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Attenuation of microglial RANTES by NEMO-binding domain peptide inhibits the infiltration of CD8⁺ T cells in the nigra of hemiparkinsonian monkey

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Abstract

Parkinson's disease (PD) is a progressive neurodegenerative disease characterized by the loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc). Despite intense investigations, little is known about its pathological mediators. Here, we report the marked upregulation of RANTES (regulated on activation, normal T cell expressed and secreted) and eotaxin, chemokines that are involved in T cell trafficking, in the serum of hemiparkinsonian monkeys. Interestingly, 1-methyl-4-phenylpyridinium (MPP⁺), a Parkinsonian toxin, increased the expression of RANTES and eotaxin in mouse microglial cells. The presence of NF- κ B binding sites in promoters of RANTES and eotaxin and down-regulation of these genes by NEMO-binding domain (NBD) peptide, selective inhibitor of induced NF- κ B activation, in MPP⁺-stimulated microglial cells suggest that the activation of NF- κ B plays an important role in the upregulation of these two chemokines. Consistently, serum ELISA and nigral immunohistochemistry further confirmed that these chemokines were strongly upregulated in MPTP-induced hemiparkinsonian monkeys and that treatment with NBD peptides effectively inhibited the level of these chemokines. Furthermore, the microglial upregulation of RANTES in the nigra of hemiparkinsonian monkeys could be involved in the altered adaptive immune response in the brain as we observed greater infiltration of CD8⁺ T cells around the perivascular niche and deep brain parenchyma of hemiparkinsonian monkeys as compared to control. The treatment of hemiparkinsonian monkeys with NBD peptides decreased the microglial expression of RANTES and attenuated the infiltration of CD8⁺ T cells in nigra. These results indicate the possible involvement of chemokine-dependent adaptive immune response in Parkinsonism.

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Keywords

Parkinson's disease; Hemiparkinsonian monkeys; Neuroinflammation; RANTES; T cell infiltration; Microglia

Introduction

Parkinson's disease (PD) is the most common neurodegenerative movement disorder, which is characterized by progressive loss of DA neurons in the ventral midbrain. Among many of its etiological factors, the role of inflammation in the pathogenesis of PD remains elusive. In a normal adult brain, the crosstalk between the peripheral immune system and the brain is transient, and there is no evidence that it leads to the brain inflammation. However in chronic neurodegeneration, when disease becomes more widespread involving distant regions of the brain and periphery, a growing body of evidence suggests that the brain-resident microglia are not only activated (Roy et al., 2006, Roy et al., 2008), but happen to be "primed" by the systemic inflammation leading to the exaggerated synthesis of pro-inflammatory molecules (Perry et al., 2007, Cunningham et al., 2009, Field et al., Lee et al., 2011, Khasnavis et al., 2014). Earlier we have shown that microglial activation plays critical role in the development of PD-related in mice and monkeys (Roy et al., 2006, Ghosh et al., 2007, Ghosh et al., 2009). In numerous studies, we and others have shown that microglial cells can be activated by the chronic infiltration of peripheral inflammatory T cells (Dasgupta et al., 2003, 2005, Dasgupta et al., 2007) as well as various toxic molecules circulating from the peripheral tissue to the brain via humoral routes (Jana et al., 2009, Jana and Pahan, 2009). Once microglia are activated, it can generate wide range of cytotoxic molecules including NO (Pahan et al., 2001, Roy et al., 2006), ROS (Roy et al., 2008), different cytokines (Pahan et al., 1997, Hanisch, 2002), and chemokines (Kremlev et al., 2004) that significantly contribute to the pathology of the disease.

RANTES (Hu et al., 1999) and eotaxin (Wei et al., 2013) are two such important inflammatory chemokines that are produced by T cells and antigen-presenting cells such as macrophages and microglia. RANTES, a pro-inflammatory chemokine, is a 68 amino acid-long protein that induces the migration and homing of classical lymphoid cells such as T cells and monocytes, and other immune cells including basophils, eosinophils, natural killer cells, dendritic cells, and mast cells. RANTES acts by promoting leukocyte infiltration to sites of inflammation (Appay and Rowland-Jones, 2001). Eotaxin is another small inflammatory chemokine with 71 amino acids, which leads to the infiltration of similar range of mononuclear cells in the site of inflammation (Wada et al., 1999). In addition to that, eotaxin has also been reported to induce other neurotoxic events in brain including hippocampus-dependent cognitive impairment and decreased neurogenesis (Villeda et al., 2011).

Our ELISPOT analyses of different chemokines revealed that both RANTES and eotaxin were produced at supraphysiological levels in the blood of MPTP-induced hemiparkinsonian monkeys. Since the nonhuman primate model of PD closely mimics idiopathic PD patients in terms of different pathological hallmarks of this disease, we were interested to study the

role and regulation of these chemokines. While looking at the promoters of RANTES and eotaxin, we found the presence of two DNA binding sites for NF- κ B located close to the start site. Since, the inhibition of NF- κ B activation reduces the induction of proinflammatory molecules (Ghosh et al., 2007), we examined the role of NF- κ B in the upregulation of RANTES and eotaxin. Activation of NF- κ B requires the activity of I κ B kinase (IKK) complex containing IKK α and IKK β and the regulatory protein NF- κ B essential modifier (NEMO) (May et al., 2000). We have demonstrated that selective inhibition of NF- κ B activation by NEMO-binding domain (NBD) peptide protects dopaminergic neurons in MPTP-intoxicated mice (Ghosh et al., 2007) and monkeys (Mondal et al., 2012). Here, we demonstrate that NF- κ B activation induces the upregulation of both RANTES and eotaxin in cultured microglial cells. We also observed marked upregulation of microglial RANTES and infiltration of CD8⁺ T cells in the nigra of hemiparkinsonian monkeys. Interestingly, treatment of these animals with NBD peptide decreased the expression of microglial RANTES and attenuated the infiltration of CD8⁺ T cells in the nigra, suggesting that NF- κ B-dependent RANTES and infiltration of CD8⁺ T cells may play a role in the disease process of PD.

Experimental procedures

Reagents

Human chemokine array kit (Enzyme-Linked ImmunoSpot; ELISPOT) was purchased from Raybiotech. Human chemokine ELISA array kit was purchased from SABiosciences. Anti-CD3, CD4 and CD8 antibodies were purchased from e biosciences. Rabbit anti-TH antibody was purchased from Calbiochem (a Millipore Company). Anti-Iba-1 antibody was purchased from Abcam. Cy2- and Cy5-conjugated antibodies were obtained from Jackson Immuno Research Laboratories (West Grove, PA).

Semi-quantitative RT-PCR analysis

Total RNA was isolated from hippocampus using Ultraspec-II RNA reagent (Biotecx Laboratories, Inc., Houston, TX) following the manufacturer's protocol. To remove any contaminating genomic DNA, total RNA was digested with DNase. RT-PCR was carried out as described earlier (Jana et al., 2007, Brahmachari et al., 2009, Ghosh et al., 2009) using a RT-PCR kit (Clontech, Mountain View, CA) and primers.

Real-time PCR analysis

DNase-digested RNA was analyzed by real-time PCR in the ABI-Prism7700 sequence detection system (Applied Biosystems, Foster City, CA) as described earlier (Jana et al., 2007, Brahmachari et al., 2009, Ghosh et al., 2009, Khasnavis and Pahan, 2012) using TaqMan Universal Master mix and optimized concentrations of FAM-labeled probes and primers. Data were processed using the ABI Sequence Detection System 1.6 software.

Subjects and MPTP intoxication

Fifteen female rhesus monkeys (6–8 years old; 5–7 kg) were used in this study. All animals were singly housed with a 12-h light/dark cycle. Purine monkey chow and water were available ad libitum. Diets were supplemented with fruit during the testing sessions. The

study was performed in accordance with federal guidelines of proper animal care and with the approval of the IACUC. Monkeys were intoxicated with MPTP according to protocol described previously by Kordower and colleagues (Kordower et al., 2000). Briefly, animals were tranquilized with ketamine (10 mg/kg, i.m.) and then maintained on an anesthetic plane with isoflurane (1–2%). The animals were put in the supine position. For each injection, a right-sided incision was made along the medial edge of the sternocleidomastoid muscle. The carotid sheath was opened and the common carotid artery, internal jugular vein, and vagus nerves were identified. The common carotid was exposed below the carotid bifurcation. The external carotid artery was then ligated. A 27 gauge butterfly needle was inserted into the common carotid artery in a direction retrograde to blood flow; for each injection, 20 ml of saline containing 3 mg of MPTP-HCl (Sigma) was infused at a rate of 1.33 ml/min (15 min). After the infusion was completed, 3 ml of saline was delivered, and then the incision was closed.

NBD peptides and their use in hemiparkinsonian monkeys

NBD peptides (> 99% pure) were synthesized in the custom peptide synthesis facility of Peptide 2.0 (Chantilly, VA). Wild type (wt) NBD peptide contains the Antennapedia homeodomain (lower case) and IKK β (upper case) segments. Positions of W→A mutations are underlined. wtNBD: drqikiwfqnrmkwkkLDWSWL; mNBD: drqikiwfqnrmkwkkLDASAL. Monkeys were treated with either wtNBD or mNBD peptide (1.0 mg/kg body wt/2 day) in saline through i.m. injection from 7 d after the first intracarotid injection (Fig. 1).

Immunohistochemistry

At the end point, monkeys were anesthetized with pentobarbital (25mg/kg intravenously) and killed via perfusion with 0.9% saline. The brain was removed, immersed in ice-cold saline for 10 min, and slabbed on a monkey brain slicer (Mondal et al., 2012). Slabs were sectioned frozen (40 μ m) on a sliding knife microtome. Tissue sections were stored in a cryoprotectant solution at 4°C before use. For TH staining, midbrain sections were immunostained with rabbit polyclonal anti-human TH antibody (1:1000 dilution: Calbiochem) and visualized by using immunofluorescence method as described earlier (Mondal et al., 2012). For other immunofluorescence staining, anti-human CD3 (1:500), rat anti-human CD8 (1: 500), and anti-human CD4 (1:500) antibodies were used. Samples were mounted and observed under a Olympus BX-51 fluorescence microscope.

Statistical analysis

All values are expressed as means $\pm$ SEM. Differences among means were analyzed by one-way ANOVA or Kruskal-Wallis test (comparison among all four groups) and post-hoc pairwise comparison. In other cases, two sample t tests were also used to compare control vs MPTP and MPTP vs (MPTP+wtNBD).

Results

Upregulation of RANTES and eotaxin in the serum of hemiparkinsonian monkeys

Chemokines play important role in the progression of many immunomodulatory disorders. However, their role in the progression of PD was poorly understood. Briefly, monkeys were injected with MPTP in the right common carotid artery (Fig. 1). Before MPTP injection, blood was collected and later assayed as control samples. We purchased fifteen female rhesus monkeys (5–7 kg) in order to include five monkeys for each of MPTP, MPTP-wtNBD and MPTP-mNBD groups. Out of fifteen monkeys, ten monkeys exhibited parkinsonian posture and left hand freezing on 7th d of the first intracarotid injection of MPTP. Therefore, these ten monkeys were distributed among three groups: MPTP (n=4), MPTP-wtNBD (n=4) and MPTP-mNBD (n=2). For chemokine experiments, we considered only the first two groups with same number of monkeys for easy statistical analysis. Interestingly, our ELISPOT analyses (Fig. 2A & 2B) followed by the quantitative densitometric analyses (Fig. 2C) clearly indicated that the expression of RANTES and eotaxin was markedly upregulated in the serum of MPTP-intoxicated monkeys, suggesting that these two chemokines might play a role in the progression of PD. Apart from RANTES and eotaxin, other inflammatory chemokines including IP-10, IL-8, MIP-1 α , MIP-1 β , and MIP-5 were also moderately upregulated in the serum of MPTP-treated monkey (Fig. 2C), as compared to control (Fig. 2A–C).

MPP⁺ stimulates the upregulation of RANTES and eotaxin in mouse microglia via activation of NF- κ B

Next, we were interested to identify the cell type that produced chemokines in hemiparkinsonian monkeys. Recently we have demonstrated chronic activation of microglia in the nigra of hemiparkinsonian monkeys (Mondal et al., 2012). Therefore, we examined if microglia were capable of producing chemokines in response to Parkinsonian insult. MPP⁺, an etiological toxin of PD, has been previously reported to activate microglial cells (Roy et al., 2012). Here, we examined the effect of MPP⁺ on the expression of RANTES and eotaxin in BV-2 microglial cells. Briefly, BV-2 microglial cells were treated with 2 μ M of MPP⁺ for different time periods followed by mRNA analysis of RANTES and eotaxin by semi-quantitative and quantitative real-time PCR. We observed that MPP⁺ markedly upregulated the mRNA expression of both RANTES and eotaxin in microglial cells at different time points of stimulation (Fig. 3A–3B).

Next, we wanted to study the molecular mechanism by which MPP⁺ stimulated the expression of these chemokines. Activation of classical NF- κ B (p65/p50) is often believed to be involved in the upregulation of inflammatory molecules (May et al., 2000, Ghosh et al., 2007). Therefore, we investigated whether activation of NF- κ B was involved in the upregulation of RANTES and eotaxin. While analyzing the promoter of RANTES, we found two conserved NF- κ B binding sites within 100 bp upstream from the transcription start site (Fig. 3C); one is between –39 to –57 bp and another between –53 to –71 bp. Similarly, the promoter of eotaxin also contains one conserved NF- κ B binding site between 118 to 132 bp upstream from the transcription start site (Fig. 3D), suggesting that NF- κ B may regulate the expression of these two chemokines. To confirm the role of NF- κ B in the upregulation of

RANTES and eotaxin, we used NBD peptide, a specific inhibitor of induced NF- κ B activation. At first, we examined if NBD peptide was capable of inhibiting MPP⁺-induced NF- κ B activation in BV-2 microglial cells. Due to the facts that NBD peptide targets the IKK complex and that IKK β is responsible for the degradation of I κ B α (Hayden and Ghosh, 2012), we tested the effect of NBD peptide on the activation of IKK β . As expected, MPP⁺ treatment induced the phosphorylation of IKK β (Fig. 3E–F). However, wild type (wt), but not mutated (mt), NBD peptide suppressed MPP⁺-induced phosphorylation of IKK β (Fig. 3E–F). On the other hand, the level of total IKK β remained unchanged during these treatment conditions (Fig. 3E–F). These results suggest that wtNBD is capable of suppressing MPP⁺-induced activation of NF- κ B in microglial cells.

Next, we examined the effect of NBD peptides on MPP⁺-induced mRNA expression of RANTES and eotaxin in BV-2 microglial cells. We observed significant inhibition of MPP⁺-stimulated mRNA expression of RANTES and eotaxin in microglial cells by wtNBD peptide (Fig. 3G–H). These results were specific as mtNBD peptides had no effect on MPP⁺-induced expression of RANTES and eotaxin (Fig. 3G–H). Moreover, our ELISA (Fig. 3I) and immunocytochemical (Fig. 3J) analyses also showed that wtNBD, but not mtNBD, peptide inhibited the expression of RANTES protein in MPP⁺-stimulated microglial cells. Taken together, our results suggest that NF- κ B indeed plays an important role in the upregulation of these chemokines in microglial cells in response to Parkinsonian toxin.

Effect of NBD peptide treatment on circulatory levels of RANTES and eotaxin in hemiparkinsonian monkeys

Next, we determined if the expression of these two chemokines were also regulated *in vivo* by NF- κ B in MPTP-induced hemiparkinsonian monkeys. Briefly, 7 days after MPTP infusion, monkeys exhibiting hemiparkinsonian symptoms received either saline (n=4) or wtNBD (n=4) via intramuscular (i.m.) injection. After 30 d of treatment, blood was collected and analyzed for different chemokines by ELISA. Similar to ELISPOT results, we observed a marked increase in RANTES (Fig. 4A) and eotaxin (Fig. 4B) in the serum of MPTP-treated hemiparkinsonian monkeys, whereas treatment with wtNBD significantly inhibited the level of these two chemokines. Apart from these two chemokines, we also analyzed the levels of other chemokines. The levels of IL-8 (Fig. 4C), MIP-1 α (Fig. 4D), MIP-1 β (Fig. 4E), and MIG (Fig. 4F) were also upregulated in the serum of MPTP-treated monkey and the treatment with wtNBD peptide significantly down-regulated the levels of these chemokines. However, the expression of ITAC (Fig. 4G), IP-10 (Fig. 4H) and MCP-1 (Fig. 4I) remained virtually unchanged in all groups. Taken together, our results clearly suggest that NF- κ B is required for the upregulation of RANTES and other chemokines *in vivo* in serum of hemiparkinsonian monkeys.

Effect of NBD peptide in the attenuation of microglial RANTES in the nigra and striatum of MPTP-induced hemiparkinsonian monkey brain

At first, we examined if NBD peptide was capable of attenuating the activation of NF- κ B in the nigra of hemiparkinsonian monkeys. MPTP intoxication markedly induced the activation of NF- κ B in the nigra of hemiparkinsonian monkeys as monitored by the phospho-IKK β

immunostaining (Fig. 5A–B). However, wtNBD peptide treatment markedly inhibited the phosphorylation of IKK β (Fig. 5A–B), suggesting the efficacy of NBD peptides *in vivo* in the nigra of hemiparkinsonian monkeys. Next, we were interested to study the types of cells that expressed such exaggerated levels of RANTES. Since, microglia are the primary immune cells in the brain that are capable of producing a wide range of inflammatory cytokines and chemokines (Hu et al., 1999), we examined the expression of RANTES in nigral microglia of MPTP-intoxicated monkey. Double-label immunohistochemical analyses (Fig. 5C–D) followed by counting (Fig. 5E) clearly indicated that microglia strongly expressed RANTES in the nigra of hemiparkinsonian, but not control, monkey, suggesting that RANTES is upregulated centrally in the nigra of hemiparkinsonian monkeys. Similar to that observed in serum, wtNBD peptide treatment significantly reduced the level of microglial RANTES in the nigra of hemiparkinsonian monkeys. In the striatum, another region of the basal ganglia that is affected in PD, we also observed upregulation of RANTES in hemiparkinsonian monkeys (Fig. 6A–6C). The treatment with wtNBD peptide markedly inhibited the microglial expression of RANTES. Together, these results suggest that RANTES is centrally upregulated *in vivo* in the basal ganglia of hemiparkinsonian monkeys via NF- κ B-dependent mechanism.

Effect of NBD peptide on the infiltration of T cells in the nigra of MPTP-induced hemiparkinsonian monkey brain

RANTES and other chemokines like eotaxin are known to induce the migration and homing of inflammatory lymphoid cells such as T cells and monocytes in the site of inflammation, eventually triggering the production of a wide range of proinflammatory molecules. Since, substantia nigra is a primary target of neurodegeneration in PD, we determined whether MPTP intoxication mediated the infiltration of inflammatory T cells in the nigra. Our dual immunofluorescence analyses of CD3 (*green*) and tyrosine hydroxylase (TH) (*red*) clearly displayed a typical CD3-immunoreactive inflammatory cuffing in the nigra of hemiparkinsonian (Fig. 7B), but not control (Fig. 7A), monkey, suggesting that the infiltration of T lymphocytes is a pathological feature in hemiparkinsonian monkey. This is consistent to that observed in the nigra of PD patients (Brochard et al., 2009). In contrast, similar to that observed in the regulation of RANTES, the treatment with wtNBD peptide attenuated the infiltration of CD3⁺ T cells in the nigra of hemiparkinsonian monkey (Fig. 7), suggesting that the infiltration of peripheral lymphocytes into the nigra of hemiparkinsonian monkey depends on the activation of NF- κ B. While analyzing the types of infiltrating T cells, our dual DAB-labeling method clearly showed that both CD4⁺ (light brown) and CD8⁺ T (black or dark brown) cells were infiltrated in the nigra of hemiparkinsonian, but not in control, monkey (Fig. 8A). Although, CD4⁺ T cells were found to be more abundant than CD8⁺ T cells (Fig. 8A) near blood vessel, more CD8⁺ T cells were detected in the deep parenchyma far from the lumen of blood vessels (Fig. 8B) in the nigra of MPTP-treated monkey brain. However, treatment with wtNBD peptide significantly attenuated the infiltration of both CD4⁺ and CD8⁺ T cells near the blood vessel (Fig. 8A & 8C) as well as in deep parenchyma (Fig. 8B & 8D) of ventral midbrain. Taken together, our results suggest that T cells are infiltrated into the nigra of hemiparkinsonian monkeys and that wtNBD peptide treatment is capable of suppressing the infiltration of T cells.

Discussion

PD is a progressive age-related neurodegenerative disease with unclear etiology. This disease sometimes progresses ruthlessly leaving its victims bound to the wheelchair or dependent on caregivers. The actual cause of PD is not known. The major obstacle of studying the pathologies of this disease is the absence of an appropriate model that resembles the human form of PD. Originating from the same primitive anthropoid ancestor, monkeys and humans are most alike in terms of their immunological regulation. Therefore, in situations where it is desirable but impossible to study human neuroimmune responses *in vivo*, monkeys provides an important alternative. A growing body of evidence supports that the alteration of cellular and humoral immunological components are very common in PD patients. However, the role of adaptive immunity in the pathogenesis of this disorder has so far remained elusive (Czlonkowska et al., 2002, Hunot and Hirsch, 2003, Laurie et al., 2007). Chemokines are important mediators of adaptive immunity and unfortunately little is known about the role of chemokines in regulating the pathogenesis of PD. Here, we delineate marked upregulation of different chemokines including RANTES, eotaxin, MIP-1 α , MIP-1 β , and TARC in the blood of hemiparkinsonian rhesus monkeys. Among all chemokines, the expression of RANTES was the maximum, which is also an important regulator of T cell-mediated immunological responses. Therefore, we were prompted to investigate the role of RANTES in the alteration of the adaptive immune response in PD. It is becoming clear that chronic microglial activation is associated with progressive loss of DA neurons in the ventral midbrain. Upon activation, microglia produce a broad-spectrum of inflammatory molecules including nitric oxide, reactive oxygen species, and proinflammatory cytokines. Here, we also demonstrate the upregulation of a diverse set of chemokines in hemiparkinsonian monkeys. Our double labeling immunohistochemical analyses revealed RANTES upregulation in microglia of substantia nigra and striatum of MPTP-intoxicated monkey as compared to normal monkey. Once produced, RANTES is known to be involved in the recruitment of both CD4⁺ T helper cells and CD8⁺ cytotoxic T cells in the site of inflammation (Fahy et al., 2001), facilitation of their crosstalk with the antigen presenting cells (Terme et al., 2008), and exacerbation of the disease outcome via systemic inflammation (Denes et al.). Accordingly, we observed infiltration of both CD4⁺ and CD8⁺ T cells in the nigra, forming a perivascular cuffing around the blood vessel. During chronic inflammation, inflammatory monocytes and lymphocytes migrate to the deep parenchymal region to modulate disease severity. Interestingly, we observed the migration of CD8⁺ T cells to the deep brain parenchyma of hemiparkinsonian monkeys. It has been reported the increased infiltration of CD8⁺ T cells over CD4⁺ T cells in PD brain, supporting the existence of disease-associated shift to a Tc1/Th1-type immune response (Brochard et al., 2009), which may contribute to the harmful brain inflammatory reaction in PD. During inflammation, CD8⁺ cells are known to produce RANTES, MIP-1 α , and MIP-1 β (Hadida et al., 1998), which are also responsible for the recruitment and cytolytic function of CD8⁺ cells. Our ELISPOT followed by ELISA analyses revealed the significant upregulation of these chemokines, further supporting the possibility of CD8⁺ T cell-mediated cytotoxic response in the nigra of PD brain.

Activation of NF- κ B is possibly the most important regulator of inflammation as it controls the transcription of most of the proinflammatory molecules in wide range of cells. However, its role in the infiltration of inflammatory T cells in the PD brain has never been explored. This study highlights the role of NF- κ B in the expression of chemokines and infiltration of inflammatory T cells in the nigra of hemiparkinsonian monkeys. Our conclusion is based on the followings: *First*, MPP⁺, an etiological toxin of PD, dose-dependently stimulated the mRNA expression of both RANTES and eotaxin, whereas NBD peptide treatment suppressed the activation of IKK β and attenuated the mRNA expression of these chemokines in MPP⁺-stimulated microglial cells. *Second*, ELISPOT and ELISA analyses revealed that the MPTP intoxication upregulated the circulatory level of RANTES, eotaxin, and other chemokines in hemiparkinsonian monkeys and the intramuscular administration of NBD peptide markedly inhibited the serum level of these chemokines. *Third*, immunohistochemical analyses further confirmed that microglia in the nigra of hemiparkinsonian monkey expressed RANTES and that NBD peptide treatment reduced the microglial expression of RANTES in these monkeys. *Fourth*, as reported in PD brains (Brochard et al., 2009), CD8⁺ and CD4⁺ T cells infiltrated into the nigra of hemiparkinsonian monkeys. However, NBD peptide treatment suppressed the recruitment of CD8⁺ T cells in the nigra of these monkeys. Recently, we have demonstrated that NF- κ B is activated in nigral glial cells of PD patients (Ghosh et al., 2007) and MPTP-intoxicated mice (Ghosh et al., 2007) and monkeys (Mondal et al., 2012). Furthermore, NBD peptide treatment inhibits nigral activation of NF- κ B, protects dopaminergic neurons in the nigra and protects locomotor activities in hemiparkinsonian monkeys (Mondal et al., 2012). Therefore, it is likely that RANTES-mediated infiltration of CD8⁺ T cells into the nigra also participate in nigral loss of dopaminergic neurons in hemiparkinsonian monkeys and that NBD peptide treatment protects the nigrostriatum by attenuating the infiltration of T cells.

In summary, our study highlights that NF- κ B is an important regulator of chemokine expression in PD brain and introduces its possible role in the altered adaptive immune response that may cause the increased infiltration and cytotoxic response of CD8⁺ T cells in the nigra of PD brain.

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Highlights

- Marked presence of RANTES in the serum of hemiparkinsonian monkeys
- RANTES was centrally upregulated in the nigra of hemiparkinsonian monkeys
- Parkinsonian toxin MPP⁺ induced RANTES in microglial cells via NF- κ B
- NBD peptide treatment decreased the microglial expression of RANTES and attenuated the infiltration of CD8⁺ T cells in nigra

Figure 1. Schematic presentation of experimental schedule

Fifteen naïve rhesus monkeys received intracarotid injection of MPTP. Only 10 monkeys displayed classical parkinsonian postures within 7d of the first MPTP intoxication. These monkeys were treated with NBD peptides (1 mg/kg body wt/2d) for 30 days via i.m. injection (n=4 for MPTP; n=4 for MPTP+ *wt*NBD; n=2 for MPTP+ *m*NBD). After 30 days of treatment, blood was collected for chemokine measurement.

Figure 2. ELISPOT analyses of different chemokines in monkey serum
Naïve female rhesus monkeys received a right intracarotid injection of MPTP. After 37 d of injection, concentrations of chemokines were determined in the serum using commercially available ELISPOT kit (Raybiotech). Briefly, serum was diluted to 1:1 with assay diluents, incubated with the blot, and detected with the biotin-labeled antibodies by chemiluminescent detection method (A, Control before MPTP injection; B, MPTP). (C) Heatmap analyses were presented after the measurement of pixel density of each spot. Results are mean of four monkeys per group. RNTS, RANTES; ETX, eotaxin.

Figure 3. MPP⁺ stimulates the expression of RANTES and eotaxin in BV-2 microglial cells
 (A) BV-2 microglial cells were treated with 2μM of MPP⁺ for 0, 2, 6, and 12 h under serum-free condition followed by semi-quantitative RT-PCR analyses for RANTES and eotaxin. GAPDH gene was analyzed as control. (B) Quantitative real-time PCR analyses of these genes were analyzed under similar condition. Results are mean ± SD of three independent experiments. *p<0.001 vs. control. Promoter analyses of (C) RANTES and (D) eotaxin were performed in MatInspector, a Genomatrix online promoter scan module. (E) BV-2 microglial cells pre-treated with 5μM wtNBD or mtNBD for 30 min were stimulated with 2μM of MPP⁺ for 30 min followed by monitoring the levels of phospho-IKKβ (p-IKK) and total IKKβ (t-IKK) by Western blot. (F) Bands were scanned and values of p-IKK/Actin and t-IKK/Actin presented as relative to control. *p<0.001 vs. control; **p<0.001 vs. MPP⁺. BV-2 microglial cells pre-treated with 5μM wtNBD or mtNBD for 30 min were stimulated with 2μM of MPP⁺ for 5 h followed by analysis of RANTES and eotaxin mRNA by RT-PCR (G) and real-time PCR (H). (I) After 24 h of stimulation with MPP⁺, supernatants were analyzed for RANTES production using RANTES ELISA kit (eBioscience). *p<0.001 vs. control; **p<0.001 vs. MPP⁺. GAPDH= glyceraldehyde-3-phosphate dehydrogenase. J)

After 24 h of stimulation with MPP⁺, cells were double-immunolabeled for Iba-1 and RANTES.

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Figure 4. ELISA analyses of chemokine in monkey serum

Naïve female rhesus monkeys received a right intracarotid injection of MPTP. After 7 d of injection, monkeys displaying classical parkinsonian postures received wtNBD peptide (1 mg/kg body wt/2d) via i.m. injection. After 30 d of treatment, blood was collected and serum concentration of RANTES (A), eotaxin (B), IL-8 (C), MIP-1 α (D), MIP-1 β (E), MIG (F), ITAC (G), IP10 (H), and MCP-1 (I) were determined by ELISA. Data are means $\pm$ SEM of triplicate assays from four monkeys per group. ^ap<0.001 vs. control before MPTP injection and ^bp<0.001 vs. MPTP.

Figure 5. Effect of wtNBD on the microglial activation of IKK β and expression of RANTES in the nigra of MPTP-intoxicated monkey

Naïve female rhesus monkeys received a right intracarotid injection of MPTP. After 7 d of injection, monkeys displaying classical parkinsonian postures received wtNBD and mNBD peptides (1 mg/kg body wt/2d) via i.m. injection. After 30 d of treatment, nigral sections were double-labeled for IBA-1 (red) and phospho-IKK β (green) (A). Cells positive for phospho-IKK β were counted in three nigral sections (two images per slide) of each of four monkeys (n=4) per group (B). *p<0.001 vs. control and **p<0.001 vs. MPTP. Nigral sections were double-labeled for IBA-1 (red) and RANTES (green) (C). Regions selected inside white box were magnified and displayed in the bottom panel for each treatment group (D). Cells positive for RANTES were counted in three nigral sections (two images per slide) of each of four monkeys (n=4) per group (E). *p<0.001 vs. control and **p<0.001 vs. MPTP.

Figure 6. Effect of wtNBD on the microglial expression of RANTES in the striatum of MPTP-intoxicated monkey

Naïve female rhesus monkeys received a right intracarotid injection of MPTP. After 7 d of injection, monkeys displaying classical parkinsonian postures received wtNBD and mNBD peptides (1 mg/kg body wt/2d) via i.m. injection. After 30 d of treatment, striatal sections were double-labeled for IBA-1 (*red*) and RANTES (*green*) (A). Regions selected inside white box were magnified and displayed in the bottom panel for each treatment group (B). Cells positive for RANTES were counted in three striatal sections (two images per slide) of each of four monkeys (n=4) per group (C). * $p < 0.001$ vs. control and ** $p < 0.001$ vs. MPTP.

Figure 7. Effect of wtNBD on the infiltration of CD3⁺ T cells in the nigra of MPTP-intoxicated monkey

Naïve female rhesus monkeys received a right intracarotid injection of MPTP. After 7 d of injection, monkeys displaying classical parkinsonian postures received wtNBD and mNBD peptides (1 mg/kg body wt/2d) via i.m. injection. After 30 d of treatment, nigral sections were double-labeled for tyrosine hydroxylase (TH; *red*) and CD3 (*green*) (A). Blood vessel of each nigral picture was spotted inside dotted box, magnified, and then displayed next to each original image (B). Results represent analysis of three nigral sections of each of four monkeys (n=4) per group.

Figure 8. Effect of wtNBD on the infiltration of CD4⁺ and CD8⁺ T cells in the nigra of MPTP-intoxicated monkey

Naïve female rhesus monkeys received a right intracarotid injection of MPTP. After 7 d of injection, monkeys displaying classical parkinsonian postures received wtNBD and mNBD peptides (1 mg/kg body wt/2d) via i.m. injection. After 30 d of treatment, nigral sections were double-labeled for CD4⁺ (DAB; *light brown*) and CD8⁺ (DAB-nickel; *dark brown or black*) (A). Infiltrations of CD8⁺ cells (dark brown) were also monitored in the deep parenchyma of ventral midbrain region (B). Cells positive for CD4 (blue bar) and CD8 (red bar) were counted around blood vessels in three nigral sections (two images per slide) of each of four monkeys (n=4) per group (C). Cells positive for CD8 were counted in the deep brain parenchyma in three nigral sections (two images per slide) of each of four monkeys (n=4) per group (D). *p<0.001 vs. control and **p<0.001 vs. MPTP.