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**Allelic imbalance of expression and epigenetic regulation
within the alpha-synuclein wt and c.209G>A gene alleles in
Parkinson Disease**

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Key Words:	Parkinson, alpha-synuclein, monoallelic, overexpression, epigenetic



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3 **Allelic imbalance of expression and epigenetic regulation within the alpha-**
4 **synuclein wt and c.209G>A gene alleles in Parkinson Disease**
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ABSTRACT

Genetic alterations in the alpha-synuclein (*SNCA*) gene have been implicated in Parkinson Disease (PD), including point mutations, gene multiplications and sequence variations within the promoter. Such alterations may be involved in pathology through structural changes or overexpression of the protein leading to protein aggregation, as well as through impaired gene expression. It is, therefore, of importance to specify the parameters that regulate *SNCA* expression in its normal and mutated state. We studied the expression of *SNCA* alleles in a lymphoblastoid cell line and in the blood cells of a patient heterozygous for p.Ala53Thr, the first mutation to be implicated in PD pathogenesis. Here, we provide evidence that: (1) *SNCA* shows monoallelic expression in this patient; (2) epigenetic silencing of the mutated allele involves histone modifications but not DNA methylation; and (3) steady state mRNA levels deriving from the normal *SNCA* allele in this patient exceed those of the two normal *SNCA* alleles combined, in matching, control individuals. An imbalanced *SNCA* expression in this patient is thus documented, with silencing of the p.Ala53Thr allele and up-regulation of the wt-allele. This phenomenon is demonstrated for a first time in the *SNCA* gene and may have important implications for PD pathogenesis.

Key words: Parkinson, alpha-synuclein, monoallelic, overexpression, epigenetic.

INTRODUCTION

The detection of a point mutation in the *SNCA* gene (OMIM accession number *163890) in Parkinson disease (PD) patients, over a decade ago, marked a turning point in the genetics of Parkinson disease. This mutation was a c.209G>A base substitution in the 4th exon of the *SNCA* gene, leading to p.Ala53Thr missense mutation. It was first identified in a large Italian PD family and three Greek PD families [Polymeropoulos et al., 1997]. This mutation is prevalent mainly in Greece, suggesting a Founder Effect [Athanassiadou et al., 1999; Papadimitriou et al., 1999]. Since then, the same mutation has been detected in a Korean PD family [Ki et al., 2007], while recently, a Swedish family carrying a *de novo* p.Ala53Thr mutation was also identified [Puschmann et al., 2009]. In addition, the identification of two different *SNCA* mutations, namely mutations p.Ala30Pro and p.Glu46Lys [Kruger et al., 1998; Zarranz et al., 2004], provides a sound genetic basis for the implication of *SNCA* point mutations in PD. All three missense *SNCA* mutations are linked to early onset familial parkinsonism with autosomal dominant inheritance, constituting a rare cause of the disease.

Important steps in PD genetics were the detection of multiplications of the *SNCA* gene in PD patients, demonstrating that even moderate but chronic elevation of the wild-type alpha-synuclein level can cause parkinsonism, in a dose dependant manner [Singleton et al., 2003; Chartier-Harlin et al., 2004; Ibanez et al., 2004]. Genomic investigation has shown that mechanisms of intra- and inter-allelic recombination with unequal crossing over are responsible for these multiplications [Ross et al., 2008]. These data, in combination with data from animal models and targeted overexpression of alpha-synuclein in animals, [van der Putten et al., 2000; Kirik et al., 2002] have established alpha-synuclein protein overproduction as a

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3 framework for understanding the molecule's involvement in the disease, at least in a
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5 number of patients.
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8 Other genetic elements of *SNCA* genetics include polymorphisms and
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10 haplotypes reported to predispose for the disease [Chiba-Falek et al., 2001; Farrer et al.,
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12 2001; Tan et al., 2004; Mueller et al., 2005]. A meta-analysis confirmed that NACP-
13
14 Rep1 promoter alleles are significantly associated with PD [Maraganore et al., 2006],
15
16 while variation in both the promoter and the 3' region of the gene definitely convey an
17
18 increased risk for PD [Fuchs et al., 2008; Pankratz et al., 2009].
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22 These data have established that altered regulation of *SNCA* gene expression is a
23
24 major contributing factor in PD pathogenesis. Elevated expression levels of *SNCA*
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26 mRNA were found in the affected regions of PD brain in sporadic PD patients [Chiba-
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28 Falek et al., 2006]. On the other hand, diminished *SNCA* mRNA levels in the substantia
29
30 nigra of PD cases were reported in early studies [Neystat et al., 1999], while later,
31
32 significant reduction was documented for substantia nigra, the putamen and the visual
33
34 cortex [Dachsel et al., 2007]. It is considered that either aggregation and/or impaired
35
36 expression are most probably the causes for lack of functional alpha-synuclein, leading
37
38 to toxic effects [Dachsel et al., 2007].
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43 Regulation of the normal *SNCA* gene expression is not well understood, but
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45 transcriptional up-regulation by specific factors has been reported [Vekrelis et al., 2003;
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47 Yang et al., 2008]. In PC12 cells, a novel regulatory region for *SNCA* transcription in
48
49 the first intron of the gene was reported [Clough et al., 2007]. Most interestingly, the
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51 same region was shown to harbour two very well known genetic elements: (1) a
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53 functional GATA binding motif in intron 1, while GATA 2 transcription factor seems
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55 to regulate the expression of the human endogenous neuronal *SNCA* [Scherzer et al.,
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57 2008] and (2) a predicted iron responsive element (IRE) in the 5' untranslated region,
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3 that is present in the human *SNCA* mRNA [Friedlich et al., 2007], but not in mRNAs of
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6 the mouse or rat gene homologues [Olivares et al., 2009].
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8 Even less is known about the regulation of expression of the mutated *SNCA*
9
10 gene. Surprisingly, the p.Ala53Thr and p.Ala30Pro alleles were found to be silenced to
11
12 different degrees, as compared to the expression of the normal counterpart in the same
13
14 patient, in various cases [Markopoulou et al., 1999; Kobayashi et al., 2003]. This inter-
15
16 allelic variation of expression, unexpected on the basis of the nature of these mutations,
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18 could cause a decrease in alpha-synuclein protein in these patients and lead the authors
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20 to conclude that a haploinsufficiency effect exists associated with the clinical severity
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22 of the disease.
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27 It is considered that elucidation of the regulation of *SNCA* gene expression is
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29 going to be critical for understanding PD pathophysiology. As a contribution towards
30
31 this end, we have studied the mode of expression of *SNCA* gene alleles in a PD patient,
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33 heterozygous for the p.Ala53Thr mutation.
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41 **MATERIALS AND METHODS**

42 43 44 45 **Patient and control individuals**

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47 A female patient, diagnosed with early onset PD, whose clinical characteristics
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49 were presented previously [Athaniassiadou et al., 1999] was the donor of blood samples
50
51 for this study. The patient is heterozygous for the p.Ala53Thr mutation in the *SNCA*
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53 gene (GenBank reference sequence number NM_007308.2) and belongs to a family
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55 with multiple PD cases in several generations, with autosomal dominant inheritance
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3 and less than 100% penetrance of the disease. Controls were normal individuals, all of
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5 matching age and gender with the patient.
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10 **Establishment of lymphoblastoid cell lines**

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12 Two Epstein-Barr virus (EBV)-transformed cell lines were used in the present
13 study. Transformation was carried out with the use of supernatant obtained from B95-8
14 lymphoblastoid cell line established from peripheral blood lymphocytes of a cotton-top
15 marmoset (*Saguinus oedipus*) following *in vitro* infection with EBV. Mononuclear cells
16 were obtained from peripheral blood by density gradient centrifugation over Ficoll-
17 Paque PLUS (GE Healthcare, Chalfont St Giles, UK). For the establishment of EBV-
18 transformed polyclonal continuous B-cell lines, 10^7 mononuclear cells were exposed to
19 1 ml of filtered (0.45 μm) supernatant of the B95-8 marmoset cell line for 1.5h at 37°C
20 in a 95% humidified 5% CO₂ atmosphere. The EBV-exposed mononuclear cells were
21 subsequently distributed to a 24-well culture plate at a cell density of 0.5×10^5 cells/ml
22 in culture medium consisting of RPMI 1640, supplemented with 20% heat-inactivated
23 fetal calf serum, 2 mM L-glutamine, 100 u/ml Penicillin/Streptomycin, 100 u/ml
24 Neomycin and 1% Phytohaemagglutinin (PHA). After about two weeks, when the T-
25 cell population has declined and transformed B-cells have taken over, cells were
26 transferred to a 25 cm² flask and were grown in the same medium minus PHA, as
27 above. When the cell number was judged to be satisfactory, cells were frozen using
28 standard media and techniques.
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53 **Cell lines and culture conditions**

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55 For resuscitation cells were placed at 37°C for 30 sec, transferred to 10 ml of
56 cold culture medium while in ice, collected by a 5 min spin at 1000 rpm and
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3 resuspended in 5 ml fresh RPMI. Cells were maintained in RPMI 1640, supplemented
4 with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), sodium
5 pyruvate (1 mM), sodium bicarbonate (50 mM), non-essential amino acids (1x) and
6 10% heat inactivated FBS, at 37°C in a humidified 5% CO₂ atmosphere. All plastics
7 were from Costar (Cambridge, MA, USA), while cell culture media and reagents were
8 supplied by Invitrogen (Carlsbad, CA, USA).
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20 **Detection of the c.209G>A sequence variation**

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22 Blood samples were collected after informed consent. DNA from blood and
23 from lymphoblastoid cell lines was extracted and mutation was detected as previously
24 described [Athanasidou et al., 1999]. Briefly, DNA was amplified in thermal cycler
25 (PTC200 MJResearch) using primers: (3), 5'-GCTAATCAGCAATTTAAGGCTAG-3'
26 (forward) and (13), 5'-GATATGTTCTTAGATGCTCAG-3' (reverse). The PCR
27 product was 216 bp. As mutation c.209G>A creates a polymorphic site for enzyme
28 Tsp45I, the mutant allele is identified by RFLP analysis (Figure 1A). RNA from
29 lymphoblastoid cell lines was extracted using Trizol reagent (Invitrogen, USA),
30 according to manufacturer's instructions. 1 µg of total RNA was used for first strand
31 cDNA synthesis in a final volume of 20 µl, using an oligo-dT (20mer) primer
32 (Invitrogen, USA) and MMLV reverse transcriptase (Invitrogen, USA). 1 µl of cDNA
33 solution, dilution 1:5, was amplified by PCR in a total volume of 25 µl, using primers:
34 (1F) 5'-ACGACAGTGTGGTGTAAGG-3' (forward) and (13R)
35 5'-AACATCTGTCAGCAGATCTC-3' (reverse). The RT-PCR product is 500 bp, and
36 the mutant allele is identified by RFLP analysis after Tsp45I digest (Figures 1B and 2A,
37 2B). Digestion of the wt allele of the *SNCA* gene with this enzyme gives 4 fragments
38 (249, 218, 24 and 9 bp), whereas digestion of the c.209G>A allele gives 5 fragments
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(249, 185, 33, 24 and 9 bp), as previously described (Polymeropoulos et al., 1997).

Bands from 33bp down to 9bp are not detectable with the present system.

Cycling conditions were as previously described [Polymeropoulos et al., 1997].

PCR and RT-PCR products were digested with the enzyme Tsp45I, (New England Biolabs) according to the manufacturer's protocol, and analysed in 3% agarose gel.

DNA and chromatin modification assays

We used 5-aza-2-deoxycytidine (5-azadC) (#A3656, Sigma-Aldrich), as inducer of DNA demethylation, and sodium 4-phenylbutyrate (4-PB) (#567616, Calbiochem-Merck) and trichostatin A (TSA) (#T8552, Sigma-Aldrich), as inhibitors of histone deacetylases (HDACs) [Chiurazzi et al., 1999].

Cells were used at a concentration of $2.5-3 \times 10^5$ cells/ml in a total volume of 10 ml per flask, and subsequently, a drug was added to a flask and was thoroughly resuspended. In all cases a control flask was left untreated.

For the 5-azadC treatment a daily dose of 1.0 μ M was added to the cell cultures and cells were harvested after 48 h treatment for RNA extraction. For 4-PBA (5 mM) and TSA (0.5 μ M) treatment a single dose was added to the cell cultures. Treatment with 4-PBA was for 24 h and with TSA was for maximum 15 h, as after that point extensive cell loss occurred.

CpG analysis for DNA methylation

2 μ g of DNA diluted in 20 μ l ddH₂O were denatured by the addition of 2 μ l NaOH 3M (freshly prepared) and incubated for 20min at 42°C. Solution A: 500 μ l of freshly prepared hydroquinone solution (27.5mg hydroquinone diluted in 12.5ml ddH₂O) was added to a sodium bisulphite solution (8ml ddH₂O, 230 μ l of 10M NaOH

and 4.05gr of sodium bisulphite). The denaturated DNA samples were then incubated for 2min at 100°C and 180 µl of the hydroquinone/sodium bisulphate solution was immediately added and mixed by pipetting. Samples were incubated in a thermal cycler (MJ Research Inc.) under following conditions: 100°C / 1 min; 55°C / 15 min; 100°C / 2 min; 55°C / 15 min; 100°C / 2 min; 55°C / 15 min; 100°C / 2 min; 55°C / 15 min; 100°C / 2 min; 55°C / 5 hours.

Removal of sodium bisulphite solution was performed using the Qiaquick PCR purification kit (Qiagen) according to the manufacturer's instructions. Samples were eluted with 45 µl of elution buffer. Subsequently 5 µl of 3M NaOH were added to each sample and samples were incubated at 37°C for 20min. Removal of NaOH solution was achieved with S-200 HR microspin columns (Amersham Biosciences) according to the manufacturer and DNA was eluted into a total volume of 50 µl. NaOH was added to a final concentration of 0.3M and samples were incubated at 37 °C for 20min. The final removal of NaOH solution was done with S-200 microspin columns and DNA samples were eluted into a final volume of 50 µl.

Following the bisulphite modification PCR reactions were performed using 5 different primers sets (1F: 5'-GGATTGTTTTTAGAGGAGGT-3'; 1R: 5'-CCAAAAAAAAACCCAAAAAAAAAA-3'; 2F: 5'-GGGAAAGAGGAAGAGGTATTAATAGGTG-3'; 2R: 5'-CCCTCTCTTAAACCCCTTCTAATC-3'; 3F: 5'-TGTAGGGAGGTTAAGTTAATAGGTG-3'; 3R: 5'-TATAATTCAACCTCAAACAAC-3'; 4F: 5'-GGTTTTTTTTGGGGATAGTTTTTT-3'; 4R: 5'-AACAACTAATCTCTCAACCCTTATAA-3'; 5F: 5'-GTTTTTGGAAATTTTGGAGAATAT-3'; 5R: 5'-CCACCTATTAACCTAACCTCCCTAC-3'). PCR conditions were the same for all

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3 sets of reactions (95°C for 15 min, followed by 95°C for 30 sec, 53°C for 30 sec and
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6 72°C for 30 sec for 40 cycles and final extension at 72°C for 7min). PCR reactions
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8 were carried out in a 50 µl volume containing 15 µl Na-bisulphite converted DNA, 5 µl
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10 PCR buffer(1X) (Promega, Madison, WI), 5 µl forward primer (10pm/µl stock), 5 µl
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12 reverse primer (10pm/µl stock), 2.4µl MgCL₂ (25mM), 3µl dNTP's (2mM), 0.6U of
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14 Taq Polymerase (Promega) and 14.3 µl ddH₂O.
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18 PCR products were electrophoresed in a 2% agarose gel to confirm size and
19
20 identity. For primer set No1 PCR products were digested by the single cutting
21
22 methylation sensitive enzyme AciI. The expected PCR product sizes were 265bp for
23
24 primer set No1, 339bp for primer set No 2, 226bp for primer set No 3, 410bp for primer
25
26 set No 4 and 384bp for primer set No 5. All PCR products were purified with the
27
28 Qiaquick PCR purification kit (Qiagen) and then sequenced (Geneservice Ltd, DNA
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30 Sequencing team, UK).
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36 Real-time PCR

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38 RNA was extracted from blood with RNeasy[®] Mini Kit (Qiagen). 800ng RNA
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40 was reverse transcribed to cDNA with PrimeScript[™], 1st strand cDNA Synthesis Kit
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42 with Oligo dT primers (Takara). For Real-time PCR dilutions 1/10 and 1/100 cDNA
43
44 were amplified using QuantiFast SYBER Green PCR (Qiagen) and the LightCycler2.0
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46 (Roche) as follows: 10sec at 95°C, 30sec at 60°C, 40 cycles.
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51 The target *SNCA* cDNA primers were: (*SNCA_F*) 5'-
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53 ACAGCAGTAGCCCAGAAGACAG-3' and (*SNCA_R*) 5'-
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55 GGCTTCAGGTTTCGTAGTCTTGATA-3'. For data normalization, the Synaptophysin
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57 (*SYP*) gene was used as internal control. This gene encodes a synaptic vesicle
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59 glycoprotein and is known to be expressed in blood at rather constant levels [Schlaf et
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1
2
3 al., 1998], while it has been used for the estimation of *SNCA* gene expression levels
4
5 previously [Chiba-Falek et al., 2006]. The *SYP* primers were: (*SYP_F*) 5'-
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7 CTGCTGGCGGACATGGAC-3' and (*SYP_R*) 5'- CCACTGTAGCTGCCGCAT-3'
8
9 (TIB, MOLBIOL).
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14

15 **Copy Number Variation (CNV) analysis**

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17 Whole genome analysis was performed by microarray analysis using
18
19 Affymetrix GeneChip Mapping 250K arrays (Array 4.0). The assays were carried out
20
21 according to the standard protocol. Briefly, 250 ng of total genomic DNA were digested
22
23 with *NspI*. Appropriate adaptors were ligated to the four base-pair overhang of the
24
25 DNA fragments. A single primer was used for amplification and PCR products were
26
27 purified. After fragmentation with *DNAse I*, products were labeled with biotin and
28
29 hybridized to the array.
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34 The arrays were washed and stained with streptavidin-phycoerythrin in the
35
36 Affymetrix fluidics station 450 and scanned in the GeneChip Scanner 3000 G7.
37
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39 The software package Affymetrix Genotyping Console 2.0 was used to perform
40
41 image collection of the scans, to measure intensities of the signals, to perform the
42
43 genotype data analysis using the BRLMM algorithm, and to perform copy number
44
45 analysis on the allele intensities of each SNP.
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RESULTS

Monoallelic expression of the *SNCA* gene

Genomic DNA, isolated by standard techniques and total RNA from the cultured, lymphoblastoid cell line produced from patient and control blood samples, were used as template for PCR and RT-PCR respectively. PCR was done in order to verify the p.Ala53Thr/wt genotype and RT-PCR was done in order to investigate the relative level of expression of the two alleles, in the patient cell line. The amplicons produced were digested with the restriction enzyme Tsp45I, targeting the polymorphic site created by the p.Ala53Thr mutation. Figure 1A shows that the patient carries the mutation p.Ala53Thr and Figure 1B shows absence of the three-band pattern expected for the mutant allele (249bp, 218bp and 185bp), thus documenting monoallelic expression of the normal *SNCA* allele in this patient. The possibility that recombinational events on the *SNCA* gene structure, due to cell culturing, may be responsible for the lack of expression of the mutated allele, was investigated by Southern blot analysis, after digestion with enzyme *EcoRI*, that has rare cutting sites in and around *SNCA* genes' sequence and no difference was found compared to the control DNA (data not shown).

Additionally, the silencing of the mutated gene was also documented by the same RT-PCR analysis, carried out with mRNA from freshly drawn blood from the patient, and this particular finding verifies the conclusion that monoallelic expression is an inherent property of this gene in this patient and not a methodological artifact.

It is not known whether monoallelic expression of the *SNCA* gene, as documented here and previously [Markopoulou et al., 1999], is restricted to PD patients only. Differential expression of the *SNCA* gene in normal individuals has not been

1
2
3 investigated, as the coding sequences are highly conserved, rendering the attempt to
4 find out whether both alleles of the gene are actually expressed very difficult. However,
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6 if silencing of one allele is the normal state of this gene's function, the finding here of
7
8 monoallelic expression may be significant to the extent that, a defect in the silencing of
9
10 the normally repressed allele could result in *SNCA* gene overexpression in the
11
12 respective individual, possibly leading, gradually, to PD pathology.
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20 **Epigenetic silencing of the mutated *SNCA* gene allele**

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22 We investigated the possibility that the p.Ala53Thr bearing allele is silenced by
23
24 epigenetic mechanisms, in which case, gene activity could be restored *in vitro* by
25
26 inducing DNA demethylation with 5-azadeoxycytidine (5-azadC) in the patient's
27
28 lymphoblastoid cell lines, or with the use of 4-phenylbutyrate (4-PBA) and trichostatin
29
30 A (TSA), which are reversible inhibitors of histone deacetylases.
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33

34 Firstly, we cultured the cells in the presence of 5-azadC for 48 h. Our results
35
36 showed no reactivation of the silenced allele, as documented by the absence of the band
37
38 pattern characteristic of the heterozygote for the mutation. The possibility of a weak
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40 reactivation, undetectable by the system used here, cannot be excluded at this stage.
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44 Secondly, we cultured the cells in the presence of the chromatin modifiers 4-
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46 PBA for 20h and 24h or TSA for 15h. Culturing of the cells for longer times in both
47
48 cases resulted in extensive cell death. Interestingly, in Figures 2A and 2B we can see
49
50 that the silenced allele is reactivated by both types of treatment, leading to the
51
52 conclusion that an epigenetic mechanism is involved in the silencing of the mutant
53
54 allele.
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57 Furthermore, we checked the methylation state of the DNA surrounding 5'
58
59 region of the *SNCA* gene, including the CpG island, 860 bp in size and the *SNCA* gene
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3 promoter and extending well into the first intron, as part of the 5' untranslated region of
4 the gene (Figure 2C). To this purpose we used 5 partially overlapping regions, covering
5
6 the gene (Figure 2C). To this purpose we used 5 partially overlapping regions, covering
7
8 a total length 1350bp including the CpG island, according to the criteria used in the
9
10 UCSC genome browser (Figure 2C). All analysed CpG sites were found to be
11
12 unmethylated, suggesting that silencing of the patient's mutated allele does not involve
13
14 DNA methylation. This is in accordance with our result of no reactivation of the
15
16 silenced allele after 5-azadC treatment of the cultured cells derived from the patient.
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19
20 In parallel, a similar analysis of this CpG island for methylation was performed
21
22 with the DNA from 3 unrelated, normal control individuals of matching sex and age
23
24 with our patient, since allele specific DNA methylation is a key component of
25
26 processes that specify hereditary monoallelic gene expression, such as genomic
27
28 *imprinting*. Again no methylation was detected in this search in normal individuals and
29
30 this indicates that the *SNCA* gene is not imprinted and, from this point of view, its
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32 normal expression is expected to be biallelic.
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36 We, therefore, conclude that an epigenetic mechanism, namely local histone
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38 modifications, is involved in the silencing of the p.Ala53Thr mutated allele in our
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40 patient and it is most probably associated with the disease.
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46 ***SNCA* mRNA levels in condition of monoallelic expression**

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49 Next, we investigated the effect of the monoallelic expression on the total *SNCA*
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51 mRNA output in these cells. To this end, we compared the *SNCA* mRNA levels in our
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53 patient to that of the control, normal individual, using RNA from immortalized B-
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55 lymphocytes. Our results with quantitative RT-PCR show that the mRNA in the control
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57 ranged from 65.1% to 86.2% of the mRNA of the patient, documenting an increase of
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3 total mRNA in the patient between 14% and 35% of that of the normal individual
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5 (Figure 3A).
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8 Furthermore, we performed real-time PCR, with RNA from freshly drawn blood
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10 cells, from both the patient and a number of controls. We estimated the total *SNCA*
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12 mRNA levels of the patient against four different control individuals, all matching for
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14 age and gender (Figure 3B), carrying out three different experiments with each control.
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18 In multiple experiments, the real-time PCR results confirmed our previous
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20 finding, and showed an average of 2-fold increase in total *SNCA* mRNA in the patient,
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22 compared with the total *SNCA* mRNA of the four different control individuals. This
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24 shows that the normal *SNCA* allele in this patient exhibits much higher expression than
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26 the expected normal level.
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29 30 31 ***SNCA* gene copy number analysis** 32

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34 In the light of the unexpected finding that the *SNCA* gene is overexpressed in
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36 the patient's blood cells, we decided to investigate the possibility that this patient's
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38 genome carries more than two copies of the *SNCA* gene.
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42 Genomic DNA of the patient was used for gene copy number analysis. The
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44 *SNCA* gene is represented on the Affymetrix 250K SNP array by 19 SNP's. Two copies
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46 of all 19 SNPs were present, and no amplification or deletion of the *SNCA* gene was
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48 found. In the database of Genomic Variants (<http://projects.tcag.ca/variation/>) no
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50 known CNVs were present for the *SNCA* gene and there was no indication for a novel
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52 rearrangement in the DNA sample of the PD patient analysed.
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DISCUSSION

In this work, we have studied *SNCA* expression in the hematopoietic system of a PD patient, as this gene is known to be expressed in several hematopoietic lineages in adults, such as lymphocytes [Kim et al., 2004], platelets [Li et al., 2002] and erythroid lineage [Nakai et al., 2007].

In our study of a patient heterozygous for the p.Ala53Thr mutation, monoallelic expression of *SNCA* gene is documented by the detection of complete silencing of the p.Ala53Thr allele, in lymphoblastoid cell line and, importantly, in mRNA from blood of the patient. Independent reports on other patients [Markopoulou et al., 1999; Kobayashi et al., 2003] have previously shown that expression of the same *SNCA* allele as well as of the allele for the p.Ala30Pro mutation, was either absent or significantly reduced. This raises the question how common such monoallelic SCNA expression is; studies on this issue will provide important insights, however, they are complicated, because nucleotide variations in the coding sequence of the gene are rare.

Monoallelic expression of *SNCA* gene in our patient is not accompanied by DNA modification, as the respective CpG island was found to be unmethylated, while the use of 5-aza-2-deoxycytidine, a potent inducer of DNA demethylation, did not reactivate the silenced allele. The same CpG island region in non-patient controls is also unmethylated. No case of methylation of this promoter has been reported, apart from a study with alcoholic patients [Bönsch et al., 2005]. In contrast, the use of known inhibitors of histone deacetylases, such as 4-PBA and TSA, resulted in reactivation of the silenced allele, revealing, for the first time, an epigenetic type of regulation in the *SNCA* gene, through chromatin modification. How and why this chromatin modification occurs in this case, is yet unknown.

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4 Interestingly, in spite of the silencing of the mutated allele, no
5 haploinsufficiency is established for this patient. A genome scan assay to identify
6 possible copy number variation of the *SNCA* gene was negative, leading to the
7 conclusion that the single, normal allele in the patient is expressed at higher levels than
8 the two normal alleles combined, in the matching non-patient. Thus, we report a
9 situation of gene expression in which the *SNCA* gene exhibits monoallelic expression in
10 coexistence with gene overexpression.
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20 It is thus for the first time documented, that there is an overall imbalance of
21 allelic expression of *SNCA* gene, with the p.Ala53Thr allele epigenetically silenced and
22 the normal allele expressed to a much higher level than in its steady state condition in
23 the non patient. The most plausible explanation of this phenomenon is that allele
24 specific mechanism(s) of regulation of gene expression are in operation in this case.
25 Such mechanisms may involve allele specific alterations (polymorphisms), which
26 facilitate altered actions on these alleles by trans acting elements, e.g. transcription
27 factors, miRNAs or the natural antisense transcripts of the *SNCA* gene [Guo et al.,
28 2006]. Polymorphisms of this kind may occur in the newly identified elements in the
29 *SNCA* promoter, namely the GATA binding site within intron 1 [Scherzer et al., 2008]
30 or the IRE-like element in the 5' UTR [Friedlich et al., 2007] and may bring about
31 allele specific effects that are reflected in the abundance of functional mRNA.
32 Conversely, variation in the DNA sequence of the specific trans acting elements
33 themselves may be involved in the same way. If such variations exist only functional
34 experiments can shed light to their possible importance within the context of
35 differential allelic expression of the *SNCA* gene.
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58 In conclusion, it is documented that the presence of the p.Ala53Thr mutation in
59 this PD patient, except of exerting a severe effect on the alpha-synuclein protein
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3 properties, it also marks a situation of dysregulation of *SNCA* gene expression,
4 characterised by complete imbalance of allelic expression, with the mutated allele being
5 silenced and the normal allele overexpressed. Apparently, this may have important
6 implications in the pathogenesis of PD, regarding the onset and the progression of the
7 disease and requires further exploration.
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18
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20 individuals used as control, for the donation of blood samples. We also wish to thank
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22 research grants from the Cyprus Research Promotion Foundation (Project ΠΔΕ_02;
23 HU-PHARMGK) and the European Commission to GPP.
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LEGENDS TO FIGURES

Figure 1

A. PCR for the detection of the c.209G>A base change in the patient's DNA. PCR products (1 and 2) and their Tsp45I digests (3 and 4), using N: normal and P: patient DNA.

B. RT-PCR products (1 and 2) and their Tsp45I digests (3 and 4), using N: normal RNA and P: patient RNA. The normal allele is cut into fragments of 249, 218, 24 and 9 bp, and the mutant into fragments of 249, 185, 33, 24 and 9 bp. Band sizes 33, 24 and 9 bp are not detectable in this electrophoresis. M: size DNA marker ϕ X174*HaeIII. Monoallelic expression of *SNCA* gene is documented by the lack of the 185bp band in the RNA of the patient.

Figure 2

A. Reactivation of the *SNCA* silenced allele is documented by the presence of the 185bp band in the RNA from the lymphoblastoid cell line of the patient.

P: Tsp45I digestion of the RT-PCR product from patient RNA. n and m: Tsp45I digestion of PCR products of cloned normal and mutant cDNAs, for comparison.

B. RT-PCR product, un: undigested; Tsp45I digested of N: normal control and P: patient RNA, experiments a and b. M: size DNA marker ϕ X174*HaeIII.

C. The *SNCA* gene CpG island, thick, grey bar. 1350 bp were checked for methylation, from -346bp to +993 bp, with reference to the start site of transcription. Double sided arrows show the 5 partially overlapping frames used and numbers show the respective

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3 fragment sizes in bp; Size lines are NOT to scale. All CpG positions checked were
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5 unmethylated.
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10 **Figure 3**

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15 **A.** RT-PCR using RNA from the lymphoblastoid cell lines, for the quantitation of
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17 *SNCA* mRNA. N.1: RNA from normal control No.1. P: patient RNA. a: *SNCA* mRNA,
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19 b: GAPDH control mRNA. mRNA in the control ranged from 65.1% to 86.2% of the
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21 mRNA of the patient, after quantitation using the Bio-gene V.97 software program.
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25 **B.** Real Time PCR, using RNA from freshly drawn blood cells. P: patient RNA, N.2,
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27 N.3, N.4 and N.5: RNA from normal controls No.2, 3, 4 and 5, correspondingly. Values
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29 were normalized with the use of Synaptophysin (*SYP*) gene RNA, as internal control.
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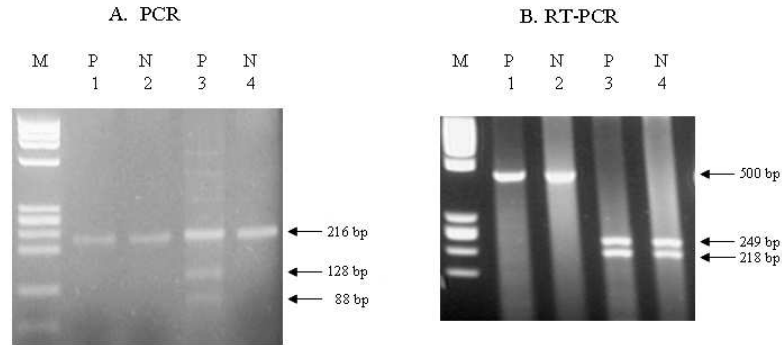


Figure 1

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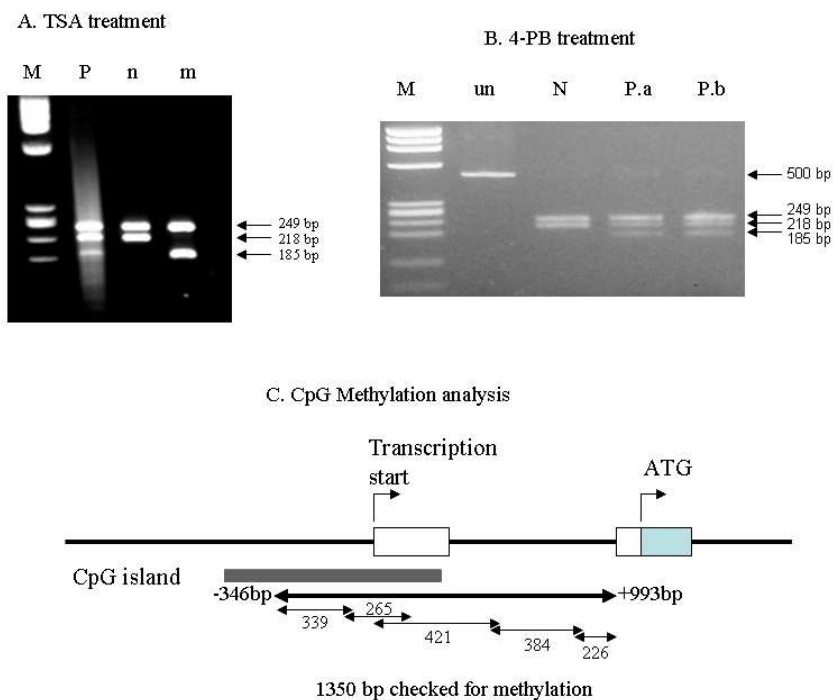


Figure 2

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- A. Reactivation of the SNCA silenced allele is documented by the presence of the 185bp band in the RNA from the lymphoblastoid cell line of the patient.
- P: Tsp45I digestion of the RT-PCR product from patient RNA. n and m: Tsp45I digestion of PCR products of cloned normal and mutant cDNAs, for comparison.
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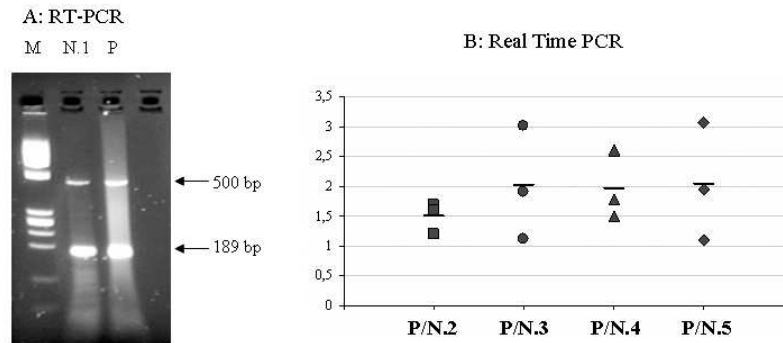


Figure 3

- A. RT-PCR using RNA from the lymphoblastoid cell lines, for the quantitation of SNCA mRNA. N.1: RNA from normal control No.1. P: patient RNA. a: SNCA mRNA, b: GAPDH control mRNA. mRNA in the control ranged from 65.1% to 86.2% of the mRNA of the patient, after quantitation using the Bio-gene V.97 software program.
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