

## ORIGINAL ARTICLE

# Capsaicin prevents degeneration of dopamine neurons by inhibiting glial activation and oxidative stress in the MPTP model of Parkinson's disease

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The effects of capsaicin (CAP), a transient receptor potential vanilloid subtype 1 (TRPV1) agonist, were determined on nigrostriatal dopamine (DA) neurons in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of Parkinson's disease (PD). The results showed that TRPV1 activation by CAP rescued nigrostriatal DA neurons, enhanced striatal DA functions and improved behavioral recovery in MPTP-treated mice. CAP neuroprotection was associated with reduced expression of proinflammatory cytokines (tumor necrosis factor- $\alpha$  and interleukin-1 $\beta$ ) and reactive oxygen species/reactive nitrogen species from activated microglia-derived NADPH oxidase, inducible nitric oxide synthase or reactive astrocyte-derived myeloperoxidase. These beneficial effects of CAP were reversed by treatment with the TRPV1 antagonists capsazepine and iodo-resiniferatoxin, indicating TRPV1 involvement. This study demonstrates that TRPV1 activation by CAP protects nigrostriatal DA neurons via inhibition of glial activation-mediated oxidative stress and neuroinflammation in the MPTP mouse model of PD. These results suggest that CAP and its analogs may be beneficial therapeutic agents for the treatment of PD and other neurodegenerative disorders that are associated with neuroinflammation and glial activation-derived oxidative damage.

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## INTRODUCTION

Parkinson's disease (PD) is characterized by the progressive degeneration of nigrostriatal dopamine (DA) neurons,<sup>1</sup> which is associated with motor dysfunctions such as slowness of movement, resting tremor and rigidity.<sup>1–3</sup> Although the exact cause of PD remains unclear, both clinical and experimental data demonstrate that reactive glia can produce NADPH oxidase-derived reactive oxygen species (ROS) and/or myeloperoxidase (MPO)-derived reactive nitrogen species (RNS), which result in oxidative damage to DA neurons.<sup>4–6</sup> Moreover, proinflammatory neurotoxic mediators, such as tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$

and inducible nitric oxide synthase (iNOS), are increased in the substantia nigra (SN) of PD patients and in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model.<sup>7–9</sup>

Transient receptor potential receptor vanilloid type 1 (TRPV1), the capsaicin (CAP) receptor, is a nonselective cation channel that causes pain and is highly expressed in sensory neurons.<sup>10</sup> TRPV1 is also present in the brain, suggesting that this receptor has an important role in the central nervous system. The activation of TRPV1 was found to modulate neuronal function,<sup>11,12</sup> control motor behavior<sup>13,14</sup> and regulate neuroinflammation.<sup>15</sup>

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Moreover, CAP regulates neurotransmitter release from nigral slices,<sup>16</sup> produces hypokinesia in parallel with a decrease in the activity of nigrostriatal DA neurons,<sup>17,18</sup> and modulates the effects of high levels of L-DOPA on motor activity in reserpine-treated Parkinsonian rats,<sup>19</sup> suggesting that TRPV1 has a functional role in nigrostriatal DA neurons. The current study sought to determine whether TRPV1 activation by CAP could rescue nigrostriatal DA neurons by inhibiting glial activation and reactive glia-derived oxidative stress in the MPTP mouse model of PD.

## MATERIALS AND METHODS

### Chemicals

The following chemicals were purchased: capsazepine (CZP; Tocris Cookson, Ellisville, MO, USA), iodo-resiniferatoxin (I-RTX; Tocris Cookson, Ellisville, MO, USA), CAP (Sigma, St Louis, MO, USA) and MPTP (Sigma). CAP, CZP and I-RTX were dissolved in ethanol and Tween-80, and then diluted with phosphate-buffered saline (PBS; 1:1:8, ethanol:Tween-80:PBS)

### Animals and drug treatments

The animal protocols and guidelines were established and approved by Kyung Hee University. All experiments were conducted according to the relevant national guidelines. Eight- to ten-week-old male C57BL/6 mice (23–25 g, Charles River Breeding Laboratory, Yokohama, Japan) were used for MPTP intoxication. MPTP (20 mg kg<sup>-1</sup>, free base; Sigma) was dissolved in PBS and then intraperitoneally injected into mice for four times at 2-h intervals. Various doses of the CAP (0.01, 0.1, 0.5, 1 and 2.5 mg kg<sup>-1</sup>) were intraperitoneally administered for 1 day starting 30 min before MPTP injection. The TRPV1 antagonists CZP and I-RTX (1 mg kg<sup>-1</sup>) were injected into the peritoneum for 30 min before the TRPV1 agonist injections. Control mice were injected with the vehicle (Supplementary Figure 1).

### Immunohistochemistry

For immunohistochemistry, mouse brain tissues were prepared as previously described.<sup>20,21</sup> Sections were washed with PBS, then permeabilized with 0.2% Triton X-100 dissolved in 0.1 M PBS containing 1% bovine serum albumin at room temperature for 30 min. Tissue sections were labeled with rabbit anti-tyrosine hydroxylase (TH; 1:2000; Pel-Freez, Brown Deer, WI, USA), rat anti-macrophage Ag complex-1 (MAC-1; 1:200; Serotec, Oxford, UK), rat anti-CD68 (ED-1; 1:1000; Serotec), mouse anti-glia fibrillary acidic protein (GFAP; 1:5000; Neuromics, Edina, MN, USA), rabbit anti-MPO (1:500; Thermo Scientific, Fremont, CA, USA) and mouse anti-8-hydroxy-29-deoxyguanosine (8-OHdG; 1:200; JaICA, Shizuoka, Japan). After overnight incubation, sections were washed and incubated with proper biotinylated secondary antibodies for 1 h. After washing, the sections were stained with an ABC kit according to the manufacturer's instructions. Stained samples were mounted on gelatin-coated slides and analyzed under a bright-field microscope (Nikon, Tokyo, Japan). To determine the immunoreactivity of the 8-OHdG-labeled area, three or four images of the SN region were obtained, thresholded using Image J (NIH, Bethesda, MD, USA), quantified and normalized by the value of the control group.

### Stereological cell counts

As previously described,<sup>20,21</sup> the total number of TH-positive neurons or MPO-ip cells was counted in the various animal groups based on

the optical fractionator method using a bright-field microscope (Olympus Optical, BX51, Tokyo, Japan) and Stereo Investigator software (MBF Bioscience, Williston, VT, USA). The actual counting of TH-labeled cells was performed within the boundaries of the optical fractionator. The estimated total number of cells was calculated according to the optical fractionator software. More than total 300 points overall of the sections of each specimen were analyzed.

### Densitometric analysis

As previously described,<sup>20–22</sup> the TH-labeled sections were imaged using a light microscope (Zeiss Axioskop, Oberkochen, Germany) coupled to a video camera (MegaPlus model 1.4i; Kodak, New York, NY, USA) and analyzed using an Image-Pro Plus system (Ver 4.0; Media Cybermetrics, Silver Spring, MD, USA). To normalize the images for differences in background illumination, the average of the background density readings from the corpus callosum was subtracted from the average of the density for each section analyzed. Then, the average of all of the sections of each animal was calculated separately before the data were statistically processed.

### Rotarod test

To measure forelimb and hindlimb motor dysfunction, mice were placed on a Rotarod system (five-lane accelerating rotarod; Ugo Basile, Comerio, Italy) as previously described.<sup>20–22</sup> For the training session, each mouse was placed onto the rotating rod (10 r.p.m.) for 20 min for 7 consecutive days before the MPTP treatment. Seven days from the last MPTP treatment, the mice were placed in a separate compartment on the rotating rod and tested at 20 r.p.m. for 20 min. The latency to fall from the rotating rod within this time period was automatically recorded by magnetic trip plates.

### Measurement of DA levels (high-performance liquid chromatography analyses)

As described,<sup>20</sup> the levels of DA in the striatum (STR) were measured using high-performance liquid chromatography consisting of an autosampler (Waters 717 Plus Autosampler, Waters Division, Milford, MA, USA) and an ESA CoulochemII electrochemical detector equipped with an mBondapak C18 column (3.9 × 300 mm × 10 μm; ESA, Chelmsford, MA, USA). Dissected striatal tissues were homogenized with 0.1 M perchloric acid and 0.1 mM EDTA, and centrifuged at 900 r.p.m. for 20 min. All supernatants were collected and eluted with a mobile phase for catecholamine analysis (Chromosystems, Munich, Germany), and the column temperature was maintained at 4 °C. The contents of DA in the STR were analyzed using a commercially available program (Breeze; Waters Corp., Tokyo, Japan).

### Measurement of MPTP and 1-methyl-4-phenyl-pyridinium levels in the STR

For sample preparation, dissected striatal tissues were collected, sonicated and immediately centrifuged at 9000 r.p.m. for 20 min, and the supernatant was saved. As described previously,<sup>21</sup> MPTP and 1-methyl-4-phenyl-pyridinium (MPP<sup>+</sup>) levels were analyzed with a liquid chromatography electrospray ionization mass spectrometry system composed of three Agilent model G1311A HPLC quaternary pumps (Palo Alto, CA, USA), a G1313A standard autosampler and a G1316A thermostated column compartment. The samples were eluted through a 150 mm × 1.5 mm inner diameter, 4 μm Zorbax Eclipse XDB-C18 (Palo Alto, CA, USA). All samples were normalized for protein content, which was determined spectrophotometrically using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA).

### *In situ* detection of O<sub>2</sub><sup>-</sup> and O<sub>2</sub>-derived oxidants

As previously described,<sup>21,22</sup> production of O<sub>2</sub><sup>-</sup> and O<sub>2</sub>-derived oxidants was assessed by detecting ethidine labeled by hydroethidine histochemistry.<sup>21,22</sup> Hydroethidine was administered intraperitoneally at 3 days after the last MPTP treatment. Brain samples were collected 15 min after hydroethidine injection and then fixed with 4% paraformaldehyde in 0.1 M phosphate buffer. After fixation, the brains were sectioned, mounted on gelatin-coated slides and counterstained with 4,6-diamidino-2-phenylindole to detect cellular nuclei. All images

were acquired using a confocal microscope (Carl Zeiss, Oberkochen, Germany) in the frame scan mode with the same exposure time.

### Immunoprecipitation and western blotting

As previously described,<sup>22</sup> SN samples were homogenized and collected, and the supernatants were centrifuged for 20 min at 13 000 g at 4 °C. The total protein (100 µg) was immunoprecipitated with anti-p47phox antibody (Ab; 1.0 µg; Santa Cruz Biotechnology, Dallas, TX, USA). The immunoprecipitated proteins were separated on an SDS-polyacrylamide gel electrophoresis gel and were then electro-transferred onto polyvinylidene difluoride membranes and blocked for 30 min at room temperature with tris-buffered saline. The immunodetection of target proteins was performed using specific monoclonal antibodies as follows: anti-phosphoserine Ab (1:5000; Invitrogen Life Technologies, Rockville, MD, USA), anti-p47phox Ab (1:1000; Santa Cruz Biotechnology) and anti-gp91phox Ab (1:4000; BD Biosciences, CA, USA). For semiquantitative analyses, Ab immunoreactivity on the membrane was analyzed using a Computer Imaging Device (Seoul, South Korea) and the accompanying software (Fujifilm, Seoul, South Korea).

### Reverse transcription PCR

After injection of the TRPV1 agonist and/or antagonists, the mice were killed at 24 h after injection of MPTP, and the SN tissues were rapidly removed. The total RNA was extracted using RNAzol B (Tel-Test, Friendwood, TX, USA) and converted to complementary DNA using Superscript II reverse transcriptase (Life Technologies, Rockville, MD, USA) according to the manufacturer's instructions. PCR was performed with the following primer sequences: 5'-CTGCTGGTGG TGACAAGCACATTT-3' (forward) and 5'-ATGTCATGAGCAAAGG CGCAGAAC-3' (reverse) for iNOS; 5'-GCGACGTGGAAC TGCCAGA AGAG-3' (forward) and 5'-TGAGAGGGGAGGCCATTTGGGAAC-3' (reverse) for TNF-α; 5'-GCAACTGTTCCCTGAACTCAACT-3' (forward) and 5'-ATCTTTTGGGGTCCGTCAACT-3' (reverse) for IL-1β; and 5'-GCAATTATTCCCATGAACG-3' (forward) and 5'-GGCCTCACTAAACCATCCAA-3' (reverse) for 18s ribosomal RNA. Annealing was performed at 56 °C for 30 s (for iNOS, TNF-α and IL-1β), and PCR reaction was ended with a final extension at 72 °C for 90 s. The PCR products were separated by electrophoresis on 1.5% agarose gels, visualized with ethidium bromide and photographed. For semiquantitative analyses, the photographs were scanned using a Computer Imaging Device and the accompanying software (Fujifilm).

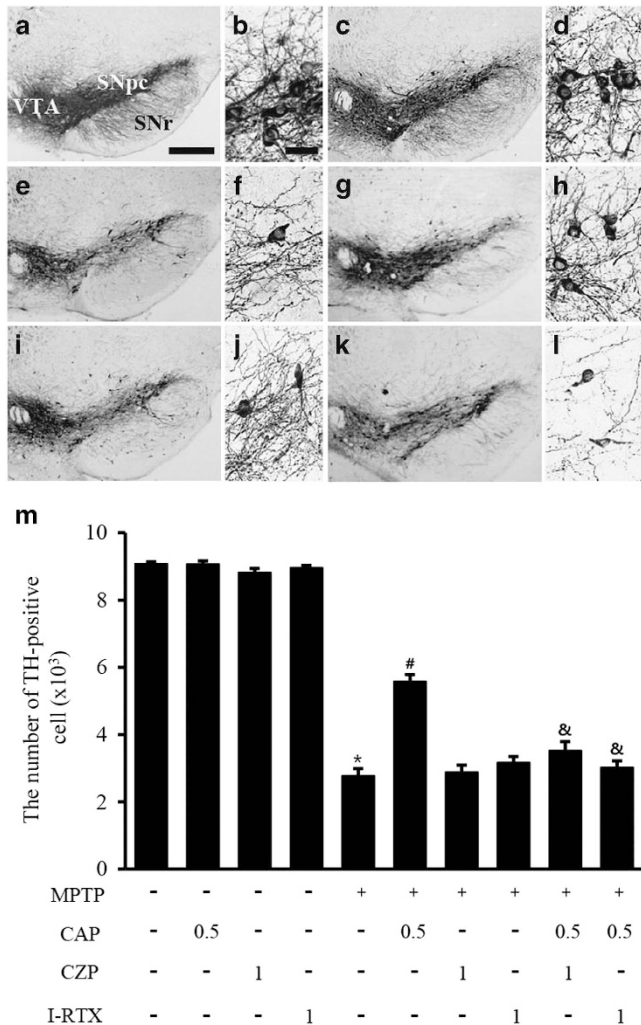
### Statistical analysis

All data are represented as the means ± s.e.m. Statistical significance ( $P < 0.05$  for all analyses) was assessed using analysis of variance (InStat 3.05, GraphPad, San Diego, CA, USA) followed by Student–Newman–Keuls analyses.

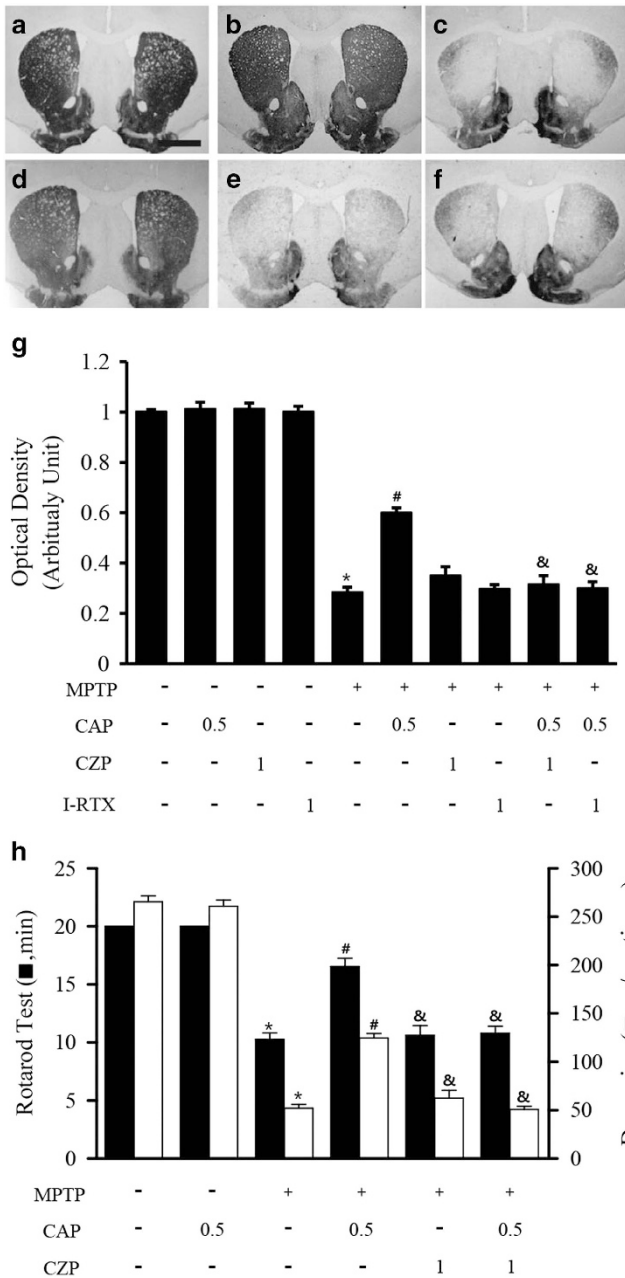
## RESULTS

### CAP rescues nigrostriatal DA neurons *in vivo* in MPTP-lesioned mice

Similar to our recent reports,<sup>21,22</sup> the TH immunohistochemical analysis revealed that MPTP exerted significant neurotoxicity on TH-positive cell bodies in the SN (Figure 1e and f) and its fibers in the STR (Figure 2c) at 7 days compared with control PBS (intact)-treated mice (Figures 1a,b and 2a). The results of the stereological counts and densitometric



**Figure 1** Capsaicin (CAP) attenuates 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxicity in the substantia nigra (SN) *in vivo*. Seven days after the last MPTP injection, animals were killed, and their brain tissues were processed for immunostaining with a tyrosine hydroxylase (TH) antibody for dopamine (DA) neurons: phosphate-buffered saline (PBS) (a, b), CAP (0.5 mg kg<sup>-1</sup>) alone (c, d), MPTP (e, f), MPTP+CAP (g, h), MPTP+CAP+capsazepine (CZP) (i, j) and MPTP+CAP+iodo-resiniferatoxin (I-RTX) (k, l). (m) The numbers of TH-positive cells in the SNpc. Bars represent the means ± s.e.m. of five to six animals per group. \* $P < 0.001$  significantly different from control, # $P < 0.001$ , significantly different from MPTP. & $P < 0.001$  significantly different from MPTP and CAP. SNpc, substantia nigra pars compacta; SNr, substantia nigra reticulata; VTA, ventral tegmental area. Scale bars, 300 µm (a, c, e, g, i, k); 50 µm (b, d, f, h, j, k).



**Figure 2** Capsaicin (CAP) prevents 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxicity in the striatum (STR) *in vivo*. The STR tissues obtained from the same animals as used in Figure 1 were immunostained with a TH antibody for dopamine (DA) fibers: phosphate-buffered saline (PBS) (a), CAP alone (b), MPTP (c), MPTP+CAP (d), MPTP+CAP+CZP (e) or MPTP+CAP+I-RTX (f). (g) The optical density of the TH-positive fibers in the STR. \* $P < 0.001$ , significantly different from control, # $P < 0.001$ , significantly different from MPTP, & $P < 0.001$ , significantly different from MPTP and CAP. Scale bars, 500  $\mu\text{m}$ . (h) After the rotarod test, striatal tissues were dissected from the animals to determine the dopamine level in the striatum. Bars represent the means  $\pm$  s.e.m. of five to six animals per group. \* $P < 0.001$ , significantly different from control, # $P < 0.001$ , significantly different from MPTP, & $P < 0.001$ , significantly different from MPTP and CAP (analysis of variance and Student–Newman–Keuls analysis).

analyses showed a significant reduction in the number of TH-positive cells by 72% in the SN (Figure 1m) and the density of TH-positive fibers by 77% in the STR (Figure 2g) in the MPTP-treated mice compared with PBS-treated control mice.

To explore the effects of CAP on the MPTP-induced neurotoxicity of nigrostriatal DA neurons, various doses of CAP (0.1–2.5  $\text{mg kg}^{-1}$ , intraperitoneal) were administered 30 min before the first MPTP injection. TH immunohistochemistry revealed dose-dependent CAP neuroprotection on nigrostriatal DA neurons at 7 days post MPTP treatment (Supplementary Table 1). Based on this result, a 0.5  $\text{mg kg}^{-1}$  dose of CAP was chosen for further studies and also effectively increased the number of TH-positive cells by 38% (Figure 1g,h and m;  $P < 0.01$ ) and density of TH-positive fibers by 36% (Figure 2d and g;  $P < 0.001$ ). Neuroprotection afforded by CAP was almost completely reversed by pretreatment with either of the TRPV1 antagonists, CZP (Figures 1i,j,m and 2e,g) or I-RTX (Figures 1k–m,2f and g), indicating TRPV1 involvement. CAP alone did not affect the TH immunoreactivity in the SN (Figure 1c and d) and STR (Figure 2b).

Next, we hypothesized that CAP neuroprotection may result from the altered metabolism of MPTP to  $\text{MPP}^+$  *in vivo*. Because striatal levels of  $\text{MPP}^+$ , an active toxic metabolite of MPTP, represent MPTP neurotoxicity,<sup>23,24</sup> we measured the difference in striatal MPTP and  $\text{MPP}^+$  levels 2 h after the last injection of MPTP in the presence or absence of CAP (Table 1). The results showed no substantial difference in the striatal levels of MPTP and  $\text{MPP}^+$ , ruling out the possibility that CAP affects MPTP conversion to  $\text{MPP}^+$  *in vivo*.

### CAP improves motor behavior by restoring striatal DA depletion in MPTP mice

To determine CAP effects on behavioral recovery in MPTP-lesioned mice, the latency to falling on the rotarod apparatus was analyzed at 7 days after the last MPTP injection in the absence or presence of CAP.<sup>21,22</sup> In MPTP-lesioned mice, robust motor deficits were evident, as determined by decreased sustained rotarod time to  $9.62 \pm 0.53$  min, a 53% decrease compared with the PBS-treated control ( $P < 0.001$ ; Figure 2h). In the CAP-treated MPTP-lesioned mice, the latency to fall was significantly increased up to  $16.27 \pm 0.45$  min ( $P < 0.001$ ; Figure 2h), which is indicative of behavioral rescue. This behavioral improvement was almost completely reversed by pretreatment with CZP ( $10.12 \pm 0.42$  min,  $P < 0.001$ ; Figure 2h).

In parallel with motor recovery, CAP produced an increase in striatal DA function. The high-performance liquid chromatography analysis revealed an MPTP-induced decrease in striatal DA levels by 72% compared with the PBS controls (Figure 2h). Compared with MPTP only, CAP resulted in a 42% increase in striatal DA levels ( $P < 0.001$ ; Figure 2h). The increase in STR DA levels was almost completely reversed by CZP pretreatment ( $P < 0.001$ ; Figure 2h). CAP or CZP alone did not affect the latency to fall or STR DA levels.

**CAP inhibits microglial activation and the expression of proinflammatory mediators in the SN *in vivo***

Because activated microglia can cause degeneration of DA neurons in the MPTP model,<sup>21,22,25</sup> we wondered whether CAP could exert neuroprotection via inhibition of microglial

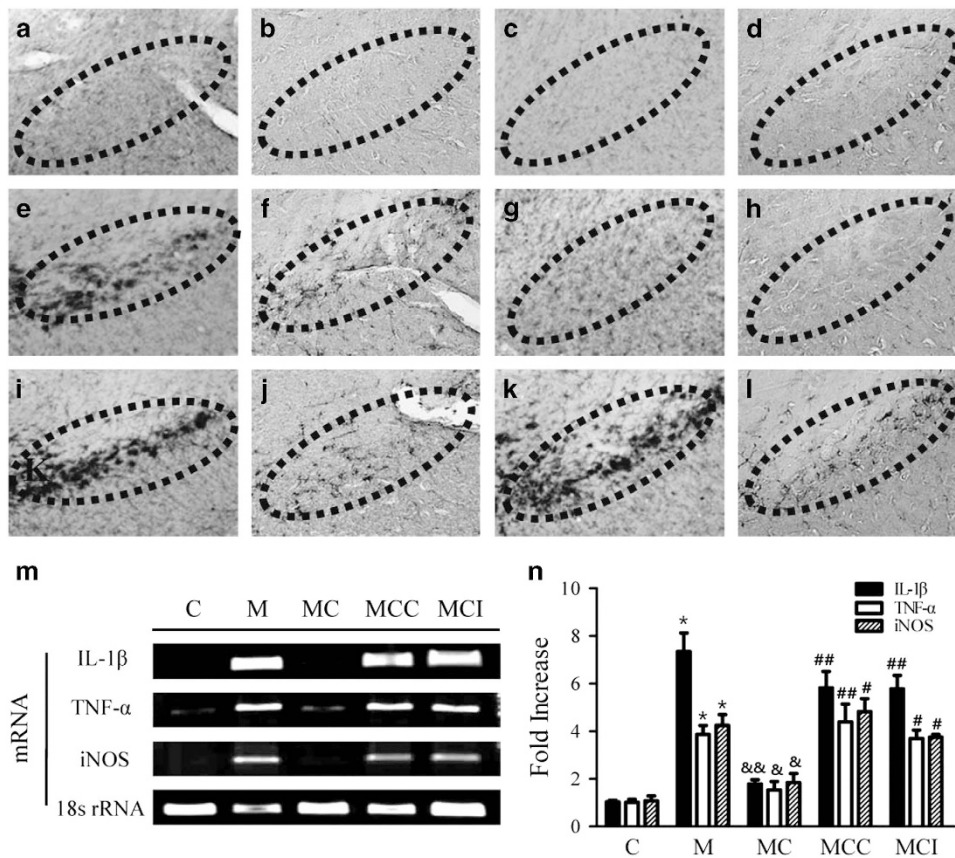
**Table 1** The effects of TRPV1 agonist on MPTP and MPP<sup>+</sup> levels ( $\mu\text{g mg}^{-1}$  protein) in the striata of C57 BL/6 mice

	Con	MPTP	MPTP+capsaicin
MPTP	0	2.5±0.9	2.7±0.5
MPP <sup>+</sup>	0	3.4±0.2	3.4±1.2

Abbreviation: MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. For capsaicin treatment, mice received a single injection/days of capsaicin ( $1 \text{ mg kg}^{-1}$  body weight) into the peritoneum, beginning at 30 min before first MPTP injection. After pretreatment, mice received four intraperitoneal injections of MPTP ( $20 \text{ mg kg}^{-1}$  body weight) at 2 h intervals. Striatal tissues were removed at 2 h after the last MPTP injection, and MPTP and MPP<sup>+</sup> levels were measured by liquid chromatography electrospray ionization mass spectrometry (LC/ESI-MASS). Note that there are no changes of striatal MPP<sup>+</sup> levels in the absence and presence of TRPV1 agonists.

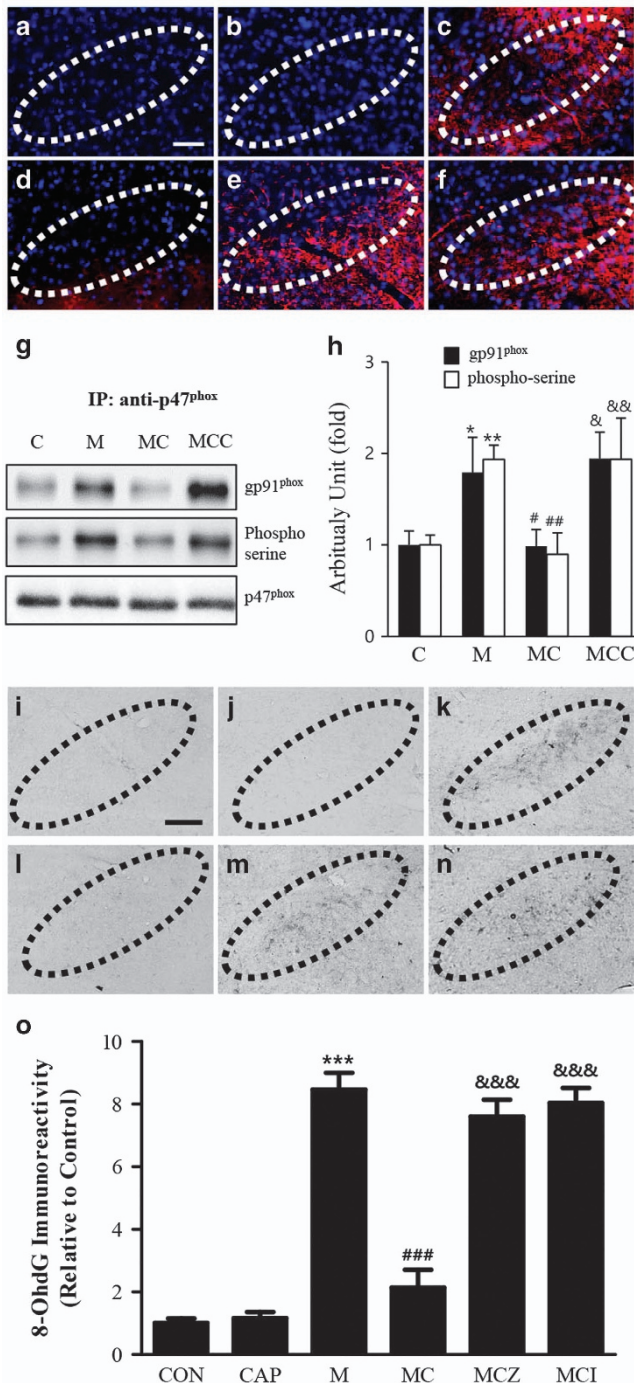
activation in the SN. Immunohistochemical analysis showed numerous MAC-1-labeled (reactive) microglia in the SN of MPTP-treated mice (Figure 3e), compared with control mice treated with PBS (Figure 3a). CAP treatment significantly attenuated MAC-1-labeled microglia in the MPTP-treated SN (Figure 3g), which was markedly reversed by CZP (Figure 3i) or I-RTX (Figure 3k). ED1-labeled phagocytic microglia<sup>26,27</sup> exhibited larger cell bodies with short processes in the MPTP-treated SN, which is indicative of an activated state (Figure 3f), compared with the control mice treated with PBS (Figure 3b). CAP treatment significantly attenuated the ED1-labeled microglia in the MPTP-lesioned SN (Figure 3h), which was markedly reversed by CZP (Figure 3j) or I-RTX (Figure 3l). However, CAP had no effects on MAC-1- (Figure 3c) and ED1-labeled microglial activation (Figure 3d).

Several lines of evidence strongly suggested that microglia-derived proinflammatory and/or cytotoxic factors, including IL-1 $\beta$ , TNF- $\alpha$  and iNOS, are implicated in the degeneration of



**Figure 3** Capsaicin (CAP) blocks microglial activation and the expression of proinflammatory mediators in the substantia nigra (SN) *in vivo*. Animals receiving phosphate-buffered saline (PBS) as a control (a, b), CAP ( $0.5 \text{ mg kg}^{-1}$ ) alone (c, d), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (e, f), MPTP+CAP (g, h), MPTP+CAP+CZP (i, j) and MPTP+CAP+I-RTX (k, l) were killed 3 days after the last MPTP injection. The brain tissues of the mice were removed and immunostained with antibodies for MAC-1 (a, c, e, g, i, k) and ED-1 (b, d, f, h, j, l) to identify microglia.  $n=5-7$ . Dotted lines indicate SNpc. Scale bar, 100  $\mu\text{m}$ . (m) Reverse transcription PCR showing the messenger RNA expression of proinflammatory cytokines in the SN. The total RNA was isolated from the SN at 1 day after the last injection of MPTP or vehicle in the absence or presence of CAP and/or CZP and I-RTX, TRPV1 antagonists. (n) Graphic representation of the means  $\pm$  s.e.m. of three to four samples. C, Control; M, MPTP; MC, MPTP and CAP; MCC, MPTP and CAP and CZP; MCI, MPTP and CAP and I-RTX. \* $P<0.01$ , significantly different from C. \*\* $P<0.01$ , & $P<0.05$ , significantly different from M. # $P<0.05$ , ## $P<0.01$ , significantly different from MC (analysis of variance and Student–Newman–Keuls analysis).

nigrostriatal DA neurons in the MPTP model of PD.<sup>8,21</sup> Thus, we determined whether CAP might regulate the expression of IL-1 $\beta$ , TNF- $\alpha$  and/or iNOS in the MPTP-lesioned SN, resulting in neuroprotection. The results of reverse transcription PCR showed that CAP attenuated MPTP-induced messenger RNA expression of IL-1 $\beta$  by 68% ( $P < 0.05$ ), TNF- $\alpha$  by 52% ( $P < 0.01$ ) and iNOS by 45% ( $P < 0.01$ ) in the SN (Figure 3m and n). The effects of CAP were reversed by treatment with CZP and I-RTX in the MPTP-lesioned SN (Figure 3m and n), suggesting TRPV1 involvement.



### CAP reduces microglial NADPH oxidase-derived oxidative stress in the MPTP-lesioned SN *in vivo*

We recently demonstrated that MPTP induced microglia-derived production of ROS, such as hydrogen peroxide and superoxide, through NADPH oxidase activation, resulting in degeneration of DA neurons in the SN.<sup>20,21,28</sup> Accordingly, we examined the effect of CAP on MPTP-induced oxidant production in the SN. Hydroethidine histochemical staining revealed significant increases in ethidium (that is, fluorescent product of oxidized hydroethidine) in the MPTP-lesioned SN (Figure 4c) compared with PBS-treated control mice (Figure 4a). In the CAP-treated MPTP-lesioned SN, the *in vivo* production of oxidants was significantly reduced (Figure 4d), which was reversed by the TRPV1 antagonists CZP (Figure 4e) and I-RTX (Figure 4f), indicating TRPV1 involvement. As controls, CAP alone had no effect on the production of oxidants (Figure 4b).

Because microglial NADPH oxidase is a significant source of ROS in the MPTP-lesioned SN *in vivo*,<sup>6,7</sup> we sought to determine whether CAP could exert neuroprotection by inhibiting NADPH oxidase-derived ROS production and the subsequent oxidative stress in the MPTP-lesioned SN.<sup>22</sup> Western blot analysis revealed that MPTP significantly upre-

**Figure 4** Capsaicin (CAP) reduces 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced oxidant production and activation of NADPH oxidase in the substantia nigra (SN) *in vivo*. (a–f) Animals receiving phosphate-buffered saline (PBS) as a control (a), CAP (0.5 mg kg<sup>-1</sup>) alone (b), MPTP (c), MPTP+CAP (d), MPTP+CAP+CZP (e) or MPTP+CAP+I-RTX (f) were killed 3 days after the last MPTP injection. SN tissues were prepared for hydroethidine histochemistry to detect oxidant production. Five to seven animals were used for each experimental group. Dotted lines indicate the SNpc. (g) Western blot analyses showed the phosphorylation of cytosolic subunits p47<sup>phox</sup> and p47<sup>phox</sup>-gp91<sup>phox</sup> binding 3 days after MPTP injection, indicating activation of NADPH oxidase in the SN. The animals were killed 3 days after the last MPTP administration, and the SN tissues were isolated. Tissue lysates were immunoprecipitated with an anti-p47<sup>phox</sup> antibody and analyzed by immunoblotting with antibodies against p47<sup>phox</sup>, gp91<sup>phox</sup> and phospho-serine. The total p47<sup>phox</sup> level indicates protein-loading control. (h) The graph represents phospho-serine and gp91<sup>phox</sup> levels expressed relative to the total p47<sup>phox</sup>. Means  $\pm$  s.e.m. of four samples. \* $P < 0.01$ , \*\* $P < 0.001$ , compared with PBS; # $P < 0.01$ , ## $P < 0.001$ , compared with MPTP only; & $P < 0.01$ , && $P < 0.001$ , compared with MPTP and CAP. (i–n) The SN tissues obtained from the same animals used in a–f were prepared for 8-OHdG immunostaining to detect oxidative damage in the SN. Control (i), CAP (0.5 mg kg<sup>-1</sup>) alone (j), MPTP (k), MPTP+CAP (l), MPTP+CAP+CZP (m) or MPTP+CAP+I-RTX (n). (o) Immunoreactivity of 8-OHdG in the SN was measured using Image J.  $n = 5–7$ . Note that CAP prevented MPTP-induced DNA damage in the SN, which was reversed by the TRPV1 antagonists (CZP or I-RTX). C, Control; CAP, capsaicin; M, MPTP; MC, MPTP+CAP; MCC, MPTP+CAP+CZP. \*\*\* $P < 0.001$ , significantly different from controls; ### $P < 0.001$ , significantly different from MPTP only, &&& $P < 0.001$ , significantly different from MC (analysis of variance and Student–Newman–Keuls analysis).

gulated the levels of both p47<sup>phox</sup> phosphorylation and p47<sup>phox</sup>-gp91<sup>phox</sup> complexes in the SN compared with PBS-treated controls (Figure 4g and h), indicating the activation of NADPH oxidase. In the CAP-treated MPTP-lesioned SN, the levels of both p47<sup>phox</sup> phosphorylation and the p47<sup>phox</sup>-gp91<sup>phox</sup> complex were significantly decreased compared with the levels in the SN of mice treated with MPTP alone (Figure 4g and h). These effects of CAP were reversed by CZP (Figure 4g and h). CAP alone did not affect the levels of phospho-p47<sup>phox</sup> or p47<sup>phox</sup>-gp91<sup>phox</sup> complex formation (data not shown).

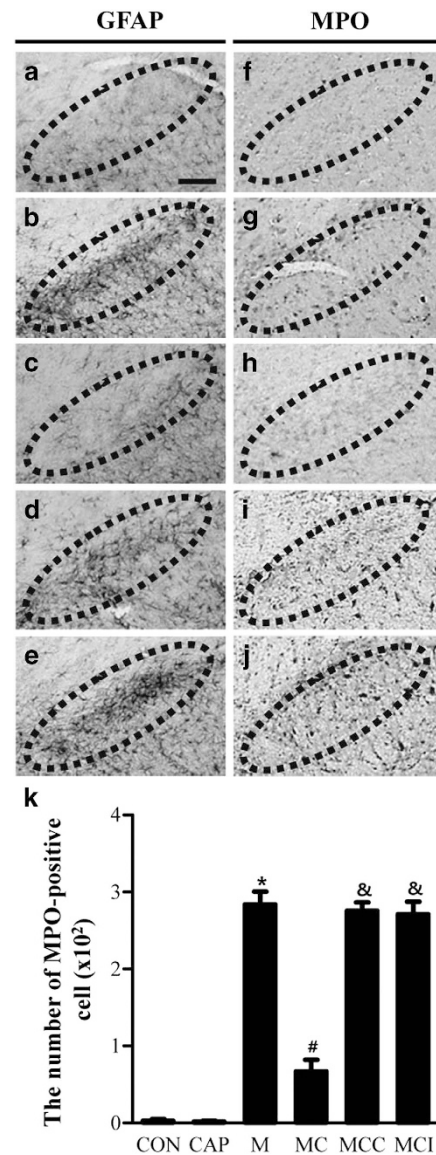
To explore CAP effects on oxidative stress to DNA in the MPTP-lesioned SN *in vivo*, we measured the levels of 8-OHdG, a marker of oxidative nucleic acid damage<sup>21,22</sup> using immunostaining (Figure 4a–o). 8-OHdG-immunopositive cells were significantly increased in the SN at 3 days post MPTP ( $P < 0.001$ ; Figure 4k) compared with the PBS-treated SN (Figure 4i). In the CAP-treated MPTP-lesioned SN, the oxidative DNA damage was markedly attenuated ( $P < 0.001$ ; Figure 4l), which was reversed by the TRPV1 antagonists, CZP ( $P < 0.001$ ; Figure 4m) and I-RTX ( $P < 0.001$ ; Figure 4n), indicating TRPV1 involvement. As controls, CAP alone did not affect DNA damage (Figure 4j).

#### CAP inhibits astroglial MPO expression in the MPTP-lesioned SN *in vivo*

We recently reported that MPTP induced MPO expression in astrocytes in the SN *in vivo*.<sup>21,22</sup> Given the reduced MPTP neurotoxicity in MPO-deficient mice,<sup>4</sup> we wondered whether CAP could exert neuroprotection by modulating astroglial activation and MPO expression in the MPTP-lesioned SN *in vivo*. The immunohistochemical analysis revealed a significant increase in the expression of GFAP-labeled reactive astrocytes in the MPTP-lesioned SN (Figure 5b) compared with the PBS-treated SN (Figure 5a). In the CAP-treated MPTP-lesioned SN, few GFAP-labeled reactive astrocytes were expressed (Figure 5c). These effects of CAP were reversed by CZP (Figure 5d) and I-RTX (Figure 5e). In line with our recent findings,<sup>21,22</sup> immunohistochemical staining showed a significant increase in the expression of MPO-positive cells in the MPTP-lesioned SN (Figure 5g) compared with the PBS-treated control SN (Figure 5f). In the CAP-treated MPTP-lesioned SN, few MPO-positive cells were expressed (Figure 5h). Stereological counting of MPO-positive cells in the SN supported these results, revealing 279-fold increases in the number of MPO-positive cells in the MPTP-lesioned SN compared with the PBS-treated control SN (Figure 5k). CAP significantly decreased the number of MPO-positive cells by 65% ( $P < 0.01$ ; Figure 5k), which was reversed by CZP and I-RTX (Figure 5i–j).

#### DISCUSSION

In the present study, we demonstrated the *in vivo* effects of CAP in the MPTP mouse model of PD. TRPV1 activation by CAP inhibits brain inflammation and oxidative stress, which rescues DA neurons and improves motor deficits in the MPTP



**Figure 5** Capsaicin (CAP) inhibits 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced astroglial activation and the expression of MPO in the substantia nigra (SN) *in vivo*. The SN tissues obtained from the same animals as used in Figure 3 were immunostained with a GFAP antibody for astrocytes (a–e) and an MPO antibody for myeloperoxidase immunoreactivity (f–j). Animals that received PBS as a control (a, f), MPTP (b, g), MPTP+CAP (c, h), MPTP+CAP+CZP (d, i) or MPTP+CAP+I-RTX (e, j) were killed 3 days after the last MPTP injection. Dotted lines indicate the SNpc. (k) The number of MPO-positive cells in the SN was counted.  $n = 5–7$ . C, control; CAP, capsaicin; M, MPTP; MC, MPTP and CAP; MCC, MPTP and CAP and CZP; MCI, MPTP and CAP and I-RTX. \* $P < 0.01$ , significantly different from controls; # $P < 0.01$ , significantly different from MPTP only, & $P < 0.01$ , significantly different from MC (analysis of variance and Student–Newman–Keuls analysis).

mouse model of PD. Accompanying neuroprotection and behavioral recovery, CAP not only inhibited NADPH oxidase and astroglial MPO but it also decreased ROS/RNS production and oxidative damage to DNA. The beneficial effects of CAP

were reversed by the TRPV1 antagonists, CZP and/or I-RTX, providing evidence for the involvement of TRPV1 in CAP neuroprotective events.

Glial cells comprising microglia and astrocytes are generally beneficial to neuronal functions in the brain. However, under neuropathological conditions, reactive glial cells are the primary producers of several potentially neurotoxic factors, including ROS/RNS and/or proinflammatory cytokines,<sup>4,7,22,29,30</sup> ultimately causing neurodegeneration.

Reactive microglia can cause degeneration of DA neurons in the SN during inflammation by producing NADPH oxidase-derived ROS.<sup>31,32</sup> Many clinical and experimental data have demonstrated the existence of reactive microglia and increased ROS generation by activated NADPH oxidase originating from reactive microglia in the SN of PD patients<sup>33,34</sup> and in the SN of the MPTP mouse PD model.<sup>21,22,35</sup> NADPH oxidase is an enzyme that catalyzes ROS production and consists of the cytosolic component p47phox and the membrane component gp91phox.<sup>36,37</sup> NADPH oxidase activation can be assessed by the increased levels of both p47phox phosphorylation and p47phox-gp91phox complexes,<sup>38</sup> which are the consequences of upregulated ROS.<sup>6,7,21</sup> In the SN of both PD patients and MPTP-treated PD model mice, the levels of oxidative damage to DNA are significantly increased.<sup>21,39</sup> Similar to our recent data,<sup>22</sup> the activation of NADPH oxidase was evident in the MPTP-treated SN, which leads to increased ROS production and results in DNA damage, as visualized by hydroethidine staining and 8-OHdG immunostaining in the SN, respectively.<sup>20–22</sup> In CAP-treated MPTP-lesioned SN, there was a decrease in NADPH oxidase activation, ROS production and oxidative damage on nucleic acid, which could be reversed by TRPV1 receptor antagonists. Our results collectively suggest that CAP contributes to neuroprotection in the MPTP-treated SN by blocking activation of NADPH oxidase and oxidative damage via TRPV1.

Microglia-derived inflammatory factors may trigger death of nigrostriatal DA neurons. Among such inflammatory factors capable of inducing neurodegeneration are iNOS<sup>8</sup> and proinflammatory cytokines, including TNF- $\alpha$  and IL-1 $\beta$ .<sup>40</sup> These inflammatory mediators are increased in the brains of PD patients and are responsible for the degeneration of DA neurons in the MPTP-treated SN.<sup>21,22</sup> The present results demonstrate that in the MPTP-treated SN, the expression levels of iNOS, TNF- $\alpha$  and IL-1 $\beta$  messenger RNA are increased. In the CAP-treated MPTP-lesioned SN, however, the expression of these three molecules was attenuated, which could be reversed by TRPV1 antagonists, indicative of TRPV1-induced neuroprotection. A recent report demonstrated that SA13353, a TRPV1 agonist, inhibited LPS-induced TNF- $\alpha$  and IL-1 $\beta$  production *in vivo* and reduced the production of inflammatory cytokines in an EAE mouse model.<sup>41</sup> Moreover, SA13353 prevented ischemic injury through suppression of TNF- $\alpha$  messenger RNA expression.<sup>42</sup> Collectively, these results suggest that TRPV1 activation by CAP rescues nigrostriatal DA neurons in the MPTP-lesioned SN by reducing MPTP-induced expression of iNOS and proinflammatory cytokines.

Reactive microglia are not the only harmful factor to DA neurons in the MPTP mouse model of PD. Astrocytes, when activated, produce neurotoxic factors and are detrimental to the survival of DA neurons.<sup>4,22,29,30</sup> Among them, reactive astrocyte-derived MPO is a significant source of cytotoxic ROS/RNS, which cause death of DA neurons in the MPTP-lesioned SN.<sup>21,22</sup> This can be observed in the SN of PD patients and of the MPTP mouse model of PD.<sup>4</sup> Alternatively, MPO secretion can cause damage to nigral DA neurons via microglial activation.<sup>43</sup> There was a significant increase in the expression of both MPO+ and GFAP+ cells in the MPTP-treated as analyzed by immunostaining. In the CAP-treated MPTP-lesioned SN, the number of MPO+ cells was significantly decreased, which could be reversed by TRPV1 antagonists. The current findings suggest that TRPV1 activation by CAP rescues DA neurons by inhibiting astroglial MPO expression and the resultant oxidative damage in the MPTP-lesioned SN.

In summary, the present study shows that TRPV1 activation by CAP suppresses glial-derived oxidative stress and rescues DA neurons in the MPTP-lesioned SN. Taken together with our recent data, we tentatively propose that TRPV1 may be a possible target for the treatment of PD associated with neuroinflammation and oxidative stress.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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