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Research Paper

BDNF over-expression induces striatal serotonin fiber sprouting and increases the susceptibility to L-DOPA-induced dyskinesia in 6-OHDA-lesioned rats

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ABSTRACT

In addition to its role in neuronal survival, the brain neurotrophic factor (BDNF) has been shown to influence serotonin transmission and synaptic plasticity, events strongly implicated in the appearance of L-DOPA-induced dyskinesia (LID), a motor complication occurring in parkinsonian patients after long-term treatment with the dopamine precursor.

In order to evaluate a possible influence of BDNF in the appearance of LID, 6-OHDA-lesioned rats received a striatal injection of different concentrations of an adeno-associated viral (AAV) vector over-expressing either BDNF or GFP, as control vector. Eight weeks later, animals started to receive a daily treatment with L-DOPA (4–6 mg/kg plus benserazide 4–6 mg/kg, s.c.) or saline, and dyskinesias, as well as L-DOPA-induced rotations, were evaluated at several time-points. Moreover, molecular changes in striatal D1 receptor-dependent cAMP/PKA and ERK/mTORC signaling pathways, as well as, sprouting of striatal serotonin axons, were measured. Results showed that the AAV-BDNF vector injection induced striatal over-expression of BDNF, as well as striatal and pallidal serotonin axon hyperinnervation. Moreover, rats that over-expressed BDNF were more prone to develop LID and L-DOPA-induced rotations, compared to the GFP-treated control group. Finally, rats that over-expressed BDNF showed increased levels of striatal D1R-dependent signaling phospho-proteins in response to L-DOPA administration. This study suggests that BDNF over-expression, by inducing changes in pre-synaptic serotonin axonal trophism, is able to exacerbate maladaptive responses to L-DOPA administration.

1. Introduction

L-DOPA-induced dyskinesia (LID) is a troublesome side effect that develops in the vast majority of parkinsonian patients under chronic L-DOPA treatment. In recent years, the serotonin system has emerged as a key player in the appearance of LIDs and became a possible target for pharmacological therapies (Bastide et al., 2015; Carta et al., 2007). Indeed, an increasing body of experimental evidence suggests that serotonin neurons can convert L-DOPA into dopamine (DA) and mediate its release as a "false" transmitter. Due to the lack of pre-synaptic mechanisms able to fine-tune L-DOPA-derived DA release, serotonin neurons would contribute to swings in extracellular DA levels after oral administration of L-DOPA, causing, in turn, a pulsatile stimulation of striatal DA receptors, a key event in the appearance of abnormal movements (Arai et al., 1995; Carta et al., 2007; Carta and Tronci, 2014; Iderberg et al., 2015; Tanaka et al., 1999). In agreement with this scenario, toxin lesion or pharmacological blockade of serotonin neuronal activity lead to almost full suppression of LID in experimental

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animal models of Parkinson's disease (PD) (Beaudoin-Gobert et al., 2015; Carta et al., 2007; Muñoz et al., 2008). In line with this evidence, it has been recently demonstrated that LIDs are correlated with the state of the serotonin innervation at striatal level in the rat experimental model of PD as well as in parkinsonian patients; in fact, sprouting of serotonin neuron terminals can further exacerbate development of LID in parkinsonian rats (Rylander et al., 2010).

Rylander et al. have shown that LID correlates with striatal levels of brain-derived neurotrophic factor (BDNF) in 6-OHDA-lesioned rats; this result is in agreement with several studies, which reported that behavioral sensitization to L-DOPA in 6-OHDA-lesioned rats was associated to an upregulation of mRNA levels for BDNF and TrkB receptor in the frontal cortex and striatum (Bordet et al., 1997; Guillin et al., 2003, 2001). Furthermore, several studies suggested that BDNF can produce local trophic effect on serotonergic neurons in the adult intact rat brain, when directly injected as protein (Goggi et al., 2002; Mamounas et al., 2000).

Based on the above evidence, we speculated that striatal injection of an adeno-associated viral (AAV) vector coding for the rat BDNF gene in 6-OHDA-lesioned rats, by over-expressing the neurotrophin, would induce sprouting of serotonin axons, in turn, promoting maladaptive responses to L-DOPA administration and worsening of LID. Here, sprouting of serotonin axons was measured in terms of SERT immunoreactivity, which is considered a reliable index of increased density of 5-HT-positive fibers or axonal varicosities (Bez et al., 2016; Descarries et al., 1995; Rylander et al., 2010). Moreover, based on the existence of a correlation between LID and D1 receptor-dependent cAMP/PKA and ERK/mTORC signaling pathways (Brugnoli et al., 2016; Decressac and Björklund, 2013; Santini et al., 2009b, 2007; Subramaniam et al., 2011), we performed biochemical analysis in order to clarify the molecular features underlying the behavioral effects observed in this study.

2. Material and methods

2.1. Production of the recombinant AAV viral vector

Transfer plasmids carrying adeno-associated viral (AAV5) inverted terminal repeat coding for either rat BDNF or enhanced green fluorescent protein (GFP), downstream of a cytomegalovirus enhancer hybrid synthetic chicken β-actin (CBA) promoter, were generated. Transfection into HEK 293 cells was carried out using the calcium phosphate method, and included the appropriate transfer plasmid encoding-enhanced BDNF or GFP and the packaging plasmids pDP5 encoding for the AAV5 capsid proteins. Cells were transfected with 2.5 mg of DNA with equimolar amounts of helper and transfer DNA. Transfected cells were incubated for 3 days before being harvested in phosphate buffered saline–EDTA. The cell pellet was treated with a lysis buffer (50 mM Tris, 150 mM NaCl, pH 8.4) and lysed through freeze–thaw cycles in a dry ice/ethanol bath. The lysate was then treated with 21 U/ml benzonase (Sigma) for nuclear digestion. The crude lysates were purified first by ultracentrifugation (1.5 h at 350,000*g* at 18 °C) in a discontinuous iodixanol gradient and the virus-containing fractions were purified with ion-exchange chromatography using fast protein liquid chromatography. The virus suspension was then concentrated using a concentrator (Millipore Amicon Ultra, 100 kDa molecular weight cut-off) at 1500 g and 18 °C in two consecutive steps by adding phosphate buffered saline. The physical titres of recombinant AAV5 vectors were determined using dot blot quantification as described previously (Zolotukhin et al., 1999). Genome copy titres were determined using real-time quantitative polymerase chain reaction PCR, and the following vector concentrations were used: 1.2×10^{14} and 3.1×10^{13} genome copies/ml for BDNF and GFP, respectively. The AAV-GFP vector was injected as a control at a dilution that matched the number of genome copy per milliliter of the transgene of interest.

2.2. Animals

Adult male Sprague–Dawley rats (275–300 g; Harlan, Italy) were used in the present study and housed on a 12 h light/dark cycle (light on 7:00–19:00) with free access to food and water. All animal work was performed in accordance with regulations set by the European Union (EEC Council 86/609; D.P.R. 116/92).

2.3. Surgical procedures

2.3.1. Experimental parkinsonism

All 6-OHDA injections were conducted using a stereotaxic frame with an attached Hamilton syringe and under general anesthesia, induced by an i.p. injection of a 20:1 mixture of Fentanest (Pfizer, Italy) and Dormitor® (Orion pharma, Italy) at a volume range of 1.4–1.6 ml. The animals received 6-OHDA injection into the middle forebrain bundle (16 μg free base in 4 μl in 0.02% L-ascorbic acid in 0.9% saline), at the following coordinates relative to the bregma (Paxinos and Watson, 2007): AP: − 4.4 mm, ML: − 1.2 mm; DV: − 7.8 mm from the dura, in order to achieve a complete lesion of the nigrostriatal pathway. Injection speed was 1.0 μl/min and the syringe was kept in place for an additional 3 min period before it was slowly retracted.

2.3.2. AAV vector injection

Surgical procedure for AAV vector injection was performed under general anesthesia, as used for 6-OHDA. To give less damage to the brain, a glass capillary (outer diameter 60–80 μm) was fitted onto the needle of a 5 μl Hamilton syringe. Rats received 4 μl (2 μl/site; 1 μl/deposit) of the AAV-BDNF or AAV-GFP solution into the 6-OHDA-lesioned striatum at the following coordinates (flat skull position) relative to the bregma (Paxinos and Watson, 2007): AP/ML = + 1/− 2.8 mm, DV = − 5/− 4 mm; AP/ML = + 0.2/− 4 mm, DV = − 5.5/− 4.5 mm from the dura surface. Injection speed was 0.5 μl/min and the needle was left in place for an additional 3 min period before it was slowly retracted.

2.4. Experimental design

2.4.1. Effect of BDNF over-expression on LID

In order to have a complete picture of the effect of BDNF over-expression on development of dyskinesia, we performed different consecutive experiments with escalating dilutions of the vector. Thus, we started with an undiluted vector and then we progressively lowered the concentration, up to 3% of the initial concentration. This was done because it has been demonstrated that, depending on the dose, neurotrophic factors may have a dual action on specific brain functions (Arias et al., 2014; Mamounas et al., 2000; Paredes et al., 2007). Thus, in a first set of experiments (Exp. 1, Fig. 1), three weeks after the 6-OHDA lesion ($n = 20$), rats were subjected to the stepping test in order to include in the study only rats with a severe impairment (stepping test score between 0 and 2, data not shown). One week later, rats having a mean not higher than two steps were divided into two well-matched groups and subjected to the striatal injection of an undiluted AAV vector solution for the expression of BDNF, or GFP (as a control). Eight weeks after, rats that over-expressed BDNF or GFP were chronically treated with L-DOPA (6 mg/kg plus benserazide 6 mg/kg, s.c., $n = 8$/group), and abnormal involuntary movements (AIMs) were scored for 3 weeks. L-DOPA-induced rotational behavioral was also evaluated at day 7 and 15 of L-DOPA treatment. At the end of the behavioral tests, rats were killed and the brain processed for the tyrosine hydroxylase (TH) immunohistochemical evaluation.

One more group of 6-OHDA-lesioned rats (Exp. 2, Fig. 1) were injected with different AAV vector dilutions for BDNF (10% and 20%) or GFP, and LID was evaluated eight weeks after the virus injection. For this experiment we also used a lower dose of L-DOPA (4 mg/kg plus benserazide 4 mg/kg, s.c.) to better highlight the difference in AIM

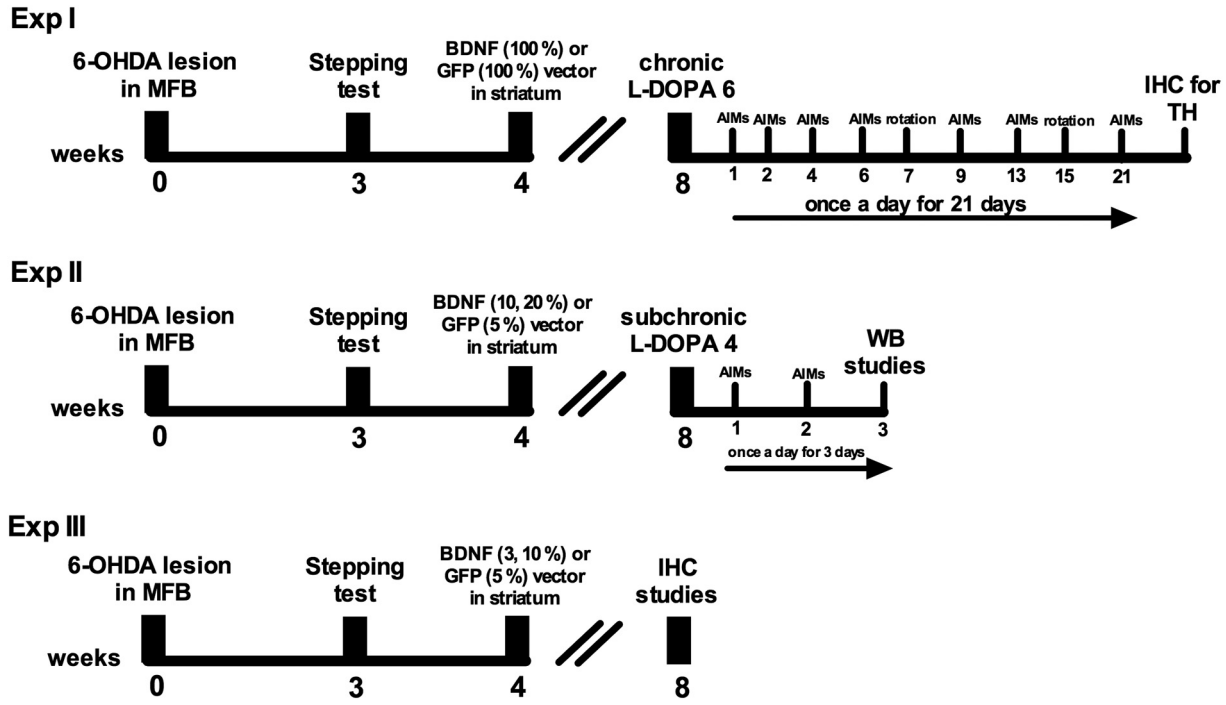

Fig. 1. Schematic representation of the experimental plan. Exp (experiment); MFB (middle forebrain bundle); AIMs (abnormal involuntary movements); IHC (immunohistochemistry); WB (Western blotting).

expression between the BDNF or GFP over-expressing rats (n = 6–10/group). AIMs were evaluated for two consecutive days after L-DOPA administration and, at day 3, rats were killed 30 min after L-DOPA injection and the striatum dissected for biochemical studies, while the midbrain processed for TH immunohistochemical evaluation. Finally, a third group of 6-OHDA-lesioned rats (Exp. 3, Fig. 1) was injected with two different AAV-BDNF vector solutions (3% and 10%) or GFP (as a control) (n = 7/group); rats were then sacrificed eight weeks after AAV vector injection, without any L-DOPA challenge, for the evaluation of the striatal serotonin terminals innervation and BDNF over-expression by immunohistochemistry.

2.5. L-DOPA-induced dyskinesia

AIMs were evaluated according to the rat dyskinesia scale described in detail previously (Lundblad et al., 2002; Muñoz et al., 2009; Tronci et al., 2014). Briefly, rats were placed individually in transparent plastic cages without bedding material and scored every 20 min following drug injection for the entire time course of dyskinesias. AIMs were classified into three subtypes according to their topographic distribution as forelimb, orolingual and axial behaviors. The severity of each AIM subtype was assessed using scores from 0 to 4 (1: occasional, i.e. present < 50% of the time; 2: frequent, i.e. present > 50% of the time; 3: continuous, but interrupted by strong sensory stimuli; 4: continuous, not interrupted by strong sensory stimuli).

2.6. L-DOPA-induced rotation

L-DOPA-induced rotation test was performed at day 7 and 15 during L-DOPA treatment. Right and left full body turns were recorded over 120 min after L-DOPA injection, using automated rotometer bowls (AccuScan Instrument Inc., Columbus, Ohio) (Tronci et al., 2012). Data are expressed as net full body turns per min, where rotation contralateral to the side of the lesion was given a positive value.

2.7. Stepping test

Stepping test was performed as previously described (Tronci et al., 2013). The number of adjusting steps was counted while the rat was moved sideways along a table surface (90 cm in 5 s), in the forehand and backhand direction, for both forelimbs.

2.8. Immunohistochemistry

Perfused brains (ice-cold 4% paraformaldehyde in phosphate buffered saline (PBS)) were transferred to 25% sucrose in PBS for cryoprotection overnight, and sections of 40 μm were obtained using a freezing microtome (Leica). Free-floating immunohistochemistry was performed as previously described (Tronci et al., 2012). Briefly, sections were incubated at room temperature with the primary antibody, rabbit *anti*-TH (1:1000, Chemicon), rabbit *anti*-BDNF sc-546 (1:1000, Santa Cruz), mouse *anti*-SERT (1:1000, Chemicon), and rabbit *anti*-GFP (1:20,000, Abcam). On the second day, sections were incubated for 1 h in a 1:200 dilution of specific biotinylated secondary antibody. After rinsing, sections were treated with avidin-biotin complex (ABC Elite Kit, Vector Laboratories) in KPBS for 1 h. The colour reaction was developed by incubation in 25 mg/ml 3,3′-diamminobenzidine and 0.005% H_2O_2.

TH immunohistochemistry was performed to verify the dopaminergic lesion; all rats were found to have an almost complete loss of the striatal and nigral TH signal (> 90%) (data not shown).

SERT-positive innervation was assessed in coronal section of striatum (at rostral, medium and caudal level) and globus pallidus of AAV-BDNF- and AAV-GFP-injected rats eight weeks after virus injection and without any L-DOPA challenge. Optical density was determined using the image analysis software ImageJ (Tronci et al., 2015).

2.9. Biochemical studies

For the determination of striatal BDNF levels, striatal proteins were extracted from rats as previously described (Guida et al., 2015). Samples were sonicated in a lysis buffer (320 mM sucrose, 50 mM Tris HCl

pH 7.5, 50 mM NaCl, 1% Triton X-100, 5 mM β-glycerol phosphate, 1 mM Na3VO4, 5 mM NaF, protease inhibitor cocktail), and incubated on ice for 30 min. Samples were spun at 12,000 g × 10 min and the supernatant transferred to fresh microfuge tube. Aliquots of the homogenate were used for protein determination using Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA). Equal amounts of total proteins (30 μg) for each sample were loaded onto 10% polyacrylamide gels, separated by SDS-PAGE and transferred overnight to membranes (PVDF; Amersham Pharmacia Biotech, Uppsala, Sweden). The membranes were immunoblotted overnight using selective antibodies against BDNF (1:1000, Santa Cruz Biotechnology). BDNF optical density values were normalized using antibodies against GAPDH (1:1000, Santa Cruz Biotechnology). For the evaluation of the striatal pThr202/Tyr204-ERK1/2, pSer240/244-S6 and pSer845-GluR1 levels, rat striata were processed according to a previous protocol (Subramaniam et al., 2011). Briefly, samples were sonicated in 1% SDS and boiled for 10 min. Aliquots of the total homogenate were used for protein determination using Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA). Equal amounts of proteins (30 μg) for each sample were loaded onto 4–20% polyacrylamide gels. Proteins were separated by SDS-PAGE and transferred to PVDF membranes (Bio-Rad Laboratories) by means of "The Trans-Blot Turbo Transfer System" (Bio-Rad Laboratories). Membranes were immunoblotted overnight using selective antibodies against pSer240/244-S6, pThr202/Tyr204-ERK1/2 (each diluted 1:1000, Cell Signaling Technology, Beverly, MA) and pSer845-GluR1 (1:1000, PhosphoSolutions, Aurora, CO). Then, in order to remove primary antibodies, membranes were treated with stripping solution (StripAblot-EuroClone S.p.A-Milan, Italy), according to the manufacturer's protocol. Antibodies against S6, Thr202/Tyr204-ERK1/2 (both diluted 1:1000, Cell Signaling Technology) and GluR1 (diluted 1:1000, Chemicon, Temecula, CA, USA), that are not phosphorylation state specific, were used, on the stripped membranes, to estimate the total amount of proteins. Blots were then incubated in horseradish peroxidase-conjugated secondary antibodies and target proteins visualized by ECL detection (Pierce, Rockford, IL), followed by quantification through the "Quantity One" software (Bio-Rad).

Representative blots shown in the figures arise from cut out bands and pasted for reassembling the image.

2.10. Statistical analysis

Statistical analysis was performed using GraphPad software. Group comparisons were performed using either one-way factorial ANOVA or two-way ANOVA, where appropriated, followed by Newman-Keuls or Bonferroni multiple comparisons tests. For dyskinesia evaluation, the main effect was verified by nonparametric Kruskal–Wallis test. We reported actual *p*-values from the ANOVAs. Statistical significance was set at $p < 0.05$. All values are presented in the study as mean ± SEM.

2.11. Drugs

All the drugs were diluted in 0.9% sterile saline. 6-OHDA and benserazide were purchased from Sigma-Aldrich; L-3,4-dihydroxyphenylalanine methyl ester hydrochloride (L-DOPA) was purchased from Research Organics, Cleveland, OH.

3. Results

3.1. Effect of BDNF over-expression on L-DOPA-induced dyskinesia and rotation

A first experiment was performed using an undiluted BDNF vector (Exp. 1). As shown in Fig. 2A, repeated administration of L-DOPA (6 mg/kg, plus benserazide 6 mg/kg, s.c., $n = 8$/group) induced development of dyskinesia in both BDNF and GFP over-expressing rats during the course of the treatment. However, rats that over-expressed BDNF showed a higher susceptibility to develop dyskinesia, during the first four L-DOPA injections, as compared to GFP control animals, while this difference disappeared when LID reached a plateau in the two groups. Two-way ANOVA repeated measures: Time: $p < 0.05$; Treatment: $p < 0.05$; Time × Treatment: $p = 0.22$. Post hoc Bonferroni multiple comparisons test: $^{*}p < 0.05$ vs the same time point of GFP 100% + L-DOPA 6 group ($n = 8$/group).

Rotational behavior test was performed at day 7 and 15 of L-DOPA chronic administration. BDNF over-expressing rats showed increased rotational behavior contralateral to the lesioned striatum, in response to L-DOPA, as compared to GFP over-expressing rats; that effect was maintained at day 15, when the difference in dyskinesia between the two groups was no longer evident ($n = 8$/group; Fig. 2B–C and D–E). For Fig. 2B–D, *t*-test: $^{*}p < 0.05$ vs GFP 100% + L-DOPA 6; for Fig. 2C–E, two-way ANOVA followed by post hoc Bonferroni multiple comparisons test: $^{*}p < 0.05$ vs the same time point of GFP 100% + L-DOPA 6 group ($n = 8$/group).

In order to evaluate whether lower concentrations of the BDNF vector were able to produce similar effect on dyskinesia, we also used the AAV-BDNF vector at 10% and 20% concentrations. For these experiments a lower dose of L-DOPA (4 mg/kg, plus benserazide 4 mg/kg, s.c.) was used in the attempt to better highlight the differences in the AIMs score between BDNF and GFP over-expressing rats (Exp. 2). As shown in Fig. 3A, results showed that all the different dilutions of the BDNF vector made the rats more prone to develop dyskinesia as compared to the GFP-control group ($n = 7$–10/group). One-way ANOVA followed by Newman-Keuls Multiple Comparison Test: $^{*}p < 0.05$ vs GFP 5% + L-DOPA 4 ($n = 8$–10/group).

To note, Western blot analysis of BDNF over-expression into the striatum ipsilateral to the injection side showed similar protein levels for both dilutions (Fig. 3B). One-way ANOVA followed by Newman-Keuls Multiple Comparison Test: $^{*}p < 0.05$ vs GFP 5% + L-DOPA 4; $\hat{}p < 0.05$ vs the corresponding intact side ($n = 8$–10/group).

3.2. Influence of BDNF over-expression on striatal D1 receptor-dependent signaling pathways

The day after the last AIM test, rats from Fig. 3 were treated with L-DOPA (4 mg/kg plus benserazide 4 mg/kg, s.c.) and sacrificed 30 min later, in order to collect striatal tissues for Western blotting analysis. Thus, the impact of BDNF over-expression on striatal D1 receptor-dependent signaling pathways was evaluated by measuring *p*-GluR1 and *p*-ERK, downstream targets of PKA activation, and *p*-S6, a downstream effector of mTORC1 activation. Rats were killed after the third L-DOPA administration with the intention to evaluate striatal molecular changes when the difference in AIMs score between the two experimental groups was still evident.

Since the two different dilutions of the AAV-BDNF vector used in this experiment (10% and 20%) showed the same effect on LID and similar levels of BDNF protein over-expression in the striatum (Fig. 3), these two groups of rats were considered as one. Results showed that BDNF over-expression significantly affected PKA activity, as observed by the higher GluR1 phosphorylation levels at Ser845 residue in the striatum ipsilateral to the 6-OHDA lesion and to the virus injection side, compared to GFP-treated group (Fig. 4A; $n = 6$–17/group). To note, although not statistically significant, *p*-GluR1 levels seemed to increase also in the striatum contralateral to the virus injection. One-way ANOVA followed by Newman-Keuls Multiple Comparison Test: $^{*}p < 0.05$ ($n = 6$–17/group).

No differences were found on *p*-Thr202/Tyr204-Erk levels in the lesioned striatum injected with BDNF compared to the GFP-injected groups (Fig. 4B; $n = 6$–17/group). One-way ANOVA followed by Newman-Keuls Multiple Comparison Test: $p > 0.05$ ($n = 6$–17/group). Conversely, a significant increase on the *p*-Ser240/244-S6, a downstream target of mTORC1 pathway was observed in the lesioned striatum of BDNF over-expressing rats compared to GFP alone or GFP

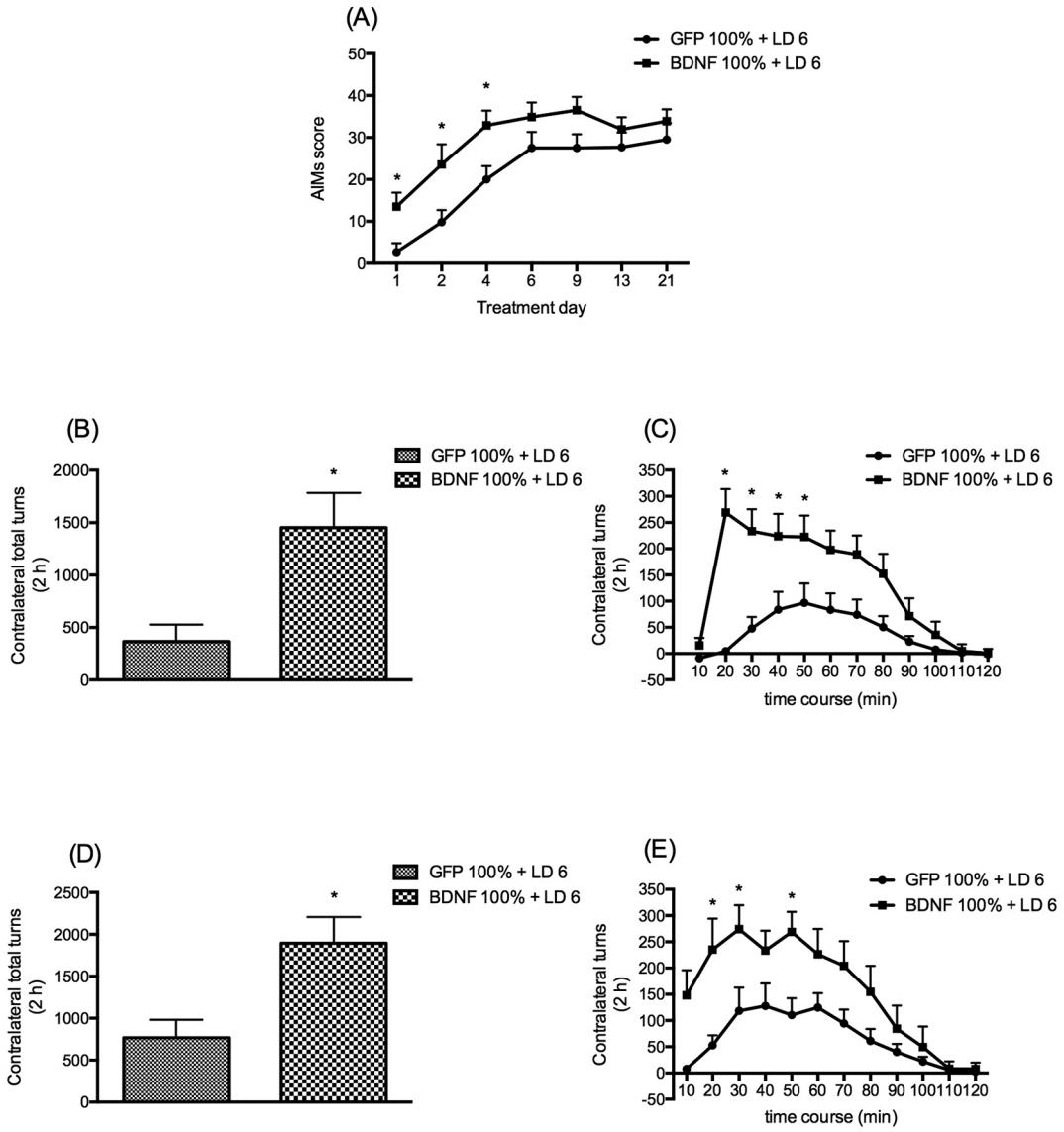

Fig. 2. (A) Unilateral striatal injection of AAV-vector for the BDNF gene (undiluted solution) in 6-OHDA-lesioned rats increased the susceptibility of rats to develop AIMs during the first four L-DOPA (6 mg/kg plus benserazide 6 mg/kg, s.c.) administrations, compared to the GFP 100% + L-DOPA 6 treated group. Two-way ANOVA repeated measures: Time: $p < 0.05$; Treatment: $p < 0.05$; Time × Treatment: $p = 0.22$. Post hoc Bonferroni multiple comparisons test: $^{*}p < 0.05$ vs the same time point of GFP 100% + L-DOPA 6 group ($n = 8$/group). The main effect was also verified by nonparametric Kruskal–Wallis test.

(B–C; D–E) Rats that over-expressed BDNF also showed higher L-DOPA-mediated contralateral turning behavior at day 7 (B–C) and 15 (D–E) of L-DOPA chronic treatment, compared to the GFP + L-DOPA 6 treated group. For Fig. 2B–D, *t*-test: $^{*}p < 0.05$ vs GFP 100% + L-DOPA 6; for Fig. 2C–E, two-way ANOVA followed by post hoc Bonferroni multiple comparisons test: $^{*}p < 0.05$ vs the same time point of GFP 100% + L-DOPA 6 group ($n = 8$/group).

plus L-DOPA-treated rats (Fig. 4C; $n = 6$–13/group). One-way ANOVA followed by Newman-Keuls Multiple Comparison Test: $^{*}p < 0.05$ vs the lesioned side of GFP 5%; $\hat{p} < 0.05$ vs the lesioned side of GFP 5% + L-DOPA 4; $^{\#}p < 0.05$ vs the corresponding intact side ($n = 6$–13/group).

3.3. Effect of BDNF over-expression on serotonin axonal sprouting

A separate group of 6-OHDA-lesioned rats was used to evaluate the effect of BDNF over-expression on serotonin axonal sprouting at striatal and pallidal level (Exp. 3). For this purpose, rats received, in the striatum ipsilateral to the lesion, an AAV vector preparation of BDNF at 10% or GFP (5%, as a control) ($n = 7$/group). One more group of rats received AAV-BDNF at 3%, in order to evaluate the effect on serotonin innervation induced by a lower concentration of the protein. BDNF 3% was previously tested in a separate group of lesioned rats on induction of LID, and, as observed for the higher concentrations, BDNF 3% also increased susceptibility of rats to develop AIMs (One-way ANOVA: AIM score at 1st day of L-DOPA: 1.07 ± 0.49 vs 7.13 ± 2.74 for GFP 5% plus L-DOPA 4 mg and BDNF 3% plus L-DOPA 4 mg, respectively, $p < 0.05$; AIM score at 2nd day of L-DOPA: 4.64 ± 1.71 vs 19.13 ± 6.97 for GFP 5% plus L-DOPA 4 mg and BDNF 3% plus L-DOPA 4 mg, respectively, $p < 0.05$; $n = 7$/group).

Rats that over-expressed BDNF (3% and 10%) or GFP ($n = 7$/group) were sacrificed eight weeks after virus injection without any L-DOPA challenge. Optical density of SERT expression was measured at three different striatal levels and at globus pallidus level. Results showed that 6-OHDA lesion did not modify the expression of SERT compared to the intact side, as demonstrated in the GFP-treated group (Fig. 5A–B–C). Interestingly, both dilutions of the AAV-BDNF vector induced a significant serotonin terminal sprouting in the rostral and medial striatum and globus pallidus. BDNF 3% also increased serotonin sprouting at caudal striatum. High magnification images from striatum and globus pallidus showed that the effect of BDNF over-expression on SERT

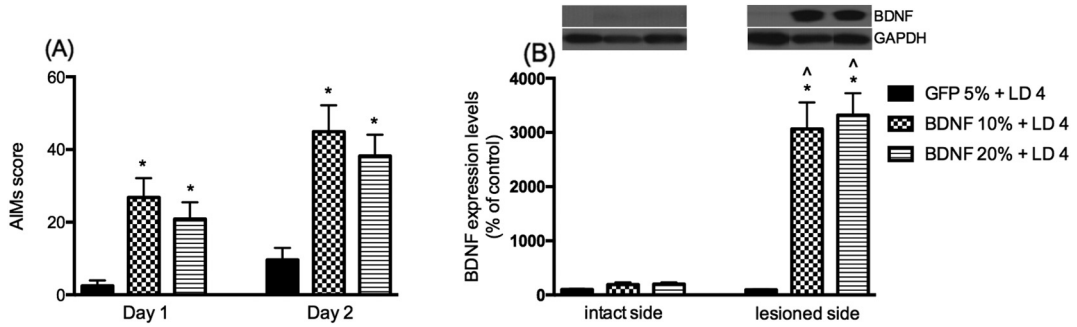

Fig. 3. (A) Unilateral striatal injection of AAV vector for the BDNF gene (10% and 20%) in 6-OHDA-lesioned rats increased the susceptibility of rats to develop AIMs at day 1 and day 2 of L-DOPA (4 mg/kg plus benserazide 4 mg/kg, s.c.) administrations, compared to the GFP 5% + L-DOPA 4 treated group. No difference was observed between the two different BDNF dilutions on AIMs expression. One-way ANOVA followed by Newman-Keuls Multiple Comparison Test: $^{*}p < 0.05$ vs GFP 5% + L-DOPA 4 (n = 8–10/group). The main effect was also verified by nonparametric Kruskal–Wallis test. (B) Western blotting quantification of striatal BDNF expression showed the same amount of protein for both dilutions of the vector and high levels of the protein compared to the GFP 5% + L-DOPA 4 treated group. One-way ANOVA followed by Newman-Keuls Multiple Comparison Test: $^{*}p < 0.05$ vs GFP 5% + L-DOPA 4; $\hat{p} < 0.05$ vs the corresponding intact side (n = 8–10/group).

immunoreactivity is associated with increased density of serotonin fibers (Fig. 5A′–B′). No difference was found in the intact side among the experimental groups (Fig. 5A–B–C). One-way ANOVA followed by Newman-Keuls multiple comparisons test: $^{*}p < 0.05$ vs the corresponding intact side; $^{\#}p < 0.05$ vs the lesioned side of GFP 5% group (n = 6–7/group).

3.4. Efficient viral vector-mediated over-expression of BDNF in the striatum

The AAV vector-mediated over-expression of the BDNF protein in the striatum ipsilateral to the 6-OHDA lesion was examined by immunohistochemistry, eight weeks after virus delivery (Exp. 3). As showed in Fig. 6A, the over-expression affected a large portion of the striatum both in BDNF 3% and 10% injected rats (n = 7/group). However, high magnification images of the striatum revealed a higher number of cells with intense BDNF staining in rats treated with BDNF 10%, compared to those injected with BDNF 3%. No cells expressing high intense BDNF staining were found in rats injected with AAV-GFP (Fig. 6B); by contrast, the efficiency of AAV-GFP infection was confirmed by GFP immunohistochemistry (data not shown) (n = 7).

Interestingly, the portion of the striatum that over-expressed BDNF appeared to overlap the extension of the serotonin terminals sprouting (Fig. 5), remarking a BDNF-mediated increase of serotonin innervation.

4. Discussion

This study demonstrates for the first time that striatal injection of an AAV vector coding for the rat BDNF gene induced long-term over-expression of the neurotrophin, as well as striatal and pallidal serotonin axon hyperinnervation in 6-OHDA-lesioned rats, as measured by SERT immunostaining. Moreover, administration of L-DOPA to these rats resulted in increased susceptibility to development of LID. The exacerbation of LID in the BDNF over-expressing rats was also accompanied by up-regulation of post-synaptic markers of dyskinesia at striatal neurons.

BDNF is already known to have trophic effects on serotonin neurons in intact rats; indeed, increased serotonin axon innervation has been reported upon short-term exposure to the protein (Bregman et al., 1997; Mamounas et al., 2000, 1995). Here, we demonstrate that chronic over-expression obtained by an AAV vector produced similar effect also in DA depleted animals. Moreover, our results directly linked the augmented striatal BDNF expression to the increased susceptibility to development of AIMs. This event is likely produced via the trophic effect that BDNF exerts on striatal serotonin neuron innervation, further supporting the key role of serotonin neurons in the appearance of LID. Indeed, several previous studies demonstrated the ability of serotonin neurons to contribute to unregulated DA release after L-DOPA administration (Carta et al., 2007; Carta and Tronci, 2014; Lindgren et al., 2010). In support of a pivotal role of this source of DA release in the appearance of LID, toxin lesion or pharmacological blockade of serotonin neurons suppressed dyskinesia in parkinsonian rats and MPTP treated monkeys (Beaudoin-Gobert et al., 2015; Carta et al., 2007; Muñoz et al., 2008). In light with these results, serotonin terminal sprouting would increase the buffering capacity for the exogenous L-DOPA, and further dysregulate DA release; this, in turn, would lead to the worsening of AIMs, as observed in the present study. Interestingly,

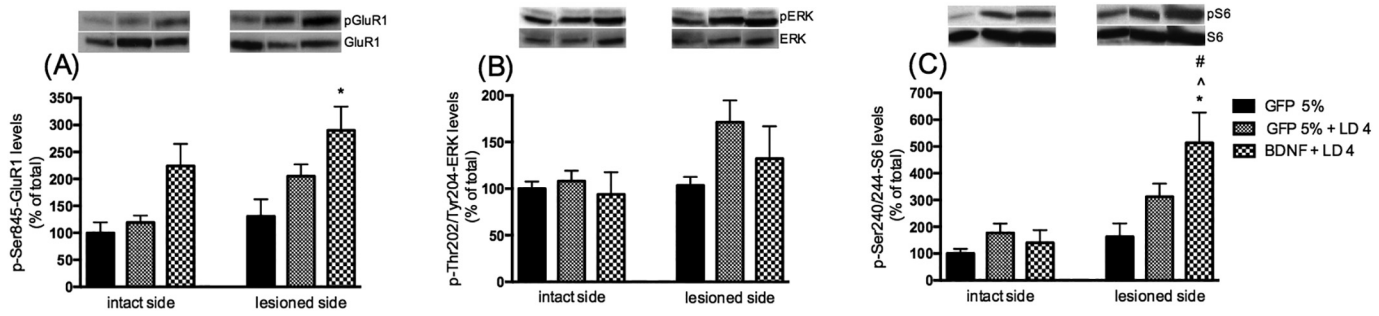

Fig. 4. For Western blotting studies, rats that over-expressed BDNF at 10% and 20% were analyzed as one group given the similar values of AIMs and BDNF striatal expression observed. (A) Results showed that, in BDNF plus L-DOPA (4 mg/kg plus benserazide 4 mg/kg, s.c.) treated rats, levels of *p*-Ser845-GluR1, an index of the cAMP/PKA signaling pathway activation, were significantly increased in the striatum that over-expressed BDNF, compared to the lesioned side of GFP 5% group; one-way ANOVA followed by Newman-Keuls Multiple Comparison Test: $^{*}p < 0.05$ (n = 6–17/group). (B) Analysis of *p*-Thr202/Tyr204-ERK striatal levels showed no differences among the experimental groups; one-way ANOVA followed by Newman-Keuls Multiple Comparison Test: $p > 0.05$ (n = 6–17/group). (C) Analysis of *p*-Ser240/244-S6 levels, an index of ERK/mTORC1 pathway activation, showed higher levels in BDNF over-expressing rats compared to both GFP 5% and GFP 5% + L-DOPA 4 treated groups, in the injected striatum; one-way ANOVA followed by Newman-Keuls Multiple Comparison Test: $^{*}p < 0.05$ vs the lesioned side of GFP 5%; $\hat{p} < 0.05$ vs the lesioned side of GFP 5% + L-DOPA 4; $^{\#}p < 0.05$ vs the corresponding intact side (n = 6–13/group).

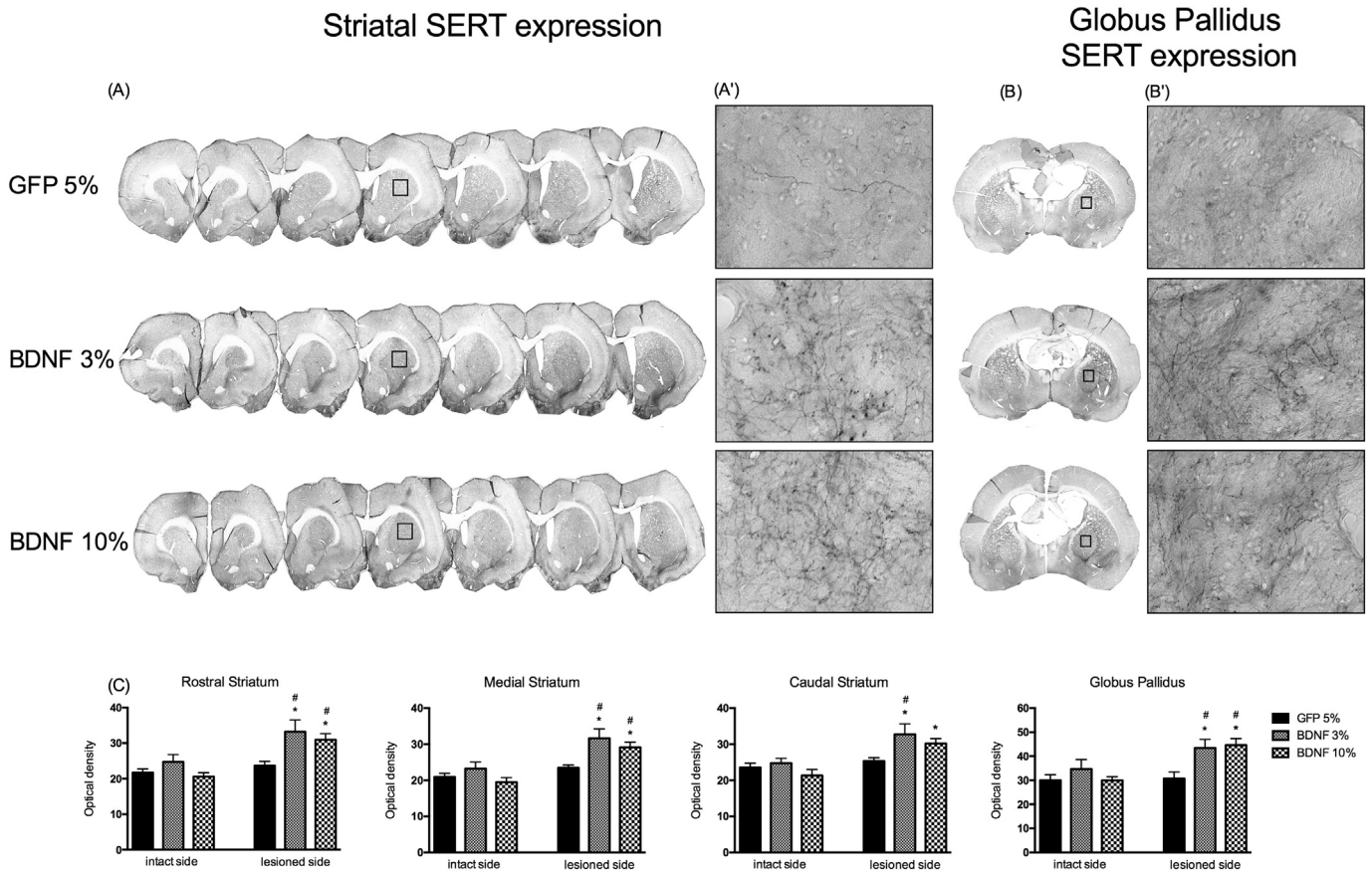

Fig. 5. (A–B) Representative photomicrographs of SERT expression in coronal sections of the striatum and globus pallidus of rats that over-expressed BDNF (3% and 10%) or GFP. (A′–B′) 60 × magnification insets from striatum and globus pallidus showing SERT positive fibers. Rats were killed eight weeks after virus injection without any L-DOPA challenge. (C) Optical density analysis for SERT expression in the striatum that over-expressed BDNF showed that both dilutions of BDNF significantly increased SERT levels in the rostral and medial striatum, as well as in the globus pallidus, compared to the GFP control group. BDNF 3% also increased SERT expression at striatal caudal level. One-way ANOVA followed by Newman-Keuls multiple comparisons test: $^{*}p < 0.05$ vs the corresponding intact side; $^{\#}p < 0.05$ vs the lesioned side of GFP 5% group (n = 6–7/group).

PD patients with dyskinesia have been found to have a more preserved serotonin innervation in caudate-putamen and globus pallidus, as compared to non-dyskinetic subjects, highlighting the clinical relevance of the animal studies (Politis et al., 2014). However, nothing is known as to why dyskinetic patients present a relatively preserved striatal serotonin innervation, while non-dyskinetic subjects show a reduction. Our study may suggest a possible role of BDNF in this event.

Previous studies have shown that L-DOPA treatment can induce BDNF expression in striatum, cortex and subthalamic nucleus of parkinsonian animals (Guillin et al., 2003, 2001; Zhang et al., 2006). Moreover, Rylander et al. (2010) showed that L-DOPA administration can induce sprouting of striatal serotonin axons in 6-OHDA-lesioned rats. Of note, this response was also associated with increased depolarization-induced [^{3}H]DA release and with a stronger release potentiation by BDNF. Thus, the over-expression of BDNF that we obtained by an AAV vector, is likely to exacerbate a process that would gradually take place overtime upon daily L-DOPA administration, leading to increased false transmitter release of DA from serotonin neurons and post-synaptic DA receptor hypersensitization. A significant difference in AIM score between rats that over-expressed BDNF and

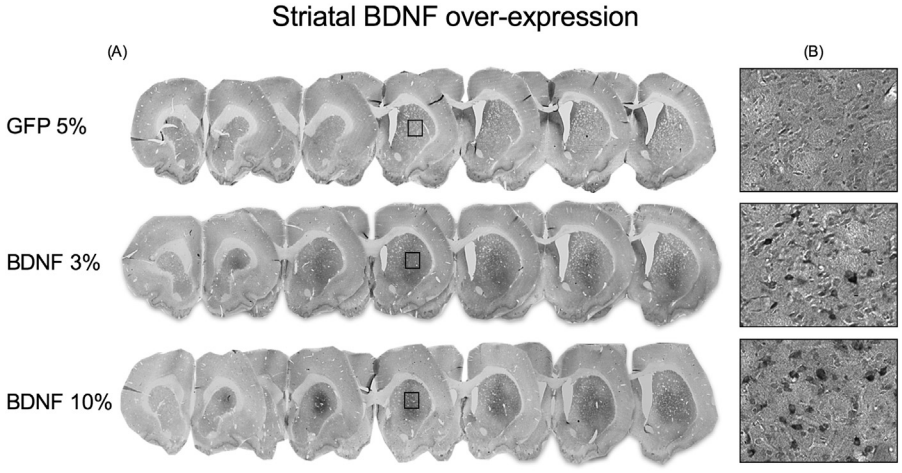

Fig. 6. (A) Representative photomicrographs showing the extension of the BDNF over-expression in striatal coronal sections of 6-OHDA-lesioned rats unilaterally injected with the AAV vector for BDNF (3% and 10%) or GFP 5%. (B) 20 X magnification photomicrographs showing striatal neurons that over-expressed BDNF in BDNF (3% and 10%) or GFP 5% treated groups.

controls was present only for the first week of treatment, while AIM scores were similar among groups as a plateau was reached (at least for the undiluted vector). This event is likely to be due to the fact that, after a certain number of L-DOPA treatments, similar effect on LID was achieved in control animals via the L-DOPA-induced endogenous BDNF. In addition, the increased rotation observed in the BDNF over-expressing rats may have partially masked the pro-dyskinetic effect of L-DOPA.

Our study also showed that the increased vulnerability of BDNF over-expressing rats to development of LID is accompanied by higher striatal levels of phospho-protein typically associated with dyskinesia; in fact, BDNF over-expressing rats showed increased D1 receptor-dependent cAMP/PKA and mTORC signaling cascades in response to L-DOPA administration, compared to GFP-treated control group (Pavón et al., 2006; Santini et al., 2007; Subramaniam et al., 2011; Westin et al., 2007). Unlike previous studies, in the present work these canonical markers of dyskinesia were not different between the GFP L-DOPA treated group and the GFP drug-naive animals, although there was a clear trend. This discrepancy may conceivably be due to several factors, including the low L-DOPA dose (4 mg/kg), the few L-DOPA treatments, the different animal model, or the different approach for marker quantification used in this study compared to others (Lebel et al., 2010; Rylander et al., 2009; Santini et al., 2009a; Westin et al., 2007).

It is worth to point out that BDNF is known to affect other key events involved in the appearance of dyskinesia beside induction of serotonin axon sprouting, such as NMDA receptor trafficking and channel activity, as well as striatal synaptic plasticity (Gardoni et al., 2006; Lu, 2003; Suen et al., 1997; Woo et al., 2005; Xu et al., 2006). Moreover, it has also been demonstrated that BDNF controls DA D3 receptor expression in rodent models of PD (Guillin et al., 2003, 2001; Razgado-Hernandez et al., 2015). Furthermore, BDNF is able to potentiate D3-mediated motor responses, and reverse striatal spine loss (Razgado-Hernandez et al., 2015). Hence, the influence of BDNF on dyskinesia may be due not only to its ability to affect the state of the striatal serotonin innervation, but also to its ability to affect other key post-synaptic events involved in dyskinesia. Further studies are required to investigate this possibility.

The present approach for studying the role of BDNF in dyskinesia appears to induce supra-physiological levels of this neurotrophin; in this sense, one may argue that such levels are never reached in physiological or pathophysiological conditions. While this remains to be further investigated, it is likely that only a low percentage of the BDNF produced by the integrated virus is physiologically active, or, in other words, is able to activate the BDNF receptors, promoting pre- and post-synaptic alterations at striatal level. In line with this interpretation, no difference in terms of dyskinesia, or SERT immunoreactivity, was seen among the groups treated with different vector dilutions. This is an important observation, as BDNF has been previously seen to produce biphasic effects on serotonin axon sprouting and neurotransmitter release when acutely administered to intact rats at different concentrations (Mamounas et al., 2000; Paredes et al., 2007).

In conclusion, our data indicate that BDNF over-expression, by inducing changes in pre-synaptic serotonin axonal trophism, is able to exacerbate maladaptive responses to L-DOPA administration, suggesting a possible pathophysiological role of BDNF in the appearance of dyskinesia, at least in the rat model of PD.

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