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Research article

Transcriptional regulation of the α-synuclein gene in human brain tissue

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HIGHLIGHTS

- We identified a new binding site for GATA2 within the 5′-promoter of *SNCA*.
- zinc finger proteins ZSCAN21 occupy a specific region within human *SNCA* intron 1.
- zinc finger proteins GATA2 occupy a specific region within human *SNCA* intron 2.
- No SNPs or mutations were found within the binding sites of ZSCAN21 and GATA2.

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ABSTRACT

The transcriptional regulation of the gene encoding α-synuclein (*SNCA*) is thought to play a critical role in the pathogenesis of Parkinson's disease (PD), as common genetic variability in this gene is associated with an elevated risk of developing PD. However, the relevant mechanisms are still poorly understood. So far, only few proteins have been identified as transcription factors (TFs) of *SNCA* in cellular models. Here we show that two of these TFs bind to the DNA in human brain tissue: the zinc finger protein ZSCAN21 occupies a region within *SNCA* intron 1, as described before, while GATA2 occupies a specific region within intron 2, where we have identified a new binding site within the complex structure of the 5′-promoter region of *SNCA*. Electrophoretic mobility shift assays confirmed these binding sites. Genetic investigations revealed no polymorphisms or mutations within these sites. A better understanding of TF-DNA interactions within *SNCA* may allow to develop novel therapies designed to reduce α-synuclein levels.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease. About 5% of the cases show a Mendelian inheritance pattern. The *SNCA* gene and its product α-synuclein are linked both pathologically and genetically to PD. It is the main component of Lewy bodies which are pathological hallmarks of familial and sporadic forms of PD [1]. The fact that not only point mutations in the *SNCA* gene [2–4] but also multiplications of the *SNCA* locus [5,6] can cause rare familial forms of PD indicates that an increased expression level of wild-type α-synuclein is sufficient to cause PD.

In addition, genome-wide association studies revealed that *SNCA* is not only linked to the rare familial forms of PD, but also to the more common sporadic cases [7]. A number of polymorphisms in the 5′-promoter region and 3′-untranslated region (UTR) of *SNCA* were identified which are associated with both an increased risk to develop PD [8,9] and with significantly higher *SNCA* expression levels in blood and brain [10]. Thus, single nucleotide polymorphisms (SNPs) in the promoter region could affect the binding of transcription factors (TFs) or other regulatory elements.

Despite the importance of *SNCA* expression, there is a paucity of knowledge about TFs of *SNCA*. Eight regulatory regions responsive to the basic leucine zipper domain TF C/EBPβ have been described in the *SNCA* promoter which may mediate increased expression of *SNCA* after dopamine-induced cell stress in SH-SY5Y neuroblastoma cells [11]. Other TFs identified to modulate *SNCA* expression *in vitro* are the TF zinc finger and SCAN domain containing ZSCAN21

Abbreviations: PD, Parkinson's disease; TF, transcription factor; SNP, single nucleotide polymorphism; TFBS, transcription factor binding sites; siRNAs, small interfering RNAs; IP, immunoprecipitation; qRT-PCR, quantitative real-time PCR; ChIP, chromatin immunoprecipitation; bp, base pairs; EMSA, electrophoretic mobility shift assay; UTR, untranslated region.

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[12], as well as the TF zinc finger protein GATA2 [13]. The latter ones have been shown to activate *SNCA* expression in cellular models. Small interfering RNAs (siRNAs) against ZSCAN21 or GATA2, respectively, reduce α-synuclein levels. Binding sites for both TFs within the 5′-promoter of *SNCA* have been identified [12,13].

In the present study, we have focused on C/EBPβ, ZSCAN21 and GATA2. We have investigated, whether these TFs bind specifically to the DNA at the *SNCA* locus in human brain cells, since all previous work has been done with model systems. We could confirm some of the previous results and we present additional findings concerning TF-DNA interactions in human brain cells.

2. Materials and methods

2.1. Human post mortem tissues

Brain tissue was provided by the German Brain Bank "BrainNet". Only tissue from neurologically healthy donors was used. Donors were at the age of 67–78. Males and females were included equally. Four tissue samples from different areas of the brain (frontal cortex, cingulate gyrus and medulla oblongata) were used. The project was approved by the Ethics Committee of the Faculty of Medicine of the University of Tübingen.

2.2. DNA for genotyping

DNA was obtained from the Biobank of the Hertie Institute for Clinical Brain Research. Only DNA from patients suffering from idiopathic PD was used. Patients were at the age of 25–87. Males and females were included equally. DNA was extracted from the patients' blood after informed consent following our established protocol (Supplementary material).

2.3. Western blotting and immunoprecipitation (IP) assays

Nuclear proteins were isolated from human brain tissue using the DUALXtract Nuclear and cytoplasmic protein extraction kit (Dualsystems Biotech). Lysates were mixed with Laemmli buffer, denaturated at 85 °C for 20 min and subsequently run on 10% SDS polyacrylamide gels. Proteins were then transferred to PDVF membranes. Membranes were incubated with antibodies directed against GATA2, C/EBPβ or ZSCAN21 (all 1:1000, Santa Cruz Biotechnology). Membranes were probed with HRP-conjugated secondary antibodies and developed with Immobilon™ Western HRP Substrate Peroxide Solution (Millipore) followed by exposure to autoradiographic films (Hyperfilm™ ECL, Amersham). For the negative controls bovine serum albumin was used instead of nuclear proteins.

For the IP assays, lysates were incubated with 2 μg of antibodies directed against ZSCAN21 (Santa Cruz Biotechnology) followed by adding Protein A/G agarose beads and another incubation period. For the negative controls an irrelevant antibody of the same isotype was used. Immune complexes bound to agarose beads were then pelleted by centrifugation, subsequently washed three times in Tris buffer, denaturated in Laemmli buffer and resolved by Western blotting as described.

2.4. Bioinformatics

Promoter analysis and prediction of potential transcription factor binding sites (TFBS) was performed using MatInspector software version 8.0.4 with MatInspector library version 8.3 from Genomatix.

2.5. Primers

Quantitative real-time PCR (qRT-PCR) primers were designed to amplify 75–275 bp fragments according to the binding sites identified with MatInspector. Amplification products for high resolution melting and sequencing analyses ranged from 80 to 150 bp. All primers were designed using Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast), synthesized and HPLC-purified by Metabion International. Sequences can be found in Supplementary material.

2.6. Chromatin immunoprecipitation (ChIP) assays and qRT-PCR

ChIP assays were performed as previously described [14] using approximately 0.5 cm^3 of human brain tissue and antibodies directed against GATA2, C/EBPβ or ZSCAN21 (all from Santa Cruz Biotechnology). The DNA concentration was measured relatively by qRT-PCR. qRT-PCR was performed in duplicates for each sample and input DNA (=genomic DNA) using LightCycler480 SYBR Green I Master (Roche) according to the manufacturer's protocol in a Light cycler 2.0 RT-PCR system (Roche). For every binding site investigated, relative differences in DNA quantity were calculated by subtracting the C_t-value of the chromatin immunoprecipitated sample from the C_t-value of the input DNA. Expression levels of the target sequences were normalized to a housekeeping gene (amyloid precursor protein). PCR efficiency was measured for every approach and differences were considered in the calculations. C_t values were calculated by the Light cycler 3 software version 3.5 (Roche).

2.7. Electrophoretic mobility shift assays (EMSA)

Double-stranded DNA probes were designed according to the TFBSs identified with ChIP assays for GATA2 (5′-GGCCCCGGTGTTATCTCATTCTTTT-3′) and ZSCAN21 (5′-GACGAGGGGTAGGGGGTGGTCCC-3′). Oligonucleotides were synthesized and 3′-labeled with biotin by Metabion International. Assays were performed using the Gel Shift Chemiluminescent EMSA Assay Kit (Active Motif) according to the manufacturer's instructions. For every approach 20 fmol labeled oligonucleotides were incubated in 1X binding buffer (Active Motif), 50 ng poly(dI/dC), 14.3% Glycerol, 200 mM KCl, 17.9 mM $MgCl_2$ and 18 μg of nuclear extract at room temperature for 20 min and subsequently run on a 4% non-denaturating polyacrylamide gel. Probes were then transferred on a nylon membrane (Sigma), cross-linked and developed using the reagents included in the kit, followed by exposure to Hyperfilm™ ECL autoradiographic films (Amersham).

2.8. Genotyping

High resolution melting analyses were performed with High Resolution Melting Master (Roche) according to the manufacturer's protocol in a Light cycler 2.0 RT-PCR system (Roche). Optimal primer concentration was found to be 0.25 μM and $MgCl_2$ concentration to be 1.25 mM. For every approach approximately 50 ng DNA was used and melting analyses were performed after 45 amplification cycles. A sample set of 30 patients was analyzed with every run. Subsequently the melting curves of all samples were visualized in one graph using Light cycler 3 software version 3.5 (Roche). Melting curves which differed from the majority of curves were considered as "suspicious".

Samples with suspicious melting curves were genotyped by performing PCR amplification, followed by Sanger sequencing of the purified PCR fragments using BigDye Terminator v3.1Cycle Sequencing kit (Applied Biosystems) in an ABI PRISM 3130xl