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LETTER TO THE EDITOR

Dopamine neuron degeneration induced by MPP^+ is independent of CED-4 pathway in *Caenorhabditis elegans*

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Dear Editor,

Parkinson's disease (PD) is a common neurodegenerative disease characterized by progressive loss of dopamine neurons in the substantia nigra, and manifests the cardinal clinical symptoms of resting tremors, rigidity, bradykinesia, hyperkinesias and abnormal posture. In the 1980s a synthetic heroin contaminant, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), was discovered as a cause of parkinsonism in a group of drug addicts. Since then, mechanisms involved in 1-methyl-4-phenylpyridinium (MPP^+)-induced neurotoxicity have been extensively studied in various animal models and cell cultures. MPTP is highly lipophilic and can easily cross the blood-brain barrier and cell membrane. MPTP is metabolized in glial cells by monoamine oxidase type B to its active ionic metabolite MPP^+, which is then selectively taken up by dopamine neurons via dopamine transporter (DAT). Hitherto, it has not been clearly defined whether the cell death induced by MPP^+ is apoptotic and/or necrotic. Contradictory results have been obtained from the treatment with different doses of MPP^+ in different culture systems [1-4]. This study, therefore, aims at determining the cell death mechanisms underlying MPP^+-induced dopamine neuron degeneration *in vivo* in a living animal model of *Caenorhabditis elegans*.

C. elegans hermaphrodite contains eight dopamine neurons: four symmetrically arranged cephalic cells (CEPs, two dorsal and two ventral), two bilateral anterior deirids (ADEs) in the head, and two bilateral posterior deirids (PDEs) near the vulva. The processes of dopamine neurons run through the body and tail. Since *C. elegans* is transparent, using the green fluorescent protein (GFP) attached to the DAT gene promoter (P*dat-1::GFP*) we can visualize the bodies and processes of dopamine neurons to study dopamine neuron degeneration *in vivo*. A number of mutant alleles related to apoptotic cell death and necrotic cell death genes are available in *C. elegans*, which afford us an opportunity to investigate the relationship between MPP^+-induced dopamine neuron degeneration and cell death pathways *in vivo* using the living animal model.

In order to investigate MPP^+-induced neurotoxicity in dopamine neurons of *C. elegans in vivo*, we synchronized P*dat-1::GFP* reporter strain BZ555 at L1 stage and incubated the worms in various concentrations of MPP^+ (from 0.25 to 1.0 mM) and observed the GFP-labeled dopamine neurons 24, 48, and 72 h after MPP^+ treatment. Dramatic changes of GFP fluorescence in dopamine neurons were detected in MPP^+-treated worms, but not in vehicle-treated worms. By 24 h after MPP^+ treatment, the GFP signal of CEP and ADE dendrites was significantly reduced or lost with retention of GFP in cell soma (Figure 1A-i, ii, iii). In addition, blebbing along the CEP and ADE processes and swelling in the dopamine cell bodies were observed in some worms (Figure 1A-ii). By 48 h, GFP was completely lost in a part of dopamine neurons. By 72 h after incubation with 0.5 mM MPP^+, we found that a few of the worms survived even though only one or two dopamine neurons were spared. Abnormal behaviors were detected in the MPP^+-treated worms. At the beginning of MPP^+ treatment, the worms displayed a slight increase of mobility, but later they showed a significant reduction of mobility and a significant increase of lethality (Figure 1A-iv). The loss of GFP in dopamine neurons was closely correlated with the reduction of mobility.

In order to elucidate whether MPP^+ is selectively taken up by DAT in *C. elegans*, we co-treated P*dat-1::GFP* reporter strain BZ555 with MPP^+ and the DAT inhibitor mazindol. Our result showed that exposure of worms to

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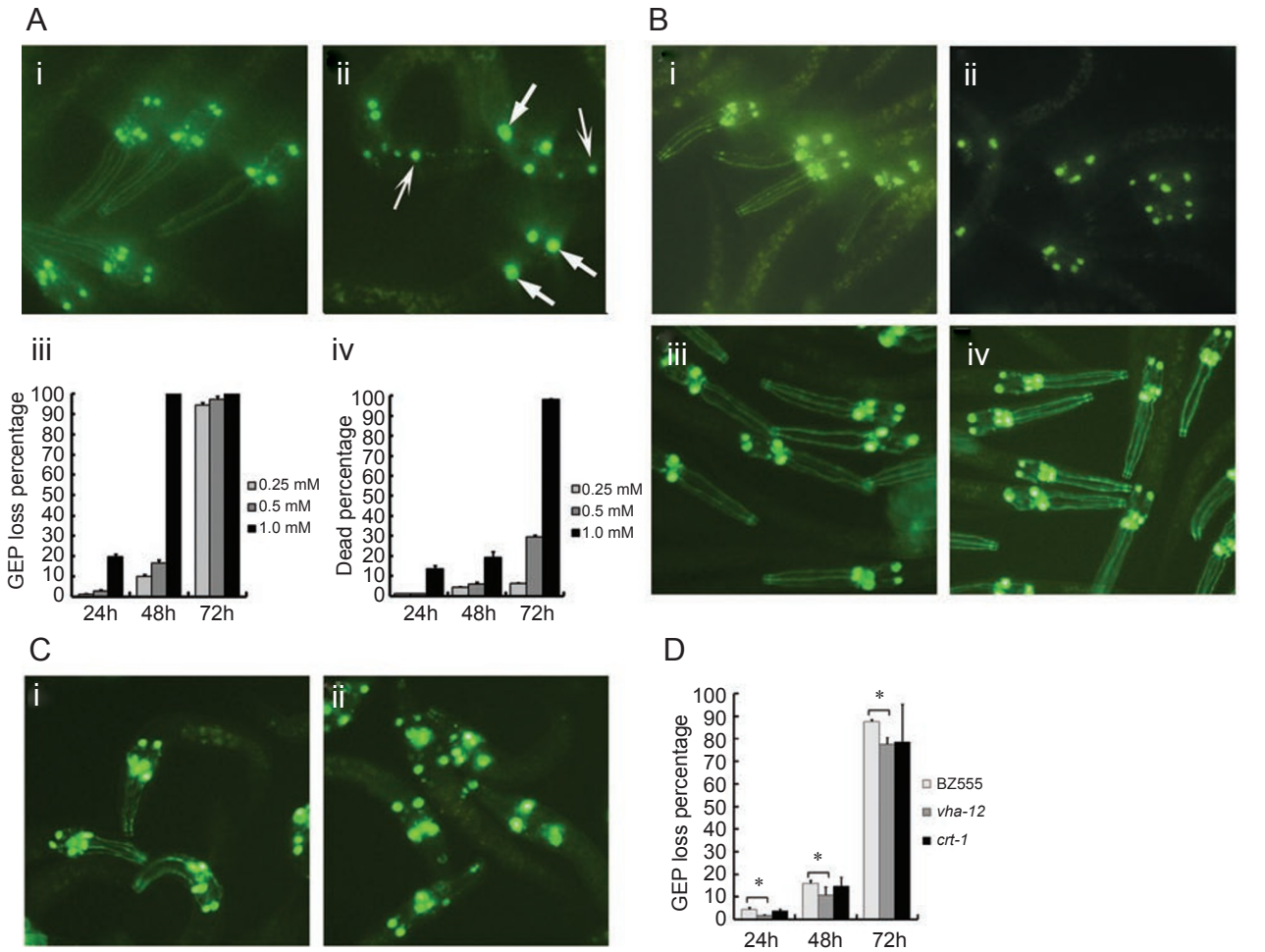

Figure 1 (A) The untreated BZ555 showed intact GFP in the dendrites of ADEs and CEPs **(A-i)**. After incubation in 1.0 mM MPP^+ for 24 h, BZ555 showed significant reduction or complete loss of GFP in the dendrites of ADEs and CEPs **(A-ii)**. Blebbings along the dendrites of CEPs were marked by thin arrows and the dopamine neurons with increased diameter were indicated by thick closed arrows **(A-ii)**. Percentage of worms showing GPF loss in the dendrites of ADEs and CEPs after various concentrations of MPP^+ treatment at different time points is presented. Error bars represent standard deviations **(A-iii)**. Dead rate of worms after various concentrations of MPP^+ treatment showed a dose-dependent manner at different time points **(A-iv)**. Data are depicted as means from three independent experiments with duplicate determinations (at least 200 animals each). Error bars represent standard deviations. **(B)** Coexposures of BZ555 to 0.5 mM MPP^+ and 0.1 mM mazindol for 48 h: mazindol could completely protect dopamine neurons from degeneration **(B-i)**. Exposure of BZ555 to 0.5 mM MPP^+ alone for 48 h **(B-ii)**. After incubation in 1.0 mM MPP^+ for 48 h, *dat-1* mutants showed intact GFP in the dendrites of ADEs and CEPs **(B-iii)**, which was indistinguishable from untreated *dat-1* mutants **(B-iv)**. **(C)** The untreated *ced-4* mutants showed intact GFP in the dendrites of ADEs and CEPs **(C-i)**. After incubation in 0.5 mM MPP^+ for 48 h, *ced-4* mutants showed significant reduction or complete loss of GFP in the dendrites of ADEs and CEPs **(C-ii)**. **(D)** Compared with the BZ555 control, *vha-12* mutants showed some resistance to MPP^+ (0.5 mM)-induced loss of GFP florescence in the dendrites of ADEs and CEPs, while *crt-1* mutants showed no difference (*P<0.05, *t* test). Data were depicted as means from three independent experiments with duplicate determinations (at least 200 animals each). Error bars represent standard deviations.

0.1 mM mazindol could completely protect dopamine neurons from 0.5 mM MPP^+-induced degeneration. GFP fluorescence remained intact in all worms, and there were no apparent behavioral defects regardless of the duration of MPP^+ treatment (up to 72 h) (Figure 1B-i, ii). Furthermore, we crossed BZ555 into *dat-1* (*ok157*) mutant worms and examined the fate of dopamine neurons in this mutant background after MPP^+ treatment. The *dat-1* (*ok157*) contains a deletion of 1 836 bp, resulting in a null mutation. We found that the dopamine neurons of *dat-1* (*ok157*) mutants were completely resistant to even higher concentrations of MPP^+ (up to 4.0 mM) (Figure 1B-iii) by showing intact GFP in the dendrites and cell bodies, which were indistinguishable from untreated *dat-1* (*ok157*) mutant worms (Figure

1B-iv). Thus, our *in vivo* studies demonstrated that either DAT inhibitor mazindol or *dat-1* (*ok157*) mutants could completely protect worms from MPP+-induced dopamine neuron degeneration, confirming that DAT-1 is necessary and indispensable for the uptake of MPP+ by dopamine neurons. Unexpectedly, although the dopamine neurons in the *dat-1* (*ok157*) mutants showed no obvious damage after MPP+ treatment, the *dat-1* (*ok157*) mutant worms still died. This result suggests that the toxicity of MPP+ is not restricted to dopamine neurons in *C. elegans* and MPP+-induced injury to other cells or tissues may lead to the death of worms. Non-neuronal monoamine transporters such as organic cation transporter OCT1, OCT2 and extraneuronal monoamine transporter EMT can efficiently transport MPP+ in mammals. OCT1 is confined to liver, kidney, and intestine; OCT2 has been detected in kidney and brain, while EMT has a broad tissue distribution [5]. *C. elegans* has the homologs of OCT1, OCT2, and EMT. So we can speculate that MPP+ is also taken up by these non-neuronal transporters other than DAT in *C. elegans*.

In *C. elegans* the programmed cell death pathway involves genes of *ced-9*, *ced-4*, *ced-3*, *egl-1*, and *ced-13,* which encode a Bcl-2-like cell death inhibitor, an Apaf-1-like adaptor protein, caspase, and two BH3-only proteins, respectively. CED-4 is normally localized to mitochondria, where it physically interacts with CED-9. When cells undergo programmed cell death, EGL-1 interacts with a protein complex composed of CED-9 and CED-4; then released CED-4 translocates to the nuclear membrane, where it activates CED-3, resulting in programmed cell death. Loss-of-function mutations in *egl-1*, *ced-4*, and *ced-3*, and a gain of function mutation in *ced-9*, can block most, if not all, programmed cell death that occurs during *C. elegans* development [6]. To investigate whether CED-4 is involved in MPP+-induced dopamine neuron degeneration, we crossed P*dat-1::GFP* reporter strain BZ555 into *ced-4* (*n1162*) mutants and examined the changes of dopamine neurons after MPP+ treatment. Loss-of-function mutations in *ced-4* could not block MPP+-induced dopamine neuron degeneration (Figure 1C). This result implies that CED-4 programmed cell death pathway is not required for the pathological cell death induced by MPP+ in *C. elegans*. Since the activation of CED-3 requires CED-4, CED-3 cannot be activated in *ced-4* (*n1162*) loss-of-function mutants. It has been shown that neither CED-3 nor its activator CED-4 appears to play a role in 6-OHDA-induced dopamine neuron degeneration. Our finding that loss-of-function of CED-4 did not block MPP+-induced dopamine neuron degeneration in *C. elegans* also indirectly implies that a caspase-independent cell death pathway may exist. It has been thought that inhibition of caspase is a potential therapeutic approach for PD. However, data from ours and others suggest that caspase inhibition is not sufficient to rescue dopamine neurons from cell death [1-4]. Once caspase pathway is inhibited, caspase-dependent cell death pathway may switch to caspase-independent cell death pathway.

In addition to the BH3-only protein EGL-1, a newly found BH3-only protein, CED-13, can also physically interact with CED-9 and might also promote programmed cell death. It is likely that EGL-1 and CED-13 may have the same function, but respond to different stimuli [7]. To determine whether CED-13 is involved in cell death induced by MPP+, we crossed P*dat-1::GFP* reporter strain BZ555 into *ced-13* (*gk260*) mutants and examined the changes of dopamine neurons after MPP+-treatment. We found that the *ced-13* mutation could not block or alleviate the MPP+-induced loss of GFP in the dendrites of ADEs and CEPs (see Supplementary information, Figure S1).

Up to now there are about 20 genes known to be associated with pathological cell death in *C. elegans,* but most of them are not expressed in dopamine neurons [8]. According to the gene expression patterns, *vha-12* and *crt-1* are most likely to be involved in dopamine neuron death [8]. It is known that the vacuolar H (+)-ATPase (*vha-12*), a pump that acidifies lysosomes and other intracellular organelles, is broadly required for necrosis caused by deleterious mutations in a diverse set of genes (*mec-4, deg-3*) and by hypoxic conditions in *C. elegans* [9]. Loss-of-function mutation in *crt-1*, a gene encoding calreticulin, partially suppresses necrotic cell death induced by *mec-4* and *deg-1* in *C. elegans* [10]. To clarify whether CRT-1 and/or VHA-12 are involved in MPP+-induced dopamine neuron degeneration, we crossed P*dat-1::GFP* reporter strain BZ555 into *vha-12* (*ok821*) mutants or *crt-1* (*ok938*) mutants and examined the fate of the dopamine neurons after MPP+ treatment. The *vha-12* mutants showed less vulnerability to low concentration (0.5 mM) of MPP+-induced loss of GFP in the dendrites of ADEs and CEPs as compared with the wild-type P*dat-1::GFP* reporter strain BZ555 (Figure 1D), suggesting that the necrosis machinery may contribute to MPP+-induced dopamine neuron degeneration in *C. elegans*. *vha-12* mutants showed no difference in vulnerability to higher concentrations of MPP+-induced loss of GFP in the dendrites of ADEs and CEPs (see Supplementary information, Figure S2). We did not achieve complete blockage of MPP+-induced dopamine neuron degeneration, which is consistent with the previous report showing that *vha-12* deficiency cannot completely block necrosis [9]. One of the core features of necrosis is a gain in cell volume (oncosis), while the main feature of apoptosis is the reduction of cell volume (pyknosis). We detected an increase in dopamine neuron diameter after MPP+ treatment (Figure 1A-ii), which supports our find-

ing that necrosis contributes to MPP^+-induced dopamine neuron degeneration in *C. elegans*. However, *crt-1* mutants showed no difference in vulnerability to MPP^+-induced loss of GFP in the dendrites of ADEs and CEPs (Figure 1D and see Supplementary information, Figure S2). A previous study has shown that loss-of-function mutation in *crt-1* could not suppress necrotic cell death induced by *deg-3* in *C. elegans* [10]. This result indicates *crt-1* may not play a critical role in necrotic cell death.

In conclusion, using the MPP^+-treated *C. elegans* model of PD we documented that mazindol could completely protect worms from MPP^+-induced dopamine neuron degeneration and that such MPP^+-induced injury was not restricted in dopamine neurons in *C. elegans*. Further, we demonstrated that inactivation of *ced-4* could not block MPP^+-induced dopamine neuron degeneration, indicating that a caspase-independent cell death pathway may exist in *C. elegans*. Moreover, we documented that loss-of-function *vha-12* mutant showed less vulnerability to MPP^+, suggesting that the necrosis machinery may play a role in MPP^+-induced dopamine neuron degeneration in *C. elegans*. Experimental materials and methods are depicted in the Supplementary information, Data S1.

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(**Supplementary information** is linked to the online version of the paper on the Cell Research website.)