Of PARK genes and lncRNAs – possible molecular mechanisms behind Parkinson's disease

Ph.D. Thesis

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II. **Boros, F. A.**, Maszlag-Török, R., Vécsei, L., & Klivényi, P. (2020). Increased level of NEAT1 long non-coding RNA is detectable in peripheral blood cells of patients with Parkinson's disease. Brain Research, 1730, 146672.
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II. **Boros, F. A.**, & Vécsei, L. (2019). Immunomodulatory effects of genetic alterations affecting the kynurenine pathway. Frontiers in Immunology, 10, 2570.
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V. Salamon, A., Maszlag-Török, R., Veres, G., **Boros, F. A.**, Vágvölgyi-Sümegi, E., Somogyi, A., ... & Zádori, D. (2020). Cerebellar Predominant Increase in mRNA Expression Levels of Sirt1 and Sirt3 Isoforms in a Transgenic Mouse Model of Huntington's Disease. Neurochemical Research, 1-10.
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List of abbreviations

AD: Alzheimer's disease

B2M: Beta-2-microglobulin

CCK-8: Cell Counting Kit-8

cDNA: complementary DNA

ceRNA: competing endogenous RNA

CNS: central nervous system

DBS: deep brain stimulation

EOPD: early onset PD

FACS: fluorescence-activated cell sorting flow cytometry

FBS: fetal bovine serum

FTD: frontotemporal dementia

GWA: genome wide association

HD: Huntington's disease

HK2: Hexokinase 2

HSF1: Heat shock factor 1

i.p.: intraperitoneal

LD: linkage disequilibrium

LDD: long disease duration

lncRNA: long non-coding RNA

LOPD: late onset PD

LRRK2: Leucine-rich repeat kinase 2

MAPT: Microtubule Associated Protein Tau

miRNA: micro RNA

mito-mRNA: mitochondrial protein coding messenger RNA

MPP+: 1-methyl-4-phenylpyridinium

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

mtDNA: mitochondrial DNA

NEAT1: Nuclear Enriched Abundant Transcript 1; Nuclear Paraspeckle Assembly Transcript 1

NEAT1L: NEAT1 long isoform

NEAT1S: NEAT1 short isoform

NLRP3: nucleotide oligomerization domain-like receptor protein with pyrin domain containing 3

PBS: phosphate-buffered saline

PCR: Polymerase chain reaction

PD: Parkinson's disease

PQ: paraquat

PSP: progressive supranuclear palsy

RAB3IP: RAB3A-interacting protein

RFLP: restriction fragment length polymorphism

ROS: reactive oxygen species

RT-qPCR: quantitative reverse transcription PCR

SDD: short disease duration

SFN: sulphoraphane

SNCA: synuclein-alpha (gene)

SNP: single nucleotide polymorphism

tBHP: t-butyl hydroperoxide

TCEANC2: Transcription Elongation Factor A N-Terminal and Central domain Containing 2

TUG1: Taurine up-regulated gene 1

WB: Western blot

α-syn: alpha-synuclein (protein)

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease worldwide. It affects 1 percent of the population over the age of 65 years, and the prevalence of the disease rises with senescence [1]. PD is caused by the irreversible loss of dopaminergic neurons in the substantia nigra. Destruction of these cells leads to the characteristic motor symptoms: tremor, bradykinesia and rigidity. These are usually accompanied by various non-motor symptoms - such as dysfunction of the autonomic nervous system, sleep and mood disorders, gastrointestinal problems – aggravating the disease and increasing the burden on not only the patient but on family members and society as well.

To date, no rapid diagnostic test is available for PD and diagnosis of the disease is based on the presence of the cardinal motor symptoms described above. However, by the time those manifest, the majority of the dopaminergic neurons are irrevocably lost. Moreover, non-motor symptoms often appear in the years that precede the manifestation of motor symptoms, implying that pathological processes start at an even earlier age [2]. Despite the intensive research focusing on development of disease-modifying therapies [3], so far curing PD is not attainable, and only symptomatic treatment is available. In light of the devastating symptoms, high prevalence, lack of diagnostic test and curative treatment, there is an urgent need to identify possible biomarkers and new therapeutic targets for PD. Various animal and cell models of the disease are used in PD research however, considering the complex and yet unclarified pathomechanism of the disease it is not surprising that none of the models in use are capable of fully reproducing the pathological hallmarks of the disorder. One of the most widespread type of models used is those generated by toxins impairing mitochondrial function such as MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)/MPP+ (1-methyl-4-phenylpyridinium) and paraquat (PQ). However, results obtained from these models often seem conflicting, calling attention to the possible shortcomings of different toxin models.

1.1. Etiology of Parkinson's disease – with a focus on the genetic background

PD is a complex multifactorial disease for which the exact pathomechanism is still not fully elucidated. Though age has been recognized as the most important risk factor [4], various environmental and lifestyle factors trigger and/or facilitate the disease [5] and several genetic alterations have been found to be related to the disorder. So far

approximately 90 genomic loci have been identified at which genetic variability(ies) occur that are reported/proposed to be linked to the disease [6]. Several genes located at these loci are designated 'PARK', referring to their relationship with PD. Among these, variations of only a handful of genes have been identified to be in direct, causal relationship with the development of the disease. These can account for both familial and the more common sporadic PD cases. Sporadic cases represent 85-90% of all PD cases, while the percentage of PD showing familial inheritance is 10-15%. Pathogenic single gene mutations (monogenic form of PD) account for approximately 30% of familial cases and for only 3-5% of sporadic PD. However, a growing body of evidence suggests the role of genetic factors in 60% of the sporadic cases as well [7]. Unlike the pathogenic mutations of monogenic PD, these genetic variants do not necessarily show a clear association with the occurrence of the disease, rather they are mostly proposed to have disease modifying effect. Some of these are risk factors that are proposed to increase the chance of developing the disease. Others are proposed to play a protective role in PD pathogenesis. Such variants are mainly single nucleotide polymorphisms (SNPs) and their linkage to the disease is most often suggested based on results of genome wide association (GWA) studies. These studies involve large numbers of participants, thus the homogeneity of the study groups is often compromised. As allele frequencies of SNPs often vary among different populations, often it is difficult to apply data obtained from a certain population to individuals of a different origin [8]. Validating results of GWA studies in smaller but more defined patient and control cohorts is therefore necessary and highly warranted.

In a recent publication Benson classified PARK genes into three groups based on inheritance pattern and clinical manifestation of the disease and cellular functions of the encoded proteins [2]. Based on this classification *PARKIN (PARK2)*, *PINK1 (PARK6)*, *DJ-1 (PARK7)*, *FBXO7 (PARK15)*, *VPS13C (PARK23)*, and *ATP13A2* (*PARK9*) constitute the first group. PD-linked loss of function mutations of either of these genes show autosomal recessive inheritance pattern. The proteins encoded by these genes play important roles in marking and clearing malfunctioning mitochondria and sustaining mitochondrial health. Malfunctioning of these proteins leads to PD symptom appearance earlier than as it would be expected in the case of sporadic PD. According to Benson, group II comprises *SNCA (PARK1* and *4*), *LRRK2 (PARK8*), and *VPS35 (PARK17*), genes of which mutations cause autosomal dominantly inherited PD with late symptom onset showing features that resemble idiopathic PD. Further common feature of these genes is that they encode proteins that regulate intracellular membrane trafficking [2]. According to

Benson the third group consists of *DNAJC6 (PARK19)* and *SYNJ1 (PARK20)*, genes of which variants are associated with recessively inherited disease with early symptom appearance leading to a rapid progress accompanied by cognitive decline. Both *DNAJC6* and *SYNJ1* take part in the regulation of clathrin coat removal from internalized presynaptic vesicles, which is impaired due to mutations of the genes [2].

In the following paragraphs we briefly summarize data on some of the PARK genes which are included in the studies described in this thesis.

SNCA (synuclein-alpha) was the first gene whose genetic alterations were identified to lead to autosomal dominant PD, thus becoming the first to be designated as a 'PARK' gene [9]. The gene encodes the 140 amino acid α-synuclein (α-syn), which is the major component of Lewy bodies [10]. The accumulation of the protein is proposed to increase dopamine toxicity by an as yet unknown manner, contributing to the selective loss of dopaminergic neurons in PD [11]. Since the identification of the first *SNCA* variant as a cause of familial PD and that the aggregation of the protein is a cardinal pathological hallmark of the disease, several further variants of the gene were recognized or proposed to increase, or in some cases to decrease the risk of developing the disease (reviewed: [12]).

A couple of years following the identification of *SNCA* as a PD related gene, the involvement in the disease of another gene *LRRK2* (Leucine-rich repeat kinase 2 *alias PARK8*) was described in 2002 [13]. During the past two decades numerous mutations of the *LRRK2* gene have been identified, making this gene the most common cause of both familial and sporadic PD [14][15]. LRRK2 has been shown to be involved in various cellular functions, though its exact physiological function needs further elucidation. Among the various functions in which LRRK2 is implicated in is a role as scaffolding protein, modulation of neurite outgrowth, involvement in cytoskeleton maintenance, intracellular vesicle trafficking, lysosome homeostasis, endolysosomal trafficking and autophagy (reviewed in [16][17]).

The long arm of chromosome 17 gives place to a common inversion of approximately 900 kb length which results in two haplotypes, H1 and H2 [18]. This inversion site incorporates several genes [19], and one of the most studied among them is *MAPT* (Microtubule Associated Protein Tau) due to its linkage with several neurodegenerative disorders [20]. The more common H1 haplotype was found to result in an increased *MAPT* expression level because of its higher transcriptional activity due to more frequent transcription initiation [21][22]. In line with this, the H1 inversion polymorphism has been associated with several neurological diseases which have the

common characteristic of accumulation of MAPT neurofibrillary tangles in neurons, such as frontotemporal dementia (FTD), progressive supranuclear palsy (PSP), Alzheimer's disease (AD) and PD – however, the association with the latter is still a matter of debate.

The PARK10 locus is situated on the short arm of chromosome 1. Its relation with PD was first described in 2002 in a large Icelandic family [23]. Similarly to the case of the *MAPT* gene, a linkage disequilibrium (LD) block of 100 kb in the PARK10 region was reported to be associated with PD [24]. One of the genes located in this region is *TCEANC2* (Transcription Elongation Factor A N-Terminal and Central domain Containing 2), the exact function of which is still unknown.

1.2. Long non-coding RNAs in Parkinson's disease

Indubitably, PD-related genes encode proteins that act in diverse cellular pathways, including mitochondria maintenance, energy homeostasis, synaptic transmission, vesicle transport, protein transport and degradation, autophagy, lysosome function, and α-syn clearance (reviewed: [2]). Identifying common traits behind the diverse mechanisms which lead to PD is crucial for the better understanding of the disease. Identifying a factor that acts at a meeting-point of these processes could lead to the identification of a therapeutic target for intervening in PD with various genetic backgrounds. Due to their diverse functions, long non-coding RNAs (lncRNAs) have recently emerged as possible regulatory hubs of complex molecular changes affecting PD development.

lncRNAs are RNA transcripts produced by RNA polymerase II that are more than 200 nucleotides in length and although they are often polyadenylated and capped [25], they are not translated into proteins. lncRNAs are known to exert regulatory roles on gene expression on various levels (reviewed: [26]). They can control gene expression by recruiting complexes to specific chromosomal regions for histone modification and for modulating transcription factor activity. At the post-transcriptional level they can take part in mRNA modifications, regulate mRNA decay and alternative splicing, and also can act as competing endogenous RNAs (ceRNAs) and sponge micro RNAs (miRNAs) (reviewed: [26]).

Considering the wide range of mode of action by which they affect cellular homeostasis, it is not surprising that lncRNAs have gained attention in relation to neurodegenerative diseases (reviewed: [27][26][25]). A relatively large number of lncRNAs have been implicated in neurodegeneration on the basis of several criteria. In

most cases however the causal role of these RNAs in disease development has not been firmly established and the mechanisms of action are disputed [28].

1.3. NEAT1 in Parkinson's disease

With regard to PD, among lncRNAs NEAT1 attracted particular interest, since its expression was found to be elevated in different brain regions of PD patients [29]. Further pieces of information on the possible role of NEAT1 in PD became available in the literature during the progress of research described here [30][31][32][33][34][35][36]. These will be discussed in detail in a later chapter of the thesis. Here I will summarize basic information available on NEAT1 discovery, structure and function.

NEAT1 (Nuclear Enriched Abundant Transcript 1, later changed to Nuclear Paraspeckle Assembly Transcript 1) lncRNA was first described as a virus inducible non-coding RNA (*alias* VINC), as it was first identified as a transcript which was up-regulated by *Japanese encephalitis*- and *Rabies virus* in mouse brain [37]. Since its identification, NEAT1 has been found to be a highly abundant nuclear RNA [38]. In human, it is transcribed from the long arm of chromosome 11, from the multiple endocrine neoplasia (MEN) type I locus [39]. The lncRNA exists in two isoforms: a 3 684 nucleotide short variant (NEAT1_1, *alias* MENepsilon), and a longer isoform which is 22 743 (NEAT1_2, *alias* MENbeta) [39]. In the following descriptions for clarity I will use NEAT1S (as for short) and NEAT1L (as for long) designations for the short and long isoform, respectively. While there is a general agreement on the expression of the two NEAT1 variants, the production of further variants is unclear [40][41][42][43]. The two NEAT1 isoforms are transcribed by RNA polymerase II from the same promoter under the same transcriptional control. The full length of NEAT1S corresponds to the 5' end sequence of NEAT1L, thus distinct investigation of NEAT1S poses a great challenge. This may be partly the reason why although NEAT1S is generally observed in higher quantities and is present in a wider range of tissues, its exact function and cellular localization is less clear compared to the longer isoform. NEAT1L is inarguably one of the main components of paraspeckles, the subnuclear ribonucleoprotein complexes localizing in the interchromatin space of cells [44][45]. Paraspeckles are important regulators of transcription and RNA processing *via* their ability to retain RNAs and proteins in the nucleus, modulating RNA editing and splicing and sponging miRNAs (reviewed in [46]). NEAT1L folds end-to-end within paraspeckles, so that the core of the RNA is localised in the centre and its 3' and 5' ends in the periphery. Considering that the 5'end of the longer isoform is identical to NEAT1S, it

is plausible that the short isoform is also situated in paraspeckles [47]. However, recently various studies refuted this notion by detecting NEAT1S in the cytoplasm in foci termed 'microspeckles' [47][48][49]. Further findings such as the identification of defects in mouse female reproductive tissue development caused by the absence of the long, but not the short isoform [50][51] and the disruption of paraspeckle formation caused by the knockdown of NEAT1L despite the presence of intact NEAT1S [52] give ground to the suggestion that while NEATL is indispensable, NEAT1S may be only a byproduct of transcription or RNA editing [50]. However, the fact that NEAT1S overproduction promotes cell resistance against oxidative stress [53] and that the two isoforms differ in accumulation and effects in various types of cancer [54][55][56][43] argues against this assumption. The different cellular localization and the relatively higher abundance of the shorter isoform [48] strengthen the concept of a distinct as yet not clarified role of NEAT1S.

In accord with its diverse functions and roles, NEAT1 expression is regulated by various factors, most of them intervening on the level of gene transcription, RNA stabilization and 3'-end processing (reviewed: [57]). NEAT1 expression has been shown to be regulated by various factors related to tumorigenesis such as p53, BRCA1, E2F1, CARM1; to immune response, such as STAT3; to cellular response to oxidative stress, such as Nrf2 [58] and to stress response such as HIF2α (Hypoxia-inducible factor 2 alpha) and HSF1 (Heat shock factor 1) (for a review see: [57]).

A recent work of Wang and colleagues revealed an intensive cross-regulation between paraspeckles and mitochondria [59]. Mitochondrial stressors and/or depletion of mitochondrial proteins result in increases in NEAT1 expression and consequent changes in the number and shape of paraspeckles, leading to enhanced retention of mRNAs encoding mitochondrial proteins (mito-mRNA) in the nucleus. The other way around, silencing of NEAT1 also altered mito-mRNA retention which also impacted mitochondrial function and dynamics [59]. Several studies have reported decreased mitochondrial DNA copy number in PD patients compared to healthy controls [60][61], therefore the finding of such a close connection between paraspeckles and mitochondria raise intriguing questions.

The role of mitochondrial dysfunction in the pathomechanism of PD is well established [61] and there is a growing body of evidence for the involvement of the immune system as demonstrated by increased levels of inflammatory markers [62][63][64]. Considering the various immunomodulatory and stress response related factors that affect NEAT1

expression and the intensive crosstalk between paraspeckles and mitochondria, the association of NEAT1 and PD seems well grounded.

Until very recently, however, most studies on NEAT1 function concerned the role of the lncRNA in tumorigenesis. As these data are out of the scope of the thesis here we mention only that in many aspects NEAT1 seems to have diverse roles in different forms of cancer, acting in some cases more as a specific oncogenic, while in other cases as a tumor suppressor factor.

The first studies on changes in NEAT1 expression in PD date back only a few years. In 2017 Kraus and colleagues reported up-regulated NEAT1 expression in the anterior cingulate cortex of PD patients compared to control samples [29]. During my PhD work NEAT1 attracted more and more interest in PD research. In the past few years NEAT1 up-regulation was also detected in *post mortem* human *substantia nigra* PD samples [30], and an increasing body of evidence has been accumulated on the possible involvement of this lncRNA in PD based on studies conducted on *in vitro* and *in vivo* models of the disease. In several aspects however, these data are controversial and a key question remains unanswered: does a change in NEAT1 level have a direct effect on PD (and if so, does it alleviate or aggravate the condition), or is NEAT1 lncRNA merely a bystander in PD pathogenesis without being actively involved in the course of the disease? Part of the aims of my thesis work was to help finding answers to these questions.

2. Aims

1st aim: Evaluate the frequency of specific PARK gene mutations in Hungarian samples. We selected 10 variants of 4 PARK genes and performed experiments to determine whether:

A: The frequencies of these SNPs differ among PD patients and non-PD controls in the Hungarian population.

B: Do any of the analysed SNPs have a disease modifying effect in the Hungarian population - if yes, is it a protective or a risk variant?

2nd aim: To determine if changes in the level of any lncRNA implicated in neurodegeneration can be detected in peripheral blood samples of PD patients.

A: Determination of which of 41 selected lncRNAs are detectable in altered level in samples of PD patients by using a three-step analysis with increasing sample number and decreasing target RNA number.

B: Analysis and comparison of any differences in the expression of any of the detectable lncRNAs between PD patient and control groups, and how this relates to PD progression.

3rd aim: Find and establish *in vitro* and *in vivo* PD models in which the altered level of identified lncRNA can be modeled and use these to answer questions on the possible molecular role of the lncRNA.

A: As we identified NEAT1 level as being altered in PD samples, by the following experiments we wanted to set up neuroblastoma cell *in vitro* and mouse *in vivo* PD models and determine conditions which result in increased NEAT1 expression.

B: With the models we intended to determine whether increasing NEAT1 expression has an effect on cell viability, apoptosis and mitochondrial DNA content.
With the information obtained by the above experiments we wanted to contribute to the answer on whether NEAT1 has a protective or pathogenic role in PD.

3. Methods

3.1.Biological samples used

In the studies described here I used human, mouse and cell samples.

3.1.1. Human samples: Blood samples of PD patients and controls were used for genotype analysis for detecting PARK gene SNPs and for lncRNA determination. For genomic DNA and RNA analysis peripheral venous blood was drawn in 5 ml EDTA containing blood collection tubes from the participants of the patient and control group. Collected samples were stored in the Biobank of The University of Szeged, Neurological Clinic. Both PD patients and non-PD participants were Hungarians of Caucasian origin. The diagnosis of PD was set up by movement disorder specialists based on medical history and physical examination. For demographical data of participants involved in the studies see Table 1.

Table 1. Demographic data of participants involved in the assessment of PARK gene variants (Table A) and lncRNA studies (Table B)

Table A				
Gene		**n male/female)**	**Age (mean ± SD; years)**	**(EOPD/LOPD ratio)**
LRRK2	**PD**	61/63	66,5 ± 9,5	68/56
	Ctrl	61/67	64,5 ± 9,6	n.a.

SNCA* and *MAPT	**PD**	60/63	66,5 ± 9,5	67/56
	Ctrl	56/66	64,3 ± 8,8	n.a.
TCEANC2	**PD**	59/62	66,5 ± 9,6	66/55
	Ctrl	50/60	64,9 ± 8,1	n.a.

Table B					
Validation study I.		**n (male/female)**	**Age (mean ±SD; years)**	**Age at disease onset (mean ± SD; years)**	**Disease duration (mean ± SD; years)**
	Ctrl	15 (6/9)	61.3±9.9	n.a.	n.a.
	PD	18 (9/9)	60.3±5.7	52.5±5.6	7.8±5.8
Validation Study II.	**Ctrl total**	36 (16/20)	57.6±18	n.a.	n.a.
	PD total	43 (24/19)	63.3±11.4	54.8±12.6	8.4±6
	PD DBS	8 (6/2)	64.3±7.1	53.7±10.6	9.7±4.6
	PD no DBS	35 (18/17)	63.1±12.2	55±13.1	8.1±6.2
	EOPD	27 (14/13)	57.6±9.8	47.5±10.2	9.6±6.7
	LOPD	16 (10/6)	73±5.9	66.5±4	6.4±4
	SDD	27 (15/12)	62.9±11.9	58±10.8	4.9±2.8
	LDD	15 (8/7)	63.7±10.9	49.1±13.9	14.6±5

Abbreviations: *LRRK2*: Leucine-Rich Repeat Kinase 2; *SNCA*: Synuclein Alpha; *MAPT*: Microtubule Associated Protein Tau; *TCEANC2*: Transcription Elongation Factor A N-Terminal And Central Domain Containing 2; PD: Parkinson's disease; Ctrl: control; SD: standard deviation; EOPD: early-onset PD; LOPD: late-onset PD; DBS: deep brain stimulation; SDD: short disease duration; LDD: long disease duration.

3.1.2. Mouse samples of *in vivo* PD model: Animals involved in the experiments were 10-12 weeks old C57Bl/6J male mice. The strain was originally obtained from Jackson Labs (Jackson Laboratories, Bar Harbor, ME, USA) and bred in our institutional vivarium. Mice were housed in cages under standard laboratory conditions (12-12 h light-dark cycle, free access to food and water). All animal experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) and approved by the local animal care committee.

For treatment, MPTP was dissolved in phosphate-buffered saline (PBS) and sulphoraphane (SFN) in ethanol and then diluted in saline solution. All treatments were administered *via* intraperitoneal (i.p.) injection. Upon termination animals were deeply anesthetized with isoflurane (Forane; Abott Laboratories Hungary Ltd., Budapest, Hungary), followed by thoracotomy and transcardial perfusion with artificial cerebrospinal fluid for 2 min by an automatic peristaltic pump. During dissection, the brains of the animals were rapidly removed on ice and four brain regions (brainstem, cerebellum,

striatum and cortex) were separated and cut in half (right and left side). Samples were stored at -80°C until further use. The experiments presented in this work were all carried out using the left sided samples.

3.1.3. Cell culture samples of *in vitro* PD model: For an *in vitro* model of PD the SH-SY5Y human neuroblastoma cell line was used (cells were kindly made available by the laboratory of Professor László Vigh, SZBK). Cells were cultured at 37°C at 5% CO_2 in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM-F12; Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Scientific, Waltham, MA, USA), 2 mM L-glutamine and antibiotics penicillin and streptomycin (1%-1% each).

Cell cultures were trypsinised and passaged by threefold dilution on every third day. The same incubation conditions were implemented during the treatment periods. As SH-SY5Y cells differentiate with serial passages all experiments presented here were performed with cultures no older than 20 passages. For treatments cells were seeded twenty-four hours prior to treatment at a density of $2.2*10^6$ cells on 10 cm (10 ml) or $9*10^5$ cells on 6 cm (5 ml) petri dishes for RNA and protein and for DNA analysis, respectively. For viability assays cells were distributed into 96 well plates ($5*10^4$ cells/100 ul). For FACS analysis $2.2*10^6$ cells were plated into 10 cm diameter petri dishes.

For cell treatments MPP+ and PQ were dissolved in PBS, while SFN was dissolved in ethanol and then diluted in PBS.

3.2.DNA, RNA and protein preparation from biological samples

3.2.1. Human samples: Genomic DNA was isolated from 500 ul peripheral blood samples by the standard desalting method [65]. The extracted DNA was stored at -20°C until further analysis.

For RNA preparation TRI Reagent was used according to the manufacturer's protocol (Sigma-Aldrich, St. Louis, Missouri, USA). Samples were then stored at -80°C until further use.

3.2.2. Mouse samples: Frozen brain tissue samples were homogenized with an ultrasound homogenizer (UP100H, Hielscher Ultrasound Technology, Germany; amplitude: 100%, cycle: 0.5) in TRI Reagent (Sigma-Aldrich, St. Louis, Missouri, USA). RNA and DNA for mitochondrial DNA copy number determination were isolated following the instructions of the manufacturer (Sigma-Aldrich, St. Louis, Missouri, USA).

Until further analysis DNA and RNA samples were stored at -20°C and -80°C, respectively.

3.2.3. Tissue culture samples: For DNA isolation, after repeated washes with PBS, cells were scraped in PBS and collected by centrifugation in Eppendorf tubes. For total DNA isolation the phenol-chloroform method was implemented. The isolated DNA was stored at -20°C until further analysis.

For RNA extraction cells on 10 cm plates were lysed in 750 ul TRI Reagent (Sigma-Aldrich, St. Louis, Missouri, USA), scraped and collected into Eppendorf tubes. Subsequent RNA isolation was done following the instructions of the manufacturer (Sigma-Aldrich, St. Louis, Missouri, USA).

For protein analysis cells were collected in PBS by centrifugation and the pellet was stored at -20°C until further use.

DNA and RNA concentration of the samples was determined using Maestro NanoDrop micro-volume spectrophotometer.

3.3. Methods used for analysis of DNA, RNA and protein samples

3.3.1. SNP analysis: For the detection of specific PARK gene mutations in genomic DNA samples restriction fragment length polymorphism (RFLP) and TaqMan allelic discrimination methods were implemented.

Polymerase chain reaction (PCR) followed by RFLP analysis was implemented for the genotyping of R1628P and G2385R *LRRK2* variants. The sequences of the primers used for generating PCR products are listed in Table 2, for cycling conditions please see Table 3. In the case of the G2385R variant PCR amplification yielded a PCR product of 170 bp. If the mutation was present in homozygous form, digestion with AccI restriction enzyme (Thermo Scientific, Waltham, MA, USA) (overnight incubation at 37°C) resulted in two (123 and 47 bp) fragments. Heterozygous samples were partially digested resulting in three fragments (170,123 and 47 bp), while wild-type G2385R samples remained undigested, yielding one, 170 bp DNA fragment.

For the detection of the R1628P variant BstUI restriction enzyme (Thermo Scientific, Waltham, MA, USA) was used under the same incubation conditions. In contrast to the G2385P variant, homozygous mutant R1628P PCR samples remained undigested, resulting in one, 419 bp fragment. The partial digestion of heterozygous samples yielded three bands (419, 263 and 156 bp), while digestion of homozygous wild-type samples resulted in the generation of two (263 and 156 bp) DNA fragments.

After digestion DNA fragments were separated by agarose gel electrophoresis (2% SeaKem LE Agarose, Lonza, Basel, Switzerland). The bands were visualized with ECO Safe alternative gel stain (Pacific Image Electronics, Torrance, CA, USA).

For the analysis of R1398H, N551K, S1647T and rs1491923 *LRRK2*, and all the investigated *MAPT*, *SNCA* and *TCEANC2* variants TaqMan allelic discrimination method was implemented. Aliquots of commercially available TaqMan assays (Thermo Scientific, Waltham, MA, USA), qPCRBio Genotyping mix (PCR Biosystems, London, UK) and DNA samples were placed into wells of a 96-well PCR plate in a final reaction volume of 25 ul. For cycling conditions see Table 3.

3.3.2. Mitochondrial DNA copy number determination: For the analysis of mitochondrial DNA copy number, isolated DNA was diluted to a concentration of 1 ng/ul, and a total of 3 ng DNA was used for quantitative reverse transcription PCR (RT-qPCR) reactions. Considering that each eukaryotic cell contains hundreds to thousands of mitochondria with one to ten copies of the circular mitochondrial genome in each of them, and that the number of mitochondrial DNA copies varies not only inter-, but intra-individually showing tissue- and age specificity, determining copy number changes is quite a formidable challenge. A feasible and accepted approach is to normalize mitochondrial DNA content to a single copy nuclear gene [66]. In our experiments the nuclear gene that served for normalization was HK2 (Hexokinase 2) and B2M (Beta-2-microglobulin) in mouse and SH-SY5Y cell samples, respectively. For mitochondrial genes, specific primers targeting the mouse 16S gene and human $tRNA^{Leu(UUR)}$ were used. RT-qPCR was carried out by SYBER green detection (RT2 SYBR Green Mastermix (qPCRBIO)) in an end reaction volume of 10 ul.

3.3.3. Determination of lncRNA levels: For analysis of lncRNA expression changes in human blood samples RNA samples were first converted into complementary DNA (cDNA). In the preliminary and first validation study cDNA synthesis was carried out from 500 ng of extracted RNA with the use of RT2 First Strand Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. RT-qPCR was carried out with the use of specifically designed Custom RT2 PCR Array (Qiagen, Hilden, Germany) containing lncRNA specific primer pairs. Two arrays were designed and used for groups of 41 and 12 lncRNAs. RT2 SYBR Green Mastermix (Qiagen, Hilden, Germany) and equal volume aliquots of cDNA samples were placed into the wells of a 96-well plate in 25 ul final volume. For validation study II. commercially available NEAT1 and TUG1 (Taurine up-regulated gene 1) gene-specific primers were obtained from Qiagen and used

according to the instructions of the manufacturer. 1000 ng RNA was converted into cDNA with the use of Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific). RT2 SYBR Green Mastermix (qPCRBIO) was used for Real-time PCR reaction, the final volume of each reaction mix was 25 µl. Cycling conditions: Table 3.

For lncRNA expression analysis of mouse and cell tissue culture experiments 1 ug and 2 ug RNA was used for cDNA synthesis, respectively. Prior to cDNA synthesis, genomic DNA was removed with digestion with RNase free DNase I following the instructions of the manufacturer (DNase I, RNase free, Thermo Fisher Scientific Inc., Marietta, OH, USA). For reverse-transcription the RevertAid First Strand cDNA Synthesis Kit was implemented (Thermo Fisher Scientific Inc., Marietta, OH, USA). qPCR reactions were carried out with SYBER green detection (RT2 SYBR Green Mastermix (qPCRBIO)) in a final volume of 20 ul. 18S rRNA was used as a housekeeping gene. In the case of animal experiments commercially available 18S rRNA primers were purchased from Applied Biosystems (Carlsbad, CA, USA) and for PCR reactions TaqMan probe mix (qPCRBIO) was used.

All real time PCR reactions were carried out in a CFX96 thermocycler (Bio-Rad). Primer sequences and cycling conditions are listed in Table 2 and 3, respectively.

3.3.4. Immunoblot analysis of proteins – Western blot: For western blotting (WB) a total of 30 ug (8 ul) of protein per sample was run on a 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) electrophoresis, following which the proteins were transferred to 0.2 um nitrocellulose membrane (Thermo Fisher Scientific). Transfer was carried out on a Bio-Rad blotting system in 2 hours at 200 mA.

Following transfer the membranes were soaked in 5% milk in TBST (10mM Tris-HCL pH 8.0, 150mM NaCL, 0,05% Tween 20) for two hours to block protein binding sites. Blots were exposed to primary antibodies at 4°C overnight**.** Anti-PINK1 rabbit antibody was from Invitrogen (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA, Cat.no.:PA5-85930 dilution: 1:1000). Rabbit anti-β-actin antibody used as an internal control was from Sigma-Aldrich (St. Louis, Missouri, USA; Cat. no.: A5160) and used in 1:3000 dilution. After removal of the primary antibodies membranes were washed with TBST four times for 10 minutes each. Next the membranes were exposed to the secondary antibodies (goat anti-Rabbit IgG antibody diluted in 1:10000, Invitrogen, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA; Cat.no.:A27036 and DAKO P0448 Polyclonal Goat Anti Rabbit antibody diluted in 1:10000, Agilent, United States for PINK1 and for β-actin, respectively) for 1 hour, incubated at room temperature. Following

repeated washes with TBST (4 times 10 minutes each) the blots were developed using chemiluminescence and visualized with LI-CoR C-DIGIt Chemiluminescence Western Blot Scanner.

3.4. Further methods used

3.4.1. Determination of cell viability: Cell viability measurements were carried out with Cell Counting Kit-8 (CCK-8) according the instructions of the manufacturer (Sigma-Aldrich, St. Louis, Missouri, USA). In brief, cells in 96 well plates were treated with different reagents to induce neurodegeneration and/or neuroprotection as described in the text. Following the treatment, culture medium was carefully aspirated and was substituted with a mixture of fresh medium and CCK-8 assay. Cells were incubated for two hours at 37°C. For absorbance measurement a Gen5™ Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA) was used. Absorbance was measured at 450 nm and 650 nm in order to exclude the differences originating from background absorbance. Changes in cell viability were calculated with the use of the difference on absorbance at 650 nm and 450 nm.

3.4.2. Analysis of apoptosis by fluorescence-activated cell sorting flow cytometry: For apoptosis analysis Annexin V-FITC Apoptosis Detection Kit was implemented (eBioscience™, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). Following treatments as described in the text, both adherent and floating cells were collected, washed with PBS and resuspended in 300 ul 10X Binding Buffer. Consequently 5 ul Annexin V-FITC was added and the samples were incubated for 15 minutes at room temperature. Following this, 5 ul propidium iodide (PI) solution (8 ng/ul) was added to the samples and kept on ice. Cells were analyzed by fluorescence-activated cell sorting (FACS) flow cytometry on a BD FACS Calibur flow cytometer. Data was analyzed by the CellQuest pro software.

3.5. Statistical analysis

For the analysis of genotype and allele frequencies Chi-square ($\chi 2$) test or Fisher's test was used. Odds ratio (OR) with a 95% confidence interval (95% CI) was calculated for the analysis of the association between PD and genotype frequencies.

Statistical analysis of the PCR results of validation study I. was performed using RT2 PCR analysis web portal (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php). For the statistical analysis of all other PCR results presented in this work GraphPad Prism 6.01 statistics software was used. For the analysis of gene expression the $\Delta\Delta$Ct method was

implemented. ΔCt is equal to the difference between a gene of interest and the average of reference gene, ΔΔCt was calculated as ΔCt (patient) – average ΔCt (control) and fold change was determined as $2^{-(\Delta\Delta Ct)}$ value (Livak and Schmittgen, 2001). In order to identify the outliers among 2−ΔΔCt replicates the ROUT method was used.

Relative mitochondrial DNA (mtDNA) copy number was determined based on the Ct values of the investigated genomic and mitochondrial genes. mtDNA content was calculated using the formula $2x2^{\Delta Ct}$, where ΔCt is the Ct value of the mitochondrially encoded gene extracted from the Ct value of the nuclear gene. Relative mtDNA content is equal to (mtDNA content of treated sample)/(mtDNA content of control sample) [66].

For the analysis of data distribution D'Agostino and Pearson omnibus normality test was used. In case of normal distribution unpaired t-test was implemented, while in the case of non-normal distribution Mann-Whitney U test was performed. P value under 0.05 was considered significant. In study settings of multiple comparisons, Bonferroni correction was implemented.

For multiple comparisons one-way ANOVA or the non-parametric Kruskal-Wallis test was implemented depending on data distribution. For correction of multiple comparisons Dunn's test or Tukey's test was implemented after performing one-way ANOVA or Kruskal-Wallis test, respectively.

Table 2. Sequences of primers used in PCR reactions

Primer name	Primer sequence (5'-3')	Reference
LRRK2 - R1628P	FW: TTCTGACTACTTTCACTGAG	[67]
	REV: GGAGGTTTA CACTAGAAGC	
LRRK2 - G2385R	FW: TAGCCCTGTTGTGGAAGTG	
	REV: TTCAGAGGCAGAAAGGAAG	
Human nuclear DNA - B2M	FW: TGCTGTCTCCATGTTTGATGTATCT	[66]
	REV: TCTCTGCTCCCCACCTCTAAGT	
Mouse nuclear DNA - HK2	FW: GCCAGCCTCTCCTGATTTTAGTGT	[68]
	REV: GGGAACACAAAAGACCTCTTCTGG	
Human mtDNA - tRNALeu(UUR)	FW: CACCCAAGAACAGGGTTTGT	[66]
	REV: TGGCCATGGGTATGTTGTTA	
Mouse mtDNA - 16sRNA	FW: CCGCAAGGGAAAGATGAAAGAC	[68]
	REV: TCGTTTGGTTTCGGGGTTTC	
human NEAT1 total	FW: GGGCCATCAGCTTTGAATAA	[30]
	REV: GGTGGGTAGGTGAGAGGTCA	
human 18S	FW: GCTTAATTTGACTCAACACGGGA	[69]
	REV: AGCTATCAATCTGTCAATCCTGTC	
mouse NEAT1 total	FW: TTGGGACAGTGGACGTGTGG	[49]

	REV: TCAAGTGCCAGCAGACAGCA	
mouse NEAT1L	FW: GCTCTGGGACCTTCGTGACTCT	
	REV: CTGCCTTGGCTTGGAAATGTAA	

Table 3. Cycling conditions of RT-qPCR reactions. FW: forward; REV: reverse.

Study	Gene/Gene variant	Cycling conditions
Assessment of genotype distribution of PARK genes	*LRRK2* - R1628P	95°C for 10 min; 48x (95°C for 30s; 56.2°C for 45s); 72°C for 5 min
	LRRK2 - G2385R	95°C for 10 min; 45x (95°C for 30s; 54.2°C for 45s); 72°C for 5 min
	SNCA (rs258398; rs356186), MAPT (rs1052553), LRRK2 (R1398H)	95°C for 10 min; 40x (92°C for 15s; 60°C for 1min)
	LRRK2 (S1647T)	95°C for 10 min; 45x (92°C for15s; 58.4°C for 1min)
	LRRK2 (N551K)	95°C for 10 min; 40x (92°C for 15s; 59°C for 1min)
	LRRK2 (rs1491923)	95°C for 10 min; 40x (92°C for 15s; 58°C for 1min)
In vitro experiments	human NEAT1 total and 18S	50°C for 2 min; 95°C for 2 min; 40x (95°C for30s; 57°C for 45s; 72°C for 30s)
In vivo experiments	Mouse NEAT1 total and NEAT1L	50°C for 2 min; 95°C for 2 min; 40x (95°C for 30s; 63°C for 45s; 72°C for 30s)
	Mouse 18S	95°C for 10 min; 40x (95°C for 15s; 60°C for 1min)
Mouse mtDNA copy number	Mouse nuclear DNA - HK2; mouse mt DNA - 16sRNA	50°C for 2 min; 95°C for 2 min; 40x (95°C for 30s; 65.2°C for 45s; 72°C for 30s)
Cell mtDNA copy number	Human nuclear DNA - B2M; Human mitochondrial DNA - tRNALeu(UUR)	50°C for 2 min; 95°C for 2 min; 40x (95°C for 30s; 60.1°C for 45s; 72°C for 30s)

4. Results

4.1. Evaluating the frequencies of PARK gene SNPs in the Hungarian population

We analyzed the frequency of six mutations of the *LRRK2* (R1628P, G2385R, S1647T, R1398H, N551K and rs1491923), two SNPs of the *SNCA* gene (rs356186 and rs2583988) and variants of the *MAPT* (1052553) and the *PARK10* locus (rs10788972).

For the analysis of two *LRRK2* variants, R1628P and G2385R, PCR followed by RFLP analysis was implemented. For the analysis of R1398H, N551K, S1647T and rs1491923 *LRRK2* variants, and all the investigated *MAPT*, *SNCA* and *TCEANC2* alleles the TaqMan allelic discrimination method was used.

4.1.1. Putative risk factor LRRK2 mutations (G2385R, R1628P, S1647T and rs1491923): The G2385R and R1628P SNPs were reported to have a risk increasing effect in the development of PD, however, they were found to be absent or extremely rare in Caucasian populations. In accord with this, we did not find any of these SNPs to be present in either of our study groups.

The S1647T substitution is a result of a T to A change in exon 34. The minor allele (A) of the variant was found to increase the risk of developing PD in various Asian populations; however, such relation has not been identified in people of Caucasian origin. The genotype and allele distribution of this variant was similar in both our study groups. The difference was not significant when comparing either early onset PD (EOPD; disease onset ≤60 years) or late onset PD (LOPD; disease onset >60 years) patient subgroups to controls, or female patients to healthy controls. However, when examining the genotype distribution of male patients in comparison with the corresponding control group, a trend towards higher AA frequency could be observed in the latter. Comparing allele frequencies of the same groups revealed the minor (A) allele to show significantly higher frequency among healthy male individuals ($\chi 2 = 6.06$; $p = 0.014$).

The SNP rs1491923 is an A to G change (indicated in reverse orientation), in a locus 0.17Mb upstream of the *LRRK2* gene and the results of a GWA study recently proposed its role as a susceptibility factor of PD [70]. We found both genotype and allelic distribution of this variant to be similar in our patient and control group. Comparison of subgroups generated by separating PD and control study groups by gender or by the age at disease onset did not reveal significant difference either in genotype or in allele frequencies.

4.1.2. Protective LRRK2 variants (R1398H and N551K): The R1398H and N551K *LRRK2* variants were found to diminish the increased risk of the disease when co-occurring with the G2385R and/or R1628P variants [71]. No significant difference between either the genotype or allele frequencies of the R1398H or N551K variants was detected between our control and PD groups. Following stratification by gender or by age at disease onset, both allele and genotype frequencies remained similar. Except for one case in our group of healthy controls, the R1398H and N551K substitution always occurred simultaneously, thus these variants were found to be in LD.

4.1.3. *SNCA* and *MAPT* gene variants: The rs356186 variant of *SNCA* is a G to A change in an intron of the *SNCA* gene. The minor A allele is proposed to have a protective effect in PD. Comparing the genotype distribution of our control and patients' groups there was a significant difference ($\chi2 = 7.65$; $p = 0.022$) due to the higher relative frequency of the AA genotype among healthy participants in comparison to patients (AA vs. GG + AG. Fisher's test: $p = 0.019$, OR: 0.12, CI (95%): 0.014−0.95). Comparing the LOPD group to healthy controls also yielded a significant difference in genotype distribution ($\chi2 = 6.14$; $p = 0.046$), which is a consequence of higher frequency of AG genotype among LOPD patients (AG vs. GG + AA. $\chi2 = 5.07$; $p = 0.024$). No significant difference in genotype or allele distribution could be detected in other study setups. In addition, no significant difference was found in genotype or allele frequency of the rs2583988 SNP of *SNCA* and the studied *MAPT* variant (rs1052553) in either comparison.

4.1.4. *TCEANC2* gene variant (rs10789972): Allele and genotype distribution of the rs10789972 SNP was similar in both PD and control groups. No significant difference was revealed when analyzing these study groups, either when analyzing the EOPD, LOPD, male or female patients in contrast to the corresponding control groups.

4.2.Analysis of changes in lncRNA level in PD blood samples

4.2.1. Out of 41 lncRNAs related to neurodegeneration in the level of one is detectable change in PD blood samples: Following a detailed review on published data on lncRNAs in neurodegenerative disorders we chose 41 transcripts (Table 4.) with the aim of attempting to detect these in peripheral blood of PD patients and control individuals (n= 3 in each group) by qRT-PCR. Previous studies indicated that nine of the investigated transcripts are linked directly to PD (RP11-101C11.1, RP11-409K20.6, RP11-124N14.3, RP11-79P5.3, AC004744.3, RP11-542K23.9, PCA3 [72], NEAT1 [29] and MALAT1 [73]), while others were found to be associated with AD (BC200, BACE1-AS [74][75]), Huntington's disease (HD) (MEG3, TUG1 (Taurine Up-Regulated Gene 1), LINC00341, HAR-1A [76][77][78][79]), and/or are involved in mechanisms likely related to neurodegeneration. Following repeated RT-qPCR assays for these RNAs using primers commercially available we excluded from further analyses the lncRNAs with a Ct larger than 35, since the low expression level makes their detection by this technique unreliable. The lncRNAs which were considered to be detectable in our first approach (RP11-409K20.6, GAS5, RP11-124N14.3, LINC00341, PINK1-AS, NEAT1, MALAT1, MTOR-AS1, TUG1, BC200, PTENP1-AS, MEG3) were then investigated in larger groups of

healthy and PD samples (n = 15 and 18, respectively) (validation study I.). In this 'validation study I.' we found the level of BC200, PTENP1-AS and MEG3 to be below reliable detection level (Ct over 35), thus we excluded these lncRNAs from further analysis. For reliable comparison of expression levels we selected GAS5 as an internal control for reference, since the expression level of this transcript showed minimal variation in both study groups. This observation is in line with the findings of Kraus *et al.* (2017) and Santoro *et al.* (2016), who also noted the stable expression of this lncRNA [29] [80]. Using GAS5 as an internal standard for comparison we found significant up-regulation of the expression of NEAT1 among PD patients (fold increase=1.93; p=0.035) compared to the control group. Similarly, up-regulation of TUG1 lncRNA was observed among PD patients compared to control individuals (fold increase = 1.71; p = 0.036). Besides these two transcripts, no significant difference was detected in the expression of any other lncRNAs with regard of PD.

Based on the results of validation study I., we set up a further set of comparisons (validation study II.) with the aim of investigating the expression of NEAT1 and TUG1 in study groups including larger numbers of participants (PD patients n= 43; controls n= 36).

Using GAS5 as normalization standard we detected a significant up-regulation of NEAT1 expression among PD patients compared to controls (fold increase=1.62; p= 0.0019; Figure 1/A).

Table 4. Neurodegeneration implicated lncRNAs included in the preliminary study (control n=3, PD n=3)

RP11-101C11.1	*BCYRN1 (BC200)*	*DLX6-AS1*	*UCHL1-AS1*
RP11-409K20.6	*ATXN8OS*	PTENP1-AS	*SOX2-OT*
SCOC-AS1	BDNF-AS	MALAT1	BACE-AS1
RP11-124N14.3	HAR1A	*HOXA11-AS*	GAS5
RP11-79P5.3	HAR1B	*HOXA-AS2*	*HOTAIR*
LOC339568	NEAT1	*HOXA-AS3*	*SIX3-AS1*
AC004744.3	*DGCR5*	*MEG9*	ST7-AS2
RP11-542K23.9	MEG3	*TUNAR*	RBM5-AS1
LOC338797	TUG1	TMEM161B-AS1	LINC00853
PCA3	LINC00341	MTOR1-AS1	ST7-AS1
LINC01262			

Bold: lncRNAs reported to have altered expression in PD [72]. Italics: lncRNAS detected in low level (Ct>40).

4.2.2. NEAT1 lncRNA level change in PD samples in relation with disease history: Intriguingly, deep brain stimulation (DBS) treatment was reported to influence lncRNA expression in leukocytes [81], thus we performed comparisons with respect to this treatment as well (Figure 1/B-D). Significant up-regulation of NEAT1 expression was detected in the case of both groups of PD including and not including DBS patients when compared to the control cohort (fold increase = 1.61 and 1.62; p = 0.0021 and 0.0071, respectively). The comparison of NEAT1 expression levels of patients with and without DBS did not reveal significant difference.

The expression of the lncRNA was significantly up-regulated in both EOPD and LOPD group as compared to the control group (fold change= 1.5 and 1.82; p= 0.0181 and 0.0073, respectively), but no significant difference was observed between the EOPD and LOPD group (Figure 1/E-G). Analysis of subgroups generated based on gender revealed significantly up-regulated NEAT1 level among female PD patients when compared to female control individuals (fold increase= 1.72; p= 0.0073). Though it did not reach significance level, up-regulation could also be observed among male PD patients compared to the corresponding control subgroup (Figure 1/H and I).

In the comparison of short disease duration (SDD; disease duration<10 years) *versus* long disease duration (LDD; disease duration ≥10 years) subgroups, slight up-regulation of NEAT1 was observed in the latter; however, the difference was not significant. The analysis of these subgroups in comparison to the control group revealed significant NEAT1 up-regulation in both cases (SDD *vs.* control: fold change= 1.57, p= 0.028; LDD *vs.* control: fold change= 1.74, p=0.0008). The difference was more prominent when comparing the LDD group to controls (Figure 1/J-L).

Following Bonferroni correction in order to adjust for multiple comparisons the difference between the control group and PD group, patients with DBS or the LDD group remained significant.

In contrast with NEAT1, we detected no significant difference in the expression of TUG1 in either of the above described comparisons when RNA level changes in relation to GAS5 control were compared in larger control and PD groups, despite the fact that in validation study I. this RNA also was detected as being elevated in PD samples.

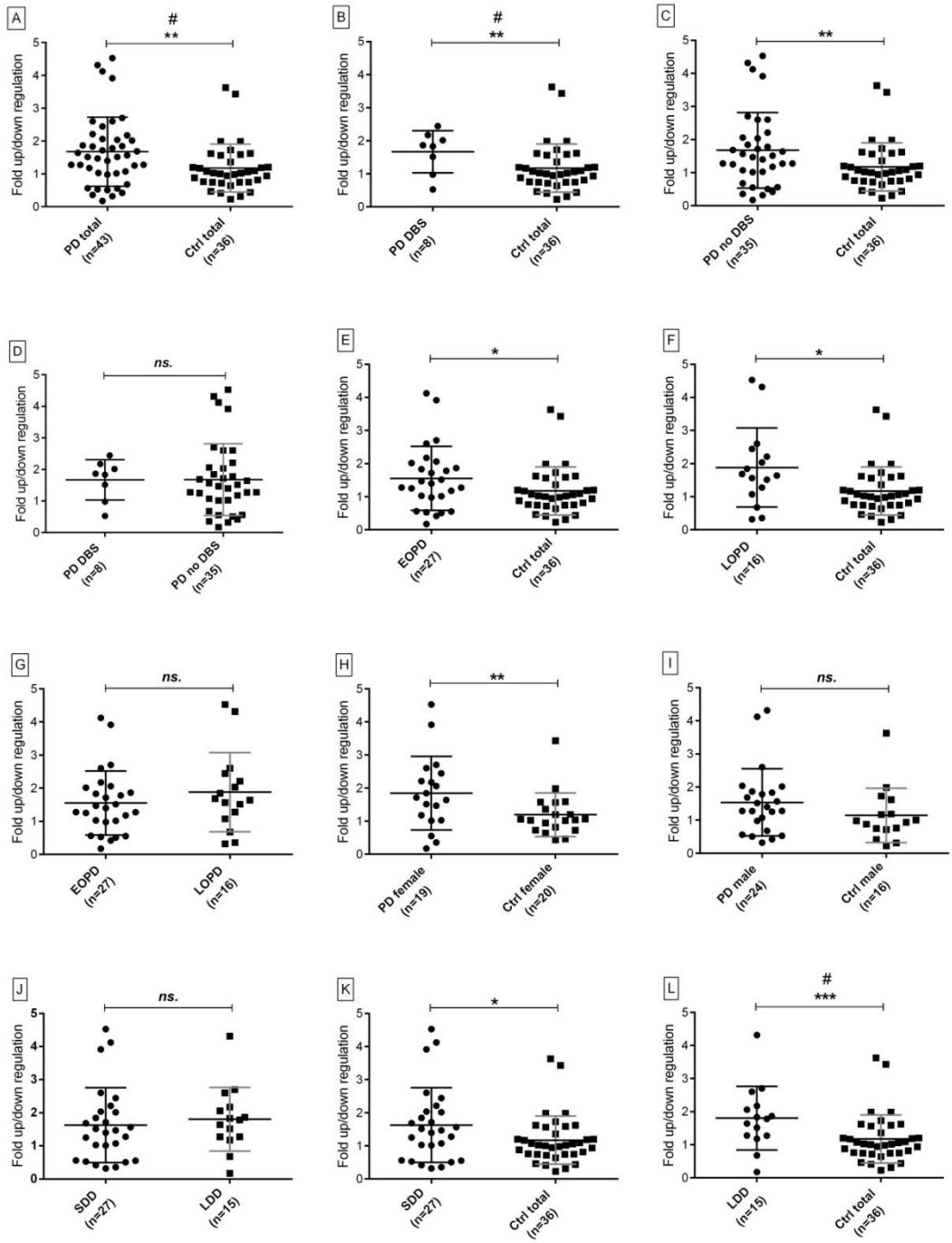

Figure 1. Comparisons of NEAT1 lncRNA level between controls and PD patients in validation study II. Fold regulations are shown with standard deviation**.**

Abbreviations: PD: Parkinson's disease; Ctrl: control; DBS: deep brain stimulation; EOPD: early onset Parkinson's disease; LOPD: late onset Parkinson's disease; SDD: short disease duration; LDD: long disease duration; ns.: non-significant; *: $p < 0,05$; **: $p < 0,01$; ***: $p < 0,001$; #: p value significant after Bonferroni correction.

4.2.3. <u>Two major NEAT1 isoforms can be detected in peripheral blood:</u> While there is a general agreement among researchers in the field on the generation of the two - short

and long - NEAT1 variants, the existence of further isoforms is unclear. Though the Human Genome Ensemble (GRCH38.p13) database lists nine NEAT1 splice variants, data regarding the natural occurrence of these is scarce and their existence remains a question. We aimed to determine whether any of the splice variants indicated in the database is detectable in human peripheral blood samples. We designed primers specific to the sequences surrounding introns, and carried out PCR reactions with them in various combinations. We then analyzed the PCR products by agarose gelelectrophoresis. Based on the sizes of the amplicons the different variants could be distinguished. Apart from the two major, short and long isoforms, we did not convincingly detect any other NEAT1 variants in human peripheral blood. If any spliced form of NEAT1 is present in blood samples it is at such a low level that it is undetectable by the technique used.

We also attempted to determine whether the NEAT1S and NEAT1L forms are differently represented in control and PD samples. The determination of the exact ratio of the two lncRNA forms itself is a challenge as no specific primer can be prepared for NEAT1S only. Comparing PCR products obtained by the use of three primer pairs (one for NEAT1 total and two pairs for different regions of NEAT1L) we found that NEAT1 total level was 6-8 fold higher than the level of NEAT1L, indicating that the shorter isoform is present in the samples in higher quantity. A comparison of the ratio of total and NEAT1L lncRNA levels in a small number of selected human samples suggests that in those samples which show increased NEAT1 level this most probably results from the increase of NEAT1L. Our results obtained by determining NEAT1 lncRNA level in brain samples of MPTP induced mouse model are in accord with this observation (see later).

4.3.*In vitro* cell based assay for exploring the mechanism of NEAT1 function

4.3.1. NEAT1 is up-regulated in SH-SY5Y neuroblastoma cells by MPP+ treatment in a dose- and time-dependent manner: Recent reports published partly during the course of my PhD work demonstrated NEAT1 up-regulation upon various toxin treatments [30][31][32][35][33][34][36]. In order to investigate the effects of MPP+ treatment on NEAT1 expression changes and set up experimental conditions which permit the modification of NEAT1 expression level, we treated SH-SY5Y neuroblastoma cells with two different doses of MPP+ (0.5 mM and 1 mM) for 6, 20 and 24 hours. In the case of 0.5 mM MPP+ treatment, NEAT1 up-regulation showed clear time-dependency: after 6 hours of treatment no expression increase was observed, at 20 hours of treatment the expression change was modest, while after 24 hours of incubation NEAT1 expression showed 4.92 fold

change (Figure 2). In the case of the 1 mM MPP+ dose, NEAT1 up-regulation showed 3.2 fold up-regulation after 6 hours of treatment, reaching its peak at 20 hours of incubation (8.03 fold up-regulation). However, after 24 hours, NEAT1 expression decreased to that seen at 6 hours of treatment (3.54 fold up-regulation). A possible explanation for this could be that the 1 mM dose of MPP+ is highly toxic for SH-SY5Y cells, and the decline in up-regulated gene expression observed at 24 hours of MPP+ treatment is the consequence of the diminished number of viable cells. This notion is supported by our findings of cell viability as discussed later.

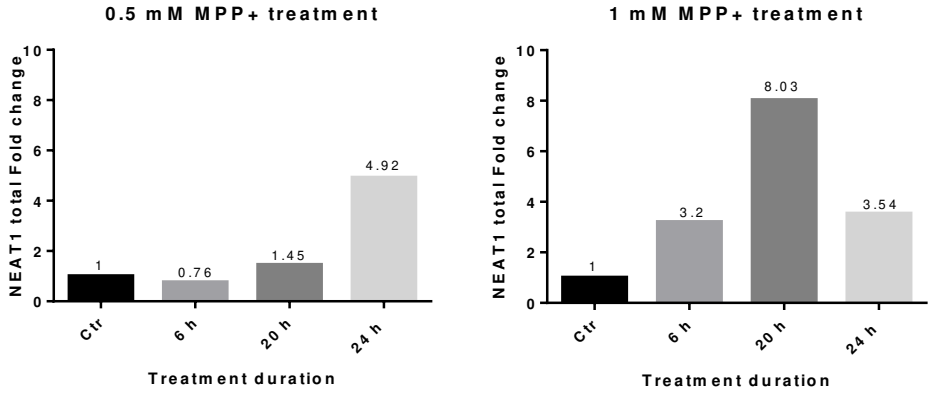

Figure 2. Change of NEAT1 total lncRNA expression in SH-SY5Y cells upon MPP+ treatment.

4.3.2. SFN treatment increased NEAT1 expression: While up-regulated NEAT1 level in *postmortem* PD samples and in PD models has been repeatedly reported, the question of whether the change in the expression of the transcript is a protective mechanism or enhances disease progression is still a matter of debate. In order to gain more insight into the role of NEAT1 up-regulation in mitochondrial maintenance and cell viability related to the disease we investigated the effects of NEAT1 up-regulation prior to toxin treatments in the SH-SY5Y cell model of PD. Recently *NEAT1* was identified as a target gene for HSF1 [82]. Upon activation of the heat shock pathway, HSF1 binds to a heat shock element in the promoter region of the gene, thus enhancing *NEAT1* expression. SFN was identified as a compound capable of enhancing the expression of the lncRNA in HeLa cells *via* activating the heat shock pathway [82].

In order to investigate the effects of SFN treatment on NEAT1 expression changes in neuroblastoma cells we treated SH-SY5Y cells with SFN at two different doses (2 uM and 10 uM) for various time durations (1, 2, 4, 6 and 24 hours). In the case of the '24+'

treatment, SFN treatment was repeated 8 hours after the start of the experiment and samples were collected 16 hours afterwards.

The 2 uM SFN treatment resulted in a modest NEAT1 total up-regulation, reaching its peak at 6 hours after start of the treatment. The 10 uM SFN dose, however, resulted in progressive and persistent NEAT1 up-regulation (Figure 3.).

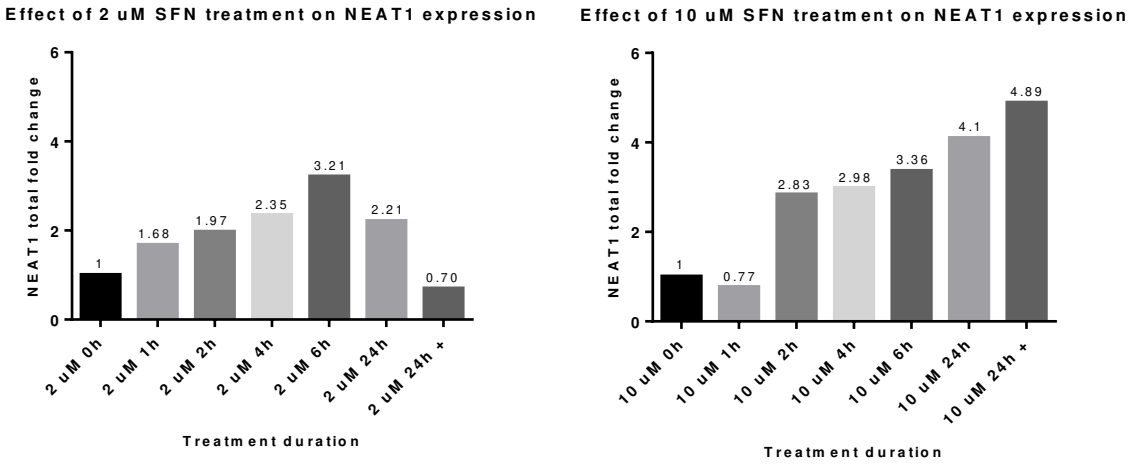

Figure 3. Change of NEAT1 total lncRNA expression in SH-SY5Y cells upon SFN treatment

4.3.3. <u>Combined treatment of SH-SY5Y cells with SFN and MPP+ has an additive effect on up-regulation of NEAT1 expression:</u> Based on our findings of the effects of MPP+ and SFN treatment on NEAT1 expression of SH-SY5Y cells we conducted further experiments to investigate how co-treatment with the compounds affects the expression of the transcript.

We treated cells with either 0.5 mM or 1 mM MPP+ combined with 10 uM SFN. Treatment with the toxin and the neuroprotective agent started at the same time point and cells were incubated with the reagents for 24 hours. Expression analysis revealed that the combined treatment with both SFN and MPP+ had an additive effect on NEAT1 up-regulation (Figure 4.). Interestingly, NEAT1 up-regulation was more prominent when SFN treatment was combined with the lower, 0.5 mM MPP+ dose, than when applied in combination with 1 mM MPP+ (fold up-regulation 15.78 and 10.52, respectively). These results are in line with the notion raised by the results of MPP+ treatment on its own, i.e. that NEAT1 expression up-regulation is moderate in 1 mM treated cells compared to the 0.5 mM treated ones due to the high toxicity of MPP+. 1mM MPP+ treatment is likely to cause excessive cell death sparing only a small number of NEAT1 expressing cells. By

demonstrating that NEAT1 expression can be modulated with SFN treatment it became possible for us to test whether this drug will increase or moderate NEAT1 effects on markers which might have relevance to PD. Through these experiments we can gain information on the role of NEAT1 in PD.

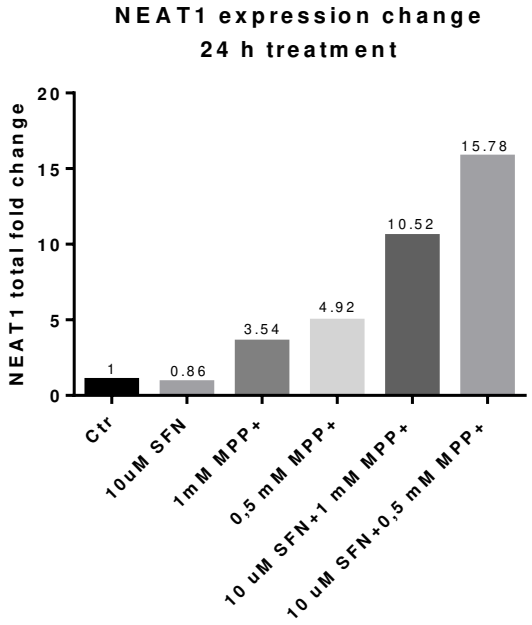

Figure 4. Change of NEAT1 total lncRNA expression in SH-SY5Y cells upon co-treatment with SFN and MPP+

4.3.4. Changes in mtDNA copy number upon MPP+ and SFN treatment: Changes in the mtDNA copy numbers have been reported in PD patients [60][83][84]. We therefore investigated changes in mtDNA copy number in the MPP+ SH-SY5Y cell model of the disease.

We treated SH-SY5Y cells with the combination of 10 uM SFN and 0.5 or 1 mM MPP+ for various durations. For the 6 hours and 24 hours treatment SFN and MPP+ were added at the same time point. In the third experiment design MPP+ was added after 6 hours of pre-treatment with SFN, followed by further incubation for 20 hours.

Our results show that MPP+ treatment decreases relative mtDNA amount in a dose and time dependent manner. In contrast, SFN treatment increases relative mtDNA content in correlation with incubation time (Figure 5.). Pretreatment with SFN was also capable of partly restoring the decrease in mtDNA copy number caused by 0.5 mM MPP+ treatment. These results indicate that the increase of NEAT1 level evoked by SFN treatment itself either does not cause a decrease in mitochondrial copy number, or, alternatively, other beneficial effects of SFN counteract it.

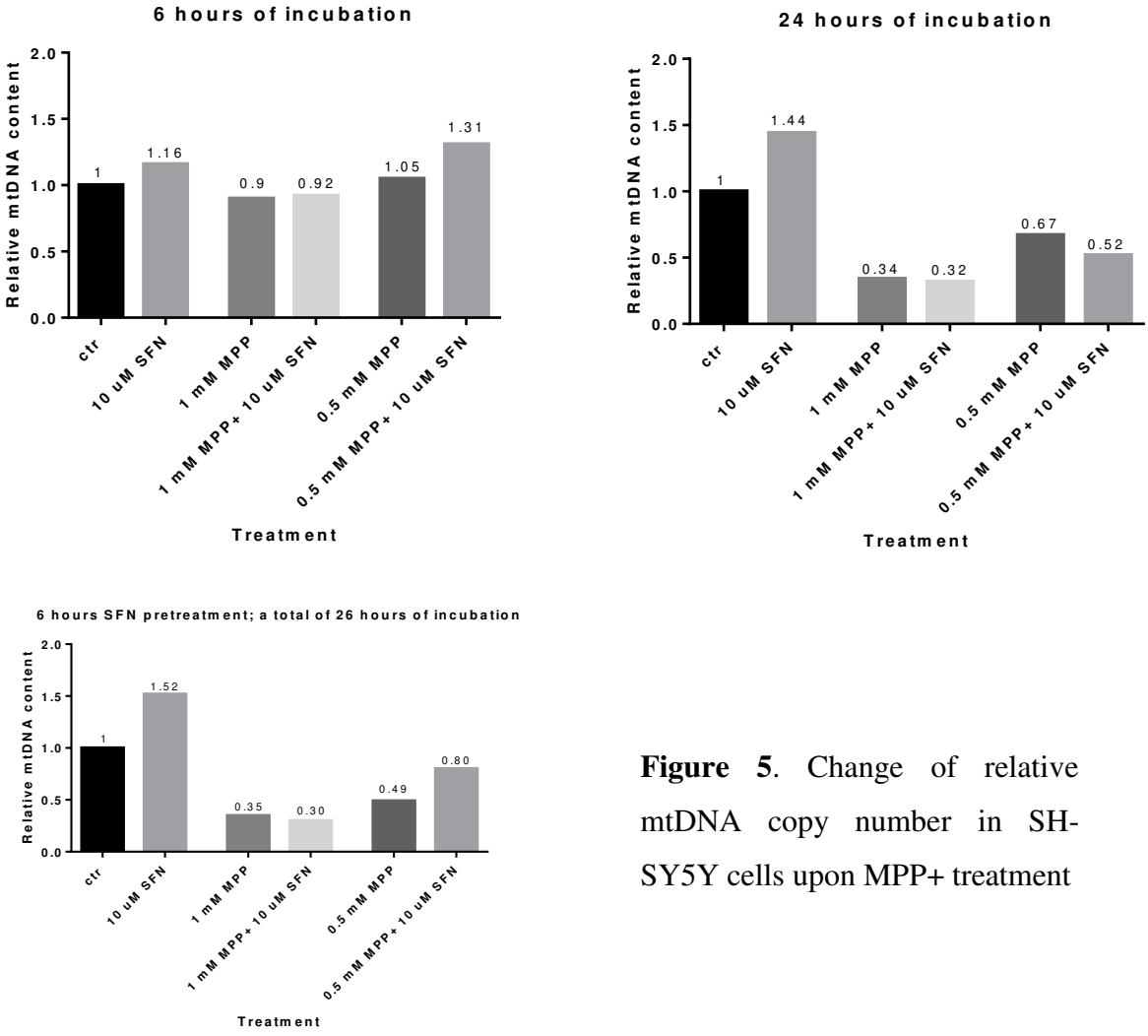

Figure 5. Change of relative mtDNA copy number in SH-SY5Y cells upon MPP+ treatment

Since MPP+ is a mitochondrial toxin, its effect on mtDNA copy number change seems straightforward. We also investigated whether the free radical generating PQ, another toxin with a different mode of action which is used for modeling the disease has similar effects on mtDNA. We treated cells with 0.1, 0.5, 1 and 1.5 mM PQ in combination with or without 10 uM SFN for 24 hours. In line with our previous results, SFN treatment on its own modestly increased relative mtDNA content (Figure 6.). However, none of the applied PQ treatments caused such a prominent mtDNA copy number decrease as seen in the case of MPP+. This result highlights the different mechanisms by which the two toxins used in modeling PD affect an important cell function.

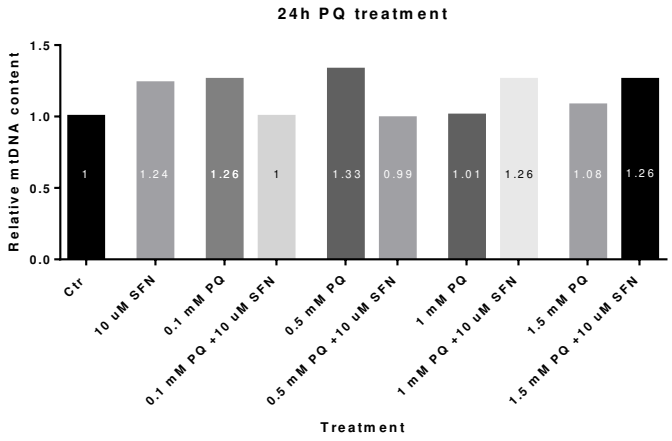

Figure 6. Change of relative mtDNA copy number of SH-SY5Y cells upon PQ treatment

4.3.5. Change in PINK1 level upon NEAT1 up-regulation: Recently NEAT1 was proposed to stabilize PINK1, thus elevating the level of the protein, resulting in an increased level of cell death [31].

We sought to investigate whether elevated PINK1 protein level can be detected upon NEAT1 up-regulation. We used WB analysis of total cellular protein extract obtained after treatment of SH-SY5Y cells with 0.25 mM MPP+ for 24 hours. WB analysis did not reveal any changes in the amount of PINK1 protein (Figure 7.). Whether this is a result of low level of NEAT1 expression change in this particular case, or, contrary to what is suggested in the literature [31], increased NEAT1 level does not affect PINK1 stability, remains to be determined.

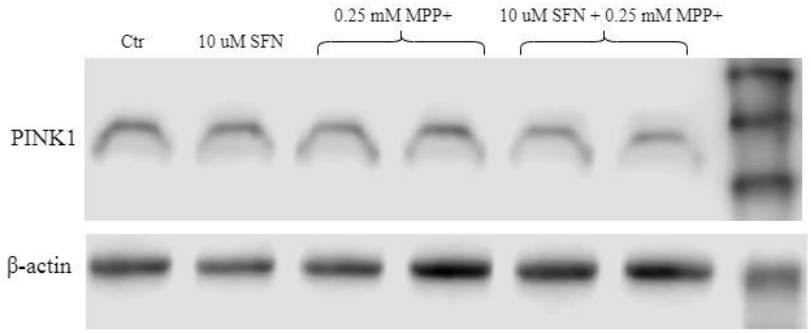

Figure 7. WB of PINK1 protein following SFN and MPP+ treatment of SH-SY5Y cells.

4.3.6. MPP+, PQ and SFN combined effects on cell viability: Finally we aimed to determine how SFN-induced NEAT1 expression change affects cell viability and whether the two toxins, PQ and MPP+ display differences in this as well.

First we treated cells with 2 uM and 10 uM SFN for 24 hours. A marked increase in cell viability was detected upon 10 uM SFN treatment (Figure 8.).

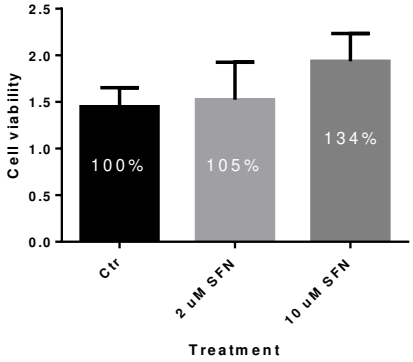

Figure 8. Effect of SFN treatment on the viability of SH-SY5Y cells.

1 and 2 mM MPP+ treatment caused a significant, approximately 60 per cent decrease in cell viability (p=0.0002 and 0.027, respectively) (Figure 9/A). In order to identify a dose that does have an effect on the cells but spares enough of them to be able to execute further expression analysis experiments, we tested lower doses of MPP+ (Figure 9/B and C), in combination with both 2 and 10 uM SFN treatment (Figure 9/C). Results revealed that the combined treatment of 10 uM SFN and low (0.002 and 0.01 mM) doses of MPP+ have a positive effect on cell viability (Figure 9/C). In fact, at very low level MPP+ seemed to have an additive effect with SFN.

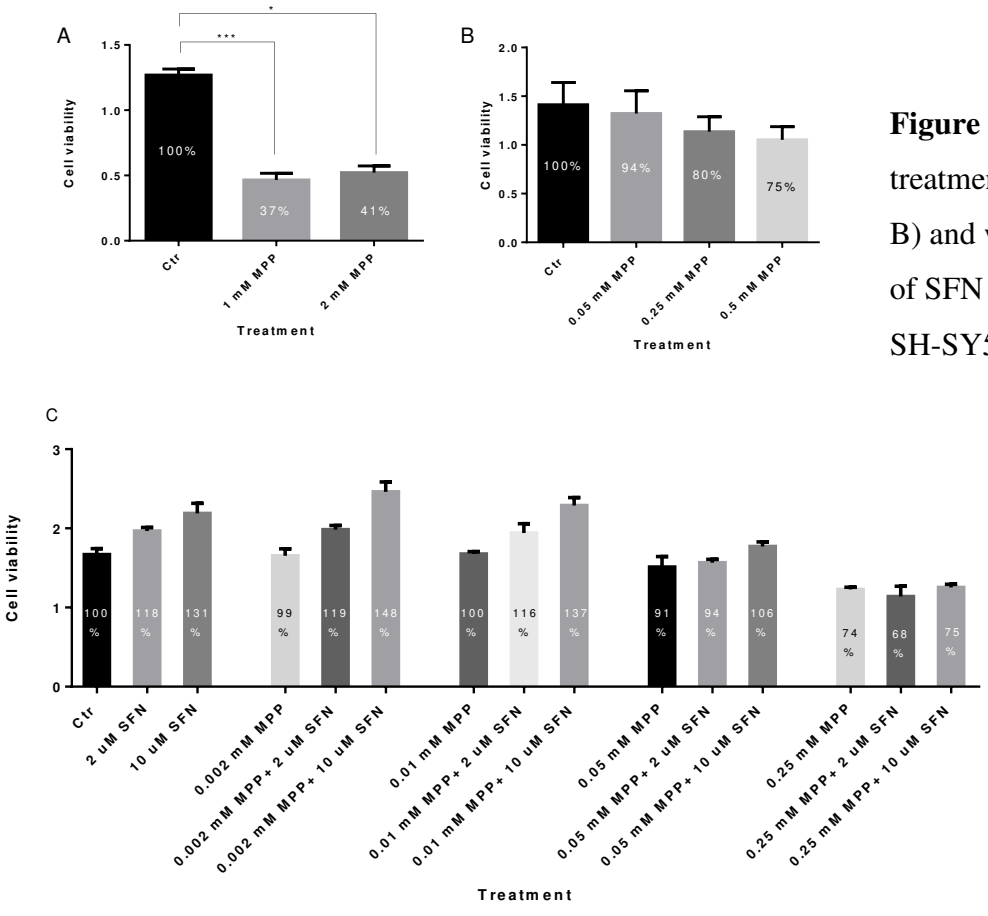

Figure 9. Effects of MPP+ treatment without (A and B) and with combination of SFN (C) on viability of SH-SY5Ycells.

In addition to MPP+, we also tested the effect of PQ, the other toxin used for PD modeling on cell viability. Cells were treated with 0.05 mM, 0.1 mM, 0.5 mM and 2.5 mM PQ for 24 hours. Cell viability results showed a significant decrease when comparing both 0.5 mM and 2.5 mM PQ treated cells to controls (p= 0.0002 and <0.0001, respectively) (Figure 10/A). Co-treatment with 10 uM SFN partly reversed the cell viability decrease due to the toxin treatment (Figure 10/B). Similarly to that seen in the case of MPP+ treatment, low dose of PQ (0.05 mM) treatment in combination with 10 uM SFN also had an additive effect on cell viability increase.

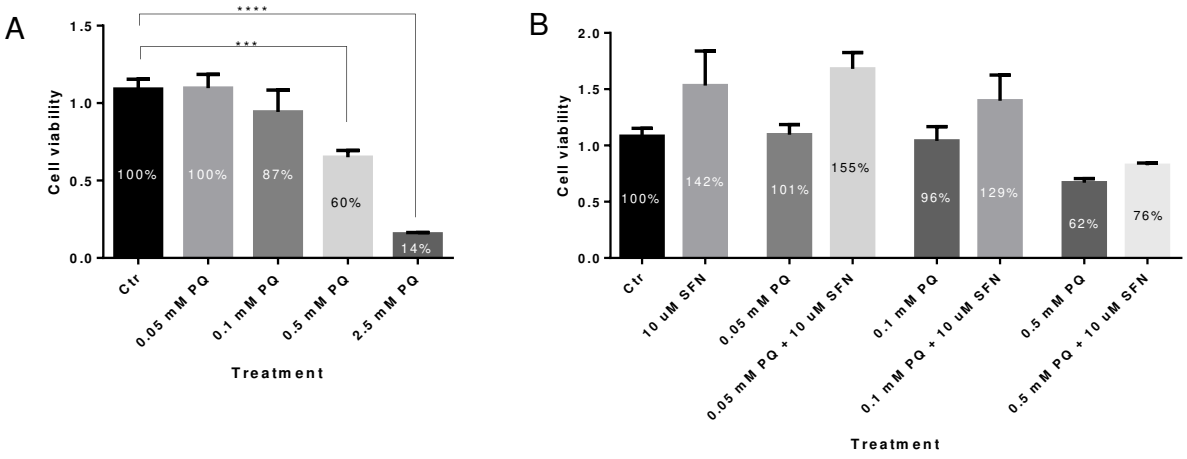

Figure 10. Effects of PQ treatment without (A) or in combination with (B) SFN on viability of SH-SY5Ycells.

4.3.7. <u>SFN treatment partially compensates apoptosis increase caused by MPP+:</u> Since SFN compensated cell viability decrease caused by low level MPP+ but could not compensate the effects of 0.25 mM MPP+ treatment, we aimed to investigate whether it has an effect on the apoptosis rate of SH-SY5Y cells treated similarly with MPP+. For this we treated cells with 0.25 mM or 0.5 mM MPP+ combined with or without 10 uM SFN for 24 hours and analyzed the level of apoptosis by Annexin V-FITC fluorescence activated cell sorting (FACS). The flow cytometry analysis revealed that SFN treatment markedly reversed the effect of MPP+ treatment at both doses as demonstrated by the decrease in the ratio of cells in the late and particularly in the early stage of apoptotic cell death (Figure 11.).

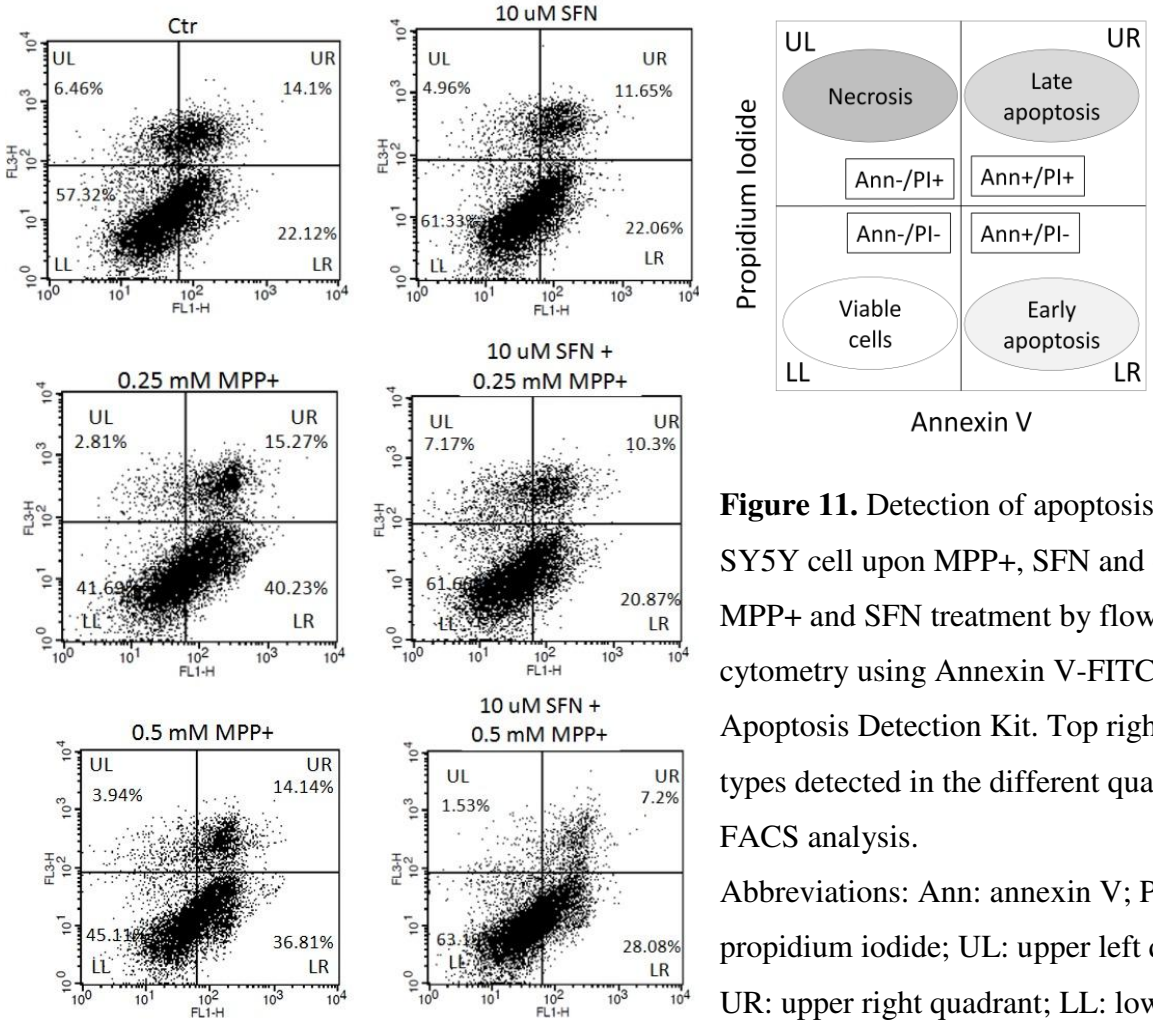

Figure 11. Detection of apoptosis of SH-SY5Y cell upon MPP+, SFN and combined MPP+ and SFN treatment by flow cytometry using Annexin V-FITC Apoptosis Detection Kit. Top right: the cell types detected in the different quadrants of FACS analysis.
Abbreviations: Ann: annexin V; PI: propidium iodide; UL: upper left quadrant; UR: upper right quadrant; LL: lower left quadrant; LR: lower right quadrant.

4.4. *In vivo* mouse model for exploring the mechanism of NEAT1 function

The results of our *in vitro* experiments showed that in SH-SY5Y cells SFN treatment increased NEAT1 expression, improved the decreased viability caused by PQ treatment and decreased apoptosis caused by MPP+ treatment. Furthermore, we found that SFN treatment partly restored mtDNA copy number decrease caused by MPP+ treatment. In light of these *in vitro* results it became particularly interesting to find experimental conditions to enable the *in vivo* alteration of NEAT1 levels and determine the effects on mtDNA copy number in the MPTP mouse model of the disease.

4.4.1. SFN causes NEAT1 up-regulation in the mouse brain in a dose- and time-dependent manner: In order to determine whether we can manipulate NEAT1 expression *in vivo* by SFN treatment, we investigated the effect of SFN on NEAT1 expression in the mouse central nervous system (CNS). For this, we treated mice with three different doses

of SFN (D1: 2.5 mg/kg of body weight; D2: 5 mg/kg of body weight; D3: 10 mg/kg of body weight). Animals were sacrificed at four different time points: 90 minutes (D1/D2/D3 90 min groups), 6 hours (D1/D2/D3 6h groups), 12 hours (D1/D2/D3 12h groups) and 24 hours (D1/D2/D3 24h groups) after i.p. SFN injection. The control group was treated with 100 ul/10 g of body weight 14.1% EtOH solution (dissolvent for the highest implemented SFN dose) and animals were sacrificed 90 minutes after injection. All study groups consisted of 3 mice.

NEAT1 total (in these experiments we determined both NEAT1 total and NEAT1L levels) expression showed no change in any of the investigated brain areas (brainstem, striatum, cortex and cerebellum; data not shown). However, the long isoform was up-regulated in all four investigated brain areas, showing the most prominent up-regulation in striatum and brainstem samples in the 6 hours to 24 hours after treatment time range (Figure 12).

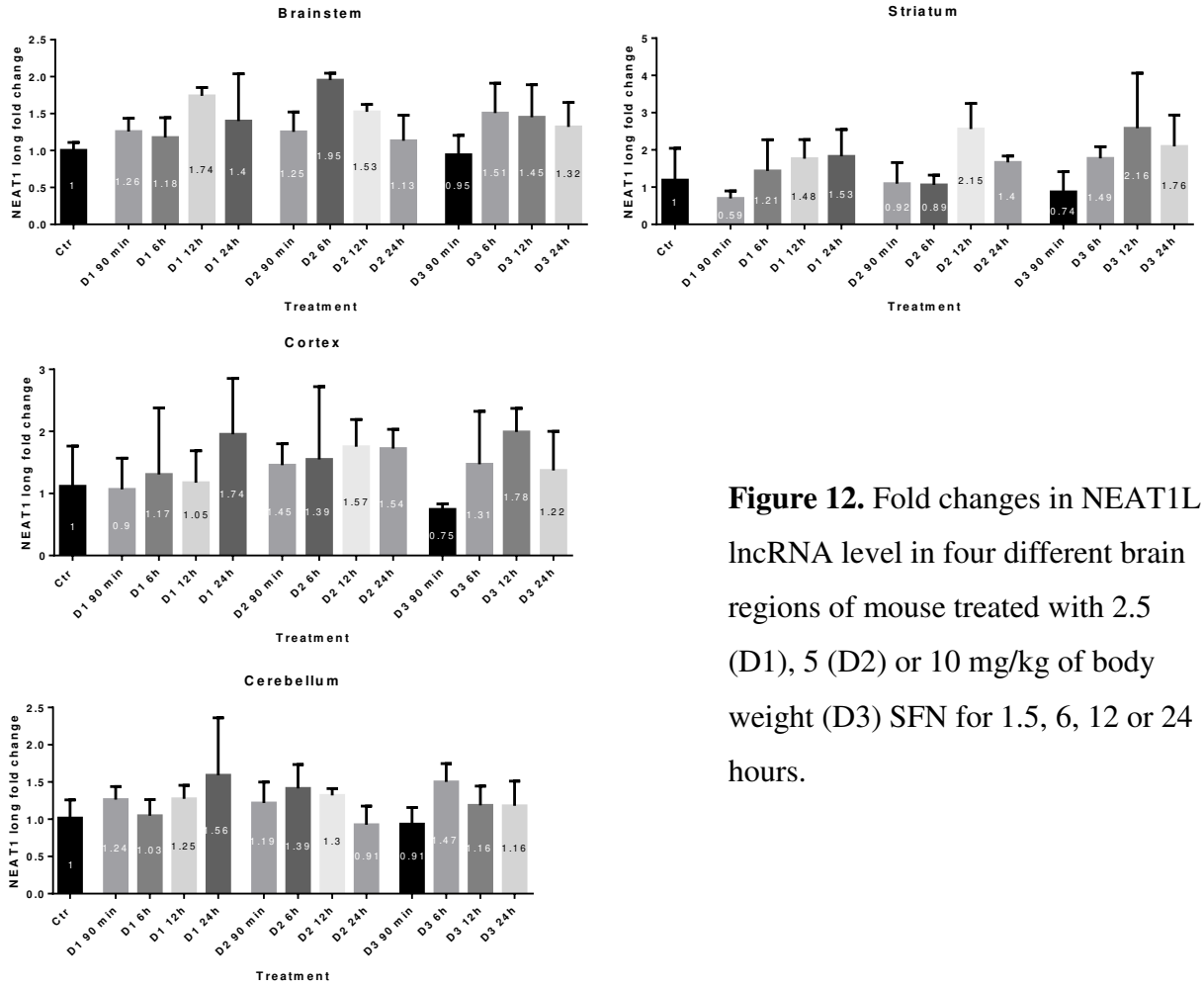

Figure 12. Fold changes in NEAT1L lncRNA level in four different brain regions of mouse treated with 2.5 (D1), 5 (D2) or 10 mg/kg of body weight (D3) SFN for 1.5, 6, 12 or 24 hours.

4.4.2. MPTP treatment up-regulates NEAT1 expression in a dose dependent manner:

MPTP treatment of mice is a well-established model of PD. MPTP is a neurotoxin selective to dopaminergic neurons in the *substantia nigra*. We aimed to investigate the effects of acute MPTP treatment on NEAT1 expression in the *substantia nigra* and brainstem of mice. Mice were treated with 15 mg/kg of body weight MPTP injected i.p. The MPTP1x group received one injection, while animals in the MPTP3x group were injected three times, with 2 hour intervals between each injection. Control animals were treated with vehicle (PBS). Animals were sacrificed 90 minutes following the last injection. Each study group consisted of 3 animals.

MPTP treatment did not cause up-regulation in NEAT1 total expression in either the striatum or brainstem of the treated animals. In contrast, separate analysis of the long NEAT1 variant revealed significant up-regulation of the transcript in both investigated brain areas. Up-regulation was more prominent in the striatum and when comparing the MPTP3x group to the corresponding control group (MPTP1x: fold up-regulation: 2.23, p=0.0014 *vs*. 1.31, p=non significant; MPTP3x: fold up-regulation: 6.49, p=0.0044 *vs.* 3.84, p=0.0001, striatum *vs.* brainstem, respectively). These results suggest that NEAT1L expression is enhanced by MPTP treatment in a dose-dependent manner (Figure 13).

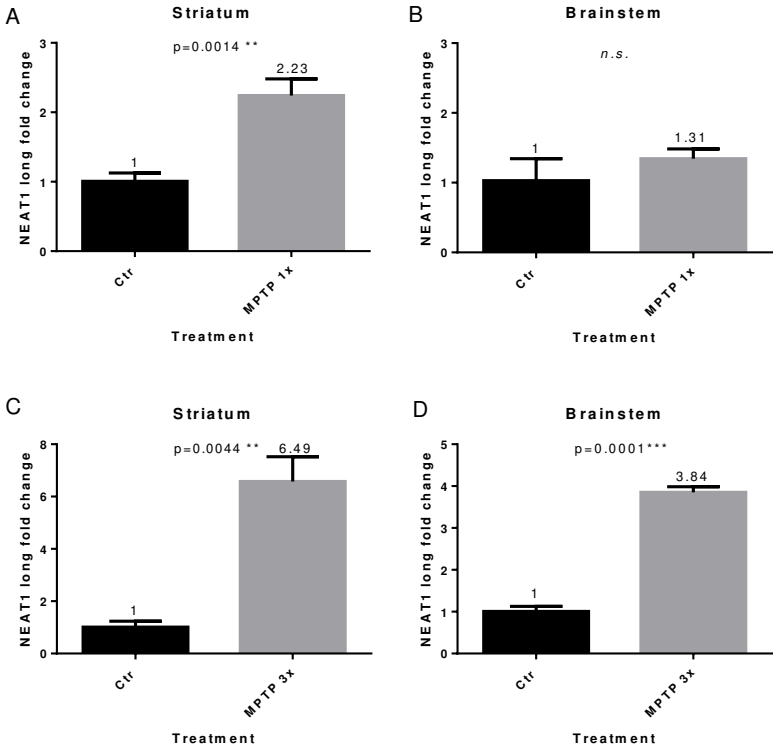

Figure 13. Effects of 1 dose (A and B) and 3 doses (C and D) MPTP treatments on NEAT1L lncRNA expression in mouse striatum (A and C) and brainstem (B and D).

4.4.3. SFN and MPTP have an additive effect on NEAT1 up-regulation: *In vitro* results show that co-treatment of cells with SFN and toxins have additive effects on enhancing NEAT1 expression. Furthermore, under certain circumstances SFN treatment is capable of partly restoring mtDNA copy number decrease provoked by toxin treatment. We aimed to translate our *in vitro* results to *in vivo* experiments as well.

Our study performed for this consisted of four study groups, with three animals in each. Based on our previous results, the MPTP treated group received 15 mg/kg of body weight toxin injections 3 times, with 2 hours between doses. In the case of the SFN+MPTP group, animals received one injection of 10 mg/kg of body weight SFN 12 hours before MPTP treatment. In order to rule out the effect of the SFN solvent, the EtOH+MPTP group was introduced. As in the case of the SFN+MPTP group, MPTP treatment was preceded with a single injection of 100 ul/10 g of body weight 14.4% EtOH solution. The control group (Ctr) was treated with PBS, the vehicle for MPTP, with the same volume and at the same time as toxin treatment was implemented. Figure 14. shows a schematic summary of the study design.

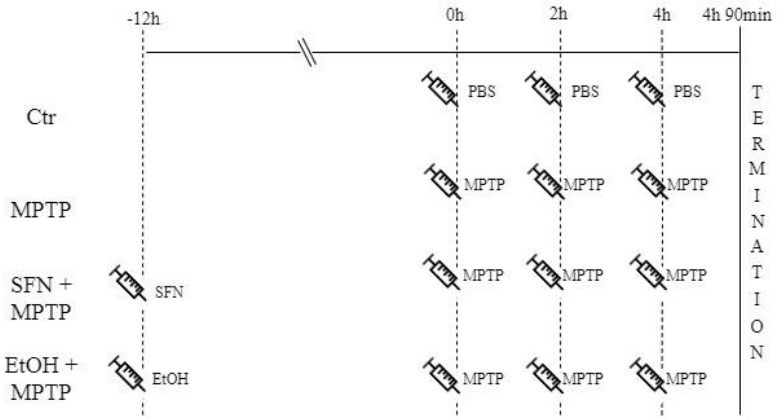

Figure 14. Schematic illustration of the experimental design of combined treatment of mice with SFN and MPTP. The syringe icon indicates treatment with the compound next to it.

Gene expression analysis revealed only subtle up-regulation in the level of NEAT1 total in the treated study groups in both striatum and brainstem samples. In contrast, NEAT1L was significantly up-regulated in all treated study groups compared to the control group in both investigated brain areas. Up-regulation was most prominent in MPTP and SFN co-treated groups (fold up-regulation: 6.92 and 5.25 in striatum and brainstem, respectively). In the case of striatum, comparison of the MPTP treated and MPTP and SFN co-treated groups also yielded a significant difference. Interestingly, NEAT1L fold up-regulation was higher in the EtOH-MPTP group compared to the MPTP treated group

(5.26 *vs.* 3.59 and 3.79 *vs* 4.39 in the striatum and brainstem, respectively). However, NEAT1L up-regulation in the EtOH-MPTP group did not reach that of the SFN-MPTP treated animals. This suggests that EtOH might have an additive effect to MPTP on NEAT1L expression increase, however the prominent expression change observed upon SFN treatment is due to the SFN itself (Figure 15.).

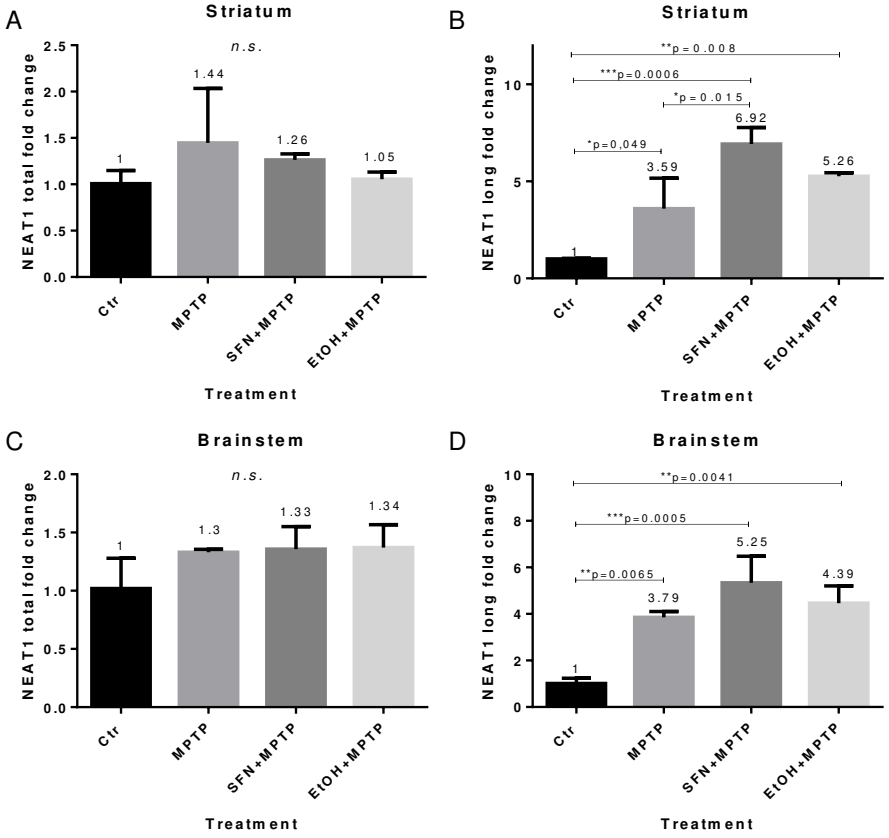

Figure 15. Expression changes of NEAT1 total (A and C) and NEAT1L (B and D) lncRNA in striatum (A and B) and brainstem (C and D) samples of mice treated with MPTP or MPTP with the combination of SFN or EtOH.

In order to determine whether the increased NEAT1 level and SFN treatment co-administered with the drug resulted in changes in mtDNA copy number we performed pilot studies on striatum samples. Our PCR based mtDNA/genome DNA ratio determination did not result in any significant difference among the groups in this experiment (Figure 16.), however, further, similar experiments on sample groups with larger number of animals are required to determine whether these changes are significant.

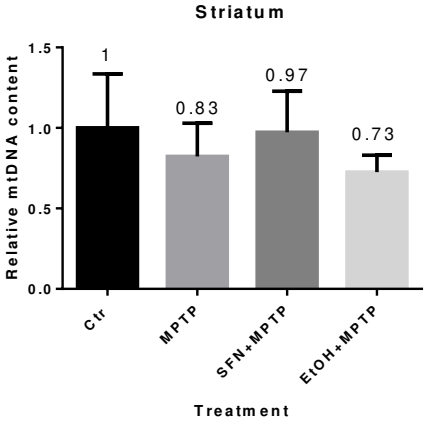

Figure 16. Changes in relative mtDNA copy number in mouse striatum upon treatment with MPTP or with the combination of the toxin and SFN or EtOH.

5. Discussion

Despite being the second most common neurodegenerative disease affecting millions of people worldwide, the exact pathomechanism underlying PD is not yet fully understood. It is generally acknowledged that PD is a multifactorial disease: besides lifestyle and environmental factors, genetic background plays a major part in the development and prognosis of the disorder. Among the PD-linked gene variants there are only a handful which are in a direct, causal relation with disease appearance. In the case of most of the gene mutations such direct association cannot be identified. There is a growing number of SNPs which - though not being pathogenic regarding the development of PD - are proposed to have a risk modifying (either risk increasing or protective) effect. The identification of such genetic contributors is primarily based on GWA studies. However, since GWA studies require a high number of participants, the heterogeneity of the study groups can easily hide differences among specific populations regarding the frequencies of specific gene variants. Also, contrasting results can be seen regarding different study groups due to variations in patterns of the non-random association of alleles at adjacent loci (linkage disequilibrium), which is often a characteristic of the population [85]. Therefore it is crucial to test the findings of GWA studies in specific, more homogenous populations.

We aimed to assess the frequency of *LRRK2*, *SNCA*, *MAPT* and *TCEANC2* gene variants in Hungarian sporadic PD patients and non-PD controls. The occurrence of ten mutations was assessed. Among these are SNPs that are considered to be risk factors, and others which are proposed to have a protective effect against PD. We selected those variants that are either the most intensively studied since their effect on disease

development has been explored in certain populations, or those which have most recently been identified as potential risk- or protective factors. To our knowledge this is the first comprehensive study focusing on these gene variants in the population of the East-Central European region.

SNCA (*PARK1* and *4*) was the first gene identified to be related to PD: its first mutation linked to the disease was identified in 1996 in a family showing autosomal dominant inheritance pattern of the disease [4]. *SNCA* is located on the long arm of chromosome 4, spanning over 114 kb. It encodes α-syn [86], a main component of Lewy bodies, which are a hallmark of PD [10] (reviewed: [2]). Besides gene copy number changes, mutations and polymorphisms of *SNCA* have also been found to be linked to PD. Rs2583988 and rs356186 are two intronic variants of the gene, the former a proposed risk, the latter a protective factor against the disease. Whether these variants have a disease modifying effect in Caucasian populations is, however, controversial: various studies report an association between the frequency of these variants and PD, while others argue against such relation [87][88][89][90][91]. Our finding of no significant association between the minor allele frequency of the rs2583988 variant and the disease is in accord with data obtained from German [90] and Irish [91] study populations.

Recently a meta-analysis was performed with the aim of determining the most relevant *SNCA* SNPs in PD [92]. Based on this meta-analysis the rs2583988 and rs356186 polymorphisms were designated as the two most interesting ones in this respect. It was also determined that in the case of the protective rs356186 variant the dominant model showed a significant difference in the analyzed study groups, thus the presence of the minor allele of this SNP in heterozygous form greatly add to the protective effect of the variant [92]. Our results strengthen the findings of the meta-analysis, since we found that the significant difference in genotype distribution between LOPD group and healthy controls is a consequence of higher frequency of the AG genotype among LOPD patients. Furthermore a significantly higher relative frequency of the AA genotype among healthy participants was detected when compared to PD patients.

Similarly to *SNCA*, *LRRK2* variants can have either protective, or risk increasing effects regarding PD. The link between *LRRK2* (*PARK8*) and the disease was first described in 2002 [13]. *LRRK2* is localized on the long arm of chromosome 12 and encodes the LRRK2 kinase - also known as dardarin – which is a large protein, built from more, than 2500 amino acids. LRRK2 is a member of the ROCO superfamily and consists of several domains, among which two - a kinase and GTPase - possess enzymatic

properties. In the past two decades numerous mutations of the gene have been identified, making *LRRK2* the most common cause of not only familial, but sporadic PD cases as well [14][15].

Besides its pathogenic mutations, wherewith the association with PD is well established, intensive research is focusing on identifying risk factor variants that modulate the disease. Out of the more than 100 SNPs of the *LRRK2* gene, two SNPs have been validated as coding susceptibility alleles of the disease in Asian populations [93]. Rs34778348 leads to a Gly to Arg substitution at the 2385th amino acid position of the protein (G2385R), while rs33949390 results in the change of the Arg at the 1628th position to Pro (R1628P). The G2385R substitution was shown to cause a 2 fold increase in PD risk, while the R1628P variant causes an even bigger increase in the possibility of disease development [94]. Both of these variants have been proven to be risk factors in the Han Chinese and other Asian populations, however to date none of these variants have been detected among Caucasians [95][96][97][98][99].

The G3285R substitution is located in the WD40 domain, towards the C terminus of the protein. Though its biological function is not fully elucidated, it has been implicated to play a role in microtubule interaction, dimerization and other protein-protein interactions [100]. Thus it can easily be assumed that mutations affecting this region of the protein cause alterations in its interactions with regulatory proteins and substrates [95]. *In vitro* studies revealed that the presence of the G2385R substitution leads to increased rate of apoptosis under oxidative stress [101]. Other studies reported increased kinase activity of the protein with the mutation [71], however there are data challenging this notion [102]. In their recent study Zhang and colleagues analyzed the crystal structure of the WD40 domain and reported mutations of the domain to mainly impair the dimerization of the protein [100].

The R1628P variant is an amino acid change in the COR domain of *LRRK2*. COR, with the adjacent Roc domain forms the tandem Roc-COR domain, accounting for the GTPase function of LRRK2. Functional analysis studies suggest decreased GTPase activity as a consequence of this variant [102]. There are data suggesting that the binding of GTP is essential for the activation of the kinase function of LRRK2, thus variations of the GTPase domain can disturb kinase activity as well. Further findings indicate that kinase activity depends on the dimeric form of the protein [102] and the COR domain is fundamental for protein dimerization [16]. Further mechanisms by which mutations affecting this protein region can modulate kinase activity are *via* the regulation of protein

conformation and autophosphorylation. In light of these, it is not surprising that similarly to G2385R, the R1628P variant was also reported to increase LRRK2 kinase activity [71]. A further mechanism proposed to cause this is the enhancement of the binding affinity of the protein with Cyclin-dependent kinase 5 (Cdk5), leading to the phosphorylation of LRRK2 at the S1627 site, resulting in higher kinase activity [103]. Furthermore, R1628P mutant cells were found to be more sensitive to oxidative stress, thus showing higher apoptotic rate compared to their wild type counterparts [104].

A change of the 1647th amino acid Ser to Thr (S1647T) of LRRK2 is also proposed to be a susceptibility factor for PD. The disease risk modifying effect of this allele was described first in a Han Chinese population [71]. However, just as in the case with the G2385R and R1628P variants, though the S1647T mutation's PD risk increasing effect was reported in Asian populations [71] [105], no such association has been found in the Caucasian populations investigated so far [106]. As this amino acid change affects the COR domain of the protein, one can easily assume that the S1647T variant can affect GTPase and kinase activity, and dimerization. However, so far no changes have been found in kinase activity in relation with the S1647T variant: further studies are thus necessary to elucidate the exact effects of this amino acid change [71].

We found no significant association between the ST1647T *LRRK2* variant and PD in our cohort, in accord with available data from the literature regarding Caucasian populations: no significant association was found in either Finnish or Greek study groups [106]. Among our male control study participants we detected the minor allele of this SNP to be more frequent as compared to PD patients. This contrasting result however could be due to the relatively small sample size.

Recent findings of GWA studies proposed a common variability 0.17 Mb upstream of the *LRRK2* gene to impact the risk of developing PD. The rs1491923 is an A to G change (forward orientation) of which the minor allele was found to be more common among PD patients compared to controls both in Caucasian and Asian populations [70]. Recently, based on their findings of an induced pluripotent stem cell (iPSC) model of the disease Marrone and colleagues proposed that this variant affects mitochondrial turnover [107]. We did not detect a significant difference in the allele frequencies of the variant between our study groups: however, the risk increasing effect of this variant cannot be ruled out. Further studies involving other independent sample groups of different populations are necessary for the clarification of the effect of this gene variant on PD.

Besides risk factor variants, the presences of other *LRRK2* SNPs are proposed to be protective against the development of the disease. The presence of either the R1398H or N551K variant (Arg to His and Asn to Lys change at the 1398th and 551th amino acid positions of the kinase, affecting the ROC domain and armadillo repeat region of the protein, respectively) was found to decrease PD risk when co-occurring with the variants G2385R or R1628P [71].

Results of a study conducted among Asian patients and controls revealed these variants to occur in LD and that both of them are significantly more frequent among PD patients [71]. Tan and colleagues also observed that the presence of these variants yielded pronounced reduction in the otherwise increased disease risk evoked by the G2385R and R1628P polymorphisms. In addition, the presence of either of the protective variants decreased the odds ratio (OR) of 1.9 to 1.5-1.6 in carriers of the R1628P variant, which shows a prominent disease risk decreasing effect. The proposed mechanism behind this observation was that by reducing the kinase function of LRRK2, the presence of the R1398H variant can compensate for the enhanced enzyme function due to the R1628P and/or G2385R amino acid changes [71].

Our findings are in line with the results of others regarding the LD of the R1398H and N551K variants, and also with data showing no significant difference in the allele frequencies of the variants among patients and controls of Greek and Finnish origin [71][106].

The PARK10 region is located on the long arm of chromosome 1. and incorporates various genes proposed or confirmed to be linked to PD [108][109][110][111]. The region was first linked to PD some 15 years ago [23], and in the last decade an LD for a block of 100 kb was recognized in this locus [24]. The rs10789972 polymorphism of the *TCEANC2* gene was found to be associated with an increased risk of PD among American patients [24], however such association was not described in respect of the Han Chinese population [112][113]. Our findings are in line with data obtained from the latter population, as we observed no association between the presence of the variant and the occurrence of PD in our study population.

MAPT encodes tau, a microtubule associated protein, the abnormal hyperphosphorylation of which causes its intracellular aggregation and formation of neurofibrillary tangles. The exact mechanism by which tau contributes to PD pathogenesis is not fully elucidated, however there is a growing body of evidence about the toxic interaction of the protein and α-syn. This can contribute to the formation of Lewy bodies,

impairment of axonal transport and disruption of cellular cargo in the cell, such as α-syn and tau themselves [20][114]. The *MAPT* gene is located at a site of an approximately 900 kb common inversion on the long arm of chromosome 17 [20]. The inversion of the 17q21 region generates two distinct haplotypes: a non-inverted H1 and an inverted H2. *In vitro* studies showed that the non-inverted H1 haplotype had up-regulated transcriptional activity resulting in enhanced expression of the *MAPT* gene [22]. Thus it is not surprising that the H1 haplotype has been associated with numerous 'taupaties', such as AD, sporadic FTD, PSP and PD. However, data regarding the association of the H1 haplotype with PD is controversial, since several studies involving subjects of different nationalities reported no, or marginal association between the presence of the haplotype and the disease (reviewed in [18]).

The rs1052553 SNP is a G to A change and was identified as a suitable marker of the 17q21 inversion [20]. Though various studies reported significant association between the H1 haplotype and PD using different tagging SNPs (reviewed: [18]), results are inconclusive. Our findings are in accord with data obtained from British [115], Swedish [116] and Taiwanese [117] populations, which found no significant association between the H1 haplotype and PD.

Considering the wide range of functions that genes linked to PD fulfill, understanding the genetic basis of the disease is a formidable challenge. Intensive research has been focusing on identifying common traits behind the diverse cellular functions affected. Recently lncRNAs emerged as possible hubs in the network of genes and pathogenesis of the disease.

We aimed to identify lncRNA expression alterations in PD that might bring us closer to the understanding of the underlying pathomechanism. After detailed review of literature data on lncRNAs involved in neurodegenerative diseases we selected 41 transcripts that were previously found to be linked either to PD [29][30][72][73], AD [74][75], HD [76][77][78][79] and/or other pathomechanisms related to neurodegeneration. We attempted to detect these lncRNAs and compare their expression levels by RT-qPCR in the peripheral blood of PD patients and control individuals. The reasoning behind the usage of peripheral blood is that the samples are easily accessible by minor invasive procedures and that leukocytes are informative in respect of both DNA based and gene expression analysis. Since they interact with most organs and tissues they are capable of displaying changes resulting from alterations throughout the body [72]. It is important to note here that there is a growing body of evidence suggesting that the

manifestation of the characteristic motor symptoms of PD is preceded years, maybe even decades by the appearance of non-motor dysfunctions not necessarily strictly related to the CNS. Moreover, once the motor symptoms are present, they are often accompanied by further non-motor symptoms. These observations and findings of changes in the levels of various inflammatory markers in the blood of PD patients [62][64] give ground to the notion that the disease has more systemic effects [118][119][120][121][122]. The findings of possible blood-based biomarkers in other neuropsychiatric and neurodegenerative diseases [123][124][125] validate such attempts in PD as well.

Out of the 41 selected transcripts we found 12 to be readily detectable, thus we investigated the expression of these transcripts in larger patient and control cohorts. We detected a significant up-regulation of NEAT1 and TUG1 in patients in comparison to control individuals. We analyzed the expression changes of these two lncRNAs in a third sample set with a larger number of participants. Results obtained from the third set of samples however did not reveal a significant difference in TUG1 expression between patients and controls. TUG1 has been shown to be up-regulated by p53 and to modulate the expression of several cell-cycle regulator genes, thus playing an important role in cell response to DNA damage [126]. Earlier enhanced TUG1 expression has been reported in HD [77] and very recently, after our results were published, TUG1 up-regulation was reported in cell and mouse models of PD. Silencing of the lncRNA exerted protective effects against neuroinflammation, oxidative stress and apoptosis both *in vitro* and *in vivo* [127]. These findings give ground to further investigations of the transcript in larger numbers of human samples.

In contrast to TUG1, in both our second and third data sets NEAT1 was found to be significantly up-regulated in PD patients on multiple comparisons. Difference in NEAT1 expression was most prominent when comparing all PD patients, PD patients with DBS treatment or LDD patients to the control group. Up-regulation of NEAT1 in PD patients is in accord with findings reporting elevated levels of the lncRNA is various brain regions of PD patients [29][30].

The findings of up-regulated NEAT1 expression in DBS treated patients is in line with experimental data showing that DBS treatment causes changes in leukocyte gene expression [81]. However, in our study the small sample size of our PD subgroup should be taken into account.

The observation that significant NEAT1 up-regulation was detected in LDD, but not in SDD patients compared to controls implies that during disease progression the

amount of the transcript increases. However, further research focusing on the role of NEAT1 in PD is needed in order to clarify whether these transcript level changes are coincidental or are in a causal relation with the disease. During the last couple of years several attempts were made to elucidate the exact mechanism of NEAT1 in neurodegeneration. Our attempts to explore the mechanism of NEAT1 action by the use of PD models are in accord with these studies.

There is a general consensus that toxins used for modeling the disease - such as MPTP/MPP+ and PQ - cause NEAT1 up-regulation [30][31][32][33][34][35][36]. Both our *in vitro* and *in vivo* data are in line with these findings, since we observed a prominent up-regulation in NEAT1 expression in MPTP and MPP+ treated mouse and SH-SY5Y cells, respectively. However, it is unclear whether NEAT1 aggravates PD pathology, acts as a protective factor, or neither of these and is only a bystander along the course of the disease. To date it seems that data obtained from *in vitro* and *in vivo* models of the disease are more suggestive of a disadvantageous role of NEAT1: observations that up-regulation of the gene facilitates the course of PD outweigh those arguing for a protective effect of the transcript (Table 5.) For the interpretation of these data, however, one has to keep in mind that the expression changes in NEAT1 level are regularly achieved by transfection of the gene into cells or by silencing the gene with siRNA treatment. Moreover, several laboratories use different toxins often in wide ranges of concentrations to induce effects resembling those seen in PD.

A recent study by Yan *et al.* reported that in the MPTP/MPP+ mouse and SH-SY5Y cell model of the disease in parallel to NEAT1 up-regulation elevated PINK1 (phosphatase and tensin homolog (PTEN)-induced kinase 1) protein level was detected. It was concluded that NEAT1 directly interacts with PINK1, stabilizing the protein by influencing its ubiquitination and preventing its degradation, thus promoting autophagy [31].

NEAT1 was also proposed to interact with various miRNAs and *via* this, to exert detrimental effects by acting as a molecular sponge decreasing the availability of the regulatory transcripts. Among the proposed targets is miR-221, one of the most abundant miRNAs in the human CNS. miR-221 plays an important role in neurite outgrowth and neuronal differentiation and was found to down-regulate the expression of various pro-apoptotic proteins thus exerting cell protective effects [128]. Decreased level of the miRNA in serum samples of PD patients has been repeatedly reported, raising the possibility of implementing the transcript as a biomarker of the disease [129][130]. In a

SH-SY5Y cell model of the disease NEAT1 down-regulation resulted in up-regulation of miR-221 and decreased reactive oxygen species (ROS) generation, resulting in improved cell viability. It was concluded that NEAT1 exerts pro-inflammatory, pro-apoptotic and cell viability decreasing effects *via* sponging miR-221 thus decreasing its availability [33].

In addition, NEAT1 was proposed to regulate neuroinflammation *via* its interaction with another miRNA, miR-124 [34]. Results of an *in vitro* PD model showed that NEAT1 silencing had anti-apoptotic and anti-inflammatory effects and improved cell viability. A direct interaction was revealed between NEAT1 and mir-124, leading to the notion that the anti-inflammatory and cell viability promoting effects of NEAT1 silencing are partly due to the lack of sponging of the micro RNA [34].

NEAT1 was also found to directly interact with miR-212-5p, influencing cell viability and apoptosis *via* the miR-212-5p/RAB3IP pathway [36]. MPP+ treatment of SK-N-SH cells led to the down-regulation of miR-212-5p and up-regulation of both NEAT1 and RAB3IP (RAB3A-interacting protein), a protein known to be involved in various cell functions such as autophagy, cell growth and apoptosis. Both NEAT1 silencing and overexpression of miR-212-5p in the cell model resulted in diminished ROS production and subsequent enhancement of cell viability and decrease of apoptosis. Dual-luciferase gene reporter assay indicated a direct interaction between the miRNA and RAB3IP mRNA, by which miR-212-5p exerts a negative regulatory effect on RAB3IP expression. Since enhanced RAB3IP expression was shown to enhance neuroinflammation leading to diminished cell viability and enhanced apoptosis in MPP+ treated SK-N-SH cells, it can easily be foreseen that NEAT1, by sponging miR-212-5p, prevents the miRNA from down-regulating RAB3IP expression, thus leading to the enhancement of inflammatory processes and consequent decrease in cell survival [36].

Via its interactions with micro RNAs NEAT1 was also proposed to affect the expression of the PD related gene *SNCA*. In SH-SY5Y cells treated with MPP+ NEAT1 knockdown resulted in the improvement of cell viability and diminishment of apoptosis [32]. Silencing of NEAT1 caused the down-regulation of *SNCA* expression: however, the protective effect of NEAT1 knockdown was abolished by over-expressing the gene. These findings led to the conclusion that up-regulation of NEAT1 is harmful in the course of PD *via* a *SNCA* related mechanism [32].

Recently a possible mechanism by which NEAT1 up-regulates *SNCA* expression was proposed [35]. Upon NEAT1 silencing in MPP+ treated SH-SY5Y cells the expression of *SNCA*, the pro-inflammatory cytokine IL-1β, NLRP3 (nucleotide

oligomerization domain-like receptor protein with pyrin domain containing 3) inflammasome, caspase-1, and the number of apoptotic cells were decreased. NLRP3 containing inflammasome is a protein complex which has been shown to play a pathologic role in neuroinflammation related to various neurodegenerative diseases, among them PD. Upon activation, inflammasomes aggravate inflammation and cell damage *via* propagating the secretion of further po-inflammatory cytokines [131]. With regard to PD, NLRP3 inflammasome was found to be activated by fibrillar α-syn, and inhibition of the inflammasome was found to have beneficial effects on motor deficits, nigrostriatal dopaminergic degeneration, and accumulation of α-syn aggregates in various rodent models of the disease [132].

NEAT1 was proposed to regulate *SNCA* expression *via* the miR-1301-3p/GJB1 pathway. GJB1 (alias connexin-32 (Cx32)) - a member of the gap junction connexin family - has recently been reported to play a cardinal role in the uptake of α-syn oligomeric assemblies in neurons and oligodendrocytes [133]. NEAT1 inhibition was suggested to arrest miR-1301-3p sponging, permitting the miRNA to exert its inhibitory effect on GJB1 expression and through this preventing α-syn induced activation of the NLRP3 inflammasome.

In contrast to the findings supporting the damaging effect of NEAT1 in PD pathogenesis, findings of Simchovitz and colleagues support the hypothesis of up-regulation of NEAT1 having a beneficial outcome in the course of the disease.

Under oxidative stress provoked by PQ and tBHP (t-butyl hydroperoxide), NEAT1 was significantly up-regulated in HEK-293T and SH-SY5Y cells [30]. Elevated NEAT1 expression was primarily due to the up-regulation of the long isoform, as a fold up-regulation of 7 was observed when investigating NEAT1L separately *vs.* fold change being 2.5 in the case of both isoforms. NEAT1L up-regulation occurred in parallel with a prominent increase in the number of paraspeckles, whereas silencing of NEAT1 diminished both the proportion of cells forming paraspeckles and the number of paraspeckles/nucleus. These findings prompted the notion of NEATL up-regulation and consequent enhanced paraspeckle formation being a cellular response to stress [30]. NEAT1 silencing not only led to decreased paraspeckle formation but also diminished mitochondrial abundance and exacerbated oxidative stress provoked cell death. Intriguingly however, the detrimental effect of NEAT1 silencing on cell viability could be reversed by treatment with an LRRK2 inhibitor. Thus NEAT1 was proposed to exert its protective effects on cell viability in an LRRK2-dependent manner. Considering the

interaction of LRRK2 with the paraspeckle proteins SPFQ and NONO, it is easily conceivable that NEAT1 acts as a *bona fide* LRRK2 inhibitor by retaining it in paraspeckles [30]. LRRK2 dysfunction has been shown to play a central role in PD pathology, and gain of function mutations leading to enhanced kinase activity have been identified as one of the main genetic contributors to the disease in both familiar and sporadic cases [134]. Thus modulating LRRK2 activity *via* NEAT1 up-regulation might be an appealing therapeutic approach. This notion is supported by the finding that enhancing NEAT1 expression in cell cultures by treatment with the PPARα agonist fenofibrate and 3-hydroxy-3-methylglutaryl-coenzyme A inhibitor simvastatin improved cell viability under oxidative stress provoked by PQ and tBHP. The finding that LRRK2 inhibitor abolishes this protective effect further strengthens the concept of NEAT1 serving as a natural LRRK2 inhibitor [30].

Table 5. Mechanisms by which NEAT1 is proposed to exert disadvantageous and protective effects in PD

NEAT1 modulation	Mode of action	Effect	Reference
NEAT1 up-regulation	stabilization of PINK1 protein	• decrease in cell survival • pro-inflammatory effect • pro-apoptotic effect	[31]
	miR-221 sponging		[33]
	miR-124 sponging		[34]
	miR-212-5p sponging		[36]
	SNCA gene expression down-regulation *via* the miR-1301-3p/GJB1 pathway		[32][35]
	LRRK2 inhibition	• enhanced paraspeckle formation • diminished oxidative stress • enhanced cell viability	[30]

Our data on the effects on NEAT1 expression changes in cellular and animal models of PD are in agreement in several aspects with findings of others and in some aspects extend those data. Similarly to others we observed NEAT1 up-regulation by MPP+/MPTP treatment in the SH-SY5Y neuroblastoma cell and mouse model of the disease. Our data obtained from the mouse PD model is also in accord with others regarding that the increase of NEAT1 level is observed mainly in the long isoform.

We could not detect an increased PINK1 protein level upon 0.25 mM MPP+ treatment in SH-SY5Y cells. The reason behind this could be the not sufficiently high dose

of MPP+ implemented. We are planning to conduct experiments with higher doses of toxin treatment and also to investigate the change in NEAT1 expression in parallel.

An important addition from our data to those already published by others is the increase in NEAT1 expression level by SFN treatment most probably through HSF1 activation. This gave us the possibility to study the effect of NEAT1 level increase evoked by toxins and a neuroprotective agent simultaneously.

Investigation of the effect of SFN on cell viability in combination with MPP+ and PQ revealed that while 10 uM SFN treatment was capable of partially restoring cell viability decrease upon 0.5 mM PQ treatment, such effect was not observed when SFN was implemented in combination with 0.25 mM MPP+. However, SFN treatment combined with either low dose PQ (0.05 mM) or MPP+ (0.002 and 0.01 mM) resulted in enhanced cell viability, an increase exceeding that observed in the case of SFN treatment on its own (142% *vs.* 155% in the case of 0.05 mM PQ treatment and 131% *vs.* 148% and 137% in the case of 0.002 and 0.01 mM MPP+ treatment, respectively). This could be due to the phenomenon termed 'preconditioning': subtoxic doses of cellular stress causing agents can trigger an endogenous neuroprotective response [135]. Thus, preconditioning the cells with low dose of MPP+ or PQ can result in enhanced improvement of cell viability upon SFN treatment. Moreover, up-regulation of heat shock proteins has been shown to have protective effects against neurodegenerative diseases (reviewed: [136]). Since SFN is a HSF inducer, induction of the heat shock pathway in combination with preconditioning with low doses of neurotoxins can also explain the additive effect of simultaneous treatments.

We also observed differences between the effects of the toxins on relative mtDNA copy number change. While both 0.5 and 1 mM MPP+ treatments led to a prominent decrease in mtDNA copy number, such change was not observed even at the highest (1.5 mM) PQ dose tested. The mtDNA decreased observed upon 0.5 mM MPP+ treatment could be partly compensated for by 6 hours pretreatment with 10 uM SFN.

While none of the implemented PQ doses had a mtDNA copy number decreasing effect, low dose (0.1 and 0.5 mM) PQ treatment caused an elevation in mtDNA copy number. A proposed mode of action of the toxin is lipid peroxidation of the mitochondrial inner membrane leading to complex I toxicity [137]. It can be assumed that sufficiently low dose PQ, which is not yet detrimental for the cells, can have a preconditioning effect and enhance mitochondrial turnover and ATP production, thus leading to an increase in the relative mtDNA copy number. The differences observed between the two toxins on

mtDNA copy number change might be due to MPP+ being a more potent mitochondrial toxin than PQ and because of the different mode of action of the compounds.

Interestingly, while no beneficial effect of 10 uM SFN treatment could be observed regarding cell viability diminishment due to 0.25 mM MPP+ treatment, results of FACS analysis indicated a prominent decrease in the ratio of apoptotic cells when 0.25 mM or 0.5 mM MPP+ treatment was combined with SFN treatment. These seemingly contrasting results could be partly explained by the different methodologies implemented. For cell viability analysis we used the CCK8 assay, a colorimetric assay which is based on the generation of a formazan dye from the tetrazolium salt WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium,monosodium salt]. The conversion requires the presence of intact electron carrier, since the reduction of WST-8 is catalyzed by dehydrogenases (CCK-8 assay protocol, Sigma-Aldrich). Changes in intracellular metabolic activity can highly influence the reduction of the assay substrates, thus changes in absorbance might not necessarily reflect the viability of the cells, but rather the metabolic changes happening within them [138][139].

Differences between toxin treatments were observed regarding not only mtDNA copy number alterations but on the effects on cell viability changes as well. SFN treatment was found to partly reverse the decrease in cell viability caused by low dose PQ treatment, however, such effect was not observed in the case of any of the MPP+ doses tested. This observation could be partly explained by the different mechanism by which these toxins exert their effects: MPTP/MPP+ is a mitochondrial toxin inhibiting complex I of the mitochondrial respiratory chain which leads to ATP synthesis disruption and excessive ROS generation. Furthermore MPTP damages dopamine storage of cells, which is a feature considered to play a key role in the selective loss of dopaminergic neurons (reviewed: [140]). On the other hand, PQ is a herbicid, which interferes with photosynthetic electron transport in plants, thus leading to superoxide production. In various experimental models of PD PQ has been linked to ROS generation and α-syn aggregate accumulation in dopaminergic neurons, although the exact mode of action by which it leads to dopaminergic cell damage is not fully elucidated [141][142]. The differences between the pathomechanisms of PQ and MPTP/MPP+ call attention to the shortcomings of toxin models accepted and widely used in PD research and might give explanation for the seemingly contrasting results obtained from different disease models. Elucidation of the complex pathomechanism of the disease is also cardinal in order to be able to establish

disease models which could mimic more precisely the underlying molecular changes of the disorder.

In conclusion, our results indicate that elevated NEAT1 level alone does not seem to have deleterious effect on apoptosis, cell viability and mtDNA copy number changes. Altogether our data do not support a primary neurodegenerative effect of NEAT1.

6. Conclusion - New findings

I. <u>We have analysed the frequency of 10 SNPs of 4 PARK genes in Hungarian sporadic PD patients and non-PD controls and determined that:</u>

1. The G2385R and R1628P risk factor *LRRK2* variants were absent both in the control and PD group.
2. Both genotype and allelic distribution of the rs1491923 *LRRK2* variant were similar in patient and control groups.
3. The minor (A) allele of the S1647T variant showed significantly higher frequency among healthy male individuals ($\chi 2 = 6.06$; $p = 0.014$) compared to the corresponding PD group.
4. The protective *LRRK2* variants (R1398H and N551K) were found to be present in LD and both occurred with similar frequencies in patient and control groups.
5. For the protective rs356186 *SNCA* variant there was a significant difference due to the higher relative frequency of the AA genotype among healthy participants in comparison to patients. LOPD group and healthy controls also show a significant difference in genotype distribution, which is a consequence of higher frequency of AG genotype among LOPD patients.
6. No significant difference was found in genotype or allele frequency of rs2583988 SNP of *SNCA* and the studied *MAPT* (rs1052553) and *TCEANC2* gene variants (rs10789972).

Our data on these SNPs are new concerning Hungarian and mostly new in respect of Caucasian population groups, and are in accord with data available on these SNPs in the literature.

II. <u>By comparing lncRNA levels in peripheral blood samples of PD patients and controls we determined that:</u>

1. NEAT1 is up-regulated in the peripheral blood of PD patients. The most prominent differences in NEAT1 expression were observed by comparing all PD patients to all control individuals (fold change = 1.62; $p = 0.0019$), PD patients with DBS to the control

group (fold change = 1.61; p = 0.0021), and LDD patients' group to control group (fold change = 1.74; p = 0.0008).

2. Apart from the two major NEAT1 isoforms (short and long) no other variants are detectable in human peripheral blood samples. Of the two forms NEAT1S is present in significantly higher levels.

   Our publication is the first report on the detection of altered NEAT1 lncRNA level in easily accessible biological samples of PD patients. *Post mortem* brain analysis of PD brain samples and data obtained in PD models by others and by us are in accord with the observed change in NEAT1 level.

III. <u>We set up a neuroblastoma cell based *in vitro* PD model and using it we determined that:</u>

1. NEAT1 up-regulation can be achieved by MPP+ treatment.
2. SNF treatment enhances NEAT1 expression of SH-SY5Y cells in a dose and time dependent manner.
3. Combined treatment of cells with MPP+ and SFN has an additive effect on NEAT1 expression up-regulation.
4. MPP+ treatment of SH-SY5Y cells results in a decrease in mtDNA copy number, while, SFN treatment increases mtDNA copy number. Pretreatment of the cells with SFN prior to MPP+ exposure is capable of partly restoring the mtDNA copy number change caused by the toxin.
5. Both MPP+ and PQ treatments cause a decrease in cell viability. In contrast, SFN increases cell viability.
6. SFN treatment can partly reverse the cell viability decrease caused by low dose of PQ treatment, however, such effect was not observed in the case of any of the MPP+ doses tested.
7. SFN treatment markedly decreased the apoptosis rate of SH-SY5Y cells treated with 0.25 and 0.5 mM MPP+.

   These findings suggest that different toxins used to mimic PD effects (MPP+ *vs.* PQ) act at least partly by different mechanisms in decreasing cell viability. The increased level of NEAT1 does not seem to have direct toxic effect on cells and NEAT1 expression up-regulation is not a direct cause of mtDNA copy number changes.

IV. <u>Using an *in vivo* mouse PD model we determined that:</u>

1. MPTP treatment of mice causes up-regulation of NEAT1L. The expression change is dose dependent and is most prominent in the striatum of the animals.
2. By altering SFN treatment time, dose and brain area dependent up-regulation of NEAT1L can be achieved.
3. SFN and MPTP have an additive effect on NEAT1 up-regulation in both striatum and brainstem samples of mice.

These findings indicate that with the use of SFN NEAT1 up-regulation can be produced in an *in vivo* PD model, permitting further studies for the exploration of the mechanism of NEAT1 action.

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Research article

Assessment of risk factor variants of *LRRK2, MAPT, SNCA* and *TCEANC2* genes in Hungarian sporadic Parkinson's disease patients

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ABSTRACT

Introduction: Parkinson's disease is the second most common neurodegenerative disease. Lifestyle, environmental effects and several genetic factors have been proposed to contribute to its development. Though the majority of PD cases do not have a family history of disease, genetic alterations are proposed to be present in 60 percent of the more common sporadic cases.

Objective: The aim of this study is to evaluate the frequency of PD related specific risk variants of *LRRK2*, *MAPT*, *SNCA* and *PARK10* genes in the Hungarian population. Out of the ten investigated polymorphisms three are proposed to have protective effect and seven are putative risk factors.

Methods: For genotyping, TaqMan allelic discrimination and restriction fragment length polymorphism method was used. *LRRK2* mutations were investigated among 124 sporadic PD patients and 128 healthy controls. *MAPT* and *SNCA* variant frequencies were evaluated in a group of 123 patients and 122 controls, while *PARK10* variant was studied in groups of 121 patients and 113 controls.

Results: No significant difference could be detected in the frequencies of the investigated *MAPT* and *PARK10* variants between the studied Hungarian PD cases and controls. The minor allele of the risk factor S1647T *LRRK2* variant was found to be more frequent among healthy male individuals compared to patients. Moreover, in the frequency of one of the investigated *SNCA* variant a significant intergroup difference was detected. The minor allele (A) of rs356186 is proposed to be protective against developing the disease. In accord with data obtained in other populations, the AA genotype was significantly more frequent among Hungarian healthy controls compared to patients. Similarly, a significant difference in genotype distribution was also found in comparison of patients with late onset disease to healthy controls, which was due to the higher frequency of AG genotype among patients.

Conclusion: The frequencies of different gene variants show great differences in populations. Assessment of the frequency of variants of PD related genes variants is important in order to uncover the pathomechanisms underlying the disease, and to identify potential therapeutic targets. This is the first comprehensive study focusing on these genetic variants in the population of East-Central European region. Our results extend the knowledge on the world wide occurrence of these polymorphisms by demonstrating the occurrence of specific alleles and absence of others in Hungarian PD patients.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease, affecting millions of people worldwide [1]. It is a multifactorial disease: several environmental, lifestyle and genetic factors have been suspected to contribute to its development. 5–10 % of PD cases are familial of which 30% is monogenic [2]. Regarding sporadic PD, only 3–5% of the cases are caused by single gene mutations. However, growing body of evidence suggest the involvement of genetic factors in 60% of the more common idiopathic PD cases as well [3].

So far over 40 human genomic loci have been proven or proposed to be related to PD [3]. Several of these are also referred as 'PARK' and a number reflecting the order of their discovery to indicate the

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association with the disease. In this study, we investigated the presence of ten variants of four PARK genes: Leucine-rich repeat kinase 2 (*LRRK2*; PARK8: R1628P, G2385R, S1647T, R1398H, N551K and rs1491923), synuclein alpha (*SNCA*; PARK1 and 4: rs356186 and rs2583988), transcription elongation factor A N-Terminal and central domain containing 2 (*TCEANC2*; PARK10: rs10788972) and microtubule associated protein tau (*MAPT*: 1052553*)* in Hungarian PD patients.

The involvement of *LRRK2* in PD was first described in a large Japanese family in 2002 [4]. Since then, several *LRRK2* mutations have been identified, and alterations of this gene have been shown to be among the major causes of both familial and sporadic PD cases. Intensive research is ongoing to identify variants of the gene that act as risk factors in the disease. Two single nucleotide polymorphisms (SNPs) have been found to increase the risk of PD in Asian populations. One of them is a Gly to Arg substitution (G2385R), the other one an Arg to Pro change (R1628 P). While both of these have been proven to be risk factors among Han Chinese, to date none of these variants has been found among Caucasians [5,6].

In addition to G2385R and R1628P, a change of the 1647th amino acid Ser to Thr (S1647T) is also proposed to be a susceptibility factor for PD [7]. Its effect of increasing PD risk was reported in Asian populations [7], however, such association has not been found in the Caucasian populations investigated so far [8].

Some of the *LRRK2* polymorphisms on the other hand have been proposed to be protective against PD. Such variants are the Arg to His and Asn to Lys changes at the 1398th and 551th positions of the protein (R1398H and N551K). The occurrence of either of these in combination with the G2385R and R1628 P allele is reported to diminish the otherwise elevated risk of the disease [7].

Recently a Genome Wide Association (GWA) Study revealed that a common variability near the *LRRK2* gene affects the risk of PD. The minor allele resulting from an A to G change (indicated in forward orientation, rs1491923) was found to be more common among both Caucasian and Asian PD patients then their healthy controls [9].

Similarly to *LRRK2*, several variants of the *SNCA* gene have been proposed to be risk-, or protective factors regarding PD. In fact, mutations of the *SNCA* gene were the first genetic variants identified as causes of autosomal dominantly inherited familial PD. Two intronic variants of the gene: rs2583988 and rs356186 are proposed risk-, and protective factors against PD, respectively. The role of these variants among sporadic PD patients of Caucasian origin is controversial. Association and also the lack of it between these variants and the disease have been reported in several studies involving Caucasian subjects [10–14].

The *MAPT* gene is located on the long arm of chromosome 17, at a site of an approximately 900 kb common inversion [15] that results in two distinct haplotypes: the non-inverted H1 and the inverted H2. The H1 haplotype has been associated with numerous diseases which are often referred as taupathies: Alzheimer's disease (AD), sporadic frontotemporal dementia, progressive supranuclear palsy (PSP) and PD. A common pathological hallmark of these is the accumulation of MAPT neurofibrillary tangles in nerve cells [15]. The association of H1 with PD is, however, still an intriguing question. Several studies involving subjects of different nationalities reported no, or marginal association between the occurrence of the H1 haplotype and PD (reviewed in [16]). SNPs suitable of marking the inversion have been identified: a G to A change (rs1052553) is an indicator of the H1 haplotype [15].

The long arm of chromosome 1 containing the PARK10 region with the locus of *TCEANC2* gene has also gathered interest concerning its role in PD. The link between PD and this region was identified first approximately 15 years ago [17], and since then, a linkage disequilibrium (LD) for a block of 100 kb was identified in the region [18]. The SNP rs10789972, located in the *TCEANC2* gene, was found to show association with sporadic PD in American population [18] but there was no association detected among subjects of Han Chinese origin [19,20].

Allelic variants of PD-related genes are found in widely different frequencies among different populations, making it difficult to clarify the genuine effect of specific variants on the development of PD in distinct populations. It is important therefore to evaluate the occurrence of specific genetic alterations in homogenous study groups of different nationalities. Information on the occurring mutations in a population can be beneficial for understanding more of the pathological mechanisms underlying the disease. Moreover, the identification of gene variants characteristic for a population might be useful also in applying the most fitting therapeutic methods and developing new therapeutic approaches.

The aim of our study was to assess the frequency of *LRRK2*, *SNCA*, *MAPT* and *TCEANC2* mutations in sporadic PD patients in Hungary. All combined, we assessed the occurrence of ten mutations which vary in their effects as some are risk factors and others are protective. We selected SNPs that are either the most intensively studied (as they have been proven to play a role in the disease in certain populations) or have been recently identified as potential risk factors. To our knowledge, this is the first throughout study focusing on the prevalence of these PARK gene variants in Hungary.

2. Material and methods

2.1. Subjects

2.1.1. LRRK2 variants

The frequencies of R1628P, G2385R, S1647T, R1398H, N551K and rs1491923 LRRK2 variants were assessed in a group of 124 sporadic PD patients (mean age: 66.5 ± 9.5 years, male-female ratio 61:63) (Table 1). Depending on the first appearance of symptoms, two groups were formed: early-onset (EOPD; disease onset ≤ 60 years) and late-onset (LOPD; disease onset > 60 years) PD patients. The EOPD group comprised 68, the LOPD 56 individuals. The age at disease onset was 51.1 ± 7.4 and 68.7 ± 5 years, respectively. The control group consisted of 128 healthy volunteers (mean age of 64.5 ± 9.6 years, male-female ratio 61:67).

2.1.2. SNCA and MAPT variants

The frequencies of the rs2583988 and rs356186 SNPs of *SNCA* and

Table 1
xxx.

Gene		Total number of participants	Age (mean ± SD; years)	Male/female ratio	Disease onset (EOPD/LOPD ratio)
LRRK2	PD	124	66,5 ± 9,5	61/63	68/56
	Control	128	64,5 ± 9,6	61/67	
SNCA and *MAPT*	PD	123	66,5 ± 9,5	60/63	67/56
	Control	122	64,3 ± 8,8	56/66	
TCEANC2	PD	121	66,5 ± 9,6	59/62	66/55
	Control	113	64,9 ± 8,1	50/60	

Demographic data of the study groups. Abbreviations: PD: Parkinson's Disease; EOPD: early-onset Parkinson's Disease; LOPD: late-onset Parkinson's Disease; SD: standard deviation.

rs1052553 variant of *MAPT* were assessed in the groups of 123 sporadic PD patients (mean age: 66,5 ± 9,5 years, male-female ratio 60:63) and 122 healthy controls (mean age: 64,3 ± 8,8 years, male-female ratio 56:66) (Table 1). Based on the appearance of the first symptoms, the patient's group was divided into two subgroups. The EOPD (disease onset ≤ 60 years) group comprised 67, the LOPD (disease onset > 60 years) group 56 patients. The age at disease onset was 51,1 ± 7,5 and 68,7 ± 5 years, respectively

2.1.3. TCEANC2 polymorphism

The frequency of the rs10789972 SNP of the *TCEANC2* gene in the PARK10 locus was evaluated among 121 sporadic PD patients (mean age: 66,5 ± 9,6 years, male-female ratio 59:62) and 113 healthy controls (mean age: 64,9 ± 8,1 years, male-female ratio 50:60) (Table 1). Among PD patients, 66 individuals reported the first disease symptoms at or under the age of 60 years (EOPD, disease onset 51 ± 7,5 years). In the case if the other 55 patients the first symptoms appeared after the age of 60 years (LOPD, disease onset 68,7 ± 5,1 years).

In all study groups, the diagnosis of PD was set up based on medical history and physical examination by movement disorder specialists. All control individuals had no history of neurological and psychiatric disorders.

Informed consent was obtained from all study participants. The study is in full accordance with the Helsinki Declaration and was approved by the Medical Research Council Scientific and Research Ethics Committee.

2.2. DNA isolation

The standard desalting method [21] was used for genomic DNA isolation from peripheral blood. The extracted DNA was stored at −20 °C.

2.3. Restriction fragment length polymorphism

For the genotyping of R1628P and G2385R variants polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) analysis was implemented. For the sequences of the primers used for generating PCR products please see [22]. Annealing temperatures and cycling conditions can be provided on request (please contact the Corresponding Author). For the investigation of G2385R and R1628 P SNPs 170 bp and 419 bp PCR products were generated, respectively. After amplification, the PCR products were digested with restriction enzymes at 37 °C overnight. *Acc*I restriction enzyme (Thermo Scientific, Waltham, MA, USA) was used for the detection of G2385R, and *Bst*UI (Thermo Scientific, Waltham, MA, USA) for R1628P. DNA fragments were then detected on 2% agarose gel electrophoresis, visualizing the bands with ECO Safe alternative gel stain (Pacific Image Electronics, Torrance, CA, USA). Wild-type G2385R samples remained undigested, resulting in one, 170 bp DNA fragment. In the case of heterozygous samples three fragments (170,123 and 47 bp), while in the case of homozygous mutants, two (123 and 47 bp) fragments could be detected. Opposite to this, in the case of the R1628P SNP digestion of homozygous wild-type samples resulted in the generation of two (263 and 156 bp) DNA fragments. The partial digestion of heterozygous samples yielded three bands (419, 263 and 156 bp), while PCR products of homozygous mutant samples remained undigested resulting in one detectable band (419 bp).

2.4. TaqMan allelic discrimination method

The analysis of R1398H, N551K, S1647T and rs1491923 *LRRK2* variants and all the investigated *MAPT*, *SNCA* and *TCEANC2* variants was performed with the use of TaqMan allelic discrimination assays obtained from Thermo Fisher Scientific (Thermo Scientific, Waltham, MA, USA). PCR reactions were run on Bio-Rad real-time thermal cycler CFX96 (Bio-Rad, Hercules, CS, USA). The reaction conditions can be obtained on request (please contact the Corresponding Author).

2.5. Statistical analysis

For statistical analysis GraphPad Prism 6.01 statistics software was used. For the analysis of genotype and allele frequencies Chi-square (χ^2) test or Fisher's test was used. Odds ratio (OR) with a 95% confidence interval (95% CI) was implemented for the analysis of the association between PD and genotype frequencies. A p value less than 0,05 was considered statistically significant.

3. Results

3.1. Putative risk factor LRRK2 mutations (G2385R, R1628P, S1647T and rs1491923)

The G2385R and R1628P variants were found to increase the risk of developing in PD, however, seem to be absent or extremely rare in Caucasian populations. In accord with this, we did not found any of these SNPs to be present in either of our study groups (Suppl. Table 2.).

The S1647T substitution results from a T to A change in exon 34. The minor allele (A) of the variant was found to be a risk factor of PD in several Asian populations. However, such relation has not been identified in Caucasian populations. The genotype and allele distribution of this variant was similar in both study groups (Suppl. Table 3.). The difference was not significant when comparing EOPD and LOPD patient subgroups to controls. When analyzing the genders separately, the comparison of female patients to healthy controls showed no significant difference regarding both genotype and allele frequencies. However, when analyzing the genotype distribution of male patients in comparison with the corresponding control group, a trend towards higher AA frequency could be observed in healthy controls. Comparing allele frequencies of the same groups revealed the minor (A) allele to be significantly more frequent among healthy male individuals ($\chi 2 = 6.06$; $p = 0.014$) (Suppl. Table 3.).

The SNP rs1491923 is an A to G change (indicated in reverse orientation), affecting a site 0.17Mb upstream of *LRRK2* gene [9]. Its role as a predisposing factor PD was proposed recently based on the results of a GWAS study [9]. We found both genotype and allelic distribution of this variant to be similar in our patient and control group (Suppl. Table 4.). Comparison of subgroups generated by separating our two main study groups (PD and control) either by gender or by the age of disease onset did not reveal significant difference either in genotype or in allele frequencies (Suppl. Table 4.).

3.2. Protective LRRK2 variants (R1398H and N551K)

The R1398H and N551K *LRRK2* variants were found to diminish the increased risk of the disease in G2385R and R1628P carriers [7]. We did not find any significant difference between either the genotype or allele frequencies of the R1398H or N551K variants between the control and PD group (Suppl. Table 5.). Allele and genotype frequencies were also similar after stratification by gender or by age at disease onset. We found these variants to be in LD, as except for one case in our group of healthy controls, the R1398H and N551K substitution always occurred simultaneously (Suppl. Table 5.).

3.3. SNCA and MAPT gene variants

The rs356186 SNP is an intronic G to A change in the *SNCA* gene, of which the minor A allele is proposed to be protective in PD. Comparing the genotype distribution of our control and patients' group there was a significant difference ($\chi^2 = 7.65$; $p = 0.022$) (Suppl. Table 6.). This intergroup difference was due to the higher relative frequency of the AA

genotype among healthy participants in comparison to patients (AA vs. GG + AG. Fisher's test: p = 0.019, OR: 0.12, CI (95%): 0.014 − 0.95). A significant difference in genotype distribution was also found when comparing the LOPD group to healthy controls (χ^2 = 6.14; p = 0.046) (Suppl. Table 6.). This difference is a consequence of higher frequency of AG genotype among LOPD patients (AG vs. GG + AA. χ^2 = 5.07; p = 0.024). No significant difference in genotype or allele distribution could be detected in other study setups.

No significant difference was found in genotype or allele frequency of rs2583988 SNP of *SNCA* and the studied *MAPT* variant (rs1052553) in either comparison (Suppl. Table 7. and 8.).

3.4. TCEANC2 gene variant

Both allele and genotype distribution of the rs10789972 SNP was similar in the PD and control group, revealing no significant difference (Suppl. Table 9.). Similarly, no significant difference was found when comparing the EOPD, LOPD, male or female patients to the corresponding control groups.

4. Discussion

The aim of our study was to assess the frequency of six *LRRK2*, two *SNCA*, a haplotype marking *MAPT* and *PARK10* variants in Hungarian sporadic PD patients. To our knowledge this is the first comprehensive study focusing on these gene variants in the population of the East-Central European region.

The *LRRK2* gene is localized on the long arm of chromosome 12. LRRK2 – also known as dardarin – is a large protein, built up of more, than 2500 amino acids. It is a representative of the ROCO superfamily and consists several domains, of which two (a kinase and GTPase) are enzymatic. Though the exact physiological function of the protein needs further elucidation, LRRK2 is suggested to serve as a scaffolding protein, to be involved in the process of neurite outgrowth, maintenance of the cytoskeleton, vesicle transport and degradation of autophagic protein (reviewed in [23]).

Among the investigated *LRRK2* variants four are putatively, or among some populations proven risk factor variants, and two SNPs have been found to have protective effects among certain circumstances.

Even today, data regarding the *LRRK2* mutations that might act as risk factors in PD is inconclusive. Out of the more than 100 SNPs in *LRRK2* gene G2385R and R1628P are the only validated coding susceptibility alleles for PD [24]. The Gly to Arg substitution at the 2385th amino acid position (G2385R) causes a two-fold increase in PD risk, while the Arg to Pro amino acid change at position 1628 (R1628P) causes an even bigger increase in the possibility of developing the disease [25]. Our results showing that both of these variants are absent in our study groups are in accord with literature data. The R1628P and G2385R substitutions have been found only in the Asian, but not in Caucasian populations [22,24,26].

The G3285R substitution is located towards the C terminus of the protein in the WD40 domain. As this domain takes part in protein-protein interactions, one might suppose that the amino acid change leads to alterations in the interactions with substrates and other regulatory proteins [5]. Functional studies revealed that under oxidative stress cells with the G2385R substitution showed a higher rate of apoptosis compared to the wild type [27]. The mutation might also increase the kinase activity of the protein, however, the data regarding this issue are inconclusive [7,28]. The R1628 P mutation affects the COR domain of the protein and there is data suggesting it to cause a diminishment in GTPase activity [28]. Besides changes in the GTPase, the R1628P substitution was also found to increase the kinase activity of dardarin [7]. This is probably because of the increased binding affinity of LRRK2 with Cyclin-dependent kinase 5 (Cdk5) due to the amino acid substitution, which leads to the phosphorylation of LRRK2 at the S1627 site, resulting in increased kinase activity of the protein [29]. Similarly to G2385R, R1628P mutant cells were found to be more prone to apoptosis under oxidative stress when compared to wild type [30].

S1647T is another variant, which the effect of increasing the risk towards PD was first spotted in a Han Chinese population [7]. This Ser to Thr substitution is located in the COR protein domain, which together with the adjacent Roc domain forms the tandem Roc-COR domain, accounting for the GTPase function of LRRK2. Existing data suggest that GTP binding is essential for the activation of kinase function of this protein, therefore mutations affecting the GTPase domain might have effects on kinase function as well [28]. Other reports indicate that the dimeric form is essential for kinase activity [28]. Considering that the COR domain is a core element in protein dimerization [31], mutations affecting this domain could have effects on kinase activity either by changes in autophosphorylation or protein conformation. However, further studies are necessary for the elucidation of the effects of the S1647T mutation, as so far no changes have been found in kinase activity in relation with this variant [7].

Our findings, that there is no significant association between the ST1647T *LRRK2* variant and PD in our cohort, is in accord with literature data available regarding Caucasian populations, as no significant association was found in Finnish and Greek study groups either [8]. Our result of higher frequency of the minor allele among male controls compared patients is in contrast with literature data. However, this conflicting result might be due to the relatively small sample size.

Rs1491923 is an A to G (forward orientation) change 0.17 Mb upstream the gene. The possible significance on developing PD of this common intronic variant was proposed by a GWA study. It was found that the minor allele of this SNP was more common among American, German and British PD patients compared to healthy controls [9]. Findings obtained by the use of an induced pluripotent stem cell (iPSC) model of idiopathic PD suggest that this variant might have detrimental effect on mitochondrial protein clearance and autophagy [32]. Though our results do not add to these findings, the possible risk effect of this variant on the disease cannot be excluded. In order to clarify such associations further genotype analysis of independent sample groups of different populations is clearly warranted.

Besides risk factor mutations there are variants of the *LRRK2* gene which seem to have a protective effect against the development of PD. Such variants are the R1398H and N551K substitutions, located in the ROC domain and armadillo repeat region of the protein, respectively. A study involving Asian patients and controls found these variants to be in LD and were significantly more frequent among PD patients [7]. The same study revealed a prominent reduction in the otherwise increased disease risk due to the presence G2385R and R1628P polymorphisms in individuals who simultaneously were carriers of either the R1398H or N551K SNPs [7]. Moreover, appearance of either of the protective variants could largely negate the risk of a R1628P carrier, resulting in an OR 1.5–1.6 instead of 1.9 [7]. This could partly be explained by the diminished kinase activity of R1398H mutant dardarin, which might be able to compensate the elevated enzyme function, a result of R1628P and/or G2385R substitutions [7]. In Caucasian population no significant difference was found in the frequencies of these variants between PD patients and healthy individuals [8]. Our observations corroborate with data published on Greek and Finnish populations [8] in finding no significant difference among PD patients and controls. Our data are also consistent with the findings of others in regard the LD these gene variants show [7].

The *SNCA* gene is located on the long arm of chromosome 4 and consisting of 10 exons it spans over 114 kb. The product of the gene is the 140 amino acid alpha-synuclein (SNCA), a major component of the PD-related Lewy bodies. Accumulation of the protein is proposed to contribute to the selective loss of dopaminerg neurons seen in PD due to the increased sensitivity of the cells to dopamine toxicity [33]. Mutations of the *SNCA* gene were the first genetic alterations identified to

cause autosomal dominant PD. Since then several SNPs within the gene have been proposed to contribute to, or, in some cases, decrease the risk of developing the disease. The SNP rs2583988 is an intronic C/T base change. The minor allele of the variant was found to occur at a significantly higher frequency among PD patients compared to controls in studies involving individuals of Caucasian origin [10–12]. However, there are also data representing for the lack of such association between PD and the polymorphism [13]. In accord with our findings, association between the minor allele frequency of rs2583988 and PD was not found among German [13] or Irish [14] patients.

Rs356186 is an A/G change (indicated in forward orientation) which is also located in the intronic region of the *SNCA* gene. The presence of the minor allele is proposed to be a protective factor against developing PD. This assumption is based on the detection of the minor allele significantly more frequent among healthy controls compared to PD patients in Irish [14], Italian [10] and populations of Northern Central and Southeastern European origin [11]. However, no significant difference was detected between controls and patients in a study involving German participants (except when comparing the frequency between female PD patients and the corresponding control individuals) [13]. Recently a meta-analysis was conducted with the aim to find the most relevant *SNCA* SNPs in PD [34]. Zang et al. analysed the significance level of the different variants from various studies, and based on that, defined the polymorphisms rs2583988 and rs356186 as recommended and most recommended *SNCA* SNPs, respectively [34]. The same study also concluded that heterozygotes of the protective *SNCA* variant (rs356186) greatly contribute to the effect of this SNP since in the overall analyzed populations the dominant model of the variants showed significant difference [34]. These findings are in accord with our data, as we found that the significant difference in genotype distribution between LOPD group and healthy controls was a consequence of higher frequency of AG genotype among LOPD patients. We also detected a significantly higher relative frequency of the AA genotype among healthy participants in comparison to patients.

MAPT gene on the long arm of chromosome 17 is located at a site of a common inversion that results in two haplotypes. The more common haplotype referred as H1, the rarer as H2 [35]. Several genes are localized in the approximately 900 kb affected region of chromosome that results in H1 and H2 haplotype formation [36]. One of the most studied one of these genes is *MAPT* due to its linkage with several disorders including neurodegeneration [37]. The H1 haplotype was found to show higher transcriptional activity being stronger at initiating transcription thus resulting in increased expression of the *MAPT* gene [38]. In accord with this, the H1 haplotype has been associated with neurological diseases such as sporadic frontotemporal dementia, PSP, AD and PD – which all share a pathological hallmarks of accumulated MAPT neurofibrillary tangles in neurons [39]. However, the role of H1 haplotype in PD risk is controversial. Several studies of various populations reported no, or only marginal significance of the variant in PD (reviewed in [37]). Our results are in accord with those which found no significant association between the H1 haplotype and PD involving British [40], Swedish [41] and Taiwanese [42] populations (reviewed [16]:).

The *TCEANC2* is one of the genes located in the *PARK10* region on chromosome 1. The gene spans approximately 58 kb and contains 6 exons. The exact function of TCEANC2 is still unknown. Data suggest its involvement in RNA processing [18]. The relationship of the *PARK10* locus and PD was first described in a large Icelandic family [17]. Since then, a LD block of 100 kb in this region was found to be associated with the disease [18]. The SNP rs10789972, located in the *TCEANC2* gene, was found to show the strongest association with sporadic PD in American population [18]. However, association between the variant and PD has not been found in Han-Chinese population [19,20]. Our findings do not indicate association of the variant and PD, however, further studies focusing on elucidating this question are strongly warranted.

5. Conclusions

A growing body of evidence suggests contribution of genetic factors in the development of PD. Besides the well established pathogenic mutations, several gene variants have been proposed to be risk factors, or, on the contrary, to play a protective role in the disease. Such assumptions are mainly based on genome wide association studies. The heterogeneity of the study groups included in these studies might cover frequency differences that might exist among different populations in respect of specific gene variants. Therefore, it is important that findings of GWA studies are tested in specific populations. Our results are from the first comprehensive study focusing on the *LRRK2*, *SNCA*, *MAPT* and *PARK10* risk and protective variants in the East-Central European region. We believe that these results represent a valuable contribution to the evaluation of the world wide significance of these genetic variants.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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Research report

Increased level of NEAT1 long non-coding RNA is detectable in peripheral blood cells of patients with Parkinson's disease

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HIGHLIGHTS

- NEAT1 lncRNA regulates cellular and mitochondrial homeostasis.
- Changes in NEAT1 level were reported in PD brain and in models of the disease.
- We detected up-regulated NEAT1 level in leukocytes of PD patients.
- NEAT1 up-regulation was most prominent among patients with long disease duration.

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ABSTRACT

Parkinson's disease (PD) is the second most prevalent neurodegenerative disorder that poses serious burden to individuals and society as well. Although a number of PD associated genetic factors have been identified, the molecular mechanism of the disease so far has not been completely elucidated. Involvement of long non-coding RNAs (lncRNAs) in the pathology of neurodegenerative disorders is attracting increased interest because of the diverse mechanisms lncRNAs affect gene expression and cellular homeostasis at different levels. We aimed to test the feasibility of detecting alterations in lncRNA levels in easily accessible samples of PD patients by routine laboratory technique. By narrowing the number of selected lncRNAs implicated in neurodegeneration and increasing the number of PD samples included, we found one out of 41 lncRNAs readily detectable in increased level in peripheral blood of PD patients. We detected NEAT1 to be significantly up-regulated in PD patients in multiple comparisons. NEAT1 is the core element of nuclear paraspeckles and it plays role in regulation of transcription, mRNA and miRNA levels, mitochondrial and cellular homeostasis. Our finding is in accord with recent data demonstrating changes in the level of NEAT1 in neurons of PD patients and in several models of the disease. However, to our knowledge this is the first study to report NEAT1 up-regulation in blood of PD patients. Identification of altered expression of this lncRNA in the periphery might help to a better understanding of the mechanisms underlying PD, and can contribute to the identification of new therapeutic targets and disease markers.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease affecting approximately 1–2% of the population over the age of 65 (Goedert, 2001). In PD leading motor symptoms, such as bradykinesia, rigidity, and tremor are often associated with non-motor symptoms, such as sleep- and mood disorders, depression and dementia. The progression of PD is a great burden for the patients, for

Abbreviations: PD, Parkinson's disease; PARK, Parkinson's disease associated genes; lncRNA, long non-coding RNA; AD, Alzheimer's disease; HD, Huntington's disease; ALS, amyotrophic lateral sclerosis; DBS, deep brain stimulation; Uchl1 AS, carboxy-terminal hydrolase L1 antisense transcript; MALAT1, Metastasis Associated Lung Adenocarcinoma Transcript 1; SNGH1, Small Nucleolar RNA Host Gene 1; NEAT1, Nuclear Paraspeckle Assembly Transcript 1; MS, multiple sclerosis; PCR, polymerase chain reaction; EOPD, early onset Parkinson's disease; LOPD, late onset Parkinson's disease; DD, disease duration; SDD, short disease duration; LDD, long disease duration; TUG1, Taurine Up-Regulated Gene 1; PTENP1-AS, Phosphatase and Tensin Homolog Pseudogene 1 Antisense RNA; SN, *substantia nigra*; NONO, non-POU domain containing octamer binding; SFPQ, splicing factor proline glutamine rich; PINK1, PTEN-induced kinase 1; PTEN, Phosphatase and tensin homolog deleted on chromosome 10

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their families and society as well. Although only a relatively small fraction of PD cases is familiar, a number of genetic factors are believed to play direct or indirect role in PD etiology. Among the Parkinson's disease associated genes (PARK) identified so far several are involved in mitochondrial energy conversion, oxidative stress response and apoptosis (reviewed in (Benson and Huntley, 2019)).

Long non-coding RNAs (lncRNAs) are frequently, although not obligatorily polyadenylated RNA polymerase II transcripts, which are over 200 nucleotides in length by definition with no identifiably open reading frame(s). In recent years lncRNAs attracted particular attention since they seem to play roles in regulating gene expression and cellular homeostasis at several levels and by diverse mechanisms. LncRNAs can modulate gene expression at transcriptional level by acting themselves as repressors or activators, by modulating regulatory factor availability, by serving as transcriptional co-factors, or by modulating RNA polymerase II activity. They can also act as post-transcriptional modulators by regulating mRNA availability, editing and degradation, or by modulating miRNA mediated functions ((Feng et al., 2006; Martianov et al., 2007; Wang et al., 2008) for a review, see (Li et al., 2019)). Additionally, lncRNAs can exert regulation through epigenetic mechanisms (Brockdorff et al., 1992; Brown et al., 1992; Butler et al., 2019). Alterations in lncRNA levels have been shown in several neurological diseases. An increasing body of evidence is accumulating the involvement of lncRNAs in Alzheimer's disease (AD), Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS) (Wang et al., 2018a) Furthermore, possible connections between specific non-coding transcripts and PD have also been suggested: Soreq *et al.* investigated the lncRNA profile of PD patients' leukocytes by whole transcriptome sequencing (Soreq et al., 2014). They found that over 6000 detected leukocyte lncRNAs, 13 had altered expression in PD patients as compared to healthy controls. The majority of these lncRNAs – 8 out of 13 - showed increased expression. In the cases of four lncRNAs, deep brain stimulation (DBS) treatment resulted in a decrease in expression. According to this study RP11-462G22.1 (lnc-FRG1-3), an anti-sense transcript of the *FRG1* gene, is significantly upregulated in PD. As the *FRG1* gene is associated with facioscapulohumeral muscular dystrophy 1, the FRG1 lncRNA might contribute to the muscle rigidity seen in PD patients. Expression of the spliceosome component U1 was also significantly upregulated in PD samples supporting the notion that the modulation of splicing might be involved in the course of the disease (Soreq et al., 2014). Strong down-regulation of the Ubiquitin carboxy-terminal hydrolase L1 antisense transcript (Uchl1 AS) in *in vitro* and *in vivo* PD models was reported in 2015 by Carrieri and colleagues (Carrieri et al., 2015). Uchl1 AS is transcribed on the same region but in opposite direction as the protein-coding *UCHL1*, alias *PARK5* gene is, which has been shown to carry mutations in rare cases of early-onset familial PD. Moreover, the expression of Uchl1 AS is under the control of the transcription factor NURR1 that is involved in the maintenance and differentiation of dopaminergic cells and for which mutations have also been associated with PD (Grimes et al., 2006; Xu et al., 2002). Normally Uchl1 AS is located in the nucleus however, under certain stress conditions it is transported to the cytoplasm, where it facilitates UCHL1 mRNA expression, leading to a rise in the level of UCHL1 protein. The overexpression of the UCHL1 protein has a neuroprotective effect, thus the increased expression of Uchl1 AS can be part of a cell-salvage mechanism (Carrieri et al., 2015).

Recently, Kraus and colleagues compared lncRNA expression levels in brain tissue of PD patients and healthy control individuals (Kraus et al., 2017). They found that out of 90 non-coding transcripts investigated in their study only 5 had significantly altered levels in patients compared to healthy controls. The lncRNA H19 upstream conserved regions 1 and 2 were significantly downregulated, while lincRNA-p21, MALAT1 (Metastasis Associated Lung Adenocarcinoma Transcript 1), SNHG1 (Small Nucleolar RNA Host Gene 1), and NEAT1 (alias TncRNA) were found to have increased expression. In line with these findings, more recently Simchovitz *et al.* reported a significant elevation in the expression level of NEAT1 in the *substantia nigra* of PD patients (Simchovitz et al., 2019).

With the aim to test the feasibility of detecting alterations in lncRNA using easily accessible samples by routine laboratory technique we compared leukocyte-derived lncRNA levels in PD patients. Data obtained by this approach might provide details on the pathological mechanism underlying this neurodegenerative disease and indicate the possibility of lncRNA detection as diagnostic marker.

Blood samples are accessible by minor invasive procedures and offer the possibility of a cheap, feasible and quick way of identifying disease-related biomarkers. Nucleated leukocytes are informative both in respect to genetic and gene expression analysis and have the advantage that they reflect the status and changes occurring throughout the body due to their interactions with most of the tissues and organs (Soreq et al., 2014). Experimental data show that impacts affecting the central nervous system, such as for example DBS, can cause changes in leukocyte gene expression (Soreq et al., 2012). Moreover, existing correlations demonstrated between peripheral blood-based and brain derived biomarkers in neuropsychiatric disorders (Harris et al., 2012), as well as identified blood-based biomarkers in AD (Fehlbaum-beurdeley et al., 2012), multiple sclerosis (MS) (Nickles et al., 2013) and schizophrenia (Harris et al., 2012) provide grounds for attempting such investigations in the case of PD as well. In this respect it is important to note that while the prominent motor symptoms of PD are primarily due to the neuronal loss in the central nervous system, more and more observations suggest that it has more systemic effects, influencing functions of the peripheral nervous system as well (Berstad and Berstad, 2017; Dzamko et al., 2015; Hawkes et al., 2007; Liu et al., 2003; Svensson et al., 2015; Weller et al., 2005; Westfall et al., 2017).

The data we report here indicate a significant increase in NEAT1 lncRNA level in peripheral blood cells of PD patients. This observation correlates well both with recently published data on the up-regulation of this lncRNA in PD models and tissue samples and also with the suggested role of NEAT1 lncRNA in cellular functions affected in PD such as mitochodrial homeostasis, oxidative stress response, apoptosis (Simchovitz et al., 2019; Wang et al., 2018b). Despite several recent reports on possible functions of NEAT1, whether it contributes primarily to neurodegeneration or neuroprotection is still unclear. It is well documented that NEAT1 increases the stability of PINK1 protein, and by this, it facilitates mitophagy. As this might affect damaged and healthy mitochondria as well, this effect is considered more to be neurodegenerative (Oe et al., 2019; Yan et al., 2018). On the other hand, in cellular and animal models, NEAT1 upregulation provides protection against oxidative stress of mitochondria by a LRRK2 mediated pathway, and based on this, its neuroprotective role is suggested (Simchovitz et al., 2019).

2. Results

2.1. LncRNAs detectable in peripheral blood samples of PD patients

By reviewing literature data we selected 41 lncRNAs which have been implicated in neurodegenerative malignancies (Table 1.). Nine of these were directly linked to PD (RP11-101C11.1, RP11-409K20.6, RP11-124N14.3, RP11-79P5.3, AC004744.3, RP11-542K23.9, PCA3 (Soreq et al., 2014), NEAT1 (Kraus et al., 2017; Simchovitz et al., 2019) and MALAT1 (Liu et al., 2017)), while others were associated with AD (BC200, BACE1-AS (Feng et al., 2018; Lukiw et al., 1992)), HD (MEG3, TUG1 (Taurine Up-Regulated Gene 1), LINC00341, HAR-1A (Chanda et al., 2018; Johnson, 2012; Johnson et al., 2010; Wang et al., 2014)), and/or were found to be involved in mechanisms that are likely related to neurodegeneration. We attempted to detect these lncRNAs in peripheral blood cells of controls and PD patients (n = 3 in each group) by qRT-PCR. Those lncRNAs, which had a Ct larger than 35 we excluded from the further analysis since the low expression level makes their detection by this technique unreliable. The levels of lncRNAs deemed to

Table 1
Neurodegeneration implicated lncRNAs included in the perliminary study (control n = 3, PD n = 3).

RP11-101C11.1	*BCYRN1 (BC200)*	*DLX6-AS1*
RP11-409K20.6	*ATXN8OS*	PTENP1-AS
SCOC-AS1	BDNF-AS	MALAT1
RP11-124N14.3	HAR1A	*HOXA11-AS*
RP11-79P5.3	HAR1B	*HOXA-AS2*
LOC339568	NEAT1	*HOXA-AS3*
AC004744.3	*DGCR5*	*MEG9*
RP11-542K23.9	MEG3	*TUNAR*
LOC338797	TUG1	TMEM161B-AS1
PCA3	LINC00341	ST7-AS1
LINC01262	MTOR1-AS1	ST7-AS2
UCHL1-AS1	GAS5	RBM5-AS1
SOX2-OT	*HOTAIR*	LINC00853
BACE-AS1	*SIX3-AS1*	

Bold: lncRNAs reported to have altered expression in PD (Soreq et al., 2014). Italics: lncRNAS detected in low level (Ct > 40), bold: lncRNAs, which were reported to have altered expression in PD by Soreq et al. (2014).

Table 2
LncRNAs included in validation study I. (control n = 15, PD n = 18) and their expression changes.

	Average Ct		Fold change (PD/Ctrl)	P value
Gene Symbol	PD	Ctrl		
RP11-409K20.6	34.21	34.63	1.53	0.88
GAS5	27.73	27.53	n.a.	n.a.
RP11-124N14.3	34.36	34.73	1.48	0.95
LINC00341	33.77	34.31	1.66	0.55
PINK1-AS	34.23	34.93	1.86	0.79
NEAT1	26.70	27.46	1.93	0.035*
MALAT1	31.95	32.36	1.52	0.07
MTOR-AS1	34.96	34.97	1.15	0.57
TUG1	30.35	30.93	1.71	0.037*
BC200	> 35	> 35	n.a.	n.a.
PTENP1-AS	> 35	> 35	n.a.	n.a.
MEG3	> 35	> 35	n.a.	n.a.

LncRNAs detected in Ct > 35 were excluded from further analysis.
Abbreviations: PD: Parkinson's disease; Ctrl: control.

be detectable in our first approach were compared within larger groups of healthy and PD samples (n = 15 and 18, respectively) (validation study I.). In this comparison we found the level of lncRNAs BC200, PTENP1-AS (Phosphatase and Tensin Homolog Pseudogene 1 Antisense RNA) and MEG3 to be below reliable detection level (Ct over 35). These RNAs therefore were omitted from the further analysis. The expression level of GAS5 showed minimal variation in both PD and control samples, therefore we selected GAS5 as an internal control for reference. Stable expression of GAS5 lncRNA has also been noted by both Kraus et al. (2017), Santoro et al. (2016). Applying GAS5 normalization, we found the expression of NEAT1 significantly up-regulated among PD patients (fold increase = 1.93; p = 0.035) compared to the control group (Table 2). Similarly, we detected a significant up-regulation of the lncRNA TUG1 by comparing PD patients to the control cohort (fold increase = 1.71; p = 0.036) (Table 2). Besides NEAT1 and TUG1, no other lncRNA was found to exhibit a significant difference in expression in comparison between this PD group and control cohort.

2.2. Comparison of NEAT1 and TUG1 lncRNA levels between study groups including larger number PD patients and controls

Based on the findings of validation study I., we investigated the expression of NEAT1 and TUG1 lncRNAs in study groups including larger number PD patients (n = 43) and controls (n = 36) (validation study II.). Applying GAS5 normalization, we found significant up-regulation of NEAT1 expression among PD patients compared to controls (fold increase = 1.62; p = 0.0019) (Suppl. Table 1, Fig. 1.) As DBS treatment was reported to influence lncRNA expression in leukocytes (Soreq et al., 2012), we compared our samples in respect of this treatment. The difference in NEAT1 expression between healthy control and PD patients was significant both in comparisons including and not including DBS patients (fold increase = 1.61 and 1.62; p = 0.0021 and 0.0071, respectively) (Suppl. Table 1, Fig. 1B and C). Between patients with- and without DBS we did not find significant difference in NEAT1 level (Suppl. Table 1, Fig. 1.). NEAT1 expression was significantly up-regulated as compared to the control group in both EOPD and LOPD group (fold change = 1.5 and 1.82; p = 0.0181 and 0.0073, respectively). Between the EOPD and LOPD group however, there was no significant difference in NEAT1 level (Suppl. Table 1, Fig. 1E–G,). Comparison of female PD patients to female control individuals revealed significantly up-regulated NEAT1 level among PD patients (fold increase = 1.72; p = 0.0073). Though the difference was not significant, up-regulation could also be observed among male PD patients compared to control male individuals (Suppl. Table 1, Fig. 1H and I).

Comparison between subgroups SDD and LDD indicated slightly up-regulated NEAT1 level among patients of the LDD group, however, the difference was not significant. Comparisons of subgroups of SDD and LDD patients to the control group revealed significant up-regulated NEAT1 RNA levels in both cases (fold change = 1.57 and 1.74; p = 0.028 and 0.0008, respectively) with more prominent NEAT1 up-regulation among LDD patients (Suppl. Table 1, Fig. 1J–L).

After performing Bonferroni correction the difference remained significant in pair wise comparisons between the control group and PD group-, or patients with DBS or the LDD group.

In the expression of TUG1 we detected no significant difference in either of the comparisons described above (Suppl. Table 2).

3. Discussion

With this study, we aimed to identify alterations in lncRNA expression in PD that could help to understand the underlying disease mechanisms, may help to find potential therapeutic targets, and can be potential biomarker for diagnosis. Our aim was also to use easily accessible sample to this analysis and reliable experimental approach that is readily available in a clinical laboratory. We selected for a first comparison 41 lncRNAs which have been reported to be related to neurodegenerative diseases. Among these 7 lncRNAs (indicated in bold in Table 1.) had already been reported by Soreq *et al.* to have altered expression in PD (Soreq et al., 2014). From these 7 lncRNAs, we detected only 2 (RP11-409 K20.6 and RP11-124 N14.3) by our assay, the other 5 had a Ct > 40 (indicated in italics in Table 1). The difference between our and the published data could be explained by the different methods used for expression monitoring: while Soreq and colleagues carried out whole-transcriptome RNA-Seq analysis, we used real-time PCR. Another explanation could be in a high inter-individual expression variability of lncRNAs (Kornienko et al., 2016), and also the small number of analysed samples. Based on the data from this preliminary study we selected 12 lncRNAs for comparison involving a larger sample number (validation study I.). In this analysis we detected low expression levels for BC200, PTENP1-AS, and MEG3, therefore these transcripts had to be considered undetectable. Our failure to detect these lncRNAs is unlikely because of technical reason. BC200 lncRNA is expressed predominantly in the brain, specifically in the hypothalamus, but shows low, or no expression in other tissues (Castle et al., 2010; Tiedge et al., 1993). MEG3 is a candidate tumour suppressor. According to lncRNA database (http://www.lncrnadb.org) MEG3 is not expressed in human white blood cells. PTENP1-AS also acts as a tumour suppressor. Poliseno and colleagues, described relatively low levels of PTENP1-AS under physiological conditions in various human tissues, including peripheral blood leukocytes (Poliseno et al., 2010). In our assays the average Ct of the PTENP1-AS transcript was only slightly

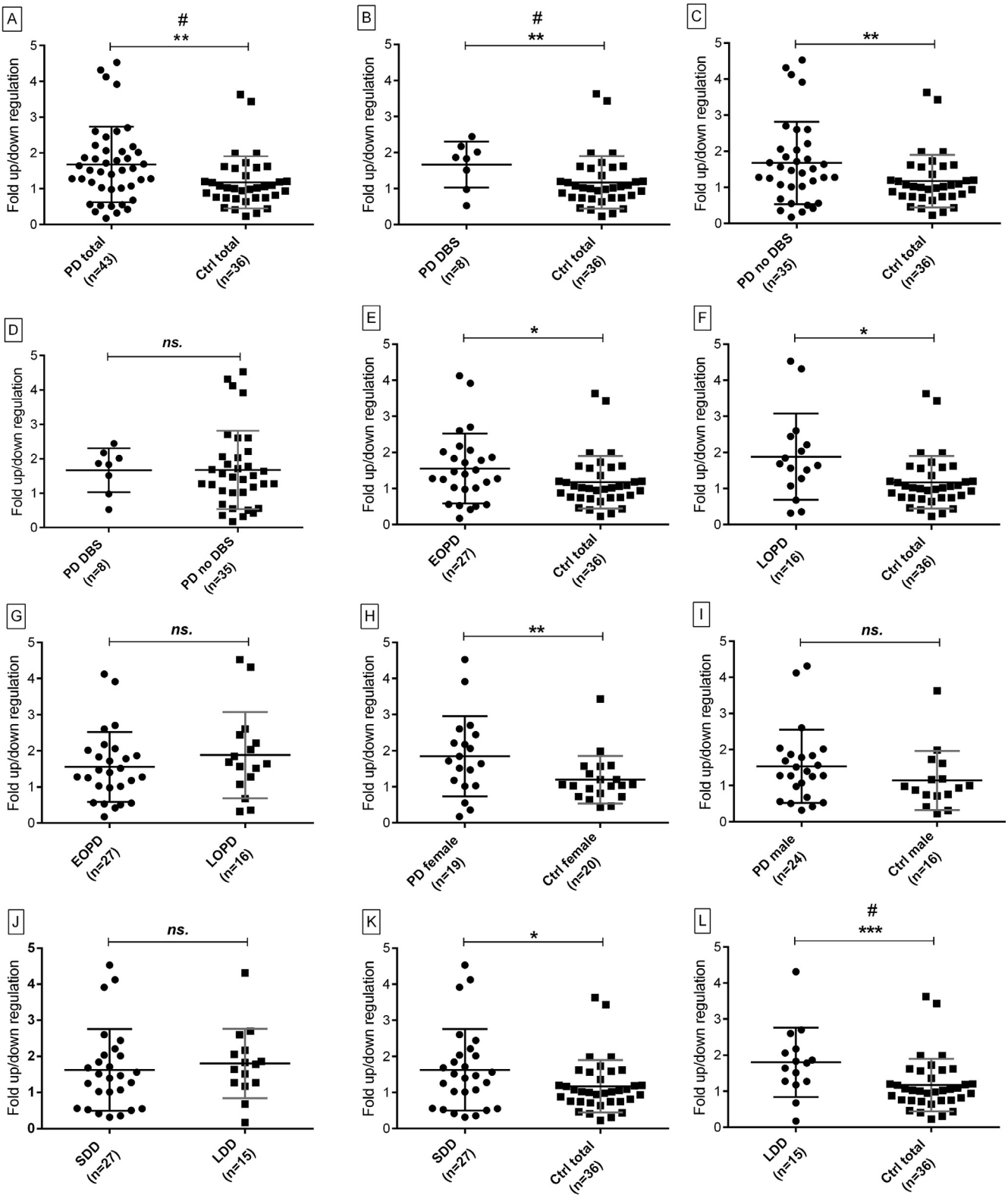

Fig. 1. Comparisons of NEAT1 lncRNA level between controls (n = 36) and PD patients (n = 43) and their subgroups. Fold regulation are shown with standard deviation. Significant up-regulation of NEAT1 expression was detected in comparisons between PD and control group (A), PD patients with or without DBS and control group (B and C). Comparison between patients with and without DBS does not shown significant difference in NEAT1 level (D). NEAT1 was found to be significantly up-regulated in EOPD and LOPD groups as compared to control group (E and F), while no significant difference was detected between EOPD and LOPD patients (G). NEAT1 was found to be up-regulated both in female PD to female control and male PD to male control comparisons (H and I), however the difference was not significant in the latter. Neither was significant difference detectable in NEAT1 level between SDD and LDD patient groups (J). NEAT1 was found significantly up-regulated in comparisons between both SDD and LDD groups and controls (K and L). The difference of NEAT1 expression between PD group *vs.* control group, patients with DBS *vs.* control group and LDD *vs.* control group remained significant after Bonferroni correction. Abbreviations: PD: Parkinson's disease; Ctrl: control; DBS: deep brain stimulation; EOPD: early onset Parkinson's disease; LOPD: late onset Parkinson's disease; SDD: short disease duration; LDD: long disease duration; *ns.*: non-significant; *: $p < 0,05$; **: $p < 0,01$; ***: $p < 0,001$; #: p value significant after Bonferroni correction.

above the threshold (37.2 ± 1.2 in the control group, and 37.9 ± 2 among PD patients).

Out of the well detectable 9 neurodegeneration-related lncRNAs, we found NEAT1 and TUG1 to be significantly up-regulated in PD patients compared to control group. We tested the expression of these two lncRNAs in a third set of samples (validation study II.). Results on this larger number of PD and control groups however revealed no significant difference in the expression of TUG1 between PD patients and non-PD controls. TUG1 lncRNA acts as transcriptional repressor, that upon DNA damage suppresses the progression of the cell-cycle (Khalil et al., 2009). It achieves this by forming PcG bodies (Polycomb group protein containing nuclear repressive foci) on the promoters of cell cycle genes in interaction with a component of the Polycomb Repressive complex 1 (PRC1) (Yang et al., 2011). With respect to neurodegenerative diseases, TUG1 was found to be up-regulated in HD (Johnson, 2012), but no other data have been reported regarding TUG1 expression in neurodegenerative diseases, including PD.

We found NEAT1 to be significantly up-regulated in PD patients in multiple comparisons. The most prominent differences were observable by comparing all PD patients to all control individuals (fold change = 1.62; $p = 0.0019$), PD patients with DBS to the control group (fold change = 1.61; $p = 0.0021$), and LDD patients' group to control group (fold change = 1.74; $p = 0.0008$). In each of these comparisons the difference remains significant after Bonferroni correction. In the case of comparison of PD patients with DBS to controls however, the small sample size of PD patients should be taken into account. The finding that the expression of NEAT1 was significantly up-regulated among LDD patients compared to control individuals suggests that the amount of the lncRNA increases with disease progression. This novel notion could be interesting in relation to PD pathology, however, whether the change in NEAT1 level is coincidental or in causative relation with disease progression remains to be explored.

In either way, NEAT1 elevated expression in PD patients deserves attention because this lncRNA has been shown to modulate cellular functions by several mechanisms and it might affect disease pathology *via* a number of these.

NEAT1 lncRNA is expressed in two forms: a 3756 nucleotide long NEAT1_1, also known as MEN ε, and as a 27 kb long NEAT1_2, alias MEN β. Although the smaller form corresponds to the 5′ end of the longer and both NEAT1 isoforms are involved in paraspeckle formation, the two differ in functions (Sunwoo et al., 2009). In this study we used primers which target the 5′ region of NEAT1, consequently permit the detection of both NEAT1 isoforms.

NEAT1 lncRNA was first identified as a virus inducible gene product that was up-regulated in mouse central nervous system after virus infection (Saha et al., 2006). More recent data demonstrated that HIV-1 (Zhang et al., 2013), influenza virus and herpes simplex virus infection also enhanced NEAT1 expression, and NEAT1 promoted the expression of antiviral genes, such as Interleukin-8 (IL-8) (Imamura et al., 2014). The involvement of NEAT1 in inflammatory processes raises the possibility that this lncRNA plays role in the reported increase of inflammatory markers in the peripheral blood of PD patients (King and Thomas, 2017). Recently various mechanisms have been described by which NEAT1 regulates cellular functions. Most of the effects mediated by NEAT1 are due to its involvement in paraspeckle formation. Paraspeckles are approximately 0.5 μm size membraneless subnuclear particles consisting of a large number of proteins and NEAT1 RNAs as a scaffold. Paraspeckles are responsible for the retention of the A-to-I hyperedited mRNAs in the nucleus (Mao et al., 2011). Through this they play a pivotal role in cellular response to stress. Under cellular stress conditions, such as transcription inhibition or exposure to the combination of lipopolysaccharide and interferon-γ, specific A-to-I hyperedited RNA types can be rapidly transported into the cytoplasm where they are used for protein synthesis (Prasanth et al., 2005). By this mechanisms the cell produces a rapid stress response since it can save time by not synthesizing *de novo* mRNAs, but using already synthesized transcripts for protein synthesis in order to respond to stress stimuli (Nakagawa and Hirose, 2012). By retaining hyperedited mRNAs in the nucleus paraspeckles play role in cell differentiation as well (Bond and Fox, 2009). Recently NEAT1 involvement in regulating mitochondrium homeostasis through the nuclear retention of mRNAs encoding proteins with mitochondrial function (mito-mRNA) has been shown. Paraspeckle-enriched mito-RNAs exhibited enhanced nucleo-cytoplasmic export in NEAT1 knockout cell suggesting a quality control mechanism that prohibit unnecessary translation of mito-mRNAs following stress (Wang et al., 2018b). Paraspeckles also regulate gene expression by retaining regulatory proteins. Sequestration and/or liberation of transcription factors to and from paraspeckles offer rapid ways for modulating gene expression.

The possible roles of NEAT1 in the human central nervous system attracted attention in the last decade. Investigation of human nucleus accumbens samples of heroin abusers showed a significant up-regulation of the gene as compared to drug-free controls (Michelhaugh et al., 2011). The association of NEAT1 lncRNA expression with neurodegenerative diseases was also examined in ALS (Nishimoto et al., 2013), HD (Johnson, 2012) and PD (Kraus et al., 2017). In 2013 Nishimoto and colleagues reported that NEAT1_2 transcript was up-regulated in ALS patients compared to the control group (Nishimoto et al., 2013). Similarly, NEAT1 up-regulation was found in HD patients (Johnson, 2012). According to a recent study by Kraus *et al.*, the lncRNA NEAT1 was significantly up-regulated in the anterior cingulate gyrus of PD patients as well as compared to healthy controls (Kraus et al., 2017). More recently Simchovitz *et al.* reported significant up-regulation of NEAT1 in the *substantia nigra* (SN) of PD patients compared to non-PD controls. In the SN, dopaminergic neurons were identified as the main NEAT1 expressers and paraspeckle forming cells. Cell culture and murine model studies showed that under oxidative stress conditions the expression of the NEAT1 transcript was enhanced. Genetic ablation of NEAT1 led to a reduction in the number of paraspeckle forming cells and also in the number of paraspeckles in the nucleus, together with a diminishment in the survival of cells pre-treated with oxidative stress agents (Simchovitz et al., 2019).

Interconnection between several PD associated genes and NEAT1 lncRNA has been demonstrated in a number of PD models. Mutations of the gene encoding LRRK2 protein is among the most frequently identified genetic alterations in both familial and sporadic PD (Kumari and Tan, 2009). LRRK2 is involved in mitophagy and LRRK2 mutation delays the arrest of dysfunctional mitochondria (see (Grünewald et al., 2019)). Simchovitz and colleagues showed that NEAT1 could serve as a natural inhibitor of LRRK2 by retaining it in paraspeckles through its interaction with non-POU domain containing octamer binding (NONO) and splicing factor proline glutamine rich (SFPQ) proteins (Simchovitz et al., 2019).

Studies of MPTP/MPP+ induced mouse and cell culture models of PD yielded seemingly contrasting results regarding the role of NEAT1 in the course of PD. Recent studies involving murine and cell models reported up-regulation of NEAT1 after treatment with MPTP or MPP+, accompanied by decreased cell viability. On the other hand, genetic inhibition of NEAT1 promoted cell survival (Geng et al., 2019; Liu and Lu, 2018; Yan et al., 2018). Various mechanisms have been described by which NEAT1 regulates apoptosis, oxidative stress and neuroinflammation (Fig. 2). Yan *et al.* showed that in PD mice both NEAT1 and a PD susceptibility gene, PTEN-induced kinase 1 (PINK1) expression was up-regulated. *In vitro* findings revealed that whereas genetic ablation of NEAT1 significantly decreased the otherwise enhanced apoptosis rate following MPP+ treatment and down regulated PINK1 expression, overexpression of the lncRNA inhibited the cycloheximide induced degradation of PINK1. The beneficial effects of NEAT1 knockdown were abolished *via* overexpressing PINK1. Based on these findings the authors proposed that by direct binding to PINK1, NEAT1 stabilizes the protein, thus mediates autophagy and neuronal injury (Yan et al., 2018). NEAT1 down-regulation was also found to decrease

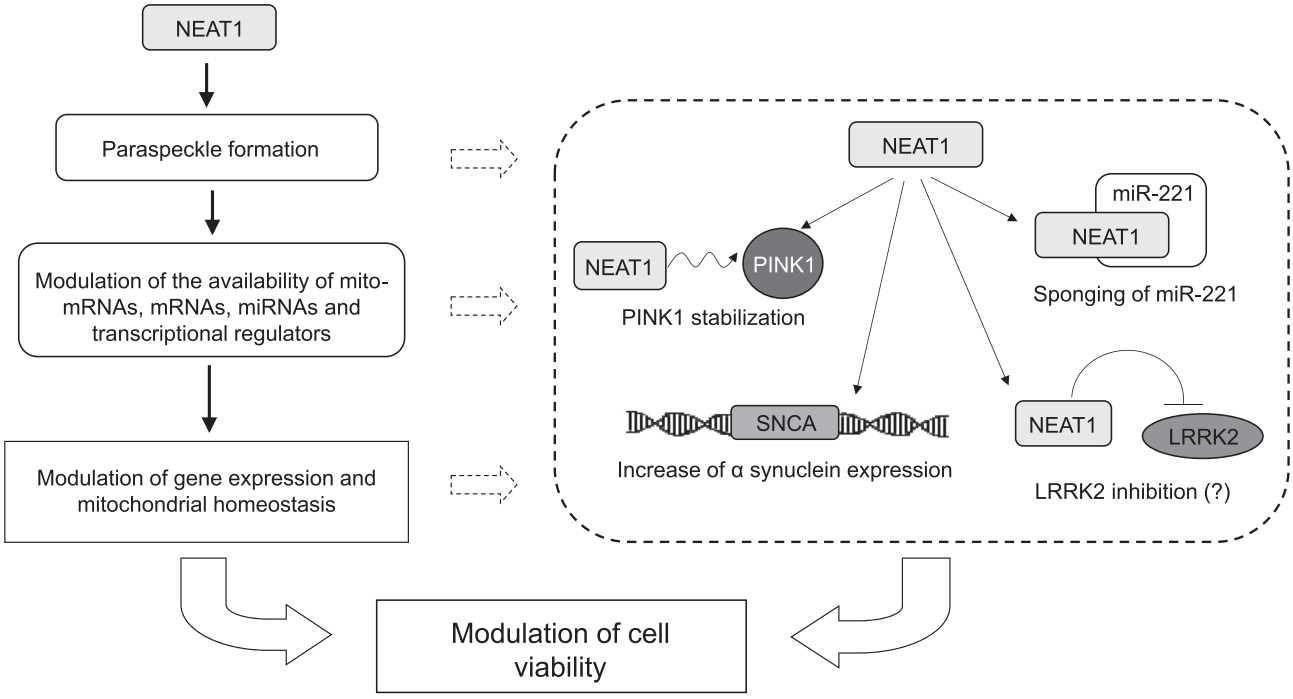

Fig. 2. Mechanisms by which NEAT1 might effect cell viability and PD. NEAT1 lncRNA, a major constituent of paraspeckles, plays divers regulatory roles by modulating the availability of mRNAs, miRNAs and transcription factors. By the nuclear retention of mito-mRNAs - mRNAs encoding proteins with mitochondrial function – NEAT1 directly affects mitochondrium homeostasis (Wang et al., 2018b). In the pathogenesis of PD (boxed) NEAT1 was suggested to participate by regulating autophagy, neuroinflammation and neuronal cell injury *via* stabilizing PINK1 (Yan et al., 2018), influencing *SNCA* expression (Liu and Lu, 2018) and sponging miR-221 (Geng et al., 2019). NEAT1 was also proposed to be a *bona fide* LRRK2 inhibitor acting *via* its nuclear retention (Simchovitz et al., 2019). Abbreviations: NEAT1: Nuclear paraspeckle assembly transcript 1; PINK1: PTEN-induced kinase 1; SNCA: α-synuclein; LRRK2: Leucine-rich repeat kinase 2; mito-mRNAs: messenger RNAs encoding proteins with mitochondrial function.

α-synuclein expression, and ablation of the lncRNA in SH-SY5Y cells increased cell viability. However, up-regulation of α-synuclein reversed the beneficial changes in apoptosis rate upon genetic inhibition of NEAT1 (Liu and Lu, 2018). NEAT1 was also proposed to take part in the course of PD *via* a miR-221 related mechanism. Recent findings revealed miR-221 down-regulation in serum samples of PD patients and proposed its potential role as a biomarker for the disease (Ding et al., 2016; Ma et al., 2016). The expression of the micro RNA was also found to be reduced in cellular models of PD and overexpression of miR-221 promoted cell viability. A direct target of miR-221 is PTEN (Phosphatase and tensin homolog deleted on chromosome 10) which in addition to its tumorsuppressor function also has been linked to the pathogenesis of neurodegenerative disorders such as AD, ALS and PD (Ismail et al., 2012). Recently a study of SH-SY5Y cells revealed the down-regulation of miR-221 in parallel with NEAT1 up-regulation following MPP+ treatment (Geng et al., 2019). On the opposite, NEAT1 knockdown caused increased expression of the micro RNA. Overexpressing miR-221 prior to MPP+ treatment led to a decrease in ROS generation, LDH release and down-regulation of IL-1β, IL-6 and TNFα. NEAT1 was identified as a molecular sponge for miR-221 that observation led to the conclusion that MPP+ induced neuronal damage alleviation by NEAT1 ablation was partly due to the decreased sponging of miR-221 by NEAT1 (Geng et al., 2019).

All combined NEAT1 seems to be in a central position to regulate several of those cellular functions which have been shown to be altered in PD. To determine, which of the effects exerted by NEAT1 through modulating mitochondrial homeostasis, apoptosis, stress response, mRNA and miRNA availability are specific to unique cells in the central nervous system or mediate functions of different cell types, among them white blood cells, requires further studies. Nonetheless, the change of NEAT1 level in peripheral blood cells in PD patients can be well accommodated with the described roles of the lncRNA and the effects it shows in PD models. However, whether the changes of NEAT1 levels that we observed in blood cells of PD patients are consequential or in causative relation with the disease needs further elucidation. Answer to this question should be sought with keeping in mind the recent shift in the paradigm of PD aetiology that instead of viewing the disease specific to the central nervous system views it as a systemic ailment.

4. Conclusions

In easily accessible peripheral blood cells of PD patients we detected an increased NEAT1 lncRNA level. In light of the diverse mechanisms by which NEAT1 affects cellular functions, the increased level of the lncRNA can be causally or coincidently linked to PD. Change in NEAT1 lncRNA expression in brain tissue of PD patients has been reported recently (Kraus et al., 2017; Simchovitz et al., 2019). Our finding that the change in NEAT1 level can be detected in blood samples might open possibilities to find signs of developing PD by investigating samples more easily accessible than the nervous tissue. By uncovering the cause and effects of the change of NEAT1 level in PD progression could lead to a better understanding of the underlying mechanisms of the disease and to the identification of new potential therapeutic targets to interfere with this devastating neurodegeneration.

5. Experimental procedure

5.1. Study design

For a preliminary study we selected 41 lncRNAs, each of which has been implicated in neurodegenerative disease (Table 1) and examined their levels in blood cells of a small number of PD patient versus control samples (n = 3 and 3) with real-time polymerase chain reaction (RT-PCR). Based on this preliminary result we narrowed the set of neurodegeneration-related lncRNAs to 12 (validation study I., Table 2) and analysed the expression levels of these lncRNAs in a second, larger

group of PD patients and non-PD controls (n = 18 and 15). Based on the results of these assays 2 lncRNAs out of the 12 were selected for further study and the level of these was investigated a third larger group of patients and controls consisting 43 and 36 individuals, respectively (validation study II.).

5.2. Participants

Both PD patients and non-PD volunteers were Hungarians of Caucasian origin. The diagnosis of PD was set up based on medical history and physical examination carried out by movement disorder specialists. Individuals with known other malignancies or with cancerous disease in their history were excluded. Validation study group I. composed of 9 women and 9 men, the mean age of the cohort was 60.3 ± 5.7 years. The average age at disease onset was 52.5 ± 5.6 years. Validation study group II consisted of 43 PD patients. The mean age of this group of patients was 63.3 ± 11.4 years, the male/female ratio: 24/19, the average age at disease onset was 54.8 ± 12.6 years. Out of the 43 participants, 6 reported first symptoms to appear before the age of 40 years (mean age at disease onset 35 ± 7.8 years). One of these patients reported the possibility of positive family history regarding PD: the main symptom of this participant was left side dominant tremor, and tremor was reported to be present in family members both of the maternal and paternal side, however, to our knowledge no definite diagnosis of PD was set up for the relatives. Genetic screening of this patient (pathogenic LRRK2, parkin and DJ-1 mutations) yielded negative results.

For data analysis patients were further divided into two cohorts: the early onset PD group (EOPD) consisted those, who had a disease onset before or at the age of 60 years (n = 27; age: 57.6 ± 9.8 years), while the late onset PD group (LOPD) consisted those with disease onset after 60 years (n = 16; age: 73 ± 5.9 years). The average age at the onset of the disease in EOPD and LOPD groups was 47.5 ± 10.2 and 66.5 ± 4.0 years, respectively. Based on disease duration (DD) patients were also separated into two subgroups. Out of the 42 patients, 27 belonged to the 'short DD' (SDD) subgroup with DD < 10 years (age: 62.9 ± 11.9 years, DD: 4.9 ± 2.8 years), while the 'long DD' - DD ≥ 10 years - subgroup (LDD) consisted of 15 individuals (age: 63.7 ± 10.9 years, DD: 14.6 ± 5.0 years). One patient had to be excluded from this analysis as no information was available upon DD. In validation study I. there were no patients who had DBS treatment. Among PD patients of validation study II., 8 out of 43 participants had gone under DBS surgery (PD DBS n = 8, age: 64.3 ± 7.1 years; PD no DBS n = 35, age: 63.1 ± 12.2 years).

Out of the 43 PD patients 26 individuals went under genetic testing regarding the monogenic forms of the disease (LRRK2, parkin and DJ-1), of which all tests yielded negative results.

The majority of patients – 30 out of 43 - presented tremor. Modified Hoehn and Yahr scale varied between 1 and 5, mean 1.9 ± 0.8. UPDRS motor score of the patients at the time of sample collection ranged from 2 to 48 points, the mean score was 12 ± 8.9 points. 36 out of 43 PD patients received levodopa as treatment, and the mean time of treatment duration at sample collection was 6.4 ± 4.1 years. Further details of clinical data are presented in Supplementary Table 4.

In validation study I. control group, the male/female ratio was 6 to 9, and the mean age was 61.3 ± 9.9 years. The validation study II. control group consisted of 36 individuals (male/female: 16/20), the mean age was 57.6 ± 18.0 years (for detailed demographic data see Suppl. Table 3).

Before participating, informed consent was obtained from each participant included in the study. The study protocol was approved by the Medical Research Council Scientific and Research Ethics Committee and was in full accordance with the Declaration of Helsinki ethical principles for medical research involving human subjects.

5.3. Methods

Blood samples were collected from members of patient and control groups in EDTA containing blood tubes. White blood cells of 5 ml blood samples were separated by centrifugation and lysed in 1 ml of TRI Reagent (Sigma) by repetitive pipetting within 2 h of collection. Samples were then stored at −80 °C. Total RNA isolation was carried out following the Trizol RNA purification protocol according to the manufacturer's instructions (Sigma). RNA concentration was determined with a MaestroNano micro-volume spectrophotometer. In the preliminary and first validation study complementary DNA (cDNA) was synthesized from 500 ng of extracted RNA with the use of RT^2 First Strand Kit (Qiagen) following the manufacturer's instructions. RealtimePCR was performed with the use of RT^2 SYBR Green Mastermix (Qiagen). Equal volumes of cDNA samples were aliquoted into the wells of a specifically designed Custom RT^2 PCR Array (Qiagen) containing lncRNA specific primer pairs (25 ul final volume). Two arrays were designed and used for the groups of 41 and 12 lncRNAs (Table 1 and 2, respectively).

For validation study II commercially available *NEAT1* and *TUG1* gene-specific primers were used (Qiagen). cDNA was synthesized from 1000 ng of RNA with the use of Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific). Real-time PCR was performed with the use of RT^2 SYBR Green Mastermix (qPCRBIO) in 25 µl final volume. PCR reactions were carried out in a CFX96 thermocycler (Bio-Rad). Cycling conditions are available upon request.

5.4. Data analysis and statistics

Statistical analysis of PCR results was performed using RT^2 PCR analysis web portal (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php) and GraphPad Prism 6.01 statistics software using the ΔΔCt method. In short, ΔCt was calculated as the difference between a gene of interest and the average of reference gene, ΔΔCt was calculated as ΔCt (patient) – average ΔCt (control) and fold change was determined as $2^{-\Delta\Delta Ct}$ value (Livak and Schmittgen, 2001). For the identification of the outliers among $2^{-\Delta\Delta Ct}$ replicates the ROUT method was used. D'Agostino and Pearson omnibus normality test was used for the analysis of data distribution. If the data showed normal distribution, we implemented unpaired *t*-test, while in the case of non-normal distribution Mann-Whitney *U* test was performed. P value under 0.05 was considered significant. Due to the multiple comparisons, Bonferroni correction was implemented. Following this, p value under 0.004 was considered significant.

6. Data availability

The data that support the findings of this study are available from the corresponding author.

CRediT authorship contribution statement

Fanni Annamária Boros: Methodology, Writing - original draft. **Rita Maszlag-Török:** Methodology, Writing - review & editing. **László Vécsei:** Writing - review & editing, Funding acquisition. **Péter Klivényi:** Conceptualization, Writing - review & editing.

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Declaration of Competing Interest

The authors declare no conflict of interest regarding the publication of this article.

Compliance with Ethical Standards

Informed consent was obtained from all individual participants included in the study. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.brainres.2020.146672.

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Review

NEAT1 on the Field of Parkinson's Disease: Offense, Defense, or a Player on the Bench?

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Abstract. Parkinson's disease (PD) is the second most common neurodegenerative disease worldwide. Considering the devastating symptoms, high prevalence, and lack of definitive diagnostic test, there is an urgent need to identify possible biomarkers and new therapeutic targets. Genes identified and/or proposed to be linked to PD encode proteins that fulfill diverse roles in cellular functions. There is a growing interest in identifying common traits which lead to the disease. Long non-coding RNAs have recently emerged as possible regulatory hubs of complex molecular changes affecting PD development. Among them, NEAT1 has attracted particular interest. It is a major component and the initiator of nuclear paraspeckles, thus regulating transcription and modifying protein functions. This review summarizes data available on the role of NEAT1 in PD. NEAT1 upregulation in PD has repeatedly been reported, however, whether this is part of a protective or a damaging mechanism is still a topic of debate. It has been proposed that NEAT1 propagates PD *via* its interaction with PINK1 and several micro RNAs and by modulating *SNCA* expression. On the other hand, findings of NEAT1 acting as a bona fide LRRK2 inhibitor argue for its protective role. These contradictory results could be due to the different disease models implemented. This calls attention to the difficulties posed by the complex patho-mechanisms of neurodegenerative disorders and the limitations of disease models. However, the potential of NEAT1 as a biomarker and as a therapeutic target for PD highly warrants further research to elucidate its exact role in this neurodegenerative disorder.

Keywords: lncRNA, NEAT1, neurodegeneration, Parkinson's disease

INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disease, affecting approximately 1-2% of the population over the age of 65 [1]. The prevalence of the disease increases exponentially with age, causing millions of deaths each year [2]. The characteristic motor symptoms of PD are often accompanied by various non-motor symptoms, exacerbating disease severity. In the absence of an early diagnostic test, PD diagnosis is based on the cardinal motor symptoms. However, by the time these manifest, the majority of the dopaminergic neurons in the *substantia nigra* have been irreversibly lost [3–5]. Despite the intensive research focusing on development of disease-modifying therapies [6], so far no effective treatment is available. Given the devastating

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symptoms, high prevalence, and lack of a specific diagnostic test, there is an urgent need to identify possible biomarkers and new therapeutic targets for PD.

PD is a complex multifactorial disease, the exact patho-mechanism of which has yet to be fully elucidated. Besides various environmental and lifestyle factors identified as triggers and/or facilitators of the disease [7], several genetic alterations have been found to be related to the disorder. In addition to 21 PARK genes described in the human genome as potential direct culprits of the disease [8], genetic variants of 26 loci have been proposed to be disease risk modifiers [9, 10]. These genes encode proteins that fulfill roles in diverse cellular functions, such as synaptic transmission, vesicle transport, protein transport and degradation, autophagy, mitochondrion maintenance and energy homeostasis [11]. There is a growing interest in identifying common traits behind the diverse mechanisms causing malfunctions which lead to PD.

Due to their versatile roles in cellular functions, long non-coding RNAs (lncRNAs) have recently emerged as possible regulatory hubs of complex molecular changes affecting PD development. lncRNAs are RNA polymerase II transcripts over 200 nucleotides in length, without long open reading frames. They are frequently polyadenylated, alternatively spliced and capped, thus having an mRNA-like structure [12]. lncRNAs have gained attention in relation to neurodegenerative diseases due to the diverse mechanisms by which they can affect cellular homeostasis [13]. lncRNAs are known to exert regulatory roles on gene expression by modulating histone post-translational modifications and transcription factor activities, participating directly in post-transcriptional mRNA modifications, acting as ceRNAs (competing endogenous RNAs) that can sponge micro RNAs (miRNAs) and possibly by several other mechanisms acting at translational and post-translation levels (for a review, see [12, 14]).

NEAT1 lncRNA has attracted particular interest in the past few years since its levels have been shown to be altered in neurodegenerative diseases (reviewed in [15]). The possibility of a direct relation between NEAT1 and PD has been strengthened by recent findings on NEAT1 effects on mitochondrial function [16], detection of elevated NEAT1 levels in postmortem PD brain samples [17, 18] and recently our research group detected elevated NEAT1 levels also in the peripheral blood of PD patients [19]. However, the questions whether a change in NEAT1 level is in causal relationship with alleviation or aggravation of PD, or alternatively, NEAT1 lncRNA is a bystander in PD pathogenesis, without being actively involved in the disease course, are still unanswered. In this review we summarize recently published data related to the possible role of NEAT1 in PD. Similarly to the seemingly contradictory views which attribute both oncogenic and tumor-suppressor roles to NEAT1 lncRNA in cancer [20, 21], recently published data suggest both protective and enhancing roles for NEAT1 in neurodegeneration. We critically review these reports with particular attention to PD in order to facilitate a clearer view on the possible involvement of this lncRNA in the disease. We hope that calling attention to the topic will help clarify contrasting data and raise questions for further research.

NEAT1: DISCOVERY, GENE STRUCTURE, EXPRESSION

NEAT1 (Nuclear Enriched Abundant Transcript 1, later changed to Nuclear Paraspeckle Assembly Transcript) lncRNA was first described in 2007 as a highly abundant nuclear RNA [22]. In human, NEAT1 is transcribed from the multiple endocrine neoplasia (MEN) type I locus on the long arm of chromosome 11 [23]. Transcription results in two NEAT1 isoforms: the shorter NEAT1_1 (alias MENepsilon) is 3 684 nucleotides, while the longer NEAT1_2 (alias MENbeta) is 22 743 nucleotides. For simplicity we will refer to the former as NEAT1S and to the latter as NEAT1L. NEAT1 related genes are specific to mammals [24] and the gene sequence is well conserved across mammalian species [25], which is an uncommon feature of lncRNAs is general [22]. Mouse NEAT1 isoforms are smaller than the human ones (3.7 and 20 kb), but are in similar relation to each other as the human ones (see more on this below).

The two NEAT1 isoforms are transcribed by RNA polymerase II from the same promoter under the same transcriptional control. NEAT1S is produced by early 3'end processing of the transcript at a canonical polyadenylation site. NEAT1L results from suppression of polyadenylation at this site. Its 3' end is formed without poly(A) tail by RNase P cleavage at a tRNA-like structure [26, 27]. Consequently, the two isoforms overlap over the full length of NEAT1S that corresponds to the 5' end sequence of NEAT1L. The proportion of the two NEAT1 isoforms produced is determined through the regulation of poly(A) addition; however, it remains to be elucidated how this process is linked to cell homeostasis.

The shorter NEAT1 isoform is generally observed in higher quantities and in a wider range of tissues. Nonetheless, the function of NEAT1S is less clear compared to that of NEAT1L which is indisputably the major structural component of paraspeckles. Paraspeckles are subnuclear ribonucleoprotein complexes within the interchromatin space in mammalian cells [28, 29]. These complexes are assembled from RNAs and various proteins many of which have RNA binding affinity. Paraspeckles play roles in regulating transcription and RNA processing by several mechanisms which include retaining RNA and proteins, modulating RNA editing and splicing and acting as sponges for miRNAs (reviewed in [30]). Knockdown of NEAT1L production results in paraspeckle elimination even in the presence of intact NEAT1S [31]. NEAT1L folds end-to-end within paraspeckles with 5' and 3' ends of the lncRNA localizing on the periphery while the core is positioned in the center of the structure. As the 5' ends of the two NEAT1 isoforms are identical, this may suggest that the short isoform is also localized in the periphery of paraspeckles [32]. However, recent findings argue against NEAT1S as a major paraspeckle component, instead revealing the short isoform to be localized in foci termed 'microspeckles' [32–34]. Mice lacking the long isoform of NEAT1 show defects in female reproductive tissue development while absence of the short isoform does not cause any obvious external or histological abnormalities [35, 36]. These findings raised the possibility of NEAT1S being a by-product without any specific role [36]. However, the observations that NEAT1L and NEAT1S accumulate differently in and have different effects on some cancer types [21, 37–39] and that overproduction of NEAT1S increases resistance of cells to oxidative stress [40] refute this notion. The observation that NEAT1S is more conserved in evolution and is generally more abundant, together with it being detected outside of paraspeckles [33] may also serve as an indirect argument for an as yet unidentified paraspeckle-independent function of this isoform.

While there is a general consensus on the production of the two NEAT1 variants, the existence of further isoform(s) is less clear. The Human Genome Ensemble (GRCH38.p13) depicts nine NEAT1 splice variants. Some of these are "annotated manually" while others are products of the "manually supervised computational pipeline". These transcripts bear small differences in their 5'regions, due to five short putative introns. As there are no reported RNA mapping results to verify the removal of these, it remains open if any of the depicted NEAT1 splice variants deserve particular attention. Among the few reports on NEAT1 isoforms Chowdhury et al. mention, 3 out of 8 NEAT1 variants to be upregulated in human endothelial cells after LPS (lipopolysaccharide) treatment [41] and Kessler et al. found differences in the expression levels of 3 variants (NEAT1-201, NEAT1-202/v2, and NEAT1-205) by comparing NEAT1 RNAs in hepato-cellular carcinoma and normal tissue samples [39].

Data on NEAT1 lncRNA expression, tissue distribution and function have been obtained primarily from mouse models which permit genome editing of the gene and from cancer related studies using tumor samples and various human cell lines. Due to space constraints these will not be reviewed here; instead we call attention only to data which exemplify the diverse, frequently contrasting effects attributed to NEAT1 lncRNAs. In the following sections we review very recent data related to possible NEAT1 functions in neurodegenerative disorders and models of these focusing primarily on PD. Excellent recent reviews on the regulation of NEAT1 lncRNA expression and the contribution of NEAT1 to tumor development can be found in [21, 42, 43].

CELLULAR FUNCTIONS AFFECTED BY NEAT1

Shortly after the description of NEAT1, it was demonstrated that the lncRNA localizes to specific nuclear ribonucleoprotein structures. Subsequent studies proved that NEAT1L knockdown leads to paraspeckle disintegration while overexpression increases paraspeckle abundance; furthermore details on the folding of the RNA within paraspeckles as well as on the protein components of the complex were revealed [32, 44]. However, the involvement of NEAT1S in paraspeckles remains disputed. NEAT1's role in paraspeckle scaffolding imply an effect on cellular functions: paraspeckles regulate transcription and RNA maturation *via* accumulation of protein factors. The amount of paraspeckles affects the retention of A-I edited RNAs, mitoRNAs (mitochondrial protein coding RNAs) and miRNAs. Changes in the level of NEAT1 modulate functions *via* these. A further mechanism of NEAT1 action which may or may not be associated with paraspeckles is acting as ceRNA by sponging miRNAs. This seems to be a major means by which NEAT1 affects carcinogenesis (reviewed in [21]).

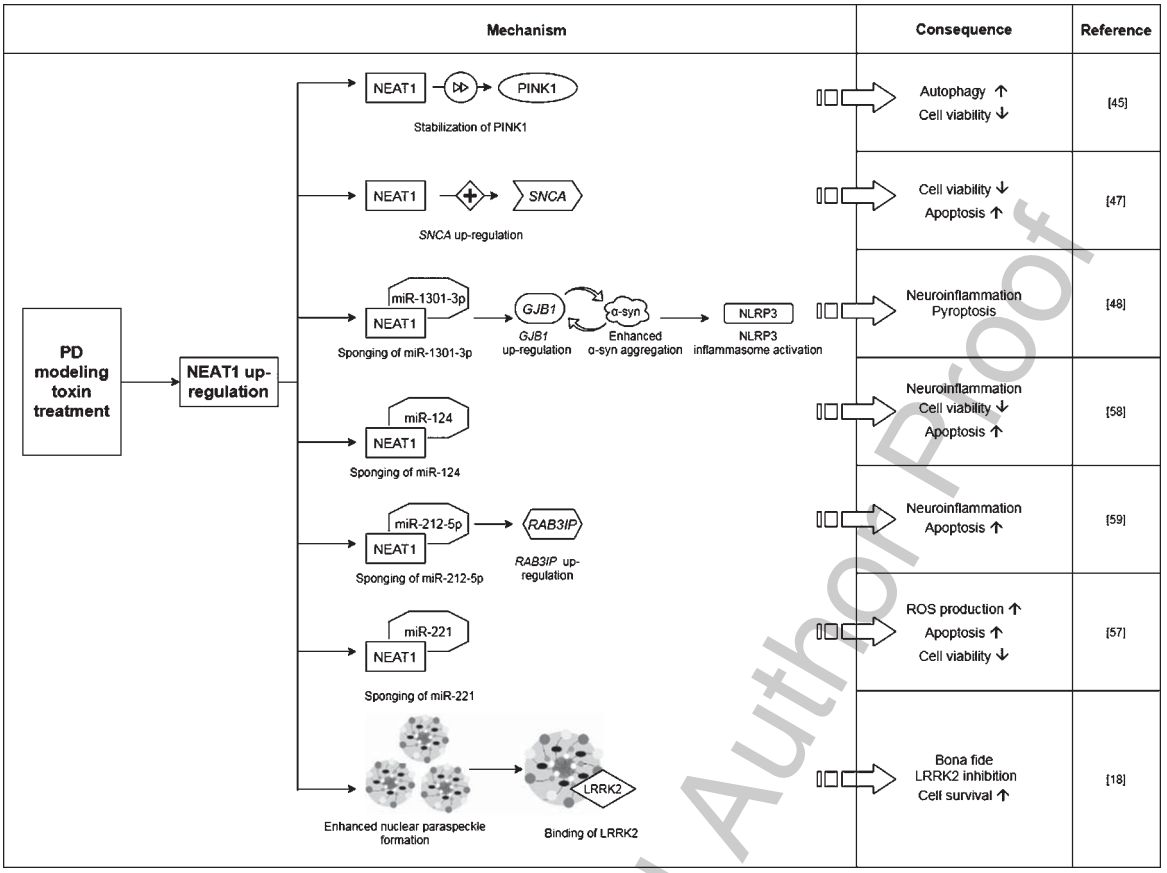

Fig. 1. Proposed mechanisms by which NEAT1 affects the course of PD. For a detailed description please see the corresponding sections of the text. NEAT1, Nuclear Paraspeckle Assembly Transcript 1; PINK1, protein phosphatase and tensin homolog (PTEN)-induced kinase 1; *SNCA*, Alpha-synuclein (gene); *GJB1*, Gap junction beta-1 (gene); α-syn, Alpha-synuclein (protein); NLRP3, NOD-, LRR- and pyrin domain-containing protein; *RAB3IP*, RAB3A interacting protein (gene); LRRK2, Leucine-rich repeat kinase 2.

Paraspeckles are dispensable under normal laboratory conditions but play essential roles when cells are placed under stress. In accord with this several cellular stressors enhance NEAT1 expression and paraspeckle formation. This is well reflected by the multitude of transcription factors known to affect NEAT1 expression. A comprehensive review on this topic was recently published by [43].

NEAT1 IN PARKINSON'S DISEASE

Altered expression of NEAT1 has been reported in various neurodegenerative diseases (reviewed in [15]), among them in PD. Elevated NEAT1 levels were reported in human postmortem brain samples of various brain areas, such as in the *substantia nigra* and anterior cingulate gyrus [17, 18]. Upregulation of the lncRNA was found to increase with progression of the disease [17]. Besides the central nervous system (CNS), elevated NEAT1 levels were also reported in the peripheral blood of PD patients [19].

In this review we summarize data available on the role of NEAT1 in PD pathogenesis obtained from *in vitro* and *in vivo* models of the disease (Fig. 1). As demonstrated by results shown below, various stressors lead to the upregulation of NEAT1 RNA; however, the role that NEAT1 plays in PD is still a topic of debate. Some of the data indicate that NEAT1 upregulation has a detrimental effect and accelerates disease progression. Other observations suggest a compensatory mechanism by which the RNA might promote cell survival and arrest disease pathology (Figs. 1–4). Finally, it may be that NEAT1 has no significant effect on PD pathogenesis and the observed changes in RNA merely reflect a bystander effect on NEAT1 in the disease process. In the following sections we summarize available data supporting either the protective or the harmful role of NEAT1

upregulation in the course of PD. Table 1 and Fig. 1 show brief summaries of reported results obtained by alterations of NEAT1 lncRNA levels using different PD models and the mechanisms assumed, respectively. Figs. 2–4 show observed effects of NEAT1 highlighting reported data in respects of PD models (animal and cellular models: Fig. 2 vs. Fig. 3) and toxins used (Fig. 3 vs. Fig. 4).

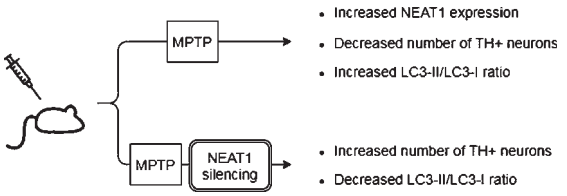

Fig. 2. Observed effects of NEAT1 in animal models of PD. For a detailed description please see the corresponding sections of the text. MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NEAT1, Nuclear Paraspeckle Assembly Transcript 1; TH, Tyrosine hydroxylase.

NEUROTOXIC NEAT1 EFFECTS

To date, seemingly more data support the notion of NEAT1 downregulation being protective against PD progression.

In a study Yan and colleagues found that treatment of mice with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) led to a rise in the expression of NEAT1, alongside an increase in the protein levels of PINK1 (phosphatase and tensin homolog (PTEN)-induced kinase 1) and LC3-II/LC3-I ratio (LC3: Microtubule-associated protein light chain 3) in the midbrain of the animals [45]. The detrimental effect of MPTP on neuronal cell survival was demonstrated by the significant decrease in the number of TH+cells (Fig. 2). The tyrosine hydroxylase enzyme catalyzes the transformation of the amino acid L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) and is a marker of dopaminergic neurons in the CNS. NEAT1 silencing significantly increased the number

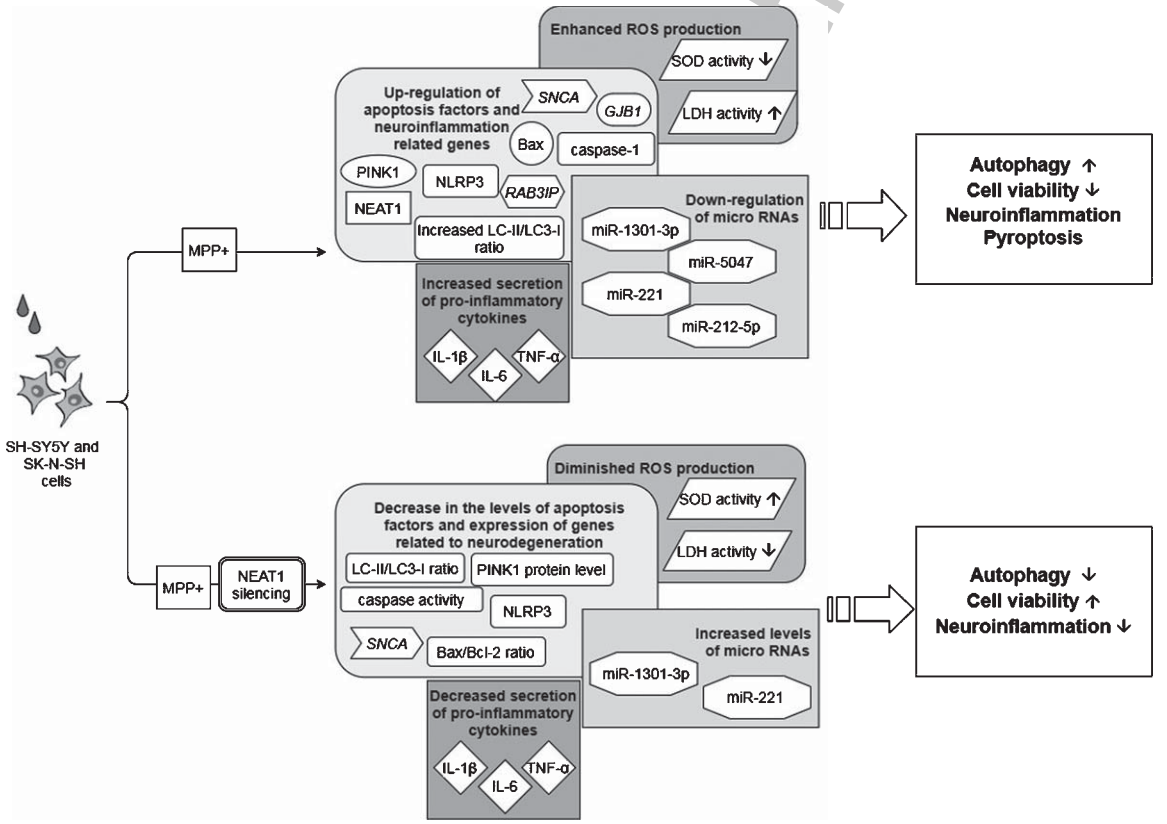

Fig. 3. Observed effects of NEAT1 in the MPP+cell model of PD. For a detailed description please see the corresponding sections of the text. MPP+, 1-methyl-4-phenylpyridinium; NEAT1, Nuclear Paraspeckle Assembly Transcript 1; PINK1, protein phosphatase and tensin homolog (PTEN)-induced kinase 1; *SNCA*, Alpha-synuclein (gene); NLRP3, NOD-, LRR- and pyrin domain-containing protein; GJB1, Gap junction beta-1; *RAB3IP*, RAB3A interacting protein (gene); ROS, Reactive oxygen species; SOD, Superoxide dismutase; LDH, Lactate dehydrogenase; IL-1β, interleukin-1β; IL-6, interleukin-6; TNF-α, Tumor necrosis factor α.

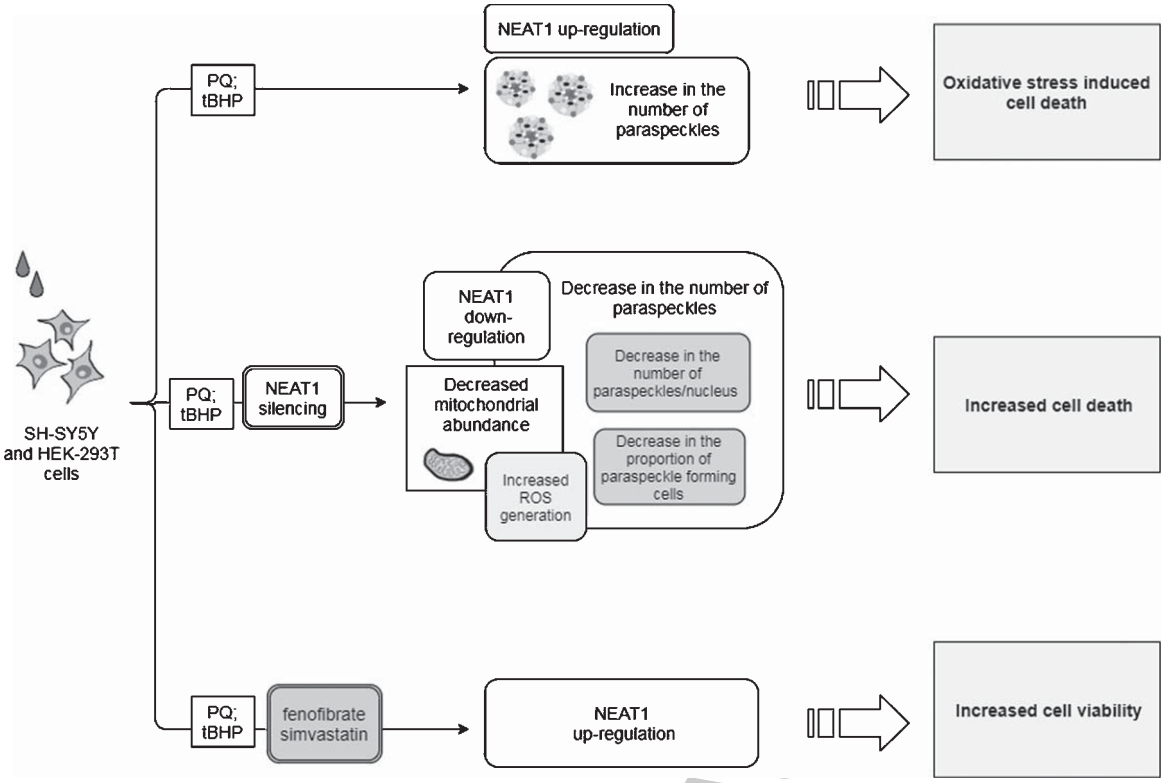

Fig. 4. Observed effects of NEAT1 in the PQ and tBHP cell models of PD. For a detailed description please see the corresponding sections of the text. PQ, Paraquat; tBHP, tert-Butyl hydroperoxide; NEAT1, Nuclear Paraspeckle Assembly Transcript 1; ROS, Reactive oxygen species.

of TH+neurons and led to a significant decrease in PINK1 protein levels. These changes were accompanied by the elevation of LC3I and decrease of LC3-II protein levels. LC3-II is an autophagosome marker, converted from the cytoplasmic LC3-I. The membrane bound LC3-II protein plays a role in the formation and elongation of the autophagosome [46]. The reduced LC3-II/LC3-I ratio is an indicator of decreased autophagy. *In vitro* studies involving the SH-SY5Y cell model of the disease yielded similar results: elevated expression of NEAT1 and PINK1 protein and increased LC3-II/LC3-I ratio were detected upon MPP+(1-methyl-4-phenylpyridinium; the active metabolite of MPTP) exposure. Conversely, knockdown of the lncRNA decreased the MPP+induced high expression of PINK1 protein, reversed the change in LC3-II/LC3-I ratio and improved cell viability (Fig. 3). Intriguingly, overexpression of PINK1 reversed the beneficial effects of NEAT1 silencing on cell survival. This observation raised the possibility that NEAT1 exerts its effects in a PINK1-dependent manner. Yan and colleagues proposed that the lncRNA might bind directly to the protein and stabilize it by influencing its ubiquitination and preventing its degradation. Elevated NEAT1 level thus leads to an increase in PINK1 level [45] (Fig. 1).

Based on these *in vivo* and *in vitro* observations, Yan et al. concluded that NEAT1 upregulation is detrimental since by stabilizing PINK1 protein the lncRNA promotes autophagy [45]. In accord with this, knocking down the lncRNA proved to be protective against MPP+/MPTP induced cell loss.

The finding on the protective effect of NEAT1 silencing was strengthened by Liu and Lu [47]. In their experiments MPTP treatment of mice led to a reduction in the number of TH+cells in the brain and NEAT1 upregulation was observed in both *in vivo* and *in vitro* models of the disease (Figs. 2 and 3). In MPP+treated SH-SY5Y cells knockdown of NEAT1 improved cell viability and diminished cell apoptosis as indicated by decreased Bax/Bcl-2 ratio and caspase activity. Upon NEAT1 silencing a downregulation in *SNCA* (Alpha-synuclein) expression was observed. Intriguingly, the beneficial effects of the knockdown of the lncRNA on cell survival and

apoptosis could be reversed by overexpressing the *SNCA* gene. These findings suggest that upregulation of NEAT1 is harmful in the course of PD *via* an α-syn related mechanism (Fig. 1).

According to a more recent study by Sun et al. [48], MPP+treatment not only caused upregulation of NEAT1 but also enhanced expression of α-syn, *GJB1* (Connexin32, Cx32; gap junction beta

Table 1
Reported results obtained by alterations of NEAT1 lncRNA levels using different PD models and the mechanisms assumed

Model organism	Toxin	Effect of toxin	NEAT1 intervention	Effect of NEAT1 intervention	Proposed NEAT1 mode of action	Reference
mouse	MPTP	increase in: - NEAT1 expression - PINK1 protein level - LC3-II/LC3-I ratio decrease in the number of TH+neurons	NEAT1 silencing	decrease in: - PINK1 protein level - LC3-II/LC3-I ratio increase in the number of TH+neurons	Stabilizes, thus increases the level of PINK1 protein	[45]
SH-SY5Y cells	MPP+	increase in: - NEAT1 expression - PINK1 protein level - LC3-II/LC3-I ratio	NEAT1 silencing	decrease in: - PINK1 protein level - LC3-II/LC3-I ratio increase in cell viability		
mouse	MPTP	increase in NEAT1 expression decrease in the number of TH+neurons	n.a.	n.a.	Upregulation of *SNCA*	[47]
SH-SY5Y cells	MPP+	increase in NEAT1 expression	NEAT1 silencing	decrease in: - Bax/Bcl-2 ratio - caspase activity downregulation of *SNCA* expression improved cell viability and diminished cell apoptosis		
SH-SY5Y cells	MPP+	enhanced expression of: - *SNCA* - *GJB* - NLR3P - IL-1β - caspase-1 - Bax downregulation of: - miR-1301-3p - miR-5047	NEAT1 silencing	decreased expression of: - *SNCA* - NLRP3 - caspase-1 - IL-1β increased miR-1301-3p expression decrease in the number of apoptotic cells	Sponges miR-1301-3p thus leads to enhanced *GJB1* expression and consequent α-syn induced NLRP3 inflammasome activation	[48]
SH-SY5Y cells	MPP+	upregulation of NEAT1 and downregulation of miR-221 expression	NEAT1 silencing	increased miR-221 expression diminished ROS generation improved cell viability and decreased apoptosis	Sponges miR-221, by this enhances ROS production, LDH release and upregulation of pro-inflammatory cytokines IL-1β, IL-6 and TNFα	[57]
SH-SY5Y cells	MPP+	NEAT1 upregulation; increased secretion of IL-1β, IL-6 and TNF-α	NEAT1 silencing	decreased levels of: - IL-1β - IL-6 - TNFα improved cell viability and decreased apoptosis rate	Sponges miR-124	[58]
SK-N-SH cells	MPP+	downregulation of miR-212-5p and upregulation of both NEAT1 and RAB3IP; decreased SOD- and increased LDH activity	NEAT1 silencing	reversed decreased SOD- and increased LDH activity diminished ROS production promotion of cell viability and reduction of apoptosis	Sponges miR-212-5p thus indirectly upregulates *RAB3IP* expression which promotes inflammatory processes and apoptosis	[59]

Table 1
Continued

Model organism	Toxin	Effect of toxin	NEAT1 intervention	Effect of NEAT1 intervention	Proposed NEAT1 mode of action	Reference
SH-SY5Y and HEK-293T cells	PQ and tBHP	NEAT1 upregulation; increased number of paraspeckles	NEAT1 silencing	decrease in the: - proportion of paraspeckle forming cells - number of paraspeckles/nucleus - number of mitochondria exacerbated oxidative stress provoked cell death	NEAT1 acts as a bona fide LRRK2 inhibitor	[18]
			NEAT1 upregulation by fenofibrate and simvastatin	increased cell viability		

NEAT1, Nuclear Paraspeckle Assembly Transcript 1; PINK, phosphatase and tensin homolog (PTEN)-induced kinase 1; TH, Tyrosine hydroxylase; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPP+, 1-methyl-4-phenylpyridinium; *GJB*, gap junction beta 1; NLR3P, nucleotide oligomerization domain-like receptor protein with pyrin domain containing 3; IL-1β, interleukin-1β; IL-6, interleukin-6; TNF-α, Tumor necrosis factor α; RAB3IP, RAB3A-interacting protein; SOD, Superoxide dismutase; LDH, Lactate dehydrogenase; *SNCA*, Alpha-synuclein gene; ROS, Reactive oxygen species.

1), NLRP3 (nucleotide oligomerization domain-like receptor protein with pyrin domain containing 3), IL-1β and apoptosis factors caspase-1 and Bax, while Bcl-2 and the miRNAs miR-1301-3p and miR-5047 were downregulated (Fig. 3).

NLRP3 containing inflammasome is a protein complex of NLRP3, ASC (Apoptosis-associated speck-like protein containing a CARD) and caspase-1, which has been identified to play a pathologic role in neuroinflammation related to various neurodegenerative diseases. Upon activation, inflammasomes provoke innate immune responses by secreting pro-inflammatory cytokines such as IL-1β and IL-18 and by promoting pyroptosis, a caspase 1-dependent cell death which contributes to the propagation of inflammation *via* the release of further inflammatory markers [49]. In murine models of PD NLRP3 inflammasome was found to be activated by fibrillar α-syn and by the degeneration of dopaminergic neurons themselves [50]. The cardinal role of inflammasome activation in PD pathology is supported by findings obtained both from studies involving animal models and human samples. Treatment with small molecule NLRP3 inhibitors inhibited inflammasome activation and effectively mitigated motor deficits, nigrostriatal dopaminergic degeneration, and accumulation of α-syn aggregates in various rodent models of the disease [50]. Further studies showed that absence of either NLRP3 or caspase 1 was protective against the development of PD symptoms and loss of neurons in the *substantia nigra* after treatment with rotenone and MPTP, respectively (reviewed in [51]).

GJB1 (alias connexin-32 (Cx32)) is a member of the gap junction connexin family. The protein has recently been reported to play a central role in the uptake of α-syn oligomeric assemblies in neurons and oligodendrocytes [52]. *In vitro* and *in vivo* models of PD demonstrated a correlation between the upregulation of GJB1 and accumulation of α-syn aggregates. The correlation is established by a positive feedback loop: *in vitro* studies demonstrated that GJB1 overexpressing cells are more prone to α-syn oligomer uptake, and both exposure to α-syn aggregates and overexpression of the *SNCA* gene leads to upregulation of *GJB1* [52]. These findings underpin the role of GJB1 in the pathophysiology of PD and raise the possibility of *GJB1* expression modulation as a feasible way of therapeutic intervention [52].

In the study of Sun and colleagues, NEAT1 knockdown in MPP+treated SH-SY5Y cells reversed the neurotoxic effects, as indicated by a significant decrease in the number of apoptotic cells and by the suppression of α-syn, NLRP3, caspase-1 and IL-1β expression (Fig. 3). Overexpression of α-syn reversed the anti-apoptotic effects of NEAT1 silencing. These findings are in line with the results of Liu and Lu as discussed earlier [47], namely that NEAT1 downregulation improves cell survival *via* decreasing α-syn expression by an as yet unidentified mechanism. Sun and colleagues proposed that the

α-syn modulating ability of NEAT1 is linked to the miR-1301-3p/GJB1 pathway [48] (Fig. 1). This was based on their findings that NEAT1 downregulation led to increased miR-1301-3p expression, while inhibition of the micro RNA diminished the protective effects of NEAT1 silencing. The latter effects were demonstrated by the increased number of apoptotic cells and by the promotion of both transcription and translation of GJB1. Reporter gene assays revealed direct interactions between both NEAT1/ miR-1301-3p and miR-1301-3p/GJB1, leading to the conclusion that the lncRNA serves as an endogenous sponge for miR-1301-3p [48]. NEAT1 silencing prevents sponging of the miRNA thus miR-1301-3p can thus exert its inhibitory effect on GJB1 expression and through this prevent α-syn induced activation of the NLRP3 inflammasome.

Besides these observations, it has been proposed that, NEAT1 affects the course of PD by another micro RNA related mechanism. miR-221 is one of the most abundant miRNAs in the human CNS, and plays an important role in promoting neurite outgrowth and neuronal differentiation [53]. A direct target of miR-221 micro RNA is PTEN (Phosphatase and tensin homolog), a tumor suppressor which has also been found to be involved in the course of various neurodegenerative diseases, such as Alzheimer's disease (AD), amyotrophic lateral sclerosis and PD [54]. Several papers have reported miR-221 downregulation in serum samples of PD patients and proposed the possibility of this RNA serving as a biomarker of the disease [55, 56]. In a study Geng et al. found that MPP+exposure of SH-SY5Y cells resulted in upregulation of NEAT1 and downregulation of miR-221 expression in a dose- and time dependent manner [57] (Fig. 3). However, NEAT1 specific siRNA treatment increased miR-221 expression and diminished reactive oxygen species (ROS) generation, which resulted in improved cell viability and decreased apoptosis. Overexpression of miR-221 prior to MPP+treatment also diminished ROS production and was accompanied by decreased lactate dehydrogenase (LDH) release and downregulation of pro-inflammatory cytokines IL-1β, IL-6 and TNFα. Based on these observations NEAT1 was proposed to act as a molecular sponge for miR-221 (Fig. 1), and the conclusion was drawn that the beneficial effects of NEAT1 silencing could be related to decreased miR-221 sponging and a consequent higher availability of the micro RNA [57].

Regulation of neuroinflammation by NEAT1 was proposed to occur *via* a further mechanism. Results of experiments by Xie et al. involving the MPP+treated SH-SY5Y cell model of the disease show that silencing of NEAT1 attenuated neuroinflammation as indicated by the decreased levels of IL-1β, IL-6 and TNFα [58] (Fig. 3). In line with findings of others, NEAT1 knockdown improved cell viability and decreased apoptosis rate. RNA pull down and immunoprecipitation assays revealed a direct interaction between NEAT1 and the micro RNA miR-124. Silencing both NEAT1 and miR-124 in MPP+exposed cells led to decreased cell viability and an increase in the levels of pro-inflammatory cytokines compared to that seen in the case on NEAT1 silencing only. These observations led to the conclusion that NEAT1 regulates MPP+induced neuronal injury in a miR-124-dependent manner [58] (Fig. 1).

According to recent findings of Liu et al., NEAT1 also interacts with miR-212-5p, thus modulating the course of MPP+induced neurodegeneration *via* the miR-212-5p/ RAB3IP miR-1301-3p and miR-221 pathway [59] (Figs. 1 and 3). Treatment of SK-N-SH cells with MPP+caused the downregulation of miR-212-5p and upregulation of both NEAT1 and RAB3IP (RAB3A-interacting protein). RAB3IP is known to be involved in various cell functions such as autophagy, cell growth and apoptosis [59]. Similarly to the observations made in the *in vitro* PD models mentioned previously, NEAT1 knockdown in MPP+exposed cells reversed the decreased superoxide dismutase and increased LDH activity and diminished ROS production, thus promoting cell viability and reducing the rate of apoptosis. Interestingly, overexpression of miR-212-5p also improved cell survival and alleviated MPP+linked inflammation and cytotoxicity. Based on their findings, Liu and colleagues suggested that similarly to the situation discussed above in relation to miRNAs miR-1301-3p and miR-221, NEAT1 acts as a molecular sponge for miR-212-5p as well, leading to the downregulation of this miRNA. Dual-luciferase reporter gene assays showed that miR-212-5p directly binds to RAB3IP mRNA and by this negatively regulates the expression of RAB3IP. In their study Liu and colleagues also showed that overexpression of RAB3IP promoted inflammatory processes and apoptosis of MPP+treated SK-N-SH cells. These findings led to the conclusion that a possible mechanism of the neuroprotective effect that NEAT1 knockdown shows against MPP+toxicity is the higher level of available miR-212-5p miRNA. The diminishment of miR-212-5p miRNA sponging with NEAT1 exerts beneficial effects on cell survival and

apoptosis by indirectly causing the downregulation of RAB3IP.

NEAT1 IN NEUROPROTECTIVE ROLE

Opposite to the studies discussed above, the findings of Simchovitz and colleagues argue for a protective role of NEAT1 upregulation in the course of PD [18]. They reported that in postmortem *substantia nigra* PD samples NEAT1 was significantly upregulated compared to healthy controls. The significant difference was found to be due to the upregulation of the long NEAT1 variant, as upregulation of NEAT1L was more prominent than the expression change of both isoforms together (fold change: 2.3 and 1.7, NEAT1L and NEAT1L+S, respectively). *In vitro* experiments yielded similar results: upon paraquat (PQ) and tBHP (t-butyl hydroperoxide) induced oxidative stress significant NEAT1 upregulation was observed in HEK-293T and SH-SY5Y cell lines, primarily due to the increased expression of the long variant (fold change: 7 and 2.5, NEAT1L and NEAT1L+S, respectively) (Fig. 4). In murine neuronal primary cultures (GSE70368), α-syn overexpressing cells also manifested upregulated NEAT1 expression as compared to their non-overexpressing counterparts.

Investigation of PQ effect on paraspeckle formation revealed that the mean number of paraspeckles in a nucleus was increased by 60% in HEK-293T cells following PQ exposure, while no change was observed either in the number of paraspeckle forming cells or in the nuclear localization of NEAT1L. Thus, upregulation of the lncRNA upon PQ exposure seemed to be in correlation with the elevation in the number of paraspeckles. In light of this, it was proposed that in PD *substantia nigra* the elevated NEAT1L expression could be a cellular response to neuronal stress in order to promote enhanced formation of paraspeckles [18]. Silencing of NEAT1 decreased both the proportion of cells forming paraspeckles and the number of paraspeckles/nucleus. In addition, this also led to a decrease in the number of mitochondria, indicating that depletion of the lncRNA also affects mitochondrial abundance (Fig. 4). Treatments with NEAT1 siRNA exacerbated oxidative stress provoked cell death; however, this could be reversed by the LRRK2 (Leucine-rich repeat kinase 2) inhibitor PF-06447475. This observation gave ground to the suggestion that NEAT1 improves cell viability by an LRRK2-dependent manner. The finding that LRRK2 protein interacts with the paraspeckle proteins NONO and SFPQ supports this assumption [18, 60]. Simchovitz and colleagues proposed that NEAT1 acts as a *bona fide* LRRK2 inhibitor *via* binding the LRRK2 protein in paraspeckles. Mutations of the *LRRK2* gene are one of the most common genetic causes of both sporadic and familial PD [61]. Several pathogenic *LRRK2* mutations have been identified to cause increased kinase activity, and overactivation of LRRK2 has been found to cause disturbances in lysosomal homeostasis, microglial overactivation, phosphorylated tau accumulation and mitochondrial function (reviewed in [61, 62]). Since LRRK2 dysfunction plays crucial role in PD pathology [63], restoration of the impaired function of the kinase is an appealing approach for the treatment of the disease. There has been intensive research focusing on the development of kinase inhibitors for PD therapy (reviewed in [64]), and the finding of NEAT1 acting as a natural LRRK2 inhibitor could make upregulation of NEAT1 a target of such drug research. The promoter region of NEAT1 lncRNA contains a PPARα (Peroxisome proliferator-activated receptor alpha) binding site thus NEAT1 expression induction could be achieved by the use of PPARα activators. Indeed, treatment with both PPARα agonist fenofibrate and 3-hydroxy-3-methylglutaryl-coenzyme A inhibitor simvastatin led to the upregulation of NEAT1 expression, leading to a more prominent rise in the amount of the long lncRNA variant. *In vitro* experiments demonstrated that administration of fenofibrate and simvastatin increased viability of PQ and tBHP treated cells (Fig. 4). In HEK-293T cells, the beneficial effect of NEAT1 upregulation on cell survival was abolished after co-treatment with PQ and LRRK2 inhibitor, strengthening the notion that NEAT1 exerts its neuroprotective effects *via* mediating LRRK2 function (Fig. 1).

Combining the results obtained from human samples and *in vitro* models of the diseases it was proposed that NEAT1 upregulation in the *substantia nigra* reflects the accumulation of the lncRNA and the enhanced formation of paraspeckles in the dying neurons, and is therefore a hallmark of neurodegeneration. Simchovitz et al. proposed that the reason behind the upregulation of NEAT1 in dopaminergic neurons could be to enhance the formation of nuclear paraspeckles as a mechanism of protecting neurons from the damage mediated by LRRK2 [18]. The fact that HOTAIR (Hox transcript antisense intergenic RNA), another lncRNA has been previously

identified as an LRRK2-dependent modifier of PD pathology also support this notion [65]. Opposite to NEAT1, however, HOTAIR was reported to enhance LRRK2 gene expression thus propagating the disease.

DISCUSSION

The diverse interaction of NEAT1 with a broad range of molecules demonstrates well the complex ways in which this lncRNA can regulate cell functions. Despite intensive research and a rapidly growing body of evidence of the involvement of NEAT1 in PD, it is still not elucidated whether this lncRNA has an ameliorating or an exacerbating effect on disease progression. The controversial results of different research groups may originate from the different disease models implemented. The observation that the effect of NEAT1 upregulation varies depending on the agent used for disease modeling raises the possibility that the contrasting results may at least partly reflect differences of causative or consequential nature of PD insults. Studies with genetic models (either knockout or transgene) of the disease which are more likely to represent pathological changes that are causative in the development of the disorder might be useful to clarify questions in this respect. This calls attention to difficulties stemming from the complex patho-mechanism behind neurodegenerative disorders: even the acknowledged and well established *in vitro* and *in vivo* models are hardly, if at all, able to mimic precisely the complexity of pathological processes. Thus, results obtained from disease models should always be interpreted with great caution.

It is worth pointing out that although in the context of PD NEAT1 downregulation improved cell viability and decreased apoptosis in MPTP/MPP+models of the disease, NEAT1 upregulation was found to have a protective effect in *in vitro* models induced by oxidative stressors such as PQ and tBPH. This implies that the effect of NEAT1 is likely context dependent. MPTP/MPP+is a mitochondrial toxin which inhibits complex I of the mitochondrial respiratory chain, resulting in the disruption of ATP synthesis and ROS generation. MPTP also damages dopamine storage of cells, a feature considered to play a key role in the selective loss of dopaminergic neurons (reviewed in [66]). PQ is a herbicid, which, by interfering with photosynthetic electron transport in plants, leads to the production of superoxide. Though PQ has been linked to the production of ROS and accumulation of α-syn aggregates in dopaminergic neurons in experimental models of PD, the exact way by which it damages dopaminergic cells is not fully elucidated [67, 68]. Such ambiguous results regarding the role of NEAT1 in different PD models could be partly due to the different pathological effects the implemented toxins exert.

The role of NEAT1 is controversial not only in PD, but in cancer and other neurodegenerative diseases as well, such as Huntington's disease (HD) and AD.

Sunwoo et al. found NEAT1 to be upregulated in brain samples of both HD patients and the R6/2 HD mouse model of the disease. However, various *in vitro* models, such as mutant huntingtin (mHtt)-transfected neuro2A cells and mouse striatal neuron-derived cell lines (STHdh) did not show upregulation of the lncRNA. Despite the fact that no change was observed in NEAT1 expression in the above *in vitro* HD models, transfection with the NEAT1 short isoform vector in the mouse neuroblastoma cell line Neuro2A improved cell viability under H_2O_2-induced oxidative stress [69]. These ambiguous findings were proposed to reflect the lack of *in vitro* models' ability to portray the complex underlying pathophysiological mechanisms of HD [69]. This again calls attention to the complexity of neurodegenerative diseases and might offer explanation for the seemingly controversial results acquired from studies implementing different models.

The finding that NEAT1 transfection improved cell viability in H_2O_2-induced oxidative stress is in line with the findings of Simchovitz et al., who also found that NEAT1 upregulation increased cell viability after treatment with ROS generators PQ or tBHP [18].

Chanda and colleagues detected consistent and significant upregulation of NEAT1 not only in animal models, but also in mHtt expressing *in vitro* models of the disease. Knockdown of NEAT1 led to a significant decrease in mHtt aggregates and decreased expression of *TP53* (Tumor protein 53) [70].

In addition to HD, NEAT1L (but not NEAT1S) upregulation was reported by Chang et al. in other polyglutamine (polyQ) repeat diseases, such as spinocerebellar ataxia types 1, 2 and 7 [71]. Upregulation of NEAT1 in mHtt expressing SH-SY5Y cells was protective against mHtt induced toxicity, while inhibition of the lncRNA decreased cell viability. Interestingly, NEAT1 silencing not only increased mHtt sensitivity of the cells but also augmented viability upon treatment with the mitochondrial toxin 3-nitropropionic acid (3-NP) [71].

Some of the observations made using AD models seem to be more directly linked to and supporting

the beneficial role of NEAT1 silencing in MPTPT/MPP+PD models. In *in vitro* models of AD Aβ (amyloid beta)-exposure enhanced NEAT1 expression, and knockdown of the lncRNA promoted cell viability and diminished apoptosis [72]. NEAT1 was identified as a decoy for miR-107, and the lncRNA was proposed to aggravate Aβ-induced cell damage by sponging the micro RNA [72].

Recently Huang and colleagues proposed a further mechanism by which NEAT1 regulates Aβ metabolism and modifies AD pathology [73]. In the APP/PS1 transgenic mouse model, NEAT1 overexpression was found to exacerbate Aβ production, whereas knockdown of the lncRNA inhibited the generation of amyloid deposits [73]. In the same animal model knockdown of the lncRNA led to an increase in the levels of PINK1 as well as those of other autophagy markers such as P62, OPTN and LC3. NEAT1 overexpression promoted the ubiquitination and consequent degradation of PINK1—just the opposite of what was seen in PD models, where NEAT1 was identified as a stabilizer of the protein [45]. Based on their findings Huang et al. proposed that *via* facilitating PINK1 degradation, NEAT1 causes the inhibition of autophagy signaling thus impairing Aβ clearance. This results in the accumulation of amyloid aggregates and propagates disease pathology [73].

NEAT1 was also proposed to modulate AD pathology by epigenetic regulation of various genes due to its interaction with the PC300/CBP lysine acetyltransferases [74]. Knocking down the lncRNA affected both the acetylation and crotonylation of H3K27, thus impacting the transcription of several genes involved in endocytosis. *In vitro* studies involving the human astrocytic U251 cell line showed that inhibition of NEAT1 impeded Aβ uptake and degradation, suggesting a negative role of the lncRNA in AD pathology [74].

Changes in NEAT1 level and the responses presumably evoked by this have been reported to affect several further neurological conditions: NEAT1 upregulation was observed in hypoxic-ischemic brain damage (HIBD). The change in NEAT1 expression was proposed to be part of a protective response reaction [75]. In neonatal HIBD mice, NEAT1 was identified to competitively bind to the micro RNA miR-339-5p. Sponging of miR-339-5p led to the upregulation of homeobox A1 (HOXA1), promoting of cell viability and decreased apoptosis.

NEAT1 silencing was also reported to have a beneficial effect on age-related memory impairment [76]. Knockdown of NEAT1 caused disruption of histone 3 lysine 9 demethylation (H3K9me2), a repressive histone modification mark which increases with age in rodent hippocampus [76]. NEAT1 overexpression led to memory impairment of young mice, similar to that observed in their older counterparts. NEAT1 knockdown, on the other hand, improved behavior test–associated memory of mice of both age groups.

NEAT1 depletion was reported to ameliorate memory impairment related to AD as well: knockdown of NEAT1 led to improvement of learning and cognitive functions of APP/PS1 transgenic mice [73]. The question of whether these effects could be causally linked to the changes in NEAT1 expression and whether they relate to the effects observed in PD models remains to be answered.

In addition to NEAT1 various other lncRNAs play role in pathological processes of PD as it has been indicated and/or proved byfindings of numerous *in vivo* and *in vitro* studies (recent reviews on these: [14, 77, 78]). Several lncRNAs are implied to have protective effects against disease development (including UCHL1-AS, MAPT-AS1, Mirt2), while others are likely to play a detrimental role (such as HOTAIR, MALAT1, lincRNA-p21, BACE1-AS, HAGLROS and SNHG1) ([14, 78] and references in there). The mode of action of these transcripts resemble those proposed for NEAT1: among them are regulation of SNCA expression and α-syn aggregation by MALAT1 (alias NEAT2) [79] and SNHG1 [80], respectively, regulation of MAPT promoter activity by MAPT-AS1 [81], enhancement of UCHL1 gene (alias PARK5) *via* its anti-sense pair UCHL1-AS [82] and modulation of LRRK2 mRNA stability through HOTAIR [65]. Besides transcriptional and post-transcriptional regulation of PARK genes, lncRNAs can influence processes related to neuroinflammation partly *via* their interaction with miRNAs (such as Mirt2 lncRNA and miR-101 [83]; lincRNA-p21 and miR-1277-5p [84]). Further modes of action of PD related lncRNAs are autophagosomy system balance maintenance, oxidative stress and dopaminergic cell loss [85, 86] (reviewed in [14] and [78]).

CONCLUSION

Despite the fact that PD is one of the most common neurodegenerative diseases worldwide, causing tremendous burden not only on the individual but on society as well, the exact underlying patho-

mechanism of the disease is still unknown. In the past few years lncRNAs have emerged as intriguing subjects of PD research due to the diverse functions they fulfill. Among lncRNAs, NEAT1 attracted particular interest, since its expression was found to be elevated both in different brain regions and also in peripheral blood of PD patients. Upregulation of NEAT1 has been detected in various *in vitro* and *in vivo* models of the disease however data on whether its role in disease progression is protective or detrimental is conflicting. Upregulated NEAT1 level was proposed to have a damaging effect *via* the interaction of the RNA with PINK1 protein and various micro RNAs such as miR-1303-3p, miR-124, miR-212-5p and miR-221 and by the upregulation of *SNCA* expression. On the other hand, results of Simchovitz et al. argue for the protective role of NEAT1, based on the finding that the lncRNA acts as a natural LRRK2 inhibitor.

The effects of NEAT1 on disease progression are contradictory in other neurodegenerative diseases such as HD and AD as well. The cause of this could be in the different models implemented by different research groups. Due to the complexity of these disorders, to date no *in vitro* or *in vivo* model exists that is capable of precisely mimicking the pathological mechanisms of neurodegeneration. Inconsistent data regarding NEAT1 effects also imply that the RNA acts in context dependent modes: based on the toxin used for modeling PD, both NEAT1 upregulation or knockdown can prove to be protective. Research aiming to clarify the role and mode of action of this lncRNA in PD is highly warranted, since NEAT1 shows promise to emerge as both a promising biomarker and a potential therapeutic target for this neurodegenerative disease.

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CONFLICT OF INTEREST

The authors have no conflict of interest to report.

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