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Inhibition of calpain activation protects MPTP-induced nigral and spinal cord neurodegeneration, reduces inflammation, and improves gait dynamics in mice

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Abstract

Parkinson's disease (PD) is the most common neurodegenerative movement disorder, resulting in dopaminergic (DA) neuronal loss in the substantia nigra pars compacta (SNpc) and damage to extranigral spinal cord neurons. Current therapies do not prevent the disease progression. Hence, developing efficacious therapeutic strategies for treatment of PD is of utmost importance. The goal of this study is to delineate the involvement of calpain-mediated inflammation and neurodegeneration in SN and spinal cord in MPTP-induced parkinsonian mice (C57BL/6N), thereby elucidating potential therapeutic target(s). Increased calpain expression was found localized to tyrosine hydroxylase (TH⁺) neurons in SN alongside with significantly increased TUNEL positive neurons in SN and spinal cord neurons in MPTP mice. Inflammatory markers Cox-2, caspase-1, and NOS-2 were significantly up-regulated in MPTP mice spinal cord as compared to control. These parameters correlated with the activation of astrocytes, microglia, infiltration of CD4⁺ / CD8⁺ T cells and macrophages. We found that subpopulations of CD4⁺ cells (Th1 & Tregs) were differentially expanded in MPTP mice, which could be regulated by inhibition of calpain with the potent inhibitor calpeptin. Pre-treatment with calpeptin (25 µg/kg, i.p.) attenuated glial activation, T cell infiltration, nigral dopaminergic degeneration in SN, and neuronal death in spinal cord. Importantly, calpeptin ameliorated MPTP-induced altered gait parameters (e.g. reduced stride length and increased stride frequency) as demonstrated by analyses of spatio-temporal gait indices using ventral plane videography. These findings suggest that calpain plays a pivotal role in MPTP-induced nigral and extranigral neurodegenerative processes, and may be a valid therapeutic target in PD.

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Conflict of interest Authors have no conflicts of interest to declare.

Keywords

axonal degeneration; calpain inhibition; extranigral degeneration; gait dynamics; neuroinflammation; spinal cord

Introduction

Parkinson's disease (PD) is a chronic progressive neurodegenerative movement disorder with unremitting loss of dopaminergic neurons in midbrain substantia nigra pars compacta (SNpc), leading to depletion of dopaminergic neurotransmission in the striatum. PD pathophysiology includes multiple extranigral and extrastriatal degenerative sites in the nervous system (e.g., spinal cord), with resulting motor dysfunction and cognitive impairment in mid to later stages of PD [1-4]. Thus far, attempts to halt the progression of PD by blocking mitochondrial and/or oxidative pathways have demonstrated limited success. Also, dopaminergic replenishment therapy may further aggravate later symptoms of the disease [5,1]. Inflammation has been implicated in the pathophysiology of many neurodegenerative diseases [6-10], and current evidences suggest that chronic inflammation may have a direct role in perpetuating the disease progression in PD. Although the mechanisms involved in this process are not clearly understood, activated microglia, the innate immune cells in the CNS, may contribute to neuronal demise [7,9]. Thus, the focus of PD research has been recently directed to targeted therapies and development of agents with multifaceted neuroprotective and anti-inflammatory efficacy.

Activated glial cells (microglia, astrocytes) present in parkinsonian SN and spinal cords may contribute to local neuronal death [11-14]. In turn, products released from damaged and/or dead neurons may activate microglia (Das et al, unpublished), perpetuating the inflammatory process. This mechanism of inflammation may be perpetuated by increased levels of astrocytic/microglial chemoattractants (cytokines and chemokines) which promote infiltration of peripheral T cells and macrophages in experimental parkinsonian animal and PD patient CNS tissues [15,11]. Thus, activation of the peripheral immune system may further exacerbate the central inflammation that propels neurodegeneration. Other pathological mechanisms in PD include mitochondrial (complex-1) dysfunction, oxidative stress [16], and persistent neuroinflammatory responses [7] which have been associated with the presence of Lewy bodies with toxic α -synuclein aggregates [17,16]. Since the degenerative process is multifactorial, blocking a single pathway has not proven effective against the progressive degeneration in PD; hence, targets involved in multiple degenerative pathways may have significant therapeutic implications.

One such target, a calcium-activated protease, calpain, has been demonstrated to be associated with many neurodegenerative diseases (e.g., multiple sclerosis [MS], Alzheimer's disease [AD], amyotrophic lateral sclerosis (ALS) including multiple degenerative pathways in PD [18,19]. Each of the proposed mechanisms for PD can lead to over-activation of calpain, which has been found to play a significant role in activation and migration of T cells into the CNS, promoting inflammation and degeneration in MS, AD and PD [11,20,12,21]. Calpain involvement has been demonstrated in postmortem human PD brain [22,23] and

spinal cord [11], as well as in diverse experimental models in vitro [24,25] and in vivo [22,26]. In addition to calpain-mediated activation of microglia and T cells, calpain plays a significant role in accumulation of aggregated toxic α -synuclein, a hallmark of PD, in neurons [27,28]. Aggregated α -synuclein has also been implicated in microglial activation, promoting inflammation in the CNS [29-31]. Thus, the persistent inflammation in PD may be critical for loss of neurons in SN and spinal cord. These findings in parkinsonian animal models and postmortem PD tissue implicated calpain as a crucial therapeutic target.

In the current study, we tested the benefits of calpain inhibition with the potent pharmacological inhibitor calpeptin against nigral and extranigral degeneration in spinal cord of 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP)-induced parkinsonian mice. Our results indicate increased inflammatory responses, elevated glial activation and calpain activity, and neuronal death in the MPTP-exposed mice. The efficacy of calpeptin was tested against inflammatory processes, nigral/spinal cord degeneration, and altered gait dynamics in MPTP-exposed mice; calpain inhibition slowed the disease progression and improved function. Findings from this study indicate calpain may be a crucial therapeutic target, as it modulates multifactorial pathways in PD disease progression.

Results

Attenuation of calpain activation by calpeptin protects MPTP-induced SN degeneration

Brain samples (pooled SN) from different groups of mice (control, calpeptin alone, MPTP, and calpeptin + MPTP; $n = 12-18$ in each group) were analyzed at 7 days post-MPTP; the involvement of calpain in MPTP neurotoxicity was evaluated along with the calpain inhibitor calpeptin (Fig. 1). Analyses of SN samples showed an MPTP induced increase in 80 kDa (55%) and active 76 kDa (35%) forms of m-calpain compared to controls ($*p < 0.05$). Pre-treatment with calpeptin (25 $\mu\text{g/kg}$) significantly attenuated the activation of calpain compared to MPTP-exposed mouse SN ($@p < 0.05$). There was also an enhanced formation of 145 kDa calpain specific SBDP in MPTP-exposed mouse SN (46%) compared to controls ($*p < 0.05$); calpeptin pre-treatment ameliorated this effect (Fig. 1a). Furthermore, representative immunofluorescent images as shown in Fig. 1b illustrated prominent staining of active m-calpain in SN of MPTP mice co-localized with TH IR; active calpain IR was minimal in TH-positive control SN neurons. The active calpain IR in TH-positive neurons (SN) of calpeptin pre-treated mice was significantly attenuated. These data suggest the involvement of calpain in MPTP-induced neurodegeneration, and calpain inhibition as a means of neuroprotection for SN neurons. Semiquantitative analysis of TH IR in SN (10 μm sections) indicated substantial loss of TH IR in SN of mice after MPTP injections (about 55% reduction) compared to controls ($*p < 0.05$). Calpeptin-control animals did not show any significant changes compared to control. Pretreatment with calpeptin 30 min prior to MPTP injections showed significant attenuation of the reduction in TH IR in SN (28% compared to control) in the calpeptin + MPTP group ($@p < 0.05$) (Fig. 1c).

Calpeptin protects against MPTP-induced neuronal death and axonal degeneration in spinal cord

Multiple aspects of MPTP-induced degeneration were tested in mouse spinal cord sections (5 μ m) from the experimental groups. Earlier reports have demonstrated that MPTP administration in mice induces neurodegeneration in both, brain and spinal cord [18,32]. Thus, we examined protective efficacy of calpeptin in mouse spinal neurons, using combined TUNEL and NeuN immunofluorescent staining in spinal cord slices. Immunofluorescent assays showed prominent NeuN IR and absence of any TUNEL staining in control spinal cords, demonstrating healthy sensory neurons and motoneurons in dorsal and ventral horns, respectively (Fig. 2a, b). Significant co-localization of TUNEL with NeuN, indicated greater neuronal death in both dorsal and ventral regions of MPTP mouse spinal cord (Fig. 2a, b). Marked reduction of TUNEL and NeuN co-localization sites were observed in spinal cord samples from mice pre-treated with calpeptin, signifying protection of dorsal and ventral neurons by calpeptin. Findings in spinal cord were similar in cervical and lumbar regions (Fig. 2a, b). While confirming our earlier findings on MPTP-neurotoxicity in spinal cord (Samantaray et al., 2008a), these data further demonstrated protective efficacy of calpeptin against MPTP-induced degeneration of spinal neurons.

Distal axonal degeneration in mouse spinal cord samples (n = 12-18) was examined via deNFP immunofluorescent staining, an indicator of axonal degeneration, demonstrating NFPs undergoing dephosphorylation, in cervical and lumbar spinal cord; representative immunofluorescent images are shown in Fig. 2a, b, lower panel. Absence of deNFP IR in controls indicated normal spinal cord. In contrast, intense deNFP IR was detected in cervical and lumbar spinal cords of MPTP mice (Fig. 2a, b, lower panel). Following pre-treatment with calpeptin there was decreased deNFP IR suggesting axonal preservation. Intensities of deNFP IR were similar in cervical and lumbar regions of the spinal cord.

Calpeptin ameliorates MPTP-induced neuroinflammation in SN and spinal cord

Although neuroinflammation has been implicated in MPTP-induced neurotoxicity in the brain [33], MPTP-induced inflammatory responses in the spinal cord have not been extensively evaluated. The effects of MPTP on glial cells were studied in ventral regions of cervical and lumbar spinal cord samples using immunofluorescent staining with markers of astrocytes (GFAP), microglia (Iba-1) and infiltration of peripheral macrophages (ED-2). Captured images demonstrated that activated astrocytes, microglia, and infiltrated macrophages were evident in brain and spinal cord in response to MPTP injections (Fig. 3a, b, middle panels) compared to controls (Fig. 3a, b left panels). In contrast, pre-treatment with calpeptin significantly attenuated MPTP-induced activation of astrocytes, microglia and infiltration of macrophages (Fig. 3a, b right panels).

The MPTP-induced infiltration of peripheral CD4⁺ and CD8⁺ positive T cells was found to be significantly greater in SN and spinal cord compared to controls (Fig. 4a, b). Pre-treatment with calpeptin markedly attenuated the migration and hence, the infiltration of T cell subtypes in SN and spinal cord. The data included shows cervical spinal cord (Fig. 4b) although similar results were seen in lumbar spinal cords.

To investigate whether inflammation plays a critical role in PD progression, the findings of gliosis and infiltration of CD4⁺ and CD8⁺ T cells in SN and spinal cord of MPTP mice were also analyzed for inflammatory markers by Western Blotting analysis. Significant up-regulation of Cox-2 (39%), caspase-1 (22%), and NOS-1 (112%), was found in MPTP mouse tissue compared to controls (**p* < 0.05, Fig. 4c). Pre-treatment with calpeptin significantly attenuated MPTP-induced elevated levels of these inflammatory markers (**p* < 0.05, Fig. 4c).

Calpeptin modulated MPTP-induced activation of splenic T cell subtypes

Systemic inflammatory responses may influence the CNS. Similarly, CNS reactions may affect distant lymphoid organs and immune functions by releasing neurohormones and neurotransmitters. We examined whether major peripheral T cell subpopulations are altered in control vs. calpain inhibitor treated MPTP mice. Flow cytometric analysis of splenocytes showed that an increased number of CD4⁺ T cell populations (12.6% vs. 19.9%) were detected in MPTP mice during sub-acute phases of the disease as compared to controls (Figure 5a). By contrast, CD8⁺ T cells were not visibly altered in control vs. MPTP mice as analyzed by flow cytometry. The increased number of CD4⁺ T cells was significantly reduced by calpain inhibition (19.9% vs. 12.6%). Further analyses of these data showed that a distinct subpopulation of CD4⁺ T cells were expanded in sub-acute MPTP mice (2.9% vs. 9.4%), which was also inhibited (9.4% vs. 3.2%) by calpain inhibition (Fig. 5a). Studies are underway to characterize and determine the pathogenic nature of this T cell subpopulation in vivo.

Regulatory T cells (Tregs – e.g., CD4⁺, Foxp3⁺) are known to suppress immune activation and maintain immune homeostasis and tolerance. While dysfunction of Tregs are observed in MPTP mice, administration of Tregs into MPTP mice has been shown to suppress microglial inflammatory responses as well as pathogenic functions of Th1/Th17 cells, leading to robust nigrostriatal protection [34]. Analysis of T cells from controls and MPTP mice showed that the number of Tregs decreased (3.6% vs. 1.1%), while calpain inhibition supported the growth of Treg populations (1.1% vs. 5%) (Figure 5b). These data suggest that differential expansion of peripheral CD4⁺ T cell subpopulations may influence the disease progression in MPTP mice. Studies are underway to investigate whether D3R is differentially expressed in different subpopulations of T cells and regulate inflammatory functions PD patients and MPTP-exposed mice.

Calpeptin mitigates MPTP-induced altered gait parameters in mice

Gait dynamics in MPTP-exposed mice at 7 and 14 days post-MPTP injection were significantly altered compared to the control mice (Fig. 6). All mice could walk at a speed of 25 cm/sec except the MPTP-exposed mice. At 7 days post-MPTP, 10 % of the mice failed to walk at 25 cm/sec (*F*_{1, 30} 13.6 at *P* < 0.01); this disability worsened at 14 days post-MPTP as 25 % of the mice failed to walk at 25 cm/sec (*F*_{1, 30} 17.9 at *P* < 0.01). Data represented were pooled from three independent experiments. Further analysis was performed accounting exclusively the mice that could walk successfully at 25 cm/sec. We analyzed several well-documented gait parameters that are impaired in PD including reduction in

stride length, increase in stride frequency, escalated stride length variability and increased number of foot-steps upon MPTP exposure.

MPTP-exposed mice walked with shortened stride length ($F_{3, 100}$ 13.48 at $*P < 0.0001$ and $F_{3, 96}$ 16.77 at $*P < 0.0001$) at 7 and 14 days respectively post-MPTP compared to control mice. Calpeptin pre-treatment alone had no significant effect, but in MPTP-exposed mice there was a strong tendency to attenuate MPTP-induced shorter stride length at 7 days post MPTP (Fig. 6a) which attained statistical significance at 14 days post-MPTP ($*P < 0.01$, Fig. 6b). Concomitant to MPTP-induced reduction in stride length, stride frequency was increased in MPTP-exposed mice compared to controls ($F_{3, 100}$ 13.48 at $*P < 0.0001$ and $F_{3, 96}$ 16.77 at $*P < 0.0001$) at 7 and 14 days respectively post-MPTP. Calpeptin pre-treatment alone had no significant effect, but in MPTP-exposed mice there was a strong tendency to attenuate MPTP-induced increased stride frequency at 7 days post MPTP (Fig. 6a) which attained statistical significance at 14 days post-MPTP ($*P < 0.01$, Fig. 6b). MPTP-exposed mice showed significantly higher stride length variability compared to controls at day 7 post-MPTP ($F_{3, 112}$ 2.951 at $*P < 0.03$) which was not statistically significant at day 14 post-MPTP ($F_{3, 112}$ 1.086 at $P = 0.3581$); calpeptin-pretreatment did not show any statistically significant difference at $p < 0.5$ compared to MPTP-exposed mice on day 7 or 14 post-MPTP. Likewise, the total number of steps was significantly increased in MPTP exposed animals compared to controls on day 7 post-MPTP ($F_{3, 97}$ 4.900 at $*P < 0.0033$), which was no longer statistically significant on day 14 post-MPTP ($F_{3, 90}$ 1.607 at $P = 0.1934$); calpeptin pre-treatment did not cause any statistically significant difference at $p < 0.5$ compared to MPTP-exposed mice on day 7 or 14 post-MPTP on the total number of steps taken by the mice.

Discussion

While the mechanisms of neurodegeneration in SN and spinal cord in PD are incompletely understood, inflammation has been suggested to be involved in many chronic neurodegenerative diseases including PD, AD, ALS, and MS. However, cumulative effects of central and peripheral inflammatory processes have been implicated in PD progression [10]. In the current study, we have examined whether chronic, persistent inflammation rendered by microglial activation, infiltration of T cells ($CD4^+$, $CD8^+$), and calpain activation are involved in PD. Neuronal death in SN, locus coeruleus, and spinal cord of PD patients has been linked to increased levels of m-calpain [23,11,35,36] and up-regulated calpain activity [22,35,11]. Moreover, the current study demonstrates calpain inhibition by pre-treatment with calpeptin blocks several degenerative pathways in PD in multiple CNS locations. Our results indicated increased inflammatory responses, elevated calpain activity, and neuronal death in the MPTP-exposed mice. Calpeptin pre-treatment significantly attenuated spinal cord degeneration, inflammatory processes, nigral degeneration, and altered gait dynamics in a sub-acute MPTP model in C57Bl6N mice.

Spinal cord involvement in PD patients has been associated with clinical symptoms including constipation, gait disturbances, and urinary/sexual dysfunction [37]. Recent studies reported a 30% loss of spinal motoneurons in an acute MPTP PD model [18,32] and in PD patient spinal cords [11]. Spinal cord degeneration was further investigated in a mouse

model of chronic PD induced by repeated MPTP injections. In confirmation of our earlier studies of MPTP neurotoxicity [18], a significant reduction of TH positive neurons in SN was detected, with increased m-calpain expression in these neurons. Other changes in mouse spinal cord induced by MPTP, such as increased deNFP levels and activation of astrocytes/macrophages, resembled similar alterations found in human PD spinal cords [11]. More importantly, calpeptin protected spinal neurons from MPTP-induced neurotoxicity by preventing neuronal degeneration, axonal alterations, and inflammatory responses - suggesting calpain as a therapeutic target to prevent these degenerative processes.

A sustained neuroinflammatory response is the hallmark of several neurodegenerative diseases including PD [31,7,10]. Evidence continues to mount that inflammation may play a crucial role in PD neurodegeneration with resulting aggregated toxic α -synuclein [38,39]. It is known that calpain activity is regulated by calcium concentration. Increased intracellular calcium leading to aberrant calcium homeostasis has been found in PD and other neurodegenerative diseases. Thus, increased calcium may play a significant role in inflammation and subsequent neurodegeneration by activating calpain. The increased free calcium seen in PD may have originated in intracellular stores such as those in mitochondria and/or endoplasmic reticulum; or calcium may be increased due to cell membrane leakage and abnormal calcium channel function[40]. Moreover, the calcium buffering system is not properly maintained by calcium binding proteins like calbindin, calretinin and others, which are decreased in PD [41-43].

Calpain may also play a crucial role in the migration of peripheral immune cells, including T cells [44,45], into the CNS, which may promote the inflammatory response in PD. Calpain mediated activation of T cells has been found in experimental autoimmune encephalomyelitis (EAE), an animal model of MS, before the onset of the disease [46,47]. Importantly, calpain also plays a key role in α -synuclein aggregation - degrading α -synuclein and aiding in generation of its toxic forms. As demonstrated in the present study, such activation of calpain, microglia, astrocytes and T cells have been previously shown by our laboratory and others [11,48,23,22,15,49]. Activated microglia and astrocytes as found in PD secrete inflammatory factors including free radicals, Cox-2, cytokines, and chemokines (Samantaray et al., unpublished). The effects of these inflammatory cytokines have been found detrimental to neurons in vitro [50]. The chemokine, monocyte chemoattractant protein-1 (MCP-1), is released by astrocytes to recruit T cells into the CNS [51,52]. The release of these factors from activated glial cells may mediate the leakage in blood brain barrier (BBB) since the role of cytokines via activation of NF- κ B has been implicated in BBB breakdown [53-55]. The loss of BBB integrity may further contribute to eventual neuronal death with the observed infiltration of toxic T cells in SN [15,56] and spinal cord, thus contributing to neurodegeneration and PD progression [49,57].

While dopaminergic cell death in PD is believed to be influenced by innate immunity, adaptive immune responses also play a significant role in regulating PD pathology [15]. Both CD4⁺ and CD8⁺ T cells have been shown to infiltrate the brain in human PD and mouse MPTP models. In addition, CD4⁺ T cells infiltrate into the SN, promoting microglial activation and degeneration of dopaminergic neurons [15,58]. Calpain activation promotes migration of inflammatory T cells through differential regulation of cytokines and

chemokines [44]. Our findings suggest that the decreased number of activated CD4⁺ Th1 type cells as well as an increase in Treg populations in calpeptin-treated MPTP mice may have reduced the disease severity. Recently, dopamine receptor D3 (D3R) has been shown to support T cell activation and acquisition of the CD4⁺ inflammatory phenotype [58], suggesting an increased number of inflammatory CD4⁺ T cells in MPTP mice might have played a role in the pathophysiology of the disease. Taken together, our current study suggests that inhibition of calpain could be a promising strategy to suppress infiltration of inflammatory T cells responsible for neuroinflammation and neurodegeneration.

Of note, our findings also demonstrated that inhibition of calpain preserves functionality by protecting against degeneration in brain and spinal cord of MPTP-induced experimental parkinsonian mice. These measurements in rodents are useful in characterizing motor impairment, and may have clinical translational significance. In human PD, a shorter stride length along with greater variability in stride length, stride duration and swing duration have been confirmed [59-65] and recently studied in MPTP primate models [66]. Assessment of gait dynamics (DigiGait, Mouse Specifics) provided several advantages: (1) the study demonstrated gait initiation abnormalities and gait maintenance; and (2) this method does not require any prior learning skills other than acclimatization to the environment, suggesting the gait indices are measures of motor impairment/restoration. DigiGait analysis has been previously reported in acute MPTP mouse models [67] and progressively increased MPTP dosing [68]. We found several spatio-temporal gait indices altered in 7 days post sub-acute MPTP, which were still impaired until 14 days post sub-acute MPTP; calpeptin pre-treatment had significant protective efficacy, especially at the later time-point.

The current study has the following limitations: (1) we applied a pre-treatment administration of calpeptin; however, more clinically relevant post-treatment regimens are underway with advanced calpain inhibitors; (2) the sub-acute MPTP model does not demonstrate toxic α -synuclein aggregation; (3) calpeptin is not clinically tested for therapeutic efficacy in humans; and (4) Digigait analyses of the 7-day post MPTP and 14-day post MPTP mice were performed on different mouse populations.

In conclusion, the findings of inflammatory neuronal death/axonal degeneration in spinal cord and brain of MPTP-induced PD mice appear linked to pathological levels of calpain up-regulation and activity. Calpeptin, a potent inhibitor of calpain, rendered significant protection against multiple parameters of neurodegeneration and neuroinflammation in brain and spinal cord against MPTP-neurotoxicity. In addition, a novel CD4⁺ T cell subtype was generated in the spleen of MPTP-induced PD mice compared to controls – also inhibited by calpeptin. PD therapeutics based on mechanisms outside symptomatic dopaminergic treatment/replenishment may render neuroprotection to the CNS in PD. Calpain inhibition may offer such a treatment choice as evidenced in this study.

Materials and Methods

Animals

Adult male C57BL/6N mice (10 weeks old, weighing 22-25 g) from Charles River Laboratories (Wilmington, MA) were used in this study. Animals were housed (4/cage)

under standard conditions (12 h light–dark cycles, 23°C, and 55% relative humidity) with ad libitum access to food and water. Mice were handled and cared in accordance with the National Institutes of Health (NIH, Bethesda, MD) *Guide for the Care and Use of Laboratory Animals* (NIH publication 80-23, revised 1996) and approved by the Institutional Animal Care and Use Committee (IACUC) of the Medical University of South Carolina (Charleston, SC). MPTP was handled according to recommendations and precautions [69-71].

Sub-acute MPTP model and calpeptin treatment

Mice were divided into following groups: (1) control+saline (n = 4); (2) control+calpeptin (n = 4); (3) sub-acute MPTP (n = 6) and (4) calpeptin+sub-acute MPTP (n = 6). Experiments were repeated twice, and data sets were pooled from 3 independent experiments for statistical analyses. To induce the sub-acute MPTP model, single injections per day of 25 mg/kg of MPTP (Sigma, St. Louis, MO, USA) were injected (i.p) for 5 consecutive days. This regimen provided a high rate of animal survival (> 90%); apoptotic mode of cell death as originally suggested [72], and ~40-50% dopaminergic cell loss on the 7th day after the last MPTP injection [69]. To test neuroprotective efficacy, 25 µg/kg of calpeptin (EMD Biosciences, Calbiochem, Gibbstown, NJ, USA) was injected 30 min prior to each MPTP administration. Calpeptin was routinely dissolved as 2.5 mg/mL DMSO as stock and diluted 1000-fold in saline to attain the optimum concentration of 25 µg/kg in mice (s.c. 200-250 µL/mice). This dose was chosen as a median dose between a less efficacious 10 µg/kg and more toxic 100 µg/kg. A cumulative dose of 125 µg/kg calpeptin over five days was well-tolerated and did not alter mouse survival. Mice from all groups were recorded for gait parameters on 7 and 14 days post-MPTP during treadmill walking at various speeds using a ventral plane videography instrument (Mouse Specifics, Quincy, MA, USA).

Tissue processing

For all biochemical and histofluorescent assays, mice were sacrificed on the 7th day after the last injection. Whole brain and spinal cord tissues were dissected and freshly frozen at –80 °C for further analysis.

Western Blot Analysis

Brain (micro-punched SN) and spinal cord (cervical and lumbar) tissues were homogenized in an ice-cold homogenizing buffer (50 mM Tris-HCl, pH 7.4; 5 mM EGTA) with freshly added phenylmethylsulfonyl fluoride (1 mM). Protein concentration was estimated with Coomassie Plus™ Protein Assay Reagent (Pierce, Rockford, IL) at 595 nm. Samples were equilibrated (1:1 v/v) in sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5 mM β-mercaptoethanol, 10% glycerol), boiled and briefly centrifuged. Supernatants were diluted to a final protein concentration of 1.5 mg/mL with sample and homogenizing buffer mix (1:1 v/v) containing bromophenol blue dye (0.01%). Protein samples were resolved in either 4–20 % sodium dodecyl sulfate–pre-cast gradient gel (Bio-Rad Laboratories, Hercules, CA, USA) at 100 V for 1 h or 7.5 % (for spectrin breakdown products or SBDP) for 90 minutes and transferred to the Immobilon™-polyvinylidene fluoride microporous membranes (Millipore, Bedford, MA, USA) in Genie transfer apparatus. Membranes were blocked in

5% non-fat milk in Tris-buffer (20 mM Tris-HCl, pH 7.6, 0.1% Tween-20) and incubated overnight at 4°C with appropriate primary IgG antibodies. Antibodies used were: mouse monoclonal anti-Cox-2, and anti-NOS2; rabbit polyclonal anti-caspase-1 (all diluted 1:250 before incubation) obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); mouse monoclonal anti- α -spectrin (1:10,000; Biomol. International, Plymouth Meeting, PA, USA); and rabbit polyclonal anti-m-calpain (1:500) raised and characterized in the lab [73]. Bound primary antibodies were visualized with corresponding secondary horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (1:2000; MP Biomedicals, Cappel, Solon, OH, USA), and detected with enhanced chemiluminescence reagents (ECL or ECLplus, Amersham, UK); images were captured on Alpha-Innotech with FluorChem FC2 Imaging System (Cell Biosciences Inc., Santa Clara, CA, USA). Immunoblots, except those for spectrin, were re-probed with mouse monoclonal anti- β -actin (1:15,000; Sigma) antibody, as a protein loading control. The immunoreactive bands of interest were quantified by densitometric analysis using ImageJ 1.45 software.

Immunohistofluorescent Staining

Immunofluorescent staining or terminal deoxynucleotidyl transferase recombinant-mediated dUTP nick-end labelling (TUNEL) was performed according to earlier described protocols [74,11]. Brain and spinal cord samples were warmed to -18 °C and cut into thin (10 μ m for midbrain through SN and 5 μ m for spinal cord) sections in a Leica CM1850 cryostat (Leica, Deerfield, IL). Sections were fixed in 95% ethanol, rinsed in phosphate-buffered saline (PBS, containing 137 mM NaCl, 2.7 mM KCl, 11.9 mM phosphates, pH 7.4) and stored in the same buffer at 4 °C for *in situ* immunofluorescence staining. Slices were blocked for 1 h in PBS containing 2% horse, goat or sheep serum (Sigma) and incubated with respective primary IgG antibodies, including mouse monoclonal anti-tyrosine hydroxylase (1:100), anti-NeuN (1:100) and anti-GFAP (1:400) from (Chemicon International, Temecula, CA, USA); rabbit polyclonal active calpain (1:100) [73,74]; mouse monoclonal anti-pan-neuronal neurofilament non-phosphoneurofilament specific marker or deNFP, (1:1000) from (Covance, Inc., Emeryville, CA, USA); mouse monoclonal anti-macrophage protein CD163 or ED-2 (1:100) from Santa Cruz Biotechnology); and goat polyclonal anti-microglial or Iba-1 (1:500) from (Abcam, Cambridge, MA, USA). Unbound primary antibodies were washed off by 3 $\times$ 5 min rinses in PBS. Bound primary antibodies were visualized by incubation with either fluorescein isothiocyanate (FITC) - or Texas Red-conjugated corresponding secondary IgG antibodies (1:100; Vector Laboratories, Burlingame, CA).

For TUNEL assays, sections pre-fixed in 95% ethanol, were further fixed in 4% methanol-free formaldehyde (Polysciences, Warrington, PA, USA), washed in PBS, equilibrated in TdT buffer (Apoptosis Detection System, Promega, Madison, WI), saturated with digoxigenin labeled nucleotides (Roche, Indianapolis, IN, USA) and recombinant TdT (Promega), and then incubated at 37 °C for 1 h in a humidified Omnislide thermal Cycler (Hybaid Ltd., UK). TUNEL reaction was stopped by immersing in 2x NaCl/Na-citrate (SSC) at room temperature for 15 min. [58]Unincorporated nucleotides were removed with 3 $\times$ 5 min washing in PBS. To detect spinal cord neuronal death, slices were double stained with primary IgG antibodies (NeuN, 1:100). TUNEL staining was visualized with anti-digoxigenin in rhodamine-coupled, Fab fragments (1:100; Roche).

All sections were rinsed in PBS and distilled water, mounted with anti-fade medium Vectashield™ (Vector Laboratories, Burlingame, CA, USA). The images were viewed using an Olympus BH-2 microscope (Olympus, Melville, NY) at 200x magnification at a pixel resolution of 1280 × 1024. Images were captured with a Dage CCD100 integrating camera (DAGE-MTI, Michigan City, IN, USA) and a Flashpoint 128 capture board (Integral Technologies, Indianapolis, IN). Image capture was performed on a Pentium IV Imaging workstation (Dell Computers, Round Rock, TX, USA), using Magna Fire Image Pro Plus software (Media Cybernetics, Silver Spring, MD, USA). Image formatting was done using Photoshop software (Adobe, San Jose, CA).

Flow Cytometry

Pooled splenocytes (2×10^5 cells/group, $n = 3-4$ / group) obtained from naive control, MPTP and calpeptin treated mice were stained with CD8-FITC (BD Biosciences, San Jose, CA) and CD4-PerCP (BD Biosciences, San Jose, CA) as described [20]. Flow cytometric two-parameter dot plots and quadrant statistics were generated using FACScan and CellQuest software (BD Biosciences, Mountain view, CA). For intracellular staining, cells were fixed and permeabilized using Fix and Perm reagents (BD Biosciences, San Jose, CA), and then incubated with specific antibodies [75]. Briefly, 5×10^5 cells per group were re-suspended in 0.5 mL of Fixation/Permeabilization Buffer (BD) and incubated at 2-8° C for 30 minutes. After washing, the cell pellet was re-suspended in Permeabilization/Wash Buffer and stained with Foxp3-APC (BD Biosciences, San Jose, CA). Cells were then analyzed on FACScan using CellQuest software as described above.

Gait Dynamics

Gait was assessed (DigiGait; Mouse Specifics) 7 or 14 days following the final injection of MPTP employing ventral plane videography, following previously described protocols [67,76]. Briefly, the digital images of each of the paws (right fore, left fore, right hind and left hind) of mouse were taken at 150 frames/sec at a speed of 25 cm/sec. Mice that failed to run at the speed of 25 cm/sec were excluded from the study for fair comparison between experimental groups. Mice were tested individually on the treadmill, bounded by an acrylic compartment (5 cm in width, 25 cm in length) and the spatio-temporal measurements were assessed by the area of the paw relative to the treadmill belt at each frame. Multiple gait parameters including reduction in stride length, increase in stride frequency, escalated stride length variability and increased number of foot-steps upon MPTP exposure were analyzed [67].

Statistical analysis

The data were obtained from three independent experiments; $n = 4-6$ mice in each group. The immunoreactive bands of Western blotting were quantified by densitometric analysis; the mean $\pm$ S.E.M. of arbitrary units (A.U.) was plotted. Assessment was done using Stat View software (Abacus Concepts) and the difference was calculated with one-way ANOVA with Fisher's protected least significant difference *post hoc* test at 95% confidence interval. Changes between groups were considered significant at * $p < 0.05$ compared to control or @ $p < 0.05$ compared to calpeptin.

For statistical analyses of the gait parameters, each of the four mouse paws were considered as independent, and a one-way ANOVA was performed with Tukey's multiple comparison at α values of 0.05 and 0.01.

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Abbreviations

DA	dopaminergic
deNFP	dephosphorylated neurofilament protein
GFAP	glial fibrillary acidic protein
IR	immunoreactivity
L-DOPA	L- 3,4- dihydroxyphenylalanine
MPTP	1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine
PD	Parkinson disease
SN	substantia nigra
TH	tyrosine hydroxylase
SBDP	spectrin break down product

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Figure 1.

Calpeptin pre-treatment mediated protection in SN of MPTP-exposed mice: (a) Calpain expression (pro-enzyme, 80 kDa and active enzyme, 76 kDa) and activity in pooled SN were found to be significantly up-regulated in MPTP mice compared to controls (* $p < 0.05$); calpeptin (25 $\mu\text{g/kg}$) pre-treatment significantly attenuated both calpain expression and activity (@ $p < 0.05$). Quantitative data are present in lower panel. (b) Immunohistochemical staining depicted intense co-localization of active calpain-IR in TH-positive neurons in SN of MPTP mice (middle panel) compared to controls (upper panel), which was significantly attenuated by calpeptin pre-treatment (lower panel). Images were captured at 200x magnification. (c) Semi-quantitative analysis of TH-IR in SN indicated significant loss in MPTP mice (middle panel) compared to controls (upper panel); calpeptin pre-treatment significantly attenuated the loss (lower panel). Images were captured at 100x magnification.

Figure 2.

Calpeptin pre-treatment mediated protection in spinal cord of MPTP-exposed mice: Intense co-localization of TUNEL in NeuN-positive neurons indicated neuronal death in both dorsal (upper panel) and ventral (middle panel) spinal cord of MPTP mice, which was significantly attenuated by calpeptin (25 μg/kg) pre-treatment in both cervical (a) and lumbar (b) spinal cord sections. Distal axonal degeneration as indicated by deNFP IR was significantly higher in MPTP mice (lower panel) compared to controls, which was significantly attenuated with calpeptin pre-treatment in both cervical (a) and lumbar (b) spinal cord sections. Images were captured at 200x magnification.

Figure 3.

Calpeptin pre-treatment attenuated neuroinflammation in spinal cord of MPTP-exposed mice: Reactive gliosis (GFAP IR indicating astrogliosis, upper panel; Iba-1 IR representing microgliosis, middle panel; and ED-2 IR indicating infiltrated macrophages, lower panel) was significantly higher in MPTP mice compared to controls in both cervical (a) and lumbar (b) spinal cord; calpeptin (25 μg/kg) pre-treatment significantly attenuated these responses. All images were captured at 200x magnification.

Figure 4.

Calpeptin pre-treatment attenuated T cell infiltration and inflammatory markers in SN and spinal cord of MPTP-exposed mice: Infiltration of T cells as indicated by the presence of CD8⁺-positive cells in SN (a) and CD4⁺-positive (upper panel) and CD8⁺-positive cells (lower panel) in spinal cord (b) was significantly increased in MPTP mice; calpeptin (25 µg/kg) pre-treatment significantly attenuated the infiltration of T cells in both SN and spinal cord. All images were captured at 200x magnification. (c) A battery of inflammatory markers (70-72 kDa Cox-2, 45 kDa Procaspase-1 and 116 kDa NOS2) was significantly enhanced in spinal cord of MPTP mice; which was significantly attenuated by calpeptin pre-treatment. Quantitative data are present in lower panel.

Figure 5.

Splenocytes obtained from acute/sub-acute MPTP mice, calpeptin treated MPTP mice, and untreated control mice were stained with PerCP labeled CD4⁺ and FITC labeled CD8⁺ antibodies, and analyzed by flow cytometry (a) as described in the methods. Cells were also stained with CD4-PerCP and Foxp3-APC, and CD4⁺ cells were gated and analyzed for the presence of Foxp3 regulatory T cells (b). Matched isotype controls were used in each analysis.

Figure 6.

Calpeptin prevented MPTP-induced altered gait dynamics: (a-b) Combined gait analysis of all limbs demonstrated significantly reduced stride length in MPTP mice compared to controls on day 7 post-MPTP (* $p < 0.01$), which remained inhibited until 14 days post-MPTP (* $p < 0.01$). Calpeptin (25 $\mu\text{g/kg}$) treatment alone had no effect, pre-treatment in MPTP mice showed restored stride length, which was not significant at $p < 0.05$ on day 7 post-MPTP, but significant on day 14 post-MPTP (@ $p < 0.01$). (c-d) Concomitantly, stride frequency was increased in MPTP mice compared to controls on days 7 and 14 post-MPTP (* $p < 0.01$); calpeptin pre-treatment significantly attenuated the MPTP-induced increased stride frequency at 14 day post-MPTP (* $p < 0.01$).