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Anti-inflammatory and neuroprotective effects of PGE₂ EP4 signaling in models of Parkinson's disease

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Abstract

Inflammation is a ubiquitous factor accompanying normal aging and neurodegeneration, and recent studies indicate a major contribution of inducible cyclooxygenase (COX-2) and its downstream prostaglandin signaling pathways in modulating neuroinflammatory responses and neuronal function. We have previously shown that the prostaglandin PGE₂ receptor EP4 suppresses innate immune responses in models of systemic inflammation. Here we investigated the role of the EP4 receptor in models of Parkinson's disease (PD). Systemic co-administration of the EP4 agonist ONO-AE1-329 with the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) prevented loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) without significant changes in glial activation, suggesting a potent neuroprotective effect of EP4 signaling in this acute model of DA neuronal loss. Cell-specific conditional ablation of

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Conflict of interest

The authors declare that they have no conflict of interest.

Authors' contributions

SSP designed and performed all MPTP and cell culture experiments, and prepared the manuscript. KS performed ELISA analysis of conditioned media from microglia and BV2 cells. ACG assisted with sectioning of brains, and immunostaining and image analysis of brain sections. JUJ performed isolation of the peritoneal macrophages used in this study and assisted in gene expression analysis. QW assisted with logistics of various experiments, in particular the luminex analysis of conditioned media from microglia. NK performed HPLC analysis of dopamine and associated metabolites from striatal brain sections. AMB collaborated on this study by lending stereology and HPLC equipment from her lab and advised in the design of experiments. KIA provided the resources to conduct the majority of experiments, and provided guidance on experimental design and manuscript preparation. All authors have read and approved the final version of the manuscript.

EP4 in Cd11bCre;EP4^{lox/lox} mice exacerbated MPTP-associated glial activation and T-cell infiltration in SNpc, consistent with anti-inflammatory functions of microglial EP4 signaling. In vitro, in primary microglia stimulated with oligomeric α -synuclein, EP4 receptor activation suppressed generation of pro-inflammatory and oxidative stress factors. Taken together, these findings suggest a dual neuroprotective and anti-inflammatory mechanism of action by the EP4 receptor in models of PD.

Background

Parkinson's Disease (PD) pathophysiology is primarily characterized by the accumulation of misfolded protein aggregates of α -synuclein and degeneration of neurons, in particular the dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) [1]. The mechanisms driving these processes, however, remain poorly understood [2]. Recent studies have identified non cell-autonomous mechanisms such as inflammation and oxidative stress as major contributors to disease pathology in various neurodegenerative diseases including PD [3-6]. Neurotoxicity in PD patients and animal models of PD is accompanied by significant increases in microgliosis and astrogliosis [7, 8]. Reactive microglia and astrocytes influence the local tissue environment by secreting inflammatory factors such as cytokines, chemokines, and reactive oxygen and nitrogen species [9-11]. These inflammatory mediators have been suggested to promote the aggregation of α -synuclein *in vitro* and result in secondary neurotoxicity to DA neurons *in vivo* [12-15]. Consistent with these observations, inhibiting inflammation is neuroprotective in animal models of PD and may exert preventive effects in human PD [16].

Prostaglandin E₂ (PGE₂) is a lipid signaling molecule important in regulating a broad range of physiological functions including vascular and bronchiolar tone, fever response, and renal function. In the brain, activity of the cyclooxygenases COX-1 and COX-2 results in the downstream generation of PGE₂ by neurons and glia. PGE₂ elicits cell-specific effects by binding to E-prostanoid (EP) receptors EP1 through EP4, which are G-protein coupled receptors with distinct downstream signaling cascades. In neurons, PGE₂ elicits neurotoxicity through the EP1 receptor [17, 18] and neuroprotection through the EP4 receptor [19], while in microglia PGE₂ can play pro- or anti-inflammatory roles depending on which EP receptor is activated [20-23]. Conditional deletion of the PGE₂ EP4 receptor in microglia increases brain inflammation in a model of innate immunity [22] and Alzheimer's disease [40]. Interestingly, PGE₂ is significantly elevated in the substantia nigra and CSF of PD patients, suggesting it may play a role in PD pathogenesis [24]. Given the anti-inflammatory effects of EP4 signaling noted in models of neuroinflammation characterized by an innate immune response, we investigated the role of the EP4 receptor in models of PD.

Administration of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is an established model of experimental PD characterized by a loss of dopaminergic (DA) neurons selectively in the substantia nigra pars compacta (SNpc). In addition, MPTP causes an immediate and localized neuroinflammatory response in the SNpc that precedes the loss of DA neurons, suggesting an interaction between neuroinflammation and neurodegeneration acutely in this model. α -synuclein was the first gene to be associated

with PD [25], and encodes a protein highly enriched in presynaptic terminals that regulates vesicle exocytosis and synapse maintenance [26-28]. α -synuclein is also the predominant component of PD-associated intraneuronal Lewy bodies [29]. Studies indicate that α -synuclein aggregates can activate microglia and astrocytes and induce production of the pro-inflammatory cytokine Interleukin-1 β (IL-1 β) and reactive oxygen species (ROS) *in vitro* [14, 15, 30, 31]. ROS injure DA neurons *in vitro* [13] and inhibiting ROS production reduces DA neurodegeneration *in vivo* [32].

In this study, we investigated the role of PGE₂ EP4 signaling in modulating neuroinflammation *in vivo* in the MPTP model, and *in vitro* using α -synuclein oligomers. Our findings support a dual neuroprotective and anti-inflammatory role of EP4 signaling in these PD models.

Methods

Materials

The EP4 agonist ONO-AE1-329 was a generous gift from ONO Pharmaceuticals (Osaka, Japan). Its specificity for the EP4 receptor has been established previously (Suzawa et al., 2000). Purified recombinant human wt α -synuclein was purchased as a lyophilized powder from rPeptide (Athens, GA) and resuspended in PBS at a concentration of 1 mg/ml. Aggregated α -synuclein was generated by incubation in PBS for 7 days at +37°C with continuous shaking at 250 rpm (Zhang et al., 2005). Cell culture media, supplements, and antibiotics were purchased from Life Technologies (Grand Island, NY).

Animals and treatments

This study was conducted in accordance with the National Institutes of Health guidelines for the use of experimental animals and protocols were approved by the Institutional Animal Care and Use Committee at Stanford University. C57BL/6J EP4^{lox/lox} mice (Schneider et al., 2004) were kindly provided by Drs. Richard and Matthew Breyer (Vanderbilt University School of Medicine, Nashville, TN). C57BL/6J Cd11bCre mice (Boillee et al., 2006) were kindly provided by Dr. G. Kollias (Alexander Fleming Biomedical Sciences Research Center, Vari, Greece) and Dr. Donald Cleveland (University of California San Diego, San Diego, CA). All mice were housed in an environment controlled for lighting (12h light/dark cycle), temperature, and humidity, with food and water available *ad libitum*. For MPTP studies, male C57B/6J mice received i.p. injections of MPTP-HCl (Sigma-Aldrich, St. Louis, MO) dissolved in saline (0.9% NaCl) at 2 hr intervals as described in detail previously (Jackson-Lewis and Przedborski, 2007). Two acute MPTP injection paradigms were used: 3 injections at a dose of 20 mg/kg or 4 injections at a dose of 16 mg/kg. For the EP4 agonist experiment, AE1-329 was dissolved in saline and injected s.c. at 0.1 mg/kg once a day for 6 days following MPTP treatment. The first EP4 agonist injection was given 12 hr after the last MPTP injection to avoid interference with the metabolism of MPTP/MPP⁺. Mice were sacrificed on day 7 post-MPTP injection and brains were harvested for tissue analysis without perfusing the mice. All mice were between 1.5-3.5 months old at the time of the first injection.

Immunohistochemistry

Brains were postfixed in 4% paraformaldehyde at +4°C for 24h, transferred to a 30% sucrose solution at +4°C for 72 hr, and sectioned on a freezing microtome into 40 μ m coronal sections. Free-floating brainstem sections were collected in six separate series and processed for immunohistochemistry. The following primary antibodies were used at +4°C for 24 hr: anti-Iba1 (1/1000; Wako, Richmond, VA), anti-GFAP (1/1000; Dako, Glostrup, Denmark), anti-CD3 (1/200; BD Pharmingen, Franklin Lakes, NJ), and anti-TH (1/1000; Sigma-Aldrich, St. Louis, MO). Secondary antibodies used were: donkey anti-mouse AlexaFluor 488 (1/1000), donkey anti-rabbit AlexaFluor 555 (1/1000), and goat anti-hamster AlexaFluor 647 (1/200) (Life Technologies, Grand Island, NY). Images of brain sections were acquired using a Nikon Eclipse E600 microscope (Nikon Instruments, Melville, NY) and a Hamamatsu Orca-ER digital camera (Hamamatsu Photonics, Bridgewater, NJ). The SNpc was delineated using TH immunostaining to identify the A9 dopaminergic neurons. The percent area positive for Iba1 and GFAP immunostaining within the SNpc was quantified using the measurements module of Volocity 6.3 image analysis software (Improvision, Waltham, MA).

TH Stereology

For stereological counting, every sixth section spanning the entire substantia nigra was sampled, for a total of 8 sections per brain. Endogenous peroxidase activity was quenched in free-floating sections with 0.3% H₂O₂ for 20 min. Sections were immunostained with anti-TH (1/2000; Novus, Littleton, CO) at +4°C for 24 hr, incubated with biotinylated secondary donkey anti-rabbit IgG (1/1000) at room temperature for 2 hr, and incubated for 1 hr in avidin-biotin peroxidase complex (Vector Laboratories, Burlingame, CA). Visualization was performed using 0.03% 3,3'-diaminobenzidine (DAB) for 2 min at room temperature. The SNpc was delineated at low magnification (10X) and systematically sampled at a higher magnification (20X) to determine the number of TH⁺ neurons using the optical fractionator technique as described previously [33].

HPLC

A 1 mm² piece of dorsal striatal tissue was dissected 3 mm posterior to the olfactory bulb and immediately flash frozen in liquid nitrogen. Catecholamines were acid extracted in 0.3N perchloric acid, and dopamine, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were quantified by reverse phase HPLC coupled with electrochemical detection. The pellet was dried and reconstituted in 0.5N NaOH and briefly sonicated, and total protein determined using the Lowry method.

ELISA

Protein levels in cell conditioned media were measured using Enzyme linked immunosorbent assay (ELISA) experiments. ELISAs were carried out according to the manufacturer's instructions for IL-1 β (BD Biosciences, San Diego, CA), TNF α , MIP-1 α , and MCP-1 (R&D Systems, Minneapolis, MN) and quantified using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA).

Primary Microglia Isolation

Primary microglia were obtained from the brains of postnatal day 7 C57BL/6J mouse pups from Charles River Laboratories (Davis, CA). Microglia were isolated using the Neural Tissue Dissociation Kit (P), MACS Separation Columns (LS), and magnetic Cd11b Microbeads as previously described [34, 40] (Miltenyi Biotec, Auburn, CA), and kept in culture for 48-72 hr before being used for an experiment.

Cell culture

Primary microglia and murine immortalized microglial BV-2 cells were grown in DMEM supplemented with 10% heat inactivated FBS (HyClone, Logan, UT) and 100 U/ml each of penicillin and streptomycin. Cells were grown in a cell incubator (NuAire, Plymouth, MN) at +37°C in a humidified atmosphere containing 5% CO₂.

Multi-analyte Luminex analysis of conditioned media

Mouse 38-plex kits were used according to the manufacturer's recommendations with modifications as described below. Briefly, microglial conditioned medium was frozen at -80°C at the designated time point and thawed on ice immediately before Luminex analysis. Samples were mixed with antibody-linked polystyrene beads on 96-well filter-bottom plates and incubated at room temperature for 2 hr followed by overnight incubation at 4°C. Room temperature incubation steps were performed on an orbital shaker at 500-600 rpm. Plates were vacuum filtered and washed twice with wash buffer, then incubated with biotinylated detection antibody for 2 hr at room temperature. Samples were then filtered and washed twice as above and resuspended in streptavidin-PE. After incubation for 40 min at room temperature, two additional vacuum washes were performed, and the samples resuspended in Reading Buffer. Each sample was measured in duplicate. Plates were read using a Luminex 200 instrument with a lower bound of 100 beads per sample per cytokine.

RNA Isolation and qRT-PCR

RNA purification from BV2 cells was performed immediately after cell isolation using Trizol (Life Technologies) and quantitative real-time PCR (qPCR) was performed as previously described [21, 34]. Briefly, total RNA was isolated using Trizol reagent (Invitrogen), treated with DNase (Invitrogen), and the reaction was terminated by heating at 65°C for 10 min. First strand cDNA synthesis was performed with 0.4 or 2 µg of total RNA from Cd11b Ab-bead sorted macrophages or tissue, respectively, combined with 4 U of Omniscript enzyme (Qiagen) and 0.25 µg of random primer in a reaction volume of 20 µl at 37°C for 1 hr. Reverse transcribed cDNA was diluted 1:10 –1:20 in RNase-free ddH₂O for subsequent qRT-PCR. The mRNA level for each target gene was quantified by SYBR Green based qPCR using the QuantiTect SYBR Green PCR kit (Qiagen). Melting curve analysis confirmed the specificity of each reaction. The standard curve method was used for quantification and gene expression level was normalized to GAPDH. Forward and reverse primers are listed in Table 1.

Statistical Analysis

Prism software (GraphPad Software, Inc., San Diego, CA) was used for statistical analyses. Data are presented as mean $\pm$ standard error of the mean and analyzed using Student's T-test (for two groups) or two-way analysis of variance with Tukey's multiple comparisons (with post-test comparisons between individual groups) two-tailed unless otherwise indicated. Results with $p < 0.05$ were considered significant.

Results

Co-treatment with EP4 agonist in MPTP model

The EP4 agonist ONO-AE1-329 was administered at a dose of 0.1 mg/kg s.c. to MPTP treated mice (20 mg/kg i.p.) once a day for 6 days. The first dose of the EP4 agonist was administered at least 12 hours after the last MPTP injection so as not to interfere with metabolism of either compound. Brains were collected on day 7 and brainstem sections spanning the SNpc were immunostained with an anti-tyrosine hydroxylase (TH) antibody. The total number of surviving TH+ neurons was counted using stereological techniques. There was no significant difference in the number of surviving TH+ neurons in the saline treated mice with or without AE1-329. Treatment with MPTP caused a 23% reduction in the number of surviving TH+ neurons in the SNpc, which was prevented by co-treatment with AE1-329 (Figure 1). Two-way ANOVA analysis showed a statistically significant effect of MPTP ($p=0.0179$) and an interaction effect between MPTP and AE1-329 treatment ($p=0.0266$) suggesting that pharmacologic activation of the EP4 receptor by AE1-329 exerts a neuroprotective effect on dopaminergic neurons in the SNpc.

Cell-specific conditional knockout of EP4 receptor in microglia

The Cre-loxP strategy was used to probe the immune function of the EP4 receptor in microglia in the MPTP model. The EP4 gene was conditionally deleted in myeloid cells by crossing C57BL/6J Cd11bCre transgenic mice to C57BL/6J EP4^{lox/lox} mice. The Cd11b driver restricts expression of Cre recombinase to myeloidlineage cells, notably microglia and macrophages, and excises genomic EP4 sequences in these cell types. The resulting Cd11bCre;EP4^{lox/lox} mice will be referred to as microglial EP4 cKO mice. The microglial EP4 cKO mice and the littermate controls (Cd11bCre;EP4^{+/+}) were treated with MPTP (4 $\times$ 16 mg/kg i.p.) and brains were harvested 7 days later. Brain slices were immunostained with an anti-TH antibody and stereology was performed to count the number of surviving TH+ neurons in the SNpc. Treatment with MPTP caused a ~25% reduction in the number of surviving TH+ neurons in the control cohort and the microglial EP4 cKO mice (Figure 2A). These data suggest that ablating the EP4 receptor in myeloid cells does not influence acute MPTP associated neurotoxicity. We further quantified the damage to dopaminergic neurons by measuring levels of dopamine and its metabolites in the striatum. MPTP treatment led to a ~75% decrease in dopamine levels in both the control cohort and the microglial EP4 cKO mice (two-way ANOVA: MPTP effect $p<0.0001$). There was no effect of genotype and no interaction effect between genotype and MPTP treatment (Figure 2B). Similarly, the dopamine turnover ratio was increased in the MPTP treated mice (two-way ANOVA: MPTP effect $p<0.0001$), and there were no differences between the genotypes (Figure 2B). These

results suggest that microglial EP4 does not play a significant role in protecting neuronal health or survival in the acute MPTP model.

Co-immunostaining with anti-TH and anti-Iba1 antibodies revealed a 500% increase in the Iba1+ area in the SNpc of MPTP treated microglial EP4 cKO mice compared to the control group (Figure 2C). A two-way ANOVA analysis showed a statistically significant effect of MPTP ($p=0.0417$), an effect of genotype ($p=0.0402$), and an interaction effect between MPTP and genotype ($p=0.0415$). There was no significant difference in the Iba1+ area between the two genotypes in the saline treated mice. Co-immunostaining with anti-TH and anti-GFAP antibodies revealed a 313% increase in the GFAP+ area in the SNpc of MPTP treated microglial EP4 cKO mice compared to the control group (Figure 2D). Two-way ANOVA showed a statistically significant effect of MPTP ($p=0.0015$) and a trend towards an interaction between MPTP treatment and genotype ($p=0.0834$). Again there was no difference in the GFAP+ area at baseline between the two genotypes. These results suggest that conditionally ablating the EP4 receptor in microglia and macrophages enhances the innate inflammatory response in the MPTP model. The neuroinflammatory response was further characterized by quantifying CD3+ infiltrating T-cells in the SNpc. Co-immunostaining with anti-TH and anti-CD3 antibodies showed a 1468% increase in infiltrating T-cells in the SNpc of MPTP treated microglial EP4 cKO mice compared to the control group (two-way ANOVA: MPTP effect $p=0.0077$, genotype effect $p=0.0151$, interaction between MPTP and genotype $p=0.0164$) (Figure 2E). These results suggest that removal of anti-inflammatory myeloid EP4 signaling increases glial activation and enhances adaptive immune cell infiltration in this model.

Treatment of microglia with α -synuclein

In vivo models of PD that exhibit both DA neuron loss and aggregation of α -synuclein are currently being developed. To model the immune effects of EP4 signaling in the context of α -synuclein, we turned to an *in vitro* approach to test whether EP4 signaling might alter microglial responses induced by aggregated α -synuclein. BV2 cells (murine microglial cell line) and primary microglia harvested from postnatal mice were treated with oligomeric recombinant wild type human α -synuclein and quantitative real-time PCR was carried out to measure gene expression levels of inflammatory and oxidative stress pathway genes. α -synuclein treatment was highly immunogenic, as evidenced by significant increases in interleukin-1 beta (IL-1 β) transcript levels that increased by 2335% compared to vehicle treated BV2 cells ($p<0.0001$). Concurrently, tumor necrosis factor α (TNF- α) transcript levels increased by 224% ($p<0.0001$), inducible nitric oxide synthase (iNOS) transcript levels increased by 484% ($p=0.0051$), cyclooxygenase-2 (COX-2) transcript levels increased by 103% ($p=0.0296$), and NADPH oxidase (gp91) transcript levels increased by 94% ($p=0.0391$) compared to the control group (Figure 3A). On the other hand, α -synuclein treatment led to a decrease of 28% in the anti-inflammatory nuclear receptor related-1 (Nurr1) transcript levels ($p=0.0245$).

We then quantified the levels of secreted pro-inflammatory cytokines IL-1 β and TNF- α , and chemokines macrophage inflammatory protein 1 α (MIP-1- α /CCL3), and monocyte chemotactic protein-1 (MCP-1/CCL2) (Figure 3B). Treatment of primary microglia with

oligomeric α -synuclein led to robust increases in secreted TNF- α (~4400%, $p<0.0001$), IL-1 β (~20,000%, $p<0.0001$), CCL3 (~550%, $p<0.0001$), and CCL2 (~500%, $p<0.0001$) compared to vehicle treated microglia. Co-treatment with the EP4 agonist AE1-329 led to a significant decrease in the secretion of TNF- α (47%, $p<0.0001$), IL-1 β (94%, $p<0.0001$), CCL3 (41%, $p<0.0001$), and CCL2 (39%, $p<0.0001$). A statistically significant interaction was observed between the EP4 agonist and oligomeric α -synuclein (two-way ANOVA: interaction effect for TNF- α , IL-1 β , CCL3 $p<0.0001$; interaction effect for CCL2 $p=0.0054$). This demonstrates that activation of the EP4 receptor dampens the α -synuclein induced inflammatory response in microglia. These results were replicated in BV2 cells as well as peritoneal macrophages (Figure 3C). As a negative control, monomeric α -synuclein did not induce secretion of TNF- α in BV2 cells (Figure 3D).

To assay a broader array of cytokines and chemokines, a 38-plex Luminex assay was then performed on conditioned media from primary microglia treated with oligomeric α -synuclein and EP4 agonist. α -synuclein treatment increased secretion of 37 out of 38 measured factors (Table 2). Co-treatment of microglia with α -synuclein and AE1-329 led to a statistically significant decrease in levels of TNF- α (86%, $p<0.0001$), CCL2 (59%, $p<0.0001$), IL-27 (37%, $p<0.0001$), IL-18 (16%, $p<0.0001$), CCL7 (47%, $p=0.0015$), Eotaxin (14%, $p<0.0001$), CCL5 (9%, $p=0.014$), CXCL10 (7%, $p=0.01$), IL-22 (7%, $p=0.003$), IL-12 (8%, $p=0.019$), CCL3 (8%, $p=0.0004$), and IL-5 (10%, $p=0.0006$) (Figure 4A). On the other hand, co-treatment with AE1-329 led to an increase in the secretion of GROA (224%, $p<0.0001$) and GCSF (257%, $p<0.0001$) (Figure 4B). Secreted VEGF levels were not affected by α -synuclein, but were significantly upregulated with AE1-329 co-treatment (397%, $p<0.0001$) (Figure 4C). A statistically significant interaction was observed between AE1-329 and α -synuclein (two-way ANOVA: interaction effect for TNF- α , CCL2, IL-27, IL-18, GROA, GCSF, VEGF $p<0.0001$; interaction effect for CCL7, Eotaxin $p<0.001$; interaction effect for CCL5, CXCL10, IL-22, IL-5 $p<0.01$; interaction effect for IL-12 $p<0.05$). A subset of inflammatory factors was upregulated by α -synuclein but was not affected by AE1-329 co-treatment (Figure 4D). These results confirm the finding that exogenous α -synuclein treatment induces a robust and broad inflammatory response in microglia. Concurrently activating the microglial EP4 receptor downregulates a large subset of these induced inflammatory factors and supports an anti-inflammatory role of the microglial EP4 receptor.

Discussion

Dysregulated neuroinflammation is a common feature in many neurodegenerative diseases including PD, Alzheimer's disease (AD), and Amyotrophic Lateral Sclerosis (ALS). The innate inflammatory response in the brain is mediated by glia and involves the secretion of soluble factors such as cytokines, chemokines, and reactive oxygen species. Controlled release of these factors is beneficial for defending against invading pathogens, but dysregulation of these processes can contribute to neuronal damage in neurodegenerative diseases. It remains to be determined whether neuroinflammation plays a causative, protective, or bystander role in the progression of neurodegenerative disorders [6].

This study investigated the role of the PGE₂ EP4 signaling pathway in regulating neurodegeneration and neuroinflammation in acute models of PD. The MPTP model was chosen for *in vivo* experiments as it exhibits highly reproducible neuronal loss in the SNpc that is accompanied by a robust neuroinflammatory response. In this study, the acute MPTP paradigm resulted in the loss of ~25% of the TH+ dopaminergic neurons in the SNpc by day 7 post treatment. The resulting neurodegeneration was accompanied by a significant increase in Iba1+ microglia, GFAP+ astrocytes, and CD3+ T-cells. The elevated inflammatory markers were localized to the SNpc and were enriched in areas of TH+ neuron loss. Co-localization of neuroinflammation and neuronal loss suggests a possible link between the two processes. Daily systemic administration of an EP4 receptor agonist for 6 days following MPTP injection completely rescued the loss of TH+ neurons in the SNpc. However, the systemic EP4 agonist did not lead to a significant change in the extent of microgliosis, astrogliosis, or T-cell infiltration into the SNpc. This indicates that the neuroprotective effect of the EP4 agonist is likely independent of MPTP-associated neuroinflammation. We have shown previously that EP4 receptors are expressed by neurons as well as endothelial cells in the brain, and EP4 elicits neuroprotection in the context of ischemia [35-37]. The data from this study suggests that systemically administered EP4 receptor agonist crosses the blood brain barrier and may directly interact with neuronal or endothelial EP4 receptors, activating intracellular pathways in a timely manner to prevent MPTP-induced degeneration of dopaminergic neurons, a process that normally take several days. However, the dysregulated inflammatory response is initiated immediately after MPTP activation, and the systemic administration of EP4 agonist may be too little too late to dampen the inflammation that is already underway.

Previous research suggests activation of microglia, astrocytes, and T-cells precedes the MPTP-associated loss of neurons [5]. Although a causal relationship has not been established between microglial activation and neuronal loss, mounting evidence suggests that microglia may contribute to PD pathology [5,38,39]. We have previously shown that the EP4 receptor is expressed by microglia and plays an anti-inflammatory role in the context of accumulating amyloid- β in a model of Alzheimer's disease [40] and in peripheral inflammation [22]. In this study, the role of innate immune EP4 was investigated using the Cre-loxP system to conditionally delete the EP4 receptor specifically in Cd11b expressing microglia and macrophages. Mice with a conditional knockout of EP4 receptor in microglia (EP4 cKO) did not show any differences in baseline levels of neuroinflammation in the SNpc. However, 7 days after MPTP injection EP4 cKO mice showed significantly greater levels of microgliosis, astrogliosis, and T-cell infiltration into the SNpc compared to the control group. This supports the hypothesis that the microglial EP4 receptor suppresses the innate inflammatory response and the consequent activation of adaptive immune responses, and deleting the microglial EP4 receptor leads to a dysregulated inflammatory reaction in response to an acute insult. It is important to note that the enhanced inflammation observed in the SNpc was not accompanied by a change in the number of surviving TH+ neurons in the SNpc. Similarly, there was no change in the striatal dopamine produced by these neurons or the dopamine turnover ratio in EP4 cKO mice compared to wild type controls injected with MPTP. These results argue against a strong interaction between microglial innate immune responses and neurodegeneration in this acute MPTP model of PD. However, this

finding does not exclude a link between chronic neuroinflammation and neurodegeneration in PD, which may be due in part to immunogenic responses to α -synuclein. To understand this, it will be important to investigate the role of inflammatory EP4 signaling in genetic models of PD that demonstrate the neuroinflammatory changes seen in patients with Parkinson's disease.

Lewy bodies, intraneuronal aggregates in the brains of PD patients, are primarily composed of the peptide α -synuclein [29]. Moreover, extraneuronal aggregates of α -synuclein are densely surrounded by activated glia [7, 12] implying that α -synuclein released from dying synapses and neurons may interact with glia in the brains of PD patients. This study investigated the effect of α -synuclein on microglia *in vitro* and demonstrated that oligomers of human α -synuclein readily induce the transcription of pro-inflammatory and oxidative stress genes in microglial cell lines. Moreover, oligomeric, but not monomeric α -synuclein, significantly increased secretion of pro-inflammatory factors from primary microglia and microglial cell lines, but this pro-inflammatory response was suppressed with EP4 receptor activation, in particular TNF- α and IL-1 β , as well as the chemokines CCL2 and CCL3 in primary microglia. Post-mortem studies have demonstrated increased levels of TNF- α and IL-1 β in the striatum of PD patients [41, 42] and microglia producing TNF- α and nitric oxide in the SNpc [43, 44]. These data suggest a potential therapeutic approach to enhance innate immune EP4 signaling to suppress α -synuclein induced inflammatory responses in PD.

We expanded upon these findings using a multi-analyte assay to measure the secretion of 38 discrete immune factors from primary microglia stimulated with oligomeric α -synuclein. 37 of the 38 factors were upregulated by α -synuclein, with the exception being VEGF. The canonical pro-inflammatory cytokines TNF- α , IL-1 β , and IL-6 were all strongly upregulated by α -synuclein in addition to the canonical chemokines CCL2, CCL3, CCL4, and CCL5. Surprisingly, other classes of molecules were also upregulated such as the colony-stimulating factors including macrophage colony-stimulating factor (MCSF), granulocyte colony-stimulating factor (GCSF), and granulocyte macrophage colony-stimulating factor (GMCSF), which promote the maturation of stem cells into monocytes and macrophages. α -synuclein significantly upregulated the secretion of interferon-gamma (IFN-gamma) and interferon- α (IFN- α) as well as the transforming growth factor beta (TGF- β). These results indicate that exogenous oligomeric α -synuclein potently and broadly upregulates the production and secretion of inflammatory factors from microglia, consistent with a role for the neuroimmune response to α -synuclein in PD.

Co-treatment of microglia with the EP4 agonist had a varied effect on α -synuclein induced secretion of immune factors. Some pro-inflammatory factors such as TNF- α , CCL2, CCL5, and IL-18 (interferon-gamma inducing factor) were significantly downregulated by EP4 co-activation, whereas others such as IL-6, CCL4, and IFN-gamma did not respond to EP4 stimulation. Only three factors were significantly upregulated by EP4 co-activation including GRO- α (CXCL1), GCSF, and VEGF. This data shows that co-stimulation with α -synuclein and EP4 agonist selectively dampens the microglial inflammatory response to α -synuclein by inhibiting the secretion of a subset of pro-inflammatory factors. Among the factors inhibited by EP4 co-activation is Eotaxin, a chemokine that increases with age and

decreases cognitive performance on behavioural tasks in mice [45]. EP4 co-activation also inhibited the secretion of CXCL10, which correlates with decreased cognitive status in Parkinson's disease patients [46]. These results imply that pharmacological activation of microglial EP4 may modulate downstream intracellular pathways that help dysregulated microglia transition back to their normal state.

Conclusions

There are currently no treatments to halt or slow the progression of neurodegeneration in PD [3]. Targeting a dysregulated microglial inflammatory response to α -synuclein through the EP4 receptor may be an effective strategy for neuroprotection in PD. Future studies examining the role of EP4 signaling need to be carried out in chronic models of Parkinson's disease characterized by α -synuclein aggregation to further elucidate the relationship between Parkinsonian pathology and microglial inflammation.

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Figure 1. Treatment with an EP4 receptor agonist rescues neuronal damage in the acute MPTP model but does not affect neuroinflammation in the substantia nigra pars compacta (SNpc)

A. Stereology of TH+ dopaminergic neurons in the SNpc. Immunohistochemical labeling of SNpc sections is shown to the left and quantification of the data is shown to the right. B,C,D. Iba1 (B), GFAP (C), and CD3 (D) immunostaining in the SNpc was visualized using fluorescence microscopy and % area was quantified using Volocity software. 2 month old male mice were given 3 i.p. injections of 20 mg/kg MPTP spaced 2 hours apart, and were injected s.c. with EP4 receptor agonist AE1-329 once a day for 6 additional days. Brains were harvested on day 7. All statistical tests were done using a two-way ANOVA analysis. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

Figure 2. Conditional deletion of EP4 in microglia and macrophages enhances SNpc neuroinflammation but does not affect neuronal damage in the acute MPTP model

2-3 month old male mice were given 4 i.p. injections of 16 mg/kg MPTP spaced 2 hours apart and brains were harvested 7 days later. A. Stereology of TH+ dopaminergic neurons in the SNpc. B. Dopamine levels were measured from striatal tissue using HPLC. Dopamine turnover ratio was calculated by adding the amounts of dopamine metabolites DOPAC and HVA, and dividing by the total amount of dopamine. C,D,E. Iba1 (C), GFAP (D), and CD3 (E) immunostaining in the SNpc was visualized using fluorescence microscopy and % area was quantified using Volocity software. All statistical tests were performed using a two-way ANOVA analysis. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

Figure 3. Oligomeric α -synuclein induces expression of inflammatory genes and secretion of inflammatory factors from microglia

A. BV2 cells were treated with oligomeric α -synuclein for 6 hrs. RNA was isolated from BV2 cells, converted to cDNA, and qPCR was used to measure gene expression levels. B. Primary microglia were harvested from P7 pups and treated with α -synuclein. ELISA of conditioned medium from microglia was performed to measure levels of secreted factors. TNF- α was measured at a 6 hr timepoint, while IL-1 β , CCL3, and CCL2 were measured at a 12 hr timepoint. C. BV2 cells and peritoneal macrophages were treated with α -synuclein for 6 hrs and conditioned medium was assayed using ELISA. D. BV2 cells were treated with monomeric or oligomeric preparations of α -synuclein for 6 hrs and conditioned medium was assayed using ELISA. Concentrations of the reagents were: Veh: 0.01% EtOH, AE1-329: 10 nM, α -synuclein: 500 nM, LPS: 10 ng/ml. All statistical tests were performed using a twoway ANOVA analysis. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

Figure 4. α -synuclein induced secretion of inflammatory cytokines and chemokines from microglia is modulated by the EP4 receptor

A. Immune factors upregulated by α -synuclein and downregulated by the EP4 receptor. B. Immune factors upregulated by both α -synuclein and the EP4 receptor. C. Growth factor VEGF is not upregulated by α -synuclein alone, but is upregulated by a combination of α -synuclein and EP4 receptor activation. D. Immune factors upregulated by α -synuclein but not affected by EP4 receptor activation. Microglia were harvested from P7 pups and treated for 6 hours with the following reagents: Veh: 0.01% EtOH, AE1-329: 10 nM, α -synuclein: 500 nM. Conditioned medium was collected and assayed using Luminex analysis. All statistical tests were done using a two-way ANOVA analysis. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

Table 1
Forward and reverse sequences of primers used for qPCR

Sequences of DNA oligos used for quantitative real-time PCR.

Gene	Forward primer	Reverse primer
COX-2	5' – TGCAAGATCCACAGCCTACC – 3'	5' – GCTCAGTTGAACGCCTTTTG – 3'
gp91 (NADPH Oxidase)	5' – CCAACTGGGATAACGAGTTCA – 3'	5' – GAGAGTTTCAGCCAAGGCTTC – 3'
iNOS	5' – TGACGGCAAACATGACTTCAG – 3'	5' – GCCATCGGGCATCTGGTA – 3'
IL-1 β	5' – CCAGGATGAGGACATGAGCACC – 3'	5' – TTCTCTGCAGACTCAAACCTCCAC – 3'
TNF- α	5' – TCATTCTGCTTGTGGCAGGG – 3'	5' – GTGGTTTGCTACGACGTGGGC – 3'
Nurr1	5' – TCTGCGCTTAGCATACAGGTCCAA – 3'	5' – CAGCAATGCAGGAGAAGGCAGAAA – 3'
GAPDH	5' – TGCACCACCAACTGCTTAG – 3'	5' – GATGCAGGGATGATGTTTC – 3'

Table 2
Upregulation of immune factors secreted by microglia upon stimulation with oligomeric α -synuclein

Fold change of secreted immune factors upon treatment of primary microglia with oligomeric α -synuclein for 6 hours. p-values were calculated using a two-tailed Student's t-test.

Factor	Fold change	p-value
TNFA	163.6	<0.0001
MIP2/CXCL2	129.3	<0.0001
MCP3/CCL7	106.2	<0.0001
IL6	102.5	<0.0001
RANTES/CCL5	97.4	<0.0001
GROA/CXCL1	27.4	<0.0001
MCP1/CCL2	26.7	<0.0001
IP10/CXCL10	25.4	<0.0001
IL22	7.2	<0.0001
MIP1A/CCL3	6.0	<0.0001
IL17A	5.4	<0.0001
MIP1B/CCL4	4.3	<0.0001
IL18	4.0	<0.0001
MCSF/CSF1	3.6	<0.0001
IL27	3.6	<0.0001
TGFB	2.9	<0.0001
IL1B	2.8	<0.0001
IL31	2.7	<0.0001
IL12P70	2.7	<0.0001
IL4	2.6	<0.0001
IL10	2.2	<0.0001
IL13	2.2	<0.0001
LIX/CXCL5	2.0	<0.0001
IL9	2.0	<0.0001
IL1A	1.9	<0.0001
IL5	1.8	<0.0001
GCSF/CSF3	1.8	<0.0001
LIF	1.7	<0.0001
EOTAXIN/CCL11	1.6	<0.0001
IL28	1.5	<0.0001
IFNG	1.5	<0.0001
GMCSF/CSF2	1.4	<0.0001
IL15/IL15R	1.4	<0.0001
IFNA	1.3	<0.0001

Factor	Fold change	p-value
IL2	1.3	<0.0001
IL3	1.2	<0.0001
IL23	1.1	0.00011
VEGF	1.0	0.84

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