Original Article

Curcumin Protects Nigrostriatal Dopaminergic Axons and Increases BDNF Immunoreactivity in the 6-OHDA-Lesioned Striatum of Mice

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Background: Brain-derived neurotrophic factor (BDNF) exerts neuroprotection upon the nigrostriatal dopaminergic (DA) neurons and their projections in animal models of Parkinson's disease (PD). Recent data indicate neuroprotective effects of curcumin in animal models of PD via anti-inflammatory and anti-oxidant actions. However, there are no studies investigating the effects of curcumin on BDNF levels in the striatum of the 6-hydroxydopamine (6-OHDA) mouse model of PD.

Objective: The present study investigated the effects of curcumin on the extent of nigrostriatal DA innervation and BDNF levels in the striatum of 6-OHDA-lesioned mice.

Materials and Methods: 6-OHDA was unilaterally injected into the right striatum of ICR male mice. Curcumin (200 mg/kg) or vehicle (dimethyl sulfoxide) was intraperitoneally administered daily for 7 days starting instantaneously after 6-OHDA injection. Seven days after 6-OHDA insult, mice were euthanized and striatal sections were collected, immunohistochemically stained, and quantitated for tyrosine hydroxylase (TH, a marker for DA neurons) and BDNF immunoreactivity.

Results: 6-OHDA injection induced a significant loss of TH-immunoreactive (-IR) axons and increased BDNF expression in the 6-OHDA-lesioned striatum. Curcumin diminished loss of TH-IR fibers and additionally promoted increases in BDNF levels in the 6-OHDA-lesioned striatum.

Conclusion: One possible mechanism by which curcumin protects nigrostriatal DA axons against 6-OHDA is through upregulation of striatal BDNF.

Keywords: Curcumin, BDNF, 6-OHDA, Nigrostriatal dopaminergic axon, Striatum

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Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease. The pathological hallmark of PD is loss of pigmented dopaminergic (DA) neurons in the substantia nigra pars compacta and their projections in the striatum[1]. Although the cause of PD remains unknown, studies in PD brains suggest that decreased expression of one or a combination of the neurotrophic factors which supports the survival nigral DA neurons might contribute directly to the loss of these neurons and the genesis of PD[2-4].

Brain-derived neurotrophic factor (BDNF) is one of the growth factors with neurotrophic activity on DA neurons in the nigrostriatal pathway. BDNF promotes the survival of cultured mesencephalic DA neurons[5]. The molecule also protects nigral DA neurons against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 1-methyl-4-phenylpyridinium ion (MPP+), as well as 6-hydroxydopamine (6-OHDA)[6,7], attenuates loss of striatal DA axons following intrastriatal injection of 6-OHDA[7], and induces sprouting of DA axons in the injured striatum[8]. In addition, decreased BDNF level in the post-mortem substantia nigra of patients with PD is suggested to be involved in the pathogenesis of PD during progression of neurodegeneration of the nigrostriatal DA neurons[3,4]. These data indicate a crucial role for BDNF in supporting the survival of nigral DA neurons in both normal and in PD.

Curcumin, a yellow pigment isolated from the rhizome of Curcuma Longa Linn (Tumeric), is one of the primary ingredients in curry powders that are used as spices and also as herbal medicine in India. Curcumin exhibits anti-inflammatory and anti-oxidant properties[9-11] and can cross the blood-brain barrier following intraperitoneal or intranasal administration[12]. In addition, it has been shown exerting neuroprotective effects against cerebral ischemia[13], traumatic brain injury[14], and amyloid beta-protein-induced damage[9]. Although cumulative evidence suggests neuroprotective effects of curcumin on the nigrostriatal pathway[10,11,15], the effect on BDNF expression in the striatum of a unilateral 6-OHDA-lesioned animal model of PD remains uninvestigated.

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The present study examined the effects of curcumin on the extent of DA axon innervation and an expression of BDNF in the striatum during early degeneration of DA axons triggered by unilateral striatal injection of 6-OHDA.

Materials and Methods

Animals

Twenty-nine male mice of the ICR strain (National Laboratory Animal Center, Thailand) were 7 weeks old at the start of experiment. They were housed individually per cage with a 12 hour light-dark cycle at a room temperature of 23°C and had access to food and water ad libitum. The animals were acclimatized to the laboratory environment for 7 to 10 days before the experiments. All experiments were performed using protocols approved by the Committee of Animal Used for Research at Faculty of Medicine, Srinakharinwirot University, Thailand.

6-OHDA lesion model and curcumin administration

Animals were divided into four groups (sham control group, n = 6; 6-OHDA group, n = 7; 6-OHDA/dimethylsulfoxide (DMSO) group, n = 7 and 6-OHDA/curcumin group, n = 9). Mice were anesthetized intraperitoneally with pentobarbital sodium (60 mg/kg) and received two stereotaxic injections of 6-OHDA hydrobromide (Sigma-Aldrich, St. Louis, MO, 5.5 µg/µl), 3 µl/injection site, or an equivalent volume of 0.9% saline containing 0.2% ascorbic acid (sham control group), into the right striatum, at a rate of 1 µl/min (coordinates: point I: AP + 0.7, ML + 2.0, DV -3.4 mm and point II: AP - 0.1, ML + 2.7, DV -3.4 mm from bregma and dura according to the atlas of Franklin and Paxinos[16]). At the completion of each injection, the needle was left in situ for a further 3 min to allow for diffusion and then withdrawn at 1 mm/min. The wound was sutured and applied with antiseptic (1% w/w iodine, Betadine). During the experiment the 6-OHDA solution was kept on ice and protected from light to minimize oxidation. Curcumin (Sigma-Aldrich, St. Louis, MO, 200 mg/kg) dissolved in DMSO (Sigma-Aldrich, St. Louis, MO) or an equivalent volume of DMSO (1 ml/kg) was injected intraperitoneally for 7 days starting immediately after the surgery and was repeated every 24 hours. Animals were euthanized on day 7 after 6-OHDA injection. Only those animals in which the needle tracts were placed at appropriate coordinates were included in the study.

Immunohistochemistry

All mice were sacrificed by an overdose of pentobarbital sodium (350 mg/kg, intraperitoneal, Sanofi, Thailand) and perfused transcardially with 30 ml of ice-cold heparinized 0.1 M phosphate buffer saline, pH 7.4 (PBS), followed by 20 ml of chilled 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) in 0.1M phosphate buffer, pH 7.4. The brains were post-fixed in the same fixative for 18 hr at 4°C and then cryoprotected with 30% sucrose in PBS at 4°C until sunk. A 1:10 series of thirty five-micrometer coronal sections were cut through the striatum, using a cryostat (Leica CM 1850, Wetzlar, Germany). The sections were stored free-floating at -20°C in cryoprotectant until used.

For tyrosine hydroxylase (TH) and BDNF immunohistochemistry, sections were rinsed in PBS to remove any cryoprotectant, and then incubated for 15 min in 0.3% H_2O_2 in 50% methanol to block endogenous peroxidase activity. After 30 min incubation in blocking buffer (PBS containing 0.3% triton X-100 and 10% normal goat serum) to reduce non-specific binding, sections were rinsed in PBS, then incubated overnight at room temperature with a polyclonal rabbit anti-TH antibody (a marker for DA neurons, Chemicon International, Temecula, CA, USA, 1:3,000 dilution) or a polyclonal rabbit anti-BDNF antibody (Chemicon International, USA, 1:450 dilution). This was followed by incubation in biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA, 1:500 for TH staining and 1:400 for BDNF staining) at room temperature and subsequently in avidin peroxidase (Sigma-Aldrich, St. Louis, MO, 1:5,000 dilution). Immunoreactivity was visualized with cobalt and nickel-intensified 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, St. Louis, MO)/H_2O_2. PBS rinses (5 min x3) were performed between each step. Finally, sections were mounted onto slides, dehydrated, and coverslipped. Negative control study was performed by omission of the primary antibody.

Quantification of TH-immunoreactive (-IR) fibers and BDNF expression in the striatum

The extent of DA innervation in the ipsilateral and the contralateral striatum was assessed by measuring the optical density (OD) of TH immunoreactivity with the image analyzing software ImageJ (National Institutes of Health, Bethesda, MD, USA). Images of the striatum of each section were captured at 4x magnification using an Olympus DP-70 digital color camera (Olympus America Inc.) mounted to an Olympus BX50 microscope. To correct for variability in lighting conditions, all images were photographed under identical conditions. After converting images to an 8-bit file, pictures were calibrated to OD values. Background levels were captured from the cerebral cortex in each section and subtracted from the total OD measurement. The OD value of each animal was the average OD from each of the nine to twelve measured sections. All analyses were performed in a blinded fashion.

The BDNF immunoreactivity in the ipsilateral and the contralateral striatum was assessed by measuring the BDNF-IR area with the image analyzing software ImageJ (National Institutes of Health, Bethesda, MD, USA) according to the method described in https://imagej.nih.gov/ij/docs/examples/stained-sections/index.html. In brief, images of the striatum of each section were captured at 4x magnification using an Olympus DP-70 digital color camera under identical microscope and camera settings. All striatal images were converted to grayscale and adjusted to threshold. The threshold area representing BDNF-IR area was measured and the percentage of BDNF-IR area was calculated. The percentage of BDNF-IR area in the striatum of each animal was the mean percentage of BDNF-IR area from nine to

twelve measured sections. All analyses were performed in a blinded fashion.

Statistical analysis

All values were expressed as mean ± SEM. One-way ANOVA with Student-Newman-Keuls *post hoc* tests was used to compare data of all animal groups from the same striatal hemisphere. Student's t-test was used to compare data in the ipsilateral and contralateral striatum within the same treatment groups. In this study significance was set at $p<0.05$.

Results

Curcumin significantly reduces 6-OHDA-induced loss of striatal TH-IR fibers

In saline-injected striatum, slight disruption of TH immunoreactivity associated with the needle tract was observed without the difference in TH immunoreactivity compared to the contralateral striatum (Figure 1A). 6-OHDA injection significantly decreased TH-IR fibers in the ipsilateral striatum compared to the saline-injected striatum ($p<0.05$) (Figure 1A, 1B, and 1C). DMSO treatment had no effect on 6-OHDA-induced loss of striatal TH-IR fibers (Figure 1A and 1D). 6-OHDA-triggered loss of striatal TH-IR fibers was inhibited by curcumin ($p<0.05$ vs. the ipsilateral striatum of sham control, 6-OHDA-, and 6-OHDA/DMSO-injected mice) (Figure 1A and 1E). TH immunoreactivity in the contralateral striatum among all animal groups was not significantly different (Figure 1A and 1B'-1E').

Curcumin promotes BDNF expression in the striatum of 6-OHDA-lesioned animals

In all animal groups, BDNF immunoreactivity was mainly localized in the striatal neurons (Figure 2B, white

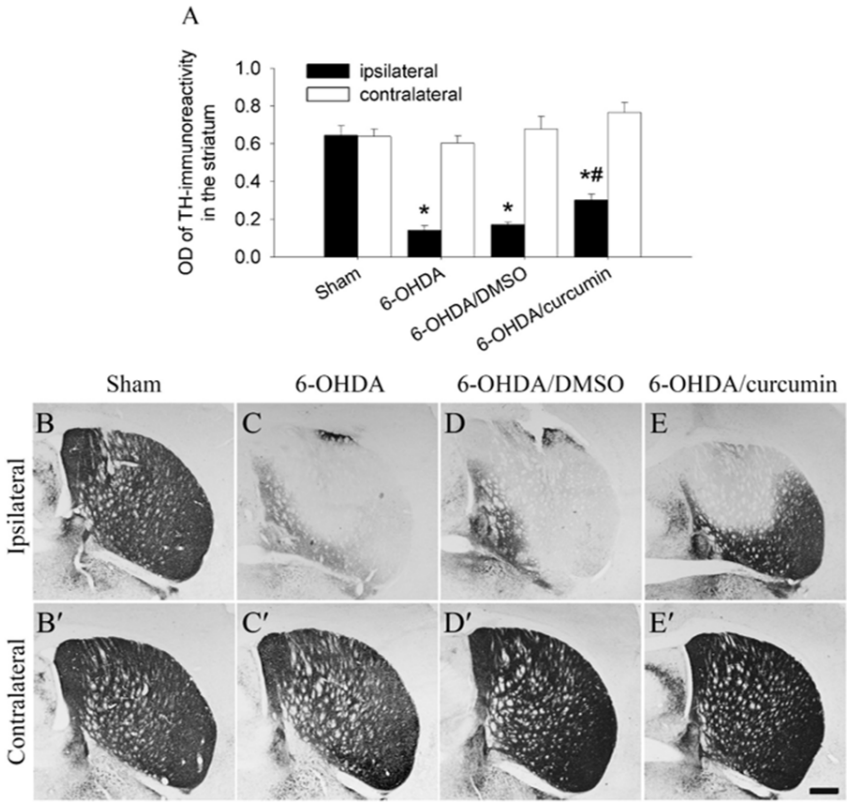

Figure 1. Curcumin diminishes loss of nigrostriatal DA axons triggered by 6-OHDA. (A), Histogram showing the OD of TH immunoreactivity in the ipsilateral striatum and the contralateral striatum of sham control, 6-OHDA-, 6-OHDA/DMSO, and 6-OHDA/curcumin-injected mice. Values are mean ± SEM. *Indicates significant difference from the ipsilateral striatum of sham mice. # Indicates significant difference from the ipsilateral striatum of 6-OHDA- and 6-OHDA/DMSO-treated animals. (B-E and B' -E'), Representative photomicrographs depicting TH immunoreactivity in the ipsilateral striatum (upper panels) and the contralateral (lower panels) of sham control (B and B'), 6-OHDA- (C and C'), 6-OHDA/DMSO- (D and D'), and 6-OHDA/curcumin-injected mice (E and E'). Scale bar in (E') represents a length of 500 µm for all photomicrographs.

arrows). BDNF-IR glial profiles in intact striatum possessed a small cytoplasmic area with slender processes and showed less intense BDNF immunostaining, indicating resting state (Figure 2B and 2C, black arrows). Seven days post-6-OHDA injection, increased number of BDNF-IR glia in the ipsilateral striatum was observed and these cells underwent morphologically transformations into activated state, including intense immunolabeling with BDNF, enlarged cell soma, more numerous as well as thickened of cell processes (Figure 2D, black arrows).

In the saline-injected striatum of sham control mice, increased BDNF immunoreactivity was observed within and in the vicinity of the needle tract. However, this did not significantly increase striatal BDNF immunoreactivity compared to that of the contralateral striatum of sham control mice (Figure 2A). Unilateral 6-OHDA injection significantly increased BDNF immunoreactivity in the contralateral and the ipsilateral striatum compared to that in the corresponding hemisphere of sham control (p<0.05) (Figure 2A, 2E, 2E', 2F, and 2F'), and this response was not affected by DMSO (Figure 2A, 2G, and 2G'). Curcumin administration to 6-OHDA-treated mice significantly increased BDNF immunoreactivity in both striatal hemispheres (p<0.05 vs. the corresponding striatal hemisphere of 6-OHDA- and 6-OHDA/DMSO-injected mice) and the BDNF level in the ipsilateral striatum was significantly greater than that in the contralateral striatum (p<0.05) (Figure 2A, 2H, and 2H'). In contrast to 6-OHDA/curcumin-treated mice, BDNF expression in the ipsilateral striatum of 6-OHDA- and 6-OHDA/DMSO-injected mice were higher but not significantly

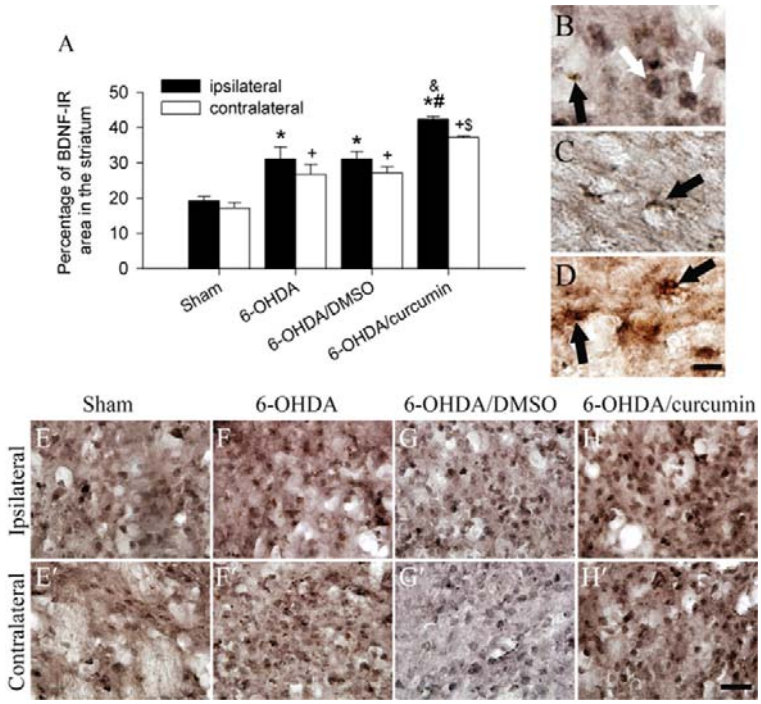

Figure 2. Curcumin increases striatal BDNF immunoreactivity in 6-OHDA-treated mice. (A), Histogram showing the percentage of BDNF-IR area in the ipsilateral striatum and the contralateral striatum of sham control, 6-OHDA-, 6-OHDA/DMSO-, and 6-OHDA/curcumin-injected mice. Values are mean ± SEM. *Indicates significant difference from the ipsilateral striatum of sham mice. # Indicates significant difference from the ipsilateral striatum of 6-OHDA- and 6-OHDA/DMSO-treated animals. + Indicates significant difference from the contralateral striatum of sham mice. $ Indicates significant difference from the contralateral striatum 6-OHDA- and 6-OHDA/DMSO-treated animals. & Indicates significant difference from the contralateral striatum 6-OHDA/curcumin-treated animals. High-power photomicrographs showing BDNF-IR neurons in the striatum of sham mice (white arrows) (B), BDNF-IR resting glia in the contralateral striatum of sham mice (black arrows) (B and C), and BDNF-IR activated glia in the 6-OHDA-lesioned striatum (black arrows) (D). (E-H and E'-H'), Representative photomicrographs depicting BDNF immunoreactivity in the ipsilateral striatum (upper panels) and the contralateral (lower panels) of sham control (E and E'), 6-OHDA- (F and F'), 6-OHDA/DMSO- (G and G'), and 6-OHDA/curcumin-injected mice (H and H'). Scale bar in (D) represents a length of 10 µm for B, C, and D photomicrographs. Scale bar in (H') represents a length of 30 µm for E-H and E'-H' photomicrographs.

different from that of the contralateral striatum (Figure 2A). A negative correlation between the OD of TH immunoreactivity with the BDNF-IR area was observed in the ipsilateral striatum (Figure 3A), while the positive correlation was noted in the contralateral striatum (Figure 3B).

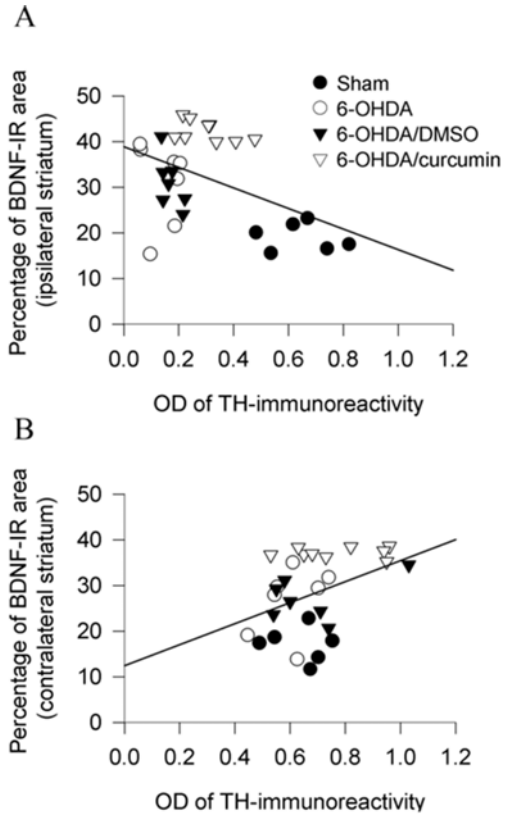

Figure 3. Correlation of the OD of TH immunoreactivity with the percentage of BDNF-IR area in the ipsilateral striatum (A) and the contralateral striatum (B) of sham control, 6-OHDA-, 6-OHDA/DMSO-, and 6-OHDA/curcumin-injected mice. Negative relationship between the OD of TH immunoreactivity and the percentage of BDNF-IR area was observed in the ipsilateral striatum (A), while positive relationship was observed in the contralateral striatum (B). Filled circles represent individual data from sham control mice; open circles represent individual data from 6-OHDA-lesioned mice; filled inverted triangles represent individual data from 6-OHDA/DMSO-treated mice; open inverted triangles represent individual data from 6-OHDA/curcumin-treated mice; solid lines are the regression lines for the OD of TH immunoreactivity and the percentage of BDNF-IR area.

Discussion

The present study shows that 6-OHDA triggers loss of striatal DA axons in the ipsilateral striatum and increases BDNF levels in the ipsilateral and contralateral striatum. Curcumin attenuates 6-OHDA-induced loss of striatal DA axons and increases BDNF levels in both striatal hemispheres of 6-OHDA-lesioned mice.

Previous study has shown that curcumin reverses the 6-OHDA-induced depletion of striatal dopamine[17]. From this data, it is not exactly known what the underlying tissue changes that associate with such benefit effect. Two possible mechanisms may be responsible for this; (1) curcumin enhances striatal dopamine levels through inhibition of monoamine oxidase (MAO)-A and MAO-B[18], leading to an increase in the amount of monoamines stored and released from DA nerve terminals; and (2) curcumin protects nigrostriatal DA axons against 6-OHDA toxin (the present study), resulting in greater striatal dopamine levels than that in lesion-only animals.

In vivo and *in vitro* data indicate neuroprotective effects of curcumin against 6-OHDA, MPTP, or MPP^+[19-21]. Several effects that may contribute to the neuroprotective properties of curcumin include inhibiting the c-Jun N-terminal kinase pathway, restoring the mitochondrial membrane potential, increasing the level of Cu-Zn superoxide dismutase, suppressing an increase in intracellular reactive oxygen species, reducing an overexpression of inducible nitric oxide synthase, and impeding the nuclear factor-kappaB translocation[19-21]. The present study has revealed new evidence that curcumin enhanced BDNF expression in the ipsilateral and contralateral striatum of 6-OHDA-lesioned mice. This is consistent with previous studies which demonstrate that curcumin upregulates BDNF levels in injured brains[14], in hippocampus and frontal cortex of diabetic db/db mice[22], and in the amygdala of animal models of depression[23]. Thus, in animal models of PD curcumin may protect nigrostriatal DA neurons via several mechanisms shown previously[19-21] and also through upregulation of striatal BDNF.

Loss of striatal DA axons in the 6-OHDA-injected striatum was associated with increased striatal BDNF expression. Consistent with these data, Carvey et al[24] observed that (1) the striatum-derived neurotrophic activity is increased after the DA denervation induced by 6-OHDA and (2) this neurotrophic activity is negatively correlated with the striatal dopamine content[24]. The increased striatal BDNF may be a compensatory reaction to the DA denervation in an attempt to protect DA axons against 6-OHDA and/or to increase DA terminal sprouting of remaining viable DA neurons. This, consequently, would help restore striatal dopamine levels.

A significant increase in BDNF expression was also evident in the contralateral striatum of 6-OHDA-, 6-OHDA/DMSO-, and 6-OHDA/curcumin-treated mice compared with that in the contralateral striatum of sham mice despite no deficit in striatal DA axons. Previous studies have shown a significant reduction in the levels of dopamine

metabolites, homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC), in the contralateral striatum of the unilateral, intrastriatal 6-OHDA-injected rats compared with the contralateral striatum of vehicle-injected rats[25]. This suggests that the lesion is indeed not unilateral[25]. Decreased HVA and DOPAC in the contralateral striatum may, at least in part, trigger BDNF upregulation in an effort to maintain HVA and DOPAC levels. This suggestion is supported by the study showing that supranigral infusions of BDNF augment striatal HVA and DOPAC levels[26].

A negative correlation between the OD of TH immunoreactivity and the BDNF-IR area was observed in the ipsilateral striatum, suggesting that injury of TH-IR axons triggers an increase in striatal BDNF. Therefore, the greater loss of the striatal TH-IR axons induces the higher levels of the striatal BDNF. However, BDNF expression in the ipsilateral striatum of 6-OHDA/curcumin group was greater than that in the ipsilateral striatum of 6-OHDA- and 6-OHDA/DMSO-treated group despite less TH-IR axonal loss. This implies that curcumin may not only protect TH-IR axons via several mechanisms[19-21], but also elevate BDNF levels. Supporting to this notion is the data showing that curcumin increased BDNF levels in the contralateral striatum of 6-OHDA/curcumin-treated mice compared with that in the contralateral striatum of 6-OHDA- and 6-OHDA/DMSO-treated groups. Therefore, increased BDNF expression in the ipsilateral striatum of 6-OHDA/curcumin group despite greater striatal TH-IR axons may result from the stimulating effect of curcumin on BDNF levels.

In contrast to the ipsilateral striatum, the positive correlation between the OD of TH immunoreactivity and the BDNF-IR area was observed in the contralateral striatum. This indicates that BDNF is required in maintaining TH-IR axons, thus the greater the striatal TH-IR axons, the greater the BDNF expression. This suggestion is supported by previous study in Wnt-BDNF knockout mice which possess decreased TH protein levels in the striatum, less dense TH-IR fibers in the dorsal striatum, and reduced TH-IR cell number in the substantia nigra compared to those in wild type[27].

Conclusion

The present study shows that curcumin protects nigrostriatal DA axons and increases BDNF content in the 6-OHDA-lesioned striatum. Increased striatal BDNF levels in the ipsilateral striatum may, in part, underlie the neuroprotective effects of curcumin in unilaterally-lesioned 6-OHDA mouse model of PD.

What is already known on this topic?

Curcumin increases BDNF expression in the in vitro study (curcumin protects rat cerebral cortical neurons against glutamate excitotoxicity by increasing BDNF level[28]) and in the in vivo studies: 1) curcumin produces antidepressant effects via increasing of BDNF protein levels in the amygdala of a mice model of depression[23]; 2) curcumin reverses the BDNF reduction in the hippocampus of rat with traumatic brain injury and increases BDNF levels in sham rats[14]; 3) curcumin reestablishes BDNF levels in hippocampus and frontal cortex of diabetic db/db mice, being higher than untreated db/db mice[22]. So far, there is no study investigating the effect of curcumin on striatal BDNF in the unilateral, intrastriatal 6-OHDA-injected rats.

What this study adds?

The present study has revealed new evidence regarding the effect of curcumin on the expression of BDNF protein in the striatum of 6-OHDA-treated mice. The results show that curcumin increased BDNF immunoreactivity in the ipsilateral and contralateral striatum of the 6-OHDA-intoxicated mice.

Acknowledgements

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Potential conflicts of interest

The authors declare no conflicts of interest.

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