Stimulation of vesicular monoamine transporter 2 activity by DJ-1 in SH-SY5Y cells

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ABSTRACT

Loss-of-functional mutation in the *DJ-1* gene causes a subset of familial Parkinson's disease. The mechanism underlying DJ-1-related selective vulnerability in the dopaminergic pathway is, however, not known. Dopamine is synthesized by two enzymes and then packed into synaptic vesicles by vesicular monoamine transporter 2 (VMAT2). In this study, we found that knockdown of DJ-1 expression reduced the levels of mRNA and protein of VMAT2, resulting in reduced VMAT2 activity. Co-immunoprecipitation and pull-down experiments revealed that DJ-1 directly bound to VMAT2, and DJ-1 was co-localized with VMAT2 in cells. Furthermore, ectopic expression of wild-type DJ-1, but not that of L166P, M26I and C106S mutants of DJ-1, increased mRNA and protein levels of VMAT2 and VMAT2 activity. Since VMAT2 and a portion of DJ-1 are localized in the synaptic membrane, these results suggest that DJ-1, but not pathogenically mutated DJ-1, stimulates VMAT2 activity in the synapse by transactivation of the *VMAT* gene and by direct binding to VMAT2 and that cysteine 106 is necessary for the stimulating activity of DJ-1 toward VMAT2.

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1. Introduction

Parkinson's disease, the most common movement disorder, is caused by gradual loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) concomitant with loss of neurochemical transport systems, including the dopamine transporter (DAT) and vesicular monoamine transporter 2 (VMAT2) [1,2]. Dopamine is synthesized from tyrosine by two enzymes, tyrosine hydroxylase (TH) and L-DOPA decarboxylase (DDC), and then transported into synaptic vesicles by vesicular monoamine transporter 2 (VMAT2). Dopamine synthesis occurs at the synaptic membrane in a complex involving TH, DDC and VMAT2 [3]. Dopamine is a highly reactive molecule that is auto-oxidized to a quinone in the cytosol, and transport of dopamine into synaptic vesicles by VMAT2 prevents its autoxidation and subsequent degeneration of dopamine neurons. While most cases of PD are sporadic, 5–10% of PD patients carry mutations with a Mendelian inheritance, and mutations in *parkin*, *DJ-1* and *PINK1* genes have been linked to autosomal recessive forms of PD [4–6].

DJ-1 was first identified by our group as a novel oncogene that transformed mouse NIH3T3 cells in cooperation with activated H-*ras* [7]. Deletion and point mutations of DJ-1 have been shown to be responsible for onset of familial Parkinson's disease, PARK7 [5]. DJ-1 is a multi-functional protein and plays roles in transcriptional regulation [8–15] and in modulation of signaling cascades [16–20] through protein–protein interaction, leading to anti-oxidative stress function [21–23]. Furthermore, we have reported that DJ-1 activated TH and DDC through direct binding to TH and DDC in an oxidative status of DJ-1-dependent manner [24] and that human DJ-1 activates *TH* gene expression in cultured human dopaminergic cells [15]. DJ-1 is preferentially localized in the cytoplasm and nucleus [7] and a portion of DJ-1 is localized in the mitochondria [22,25–27] and synapse [28].

In this study, we found that DJ-1 enhanced VMA2 activity by upregulation of VMAT transcription and by interaction of DJ-1 with VMAT2.

2. Materials and methods

2.1. Cell culture and knockdown of DJ-1

Human SH-SY5Y and SH-SY5Y cells expressing wild-type or mutant forms of DJ-1 [24] were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum. The nucleotide sequences for siRNA targeting human DJ-1 were as follows: 5′-UGGAGACGGU-

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CAUCCCUGUdTdT-3′ (upper strand) and 3′-dTdTACCUCUGCCAG UAGGGACA-5′ (lower strand). siRNA for the *luciferase* gene and non-specific siRNA (Allstar siRNA) were purchased from Greiner (Frickenhausen, Germany) and Qiagen (Valencia, CA), respectively. Twenty-five pmol of siRNA was transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the supplier's protocol.

2.2. Real-time PCR

Nucleotide sequences of primers used for real-time PCR were as follows: human DJ-1-sense: 5′-TTGTAGGCTGAGAAATCTCTGTG-3′; human DJ-1-antisense: 5′-ATCCATTCTCACTGTGTTCGC-3′; human VMAT2-sense: 5′-GCAGGCAGAGGCCATCATGT-3′; human VMAT2-antisense: 5′-GGCGATCTCAGCAATCAGCT-3′; human β-actin-sense: 5′-CCCTAAGGCCAACCGRGAAA-3′; human β-actin-antisense: 5′-AC-GACCAGAGGCATACAGGGA-3′. Forty-eight hours after transfection of siRNA into cells, total RNAs were prepared and subjected to quantitative RT-PCR (real-time PCR) analyses as described previously [15].

2.3. Vesicular monoamine transporter 2 activity assay

SH-SY5Y cells (1×10^5) in 24-well plates were washed with an uptake buffer containing 110 mM potassium tartrate, 5 mM glucose, 0.2% bovine serum albumin, 5 mM $MgCl_2$, 1 mM ascorbic acid, 10 mM pargyline and 20 mM K-HEPES (pH 7.8), and after addition of 500 μl/well of uptake buffer containing 10 mM digitonin, the cells were incubated for 10 min at 37 °C. The medium was then replaced with uptake buffer containing 5 mM Mg-ATP and 85 nM ^{3}H-dopamine in the absence of digitonin, and the cells were incubated for 45 min at 37 °C followed by addition of uptake buffer containing 5 mM $MgSO_4$. The cells were then lysed with 0.1% Triton X-100 and their radioactivity was measured.

2.4. Western blotting and antibodies

The cells were homogenized with a buffer containing 20 mM HEPES (pH 7.4), 125 mM NaCl and 1 mM EGTA using a polytron, and proteins were extracted with 1% Triton X-100 for 60 min at 4 °C. Proteins were then separated on a 10% or 12.5% polyacrylamide gel and subjected to Western blotting with respective antibodies. Proteins on the membrane were reacted with an IRDye 800- (Rockland, Philadelphia, PA) or Alexa Fluor 680-conjugated secondary antibody (Molecular Probes, Eugene, OR) and visualized by using an infrared imaging system (Odyssey, Li-COR, Lincoln, NE). Antibodies used were anti-VMAT2 (1:500, Chemicon, Temecula, CA), anti-DJ-1 (1:4000, 3E8, MBL, Nagoya Japan) and anti-actin (1:4000, Chemicon) antibodies.

2.5. In vivo co-immunoprecipitation assay

Proteins were extracted from cells as described above and immunoprecipitated with a rabbit anti-DJ-1 antibody (1:500) or normal IgG, and precipitates were analyzed by Western blotting with anti-VMAT2 (1:500, Chemicon) or mouse anti-DJ-1 antibody (1:1000, 3E8, MBL). The rabbit anti-DJ-1 antibody was prepared by us as described previously [7].

2.6. Pull-down assay

^{35}S-labeled VMAT2-FLAG-His was synthesized *in vitro* using the reticulocyte lysate of the TNT transcription-translation coupled system (Promega, Madison, WI). Labeled proteins were mixed with GST or GST-DJ-1 expressed in and prepared from *Escherichia coli* at 4 °C for 60 min in a buffer containing 150 mM NaCl, 5 mM EDTA, 50 mM Tris (pH 7.5), 0.05% bovine serum albumin and 0.1% Non-

idet P-40 (NP-40). After washing with the same buffer, the bound proteins were separated in a 10% polyacrylamide gel containing SDS and visualized by fluorography.

2.7. Indirect immunofluorescence

Cells were fixed with 4% paraformaldehyde and reacted with rabbit anti-DJ-1 polyclonal (1:100) and mouse anti-VMAT2 (1:50) antibodies. The cells were also stained with DAPI. The cells were then reacted with a rhodamine-conjugated anti-rabbit IgG or an FITC-conjugated anti-mouse IgG and observed under a Bio-imaging system (OLYMPUS, FSV100, Tokyo, Japan).

2.8. Statistical analyses

Data are expressed as mean ± S.E. Statistical analyses were performed using analysis of variance (one-way ANOVA) followed by unpaired Student's *t*-test. For comparison of multiple samples, the Tukey–Kramer test was used.

3. Results

3.1. Reduction of gene expression and activity of VMAT2 in DJ-1-knockdown cells

Human dopaminergic SH-SY5Y cells were transfected with siRNAs targeting *DJ-1* or with a negative control siRNA, and expression levels of *DJ-1* and *VMAT2* mRNAs were examined by quantitative RT-PCR (real-time PCR) (Fig. 1A). β-*actin* was used as a loading control. The levels of *VMAT2* gene expression in SH-SY5Y cells were reduced after *DJ-1* gene expression had been knocked down by siRNA to approximately 55% of the level of *DJ-1* in cells without siRNA or with negative control siRNA (Fig. 1A). A reduced expression level of VMAT2 was also observed in DJ-1-knockdown SH-SY5Y cells by Western blotting (Fig. 1B). In this case, the expression level of VMAT2 in DJ-1-knockdown cells was reduced to 40% of that in cells without siRNA or with negative control siRNA. VMAT2 activity was then examined as an indicator of incorporation of ^{3}H-labeled dopamine into SH-SY5Y cells. First, to confirm specific reaction of VMAT2, nomifensine, 3,4-dihydroxy-L-phenylalanine (3,4-DHP) and reserpine were added to cells as inhibitors for dopamine transporter, DDC and VMAT2, respectively. Since 3,4-DHP inhibits new synthesis of dopamine from tyrosine and since nomifensine inhibits re-uptake of secreted dopamine, VMAT2 incorporates exogenously added ^{3}H-dopamine and this activity should be inhibited by reserpine. As shown in Fig. 1C, incorporation of ^{3}H-dopamine was specifically inhibited by reserpine but not by 3,4-DHP and nomifensine. The effect of DJ-1-knockdown on VMAT2 activity was examined, and the results showed that knockdown of DJ-1 expression by DJ-1 siRNA, but not by control siRNA, reduced VMAT activity (Fig. 1D). These results indicate that DJ-1 positively regulatesVMA2 expression and its activity.

3.2. Association of DJ-1 with VMAT2

We have reported that DJ-1 positively regulates TH mRNA expression and TH activity by sequestering PSF, a repressor for transcription of the *TH* gene, and by direct association of DJ-1 with TH, respectively [15,24]. It has been reported that TH and DDC are associated with VMAT2 [3]. We therefore examined interaction of DJ-1 with VMAT2. Proteins extracted from SH-SY5Y cells were immunoprecipitated with an anti-DJ-1 antibody or with non-specific IgG, and the precipitates were analyzed by Western blotting with anti-VMAT2 and anti-DJ-1 antibodies. As
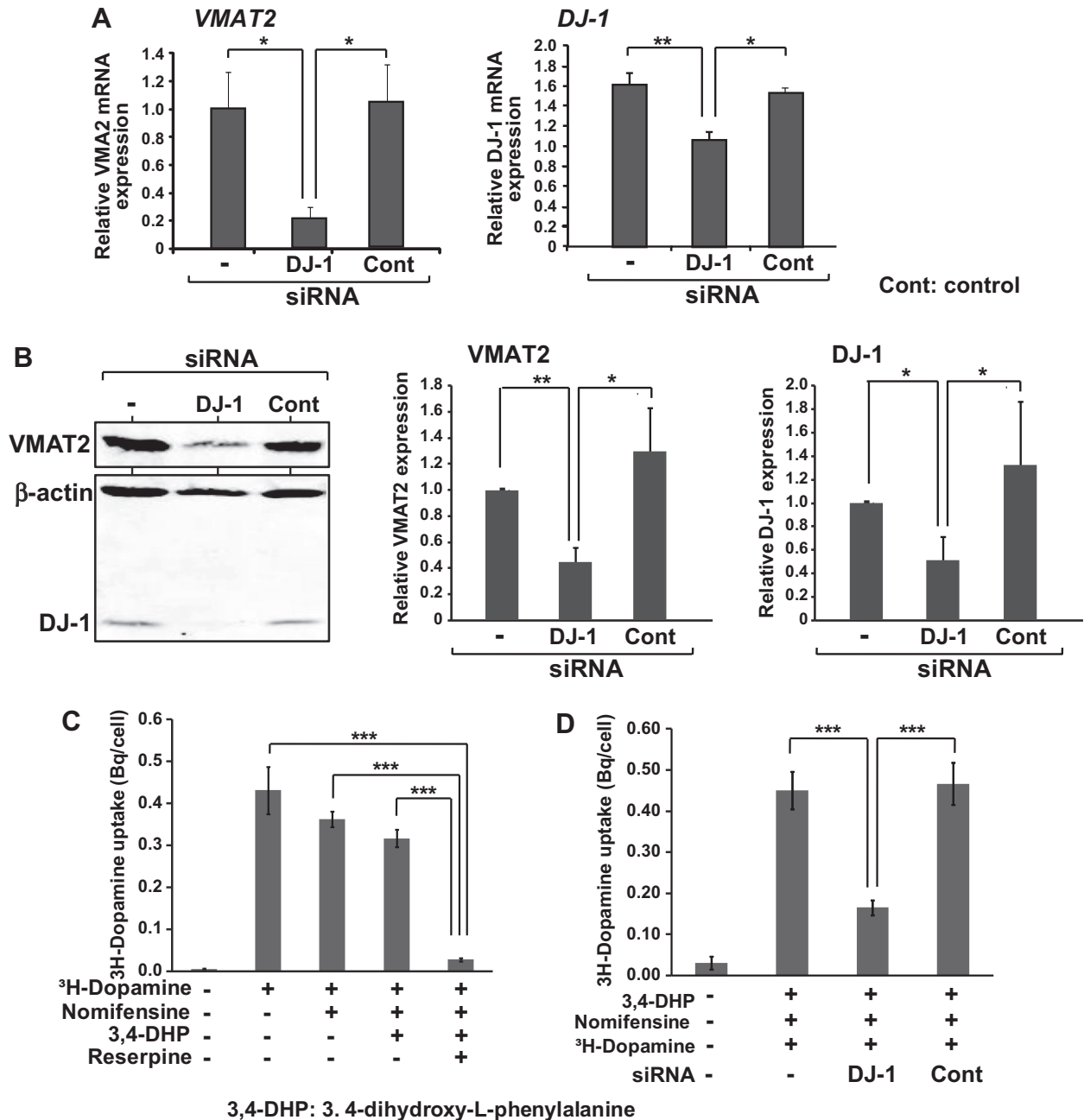
Fig. 1. Knockdown of DJ-1 expression reduces VMAT2 expression and activity. (A) SH-SY5Y cells were transfected with siRNA targeting DJ-1 or with negative control siRNA (Allstar siRNA). At 48 h after transfection, expression levels of DJ-1, VMAT2 and β-actin were examined by real-time PCR as described in Materials and methods. Relative expression of DJ-1 and VMAT2 mRNA *versus* β-actin mRNA is shown. Average results of 3 independent experiments are shown. (B) SH-SY5Y cells were transfected with siRNA targeting DJ-1 or with negative control siRNA targeting the luciferase gene. At 48 h after transfection, cell extracts were prepared and expression levels of VMAT2, DJ-1 and β-actin were examined by Western blotting with respective antibodies (left panel). Intensities of bands were quantified, and relative expression of DJ-1 and VMAT2 *versus* β-actin is shown (center and right panels). Average results of 3 independent experiments are shown. (C) SH-SY5Y cells were treated with 10 mM digitonin and then replaced with an uptake buffer containing 5 mM Mg-ATP and 85 nM ^{3}H-dopamine in the absence or presence of nomifensine, 3,4-DHP or reserpine. After incubation for 45 min, cells were lysed with 0.1% Triton X-100 and incorporated radioactivity was measured as described in Materials and methods. Average results of 3 independent experiments are shown. (D) SH-SY5Y cells were transfected with siRNA targeting DJ-1 or with negative control siRNA targeting the luciferase gene. At 48 h after transfection, cells were treated with ^{3}H-dopamine, nomifensine and 3,4-DHP and incorporated radioactivity was measured. Average results of 3 independent experiments are shown.

shown in Fig. 2A, the anti-DJ-1 antibody co-immunoprecipitated VMAT2. Pull-down experiments were then carried out using ^{35}S-labeled VMAT2-FLAG-His and GST, GST-DJ-1 or GST-TH as a positive control, and the results showed that ^{35}S-VMAT2-FLAG-His was precipitated by GST-DJ-1 and GST-TH but not by GST (Fig. 2B). Furthermore, SH-SY5Y cells were stained with anti-DJ-1 and anti-VMAT2 antibodies. As shown in Fig. 2C, DJ-1 and VMAT2 were mainly localized and co-localized in the cytoplasm (See the merged figure in which red (DJ-1) and green (VMAT2)

colors turned yellow.). These results clearly indicate that DJ-1 directly binds to VMAT2.

3.3. Stimulation of VMAT2 activity by DJ-1

Stable cell lines of SH-SY5Y cells expressing wild-type DJ-1 and mutant DJ-1, including M26I, C106S and L166P DJ-1, were established as described previously [24]. SH-SY5Y cells harboring a vector backbone were also established. M26I and L166P DJ-1 were
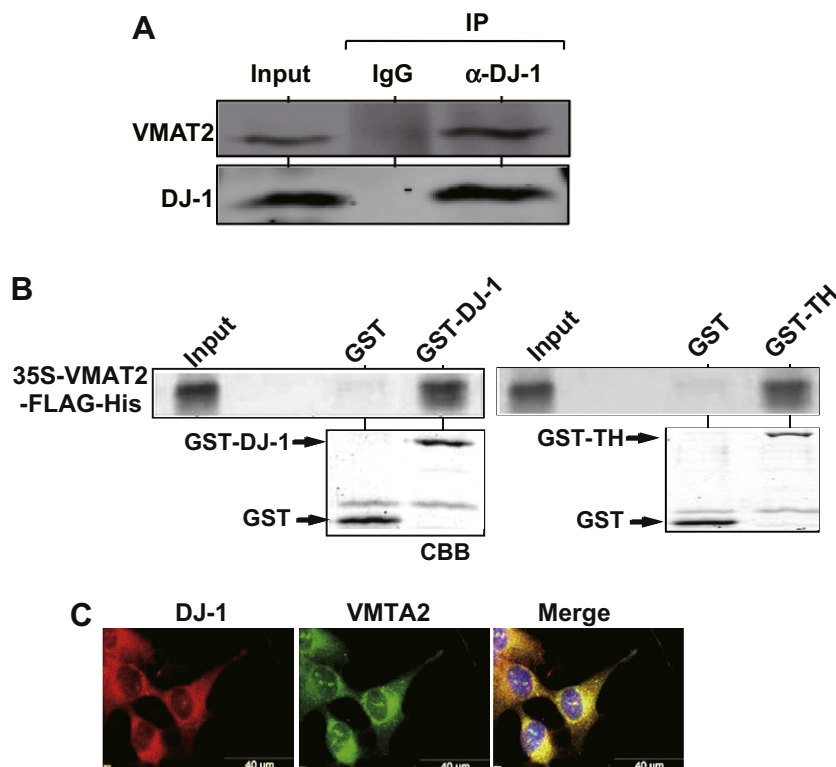
Fig. 2. Association of DJ-1 with VMAT2. (A) Cell extracts prepared from SH-SY5Y cells were immunoprecipitated with an anti-DJ-1 antibody or IgG. Precipitates were then analyzed by Western blotting with anti-DJ-1 and anti-VMAT2 antibodies as described in materials and methods. (B) GST-DJ-1, GST-TH and GST were expressed in and purified from *E. coli* and reacted with ^{35}S-labeled VMAT2-FLAG-His that had been synthesized *in vitro* using reticulocyte lysates. Pull-downed proteins were separated on gels and subjected to fluorography as described in materials and methods. (C) SH-SY5Y cells were immunostained with anti-DJ-1 and anti-VMAT2 antibodies. After reaction of the cells with secondary antibodies, cell images were obtained by using a fluorescence microscope as described in materials and methods. A merged figure is also shown.

found to be as homozygous mutations in Parkinson's disease patients [5,29]. Since cysteine at amino acid number 106 (C106) is essential for all of the functions of DJ-1 [21–23], C106S DJ-1 was also used. First, expression levels of VMAT2 mRNA and protein were examined by real-time PCR and Western blotting, respectively. As shown in Fig. 3A and B, both mRNA and protein levels of VMAT2 in wild-type DJ-1-expressing cells were higher than those in host cells, vector-containing cells and mutant-DJ-1-expressing cells. VMAT2 activity was then examined to measure ^{3}H-dopamine uptake into these cell lines and parental SH-SY5Y cells (host cells) in the presence of 3,4-DHP and nomifensine (Fig. 3C). The results showed that VMAT2 activity in wild-type DJ-1-expressing cells was higher than that in host and vector-containing cells and that VMAT2 activity in M26I and L166P-expressing cells was at the same level as that in host and vector-containing cells. VMAT2 activity in C106S DJ-1-expressing cells was lower than that in wild-type-expressing cells but higher than that in M26I and L166P-expressing cells. The levels of VMAT2 activity were parallel to those of mRNA and VMAT2 protein in various SH-SY5Y cell lines. These results indicate that wild-type DJ-1, but not Parkinson's disease-derived mutant DJ-1, stimulates VMAT2 activity and that C106 is necessary for the stimulating activity of DJ-1 toward VMAT2.
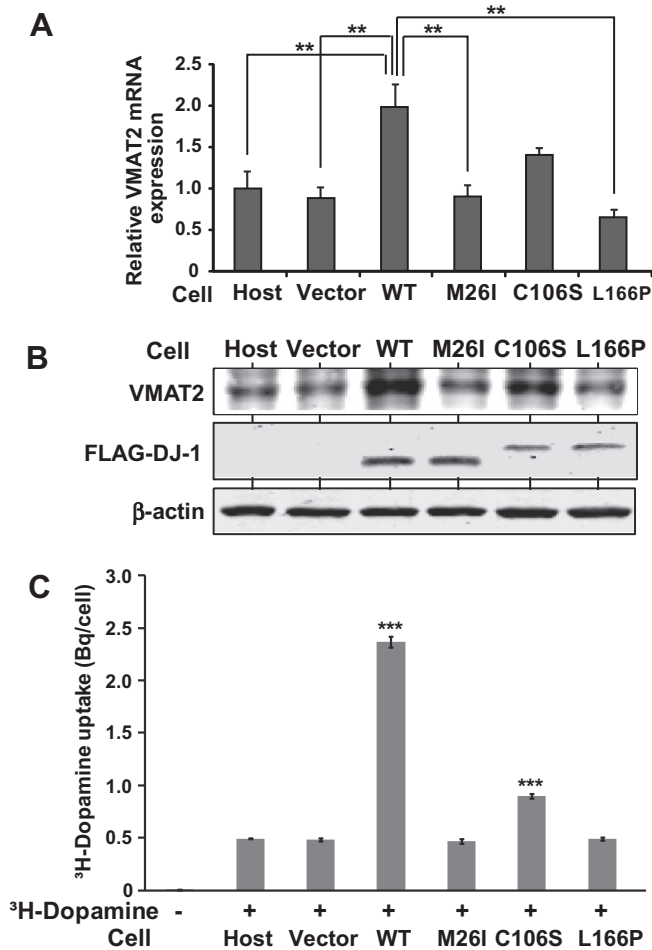
4. Discussion

In this study, we found that expression of the *VMAT2* gene and VMAT2 activity were reduced in *DJ-1*-knockdown human SH-SY5Y cells and that ectopic expression of wild-type DJ-1, but not that of L166P and M26I mutant DJ-1, which have been found in patients

with a familial form of Parkinson's disease [5,29], stimulated expression of the *VMAT2* gene and VMAT2 activity. C106 mutant of DJ-1 also reduced stimulating activity toward VMAT2. DJ-1 directly bound to VMAT2 and was co-localized with VMAT2 in cells. These results indicate that wild-type DJ-1, but not Parkinson's disease-derived mutant DJ-1, stimulates VMAT2 activity and that C106 is necessary for the stimulating activity of DJ-1 toward VMAT2.

VMAT2 is located in the synaptic membrane and transports monoamines such as dopamine into synaptic vesicles. Once dopamine is released from synaptic vesicles in the presynapse, re-uptake of excess amounts of dopamine by the dopamine transporter into the presynapse occurs. Dopamine is easily oxidized, and oxidized dopamine injures mitochondria, leading to production of reactive oxygen species (ROS) from compromised mitochondria, thereby resulting in cell injury. To avoid the effect of oxidized dopamine on cell injury, excess amounts of dopamine are again trapped into synaptic vesicles by VMAT2 [see a review 30, references therein]. Prior to storage of dopamine within synaptic vesicles, dopamine is synthesized in the synaptic terminal from tyrosine by two enzymes, TH and DDC. Although TH is localized both in the cytosol and in the membrane, a recent study showed that TH, DDC and VDAC2 are physically and functionally associated in the synaptic membrane and that synthesis and transport of dopamine are coupled [3].

Since dopaminergic neurons die in Parkinson's disease patients, the expression level of TH and TH activity are reduced. A reduced level of VMAT2 mRNA in Parkinson's disease patients has also been reported [31]. We have shown that DJ-1, a causative gene product of a familial form of Parkinson's disease, regulates TH and DDC activities: DJ-1 upregulates transcription of the *TH* gene in a hu-

be involved. We also showed that mutation of C106 of DJ-1 reduced the stimulating activity of DJ-1 toward VMAT2 expression and activity (Fig. 3). Since C106 is necessary for DJ-1 to exert its functions, including transcriptional activation of genes and stimulation of TH and DDC activities, stimulation of VMAT2 activity also needs C106.

Anti-oxidative stress reaction is a major role of DJ-1. To exert this function, DJ-1 eliminates ROS by self-oxidation of DJ-1 [21] and activates Nrf2, a master transcription factor in the redox system [11]. In terms of anti-oxidative stress reaction of DJ-1, the present study has revealed another role of DJ-1: stimulation of re-uptake of excess dopamine into synaptic vesicles.

Acknowledgments

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Fig. 3. Stimulation of VMAT expression and activity by wild-type DJ-1. (A) SH-SY5Y cells stably expressing FLAG-tagged wild-type, M26I, L166P and C106S DJ-1, SH-SY5Y cells harboring a vector and parental SH-SY-5Y cells (host) were used. Expression levels of VMAT2 and β-actin mRNA were examined by real-time PCR, and relative expression of VMAT2 mRNA *versus* β-actin mRNA is shown. Average results of 3 independent experiments are shown. (B) Cell extracts were prepared from various SH-SY5Y cells described in A, and expression levels of VMAT2, FLAG-DJ-1s and actin in cells were analyzed by Western blotting with anti-VMAT2, anti-DJ-1 and anti-β-actin antibodies. (C) VMAT2 activity in various SH-SY5Y cells described in A were examined as described in the legend for Fig. 1D. Average results of 3 independent experiments are shown.

man-specific manner [15] and enhances TH and DDC activities through protein–protein interaction [24]. As in the case of TH, the present study showed that DJ-1 activates VMAT2 expression and enhances its activity at the level of transcription and by protein–protein interaction. Although most of DJ-1 is located in the cytoplasm and nucleus [7], a subset of DJ-1 is localized in the synaptic membrane [28]. It is therefore thought that TH, DDC, VMAT2 and DJ-1 make a complex at least in the synaptic membrane to synergistically facilitate synthesis and transport of dopamine.

Pathogenic mutations of DJ-1 lost stimulating activity toward VMAT2 (Fig. 3). Although VMAT2 activity was not examined in patients with DJ-1 mutation-derived Parkinson's disease, it would be interesting to do so. Contrary to human cases of familial forms of Parkinson's disease caused by mutations of *parkin*, *pink1* and *DJ-1* genes, corresponding knockout mice, even triple knockout mice, do not show obvious phenotypes of Parkinson's disease [32]. There are no significant differences in TH, DDC and VMAT2 levels between wild-type and DJ-1-knockout mice [33]. Although the precise mechanisms underlying the absence of phenotypes in knockout mice are not clear, a compensation mechanism might

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