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Testing NF- κ B-based therapy in hemiparkinsonian monkeys

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Abstract

Parkinson's disease (PD) is the most common human neurodegenerative disorder affecting movement, balance, flexibility, and coordination. Despite intense investigation, no effective therapy is available to stop the onset PD or halt its progression. The primate model of PD is considered to be one of the best available models for human PD. Since neuroinflammation plays an important role in the pathogenesis of PD and NF- κ B, a proinflammatory transcription factor, participates in the transcription of many proinflammatory molecules, this study evaluates the ability of a peptide corresponding to the NF- κ B essential modifier (NEMO)-binding domain (NBD) of I κ B kinase (IKK) α or IKK β to protect dopaminergic neurons in hemiparkinsonian monkeys. First, we found that NF- κ B was activated within the substantia nigra pars compacta of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-intoxicated hemiparkinsonian monkeys. However, intramuscular injection of wild type NBD (wtNBD) peptide reduced nigral activation of NF- κ B and expression of inducible nitric oxide synthase, protected both the nigrostriatal axis and neurotransmitters, and improved motor functions in hemiparkinsonian monkeys. These findings were specific as mutated NBD peptide did not exhibit such effects. These results may help in the translation of NF- κ B-based therapy to PD clinics.

Keywords

Parkinson's disease; Monkey; NF- κ B; Dopaminergic neurons; Dopamine; Motor function

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease. In the vast majority of cases, it has an unclear etiology (Olanow and Tatton, 1999; Dauer and Przedborski, 2003; Hardy et al., 2006). Clinical symptoms of PD include tremor, bradykinesia, rigidity, and postural instability, which develop due to a severe diminution of nigrostriatal dopamine (DA). Pathologically, PD is characterized by gliosis and progressive degeneration of the dopaminergic neurons associated with the presence of intracytoplasmic inclusions (Lewy bodies) in the substantia nigra pars compacta (SNpc) (Dauer and Przedborski, 2003; Hardy et al., 2006).

Once patients are diagnosed with PD, there is no drug available to halt its progression. Therefore, understanding the mechanism of the disease process of PD may facilitate the

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development of effective therapeutic approaches that could halt disease progression. Although the disease mechanisms that cause PD are poorly understood, recent studies strongly support the role of neuroinflammation in nigrostriatal degeneration seen in PD (Qureshi et al., 1995; Hunot et al., 1996; Dehmer et al., 2000; Dauer and Przedborski, 2003; Gao et al., 2003; Ghosh et al., 2009). Recently we have demonstrated that NF- κ B, a transcription factor required for the transcription of most of the proinflammatory molecules, is activated in the SNpc of PD patients and MPTP-intoxicated mice and that selective inhibition of NF- κ B in mice by NEMO-binding domain (NBD) peptides protect dopaminergic neurons from MPTP toxicity (Ghosh et al., 2007). These mice results suggest that this NF- κ B-based therapeutic approach may be useful in PD. However, as we know mice results are not always translated to humans. On the other hand, similar to humans, parkinsonian monkeys also respond to dopamine replacement therapies and as observed in human PD, after long-term use, dopamine replacement also results in motor complications in monkeys (Collier et al.; Collier et al., 2003). Therefore, the monkey model of PD is considered as the best available model for human PD and it is important to examine its efficacy in a monkey model before testing NBD peptides against PD in humans.

Here we demonstrate that NF- κ B activation is induced in the SNpc of hemiparkinsonian monkeys and that intramuscular treatment of hemiparkinsonian monkeys with NBD peptide results in marked inhibition of nigral NF- κ B activation, suppression of proinflammatory molecules, significant protection of the nigral dopaminergic neurons and striatal dopamine, and improvement of locomotor activities. These results suggest that NBD peptide may be used to halt the disease progression in PD patients.

Materials and Methods

Reagents

Goat anti-human NF- κ B p65, rabbit anti-human BDNF and goat anti-human NT-3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rat anti-human CD11b antibody was purchased from Cedarlane (Burlington, NC). Sheep anti-human GDNF, rabbit anti-human GFAP and mouse anti-human HLA-DR were purchased from Dako (Carpinteria, CA). Rabbit anti-human iNOS was purchased from BD Bioscience (San Diego, CA). Cy2- and Cy5-conjugated antibodies were obtained from Jackson Immuno Research Laboratories (West Grove, PA).

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; Emborg et al., 2001; Kordower et al., 2006). 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) and mutated (m) NBD peptides contain the Antennapedia homeodomain (lower case) and IKK β (upper case) segments. Positions of W→A mutations are underlined (May et al., 2000). wtNBD: drqikiwfnrrmkwkkLDWSWL; mNBD: drqikiwfnrrmkwkkLDASAL. Monkeys were treated with either wtNBD or mNBD peptides (1.0 mg/kg body wt/2 day) in saline through i.m. injection from either 7 d after the first intracarotid injection (Figs. 2–8) or 7 d after the second intracarotid injection of MPTP (Supplementary Fig. 3).

Immunohistochemistry

At the end point, monkeys were anesthetized with pentobarbital (25mg/kg intravenously) and killed via perfusion with 0.9% saline. The brain were removed, immersed in ice-cold saline for 10 min, and slabbbed on a monkey brain slicer (Kordower et al., 2000; Kordower et al., 2006). Slabs through the head of the caudate and putamen were punched bilaterally with a 1-mm brain punch. These punches were processed for HPLC analysis (for quantification of neurotransmitters). Tissue slabs were immersed in Zamboni's fixative followed by immersion in 30% sucrose in PBS. 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 (Calbiochem) and visualized by using DAB/H₂O₂ (DAB kit, Vector Laboratories) as described (Kordower et al., 2000; Ghosh et al., 2007; Ghosh et al., 2009). Estimates of dopaminergic nigral cell number were performed bilaterally using an unbiased design-based counting method (optical fractionator, StereoInvestigator; Microbrightfield, Williston, VT). All counts were performed by a single investigator blinded to the experimental conditions. Using a random start, we outlined the substantia nigra under low magnification (1.25 $\times$ objective) and sampled 20% of the treated nigra or 5% of the intact nigra in a random but systematic manner. Quantitation of striatal TH immunostaining was performed as previously described (Kordower et al., 2000; Ghosh et al., 2007; Ghosh et al., 2009). Optical density measurements were obtained by digital image analysis (Scion, Frederick, MD).

For immunofluorescence staining, mouse anti-human HLA-DR (1:500), rat anti-human CD11b (1:250), goat anti-human NF- κ B p65 (1:100), rabbit anti-human iNOS (1:1000), and rabbit anti-human GFAP (1:1000) were used. The samples were mounted and observed under a Bio-Rad MRC1024ES confocal laser scanning microscope as described before (Khasnavis and Pahan; Ghosh et al., 2007; Ghosh et al., 2009).

HPLC analysis

On the day of the analysis, striatal tissues were sonicated in 0.2M perchloric acid containing isoproterenol (internal standard) and resulting homogenates were centrifuged at 20,000 $\times$ g for 15 min at 4°C. After pH adjusting and filtration, 10 μ l of supernatant was injected onto a Eicompak SC-3ODS column (HPLC-ECD System EiCOMHTEC-500 from JM Science Inc., Grand Island, NY) and levels of dopamine, 3, 4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were analyzed as described earlier (Roy and Pahan; Ghosh et al., 2007; Ghosh et al., 2009).

For measuring NBD peptide, brain punches (dorsomedial caudate) were homogenized in PBS containing protease inhibitor mixture (Sigma) and amyloid- β 1–42 (Bachem Bioscience) as internal standard. Homogenates were centrifuged and supernatants were passed through 100-kDa (Millipore microconYM 100) and 30-kDa (Millipore microconYM 30) protein separation filters, respectively. Flow-through samples were then analyzed in Waters 2695 separation module HPLC system using the Phenomenex Luna C18 separation

column (250 × 4.6 mm; 280-nm UV wavelength) with an isocratic gradient consisting of mobile phase A (18 mM water with 0.01 M H₃PO₄) and mobile phase B (acetonitrile with 0.01 M H₃PO₄) (1:1) at the flow rate of 0.5 ml/min.

Behavioral analysis

The animals were trained to a consistent level of performance before intracarotid injection of MPTP. Monkeys were rated thrice a week using a parkinsonian rating scale (PD scale) utilized to quantify the clinical status of the monkeys (Kordower et al., 2000; Emborg et al., 2001; Kordower et al., 2006). The scale included ratings of 10 parkinsonian features (tremor, posture, locomotion, hypokinesia, bradykinesia, balance, fine and gross motor skills, startle response, and freezing). Each test consisted of twelve total trials alternating between arms, six per side. Delaying feeding time until after the task was completed ensured animals' compliance with the test. Data collected included the time taken for the animal to move its hand into the chamber where the fruit was located (reaction time), the time taken to pick up the fruit while the hand was in the chamber (reception time) and the total time taken to move the hand into the chamber, retrieve the fruit and bring the hand back out of the panel and into the cage (total time).

Statistical analysis

All values are expressed as means ± 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. For behavioral analysis, one way repeated measure ANOVA models were used to compare each outcome measure among four groups. Pairwise comparisons were conducted with bonferroni adjustment.

Results

Characterization of NF-κB in rhesus monkeys

Activation of NF-κB has been poorly characterized in rhesus monkeys. To understand if NF-κB is activated in rhesus monkeys in a similar fashion as humans and mice, we examined the activation of NF-κB in proinflammatory cytokine-stimulated monkey macrophages. Electrophoretic mobility shift assay (EMSA) shows that both human TNF-α and IL-1β induced the activation of NF-κB in monkey macrophages (Fig. 1A). Supershift assay (Fig. 1B) indicates that the activated complex contained p65 and p50, subunits of the classical NF-κB heterodimer, as TNF-α-induced slower migrating band was ablated by antibodies against p65 and p50, but not control IgG. As evident from figure 1C, wtNBD, but not mNBD, peptide also markedly suppressed TNF-α-mediated activation of NF-κB in macrophages, indicating the involvement of IKK in the activation of NF-κB in proinflammatory cytokine-stimulated monkey macrophages.

Treatment of hemiparkinsonian monkeys

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, four monkeys demonstrated these symptoms after second intracarotid injections and one monkey did not show any symptoms even after four intracarotid injections, which was left out of data analysis. The first set of ten monkeys was distributed among three groups: MPTP (n=4), MPTP-wtNBD (n=4) and MPTP-mNBD (n=2). The other set of four monkeys was divided among two groups: MPTP (n=2) and MPTP-wtNBD (n=2). In our mouse study (Ghosh et al., 2007), mice received NBD peptide

daily via i.p. injections from one day prior to or two day after MPTP injection. However, in order to make our primate work relevant to the clinical scenario, monkeys with established hemiparkinsonian symptoms were treated with NBD peptide alternate days via intramuscular (i.m.) injection (Supplementary Figure 1).

NBD peptide inhibits the activation of NF- κ B in vivo in the substantia nigra pars compacta (SNpc) of hemiparkinsonian monkeys

Earlier we have demonstrated that after i.p. injection NBD peptide enters into the brain (Ghosh et al., 2007). Because we decided to treat monkeys via i.m., but not i.p., injection, we examined if after i.m. injection, NBD peptide also reached the brain of hemiparkinsonian monkeys. We used A β 1–42 as an internal standard (Supplementary Figure 2A). We observed a small, but distinct, peak for wtNBD peptide in dorsomedial caudate of wtNBD-treated (Supplementary Figure 2C), but not untreated (Supplementary Figure 2B), hemiparkinsonian monkeys, suggesting that after i.m. injection, NBD peptide is also capable of entering into monkey brain. Next we examined if NBD peptide inhibited the activation of NF- κ B *in vivo* in the SNpc of hemiparkinsonian monkeys. MPTP intoxication led to robust expression of RelA p65 in the SNpc of the lesion side as compared to intact normal side (Fig. 2A–C) and this p65 was principally expressed by microglia (Fig. 2A) and astrocytes (Fig. 2B). Pathways leading to the activation of NF- κ B are becoming clear. In addition to stimulus-induced nuclear translocation of NF- κ B, it has been shown that stimulus-induced phosphorylation and acetylation of p65 are also important for transcriptional activation (Ghosh and Hayden, 2008; Hayden and Ghosh, 2008). Therefore, we also examined the status of phospho-p65 and acetylated-p65. Levels of phospho-p65 (Fig. 3A & C) and acetylated-p65 (Fig. 3B & D) were markedly higher in the SNpc of the lesion side as compared to intact normal side, confirming the presence of NF- κ B in activated state *in vivo* in the SNpc of hemiparkinsonian monkeys. Earlier we have demonstrated that antennapedia homeodomain-containing NBD peptides capable of blocking NEMO/IKK interactions enter into the brain and inhibit the activation of NF- κ B in the SNpc of MPTP-intoxicated mice (Ghosh et al., 2007). Therefore, hemiparkinsonian monkeys were treated with NBD peptides via intramuscular injection. After 30 d of treatment, wtNBD, but not mNBD, peptides strongly inhibited the induction of total p65 (Fig. 3A–C), phospho-p65 (Fig. 3D & F) and acetylated-p65 (Fig. 3E & G) *in vivo* in the SNpc of MPTP-intoxicated monkeys, suggesting that NBD peptide is capable of suppressing the activation of NF- κ B *in vivo* in the nigra of hemiparkinsonian monkeys.

NBD peptide inhibits inflammation in vivo in the SNpc of hemiparkinsonian monkeys

Inflammation plays a role in the loss of dopaminergic neurons in PD and its animal model (Qureshi et al., 1995; Mogi et al., 1996; Dehmer et al., 2000; Nagatsu et al., 2000; Gao et al., 2003; Ghosh et al., 2007; Ghosh et al., 2009). Accordingly, MPTP intoxication led to marked increase in nigral iNOS protein expression and this iNOS was mainly expressed by either CD11b-positive microglia (Fig. 4A) or GFAP-positive astroglia (Fig. 4B). However, similar to the attenuation of p65 expression, wtNBD, but not mNBD, peptide markedly suppressed the expression of iNOS protein in the SNpc of hemiparkinsonian monkeys (Fig. 4A–C).

Apart from NO and iNOS, glial cells in the SNpc of MPTP-treated mice or postmortem PD brain also express proinflammatory cytokines like tumor necrosis factor α (TNF- α), interleukin-1 β (IL-1 β) and IL-6 (Mogi et al., 1996; Nagatsu et al., 2000; Sriram et al., 2006). Accordingly, these proinflammatory cytokines are found elevated in both CSF or serum of PD patients as compared to healthy subjects (Mogi et al., 1996; Nagatsu et al., 2000). Similarly, levels of TNF- α (Fig. 5A), IL-1 β (Fig. 5B), IL-6 (Fig. 5C), and IL-12 (Fig. 5D) were significantly higher in serum of MPTP-insulted monkeys than controls. In contrast,

there were no significant increases in IL-1 α (Fig. 5E) and GM-CSF (Fig. 5F), illustrating the specificity of the effect. However, wtNBD peptide treatment markedly suppressed the serum level of TNF- α , IL-1 β , IL-6, and IL-12 in hemiparkinsonian monkeys (Fig. 5A–D).

NBD peptide protects the nigrostriatum in hemiparkinsonian monkeys

Animals were processed for quantification of dopaminergic cell bodies in the SNpc and of projecting dopaminergic fibers in the striatum using TH immunostaining. Stereological counting of TH-ir neurons in nigral sections showed that MPTP-intoxication led to marked loss of SNpc TH-positive neurons (Fig. 6A–C) in the lesion side compared with the control intact side. Similarly, quantitative measurements of optical density of the TH staining in caudate nucleus and putamen revealed marked loss of striatal TH fibers (Fig. 7A–C) in the lesion side compared with the intact side. However, in hemiparkinsonian monkeys treated with wtNBD, but not mNBD, peptides, SNpc TH-positive neurons (Fig. 6A–C) and striatal TH ODs (Fig. 7A–C) were partly restored.

To determine if NBD peptide protects against biochemical deficits caused in hemiparkinsonian monkeys, we quantified levels of DA in different parts of striatum. MPTP intoxication led to marked loss of DA in dorsomedial (Fig. 8A) and ventromedial caudate (Fig. 8B), the dorsal (Fig. 8C) and ventral putamen (Fig. 8D), and the nucleus accumbens (Fig. 8E) of the lesion side compared to intact control side. In contrast, hemiparkinsonian monkeys that received wtNBD peptide showed partial preservation of DA in different parts of striatum (Fig. 8A–E). However, consistent with the TH striatal optical density measurements (Fig. 7), the wtNBD peptide exhibited greater protection in the putamen than the caudate, while the mNBD peptide had no such protective effect (Fig. 8). Although the wtNBD peptide exhibited beneficial effects in hemiparkinsonian monkeys, it was important to consider whether this peptide might elicit adverse effects on dopaminergic neurons in normal monkeys. However, in our unilateral model, we did not see any significant change in nigral dopaminergic neurons and striatal TH fiber density and DA in the intact side even after 30 d of NBD peptide treatment, suggesting that this effect did not occur.

Next we investigated if wtNBD peptide was capable of protecting striatal DA in hemiparkinsonian monkeys when treatment started very late after two intracarotid injections (Supplementary Figure 3). In this instance, wtNBD peptide remained unable to protect DA in different parts of the striatum (Supplementary Figure 3), suggesting that wtNBD treatment should be started early.

Functional improvement in hemiparkinsonian monkeys after NBD peptide treatment

The ultimate goal of neuroprotective therapies is to decrease functional impairments. Therefore, monkeys were assessed for performance on a hand reaching task, general activity, and clinical dysfunction (tremor, posture, locomotion, bradykinesia, balance, fine and gross motor skills, startle response, and freezing) based on a clinical rating scale modeled after the Unified Parkinson's Disease Rating Scale. As previously reported (Kordower et al., 2000; Emborg et al., 2001; Kordower et al., 2006), MPTP injection caused a marked decrease in performance in the left hand (contralateral to the MPTP infusion) while maintaining relatively normal function with the right hand (Table 1). Interestingly, significant improvement in functional ability was observed by wtNBD peptide treatment on both a subjective clinical rating scale and an objective operant motor test (Table 1), whereas mNBD peptide had no effect. On the other hand, we did not observe any drug related side effect (e.g. hair loss, untoward infection, hyperkinesias, psychological disturbance, vomiting, diarrhea etc) in any of these monkeys.

Discussion

Although the disease mechanisms that cause PD are poorly understood, a number of recent studies demonstrate the importance of chronic neuroinflammation in nigrostriatal degeneration in this disease. Promoter regions of many proinflammatory molecules contain the DNA binding site for NF- κ B (Saha et al., 2006; Saha and Pahan, 2006; Ghosh and Hayden, 2008; Hayden and Ghosh, 2008). Furthermore, the inhibition of NF- κ B activation reduces the induction of proinflammatory molecules (May et al., 2000; Ghosh et al., 2007; Hayden and Ghosh, 2008). These results strongly suggest that NF- κ B is an important regulator of inflammation. 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 NEMO (NF- κ B essential modifier) (May et al., 2000; Ghosh and Karin, 2002). Ghosh and colleagues (May et al., 2000) have shown that peptides corresponding to the NEMO-binding domain (NBD) of I κ B kinase (IKK) α or IKK β specifically inhibit the induction of NF- κ B activation without inhibiting basal NF- κ B activity. We have previously demonstrated that activation of NF- κ B is induced in the SNpc of MPTP-intoxicated mice and that a cell-permeable NBD peptide capable of inhibiting the induction of NF- κ B activation without altering the basal activity of NF- κ B can enter the CNS and protect dopaminergic neurons (Ghosh et al., 2007). However, due to the wide immunological gap between inbred rodent strains and heterogeneous human population, rodent results are not always translated to humans. On the other hand, rhesus monkeys are old world monkeys and both rhesus monkeys and human are thought to have diverged from a common ancestor, a primitive anthropoid, approximately 25 million years ago. Interestingly, despite 25 million years of evolutionary separation, humans and macaques apparently share about 93% of their DNA sequence (Gibbs et al., 2007). It is also important to note that the immune systems of rhesus monkeys and humans are very similar. Therefore, in situations, where it is desirable, but impossible, to study human neuroimmune responses *in vivo*, rhesus monkey provides the best available alternative.

Several lines of evidence presented in this manuscript clearly demonstrate that NF- κ B is activated *in vivo* in the nigra of hemiparkinsonian monkeys and that selective inhibition of NF- κ B activation by NBD peptide treatment results in protection of monkeys both clinically and pathologically. Our conclusions are based on the following. *First*, only the monkeys that exhibited parkinsonian posture, tremor and left hand freezing after intracarotid injection of MPTP were selected for NBD peptide treatment. *Second*, chronic inflammation is a hallmark of PD pathology. Similarly, even after 37 d of single MPTP injection, levels of NF- κ B p65, phospho-p65, acetylated-p65, and iNOS remained markedly higher in the lesioned nigra than non-lesioned nigra. However, i.m. injection of wtNBD, but not mNBD, peptide strikingly suppressed nigral expression of p65, phospho-p65, acetylated-p65, and iNOS. Similar to PD patients, hemiparkinsonian monkeys also exhibited increased serum levels of IL-1 β , TNF- α , IL-6, and IL-12 compared to normal monkeys. As expected, wtNBD peptide treatment significantly attenuated serum levels of these proinflammatory cytokines in hemiparkinsonian monkeys. *Third*, as observed in PD, nigral dopaminergic neurons disappeared and the level of dopamine (DA) decreased in hemiparkinsonian monkeys. But treatment with wtNBD, but not mNBD, peptide protected TH-positive dopaminergic neurons and restored the level of DA. *Fourth*, wtNBD, but not mNBD, peptide also reversed motor deficits in a hand-reach task. These results may help in establishing NF- κ B as a therapeutic target in PD and in translating this NF- κ B-based treatment to the clinic.

At present, no effective therapy is available to halt the progression of PD. Administration of a dopamine agonist or levodopa has been the standard treatment for PD. However, it is often associated with a number of side effects and unsatisfactory outcomes. For example, after 2 years of therapy with levodopa, 30–50% of patients begin to experience dopaminergic-related complications, including dyskinesia, wearing off, or on-off motor fluctuations. These

motor complications may eventually dominate the clinical picture. Therefore, development of NF- κ B-based therapeutic approach to halt the disease progression in hemiparkinsonian monkeys is of paramount importance. NF- κ B is a multifunctional transcription factor and in addition to its involvement in inflammation, activated NF- κ B is also known to protect cells by up-regulating several anti-apoptotic genes (Ghosh and Karin, 2002; Hayden and Ghosh, 2008). In this connection, one might expect an adverse event where NBD peptide may augment the death of dopaminergic neurons by inhibiting neuronal induction of NF- κ B activation. However, after one month of NBD peptide treatment we have seen protection, but not greater loss, of dopaminergic neurons in hemiparkinsonian monkeys. Probably, under this scenario, NBD peptide is also inhibiting the induction of NF- κ B activation in cells producing proinflammatory molecules resulting in an inhibition of proinflammatory molecule production. As a result, the inflammatory insult on dopaminergic neurons is reduced and, in turn, dopaminergic neurons probably do not need induced NF- κ B activation to survive in a noninflamed environment.

In summary, we have demonstrated that intramuscular NBD peptide treatment blocks the activation of NF- κ B and the expression of iNOS in the SNpc, protects the loss of dopaminergic neurons, improves striatal DA, and reverse motor deficits in hemiparkinsonian monkeys. These novel results should move NBD peptide therapy one step closer to PD clinics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Time (min) - 30 60 30 60

A IL-1 β - - - + +

TNF- α - + + - -

Cont IgG - - - - +

p50 Ab - - - + -

p65 Ab - - + - -

TNF- α - + + + +

B

mNBD (μ M) 0 0 0 10

wtNBD (μ M) 0 0 10 0

TNF- α - + + +

C

Fig. 1. Activation of classical NF- κ B p65:p50 heterodimer in monkey macrophages

Monkey macrophages were isolated from peripheral blood using the Ficoll-Hypaque method. Briefly, peripheral blood mononuclear cells (PBMC) were separated from erythrocytes by density centrifugation on a Ficoll (Fisher) gradient, washed and resuspended in culture media (5×10^6 cells ml^{-1}). Cells were plated in 12-well plates (1 ml/well) and allowed to adhere for 30 min. Non-adherent cells were removed and adherent cells (mostly macrophages) were washed thrice with RPMI-1640. Four h after plating, macrophages were stimulated with human TNF- α (50 ng/ml) and IL-1 β (50 ng/ml) for different time periods followed by monitoring DNA-binding activity of NF- κ B (A) in nuclear extracts by electrophoretic mobility shift assay (EMSA) using infrared-labeled consensus NF- κ B-

binding sequence (5'-AGT TGA GGG GAC TTT CCC AGG C-3' (Licor Biosciences) as described earlier (Jana and Pahan). B) For the supershift assay, nuclear extract suspended in binding buffer was incubated with 1 µg of anti-p65 Ab, anti-p50 Ab or control IgG for 30 min in ice prior to separation on a 6% polyacrylamide gel. C) Cells preincubated with wtNBD or mNBD peptide for 30 min were stimulated with TNF-α for 1 h followed by monitoring DNA-binding activity of NF-κB by EMSA. Results represent three separate experiments.

Fig. 2. WtNBD, but not mNBD, peptide treatment decreases the induction of NF- κ B p65 in the SNpc of hemiparkinsonian monkeys

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 immunostained for CD11b (red) & p65 (green) (A) and GFAP (green) & p65 (red) (B). DAPI was used to visualize nucleus. NF- κ B p65 positive cells were counted in four nigral sections (two images per slide) of each monkey [MPTP, n=4; (MPTP+wtNBD), n=4; (MPTP+mNBD), n=2] in an Olympus IX81 fluorescence microscope using the MicroSuiteTM imaging software (C). ^a $p < 0.0001$ vs control; ^b $p < 0.0001$ vs MPTP.

Fig. 3. WtNBD, but not mNBD, peptide treatment decreases the phosphorylation and acetylation of NF- κ B p65 in the SNpc of hemiparkinsonian monkeys

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 immunostained for HLA-DR (red) & phospho-p65 (green) (A) and HLA-DR (red) & acetylated p65 (green) (B). DAPI was used to visualize nucleus. Cells positive for phospho-p65 (C) and acetylated-p65 (D) were counted in four nigral sections (two images per slide) of each monkey [MPTP, n=4; (MPTP+wtNBD), n=4; (MPTP+mNBD), n=2]. ^a $p < 0.0001$ vs control; ^b $p < 0.0001$ vs MPTP.

Fig. 4. WtNBD, but not mNBD, peptide treatment decreases the expression of iNOS *in vivo* in the nigra of hemiparkinsonian monkeys

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 immunostained for CD11b (red) & iNOS (green) (A) and GFAP (green) & iNOS (red) (B). DAPI was used to visualize nucleus. Cells positive for iNOS were counted in four nigral sections (two images per slide) of each monkey [MPTP, n=4; (MPTP+wtNBD), n=4; (MPTP+mNBD), n=2] in an Olympus IX81 fluorescence microscope using the MicroSuite imaging software (C). ^a $p < 0.0001$ vs control; ^b $p < 0.0001$ vs MPTP.

Fig. 5. WtNBD, but not mNBD, peptide treatment decreases the induction of serum proinflammatory cytokines in hemiparkinsonian monkeys

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, blood was collected and serum concentration of various proinflammatory cytokines was measured by ELISA (A, TNF-α; B, IL-1β; C, IL-6; D, IL-12; E, IL-1α; F, GM-CSF) by a single investigator blinded to the experimental conditions. Data are means $\pm$ SEM of triplicate assays from four monkeys per group. ^a $p < 0.001$ vs control; ^b $p < 0.05$ vs control; ^c $p < 0.001$ vs MPTP; ^d $p < 0.05$ vs MPTP.

Fig. 6. WtNBD, but not mNBD, peptide treatment protects nigral dopaminergic neurons in hemiparkinsonian monkeys

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 immunostained for TH (A, lower magnification; B, higher magnification). C) Estimates of dopaminergic nigral cell number [MPTP, $n=4$; (MPTP+wtNBD), $n=4$; (MPTP+mNBD), $n=2$] were performed bilaterally using an unbiased design-based counting method (optical fractionator, StereoInvestigator; Microbrightfield, Williston, VT). All counting were performed by a single investigator blinded to the experimental conditions. ^a $p < 0.001$ vs MPTP-intact; ^b $p < 0.01$ vs MPTP-lesion.

Fig. 7. WtNBD, but not mNBD, peptide treatment protects striatal fibers in hemiparkinsonian monkeys

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 immunostained for TH (A). Density of TH fibers in caudate (B) and putamen (C) is expressed as relative to intact side of the MPTP group. Analysis of six different striatal sections of each monkey [MPTP, n=4; (MPTP+wtNBD), n=4; (MPTP+mNBD), n=2] was performed by a single investigator blinded to the experimental conditions. ^a $p < 0.0001$ vs MPTP-intact; ^b $p < 0.05$ vs MPTP-lesion; ^c $p < 0.001$ vs MPTP-lesion.

Fig. 8. WtNBD, but not mNBD, peptide protects striatal dopamine in hemiparkinsonian monkeys

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, dopamine levels were measured in dorsomedial caudate (A), ventromedial caudate (B), dorsal putamen (C), ventral putamen (D), and nucleus accumbens (E). HPLC quantification of dopamine was performed by a single investigator blinded to the experimental conditions. Data are means $\pm$ SEM of triplicate assays from each monkey [MPTP, n=4; (MPTP+wtNBD), n=4; (MPTP+mNBD), n=2]. ^a $p < 0.001$ vs MPTP-intact; ^b $p < 0.05$ vs MPTP-lesion.

WT/NBD, but not mNBD, peptide led to functional improvements in hemiparkinsonian monkeys

Table 1

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. Monkeys [MPTP, n=4; (MPTP+wtNBD), n=4; (MPTP+mNBD), n=2] were rated thrice a week using a parkinsonian rating scale (PD scale) utilized to quantify the clinical status of the monkeys. The scale included ratings of 10 parkinsonian features (tremor, posture, locomotion, hypokinesia, bradykinesia, balance, fine and gross motor skills, startle response, and freezing). Monkeys were not tested on these tasks during the week between MPTP intoxication and initiation of NBD peptide treatment.

Motor parameters	Before MPTP infusion		After MPTP infusion		After treatment		
					MPTP + saline	MPTP + wt NBD	MPTP + mNBD
General (Gross) motor skills							
	R Hand	0 ± 0.0	0 ± 0.0		0 ± 0.0	0 ± 0.0	0 ± 0.0
L Hand	0 ± 0.0	2.56 ± 0.11			2.47 ± 0.14	1.48 ± 0.16 *	2.48 ± 0.15
Tremor							
	R Hand	0 ± 0.0	0 ± 0.0		0 ± 0.0	0 ± 0.0	0 ± 0.0
L Hand	0 ± 0.0	1.63 ± 0.16			1.5 ± 0.14	0.39 ± 0.12 *	1.45 ± 0.1
Rigidity (upper left arm)	0 ± 0.0		2.32 ± 0.17		2.25 ± 0.22	0.96 ± 0.06	2.19 ± 0.2
Posture	0 ± 0.0		1 ± 0.0		1 ± 0.00	0.53 ± 0.05 *	0.96 ± 0.05
Fine motor function of Hands							
	R Hand	0 ± 0.0	0.485 ± 0.03		0.46 ± 0.05	0.1 ± 0.01	0.42 ± 0.05
L Hand	0 ± 0.0	2.41 ± 0.10			2.35 ± 0.12	1.33 ± 0.15 *	2.38 ± 0.11
Bradykinesia	0 ± 0.0		1.41 ± 0.69		1.3 ± 0.15	0.46 ± .05 *	1.27 ± .026
Freezing	0 ± 0.0		0 ± 0.0		0 ± 0.0	0 ± 0.0	0 ± 0.0
Total (sum means)	0 ± 0.0		11.81 ± 1.26		11.33 ± 0.82	5.44 ± 0.57 *	11.11 ± 0.99

* $p < 0.05$ vs (MPTP+saline).