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# Lipid-like components released from degenerating dopaminergic neurons trigger the dynamic migration of microglia

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ABSTRACT

In the brain, communication between neural and non-neural cells is crucial for the proper functioning of the central nervous system. Microglia play an important role in the clearance of neural cellular corpses and debris, especially under pathological conditions. It remains, however, unclear how microglia sense the degenerating neurons at a distance in order to migrate to them. In the present study, we explored the interaction between neurons and microglia using an *in vitro* model of Parkinson's disease (PD). In primary mesencephalic neuronal cultures, 1-methyl-4-phenylpridinium ($MPP^+$) induced the selective death of dopaminergic (DAergic) neurons in a dose- and time-dependent manner. Transmigration assay showed that the conditioned medium (CM) from mesencephalic cultures treated with $MPP^+$ was enough to trigger the attraction of microglia at an early as well as a late phase of neuronal damage. Microglia preferably reacted with the soluble parts separated by ultracentrifugation over the neural debris-containing pellets. This chemoattractive activity was significantly reduced by the removal of the lipidic components in CM, but not by the removal of proteins, DNA or RNA. These results suggest that as yet-unidentified lipid-like components released from dying DAergic neurons are likely to recruit microglia, and thus have a role in neuronal damage.



## 1. Introduction

Parkinson's disease (PD) is a common neurodegenerative disease characterized by abnormal motor faculties, such as muscle stiffness, tremor, postural instability and a paucity of voluntary movements. The histopathological hallmark of PD is the specific loss of the nigrostriatal dopaminergic (DAergic) neurons, the cell bodies of which reside in the substantia nigra (SN), with nerve terminals projecting to the striatum [1]. Although the pathogenesis of PD has long remained unresolved, epidemiological, clinical and genetic evidence suggests that various factors, such as oxidative stress, mitochondrial dysfunction, neurotoxic aggregates and genetic deficits are closely associated with the degeneration of DAergic neurons [2]. In particular, increasing evidence has indicated that microglial activation is involved in this progressive disease [3,4].

Microglia, the major glia of the central nervous system, are critically important as resident immunocompetent and phagocytic cells [5]. They scavenge cells in the event of infection, inflammation, trauma, ischemia and neurodegeneration by interacting with other types of brain cells [6]. Even in the resting state, microglia are rather highly dynamic, which makes them sensitive to any changes in the normal environment [7]. Microglia become rapidly activated in response to a wide range of stimuli and concomitantly display proliferation, migration, expression of immune-related antigens all with conspicuous plasticity. Such flexibility enables them to carry out adaptive functions against pathophysiological challenges that arise in the brain in neurodegenerative disease [8].

In the normal brain, the density of microglia is remarkably higher in the SN compared to other brain regions, which leads to an idea that microglia may play an especially central role in PD [9]. Several reports have suggested that there is a close link between microglial activation and PD. Specifically, HLA-DR-positive reactive microglia were reportedly detected in the SN of human PD brain [10], and activated microglia surrounded the neurons damaged by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxin that selectively destroys DAergic cells, in the human SN [11]. In addition, reactive microglia were detected in the SN of Parkinsonian monkeys after MPTP exposure [12], and a cohort of microglia came together in the vicinity of DAergic neurons in the SNpc in the early aftermath of the toxic insult [13].

Under certain pathological conditions, microglia dynamically migrate into the damaged region of the brain. To fulfill this function, microglia recognize many motility-inducing factors and are rapidly activated. However, to date little is known about the factors

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which trigger the migration of microglia to the site of damaged DAergic neurons. Although microglia possess receptors for certain "eat-me" signals on injured cells such as, these are effective as a signal only in the immediate vicinity of apoptotic cells [14]. In this regard, other signals may be required to induce microglial migration toward cells at dome distance. At present it is uncertain which types of signaling molecules, particularly from damaged DAergic neurons, enable microglia to recognize an alteration of the microenvironment, thus leading to chemoattractive migration. In the present study, we investigated microglial migration using an *in vitro* model of PD.

## 2. Materials and methods

### 2.1. Primary mesencephalic neuronal culture

Primary mesencephalic neuronal culture was performed as previously described [15]. Embryonic day 14 (E14) gestation Sprague–Dawley (SD) rats were purchased from FOLAS, and animal handling was carried out according to the Animal Welfare Guidelines of Seoul National University. All of the culture ingredients were obtained from GIBCO™ Invitrogen Corporation. On Day *in vitro* 7 (DIV7), $MPP^+$ was added to mesencephalic culture in reagent media (RM; Minimum Essential Media (MEM) supplemented with 20 mM glucose, 38 mM sodium bicarbonate, 1 mM pyruvic acid, 2% heat-inactivated fetal bovine serum (FBS) and 2% heat-inactivated horse serum (HS), 2 mM L-glutamine and antibiotic–antimycotic).

### 2.2. Primary microglia culture

Microglia were prepared from the whole brain of 1-day-old SD rats (Experimental Animal Center, Seoul National University). Brain tissues were mechanically dissociated in dissecting media (DM; Hank's balanced salt solution containing 55.5 mM glucose and 20.4 mM sucrose). After centrifugation at 500*g* for 5 min, dissociated cells were suspended in microglia media (MEM supplemented with 20 mM glucose, 38 mM sodium bicarbonate, 1 mM pyruvic acid, 10% heat-inactivated FBS, 100 μM non-essential amino acid, 2 mM L-glutamine and antibiotic–antimycotic). The culture was maintained at 37 °C in a humidified atmosphere, and the medium was changed 3 days later. In the case of DIV8-9, microglia were separated by shaking for 2 h.

### 2.3. Conditioned medium (CM) preparation

CM was obtained from mesencephalic culture treated with 30 μM $MPP^+$. Dead cells and debris were removed by centrifugation (10,000*g*, 10 min) and stored at −70 °C until use. CM was further fractionated by ultracentrifugation at 100,000*g* for 2 h and pellets were re-suspended in RM.

To characterize any substances contributing to microgial migration, the supernatant (SNT) of ultracentrifuged CM was incubated with proteinase K (50 μg/mL; Sigma), RNase A (50 μg/mL; Qiagen) and DNase I (5 units/mL; Takara) at 37 °C for 1 h. For lipid depletion, the SNT was mixed with 1 volume of chloroform or ether, placed on ice for 20 min and centrifuged at 10,000*g* for 30 min. Layers in which the lipids were excluded were transferred to new tubes and vacuumed so as to flow out toxic chloroform or ether at 40 °C for 15 min. Evaporated media were filled with distilled water to balance the ion concentrations.

### 2.4. Immunocytochemistry

After $MPP^+$ treatment, mesencephalic culture was fixed with 4% paraformaldehyde (PFA) for 10 min. Cells were incubated with 0.3% hydrogen peroxidase for 20 min, and blocked with PBS containing 1.5% serum and 0.3% Triton X-100 for 30 min. Primary antibodies against microtubule-associated protein-2 (MAP-2; Sigma) and tyrosine hydroxylase (TH; Sigma) were incubated for 2 h at room temperature. After washing with PBS, cells were incubated with anti-mouse biotinylated secondary antibody for 1 h, followed by ABC reagent (Vector) for 30 min. Immunostaining was visualized with 3,3′-diaminobenzidine (DAB; Vector). For the quantification, MAP-2 and TH-immunopositive cells were counted in 4 random fields of each well and the entire well, respectively.

Microglia were fixed with PFA and incubated in blocking solution for 30 min. Cells were labeled with an anti-ED-1 antibody (Serotec) followed by FITC-conjugated anti-mouse IgG. Then, F-actin of microglia was labeled with phalloidin-TRITC (Molecular Probe). After nuclei were stained with 4′-6-diamidino-2-phenylindole (DAPI), the cells were observed under fluorescent microscopy (Olympus).

### 2.5. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)

Apoptotic cells were determined using the *In situ* Cell Death Detection kit (Roche). After being stained with an anti-TH antibody followed by TRITC-conjugated anti-mouse IgG (Jackson), cells were incubated with the TUNEL mixture for 1 h at 37 °C. After the nuclei were counter-stained with DAPI, the cells were observed under confocal microscopy (LCM510; Carl Zeiss).

### 2.6. Detection of peroxynitrite generation

Free radical generation was monitored using dehydrorhodamine123 (DHR123; Molecular Probe). After mesencephalic cultures were treated with $MPP^+$, DHR123 was added to achieve a final concentration of 1 μM. After incubation for 1 h at 37 °C, cultures were washed with PBS and fixed with PFA. DHR123-positive neurons were first identified with TRITC filter under fluorescence microscopy, and then double labeling with TH antibody was performed.

### 2.7. Identification of cell viability

To assess cell viability, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (4 mg/mL in PBS) was added to the culture at one-tenth of the total media volume and incubated at 37 °C for 3–4 h. Media were aspirated completely and insoluble violet formazan was dissolved in DMSO. After absorbance was measured at 570 nm, the amount of MTT conversion was displayed as a percentage of the absorbance of treated wells relative to that measured in the DMSO control wells. Otherwise, calcein-acetoxymethyl (Calcein-AM; Molecular Probe) was added to the culture at a final concentration of 10 μM, and incubated for 10 min at 37 °C. After PBS washing, live cells were observed under inverted fluorescent microscopy.

### 2.8. Transmigration assay

To evaluate microglial mobility, transwells (Costar) were used for the *in vitro* migration assay. Microglia were applied to the upper insert via a 5 μm pore membrane, and CM was filled in the lower chamber. After 12 h incubation, the migrating cells were fixed with methanol and the microglia on the upper side of the membrane were removed with a cotton swab. The migrating microglia on the lower side of the membrane were stained with DAPI and captured with a camera connected to an inverted

fluorescent microscope through a 20× objective lens. To measure the number of migrated microglia, 4 random fields of each membrane were captured, and the number of cells was counted using the Image-Pro Plus 5.0 program (MediaCybernetics).

### 2.9. Statistical analysis

Data are expressed as the mean ± SEM of each group ($n$ = 4–8) in independent experiments. For a comparison of three or more groups, data were analyzed by one-way analysis of variance (ANOVA) followed by *post hoc* Bonferroni or Tukey comparisons test. Statistical analysis was carried out using GraphPad Prism 4 (GraphPad Software). A value of $p < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. $MPP^+$ induced a selective degeneration of DAergic neurons in primary mesencephalic neuronal culture

To validate the mesencephalic culture system with DAergic neurons, MPTP and $MPP^+$ were added to the culture. The pro-toxin MPTP is known to be converted to the actively toxic molecule $MPP^+$ by monoamine oxidase-B in astrocytes, after which it induces neurodegenerative processes via dopamine transporters [16]. The survival of DAergic neurons after MPTP or $MPP^+$ treatment for 48 h was determined by counting the TH-positive neurons. DAergic neurons were degenerated by $MPP^+$ in a dose-dependent manner, whereas MPTP did not affect DAergic neuronal viability (Fig. 1A). This result indicates that astrocytes were rare in this primary mesencephalic neuronal culture system.

Next, the selective $MPP^+$ insult to DAergic neurons was subsequently evaluated by quantifying the TH-positive neurons. $MPP^+$ was applied at various concentrations for 48 h. Although 50 μM $MPP^+$ evoked non-selective neuronal cell death, as shown by MTT assay, a significant decrease in the number of TH-positive neurons was observed at 10 μM and higher concentrations of $MPP^+$ (Fig. 1B). In addition, 30 μM $MPP^+$ induced a time-dependent DAergic neuronal death, and a significant DAergic loss was obvious by 24 h after $MPP^+$ treatment (Fig. 1C). TH-positive neurons were evidently shrunken and exhibited less neuritis at 48 h after 30 μM $MPP^+$ treatment (Fig. 1D). To substantiate the selective DAergic injury by $MPP^+$, the entire neuronal population was determined with MAP-2, a mature neuronal marker, by immunocytochemistry (Fig. 1C and E). These results indicate that DAergic neurons were selectively damaged by $MPP^+$ insults in both a time- and dose-dependent manner.

### 3.2. Dying DAergic neurons generated reactive oxygen species (ROS) at an early time point after $MPP^+$ treatment

DAergic injury by $MPP^+$ was determined by double-labeling with TH and TUNEL. When the mesencephalic culture was treated with 30 μM $MPP^+$ for 48 h, TH-positive neurons were merged with TUNEL signals (Fig. 2A). To determine whether damaged DAergic neurons produced free radicals in the early period after $MPP^+$ insult, DHR123, an indicator of peroxynitrite, was applied to the 30 μM $MPP^+$-treated mesencephalic culture at various time points. The production of peroxynitrite peaked 12 h after $MPP^+$ treatment and gradually decreased (Fig. 2B), and the TH-positive DAergic neurons were co-localized with the DHR123 signals (Fig. 2C). This suggests that $MPP^+$ causes oxidative stress to DAergic neurons at an early time point, which results in the degeneration of DAergic neurons.

### 3.3. Conditioned medium from $MPP^+$-treated mesencephalic cultures induced microglia migration

To explore if any communication occurred between the injured mesencephalic neurons and microglia, we examined microglial motility in response to various stimuli. Prior to the assessment of microglial migration, we monitored the morphological phenotypes of microglia, because microglia display highly motile behavior accompanied by morphological alterations, such as the formation of filopodia-like protrusions [7]. CM was collected from vehicle or $MPP^+$-treated mesencephalic cultures, and microglia were exposed to CM for 12 h. Then, F-actin was marked with phalloidin staining, and the identity of the microglia was confirmed with ED-1. CM from $MPP^+$-treated cultures robustly evoked filopodia-like formation, suggesting that CM contains an activator of microglia (Fig. 3A).

To examine whether such morphological changes consequently lead to the migration of microglia, microglia were exposed to CM using a transwell. Microglia which migrated from the upper part to the lower chamber via the 5 μm pore membrane were visualized with DAPI (Fig. 3B) and counted. The CM derived from $MPP^+$-treated culture induced microglial migration depending on the $MPP^+$ exposure time (Fig. 3C), implying that the CM from $MPP^+$-insulted DAergic neurons contains certain components eliciting motility from microglia.

### 3.4. The chemoattractants in the CM were soluble components released from damaged DAergic neurons

To characterize the substances putatively attracting the microglia, the CM was fractionated into a soluble supernatant (SNT) and insoluble pellet (PEL) by ultracentrifugation. The microglia fractionated into separated parts were enumerated by visualization with DAPI (Fig. 3D). Soluble SNT from the $MPP^+$-treated cultures induced approximately 5 times higher microglial migration than that from vehicle-treated cultures, which is comparable to the migration of the intact $MPP^+$-treated CM. However, the debris-containing pellets did not elicit any significant migration of the microglia (Fig. 3E). Thus, it appears that there are soluble and diffusible components in SNT that are responsible for the chemotactic attraction of microglia toward damaged DAergic neurons.

### 3.5. Proteins, RNA and DNA were not identified as major chemotactic factors in microglia migration

To identify the main chemoattractants, proteins, RNA and DNA in the SNT were individually depleted from the vehicle and the $MPP^+$-treated mesencephalic cultures. Proteins were digested by proteinase K (PK) treatment combined with heat inactivation, and RNA and DNAs were digested by RNase A and DNase I, respectively. None of these depletion steps exhibited any cytotoxic effects on microglia, which were examined by MTT assay (Fig. 4A). Based on transmigration assay, we found that $MPP^+$-treated SNT depleted of proteins, DNA and RNA still induced a significant migration of microglia (Fig. 4B).

### 3.6. Lipid depletion completely abrogated the chemoattractive properties of CM

Alternatively, we explored lipid components as a possible candidate, since it was reported that apoptotic cells secrete lipid-like chemoattractants that simulate monocytes [17,18]. Cell-free SNT was processed with chloroform or ether to remove the lipid components. This process did not affect microglial viability or mobility, as confirmed by MTT assay, Calcein-AM staining (Fig. 4C and D) and DAPI staining (Fig. 4E). SNT from $MPP^+$-treated culture induced

significant migration of microglia compared to SNT from vehicle-treated cultures. Lipid-depleted SNT from $MPP^+$-treated cultures, which were extracted via either ether or chloroform, markedly attenuated microglial migration to the extent of PEL or control

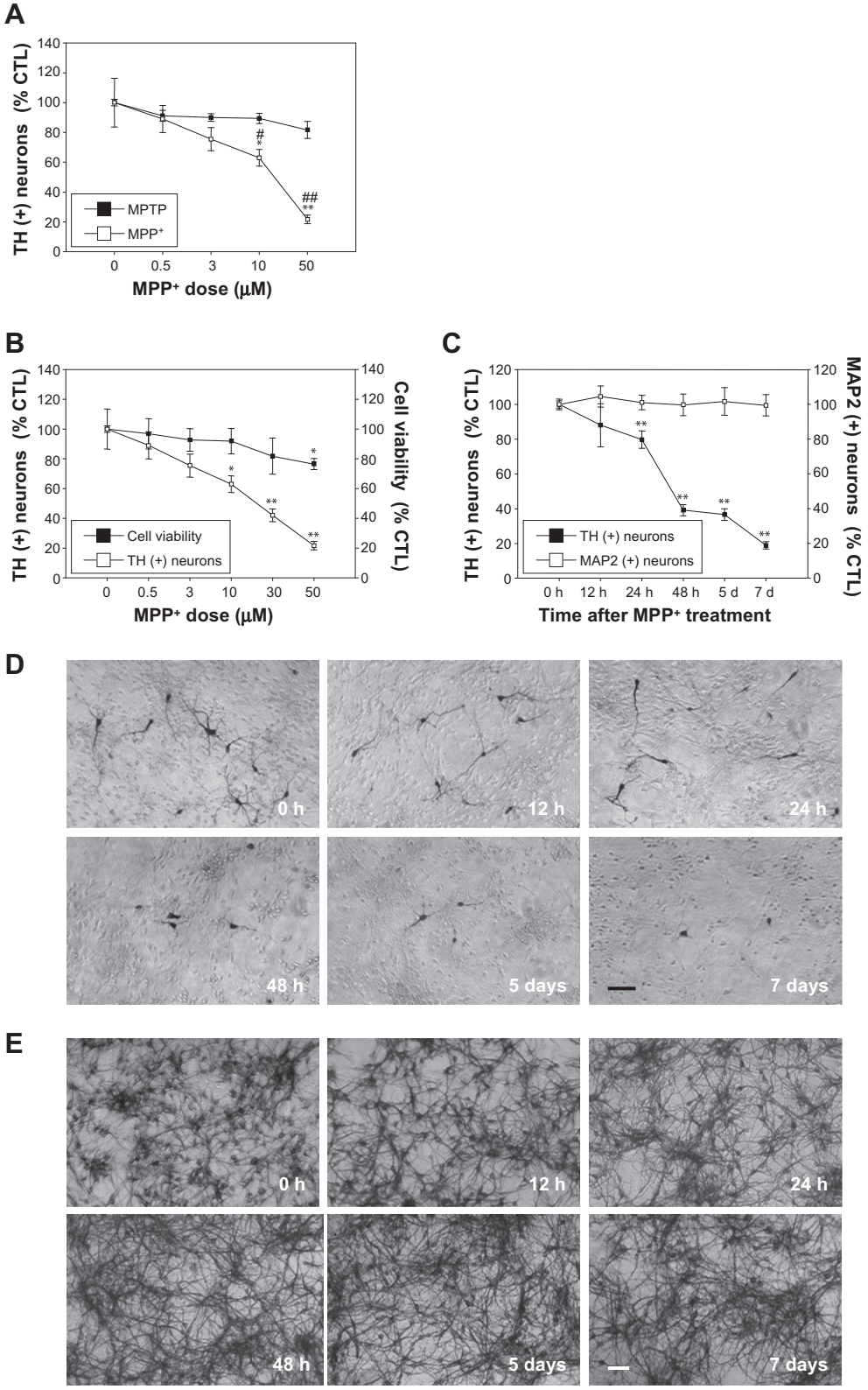


**Fig. 1.** $MPP^+$ selectively damages DAergic neurons. (A) Mesencephalic culture was treated with MPTP or $MPP^+$ for 48 h, and the DAergic neurons were estimated by counting the TH-positive neurons. $^*p < 0.05$, $^{**}p < 0.01$ *vs.* vehicle-treated control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs.* MPTP-treated culture. (B) Mesencephalic culture was exposed to various concentrations of $MPP^+$ for 48 h. Neuronal viability and the survival of DAergic neurons were assessed by MTT assay and immunostained-TH cell counting, respectively. $^*p < 0.05$, $^{**}p < 0.01$ *vs.* vehicle-treated control. (C) After mesencephalic cultures were exposed to 30 μM $MPP^+$ for various time courses, the number of DAergic neurons and the entire number of neurons were determined by (D) TH and (E) MAP-2 immunocytochemistry, respectively. $^{**}p < 0.01$ *vs.* $MPP^+$-treated cultures for 0 h. Scale bar, 50 μm.

levels (Fig. 4F). These results indicate that certain lipid-like molecules are present in the CM released from damaged DAergic neurons and trigger the recruitment of neighboring microglia.

## 4. Discussion

In the present study, it was demonstrated that microglia have the potential to migrate toward CM prepared from primary mesencephalic neurons, including DAergic neurons damaged by $MPP^+$. Microglia preferentially toward the soluble lipid-containing of CM, not the cellular debris-containing part. Although the precise mechanism underlying the release of diffusible factors from DAergic neurons after $MPP^+$ exposure is not yet elucidated, several points are worth considering.

First, our results strongly suggest that as yet unidentified soluble and lipid-like components released from $MPP^+$-treated mesencephalic culture are likely to be putative factors triggering microglial migration. In addition to the fact that factors such as ATP/ADP and various chemokines are reported to stimulate microglial motility [19,20], it is also reported that lipid-related factors act as migration-inducing chemoattractants. For example, apoptotic cells induce the migration of peripheral phagocytes via caspase-3-mediated release of a lipid attraction signal [17]. Lysophosphatidic acid, a bioactive phospholipid, activates the migration of microglia [21]. Phospholipase $A_2$ ($PLA_2$) is the rate-limiting enzyme in the conversion of membrane phospholipid to arachidonic acid and lysophospholipid. Secretory $PLA_2$ ($sPLA_2$) is able to attract human neutrophils *in vitro* by inducing the release of the lipid chemoattractants, and $PLA_2$ inhibitors markedly inhibit $sPLA_2$-induced neutrophil migration [22]. Calcium-independent $PLA_2$ ($iPLA_2$) regulates Src trafficking and microglial chemotaxis [23]. In addition, lysophosphatidylcholine (LPC), a $PLA_2$-induced

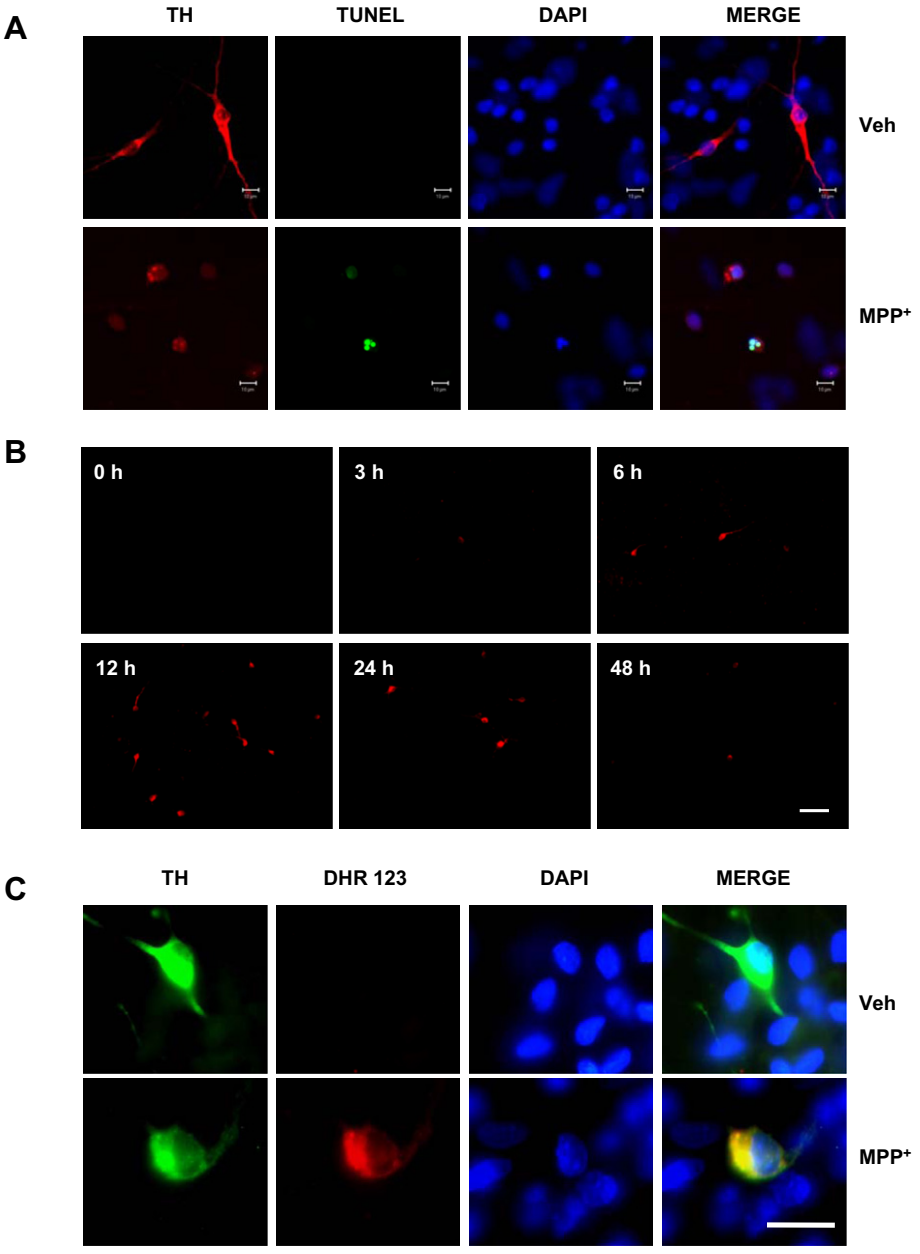


**Fig. 2.** $MPP^+$ insult generates peroxynitrite in DAergic neurons at an early time point. (A) Mesencephalic culture was double-labeled with TUNEL (green) and TH (red) after 30 μM $MPP^+$ treatment for 48 h. Scale bar, 20 μm. (B) After 30 μM $MPP^+$ treatment for various time courses, the generation of peroxinitrite was detected by DHR123. Scale bar, 50 μm. (C) After 30 μM $MPP^+$ treatment for 12 h, the culture was incubated with DHR123 (red) and subsequently double-labeled with TH (green). Scale bar, 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

hydrolysis product, elicits the migration of normal peripheral human T cells [24], and $iPLA_2$ activation and the subsequent LPC generation attract monocytes [17]. Most of all, the activity of phospholipid biosynthetic enzymes was shown to be elevated in the SN of PD patients [25]. Therefore, microglial attraction by lipid signals released from damaged DAergic neurons in PD needs to be further explored.

Next, it is of interest to note that while $MPP^+$ treatment for 12 h did not significantly induce DAergic neuronal death, the CM from $MPP^+$-treated culture for 12 h did induce a significant migration of microglia ($p < 0.01$ *vs.* control). At that time, ROS were generated in DAergic neurons by $MPP^+$ insult. These results suggest that DAergic neurons produce attractants at an early stage of $MPP^+$. In other words, not only dead but also dying neurons attract

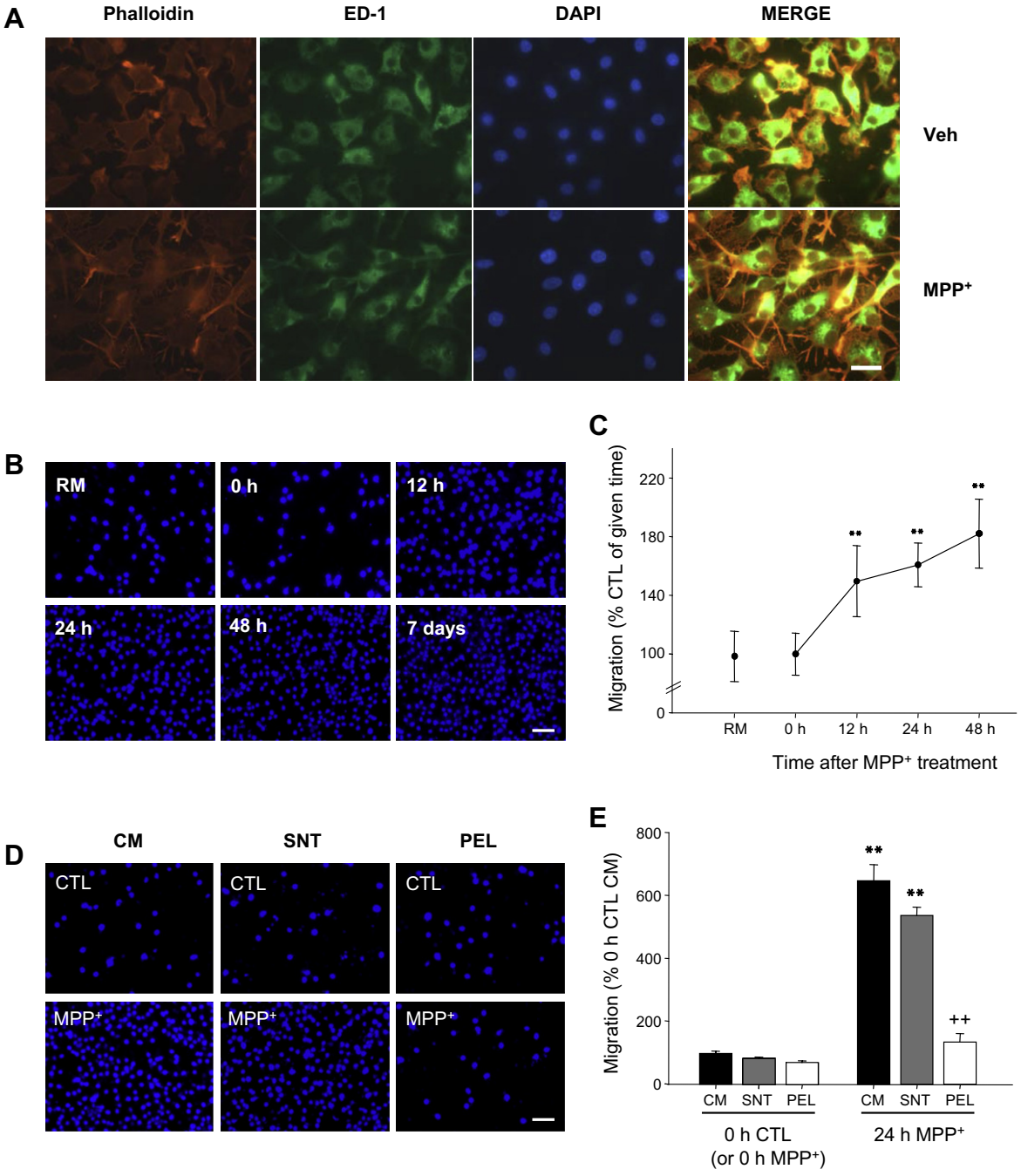


**Fig. 3.** Soluble components induce microglal migration. (A) Conditioned medium was obtained from mesencephalic culture treated with 30 μM $MPP^+$ for 24 h. After being exposed to CM for 12 h, microglia were stained with ED-1 (green) and phalloidin (red). Scale bar, 10 μm. Mesencephalic cultures were treated with vehicle or 30 μM $MPP^+$ for various time courses, and the harvested CM was incubated with microglia for 12 h. RM was used to monitor spontaneous migration. (B) Migrating cells were visualized with DAPI. Scale bar, 50 μm. (C) The net migration induced by the $MPP^+$-treated CM over the spontaneous microglial migration at each time point was represented as a percentage of the migration index. $^{**}p < 0.01$ *vs.* the induction of microglial migration at the time after MPP + treatment at 0 h. The CM from mesencephalic cultures treated with 30 μM $MPP^+$ for 24 h was fractionated into soluble and insoluble parts by ultracentrifugation. Microglia were seeded on the upper transwell insert and exposed to SNT or PEL in the lower transwell chamber for 12 h. (D) Migrated microglia were visualized with DAPI. Scale bar, 50 μm. (E) The migrating microglia are expressed as a percentage of the vehicle-treated control CM at 0 h. $^{**}p < 0.01$ *vs.* control CM for 0 h; $^{++}p < 0.01$ *vs.* CM from $MPP^+$-treated culture for 24 h. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

microglia dynamically. Indeed, phagocytosis of axotomized neurons by activated microglia is not limited to dead neurons, but includes dying neurons [26]. It is widely believed that increased oxidative stress contributes to the DAergic neuronal degeneration that occurs in PD, and several biological markers of oxidative damage are reportedly elevated in the SN of PD brains [2]. One of the major cellular targets free radicals is polyunsaturated fatty acid (PUFA). In PD, the PUFA levels are reduced in the SN, whereas there are increased levels of lipid peroxidation-related products [27]. Moreover, some lipid peroxidation products, such as 4-hydroxynonenal, can cooperate in cell recruitment [28]. Also, the oxygenation products of arachidonic acid elicit chemotaxis in human polymorphnuclear leukocytes [29]. DAergic neurons produce ROS at an early time point in the course of $MPP^+$, and they release lipid substances which apparently trigger microglial migration. This suggests that both oxidative stress-related and lipid-like products generated by $MPP^+$ insult induce the dynamic migration of microglia.

Finally, one of the challenging issues in PD is the difficulty of diagnosing this disease at an early phase, because clinical symptoms tend to not be evident early. In this study, we found that not only dead neurons, but also dying neurons, activate and attract microglia dynamically in the early period. If it indeed turns out to be the case that the migration of activated microglia is intimately related to PD progression, it might be possible to identify PD at an early stage by detecting the attracting factor as a pathological biomarker.

In conclusion, DAergic neurons are degenerated selectively by $MPP^+$ insult. The damaged DAergic neruons apparently then release certain soluble attractants, and these factors induce the

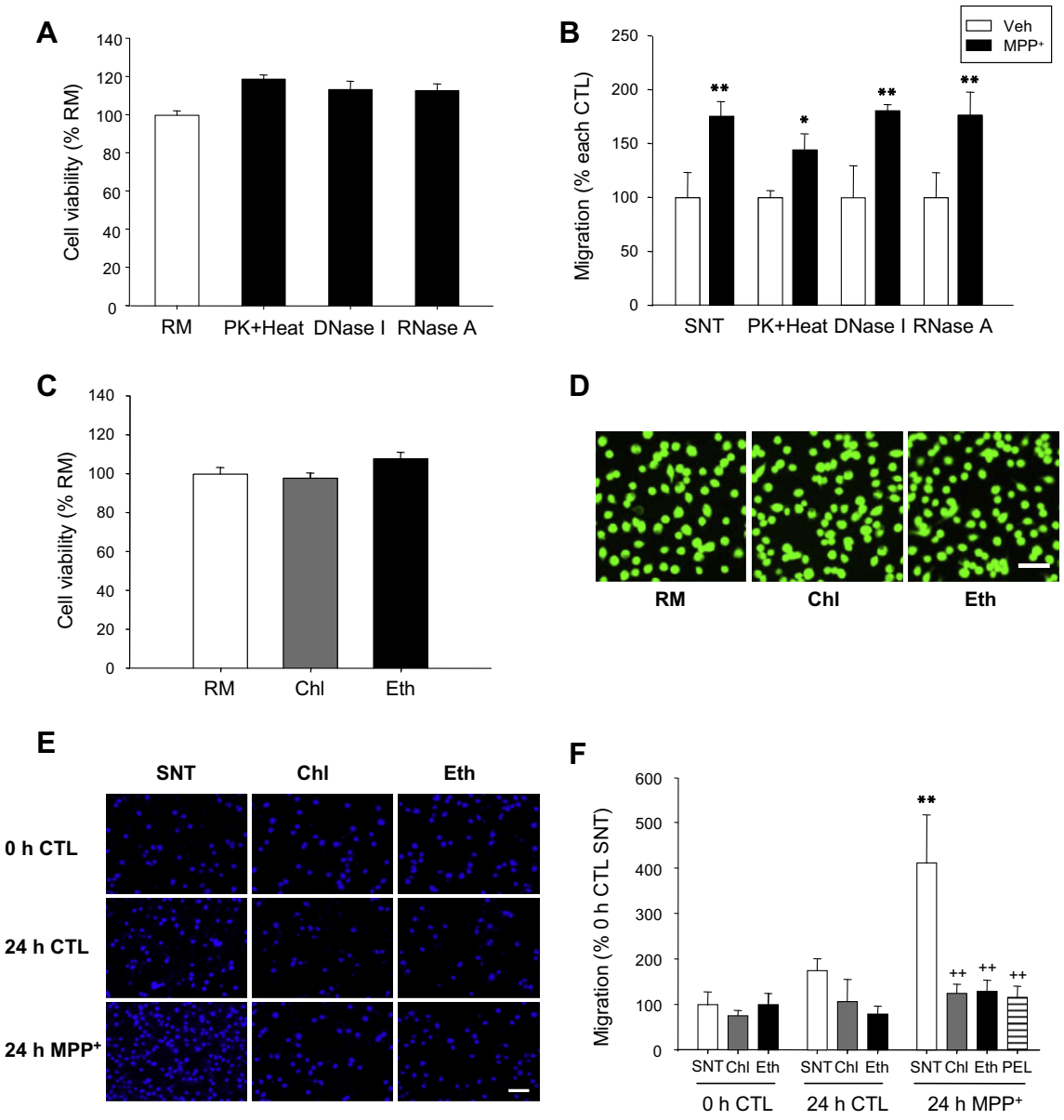


**Fig. 4.** The microglia attractants are lipid-like components. Protein-, DNA-, RNA- and lipid-depleted supernatant (SNT) was incubated with microglia for 12 h using a transwell. (A) To examine the cytotoxic effects of the protein-, DNA- and RNA-digested SNT on microglia, MTT assay was performed. (B) Migrating microglia were enumerated by counting the DAPI-positive microglia, and these are represented as a percentage of each vehicle-treated control. $^*p < 0.05$, $^{**}p < 0.01$ *vs.* each vehicle-treated control. To determine the cytotoxic effect of lipid-depleted SNT by chloroform (Chl) or ether (Eth) on microglia, (C) MTT assay and (D) calcein-AM staining were performed. Scale bar, 50 μm. (E) Migrated cells were visualized with DAPI and (F) are represented as a percentage of vehicle-treated control SNT at 0 h. Scale bar, 50 μm. $^{**}p < 0.01$ *vs.* vehicle-treated control SNT for 0 h; $^{++}p < 0.01$ *vs.* $MPP^+$-treated SNT for 24 h.

dynamic migration of microglia. Thus, the present study strongly suggests that there is a communication that takes place between damaged DAergic neurons and microglia that is mediated by diffusible lipid signals.

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