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Donepezil Regulates 1-Methyl-4-phenylpyridinium-Induced Microglial Polarization in Parkinson's Disease

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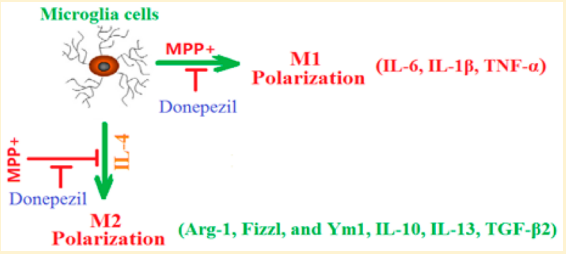
Supporting Information

ABSTRACT: 1-Methyl-4-phenylpyridinium (MPP+) induces microglial activation and degeneration of dopaminergic (DAergic) neurons. Donepezil is a well-known acetylcholinesterase inhibitor used clinically to treat cognitive dysfunction in Alzheimer's disease (AD). In the present study, we tested the hypothesis that MPP+ promotes microglial M1 polarization and suppresses M2 polarization and that this can be restored by donepezil. Results indicate that MPP+ treatment in microglial BV2 cells promotes microglial polarization toward the M1 state. However, pretreatment with donepezil inhibited MPP+-induced M1 polarization in microglia by suppressing the release of interleukin (IL)-6, IL-1β, or tumor necrosis factor (TNF)-α. Importantly, we found that MPP+ inhibited microglial M2 polarization by suppressing expression of Arg-1, Fizz1, and Ym1, which was also rescued by pretreatment with donepezil. In addition, IL-4-mediated induction of anti-inflammatory marker genes IL-10, IL-13, and transforming growth factor-β2 (TGF-β2) were significantly attenuated by MPP+ in BV2 cells, which was restored by pretreatment with donepezil in a concentration-dependent manner. Mechanistically, we found that the addition of MPP+ reduced the intensity of phosphorylated signal transducer and activator of transcription 6 (STAT6) but not total STAT6 in IL-4-stimulated BV2 cells. Importantly, pretreatment of microglial BV2 cells with donepezil 3 h prior to administration of MPP+ rescued the reduction of STAT6 phosphorylation induced by MPP+.



KEYWORDS: *Donepezil, MPP+, microglia M2 polarization, arginase-1 (Arg-1), STAT6*

Second to Alzheimer's disease (AD), Parkinson's disease (PD) is the most prominent disease producing motor manifestations and multiple nonmotor symptoms.[1] Neuroinflammatory changes, including chronic microgliosis, play a critical role in the pathological development of PD.[2] Having high levels of morphological and functional plasticity, microglia can be extensively characterized and have been shown to take on distinct phenotypes, including the M1 and M2 phenotypes, depending on their stimuli.[3] M1 and M2 microglia serve distinct functions in the regulation of the inflammatory response. Activated microglia, referred to as classical activation or the M1 phenotype, may promote neurotoxicity via the release of neurotoxic pro-inflammatory cytokines, such as IL-1β, TNF-α, IL-12, and IL-6. In contrast, it has been reported that M2-polarized microglia are neuroprotective. M2 microglia express different M2 markers, such as arginase-1 (Arg-1), resistin-like α (Retnla, Fizz1), and chitinase 3-like 3 (Chi3l3, Ym1), which also possess neuroprotective properties.[4] The prevalence of M1 over M2 microglia or macrophages has been considered to be associated with neurodegenerative pathologies, such as Alzheimer's disease (AD).[5] However, the role of microglial polarization into the pro-inflammatory M1 phenotype or the anti-inflammatory M2 phenotype is poorly understood in PD, although post-mortem studies measuring cytokine levels have suggested that both pro- and anti-inflammatory microglia may coexist in the Parkinsonian brain.[6,7]

1-Methyl-4-phenylpyridinium (MPP+) is the active neurotoxic metabolite of the Parkinsonian toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). As a lipophilic compound, MPTP itself is nontoxic and has the ability to cross the blood–brain barrier. Once inside the brain, MPTP is metabolized into the toxic cation 1-methyl-4-phenylpyridinium (MPP+) by the glial cell enzyme monoamine oxidase (MAO)-B.[8] In a fashion consistent with that of dopamine transporters, MPP+ is actively transported into dopaminergic (DAergic) neurons in the pars compacta of the substantia nigra (SN) through the plasma membrane where it then primarily kills dopamine-producing neurons.[9] MPP+ has been used extensively to model PD in a variety of *in vivo* and *in vitro* systems. It has been reported to inhibit complex I of the mitochondrial respiratory chain, inducing energy depletion and producing reactive oxygen species (ROS), such as superoxide anion ($^{\bullet}O_2^-$).[10] Activated microglia have been associated with neurotoxicity of MPTP/MPP+. For example, accumulation of activated microglia around DAergic neurons has been found in post-mortem

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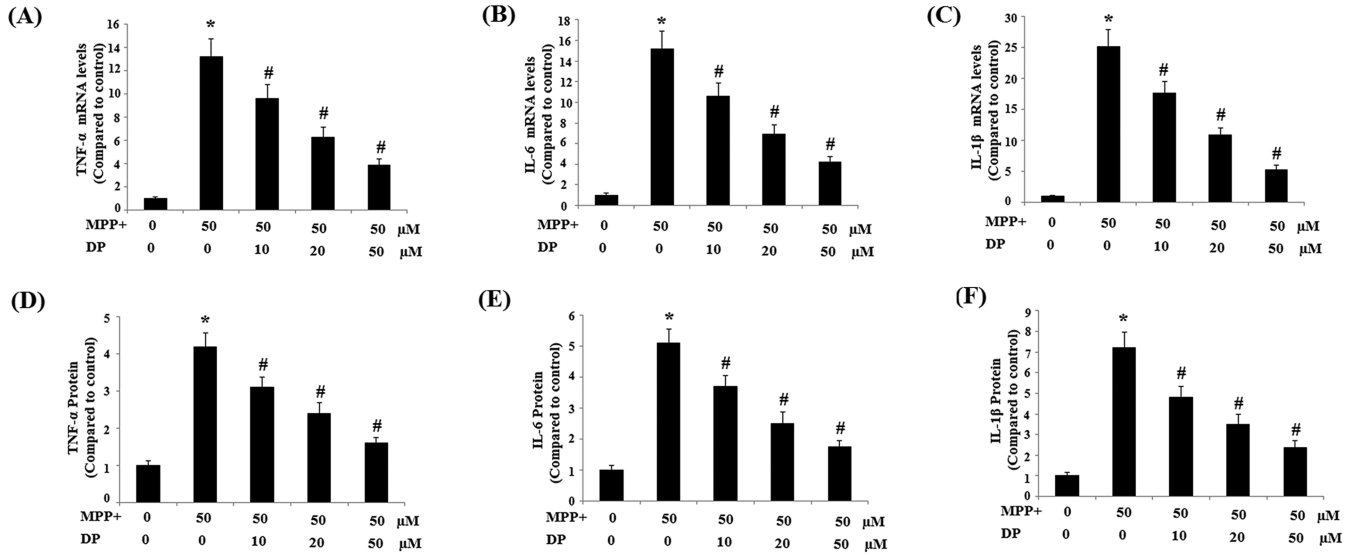

Figure 1. Effects of donepezil on MPP+-induced microglial M1 polarization. BV2 microglial cells were first treated for 3 h with donepezil at concentrations of 10, 20, and 50 μM and then with or without MPP+ (50 μM). After 24 h, total RNA was extracted from microglial cells and qRT-PCR was performed. (A) IL-6; (B) IL-1β; (C) TNFα. Supernatants were collected and ELISA was performed to determine the protein concentrations of (D) IL-6, (E) IL-1β, and (F) TNFα (*$p < 0.01$ compared with the control group and #$p < 0.01$ compared with the MPP+-treated group as determined by one-way ANOVA, followed by Tukey's multiple comparison test).

human brains with MPTP-induced Parkinsonism.[11] In addition, elevated pro-inflammatory cytokines, such as TNF-α and IL-1β, are known to be involved in DAergic neuronal death in MPTP-treated mice.[12] Together, these data imply a close association between MPP+-induced microglial activation and the degeneration of DAergic neurons. Based on these reports, we speculated that MPP+ might regulate microglial polarization.

Donepezil [*R*,*S*-1-benzyl-4-[(5, 6-dimethoxy-1-indanon)-2-yl] methylpiperidine hydrochloride], a well-known acetylcholinesterase inhibitor, has been used clinically for cognitive dysfunction in Alzheimer's disease (AD). The potential therapeutic capacity of donepezil in other neurodegenerative diseases has also been reported.[13] Although its major mechanism of action is to inhibit cholinesterase activity, which increases acetylcholine (ACh) levels and cholinergic transmission, several clinical studies have suggested that donepezil can also produce excellent neuroprotective and disease-modifying effects in AD patients.[14] Notably, increasing evidence shows that donepezil has an effect on inflammatory processes.[15] A recent study demonstrated that treatment with donepezil suppressed neuroinflammation by reducing IL-1β and cyclooxygenase-2 (COX-2) expression in the brain and the spleen, thereby suggesting that donepezil directly prevents systemic inflammation, which indicates that donepezil may not only act as a cognition-linking neurotransmitter but also suppress the pathological mechanisms associated with neurodegeneration via anti-inflammatory action.[16] Moreover, donepezil significantly attenuated the release of inflammatory mediators (prostaglandin E2, IL-1β, TNF-α, and NO) induced by amyloid-β oligomer from microglia in AD by inhibiting translocation of NF-κB.[17] In this study, we found that microglial polarization induced by MPP+ stimulation could be restored by treatment with donepezil.

RESULTS AND DISCUSSION

Cells were incubated with donepezil at concentrations of 0.1, 1, 10, 20, 50, and 100 μM for 24 h. We found that treatment with 100 μM donepezil moderately decreased cell viability, but incubation with donepezil at concentrations of 0.1, 1, 10, 20, and 50 μM had no obvious effects on cell viability compared with controls (Figure esupp 1A, Supporting Information). Therefore, we administered donepezil at concentrations of 0.1, 1, 10, 20, and 50 μM in order to examine its effects on MPP+-induced microglial polarization. The time-course of cell survival following MPP+ treatment in BV2 cells is shown in Figure esupp 1B, Supporting Information. The results indicate that incubation with 50 μM MPP+ for 24 h induced moderate MPP+ toxicity in BV2 cells. Cells were pretreated with donepezil (10, 20, and 50 μM) for 3 h followed by treatment with MPP+ for another 24 h. Three hours was chosen as the treatment time in order to allow enough time for donepezil to enter into microglial cells. In a previous study, the authors treated primary astrocytes with 20 μM donepezil for 2 h.[19]

Expression levels of the potent pro-inflammatory cytokines IL-6, IL-1β, and TNF-α were measured using real time PCR. Our results indicate that MPP+ could induce BV2 microglial cell activation by dramatically increasing the mRNA levels of these cytokines. Notably, pretreatment of microglial BV2 cells with donepezil for 3 h prior to administration of MPP+ elicited a reduced pattern of IL-6 (Figure 1A), IL-1β (Figure 1B), and TNF-α (Figure 1C) gene expression, thereby suggesting that donepezil plays a notable role in the expression of these cytokines. On the other hand, treatment with MPP+ alone considerably increased the presence of pro-inflammatory cytokines at protein levels, indicating that MPP+ can also induce an inflammatory process in microglial cells. Elevation of IL-6, IL1-β, or TNF-α protein expression levels induced by MPP+ was attenuated to control levels in microglial cells pretreated with donepezil (Figure 1D–F), suggesting that donepezil plays a role as an anti-inflammatory molecule by impacting both the gene and protein expression of pro-inflammatory cytokines.

To study the function of MPP+ in the process of microglial M2 polarization, we used IL-4 to induce expression of prototypical target genes that characterize the M2 phenotype, including Arg-1, Fizzl, and Ym1. As shown in Figure 2, real time

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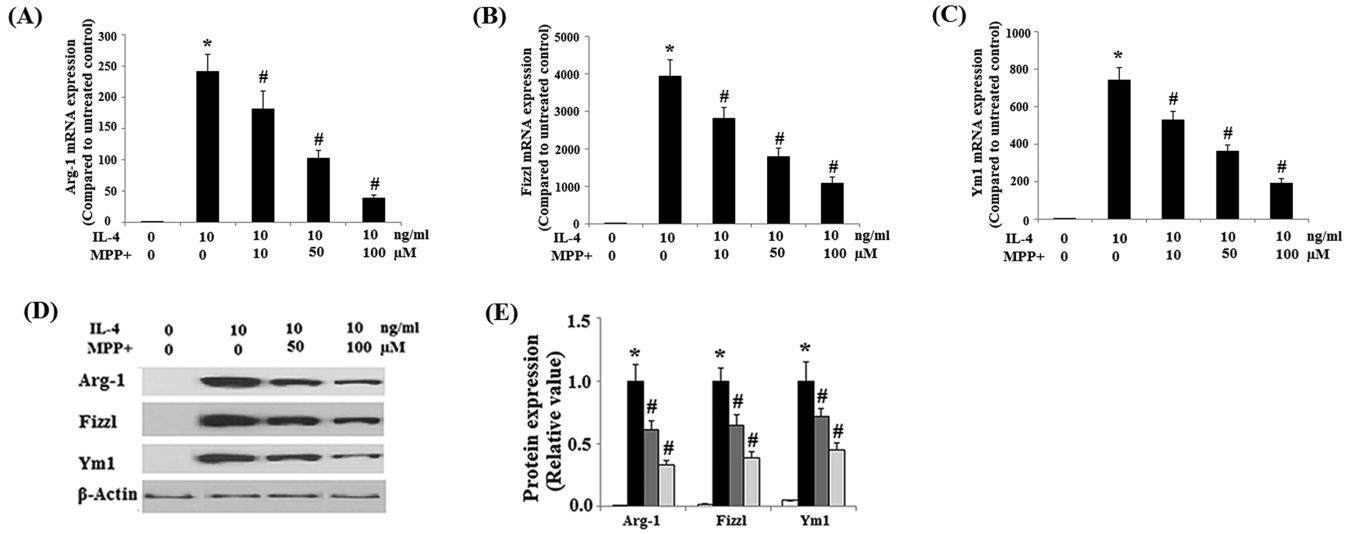

Figure 2. MPP+ suppresses IL-4-mediated microglial M2 polarization. BV2 cells were treated with IL-4 in the presence or absence of MPP+ (10, 50, 100 μM). After 24 h, total RNA was extracted from microglial cells, and qRT-PCR was performed to detect the mRNA levels of (A) Arg-1, (B) Fizzl, and (C) Ym1. In addition, Western blot analysis was performed to determine the expression of M2 marker proteins (D) Arg-1, (E) Fizzl, and (F) Ym1 (*p < 0.01 compared with the control group and #p < 0.01 compared with the IL-4 treated group).

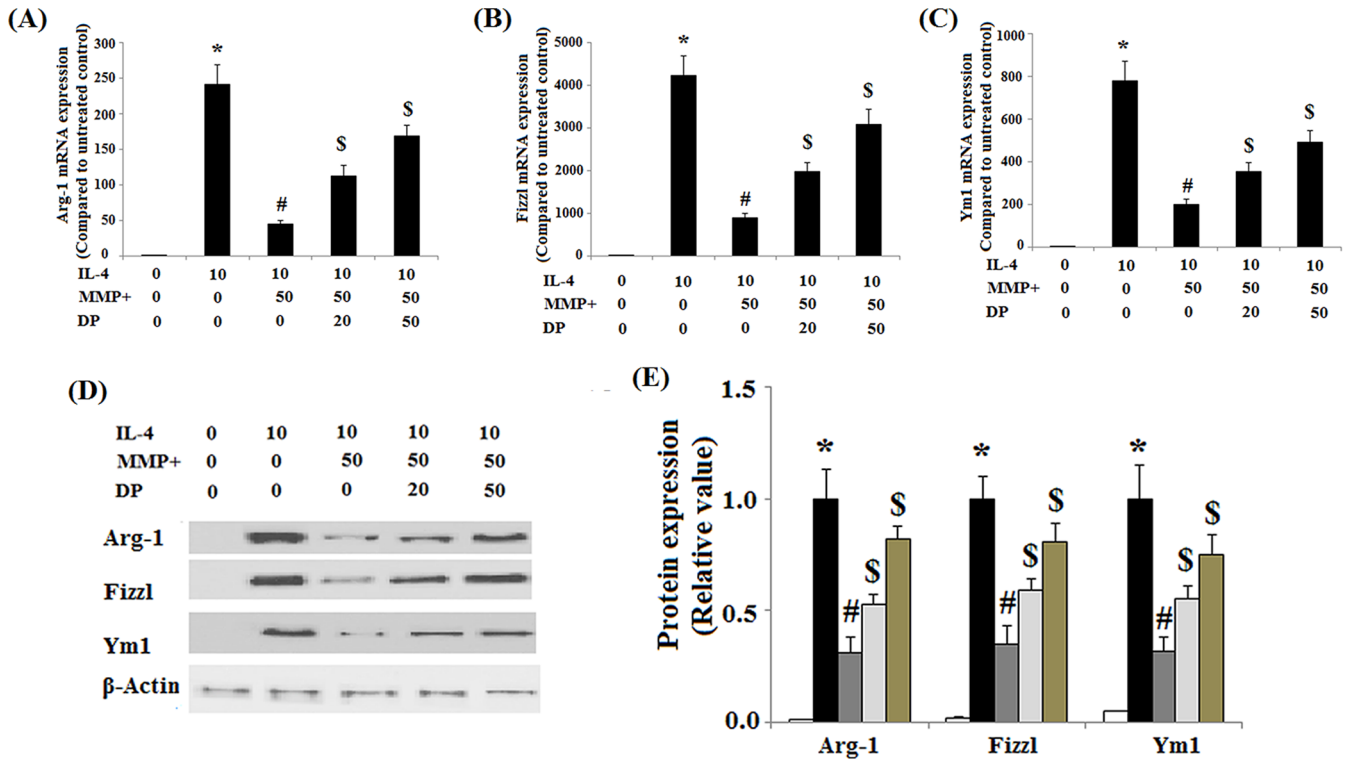

Figure 3. Donepezil abolished the inhibitory effects of MPP+ on promoting microglial M2 polarization. BV2 microglial cells were first treated for 3 h with donepezil at concentrations of 10, 20, and 50 μM and then with or without MPP+ in the presence of IL-4. After 24 h, total RNA was extracted from microglial cells and qRT-PCR was performed to detect the mRNA levels of (A) Arg-1, (B) Fizzl, and (C) Ym1. In addition, Western blot analysis was performed to determine the expression of M2 marker proteins (D) Arg-1, (E) Fizzl, and (F) Ym1 (*p < 0.01 vs untreated control; #p < 0.01 vs the IL-4 only treated group; $^{\$}p$ < 0.01 vs the IL-4 and MPP+ treated group).

PCR results indicate that IL-4-mediated induction of the characteristic M2 marker genes Arg-1 (Figure 2A), Fizzl (Figure 2B), and Ym1 (Figure 2C) was significantly attenuated by MPP+ in BV2 cells in a concentration-dependent manner. The results were verified by Western blot analysis at protein levels (Figure 2D–F). In contrast, pretreatment of microglial BV2 cells with donepezil for 3 h prior to administration of MPP + restored IL-4-mediated induction of Arg-1 (Figure 3A), Fizzl (Figure 3B), and Ym1 (Figure 3C) in a concentration-dependent manner. The results were verified at protein levels (Figure 3D–F). These results indicate that donepezil can reverse neurotoxin-induced changes in the expression of M2 activation factors.

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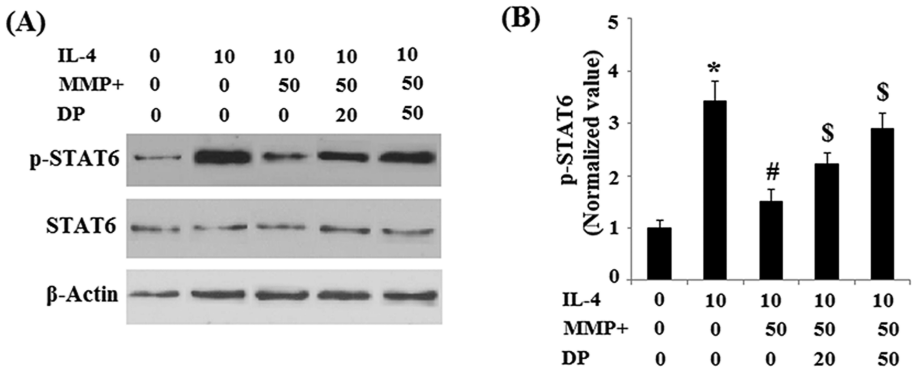

Figure 4. Donepezil abolished the inhibitory effects of MPP+ on the phosphorylation of STAT6. BV2 microglial cells were first treated for 3 h with donepezil at concentrations of 5, 10, and 20 μM and then with or without MPP+ in the presence of IL-4. Phosphorylation of STAT6 was determined by Western blot analysis (*$p < 0.01$ vs untreated control; #$p < 0.01$ vs the IL-4 only treated group; \$$p < 0.01$ vs the IL-4 and MPP+ treated group).

The expression of anti-inflammatory markers, such as IL-10, IL-13, and TGF-β2, is an important characteristic of microglial M2 polarization. Thus, we determined the gene expression of IL-10, IL-13, and TGF-β in BV2 cells. The results indicate that IL-4-mediated induction of anti-inflammatory marker genes IL-10 (Figure esupp 2A, Supporting Information), IL-13 (Figure esupp 2B, Supporting Information), and TGF-β2 (Figure esupp 2C, Supporting Information) was significantly attenuated in BV2 cells by MPP+ in a concentration-dependent manner. Importantly, pretreatment of microglial BV2 cells with donepezil for 3 h before administration of MPP+ restored IL-4-mediated induction of IL-10, IL-13, and TGF-β2 in a concentration-dependent manner (Figure esupp 3A−C, Supporting Information).

It is known that IL-4-induced M2 macrophage polarization is dependent on STAT6, a master regulator of M2 genes.[20] Therefore, we further examined whether MPP+-treatment influenced total STAT6 and STAT6 phosphorylation in IL-4-stimulated BV2 cells using Western blot analysis. As expected, obvious STAT6 phosphorylation was observed in IL-4-stimulated BV2 cells. Addition of MPP+ reduced the intensity of phosphorylated STAT6 but not total STAT6 in IL-4-stimulated BV2 cells. This result was further confirmed by a significant reduction in pSTAT6 when normalized to total STAT6 (Figure esupp4, Supporting Information). Importantly, pretreatment of microglial BV2 cells with donepezil for 3 h before administration of MPP+ rescued the reduction in STAT6 phosphorylation induced by MPP+ (Figure 4). Collectively, these results suggest that MPP+ can directly suppress IL-4-induced microglial M2 polarization in part by inhibiting STAT6 phosphorylation, and donepezil can rescue this effect.

Glial dysfunction has been shown to play an essential role in the progression of neurodegeneration in diseases such as Parkinson's disease. The density of microglial cells is highest in the substantia nigra (SN) region of the brain.[21] Thus, the neurons in this region are particularly susceptible to microglia-mediated toxicity both *in vitro* and *in vivo*. Multiple lines of evidence have shown that microglial cells are regionally activated in the SN of PD patients as well as in PD animal models.[22,23] In response to neurotoxin stimulation, microglia trigger a self-perpetuating cycle of chronic neuro-inflammation, increasing the release of inflammatory chemical substances and promoting microglial activation. Understanding inflammation in the context of M1 (classical phenotype) and M2 (alternative phenotype) activation paradigms may help clarify our interpretation of these complex and dynamic processes. In this study, we found that MPP+ promoted the M1 state but inhibited the M2 state. Consistent with our findings, previous studies have shown that increases in cytokines associated with the M1 state, such as TNF-α and IL-1β, and possibly TNF-α activation in astrocytes,[24] have been found in serum and cerebrospinal fluid from PD patients.

Conversely, M2-polarized microglia have been reported to have neuroprotective properties. Therefore, agents that polarize microglia and macrophages into an M2 skewed phenotype could present a better therapeutic option in neurodegenerative disease by avoiding caveats associated with blockage of individual M1 responses. The M2 phenotype is alternatively activated by IL-4. Under treatment with IL-4, M2 microglia express various M2 markers, including Arg-1, Fizzl, and Ym1, which have been shown to be neuroprotective.[25] In addition to IL-4, IL-10 is another M2 targeted therapy agent. A recent study reported that delivery of IL-10 into the midbrain or striatum of rodents significantly ameliorated dopaminergic neuron loss induced by MPTP or 6-OHDA intoxication in the SN region.[26] Consistently, IL-10 has also been reported to be involved in the differentiation and survival of neurons and has been suggested to exert beneficial and neuroprotective effects against 1-methyl-4-phenylpyridinium (MPP+) toxicity *in vitro* and in PD experimental models *in vivo*.[27] Importantly, administration of MPP+ has been reported to stimulate microglial activation as well as the release of proinflammatory cytokines.[28] However, the effects of MPP+ on M2-polarized microglia have not yet been reported. Our study has clearly demonstrated that MPP+ treatment suppresses M2 state polarization of microglia by inhibiting expression of Arg-1, Fizzl, and Ym1.

As an important acetylcholinesterase (AChE) inhibitor, donepezil is clinically used for the treatment of Alzheimer's disease (AD). Donepezil has also been found to exert anti-inflammatory effects in experimental animal models, although the major mechanism driving this action remains unknown. For example, donepezil inhibited production of TNF-α and interferons in vesicular stomatitis virus-infected peripheral blood leukocytes, which was accompanied by a reduction in NF-κB activation.[29] Another study showed that donepezil treatment attenuated production of nitric oxide and TNF-α in microglia and suppressed gene expression of inducible nitric oxide synthase (iNOS), IL-1β, and TNF-α by inhibiting a canonical inflammatory NF-κB signaling pathway. Inhibition of microglial activation resulting from treatment with donepezil

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occurred independent of acetylcholine and its receptor. We speculate that the inflammatory activation signaling of microglia may be one of the direct targets of donepezil in the central nervous system. Kim and colleagues report that donepezil significantly attenuated the release of inflammatory mediators (prostaglandin E2, IL-1β, TNF-α, and NO) induced by amyloid-β oligomer from microglia in AD. They also showed that donepezil decreased amyloid-β oligomer-induced up-regulation of inducible nitric oxide synthase and cyclo-oxygenase-2 protein and phosphorylation of p38 mitogen-activated protein kinase as well as translocation of NF-κB.[17] These results support our findings showing that donepezil inhibited MPP+-induced microglial M1 polarization. In contrast, the alternative M2 activation state encompasses a broad set of responses compared with the M1 activation state. Generally, the M2 activation state is associated with healing and scavenging, opposing the pro-killing state of the M1 phenotype. Another important finding in this study is that donepezil partially restored suppression of M2 microglia induced by MPP +.

Donepezil was used in *in vitro* studies in different kinds of cells at concentrations ranging from 5 to 50 μM. Notably, a recent study demonstrated that donepezil (5−40 μM) can reduce LPS-induced nitric oxide (NO) production in BV2 microglial cells in a concentration-dependent manner.[30] In addition, under oxygen-glucose deprivation (OGD) conditions, 30 μM donepezil effectively inhibited K(+) efflux via activated voltage-gated K(+) channels and decreased the rate of HEK293 cell apoptosis.[31] Pretreatment of primary astrocytes with 20 μM donepezil attenuated Aβ1−40-induced toxicity.[19]

To date, the control of microglial polarization has been largely attributed to a small group of functional factors including NF-κB, the activator protein 1 (AP-1), hypoxia-inducible factors (HIFs), signal transducers, and activators of transcription (STATs).[32] IL-4 exerts the majority of its immunological effects on macrophages through activation of STAT6 proteins signals. It is reported that STAT6 is involved in the transmission of polarization signals to the nucleus and plays a distinct role in macrophage polarization.[33] Notably, it has also been reported that STAT6/mice have a suppressed M2 macrophage phenotype, with lower levels of Arg-1 expression and higher levels of NOS2/nitric oxide.[34] Our results indicate that MPP+ treatment abolished IL-4-induced phosphorylation of STAT6, which was rescued by treatment with donepezil, thereby suggesting the involvement of STAT6 in this process.

Donepezil is a reversible and noncompetitive centrally acting acetyl cholinesterase inhibitor. The anti-inflammatory and neuroprotective effects of the drug have been reported in recent studies. The "cholinergic anti-inflammation pathway" has been implicated in these effects. Microglia express the α7 nicotinic acetylcholine receptor (nAChR).[35] However, Arias et al. have reported on the allosteric regulation of nAChR by donepezil in SH-SY5Y neuroblastoma cells but not in microglia. Interestingly, antagonists of nAChR did not significantly influence the inhibitory effects of donepezil on NO production in microglial cells.[36] Donepezil has been reported to attenuate inducible nitric oxide synthase (iNOS), IL-1β, and TNF-α gene expression through inhibition of a canonical inflammatory NF-κB signaling pathway in microglia. Notably, a recent study demonstrated that nicotinic acetylcholine receptor antagonists do not abolish the effects of donepezil on microglial inflammation, thus suggesting that donepezil-induced inhibition of microglial activation occurs independent of acetylcholine and its receptor.[30] Therefore, we speculated that promotion of microglial M2 polarization may be one of the direct targets of donepezil in the central nervous system. These findings suggest that a large gap may exist between the therapeutic dose of donepezil used clinically and the concentration of the drug that exerts this direct action on microglial cells. Future studies will help to explore the molecular mechanisms underlying this action.

MATERIALS AND METHODS

Cell Culture. The microglial BV2 cell line was maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 0.1% penicillin and streptomycin. Confluent cultures were passaged by trypsinization. To determine the effects of donepezil on MPP+-induced microglial polarization, cells were pretreated with donepezil (10, 20, and 50 μM) for 3 h followed by treatment with MPP+ for another 24 h.

Quantitative Real-Time PCR. Total RNA was extracted from cells using a Trizol plus RNA purification system (Life Technologies, USA) according to the manufacturer's instructions. Concentrations of RNA were determined using a Biospec nano spectrophotometer (Shimaduz, Japan). cDNA was synthesized using a cDNA high capacity kit (Life Technologies) following the manufacturer's instructions. Real-time PCR was performed using the SYBR Green qPCR master mix (Roche, USA).

Western Blotting Analysis. Proteins were isolated from cells using a cell lysis buffer kit (Cell Signaling, USA). Samples containing equal amounts of protein (20 μg) were analyzed using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transference onto a polyvinylidene difluoride (PVDF) membrane (Immobilon, Millipore, USA).[18] After being blocked with 5% fat-free milk in tris-buffered saline and Tween 20 (TBST), blots were incubated overnight at 4 °C with primary antibodies in a 5% BSA TBST solution. Blots were then incubated for 2 h at room temperature in a 0.5% BSA TBST solution containing horseradish peroxidase (HRP)-conjugated secondary antibodies. Immunoblots were then treated with an ECL reagent, and luminescence was detected and analyzed using the OdysseyFc Imaging System (MS Technosystems, Osaka, Japan). The following antibodies were used in this study: rabbit polyclonal antibody against Arg-1 (1:1,000, no. sc20150, Santa Cruz Biotechnology, USA); rabbit polyclonal antibody against Fizzl (1:1,000, no. ab176577, Abcam, USA); rabbit polyclonal antibody against Ym1 (1:1,000, no. 01404, STEMCELL Technologies, USA); mouse monoclonal antibody against β-actin (1:10,000, no. sc-47778, Santa Cruz Biotechnology, USA); rabbit polyclonal antibody against STAT6 (1:2,000, no. ab44718, Abcam, USA); rabbit polyclonal antibody against anti-p-STAT6 (1:1,000, no. ab125308, Abcam, USA).

Enzyme-Linked Immunosorbent Assays (ELISAs) for IL-6, IL-1β, and TNF-α. IL-6, IL-1β, and TNF-α were measured using specific ELISAs (BioLegend, San Diego, CA) according to the manufacturer's instructions. Antibodies used for ELISA were included in the ELISA kit. Both the capture antibody and the detection antibody were diluted to a concentration of 200:1. Upon completion of the indicated treatment, mouse-specific monoclonal antibody (IL-6, IL-1β, and TNF-α) was first coated onto 96-well plates. We then added standards and samples to the wells for 2 h, where IL-6, IL-1β, or TNF-α were bound to the immobilized capture antibodies. After being washed 3 times, a biotinylated anti-mouse detection antibody was added and incubated for another 1 h at room temperature, producing an antibody−antigen−antibody "sandwich" to which an avidin horseradish peroxidase solution was added for 30 min. Finally, samples were incubated with a tetramethylbenzidine solution for 15 min in the dark. Reaction with horseradish peroxidase resulted in conversion of the substrate to a blue-colored product. Addition of 2 N sulfuric acid stop solution yielded a yellow color. Absorbance recorded at 450 nm was used to index the concentration of the target proteins.

Statistical Analysis. All data, analyzed at the 95% confidence interval, are expressed as means ± SEM from at least three independent experiments. Statistical analysis was performed using

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one-way analysis of variance (ANOVA) followed by Dunnett's post hoc significance test. A value of $p < 0.05$ was considered to be a significant difference.

■ ASSOCIATED CONTENT

Supporting Information

Effects of donepezil and MPP+ on BV2 cell survival, MPP+ suppression of IL-4-mediated expression of anti-inflammatory markers, donepezil reversal of the inhibitory effects of MPP+ on anti-inflammatory markers, MPP+ inhibition of IL-4-induced phosphorylation of STAT6. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschemneuro.5b00026.

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Notes

The authors declare no competing financial interest.

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