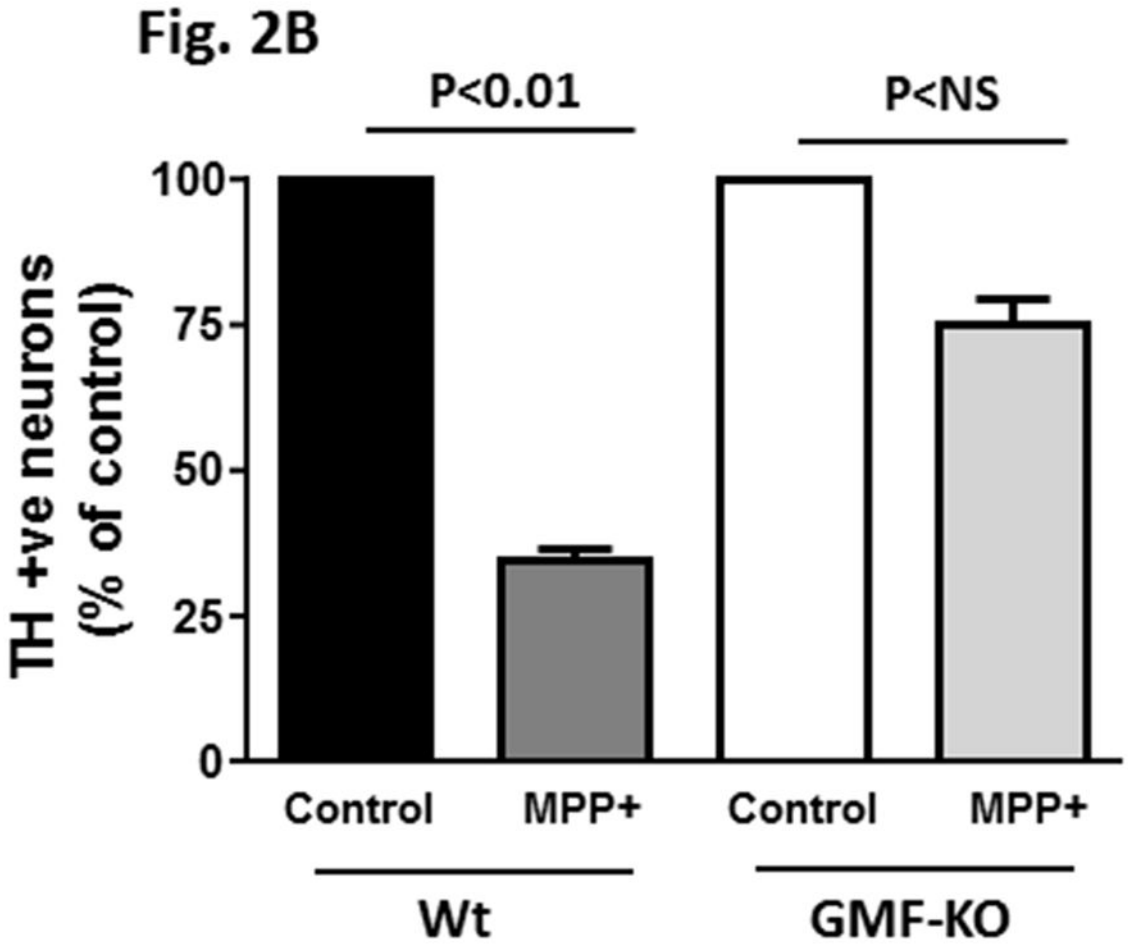
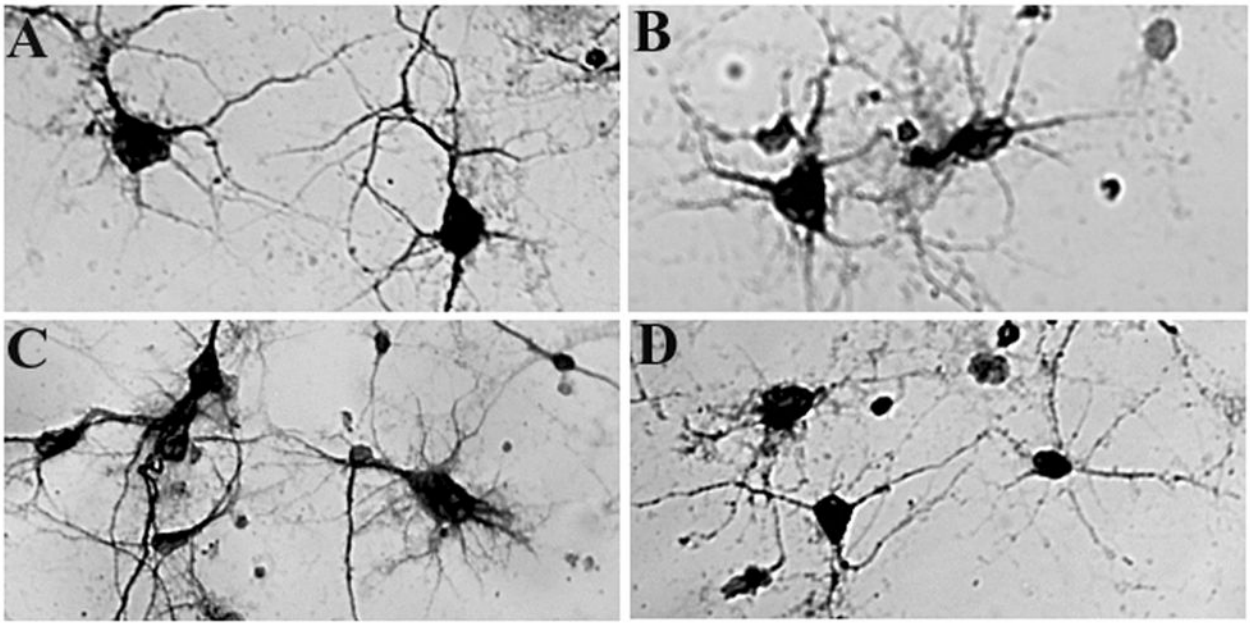


Figure 2.

Significant suppression of MPP+-induced dopaminergic neuron loss in neuron/glia cells derived from GMF-KO mice. Primary neuron-glia cultures derived from Wt and GMF-KO

mice were treated with MPP+ (5 μM) for 24 h. Representative photomicrographs show prominent TH^+ DA neuronal fiber degeneration in cultures from Wt mice compare to barely detectable degeneration in GMF-KO cells (Figure 2A). Figure 2B shows quantification of TH-positive cells.



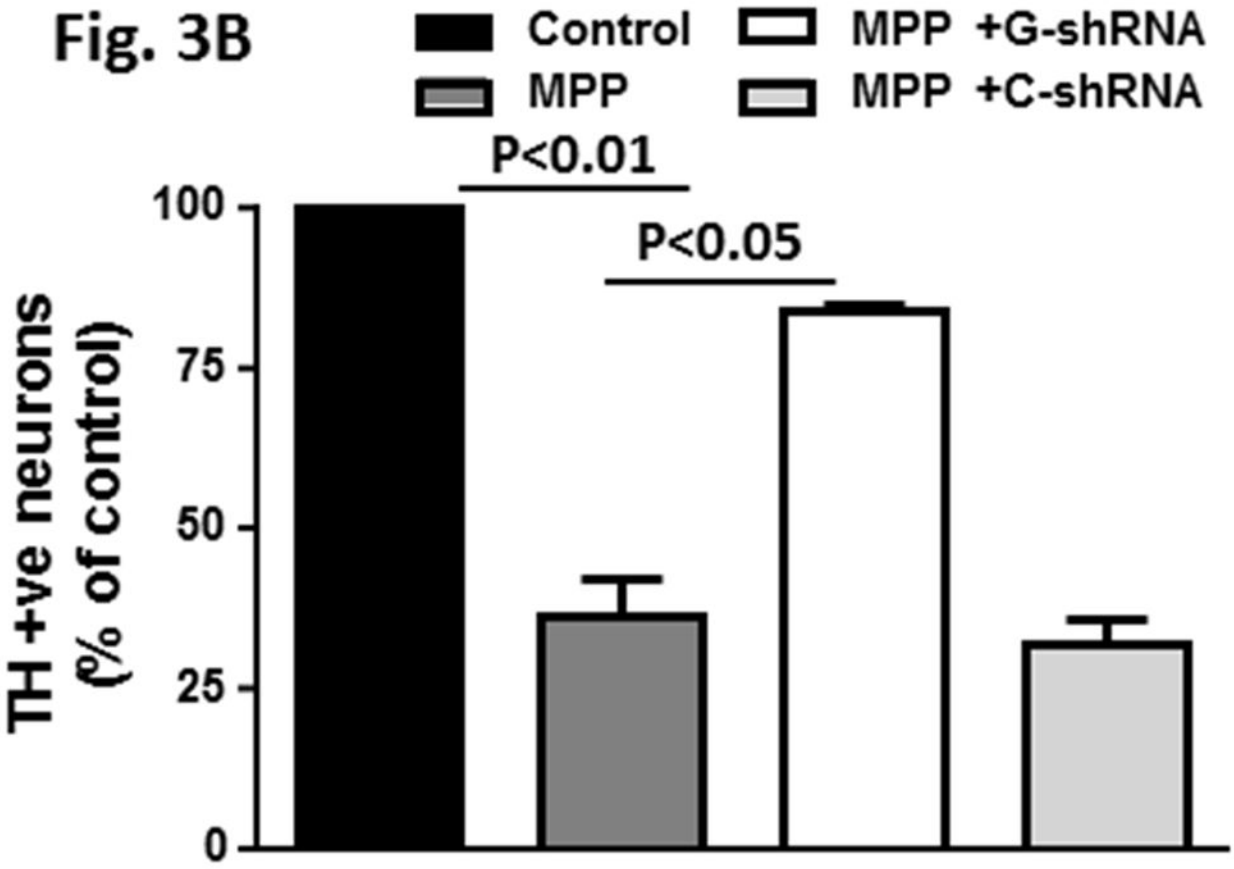


Figure 3.

GMF-downregulation protects dopaminergic neuron from MPP-induced toxicity. The neuron/glia cultures were exposed to GMF-shRNA for 48 h prior to MPP treatment. The cultures were immunostained for TH-positive neurons (Figure 2A). A, vehicle control; B, MPP (10 uM); C, MPP+ G-shRNA; D, MPP + control CshRNA. Figure 2B shows quantification of TH-positive cells. The data shown are representative of at least two independent experiments.

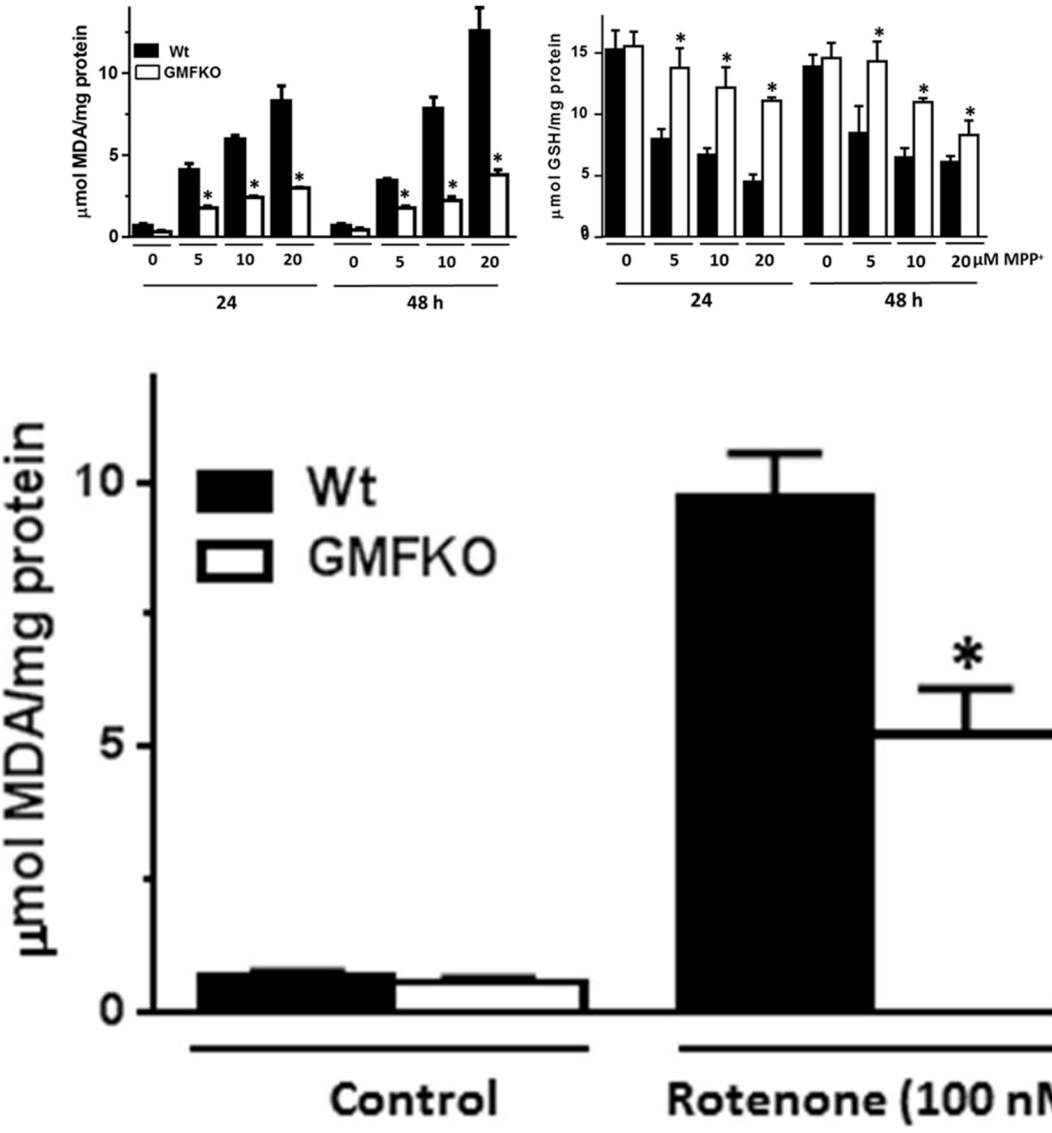


Figure 4.

Primary cultures of neuron-glia derived from Wt and GMF-KO mice were incubated with MPP^+ (0, 5, 10, 20 µM) for 24 and 48, h. Levels of MDA (A), and GSH (B), were assayed in the cell lysate. (n=4); Values are means ± SEM. *P < 0.05, compared with the Wt neuron-glia and GMF-KO neuron-glia treated with MPP^+. Primary cultures of neuron-glia derived from Wt and GMF-KO mice were incubated with rotenone (100 nM) for 24 and 48, h. Levels of MDA was assayed in the cell lysate. (n=4); Values are means ± SEM. *P < 0.01, compared with the Wt neuron-glia and GMF-KO neuron-glia treated with rotenone (C).

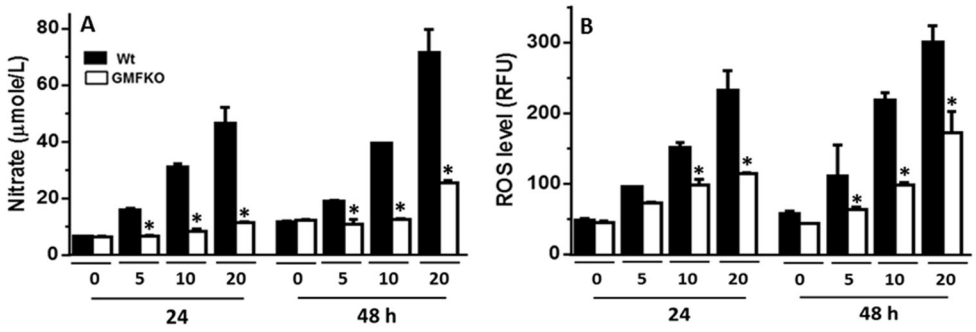


Figure 5.

Primary cultures of neuron-glia derived from Wt and GMF-KO mice were incubated with MPP^+ (0, 5, 10, 20 μM) for 24 and 48, h. NO in term of nitrate and ROS production were assayed according to manufacturer's instructions. Values are means ± SEM for n=4. *P < 0.05, compared with the Wt neuron-glia and GMF-KO neuron-glia treated with MPP^+.

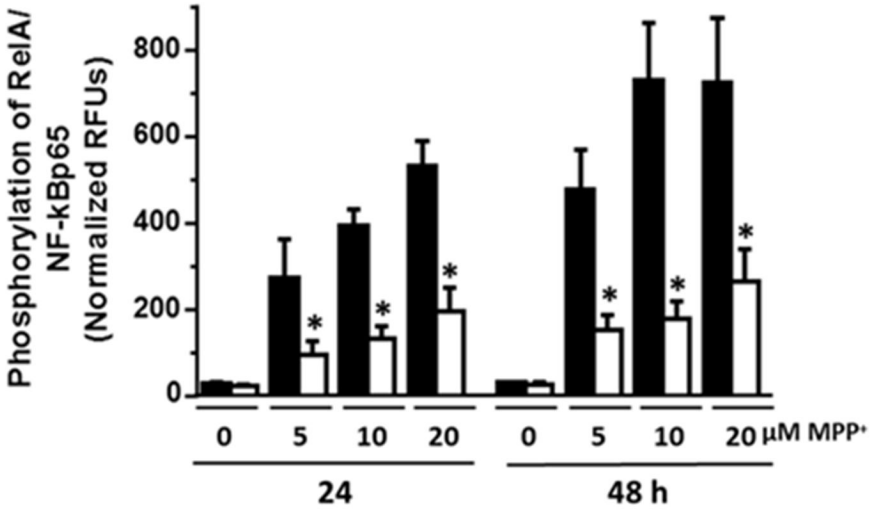


Figure 6.

Reduction in NF-κB activity in GMF-KO neuron-glia compared to Wt neuron-glia. Cells were seeded and treated with 5, 10 and 20 μM MPP^+ for 24 and 48 h and activity of NF-κB were measured by ELISA according to manufacturer's protocol. A significant time-dependent suppression of NF-κB activation following MPP^+ treatment was seen in GMF-KO neuron-glia as compared with Wt neuron-glia. Values are means ± SEM. *$P < 0.05$, compared with the Wt neuron-glia and GMF-KO neuron-glia treated with MPP^+, n=5.

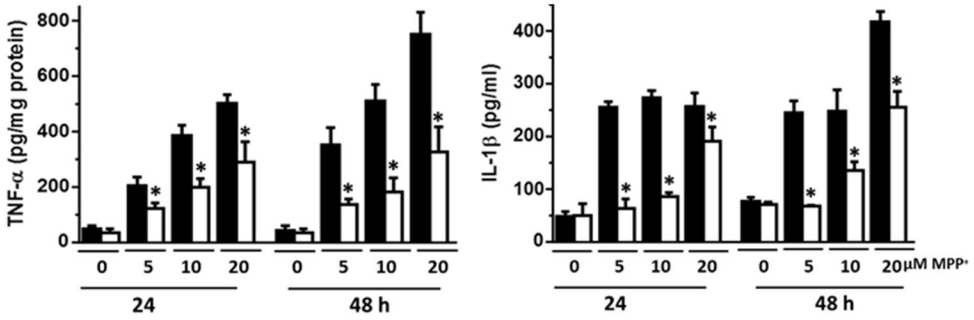


Figure 7.

Reduced expressions of inflammatory cytokines in primary cultures of GMF-KO neuron-glia compared to Wt neuron-glia following MPP^+ treatment. Cells were incubated with MPP^+ for 24 and 48 h at 5, 10 and 20 μM MPP^+. After the incubation period was over the culture media were collected for the assay of proinflammatory cytokines by ELISA. Levels of TNF-α, and IL-1β, were significantly decreased in the GMF-KO neuron-glia when compared to the Wt neuron-glia following MPP^+-treatment. Values are means ± SEM. *$P < 0.05$, compared with the Wt neuron-glia and GMF-KO neuron-glia treated with MPP^+, n=5.