Chapter 12
Therapeutic Activities of DJ-1 and Its Binding Compounds Against Neurodegenerative Diseases

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Abstract Parkinson's disease (PD) is a progressive neurodegenerative disorder that is primarily characterized by the degeneration of dopaminergic neurons in the nigrostriatal pathway. Loss-of-function mutations in the gene encoding PARK7/DJ-1 were identified in familial PD. Wild-type DJ-1 acts as an oxidative stress sensor in neural cells. Previously, we identified binding compounds of DJ-1, including UCP0045037/compound A, UCP0054278/compound B, and compound-23 (comp-23), by in silico virtual screening. These compounds prevented oxidative stress-induced dopaminergic neuronal death and restored locomotion defects in animal models of PD. In addition, these binding partners reduced infarct size in cerebral ischemia in rats. The neuroprotective effects of these compounds are lost in *DJ-1*-knockdown cells and *DJ*-1-knockout animal. These results suggest that these compounds interact with endogenous DJ-1 and then produce antioxidant and neuroprotective responses in both animal models for PD and cerebral ischemia in rats. This raises the possibility that interaction partners of DJ-1, such as UCP0045037, UCP0054278, and comp-23, may represent a novel dopaminergic neuroprotective drug for the treatment of PD.

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12.1 Introduction

12.1.1 DJ-1

DJ-1 (also known as PARK7) was first discovered as a novel oncogene (Nagakubo et al. 1997) and was later identified as a causative gene of Parkinson's disease (PD) (Bonifati et al. 2003). DJ-1 has multiple functions and plays a role in anti-oxidative stress and transcriptional regulation (Takahashi et al. 2001; Niki et al. 2003; Yokota et al. 2003; Taira et al. 2005; Canet-Aviles et al. 2004; Shendelman et al. 2004; Martinat et al. 2004; Shinbo et al. 2005, 2006; Sekito et al. 2006; Xu et al. 2005; Fan et al. 2008; Ishikawa et al. 2009, 2010). Wild-type DJ-1 plays a key role in antioxidation and neuroprotection in neuronal cells, and mutations in the *DJ-1* gene cause loss of function (Taira et al. 2005; Inden et al. 2006; Yanagisawa et al. 2008; Miyazaki et al. 2008; Yanagida et al. 2009a). We have previously demonstrated that DJ-1 administration reduced dopaminergic neuronal death and restored locomotor function in a rat model of PD (Inden et al., 2006). In addition, we have reported that intrastriatal injection of DJ-1 reduced infarct size in cerebral ischemia in rats (Yanagisawa et al. 2008). These findings suggest that DJ-1 is a pharmaceutical target for PD and cerebral ischemia.

Wild-type DJ-1 has three cysteine residues at amino acid numbers 46, 53, and 106 (C46, C53, and C106) in humans and rats (Kinumi et al. 2004) (Fig. 12.1a). A cysteine residue is oxidized from its reduced form (−SH) for sulfenation (−SOH), sulfination ($-SO_2H$), and sulfonation ($-SO_3H$). Among these three cysteine residues, C106 is the most sensitive to oxidative stress (Kinumi et al. 2004). Previously, we identified UCP0045037/compound A and UCP0054278/compound B as interaction partners of the reduced and the SO_2H-oxidized C106 region of DJ-1, respectively. These compounds were found by screening the university compound library, which contains approximately 30,000 compounds (Miyazaki et al. 2008). Like DJ-1, these compounds prevented oxidative stress-induced dopaminergic neuronal death and restored normal locomotor function in animal models of PD. In addition, they reduced infarct size in cerebral ischemia in rats (Miyazaki et al. 2008; Yamane et al. 2009; Yanagida et al. 2009b; Inden et al. 2011a). We further screened for DJ-1-binding compounds from a Zinc compound library that contains approximately 2,500,000 compounds (Kitamura et al. 2011). This screening identified compound-23 (comp-23), which protected oxidative stress-induced dopaminergic neuronal death in PD and ischemia models (Kitamura et al. 2011; Takahashi-Niki et al. 2015). These results suggest that DJ-1-binding compounds are fundamental drugs for PD therapy.

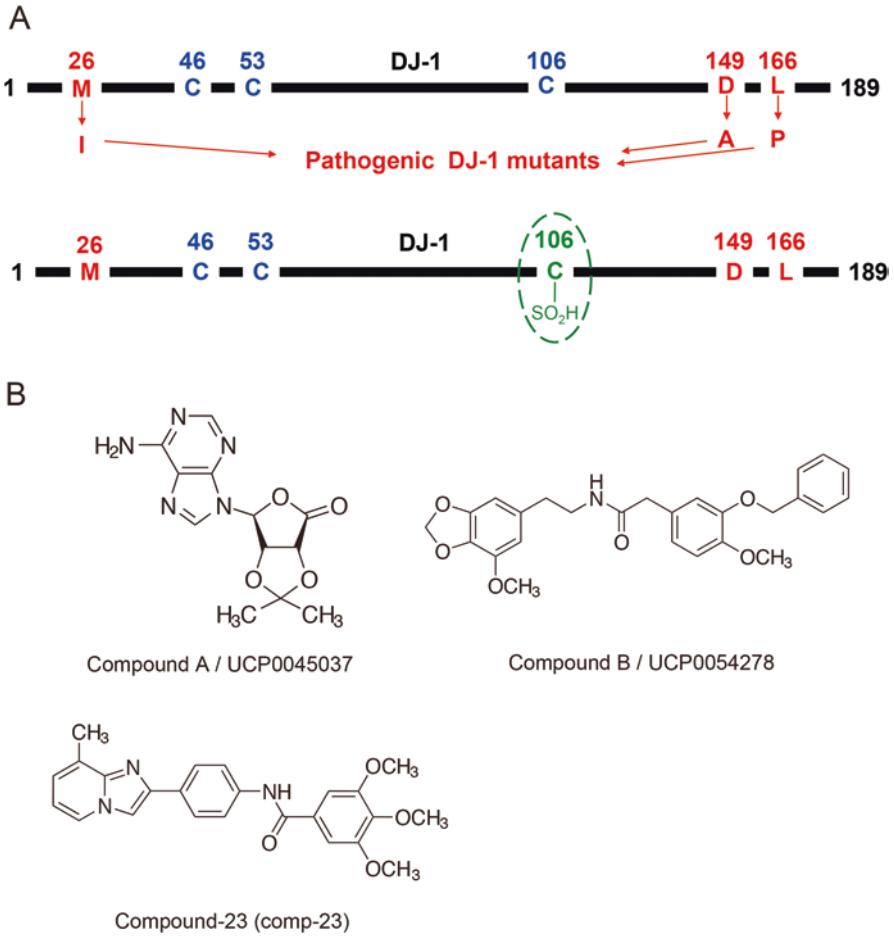

Fig. 12.1 Cysteine oxidation of DJ-1 and DJ-1-binding compounds. (**a**) DJ-1 contains three cysteine residues at amino acid numbers 46, 53, and 106 (C46, C53, and C106). (**b**) Chemical structures of the DJ-1-binding compounds UCP0045037/compound A, UCP0054278/compound B, and compound-23

12.2 DJ-1-Mediated Neuroprotection in PD Models

First, we examined the distribution of endogenous DJ-1 in rat brains treated with 6-hydroxydopamine (6-OHDA). 6-OHDA has long been used as an experimental model to study dopaminergic function in the brain and to evaluate the effects of drugs on central nervous system dopaminergic neurons (Inden et al. 2006). Endogenous expression of DJ-1 is much lower in the dopaminergic neurons of the substantia nigra after 6-OHDA treatment compared with that in glial cells. Therefore, we chose to study the protective effects of exogenous DJ-1 against 6-OHDA-induced parkinsonian in the substantia nigra. DJ-1 was introduced into the brain as

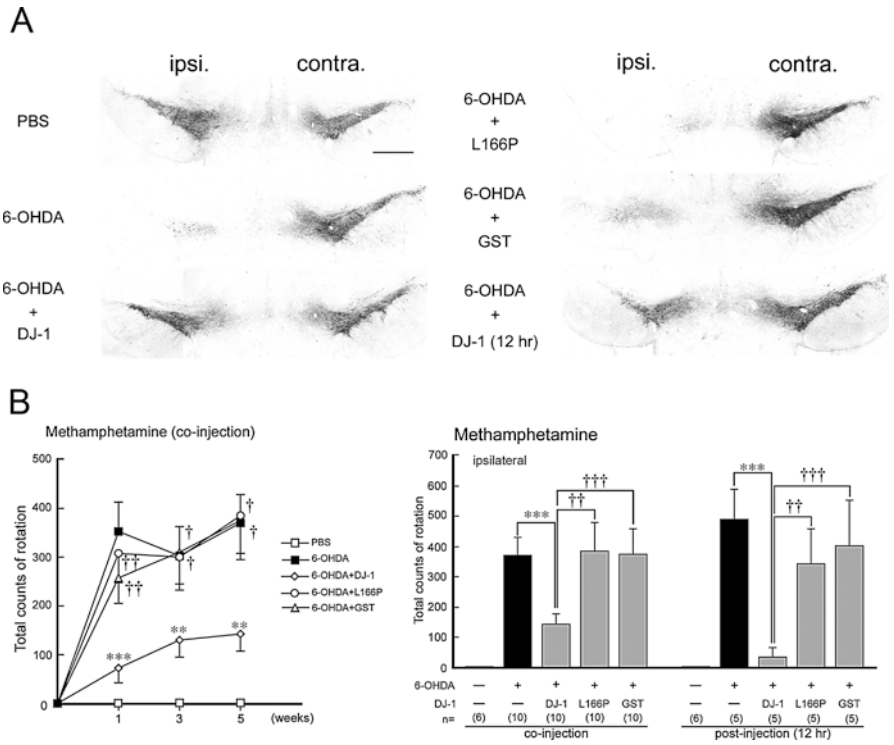

Fig. 12.2 Effect of DJ-1 in 6-OHDA-microinjected rats. (**a**) Changes in the numbers of TH-positive neurons in the substantia nigra. Midbrain slices were stained using an anti-TH antibody. Scale bar, 1 mm. (**b**) Changes in methamphetamine-induced rotational behavior in rats co- or post-treated with DJ-1 (Significance: $^{**}P < 0.01$, $^{***}P < 0.001$ vs. 6-OHDA injection alone. $\dagger P < 0.05$, $\dagger\dagger P < 0.01$, $\dagger\dagger\dagger P < 0.001$ vs. co- or post-administration of DJ-1)

recombinant glutathione S-transferase (GST)-tagged proteins, and GST was injected together with the corresponding vehicle control. Wild-type DJ-1 (DJ-1), GST-tagged mutant DJ-1 (L166P), and vehicle controls were microinjected into the substantia nigra together with 6-OHDA. Dopaminergic neurons were visualized by tyrosine hydroxylase (TH) staining (Fig. 12.2a). Quantification of TH staining revealed that 6-OHDA caused a significant loss of dopaminergic neurons in the substantia nigra and these neurons were preserved in DJ-1-treated rats. However, loss of dopaminergic neurons was not inhibited by the co-administration of L166P and GST. We also investigated whether DJ-1 could protect against 6-OHDA-induced neuronal loss 12 h after 6-OHDA injection. Administration of DJ-1 but not of L166P and GST at 12 h after 6-OHDA injection protected against 6-OHDA-induced loss of dopaminergic neurons in the substantia nigra.

Injection of dopamine-releasing methamphetamine into animals with 6-OHDA-induced unilateral lesions induced movement ipsilateral to the injection site. This rotational behavior was significantly reduced by co-administration of DJ-1 but not

of L166P or GST at 1, 3, and 5 weeks after injection (Fig. 12.2b). Further, we examined the effect of post-administration of DJ-1 in 6-OHDA-lesioned hemiparkinsonian rats. Methamphetamine-induced rotational behavior was significantly reduced by the administration of DJ-1 12 h after 6-OHDA injection.

12.3 Neuroprotective Effect of DJ-1 in Ischemia Models

Cerebral ischemia occurs as a result of a local reduction or arrest of blood supply and leads to neuronal cell death in the ischemic region. The pathophysiological mechanisms of ischemia/reperfusion injury are complex. Neurons and glial cells are lethally damaged by a number of events. These include the production of reactive oxygen species (ROS), which occurs immediately after ischemia/reperfusion, followed by more delayed postischemic inflammation and apoptosis. In addition, peri-infarct depolarization occurs within hours of ischemia, and together, these events contribute to the progression and expansion of brain injury. Controlling ROS formation is important for neuroprotection against ischemia/reperfusion injury (Yanagisawa et al. 2008). Three days after middle cerebral artery occlusion (MCAO), a regional loss of 2,3,5-triphenyltetrazolium chloride (TTC) staining was observed in the ipsilateral cerebral cortex and the striatum. This effect was reduced by the injection of DJ-1 110 min after the onset of MCAO (10 min before the reperfusion) (Fig. 12.3a) (Yanagisawa et al. 2008). Quantitative analysis showed that the infarct area was reduced in a dose-dependent manner by administration of DJ-1, and compared with the PBS- and GST-injected groups, the total infarct volume was significantly reduced by DJ-1 injection in a dose-dependent manner (Fig. 12.3b). Delayed administration of DJ-1 at 60 and 110 min after the onset of MCAO significantly reduced the infarct volume. However, the protective effect was lost when the injection was delayed for 180 min (Fig. 12.3c). After 180 min, the total infarct volume did not significantly change. To evaluate the effect of DJ-1 on behavioral function, MCAO or sham operations were performed in animals followed by injection with PBS, GST, or DJ-1 at 110 min after the onset of MCAO (Fig. 12.3d) (Yanagisawa et al. 2008). Neurological tests revealed improved functional outcomes in DJ-1-injected rats 1 day after MCAO. Improved functional outcomes continued in DJ-1-injected rats for 3, 4, 5, and 6 days after MCAO, compared with the PBS-injected group, and for 5 and 6 days after MCAO, compared with the GST-injected group. There were no differences in rotarod test performance between PBS-, GST-, and DJ-1-injected groups 1 day before MCAO (pre-performance). Two and 5 days after MCAO, the PBS- and GST-injected groups showed a marked reduction in rotarod performance, compared with pre-performance. These reductions were inhibited by DJ-1 administration, and there were significant improvements 2 and 5 days after MCAO, compared with PBS-injected and GST-injected animals (Fig. 12.3e).

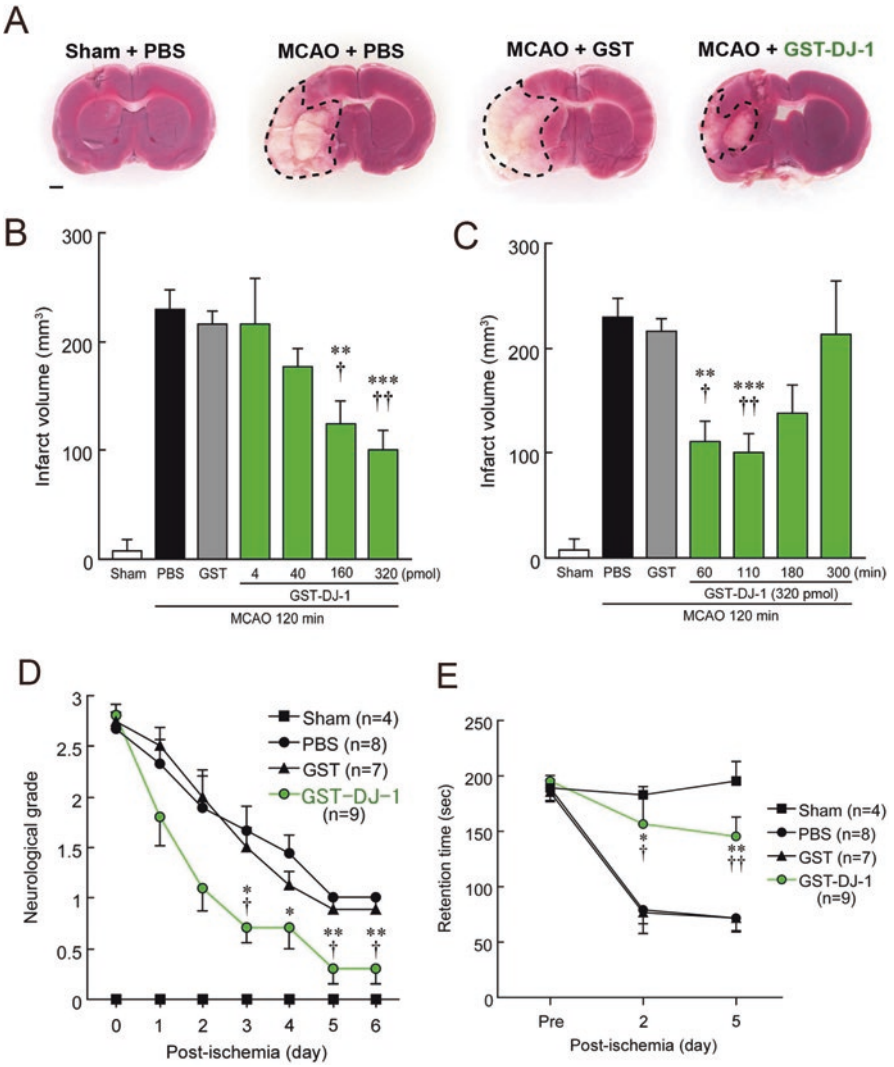

Fig. 12.3 Effect of DJ-1 in ischemia models. (**a**) Representative images of coronal brain sections at +1 mm anterior–posterior from the bregma with TTC staining 3 days after MCAO. (**b**) Dose-dependent protection by DJ-1. (**c**) Time-window of DJ-1-mediated neuroprotection. (**d**) Neurological evaluation was performed daily after reperfusion. (**e**) Spontaneous motor activity was evaluated by the rotarod test before MCAO (pre) and at 2 and 5 days after MCAO (Significance: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. PBS-injected rats. $\dagger P < 0.05$, $\dagger\dagger P < 0.01$, $\dagger\dagger\dagger P < 0.001$ vs. GST-injected rats. Scale bar: 1 mm (**a**))

12.4 DJ-1 Reduces ROS Production and Protects Against H_2O_2-Induced Cell Death

To investigate whether DJ-1 or L166P affected ROS production, we performed confocal microscopy analysis with a ROS-specific fluorogen (C-DCF) (Inden et al. 2006; Yanagisawa et al. 2008). H_2O_2-induced intracellular C-DCF fluorescence markedly increased in normal human SH-SY5Y cells treated with 100-μmol/L H_2O_2 for 1 h (Fig. 12.4). After 1 h, H_2O_2-induced intracellular ROS production was significantly inhibited by 1-μmol/L DJ-1 but not by GST or L166P (Fig. 12.4). This was also true 24 h after the H_2O_2 treatment.

12.5 Identification of DJ-1-Binding Compounds and Their Neuroprotective Effects in PD and Ischemia Models

C106 is the most sensitive cysteine residue in DJ-1 to oxidative stress. It has been reported that a reduced form and/or partial oxidation of C106 activates DJ-1, which may exert an anti-oxidative response. In contrast, excessive oxidation of DJ-1 may lead to a loss of function (Fig. 12.1a). In addition, an excessively oxidized and inactive form of DJ-1 has been observed in brains of patients with PD, Alzheimer's disease, and Huntington's disease (Bandopadhyay et al. 2004; Choi et al. 2006; Sajjad et al. 2014). Therefore, drugs that inhibit excessive oxidation of C106 may be useful for PD therapy. To search for modulators of DJ-1 binding, we performed virtual screening (in silico) using the X-ray crystal structure of the reduced and

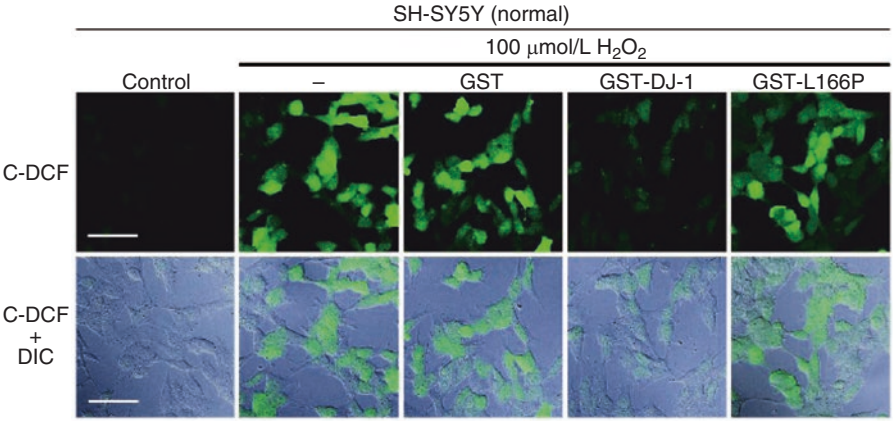

Fig. 12.4 Effect of DJ-1 on H_2O_2-induced oxidative stress in normal SH-SY5Y cells. C-DCF fluorescence (green) was markedly increased by 100-μmol/L H_2O_2 (upper row). In contrast, simultaneous treatment with GST-DJ-1 inhibited the increase in fluorescence intensity induced by H2O2 (Cell images were obtained by difference interference contrast (DIC; lower row). Scale bar: 20 μm)

SO_2H-oxidized C106 region of DJ-1 and the three-dimensional coordinate data of about 30,000 chemical compounds in the University Compound Project at the Foundation for Education of Science and Technology in Japan. Among the DJ-1-binding compounds identified in silico, UCP0045037/compound A and UCP0054278/compound B had the highest binding constant (docking score) for the reduced and SO_2H-oxidized C106 region of DJ-1, respectively (Miyazaki et al. 2008) (Fig. 12.1b).

To examine the effect of UCP0045037 and UCP0054278 on PD phenotypes in vivo, we used the 6-OHDA PD rat model. 6-OHDA was stereotaxically microinjected into the unilateral mesencephalon, and intranigral co-injection of UCP0045037 and UCP0054278 restored the 6-OHDA-induced locomotor defect and prevented 6-OHDA-induced dopaminergic neural death (Miyazaki et al. 2008). In addition, methamphetamine-induced rotational behavior was significantly reduced by co-administration of UCP0045037 and UCP0054278 7 days after injection. TH-positive neurons were preserved in the ipsilateral substantia nigra of UCP0045037- and UCP0054278-treated animals, compared with animals injected with 6-OHDA alone 10 days post-lesion.

In addition, the intrastriatal pre-injection of UCP0045037 and UCP0054278 inhibited neurodegeneration induced by MCAO and reperfusion in rats (Yamane et al. 2009; Yanagida et al. 2009b). TTC staining was markedly decreased 24 h after MCAO in the ipsilateral cerebral cortex and striatum of vehicle-injected rats, and this effect was reduced by the microinjection of UCP0045037 and UCP0054278. Each infarct area was quantitatively smaller, and the total infarct volume was significantly reduced by UCP0045037 and UCP0054278 administration, compared with vehicle-injected rats.

12.6 Effect of UCP0054278 on 6-OHDA-Induced Locomotor Defects and Dopaminergic Neuronal Death

We examined the neuroprotective effect of UCP0054278 on 6-OHDA-induced dopaminergic neuronal death in an in vivo PD model. Pretreatment or posttreatment of UCP0054278 showed a tendency to reduce the number of methamphetamine-induced rotations compared with untreated rats, but this difference was not significant. However, pre- and posttreatment with UCP0054278 significantly ameliorated the methamphetamine-induced behavioral impairment (Fig. 12.5a). To examine the effect of UCP0054278 on 6-OHDA-induced dopaminergic neuronal death, 1-mg/kg UCP0054278 was intraperitoneally injected before treatment, after treatment, or before and after treatment. TH-positive neurons were protected from the effects of 6-OHDA by pre- and posttreatment with UCP0054278 7 days after lesion (Fig. 12.5b). Stereological analysis of nigral TH-positive neurons showed that loss of dopaminergic neurons in the substantia nigra was significantly inhibited by both

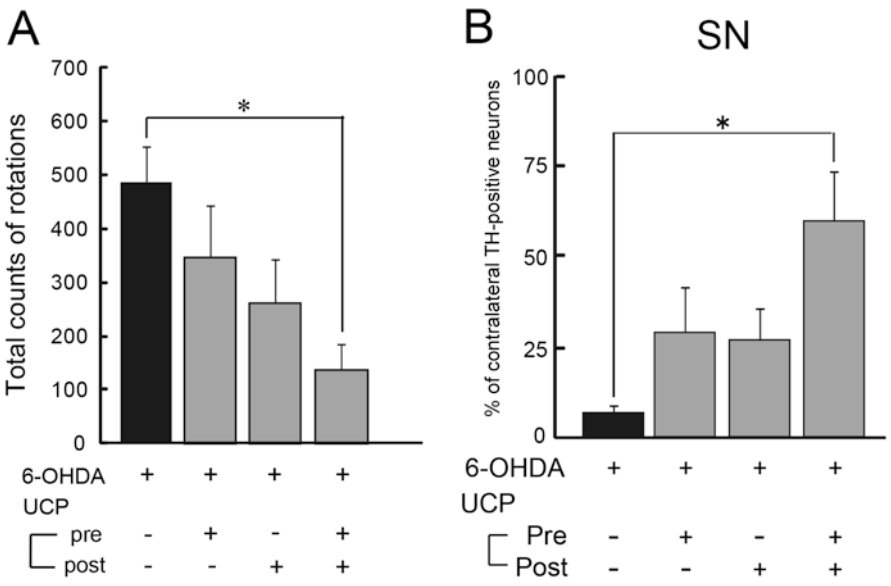

Fig. 12.5 Effect of UCP0054278 on methamphetamine-induced rotation behavior and 6-OHDA-induced dopaminergic neurodegeneration in rats with intranigral 6-OHDA lesions. (**a**) 6-OHDA-microinjected rats were intraperitoneally injected with 1 mg/kg of vehicle or UCP0054278 at 24 h and 30 min before microinjection (pre-treatment), 24, 48 and 72 h after microinjection (posttreatment) or pre- and posttreatment. Seven days later, methamphetamine-induced rotation tests were performed. (**b**) Stereological analysis of TH-positive neurons in the substantia nigra (SN) (Significance: $*P < 0.05$ vs. 6-OHDA alone)

pre- and posttreatment with UCP0054278. However, the loss of dopaminergic neurons was not inhibited by either pre- or posttreatment with UCP0054278.

12.7 Effect of UCP0054278 on Rotenone-Induced Locomotor Defects and Dopaminergic Neuronal Death

We also investigated the neuroprotective effects of UCP0054278 in the rotenone-induced PD model (Inden et al. 2011a). To identify deficits in motor coordination, rotenone-treated mice were tested weekly on the accelerating rotarod (Inden et al. 2011b). Rotenone-treated mice showed marked reductions in endurance time and ability to remain on the rotarod. Treatment with 1- and 3-mg/kg UCP0054278 significantly rescued the retention time on the rotarod (Fig. 12.6a).

To investigate whether UCP0054278 protects dopaminergic neurons in the substantia nigra from rotenone-induced damage, we treated animals with either 1- or 3-mg/kg UCP0054278 for 30 min before the oral administration of rotenone. The

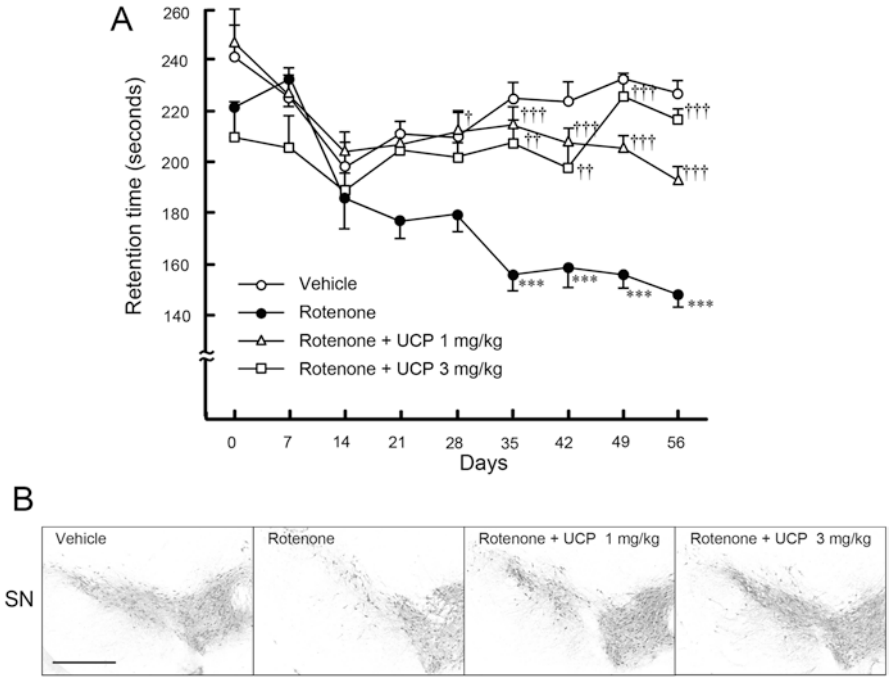

Fig. 12.6 Suppression of rotenone-induced behavioral dysfunction and dopaminergic neurodegeneration by UCP0054278. Rotenone was orally administered to C57BL/6 mice at a dose of 30-mg/kg per day for 56 days. Mice were injected with 1- or 3-mg/kg UCP0054278 once daily for 56 days, 30 min before the oral administration of rotenone. (**a**) The rotarod test was performed once every week (Significance: ***$P < 0.001$ vs. vehicle. †, $P < 0.05$, ††, $P < 0.01$, †††, $P < 0.001$ vs. rotenone alone. (**b**) Representative images of TH immunoreactivity in the substantia nigra (SN). Scale bar: 500 μm)

rotenone-induced loss of TH-positive neurons in the substantia nigra was significantly inhibited by the injection of both 1- and 3-mg/kg UCP0054278 (Fig. 12.6b).

12.8 Effect of UCP0054278 on Intracellular α-Synuclein Expression

A previous study showed that the oral administration of 30-mg/kg rotenone for 56 days produced some TH-positive neurons with a high level of cytoplasmic α-synuclein immunoreactivity in the substantia nigra (Inden et al. 2007, 2011a). To examine the effect of UCP0054278 on intracellular α-synuclein expression in the substantia nigra, we performed confocal microscopic analysis (Fig. 12.7). TH-positive neurons were clearly detected in the substantia nigra of vehicle-treated mice; however, these TH-positive neurons did not show α-synuclein immunoreactivity. Rotenone treatment reduced the number of TH-positive neurons in the

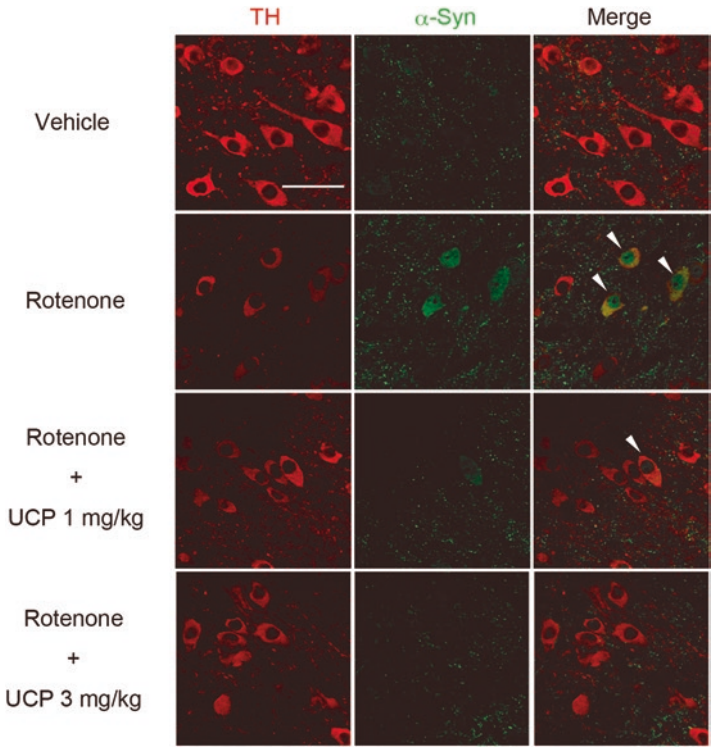

Fig. 12.7 Intracellular expression of α-synuclein in the substantia nigra. Nigral slices from mice treated with 30-mg/kg vehicle or rotenone in the absence or presence of 1- or 3-mg/kg UCP0054278 were labeled with antibodies against TH (red) and α-synuclein (green), and images were captured by laser scanning confocal microscopy. Scale bar: 50 μm

substantia nigra, but α-synuclein immunoreactivity was detected in some of the surviving TH-positive neurons. The number of neurons expressing both α-synuclein and TH was significantly increased in the substantia nigra of rotenone-lesioned mice. Interestingly, α-synuclein expression was significantly decreased in the surviving TH-positive neurons of UCP0054278-treated rotenone mice. In addition, the number of neurons expressing both α-synuclein and TH was significantly decreased by UCP0054278 treatment.

12.9 Effect of UCP0054278 on In Vitro Cultures

We previously established *DJ-1*-knockdown SH-SY5Y cells, in which the endogenous DJ-1 expression was reduced by approximately 76%. These cells serve as an in vitro model of PD (Yanagisawa et al. 2008), and we used this model to examine the effect of UCP0054278 on 6-OHDA-mediated cell death (Yanagisawa et al. 2008).

In normal human SH-SY5Y cells, 6-OHDA caused cell death after 24 h in a concentration-dependent manner (Fig. 12.8a). Cell death was significantly inhibited by UCP0054278 treatment in a concentration-dependent manner. In *DJ-1*-knockdown SH-SY5Y cells, 6-OHDA-induced apoptosis also occurred (Fig. 12.8b), but this was not rescued by UCP0054278 treatment. Similarly, in normal human SH-SY5Y cells, rotenone caused cell death after 48 h in a concentration-dependent manner. Cell death was significantly inhibited by UCP0054278 treatment in a concentration-dependent manner. However, in *DJ-1*-knockdown cells, UCP0054278 did not protect against rotenone-induced cell death. These findings suggest that UCP0054278 exerts its neuroprotective effects against 6-OHDA and rotenone through interactions with DJ-1.

In normal SH-SY5Y cells, incubation with 50-μM 6-OHDA for 1 h induced marked intracellular ROS production (Fig. 12.9a), while 25-μM 6-OHDA had only a slight effect. In *DJ-1*-knockdown cells, 25-μM 6-OHDA was sufficient to significantly enhance ROS production after 1 h (Fig. 12.9b). Simultaneous treatment with 10-μM UCP0054278 significantly inhibited 6-OHDA-induced ROS production in SH-SY5Y cells (Fig. 12.9c). In *DJ-1*-knockdown cells, the inhibitory effect of UCP0054278 on 6-OHDA-induced ROS production was lost (Fig. 12.9d). This suggests that inhibition of ROS production by UCP0054278 depends upon interaction with DJ-1.

12.10 Compound 23

We performed further screening for DJ-1-binding compounds from the Zinc compound library containing approximately 2,500,000 compounds (Fig. 12.1b). We identified compound-23 (comp-23), which prevented oxidative stress-induced death of SH-SY5Y cells and primary neuronal cells of the ventral mesencephalon, but not that of *DJ-1*-knockdown SH-SY5Y cells (Kitamura et al. 2011). Comp-23 inhibited ROS production induced by oxidative stress and prevented excess oxidation of DJ-1. In addition, comp-23 prevented dopaminergic neuronal death and restored normal locomotor function in 6-OHDA-injected rats and rotenone-treated mice. Comp-23 also reduced the infarct size of cerebral ischemia induced by MCAO in rats. The protective activity of comp-23 was stronger than that of UCP0054278. Furthermore, comp-23 inhibited MPTP-induced reduction of the rotarod retention time, dopaminergic neuronal death in the substantia nigra, and striatal dopamine levels in wild-type mice but not in *DJ-1*-knockout mice. These results indicate that comp-23 can cross the blood–brain barrier and that its effects in the brain are dependent upon interaction with DJ-1 (Takahashi-Niki, et al. 2015).

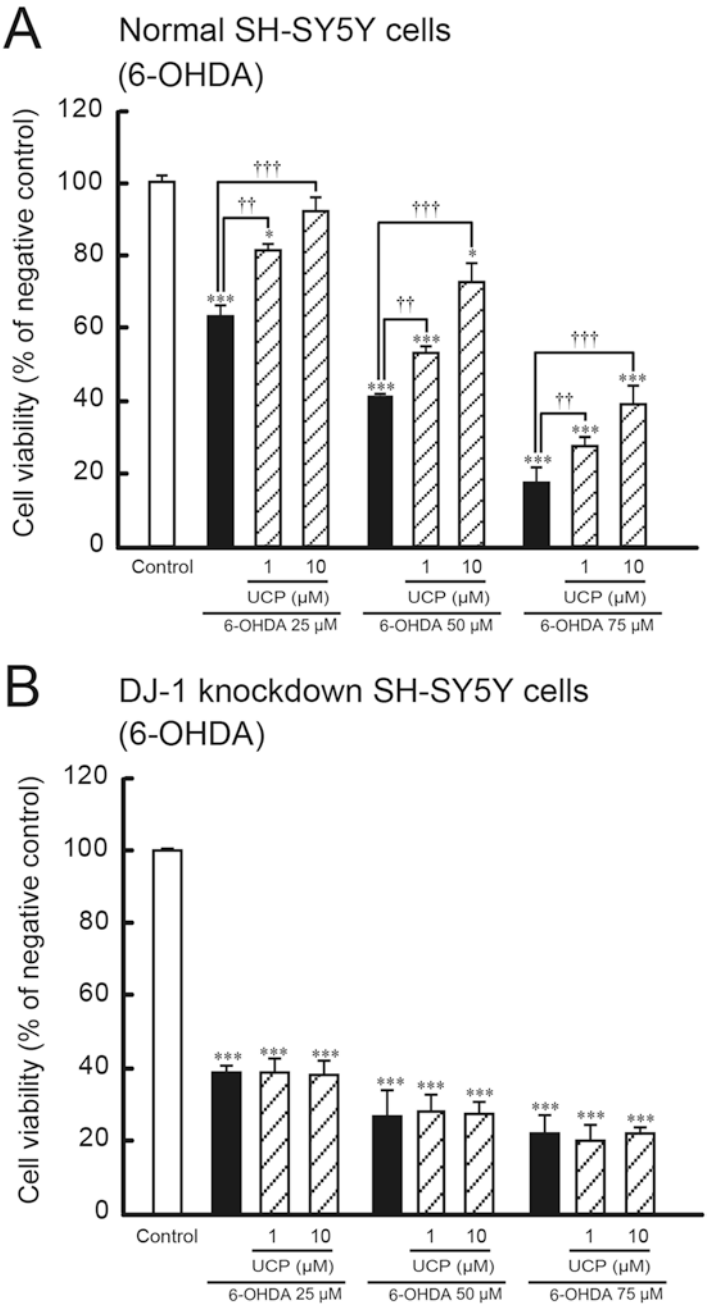

Fig. 12.8 Effect of UCP0054278 on 6-OHDA-induced oxidative stress in human SH-SY5Y cells (**a**, normal) and DJ-1-knockdown cells (**b**, DJ-1 knockdown) (Significance: *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ vs. treatment with control. ††, $P < 0.01$, †††, $P < 0.001$ vs. treatment with 6-OHDA alone)

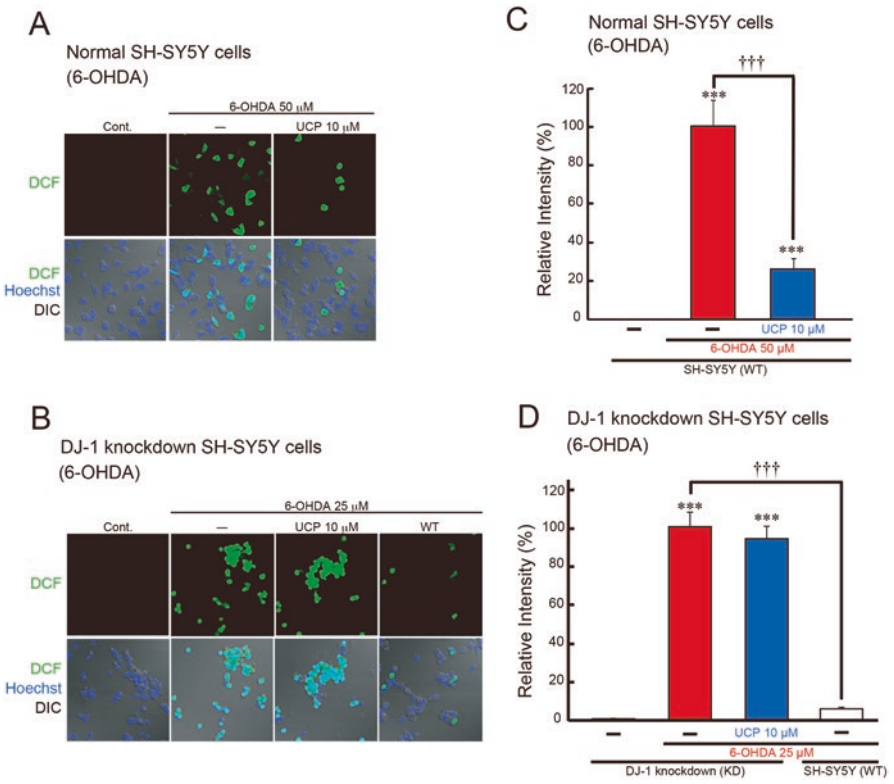

Fig. 12.9 Effect of UCP0054278 on 6-OHDA-induced ROS production. Normal and DJ-1-knockdown SH-SY5Y cells were treated with 6-OHDA in the presence of vehicle (0.01% DMSO) or 10-μM UCP0054278 for 2.5 h. Subsequently, CM-H_2DCFDA was added, and the fluorescence intensity of oxidized DCF (green) was examined by confocal microscopy. Nuclei were stained with Hoechst 33,258 (blue), and cell images were obtained by difference interference contrast (DIC) (Significance: ***$P < 0.001$ vs. vehicle. †††$P < 0.001$ vs. 6-OHDA alone. Scale bar: 50 μm)

12.11 Conclusion

DJ-1-binding compounds, including UCP0045037/compound A, UCP0054278/compound B, and compound-23, were identified by in silico screening using the university compound library and Zinc compound library. These compounds inhibited excessive oxidation of the C106 residue of DJ-1 and maintained the reduced form of DJ-1. This inhibited oxidative stress-induced neuronal cell death and restored locomotor function in animal models of PD and cerebral ischemia. These compounds were able to cross the blood–brain barrier and exerted their protective effects by interacting with DJ-1; protective functions were lost in *DJ-1*-knockdown cells and *DJ-1*-knockout animals. These DJ-1-binding compounds represent promising therapeutic targets for a wide range of neurodegenerative diseases.

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