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Biochemical and Biophysical Research Communications 318 (2004) 113–118
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BDNF is induced by wild-type α-synuclein but not by the two mutants, A30P or A53T, in glioma cell line

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Received 1 April 2004

Abstract

Parkinson's disease (PD) is one of the most prevalent neurodegenerative diseases but its etiology is unclear. α-Synuclein (α-SN) is a major component of Lewy bodies and Lewy neurites, and its missense mutations, A30P and A53T, cause familial PD. In PD, α-SN-positive glial inclusions are distributed mainly in the dorso-medial region of the substantia nigra, which contains most of the surviving dopaminergic neurons, suggesting that α-SN expression might have a neuroprotective function in glial cells. To investigate this hypothesis, we established α-SN transfected C6 glioma cell line clones and evaluated the expression of neurotrophins using semi-quantitative reverse transcription polymerase chain reaction and enzyme-linked immunosorbent assay. Brain-derived neurotrophic factor (BDNF) was induced by overexpression of wild-type α-SN but not by that of A30P and A53T. These data suggest that the pathogenic α-SN mutations, A30P or A53T, are linked to the loss of BDNF production in glial cells.

Keywords: α-Synuclein; BDNF; Glia

Parkinson's disease (PD) is one of the most prevalent neurodegenerative diseases. Although the etiology of the disease has not been resolved, it has been established that two separate missense mutations (A30P and A53T) of α-synuclein (α-SN) cause autosomal dominant PD [1,2]. α-SN is a major component of Lewy bodies and Lewy neurites, both of which are pathological characteristics of PD. In addition to neurons, astrocytes express α-SN in the affected regions of neurodegenerative diseases; they appear in the substantia nigra, the locus ceruleus, and the putamen in PD [3], and in the cerebral neocortex and the hippocampus in diffuse Lewy body disease, a Lewy body variant of Alzheimer's disease and PD [4]. Furthermore, they are detected in the cerebral cortex in Pick's disease, progressive supranuclear palsy, and corticobasal degeneration [4], and in the spinal cord in amyotrophic lateral sclerosis [5]. In PD, α-SN-positive glial inclusions appear mainly in the dorso-medial region of the substantia nigra in which dopaminergic neurons are relatively preserved [3,6]. Astrocytes might react to neurodegeneration and may provide neuroprotection by producing various neurotrophic factors such as brain-derived neurotrophic factor (BDNF). In this context we investigated the possibility that the glial expression of α-SN might be related to the production of neurotrophic factors. In this study, we evaluated the role of α-SN in inducing the expression of BDNF and nerve growth factor (NGF) in glial cells. We established two sets of rat C6 glioma cell line clones which stably expressed human α-SN and investigated the expression of neurotrophins using semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) at the mRNA level and enzyme-linked immunosorbent assay (ELISA) at the protein level.

Materials and methods

Reagents. A monoclonal anti-α-synuclein antibody was purchased from Transduction Lab (Lexington, KY). A polyclonal antibody against BDNF was raised by immunizing rabbits with synthetic

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0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.04.012

peptide as previously reported [7]. Chamber slides were obtained from Nalge Nunc International (Roskilde, Denmark). The RT-PCR kit and restriction enzymes were purchased from TaKaRa (Shiga, Japan). The BDNF ELISA kit was obtained from Promega (Madison, WI, USA). Nifedipine, BAPTA-AM, and H-89 were purchased from Nacalai tesque (Kyoto, Japan). Thapsigargin was obtained from Alomone labs (Jerusalem, Israel). BAPTA was purchased from Sigma (Missouri, USA). 2′,5′-Dideoxyadenosine, PD98059, KN-93, and Gö6976 were purchased from Calbiochem (Darmstadt, Germany).

Plasmid construction. α-Synuclein cDNA was amplified from postmortem human brain samples by RT-PCR using *Kpn*I and *Xba*I tailed primers complementary to the human α-synuclein coding region (GenBank AY049786). The nucleotide sequences of sense and antisense primers were as follows: 5′-CGGGGTACCTGGCCATTCG ACGACAGTGT-3′ (sense), 5′-TGCTCTAGAGGATGGAACATCT GTCAGCA-3′ (antisense). Appropriate restriction digestion of the PCR-amplified product was performed and the wild-type sequence was then cloned into the pcDNA3.1(+) vector (Invitrogen, San Diego, CA, USA). To introduce the A30P or A53T mutation, cDNA was subcloned into the *Kpn*I/*Xba*I sites of the pKF18K vector (TaKaRa, Japan) and subjected to site-directed mutagenesis. The oligonucleotide primer, 5′-GGCAGAAGCACCAGGAAAGAC-3′, was used for generation of the A30P mutation and 5′-GCATGGTGTGACAACAGTGGC-3′ was used for that of A53T. The PCR products were ligated into the *Kpn*I/*Xba*I sites of pcDNA3.1(+) (Invitrogen). Plasmid DNA was subsequently transformed into DH5α cells (Toyobo, Japan). The resulting colonies were screened by PCR, and positives were selected, grown in liquid medium, and sequenced to verify the presence of the mutation (data not shown).

Cell culture and stable transfection. C6 rat glioma cell lines (ATCC CCL107) were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. When cells reached 80% confluency in 60 mm culture dishes, the medium was changed to Opti-MEM I (Gibco-BRL, Rockville, USA) and cells were transfected with 8 μg of both α-SN and β-galactosidase (LacZ) DNA constructs using 10 μl of lipofectamin 2000 (Gibco-BRL, Rockville, USA). After incubating for 24 h, positive clones were selected using G418 (1300 μg/ml) for 2 weeks. Single cells were cloned in 96-well tissue culture plates. Stable transfectants established from these clones were evaluated for α-SN and LacZ expression using Western blot analysis and immunocytochemistry; two clones expressing high levels of α-SN and LacZ were used in these studies.

RNA preparation and semi-quantitative RT-PCR. To explore our hypothesis, we evaluated the expression levels of BDNF and NGF mRNA by semi-quantitative RT-PCR in all cultured cells. The expression level of β-actin mRNA was used as an internal standard to normalize the levels of BDNF and NGF mRNA. Total cell RNA preparation and RT-PCR were carried out as previously reported [8]. The nucleotide sequences of sense and antisense primers and the expected product size were as follows: BDNF: 5′-GAGCTGAGCG TGTGTGACAG-3′ (sense), 5′-CGCCAGCCAATTCTCTTTTTGC-3′ (antisense, 278 bp), NGF: 5′-GTTTTGGCCAGTGGTCGTGCAG-3′ (sense), 5′-CCGCTTGCTCCTGTGAGTCCTG-3′ (antisense, 498 bp), β-actin: 5′-TCATGAAGTGTGACGTTGAC-3′ (sense), and 5′-CCTAGAAGCATTTGCGGTGC-3′ (antisense, 285 bp). The annealing temperature is 58 °C for BDNF, 55 °C for NGF, and 60 °C for β-actin. We determined the optimal PCR cycle number within a linear range of amplification as described previously [8]: 32 cycles for BDNF, 35 cycles for NGF, and 19 cycles for β-actin. The PCR products were visualized using a UV transilluminator coupled to a CCD camera and analyzed by quantitative densitometry using a computerized image analysis program (NIH Image 1.59). The BDNF or NGF band density was compared with that of β-actin, which was used as an internal standard. Each PCR product was sequenced to confirm that they were adequately amplified (data not shown).

Immunostaining. For immunostaining of cultured cells, cloned cells were seeded into 8-well chamber slides (Nunc, NY) at 5×10^4 per well. When cells were subconfluent, they were fixed for 20 min in 4% (wt/vol) paraformaldehyde at room temperature. Cells were incubated for 15 min with blocking solution [3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS, pH 7.6) containing 0.2% Triton X-100], followed by overnight incubation with anti-α-SN antibody (1:500) in PBS containing 3% BSA at 4 °C. The next day, cells were incubated with fluorescent secondary antibody (1:2000, Alexa Fluor 488 goat anti-mouse IgG antibody, Molecular probe, Oregon, USA) for 1 h. Confocal images were obtained using the LSM 410 (Carl Zeiss, Germany).

Western blotting. Cells were washed with ice-cold PBS three times, scraped, and lysed with buffer [50 mM Tris–HCl (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, and protease inhibitor cocktail set III (Calbiochem, Germany)]. The lysates were centrifuged at 20,000*g* for 30 min and the supernatants were collected for analysis. Protein levels were determined using the method of Bradford [9] with bovine serum albumin as the protein standard. Samples containing equal amounts of protein (20 μg) were then electrophoresed on polyacrylamide gels (8–16%) (Daiichikagaku, Japan) in the presence of sodium dodecyl sulfate. Immunoblotting was carried out by transferring the proteins to polyvinylidene difluoride microporous membrane (Millipore, Bedford, MA), blocking with 5% non-fat dried milk in 50 mM Tris-buffered saline containing 0.1% Tween 20 (TBS-T, pH 7.8), and incubating overnight at 4 °C with anti-α-SN antibody. The blots were then washed in TBS-T and incubated with a horseradish peroxidase-conjugated secondary antibody (1:2000, Amersham Biosciences, NJ, USA) in TBS-T for 1 h at room temperature. The specific reaction was visualized using the enhanced chemiluminescence method (Amersham Biosciences, NJ, USA).

BDNF ELISA method. BDNF protein levels in medium and cultured cells were quantified using an ELISA kit (Promega). When cells reached 80% confluency, the medium was changed to Opti-MEM I. After incubation for 24 h, we took 100 μl medium to determine the amount of BDNF secretion and collected and washed the cells in ice-cold PBS to determine the cell-contained BDNF. Medium was centrifuged at 3000*g* (5 min, 4 °C) and the supernatant was stored at −80 °C until assayed for BDNF. The cell pellet was homogenized in lysis buffer [20 mM Tris, 137 mM NaCl, 1% Nonidet P-40, and 10% glycerol (pH 7.8)]. Homogenates were centrifuged at 13,000*g* (20 min, 4 °C) and the supernatant was stored at −80 °C until assayed for BDNF. All samples were analyzed in triplicate along with a known BDNF dilution series ranging from 0 to 500 pg/ml. ELISA was performed according to the instruction manual. Briefly, 96-well plates were coated with monoclonal anti-BDNF antibody and 100 μl of protein sample was added to each well. After 2 h incubation, plates were incubated with polyclonal anti-human BDNF antibody for 2 h and anti-IgY HRP conjugate for 1 h. For colorimetric detection, plates were incubated for 2 h with TMB one solution (Promega). The enzymatic reaction was stopped by adding phosphoric acid (1 M). The optical density of each well was measured at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). The detection limit of the ELISA kit was 15.6 pg/ml BDNF. The ELISA results were corrected with respect to the protein content in the cell lysate.

To analyze the cellular mechanism of BDNF production in our clones, when cells reached 80% confluency, the medium was changed to Opti-MEM I containing one of the following reagents: actinomycin D, nifedipine, BAPTA, BAPTA-AM, thapsigargin, 2′,5′-dideoxyadenosine, KN-93, H-89, Gö6976, and PD98059. After incubation for 24 h, we determined BDNF contents in cell lysate as described above.

Statistical analysis. Values were expressed as means ± SE. Comparison of two groups was made using one-way analysis of variance (ANOVA), and p values of <0.01 were considered to have statistical significance. Comparisons of multiple groups were done by one-way ANOVA with Bonferroni's correction for significance.

Results

Generation of α-synuclein transfectants

α-Synuclein (α-SN) was detected as a specific band at 18 kDa in α-SN transfectants. It was not detected in non-transfected clones or β-galactosidase (LacZ) transfectants (Fig. 1A). α-SN transfectants showed overexpression of α-SN by immunocytochemistry. Non-transfected and LacZ transfected cells showed no endogenous α-SN (Fig. 1B).

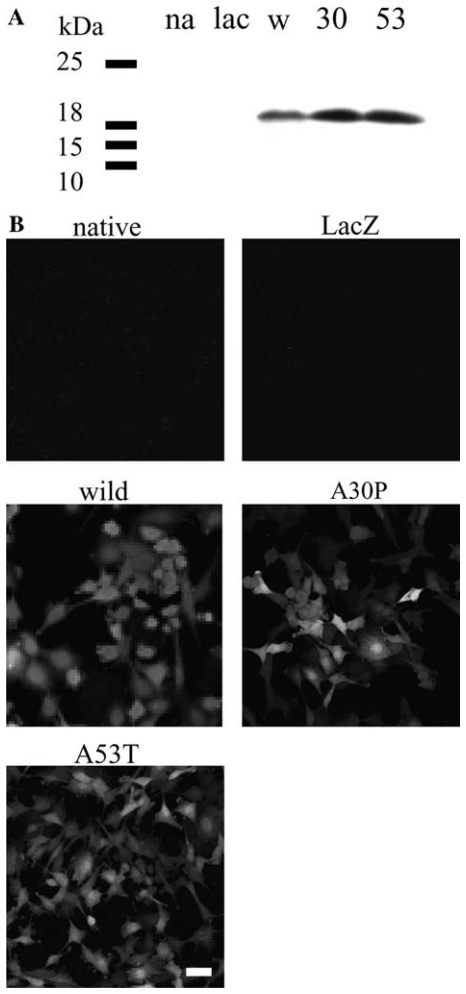

Fig. 1. Immunoblot analysis and immunocytochemistry of LacZ, α-synuclein transfected, and non-transfected C6 cells. (A) Immunoblot analysis of α-synuclein (α-SN) immunoreactivity in non-transfected (na), LacZ transfected (Lac), wild-type α-SN transfected (w), A30P mutated α-SN transfected (30), and A53T mutated α-SN transfected (53) cells. α-SN transfected, but not non-transfected or LacZ transfected, cells expressed α-SN at 18 kDa. Immunoblot analysis was performed on the detergent soluble fraction of cells (20 μg/lane). (B) Representative immunocytochemical photographs of each clone. Cells were plated out into chamber slides and immunostained with anti-α-SN antibody. Non-transfected and LacZ transfected cells did not show endogenous α-SN expression. Wild-type, and the A30P and A53T mutated forms of α-SN transfected cells showed overexpressed α-SN. Scale bar, 20 μm.

Increase of BDNF mRNA expression in wild-type α-SN transfectant

BDNF mRNA was detected in all clones and the expression was significantly increased in the wild-type α-SN transfectant (Fig. 2A). Semi-quantitative analysis revealed that BDNF mRNA levels were increased about twofold in wild-type α-SN transfectants. In contrast, neither β-actin nor NGF mRNA expression was changed (Fig. 2B). These results were confirmed in another set of stable clones (data not shown).

Increase of BDNF protein production in wild-type α-SN transfectants

To determine whether the increase in the BDNF mRNA level leads to that of the protein level, we evaluated the BDNF protein content in both the conditioned medium and the cell lysate by ELISA. The wild-type α-SN transfectant produced a significantly higher amount of BDNF compared to other clones in both the conditioned medium and the cell lysate (Table 1). The BDNF protein content in the other clones was almost under the detectable limit (conditioned medium: <15.6 pg/ml; cell lysate: <4.64 pg/protein). The results were confirmed in another set of stable clones (data not shown). In addition, the protein levels of BDNF in wild-type α-SN transfectants were significantly suppressed by 10 μg/ml actinomycin D in cell lysates (Fig. 3).

The mechanism of BDNF production

BDNF production in glial cells has been shown to be dependent on intracellular calcium and cAMP [10,11]. Therefore, we examined whether BDNF induction by wild-type α-SN is related to calcium- or cAMP-mediated signaling. We treated wild-type α-SN transfectants with the following agents and determined the BDNF content in the cell lysate by ELISA: 5 μM nifedipine as a calcium channel blocker, 10 μM BAPTA as a membrane-impermeable calcium chelator, 20 μM BAPTA-AM as a cell-permeable calcium chelator, 1 μM thapsigargin as an endoplasmic reticulum calcium depletor, and 20 μM 2′,5′-dideoxyadenosine as an adenylate cyclase inhibitor. BAPTA-AM and thapsigargin significantly inhibited BDNF production, whilst BAPTA and nifedipine did not have any effect (Fig. 4).

Next, we examined the downstream signaling of calcium in association with increased BDNF production. We treated wild-type α-SN transfectant with the following agents: 50 μM KN-93 as a calcium/calmodulin dependent kinase inhibitor, 30 μM H-89 as a protein kinase A inhibitor, 2 μM Gö6976 as a protein kinase C inhibitor, and 50 μM PD 98059 as a mitogen activated protein kinase kinase inhibitor. None of these agents inhibited the BDNF production (data not shown).

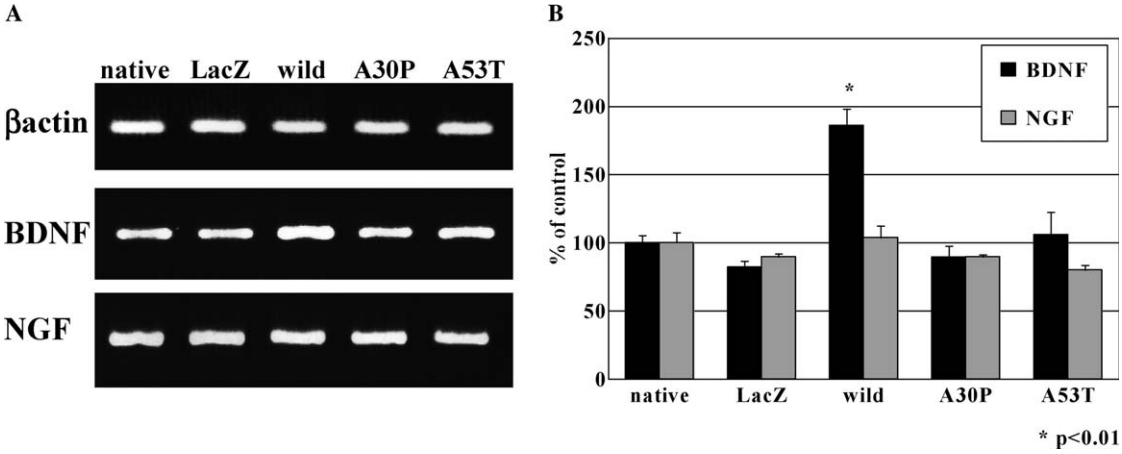

Fig. 2. RT-PCR analysis in LacZ, α-synuclein transfected, and non-transfected C6 cells. (A) The expression of BDNF and NGF mRNA was analyzed using semi-quantitative RT-PCR analysis. (B) The results of the densitometric quantification of BDNF and NGF. The optical density of each blot was normalized to that of β-actin mRNA. Data points represent the average of three experiments. Note the selective induction of BDNF mRNA in wild-type α-SN transfectants ($p < 0.01$, one-way ANOVA); error bars indicate the SE.

Table 1
BDNF secretion and production by transfectants and non-transfected cells

	Native	LacZ	Wild	A30P	A53T
Conditioned medium	<15.6	<15.6	142.6 ± 3.3	<15.6	<15.6
Cell lysate	6.82 ± 1.17	<4.64	110.6 ± 22.9	<4.64	<4.64

BDNF concentration in the conditioned medium (CM) and cell lysates was assayed by ELISA. Wild-type α-SN transfectants secreted and produced more BDNF compared to other cell lines. The detectable limit of the ELISA was 15.6 pg/ml. Data points represent the average of three plates; error bars indicate the SE. Upper, pg/ml; lower, pg/protein (mg).

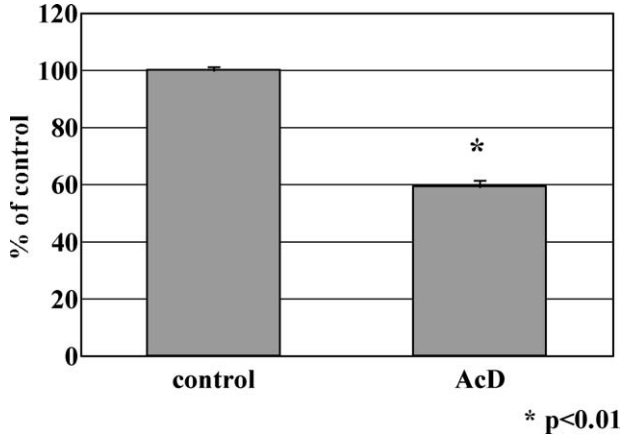

Fig. 3. Blockade of BDNF production by actinomycin D. RNA polymerase inhibitor, actinomycin D (AcD), significantly inhibited BDNF production in wild-type α-SN transfectant ($p < 0.01$, one-way ANOVA). Data points represent average of three trials; error bars indicate SE.

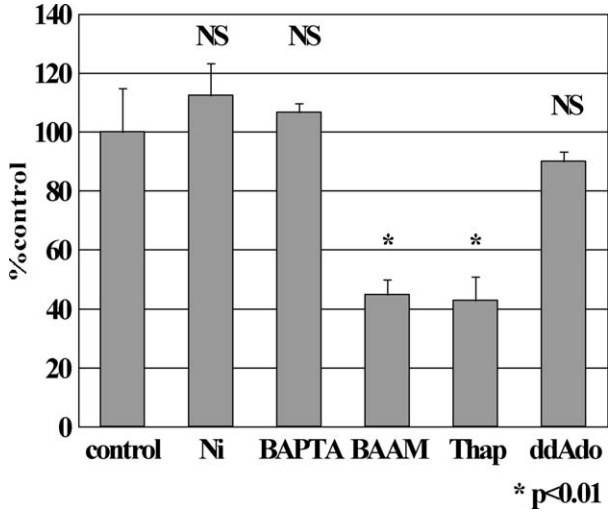

Fig. 4. Dependence of BDNF secretion and production on intracellular calcium. An intracellular calcium chelator, BAPTA-AM (BAAM), and an endoplasmic reticulum calcium depletor, thapsigargin (Thap), significantly inhibited BDNF production in wild-type α-SN transfectants ($p < 0.01$, one-way ANOVA). An extracellular calcium chelator, BAPTA, a calcium channel blocker, nifedipine (Ni), and an adenylate cyclase inhibitor, 2′,5′-dideoxyadenosine (ddAdo), had no effect on BDNF production. Data points represent the average of three experiments using ELISA; error bars indicate the SE; NS, not significant.

BDNF mRNA and protein levels in neuroblastoma cell lines expressing wild-type α-SN

To evaluate whether wild-type α-SN upregulates BDNF mRNA and protein in other types of cells, we also generated human dopaminergic neuroblastoma cell lines, SH-SY5Y, stably expressing α-SN and LacZ. We performed RT-PCR of BDNF, NGF, and β-actin and ELISA of BDNF in each SH-SY5Y cell line established. There were no differences in either

BDNF mRNA expression or protein content between wild-type α-SN transfectants and the others (data not shown).

Discussion

We evaluated the role of α-SN in glioma cell lines focusing on neurotrophin production. Overexpression of wild-type α-SN in a glioma cell line increased expression of both BDNF mRNA and protein levels, whereas that of mutant α-SN did not. In contrast to C6 glioma cell lines, overexpression of α-SN in dopaminergic neuroblastoma cell lines produced no difference in either BDNF mRNA expression or protein content between wild-type α-SN transfectants and the others. These results suggest that α-SN in glial cells but not neuronal cells can be linked to regulation of BDNF which blocks dopaminergic neuronal death [12].

Although the precise mechanism of glial inclusion formation has not been elucidated, α-SN is a major component of the glial inclusions in astrocytes in the brains of other neurodegenerative diseases as well as PD [4–6]. In PD, glial inclusions may be associated with compensatory response because they are distributed mainly in the dorso-medial area of the substantia nigra where many dopaminergic neurons are preserved [3,6]. Although the BDNF concentration is decreased in the substantia nigra and the striatum in autopsy brains of patients with PD [13], the BDNF receptor, TrkB, is highly expressed in the medial-area of the substantia nigra, compared to the other regions [14]. In this context, the increase of α-SN can lead to glial inclusion formation on one hand and it might enhance BDNF production on the other hand. Taken together with the results of the present and previous studies, α-SN can play a pivotal role in the regulation of BDNF in astrocytes and two mutants of α-SN may lose its ability.

Since wild-type α-SN transfectants showed a twofold increase in BDNF mRNA and an eightfold increase in BDNF protein compared to other cells, there was a discrepancy between the increase in BDNF mRNA and protein. As the protein levels of BDNF in wild-type α-SN transfectants were significantly suppressed by transcriptional inhibitor, actinomycin D, the increase of BDNF protein would reflect the increase of mRNA. On the other hand, it has been shown that α-SN interacts with synaptic vesicles and controls the release of synaptic vesicle contents in neurons [15–17]. Therefore, it is possible that α-SN might control the stability or the release of BDNF in addition to BDNF production in glioma cell line, although further study will be needed to clarify this point. Another possibility is that overexpression of wild-type α-SN might inhibit proteasomal activity and increase its substrates. However, since overexpression of LacZ or the mutated form of α-SN showed no increase of BDNF content, that would be unlikely.

With respect to the mechanism of the upregulation of BDNF by wild-type α-SN, the present results suggest that BDNF production in glial cells is dependent on intracellular calcium. BDNF gene has two calcium responsive elements [10,11,18]. Therefore, we evaluated the effect of calcium on the induction of BDNF expression in wild-type α-SN transfectants and found that the enhancement of BDNF production was dependent on intracellular calcium. In the downstream signaling of calcium regulation, there are several reports to show that calcium/calmodulin dependent kinase, protein kinase A, protein kinase C, and mitogen activated kinase are related to expression of BDNF in neurons or microglia [11,19–21]. However, our results indicated that none of them are closely associated with BDNF production. Therefore, BDNF upregulation by wild-type α-SN transfection might be mediated via another calcium responsive pathway. A previous report showed that the calcium binds directly to α-SN [22]. α-SN might amplify the intracellular signal transduction associated with intracellular calcium by both binding directly to calcium and increasing the transcription of BDNF mRNA. Further study is needed to elucidate this detailed mechanism.

Previous reports concerning α-SN overexpression in glial cells indicate that the overexpression of α-SN in human glial cell lines causes inclusion body formation and cells overexpressing α-SN are vulnerable to oxidative stress [23,24]. In our experiments, however, wild-type α-SN transfectants were resistant to oxidative stress, and the mutated form of α-SN transfectants lost this property (data not shown). This protective effect of wild-type α-SN might be dependent on BDNF, but the detailed mechanism needs to be resolved. Furthermore, none of these transfectants formed inclusion bodies. These discrepancies might be due to the amount of α-SN expressed and the difference in cell lines used in the experiments.

In conclusion, our study showed that wild-type α-SN increased BDNF production in glioma cell lines. Only wild-type α-SN had this function, and it was absent in mutated forms of α-SN. In this context, BDNF regulation by the α-SN genotype may be, at least partially, involved in the pathomechanism of familial PD with A30P and A53T.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and grants from Japan Society for the Promotion of Science, Ministry of Health, Labour and Welfare, and the Smoking Research Foundation.

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