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Protective effects of metallothionein against dopamine quinone-induced dopaminergic neurotoxicity

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Abstract **Dopamine (DA) quinone as DA neuron-specific oxidative stress conjugates with cysteine residues in functional proteins to form quinoproteins. Here, we examined the effects of cysteine-rich metal-binding proteins, metallothionein (MT)-1 and -2, on DA quinone-induced neurotoxicity. MT quenched DA semiquinones in vitro. In dopaminergic cells, DA exposure increased quinoproteins and decreased cell viability; these were ameliorated by pretreatment with MT-inducer zinc. Repeated L-DOPA administration markedly elevated striatal quinoprotein levels and reduced the DA nerve terminals specifically on the lesioned side in MT-knockout parkinsonian mice, but not in wild-type mice. Our results suggested that intrinsic MT protects against L-DOPA-induced DA quinone neurotoxicity in parkinsonian mice by its quinone-quenching property.**

Keywords: Dopamine quinone; Metallothionein; Parkinson's disease; Zinc

1. Introduction

Dopamine (DA) is stable in the synaptic vesicle under normal physiological conditions. However, damaged DA neurons may release excess cytosolic DA outside the synaptic vesicle, inducing neurotoxicity through the generation of reactive oxygen species (ROS) and reactive DA quinones [1]. DA quinones are cytotoxic through their interaction with the sulfhydryl group of cysteine amino acids in various bioactive molecules, resulting predominantly in the formation of 5-cysteinyl-DA [1,2]. Since cysteine sulfhydryl groups often form at the active site of functional proteins, covalent modification of cysteine residues by quinones to form 5-cysteinyl-DA irreversibly alters or inhibits protein function. Indeed, DA quinone covalently binds to key molecules in DA neurons including tyrosine hydroxylase (TH), DA transporter (DAT), and parkin protein, consequently inactivating those molecules [3–5]. The neurotoxicity of DA quinones formed via auto-oxidation or enzymatic oxidation has received attention recently as dopaminergic neuron-specific oxidative stress. In Parkinson's disease, L-DOPA therapy is a standard approach as it is designed to replenish the loss of DA from dopaminergic neurons. Despite the marked benefits of L-DOPA, long-term use may cause adverse effects, especially motor fluctuations including the wearing-off phenomenon and dyskinesia, as well as psychiatric symptoms [6]. In advanced Parkinson's disease, L-DOPA does not completely replenish DA because few dopaminergic neurons remain in the nigrostriatal pathway. The toxicity of L-DOPA and DA has been well documented. We and other groups demonstrated that L-DOPA itself is a potential L-DOPA quinone radical and that the repeated administration of L-DOPA increases lipid peroxidation in the striatum of parkinsonian mice [7–10]. Controversy remains, however, regarding the non-toxic or protective effects of L-DOPA in naive animals or moderately lesioned parkinsonian models [8,11].

DA-induced formation of DA quinones and the consequent dopaminergic cell damage in vitro and in vivo are successfully prevented by treatment with superoxide dismutase, glutathione (GSH), and some thiol reagents due to their quinone-quenching activity [3,12–16]. The sulfhydryl group of free cysteine in GSH and thiol reagents competes with the sulfhydryl group on cysteine in functional proteins bound by DA quinones. Metallothioneins (MTs) are a family of low molecular weight, cysteine-rich (30% of the protein), ubiquitous, and inducible intracellular proteins that bind to heavy metals such as zinc, copper, and cadmium, participating in metal homeostasis and detoxification [17]. The mammalian MT family comprises four isoforms: MT-1, MT-2, MT-3, and MT-4. While MT-3 is predominantly brain-specific, expressed in neurons and stimulated glial cells [18], the two major isoforms, MT-1 and -2, are expressed in most organs including the brain. MT-1 and -2 help to regulate metal homeostasis in the brain, as well as neural protective functions in various pathological and inflammatory conditions [17,19]. In fact, MT-1 is more abundantly expressed in astrocytes than MT-3 [17]. Attention has been focused on MTs as radical scavengers because of their abundant thiol groups, which participate exclusively in the formation of metal-thiolate clusters [17,20,21]. For example, MT-1 and -2 are considered by some investigators to have a therapeutic potential by providing neuroprotection [19,22]. MT-1 and -2 genes (but not MT-3 or -4) are highly inducible by several heavy metals such as zinc, cadmium, mercury, and copper, with zinc the most potent of these in vitro and in vivo [23,24]. This

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study examined the effects of MT on DA quinone-induced neurotoxicity using DA-treated dopaminergic cells and L-DOPA-injected MT-1- and 2-knockout or wild-type (WT) hemi-parkinsonian model mice.

2. Materials and methods

2.1. ESR spectrometry of semiquinone radicals from DA

The spectra of semiquinone radicals generated in vitro from DA were recorded with an electron spin resonance (ESR) spectrometer (JES-FR30, JEOL, Tokyo, Japan) using a flat quartz cuvette as reported previously [13]. DA was dissolved in 10 mM phosphate buffer (PB, pH 7.4), and the pH was adjusted to 7.0 by adding 0.1 M NaOH at 4 °C. For the preliminary experiment on time-dependency, the pH-adjusted DA (5 mM) was immediately incubated for 0.75–10 min at 37 °C, and the spectra for these combinations were analyzed. The relative peak height of the second signal of the semiquinone radical spin adduct compared to a manganese ion signal as internal standard was evaluated, since this is directly proportional to double integration of the spectra. For the simultaneous incubation of MT-1 recombinant protein (Sigma–Aldrich, St. Louis, MO), the pH-adjusted DA (5 mM, pH 7), with or without various concentrations of MT-1 (100 nM to 50 μM) dissolved in 10 mM PB, was immediately incubated for 1 min at 37 °C, and the spectra for these combinations were analyzed. The ESR spectrometer was set as follows to estimate semiquinone radicals, magnetic field, power, modulation frequency, modulation amplitude, response time, temperature, amplitude, and sweep time: 335.5 ± 5 mT, 4 mW, 9.41 GHz, 125 μT, 0.1 s, 25 °C, 1 × 1000, and 1 min, respectively.

2.2. Cell culture

Dopaminergic CATH.a cells (ATCC; #CRL-11179), derived from mouse DA-containing neurons, were cultured at 37 °C and 5% CO_2 in RPMI 1640 culture medium (Invitrogen, San Diego, CA) supplemented with 4% fetal bovine serum, 8% horse serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were seeded in 96-well culture plates to measure cell viability, and in 6-well plates for extraction of total RNA used for the measurement of MT-1 mRNA expression, and preparation of total cell lysates to measure protein-bound quinone, at a density of 1.0×10^5 cells/cm^2.

2.3. Metallothionein induction by zinc treatment in CATH.a cells

Expression of MT-1 mRNA in CATH.a cells was examined after treatment with $ZnCl_2$ (25–50 μM) for 16–40 h by RT-PCR using specific primer sets for MT-1 genes: 5′-TCACCAGATCTCGGAATGG-3′ (20 μM) as the upstream primer and 5′-CAGGGTGGAACTGTATAGGA-3′ (20 μM) as the downstream primer. After reverse transcription of extracted total RNA from CATH.a cells, PCR was performed under the following conditions: 95 °C for 1 min, 55 °C for 1 min, for 30 cycles. Each PCR reaction mixture was electrophoresed on a 2% agarose gel, and the MT-1 signals visualized by staining with ethidium bromide. β-Actin cDNA was simultaneously amplified as an internal control.

2.4. WST-1 assay for measurement of cell survival

CATH.a cells were exposed to 100–200 μM DA diluted in H_2O for 24 h with/without pretreatment with 30 μM $ZnCl_2$ for 24 h. Cell viability was determined by a colorimetric WST-1 assay (Wako Pure Chemical Industries, Osaka, Japan), which is a modification of the standard MTT assay [13].

2.5. Animal experiments

Homozygous MT-1-, -2-KO (MT KO) mice were purchased from Jackson Laboratories (Bar Harbor, ME). The 129/Sv mice were employed as WT controls, since the KO mice were raised on a 129/Sv genetic background. All animal procedures undertaken in this study accorded strictly with the Guidelines for Animal Experiments at Okayama University Medical School. Unilateral striatal lesions were generated by intrastriatal injections of 6-OHDA as reported previously [25]. Ten micrograms of 6-OHDA was injected at two sites in the right striatum of KO and WT mice (9–10 weeks old) at the following coordinates: A + 0.7 mm, L + 2.0 mm, V + 3.0 mm; A + 0.2 mm, L + 2.4 mm, and V + 3.0 mm from the bregma according to the atlas of the mouse brain [26]. Apomorphine-induced rotation tests were performed 2 weeks after the 6-OHDA-injection to confirm lesion induction. Mice that exhibited asymmetric rotation behavior towards the contralateral side of >50 turns every 10 min after apomorphine injection (0.5 mg/kg, s.c.) were selected for subsequent experiments. Two weeks after the apomorphine test, hemiparkinsonian mice were intraperitoneally injected with L-DOPA/carbidopa (50/5 mg/kg/day) suspended in 0.5% methylcellulose once a day for 7 days. One day after the final administration, animals were used to assay protein-bound quinone formation or for immunohistochemistry.

2.6. Measurement of protein-bound quinone (quinoprotein)

CATH.a cells were exposed to 100–150 μM DA for 24 h with/without pretreatment with 30 μM $ZnCl_2$ for 24 h before preparation of total cell lysates. Cell lysates were prepared with 10 μg/ml phenylmethylsulfonyl fluoride in ice-cold RIPA buffer [phosphate buffered saline; PBS (pH 7.4), 1% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate]. For hemiparkinsonian MT KO/WT mice injected with L-DOPA/carbidopa, the striatal tissue was homogenized with 50 mM PB (pH 7.4), and then the same volume of 10% trichloroacetic acid was added for protein precipitation. The precipitate centrifuged at 1000 × g for 10 min was washed twice with ethanol, further treated with chloroform-methanol (2:1, v/v), vortexed vigorously, and centrifuged at 5000 × g for 10 min. The delipidated protein precipitate was suspended with 2 M potassium glycinate (pH 10). For detection of protein-bound quinones (quinoprotein), the NBT/glycinate assay was performed on lysates as described previously [27]. Briefly, the protein sample was added to 500 μl of NBT reagent (0.24 mM NBT in 2 M potassium glycinate, pH 10) followed by incubation in the dark for 2 h on a shaker. Absorbances were measured at 530 nm.

2.7. Tissue preparation for immunohistochemistry

Mice were transcardinally perfused with saline followed by a fixative containing 4% paraformaldehyde and 0.35% glutaraldehyde in 0.1 M PB (pH 7.4) under sodium pentobarbital anesthesia (70 mg/kg, i.p.) 1 day after L-DOPA/carbidopa treatment of hemiparkinsonian MT KO/WT mice. The perfused brains were postfixed for 24 h in 4% paraformaldehyde and then cryoprotected in 15% sucrose in PB for about 48 h. Brain snap-frozen with powdered dry ice was cut coronally on a cryostat at levels containing the mid-striatum (+0.6 to +1.0 mm from the bregma) and the substantia nigra pars contacta (SNpc) (−2.8 to −3.0 mm from bregma) at 20-μm thickness.

2.8. Immunohistochemistry

TH-immunopositive cells in the SNpc and striatum, and DAT-immunopositive cells in the striatum were stained by standard free-floating immunohistochemistry. The sections were incubated with anti-TH rabbit polyclonal antibody (diluted 1:5000; Protos Biotech, New York, NY) or anti-DAT rat monoclonal antibody (diluted 1:1000; Chemicon International, Temecula, CA) for 18 h at 4 °C, followed by incubation for 2 h at room temperature with biotinylated goat anti-rabbit IgG secondary antibody for TH (diluted 1:1000; Vector Laboratories, Burlingame, CA) or biotinylated rabbit anti-rat IgG secondary antibody for DAT (diluted 1:1000; Vector Laboratories). Following washes in 10 mM PBS containing 0.2% Triton X-100, the sections were incubated with avidin–biotin peroxidase complex (diluted 1:2000, Vector Laboratories) for 1 h at room temperature. TH- or DAT-immunopositive cells were visualized by 3,3′-diaminobenzidine, nickel, and H_2O_2. TH-immunoreactive neurons in the SNpc were counted manually under a microscope at ×100 magnification using a superimposed grid. The boundary between the SNpc and ventral tegmental area was defined as a line extending dorsally from the most medial boundary of the cerebral peduncle. Counting was performed blindly. The relative density of TH- or DAT-positive cells over the entire area containing midstriatum was assessed microscopically at ×40 magnification using a Macintosh computer-based image analysis system (NIH Image J 1.37v).

2.9. Protein measurement

Protein concentration was determined by the Bio-Rad DC protein assay kit (Bio-Rad, Richmond, CA), based on the Lowry assay, using bovine serum albumin as a standard.

2.10. Statistical analysis

Results are presented as means ± S.E.M. Statistical significance was determined by one-way or two-way ANOVA followed by post hoc Fisher's PLSD test. A P value less than 0.05 denoted the presence of a statistically significant difference.

3. Results and discussion

3.1. Effects of MT-1 on DA-semiquinone formation in vitro

DA at neutral pH produced an ESR signal consistent with semiquinone radical formation, characterized by four waves (Fig. 1A). DA incubated at 37 °C started forming these DA-semiquinone radicals immediately within 1 min, at which time the signal peaked before gradually decreasing for 10 min (data not shown). The simultaneous incubation of MT-1 recombinant protein (100 nM to 50 μM) with DA at neutral pH (5 mM) for 1 min at 37 °C dose-dependently decreased the formation of DA-semiquinone radicals (Fig. 1A,B). We reported previously that DA agonist, pergolide, also scavenges DA semiquinones [25]. The scavenging effect of MT-1 on DA semiquinones was stronger than that of pergolide, with MT-1 active at lower concentrations (1–50 μM) than pergolide (100 μM–2 mM). We also examined the effects of MT-1 on the generated DA-semiquinone radicals. Following an advance 1-min incubation of DA at 37 °C, MT-1 was added and incubated for a further 1 min at 37 °C. The postincubation of MT-1 also decreased the signal intensity of the DA-semiquinone radicals generated (data not shown), indicating a possible direct interaction of MT-1 with the DA semiquinones, probably through its abundant cysteine residues.

3.2. Effects of zinc on MT-1 mRNA expression and DA-induced neurotoxicity in CATH.a cells

DA exposure (100–200 μM) for 24 h dose-dependently induced cell death in CATH.a cells (Fig. 2B). Levels of quinoprotein formation also increased with DA treatment (100, 150 μM) for 24 h (Fig. 2C), coinciding with cell toxicity (Fig. 2B). Zinc induces MT-1 and -2 mRNA and protein expression under various conditions [22,28]. To examine whether MT-1 could protect against DA-induced neurotoxicity, we first confirmed the dose-dependent induction of MT-1 mRNA by treatment with $ZnCl_2$ (25–50 μM) for 16–24 h in CATH.a cells (Fig. 2A). Since zinc itself is toxic for CATH.a cells at high doses, we used $ZnCl_2$ at a dose of 30 μM to induce MT-1 in the subsequent experiments on DA-induced cell damage. Pretreatment of CATH.a cells with this dose significantly reduced DA-induced neurotoxicity (Fig. 2B) and blocked DA-induced elevation of quinoprotein levels (Fig. 2C).

3.3. Involvement of MT in L-DOPA-induced quinoprotein formation in hemiparkinsonian mice

We reported previously that repeated L-DOPA administration markedly increased DA-quinone formation in the striatum of hemiparkinsonian mice [25]. To clarify the involvement of MT-1 and -2 in quinoprotein formation in vivo, we examined the striatal quinoprotein levels in hemiparkinsonian mice using L-DOPA-treated MT KO and WT mice. Repeated L-DOPA administration (50 mg/kg/day for 7 days), which increases cytosolic free DA [7,25], resulted in marked elevation of striatal quinoprotein levels specifically in the ipsilateral striatum of MT KO mice (Fig. 3). In contrast,

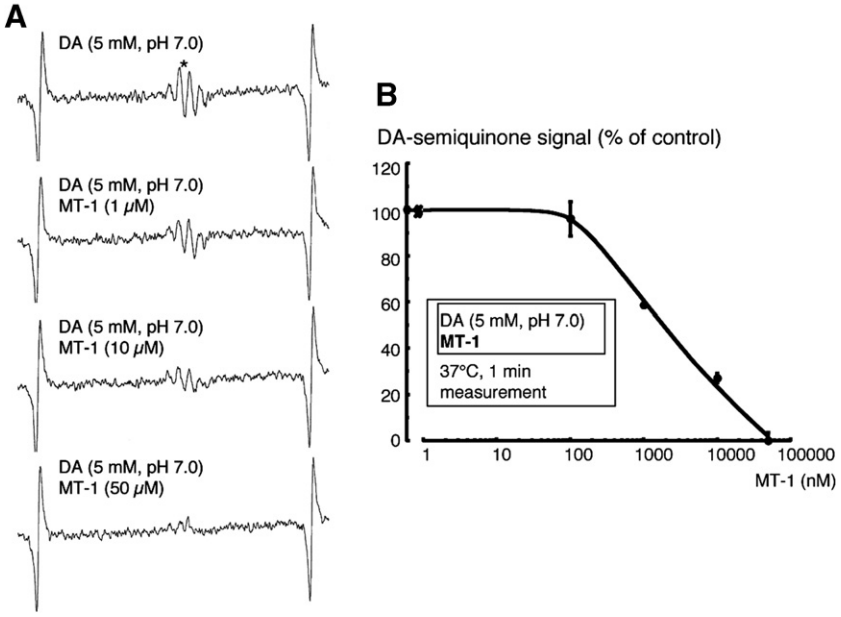

Fig. 1. Effect of MT-1 on DA-semiquinone formation in vitro. (A) Representative ESR spectra of DA-semiquinone radicals following DA incubation at neutral pH (5 mM) for 1 min at 37 °C with/without MT-1 (1, 10, or 50 μM). (B) Effects of simultaneous incubation of MT-1 on DA-semiquinone radical formation in vitro. DA at neutral pH (5 mM) was simultaneously incubated with MT-1 (100 nM to 50 μM) for 1 min at 37 °C, and then the relative signal intensity of DA-semiquinone radicals was measured by ESR spectrometry. The signal intensity was evaluated from the peak height of the second signal of the semiquinone radical spin adduct (*) relative to the height of the MnO signal. Each value represents the means ± S.E.M. expressed as a percentage of the signal intensity for DA-semiquinone radicals in four independent experiments.

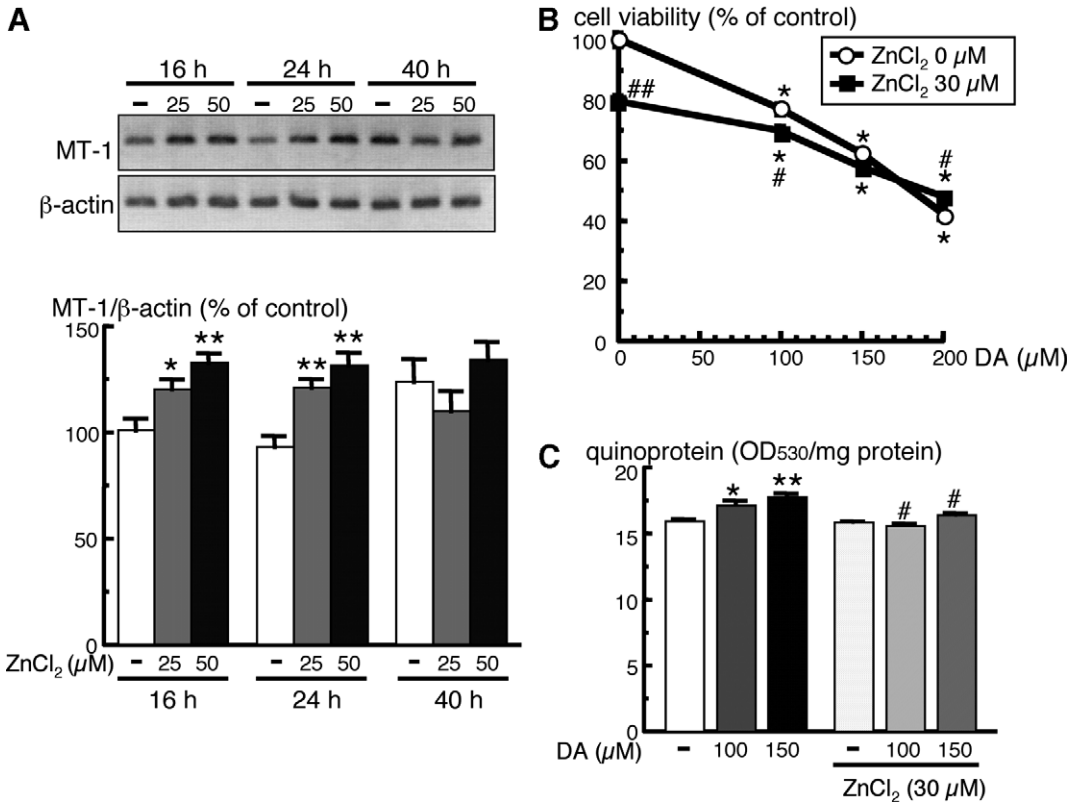

Fig. 2. Effects of $ZnCl_2$ on MT-1 mRNA expression, DA-induced neurotoxicity, and quinoprotein formation in CATH.a cells. Semiquantitative analysis of MT-1 mRNA expression by RT-PCR (A). CATH.a cells were pretreated with 25–50 μM $ZnCl_2$ for 16–24 h. Each value is expressed as the means ± S.E.M. of the band-intensity ratios corresponding to MT-1 mRNA and β-actin mRNA ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.001$ vs. untreated control group. Neuroprotective effects of $ZnCl_2$ pretreatment against DA-induced neurotoxicity (B). CATH.a cells were pretreated with 30 μM $ZnCl_2$ for 24 h and subsequently treated with 100–200 μM DA for 24 h. The cell viability was measured by WST-1 assay. Each value is expressed as the means ± S.E.M. ($n = 6$) expressed as a percentage of each control. $^{*}P < 0.001$ vs. each control group without DA treatment, $^{\#}P < 0.01$, $^{\#\#}P < 0.001$ vs. DA dose-matched controls without $ZnCl_2$. Effects of $ZnCl_2$ on DA-induced quinoprotein formation (C). CATH.a cells were pretreated with 30 μM $ZnCl_2$ for 24 h and subsequently treated with 100–150 μM DA for 24 h. Each value is expressed as the mean ± S.E.M. of OD 530/mg protein ($n = 6$). $^{*}P < 0.01$, $^{**}P < 0.001$ vs. control group without DA treatment, $^{\#}P < 0.001$ vs. DA dose-matched controls without $ZnCl_2$.

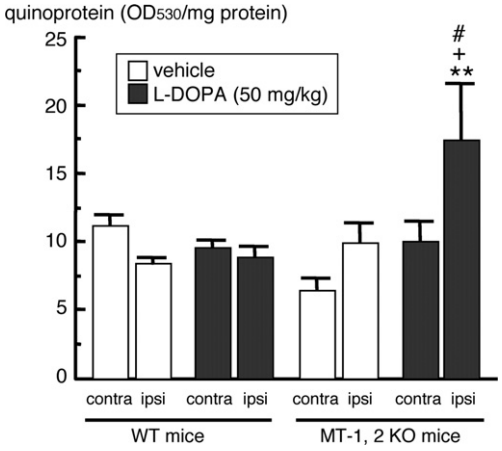

Fig. 3. Effects of repeated administration of L-DOPA/carbidopa (50/5 mg/kg/day) for 7 days on the levels of quinoprotein formation on the non-lesioned control side (contra) and 6-OHDA-lesioned parkinsonian side (ipsi) of the striatum in hemi-parkinsonian WT and MT-1, -2-KO mice. Each value is the mean ± S.E.M. of 4–6 animals. $^{**}P < 0.001$ vs. control side (contra) of each group. $^{+}P < 0.01$ vs. the same side of the vehicle-treated group. $^{\#}P < 0.01$ vs. each treated WT mice.

quinoprotein levels in the striatum of WT mice with/without L-DOPA administration were unchanged (Fig. 3). These results indicate that MT proteins protect against excess DA-induced quinone formation in the lesioned striatum. The abundant cysteines in MTs [17] possibly compete with quinoprotein formation by binding to the DA quinones. Cysteine-rich protein parkin is also susceptible to DA-induced solubility alterations [29]. Therefore, the protective effects of MTs against DA quinone-induced toxicity in vitro and in vivo could reflect the ability to bind and scavenge DA semiquinones via their cysteine residues.

The quinoprotein formation was markedly elevated specifically in the parkinsonian striatum after repeated L-DOPA administrations in our previous study [25]. However, the quinoprotein levels were unchanged in the striatum of WT 129/Sv mice with/without L-DOPA administration in the present study. This discrepancy may be due to differences in mouse strains between the tyrosinase-null albino ICR mice used in our previous work and the tyrosinase-expressing 129/Sv used here, since tyrosinase is also protective against DA-quinone toxicity [30].

3.4. Aggravation of 6-OHDA-induced dopaminergic neurotoxicity in MT-1, -2-KO mice brain

Based on the L-DOPA-induced increase in quinoprotein levels in the 6-OHDA-injected striatum of MT KO mice, we

examined dopaminergic neurotoxicity in hemiparkinsonian MT KO and WT mice that were repeatedly injected with L-DOPA. WT mice showed a significant decrease in the relative density of striatal TH-immunoreactivity on the 6-OHDA-injected ipsilateral side, and L-DOPA administration had no additional effect. In contrast, the TH-immunopositive signal was markedly reduced on the lesioned side in the striatum of MT KO mice following repeated L-DOPA administrations (Fig. 4A,B), corresponding to quinoprotein formation (Fig. 3). We also evaluated changes in DAT in the striatum and dopaminergic neuronal loss in the SNpc of both WT and MT KO mice. The relative density of striatal DAT immunoreactivity was significantly decreased on the 6-OHDA-injected side in all mice (Fig. 4C,D), as was the case for nigral TH-immunopositive dopaminergic neurons on the lesioned side (Fig. 4E,F).

In our previous study, 6-OHDA-induced toxicity to DA neurons was more severe in the same MT KO mice compared with WT mice [31]. Several reports have suggested a neuroprotective action for MTs against treatment with various dopaminergic neurotoxins [28,32]. In addition, Xie et al. [22] showed expression of the MT-1 and -2 genes following MDMA treatment and that overexpression of MT protein could protect against MDMA-induced toxicity to DA neurons in mice. Together with previous reports, our present data indicate that endogenous MT proteins provide protection against the long-term L-DOPA treatment-induced DA quinone neurotoxicity in parkinsonian models by quenching the DA quinones. Dopaminergic neurotoxicity by 6-OHDA injection was observed on the lesioned side of both WT mice and MT KO mice, although quinoprotein formation was markedly elevated specifically on the parkinsonian side of only the L-DOPA-treated MT KO mice. This discrepancy may indicate that 6-OHDA produces dopaminergic neurotoxicity not only through the production of DA quinones, but also by generating ROS such as superoxide anions from the toxin. Interestingly, even on the control side of the MT KO mice, repeated L-DOPA administrations significantly reduced the striatal TH- and DAT-immunopositive signals and the nigral TH-immunopositive neurons (Fig. 4). These results suggested that deficiencies in MT-1 and

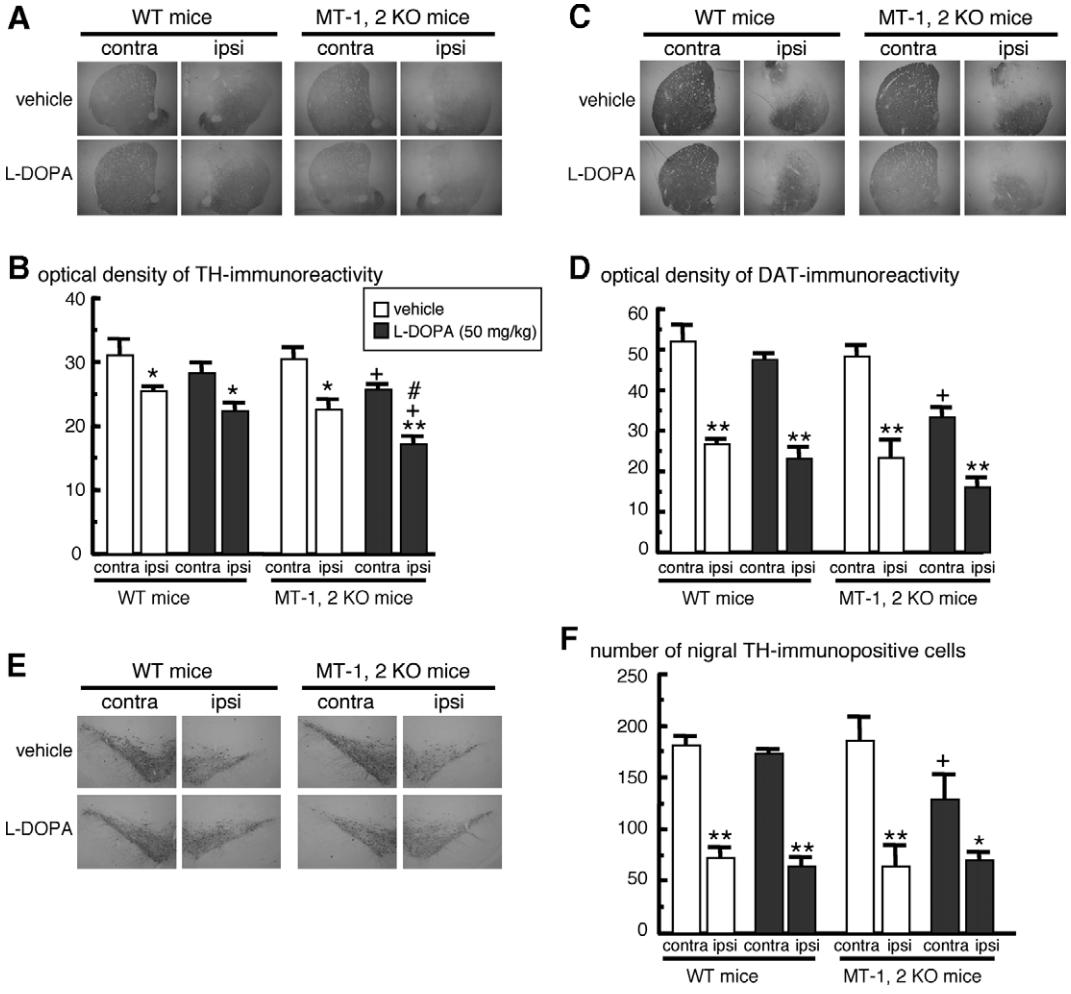

Fig. 4. Dopaminergic neurotoxicity in hemiparkinsonian WT and MT-1, -2-KO mice after repeated L-DOPA/carbidopa administration (50/5 mg/kg/day) for 7 days. Representative TH- (A) and DAT-immunostaining (C) in striatal sections, as well as TH-immunoreactive neurons in the SNpc (E) of WT (129/Sv) and MT-1, -2-KO mice. Quantification of the relative density of TH-immunopositive signals (B), DAT-immunopositive signals (D) in the striatum, and the number of TH-immunopositive cells in the SNpc (F) of WT and MT-1, -2-KO mice. Each value is expressed as the mean ± S.E.M. of optical density or the number of TH-positive cells of 5 animals per group. $^{*}P < 0.05$ and $^{**}P < 0.001$ vs. control side (contra) of each group. $^{+}P < 0.01$ vs. the same side of the vehicle-treated group. $^{\#}P < 0.01$ vs. each treated WT mice.

-2 render dopaminergic neurons more vulnerable to L-DOPA toxicity.

In conclusion, this study implicates DA-quinone neurotoxicity caused by MT dysfunction in the pathogenesis and/or progression of Parkinson's disease and other dopaminergic neurodegenerative conditions.

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