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

Summary of Ph.D. thesis

Fanni Annamária Boros M.D.

Clinical and Experimental Neuroscience Program
Doctoral School of Clinical Medicine
Faculty of Medicine
University of Szeged

Supervisors:
Péter Klivényi M.D., Ph.D., D.Sc.
László Vécsei M.D., Ph.D., D.Sc.

Szeged
2020

Original research articles directly related to the thesis

I. **Boros, F. A.**, Török, R., Vágvölgyi-Sümegi, E., Pesei, Z. G., Klivényi, P., & Vécsei, L. (2019). Assessment of risk factor variants of LRRK2, MAPT, SNCA and TCEANC2 genes in Hungarian sporadic Parkinson's disease patients. Neuroscience letters, 706, 140-145. Original paper; IF: **2.274**

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.
Original paper; IF: **2.733**

Review articles directly related to the thesis

I. **Boros, F. A.**, Vécsei, L., & Klivényi, P. NEAT1 on the field of Parkinson's disease: offense, defense or a player on the bench?
Journal of Parkinson's disease – in press; IF: **5.178**

Publications not directly related to the thesis

I. **Boros, F. A.**, Bohár, Z., & Vécsei, L. (2018). Genetic alterations affecting the genes encoding the enzymes of the kynurenine pathway and their association with human diseases. Mutation Research/Reviews in Mutation Research, 776, 32-45.
Review article; IF: **6.081**

II. **Boros, F. A.**, & Vécsei, L. (2019). Immunomodulatory effects of genetic alterations affecting the kynurenine pathway. Frontiers in Immunology, 10, 2570.
Review article; IF: **5.085**

III. **Boros, F. A.**, Klivényi, P., Toldi, J., & Vécsei, L. (2019). Indoleamine 2, 3-dioxygenase as a novel therapeutic target for Huntington's disease. Expert Opinion on Therapeutic Targets, 23(1), 39-51.
Review article; IF: **5.473**

IV. Salamon, A., Torok, R., Sumegi, E., **Boros, F.**, Pesei, Z. G., Molnar, M. F., ... & Klivenyi, P. (2019). The effect of physical stimuli on the expression level of key elements in mitochondrial biogenesis. Neuroscience letters, 698, 13-18.
Original paper; IF: **2.274**

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.
Original paper; IF: **3.038**

VI. **Boros, F.**, & Vécsei, L. (2020). Progress in the development of kynurenine and quinoline-3-carboxamide-derived drugs. Expert Opinion on Investigational Drugs, 1-25.

Review article; IF: **5.081**

Abbreviations:

PD: Parkinson's disease

GWA: genome wide association

lncRNA: long non-coding RNA

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

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

PBS: phosphate-buffered saline

SFN: sulphoraphane

i.p.: intraperitoneal

PQ: paraquat

MPP+: 1-methyl-4-phenylpyridinium

RFLP: restriction fragment length polymorphism

mtDNA: mitochondrial DNA

qRT-PCR: quantitative reverse transcription PCR

cDNA: complementary DNA

TUG1: Taurine up-regulated gene 1

WB: western blot

FACS: fluorescence-activated cell sorting

LD: linkage disequilibrium

LOPD: late onset PD

DBS: deep brain stimulation

NEAT1S: NEAT1 short isoform

NEAT1L: NEAT1 long isoform

HSF1: Heat shock factor 1

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease worldwide. It is caused by the irreversible loss of dopaminerg neurons in the substantia nigra leading to the characteristic motor symptoms: tremor, bradykinesia and rigidity. Diagnosis of the disease is based on the presence of the cardinal motor symptoms, however by the time those manifest, the majority of the dopaminergic neurons are irrevocably lost. Despite the intensive research focusing on development of disease-modifying therapies [1], so far only symptomatic treatment is available. In light of the devastating symptoms, high prevalence, lack of diagnostic test and curative treatment, there is an urging need of identifying possible biomarkers and new therapeutic targets for PD.

PD is a complex multifactorial disease for which the exact pathomechanism is still not fully elucidated. Familial inheritance of PD is observed in a smaller fraction (10-15%) of cases and the majority of cases are sporadic with suspected contribution of the genetic background [2]. Besides various environmental and lifestyle factors several genetic alterations have been found to be related to the disorder [3]. These alterations are mostly single nucleotide polymorphisms (SNPs) and their linkage to the disease is most commonly proposed based on results of genome wide association (GWA) studies involving large number of participants. Validating results of such studies in smaller but more defined patients' and controls' cohorts is necessary and highly warranted. Part of my PhD work was analysis of the occurrence of specific PARK gene variants in Hungarian population.

PD-related genes encode proteins that act in diverse cellular pathways and identifying common traits behind the diverse mechanisms which lead to PD is crucial for the better understanding of the disease. Due to their diverse functions and regulatory role on gene expression that they exert both transcriptionally and post-transcriptionally (reviewed: [4]), recently long non-coding RNAs (lncRNAs) have emerged as possible regulatory hubs of complex molecular changes affecting PD development.

NEAT1 (Nuclear Enriched Abundant Transcript 1; Nuclear Paraspeckle Assembly Transcript 1) lncRNA attracted particular interest in PD research, since its expression was found to be elevated in different brain regions of PD patients [5]. Further pieces of information on the possible role of NEAT1 in PD became available in the literature during the progress of research described in this thesis [6] [7] [8] [9] [10] [11] [12]. However, results on NEAT1 role are contradictory and key questions remain 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 my thesis work were to help finding answer 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 Hungarian population.

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

2nd aim: Determination 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 of in what type of comparison is there a difference in the expression of any of the detectable lncRNAs between PD patient – and control groups, and how does this relate 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 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 mitochondrium DNA content.

With the information obtained by the above experiments we wanted to contribute to the answer whether NEAT1 has a protective or pathogenic role in PD.

3. Methods

3.1. Biological samples

In the studies human, animal and cell samples were used. For detecting PARK gene SNPs and for lncRNA determination peripheral venous blood samples of sporadic PD patients and control individuals were used. In experiments involving animals 10-12 weeks old C57Bl/6J male mice were used. Animals were kept under standard laboratory conditions and were exposed to MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) and sulphoraphane (SFN) *via* intraperitoneal (i.p.) injection. For an *in vitro* model of PD the SH-SY5Y human neuroblastoma cell line was exposed to SFN, MPP+ (1-methyl-4-phenylpyridinium) and paraquat (PQ).

3.2. DNA, RNA and protein preparation from biological samples

DNA was isolated from peripheral venous blood and mice brain samples by the standard desalting method [13] and with the use of TRI Reagent, respectively. For DNA isolation from tissue culture samples the phenol-chloroform method was implemented. RNA extraction was carried out with the use of TRI Reagent. For protein analysis total cell lysates were prepared.

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

For the detection of specific PARK gene mutation in genomic DNA samples restriction fragment length polymorphism (RFLP; R1628P and G2385R *LRRK2* variants) and TaqMan allelic discrimination methods (in the case of R1398H, N551K, S1647T and rs1491923 *LRRK2*, and all the investigated *MAPT*, *SNCA* and *TCEANC2* variants) were implemented.

Relative mitochondrial DNA (mtDNA) copy number was determined by quantitative reverse transcription PCR (RT-qPCR) reactions. HK2 (Hexokinase 2) and B2M (Beta-2-microglobulin) nuclear genes were used for normalization in mouse and SH-SY5Y cell samples, respectively. Mitochondrium specific primers were for 16S gene and human $tRNA^{Leu(UUR)}$.

lncRNA expression changes in human blood were determined by RT-qPCR using specifically designed Custom RT2 PCR Array (Qiagen, Hilden, Germany) for groups of 41 and 12 lncRNAs (validation study I.). For validation study II. NEAT1 and TUG1 (Taurine up-regulated gene 1) gene-specific primers were obtained from Qiagen.

For lncRNA expression analysis of mouse and cell culture models qRT-PCR reactions were carried out using custom-synthethised primers and SYBER green detection. As reference18S rRNA was used. PINK1 protein level was determined by western blot (WB) using anti-PINK1 rabbit polyclonal antibodies (ThermoFisher Scientific).

Cell viability measurements were carried out with Cell Counting Kit-8 (CCK-8) (Sigma-Aldrich, St. Louis, Missouri, USA).

Apoptosis was analyzed by fluorescence-activated cell sorting (FACS) using Annexin V-FITC Apoptosis Detection Kit (eBioscience™, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA).

3.4. Statistical analysis

For the analysis of genotype and allele frequencies Chi-square ($\chi2$) 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.

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 ΔΔCt method was implemented. Relative mtDNA copy number was determined based on the Ct values of the investigated genomic- and mitochondrial genes according to Venegas *et al*. [14].

Following the analysis of data distribution by D'Agostino and Pearson omnibus normality test unpaired t-test or Mann-Whitney U test was implemented. P value under 0.05 was considered significant. In study settings of multiple comparisons, Bonferroni correction was used.

For multiple comparisons one-way ANOVA, or the non-parametric Kruskal-Wallis test was implemented. For correction of multiple comparisons Dunn's test or Tukey's test was used.

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 *MAPT* (1052553) and *PARK10* locus (rs10788972).

4.1.1. *LRRK2* variants

In accord with literature data of Caucasian populations, the G2385R and R1628P SNPs were absent in both of our study groups.

For S1647T a higher AA frequency could be observed in male patients in comparison with the corresponding control group. Comparing allele frequencies of the same groups

revealed the minor (A) allele to show significantly higher frequency among healthy male individuals ($\chi2 = 6.06$; $p = 0.014$).

There was no significant difference in the frequencies of the rs1491923, R1398H and N551K *LRRK2* variants between our patient and control groups. Except for one case in healthy controls, the R1398H and N551K substitutions always occurred simultaneously, thus these variants were found to be in linkage disequilibrium (LD).

4.1.2. *SNCA*, *MAPT* and *TCEANC2* gene variants

Comparing the genotype distribution of the rs356186 variant of *SNCA* 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 late onset PD (LOPD; disease onset >60 years) 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 was found in genotype or allele frequency of the rs2583988 SNP of *SNCA*, the studied *MAPT* (rs1052553) and *TCEANC2* (rs10789972) variants in either comparison.

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

We chose 41 non-coding transcripts to compare their levels in peripheral blood of PD patients and control individuals (n= 3 in each group) by qRT-PCR. Twelve lncRNAs which proved to be readily detectable by our first approach were then investigated in larger groups of healthy and PD samples (n = 15 and 18, respectively) (validation study I.). 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 in regard of PD.

Based on the results we set up a further comparison (validation study II.) for NEAT1 and TUG1 levels in study groups including 43 PD patients and 36 controls. Using GAS5 as normalization standard, following Bonferroni correction in order to adjust for multiple comparisons we detected a significant up-regulation of NEAT1 expression among PD patients compared to controls (fold increase=1.62; p=0.0019). Significant up-regulation of NEAT1

expression was detected when comparing the groups of PD patients with deep brain stimulation (DBS) to the control cohort (fold increase = 1.61; p = 0.0021) and also when comparing the expression of patients' with long disease duration (LDD; disease duration ≥10 years) to non-PD controls (fold change= 1.74, p=0.0008).

In contrast with NEAT1, we detected no significant difference in TUG1 expression in either of the above described comparisons when RNA level changes in relation to GAS5 control were compared in larger control and PD groups.

4.2.1. Two major NEAT1 isoforms can be detected in peripheral blood

We aimed at determining whether any of the splice variants indicated in the database is detectable in human peripheral blood samples. With the use of various primers surrounding introns, we did not detect convincingly any other NEAT1 variants apart from the two major forms (NEAT1S and NEAT1L) in human peripheral blood.

We also attempted to determine whether the NEAT1S and NEAT1L forms are differently represented in control and PD samples. 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.

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

In order to investigate the effects of MPP+ treatment on NEAT1 expression changes and to set up experimental conditions which permit the modification of NEAT1 expression level we exposed SH-SY5Y neuroblastoma cells to different doses of MPP+ (0.5 mM and 1 mM) for 6, 20 and 24 hours. NEAT1 up-regulation showed time and dose dependency. For treatment of 0.5 mM MPP+ 24 hours of incubation NEAT1 expression showed 4.92 fold change. In the case of the 1 mM MPP+ dose, NEAT1 up-regulation reached its peak at 20 hours of incubation (fold up-regulation: 8.03). However, after 24 hours, NEAT1 expression decreased to that seen at 6 hours of treatment (fold up-regulation: 3.54). 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.

4.3.2. SFN treatment increased NEAT1 expression

Recently *NEAT1* was identified as a target gene for HSF1 (Heat shock factor 1) [15]. As SFN was identified as a compound capable of activating the heat shock pathway and *via* this enhancing the expression of the lncRNA in HeLa cells [15] we investigated the effects of SFN treatment on NEAT1 expression in neuroblastoma cells. We found that 10 uM SFN dose resulted in progressive and persistent NEAT1 up-regulation.

4.3.3. Combined treatment of SH-SY5Y cells with SFN and MPP+ has an additive effect on NEAT1 expression up-regulation

Upon combined treatment with SFN and MPP+ we detected an additive effect on NEAT1 up-regulation. The increase in the RNA level 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), most probably due to the high toxicity of MPP+ at higher concentrations.

4.3.4. Changes in mtDNA copy number upon MPP+ and SFN treatment

In SH-SY5Y cells MPP+ treatment decreased relative mtDNA amount in a dose and time dependent manner. On the contrary, SFN treatment increased relative mtDNA content. Pretreatment with SFN was also capable of partly restoring the decrease in mtDNA copy number due to 0.5 mM MPP+ treatment. Consequently, these results indicate that the increase of NEAT1 level evoked by SFN treatment itself do not cause a decrease in mtDNA copy number. Or, alternatively, other beneficial effects of SFN might counteract it.

Treatment of SH-SY5Y cells with PQ in combination with or without SFN yielded similar result in that SFN treatment alone modestly increased relative mtDNA content, however, none of the applied PQ treatments caused such a prominent mtDNA copy number decrease as seen in the case of MPP+.

4.3.5. Change in PINK1 level upon NEAT1 up-regulation

We exposed SH-SY5Y cells to 0.25 mM MPP+ for 24 hours. WB analysis of total cellular protein extract did not reveal any changes in the amount of PINK1 protein.

4.3.6. MPP+, PQ and SFN combined effects on cell viability

MPP+ treatment of SH-SY5Y cells caused a significant (up to 60%) decrease in cell viability. Combined treatment of 10 uM SFN and low (0.002 and 0.01 mM) doses of MPP+ had a positive effect on cell viability. In fact at very low level MPP+ seemed to have an additive effect with SFN.

In addition to MPP+, we also tested the effect of PQ. Treatments with 0.1 mM, 0.5 mM and 2.5 mM PQ for 24 hours also resulted in decrease in cell viability while co-treatment with 10 uM SFN partly reversed this. Similarly to as 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.

4.3.7. SFN treatment partially compensate apoptosis increase caused by MPP+

Flow cytometry analysis of the level of apoptosis by Annexin V-FITC revealed that SFN treatment markedly reversed the effect of both 0.25 and 0.5 mM MPP+ treatment as demonstrated by the decrease in the ratio of cells in the late and particularly in the early stage of apoptotic cell death.

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

4.4.1. SFN causes NEAT1 up-regulation in the mouse brain in a dose and time dependent manner

In mice exposed to SFN (2.5, 5 and 10 mg/kg of body weight, 90 minutes, 6, 12 and 24 hours) the long isoform of NEAT1 was up-regulated most prominently in striatum and brainstem.

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

NEAT1 long variant was also significantly up-regulated in mice treated with MPTP. Up-regulation was more prominent in the striatum and in groups which received repeated MPTP injections (fold up-regulation: 6.49, p=0.0044 *vs* fold up-regulation: 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.

4.4.3. SFN and MPTP have an additive effect on NEAT1 up-regulation

Combined SFN and MPTP treatment of mice resulted in significant NEAT1L up-regulation in several combinations in both investigated brain areas (striatum and brainstem). 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). Interestingly, our results indicated that EtOH – used as a solvent for SFN – might have an additive effect to MPTP on NEAT1L expression increase.

Preliminary experiments involving low number of samples did not indicate significant change in mtDNA content in brain samples of MPTP exposed animals.

5. Discussion

By our studies we aimed at assessing the frequency of ten *LRRK2*, *SNCA*, *MAPT* and *TCEANC2* gene variants in Hungarian sporadic PD patients and non-PD controls.

Our finding of no significant association between the minor allele frequency of the rs2583988 variant of *SNCA* and the disease is in accord with data obtained from German [16] and Irish [17] study populations. Our results regarding the rs356186 variant are also in line with literature data [18]: the significant difference in genotype distribution between LOPD group and healthy controls is a consequence of higher frequency of 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.

Regarding the G2385R and R1628P variants of *LRRK2* (Leucine-rich repeat kinase 2 *alias PARK8*) our findings are in line with data of others [19][20]. In the case of the S1647T SNP, the minor (A) allele showed significantly higher frequency among healthy male individuals ($\chi2 = 6.06$; $p = 0.014$) compared to the corresponding PD group.

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 a 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 [21]. 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.

Our findings are in line with the results of others regarding the LD of the two protective *LRRK2* variants, R1398H and N551K, and also with data of lack of significant difference in the allele frequencies of the variants among patients and controls of Greek and Finnish origin [22] [23].

Concerning the frequency of haplotypes resulting from the 17q21 inversion which affects *MAPT* expression our findings are in accord with data obtained from British [24], Swedish [25] and Taiwanese [26] populations, which found no significant association between the H1 haplotype and PD.

The rs10789972 polymorphism of *TCEANC2* (PARK10 locus) was found to be associated with an increased risk of PD among American patients [27], however such association was not described in respect of Han Chinese population [28][29]. 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.

In light of the diverse functions of PARK genes, intensive research has been focusing on identifying common traits on which these might converge. Recently lncRNAs emerged as possible hubs in the network of genes and pathogenesis of the disease. We aimed to identify

lncRNA expression alterations in easily accessible PD samples that might serve as diagnostic markers and bring us closer to understanding the underlying pathomechanism.

Among the investigated lncRNAs we detected NEAT1 to be significantly up-regulated in PD patients in 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 [5][6].

NEAT1 lncRNA as the main component of paraspeckles is an important regulator of gene expression and cellular homeostasis at different levels (reviewed in [30]). There is a growing body of evidence of the involvement of the lncRNA in PD, however, it remains to be clarified whether the changes in the level of the transcript are coincidental or are in a causal relation with the disease. Our attempts to explore the mechanism of NEAT1 action by the use of PD models are in accord with studies of other laboratories performed in parallel with us. Among the reported mechanisms by which NEAT1 exerts detrimental effects are promotion of autophagy *via* PINK1 stabilisation [31], modulation of *SNCA* expression [8][11] and acting as a molecular sponge for various micro RNAs [9][10][11][12] and by this affecting ROS generation, neuroinflammation, autophagy, cell growth and apoptosis. On the contrary with these, NEAT1 lncRNA was also proposed to exert protective effect in PD by acting as a natural LRRK2 inhibitor [6].

Our data on the effects on NEAT1 expression changes in cellular and animal models of PD are in several aspects in agreement 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. An important addition by 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. Our experiments on cell viability revealed that SFN treatment enhances it. SFN treatment combined with either low dose PQ or MPP+ resulted in enhanced cell viability exceeding that observed in the case of SFN treatment alone. This could be due to the phenomenon termed ‘preconditioning’: subtoxic doses of cellular stress causing agents can trigger an endogenous neuroprotective response [32].

We also observed differences between the effects of the toxins used for modeling PD on relative mtDNA copy number change. While 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 decrease observed upon 0.5 mM MPP+ treatment could be partly compensated 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. Thus it can be assumed that sufficiently low dose PQ can have a preconditioning effect and enhance mitochondrial turnover and ATP production. The differences observed between the two toxins on mtDNA copy number change might be due to the different mode of action of the compounds.

Interestingly a prominent decrease in the ratio of apoptotic cells was observed when 0.25 mM or 0.5 mM MPP+ treatment was combined with SFN treatment. This result is seemingly in contrast with the observations that SFN did not neutralize MPP+ effect on cell viability, but the different methodologies might offer an explanation: the CCK8 assay used for cell viability analysis is dependent on the presence and activity of dehydrogenases, thus changes in intracellular metabolic activity can influence it, reflecting more metabolic changes happening within cells [33][34].

Our results highlighting differences in responses upon different toxin exposures (mtDNA copy number and cell viability) call attention to the shortcomings of toxin models accepted and widely used in PD research. For the elucidation of the complex pathomechanism of the disease it is cardinal to establish disease models which could mimick 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 change. Altogether our data do not support a primer neurodegenerative effect of NEAT1.

6. New findings

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

1. The G2385R and R1628P risk factor *LRRK2* variants were absent both in the control and PD group.
2. Both genotype and allelic distribution and 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. By comparing lncRNA levels in peripheral blood samples of PD patients and controls we determined that:

1. NEAT1 is up-regulated in 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. We set up neuroblastoma cell based *in vitro* PD model and using it we determined that:

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, on the contrary, SFN treatment increases mtDNA copy number. Pretreatment of the cells with SFN prior to MPP+ exposure is capable of restoring partly the mtDNA copy number change caused by the toxin.
5. Both MPP+ and PQ treatments cause a decrease in cell viability. On the contrary, 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. By the use of an *in vivo* mouse PD model we determined that:

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 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 *in vivo* PD model permitting further studies for the exploration of the mechanism of NEAT1 action.

7. Acknowledgements

I would like to thank my supervisors, Péter Klivényi M.D., Ph.D., D.Sc. (Professor and Head of the Neurology Department, University of Szeged) and László Vécsei M.D., Ph.D., D.Sc. (Former Head of the Neurology Department, University of Szeged, Member of the Hungarian Academy of Sciences), for the possibility to perform my studies at the department and for their support, guidance and feedback provided during my work.

I would like to thank Rita Maszlag-Török M.Sc., Ph.D. and Evelin Vágvölgyi-Sümegi M.Sc. for their help with experiments and for the friendly atmosphere throughout my time in the laboratory.

I would like to say thanks for colleagues at SZTE TTIK and BRC who helped to set up the cell culture model and to perform specific experiments with that. I am particularly thankful for Katalin Ökrös Gyuláné for her invaluable help with these experiments.

Last, but not least, I am grateful for my family for their patience and encouragement during both my studies and work, without their continous help and support this work would not have been possible.

8. References

[1] Savitt D, Jankovic J (2019) Targeting α-synuclein in Parkinson's disease: Progress towards the development of disease-modifying therapeutics. *Drugs* **79**, 797–810.

[2] Hamza TH, Payami H (2010) The heritability of risk and age at onset of Parkinson's disease after accounting for known genetic risk factors. *J Hum Genet* **55**, 241–243.

[3] Johnson ME, Stecher B, Labrie V, Brundin L, Brundin P (2019) Triggers, facilitators, and aggravators: Redefining Parkinson's disease pathogenesis. *Trends Neurosci* **42**, 4–13.

[4] Lv Q, Wang Z, Zhong Z, Huang W (2020) Role of long noncoding RNAs in Parkinson's disease: putative biomarkers and therapeutic targets. *Parkinsons Dis* **2020**, 1–12.

[5] Kraus TFJ, Haider M, Spanner J, Steinmaurer M, Dietinger V, Kretzschmar HA (2017) Altered Long Noncoding RNA Expression Precedes the Course of Parkinson's Disease-a Preliminary Report. *Mol Neurobiol* **54**, 2869–2877.

[6] Simchovitz A, Hanan M, Niederhoffer N, Madrer N, Yayon N, Bennett ER, Greenberg DS, Kadener S, Soreq H (2019) NEAT1 is overexpressed in Parkinson's disease substantia nigra and confers drug-inducible neuroprotection from oxidative stress. *FASEB J* **33**, 11223–11234.

[7] Yan W, Chen ZY, Chen JQ, Chen HM (2018) LncRNA NEAT1 promotes autophagy in MPTP-induced Parkinson's disease through stabilizing PINK1 protein. *Biochem Biophys Res Commun* **496**, 1019–1024.

[8] Liu Y, Lu Z (2018) Long non-coding RNA NEAT1 mediates the toxic of Parkinson's disease induced by MPTP/MPP+ via regulation of gene expression. *Clin Exp Pharmacol Physiol* **45**, 841–848.

[9] Geng L, Zhao J, Liu W, Chen Y (2019) Knockdown of NEAT1 ameliorated MPP+ -induced neuronal damage by sponging miR-221 in SH-SY5Y cells. *RSC Adv* **9**, 25257–25265.

[10] Xie SP, Zhou F, Li J, Duan S jie (2019) NEAT1 regulates MPP+-induced neuronal injury by targeting miR-124 in neuroblastoma cells. *Neurosci Lett* **708**, 134340.

[11] Sun Q, Zhang Y, Wang S, Yang F, Cai H, Xing Y, Chen Z, Chen J (2020) NEAT1 decreasing suppresses Parkinson's disease progression via acting as miR-1301-3p sponge. *J Mol Neurosci* 1–10.

[12] Liu R, Li F, Zhao W (2020) Long noncoding RNA NEAT1 knockdown inhibits MPP+-induced apoptosis, inflammation and cytotoxicity in SK-N-SH cells by regulating miR-212-5p/RAB3IP axis. *Neurosci Lett* **731**, 135060.

[13] Miller SA, Dykes DD, Polesky HF (1988) A simple salting out procedre for extracting DNA from human nucleated cells. *Nucleid acids Res* **16**, 1215.

[14] Venegas V, Wang J, Dimmock D, Wong LJ (2011) Real-time quantitative PCR analysis of mitochondrial DNA content. *Curr Protoc Hum Genet* 1–12.

[15] Mohammad Lellahi S, Rosenlund IA, Hedberg A, Kiær LT, Mikkola I, Knutsen E, Perander M (2018) The long noncoding RNA NEAT1 and nuclear paraspeckles are up-regulated by the transcription factor HSF1 in the heat shock response. *J Biol Chem* **293**, 18965–18976.

[16] Mueller JC, Fuchs J, Hofer A, Zimprich A, Lichtner P, Illig T, Berg D, Wüllner U, Meitinger T, Gasser T (2005) Multiple regions of alpha-synuclein are associated with Parkinson's disease. *Ann Neurol* **57**, 535–541.

[17] Ross OA, Gosal D, Stone JT, Lincoln SJ, Heckman MG, Irvine BG, Johnston JA, Gibson JM, Farrer MJ, Lynch T (2007) Familial genes in sporadic disease: common variants of α-synuclein gene associate with Parkinson's disease. *Mech Ageing Dev* **128**, 378–382.

[18] Zhang Y, Shu L, Sun Q, Pan H, Guo J, Tang B (2018) A comprehensive analysis of the association between SNCA polymorphisms and the risk of Parkinson's disease. *Front Mol Neurosci* **11**, 1–12.

[19] Toft M, Haugarvoll K, OA R, MJ F, JO A (2007) LRRK2 and Parkinson's disease in Norway. *Acta Neul Scand* **115**, 72–75.

[20] Kumari U, Tan EK (2009) LRRK2 in Parkinson's disease: genetic and clinical studies from patients. *FEBS* **276**, 6455–6463.

[21] Simon-sanchez J, Schulte C, Bras JM, Sharma M, Gibbs R, Berg D, Paisan-ruiz C, Lichtner P, Scholz SW, Hernandez DG, Kruger R, Federoff M, Klein C, Goate A, Perlmutter J, Bonin M, Nalls MA, Illig T, Gieger C, Houlden H, Steffens M, Okun MS, Cookson M, Foote KD, Fernandez HH, Traynor BJ, Schreiber S, Arepalli S, Zonozi R, Gwinn K, Brug M van der, Lopez G, Chanock SJ, Schatzkin A, Park Y, Hollenbeck A, Gao J, Huang X, Wood NW, Lorenz D, Deuschl G, Chen H, Riess O, Hardy JA, Singleton AB, Gasser T (2009) Genome-wide association study reveals genetic risk underlying Parkinson's disease. *Nat Genet* **41**, 1308–1312.

[22] Tan E, Peng R, Teo Y, Tan LC, Angeles D, Ho P, Chen M-L, Lin C-H, Mao X-Y, Chang X-L, Prakash KM, Liu J, Au W, Le W-D, Jankovic J, Burgunder J-M, Zhao Y, Wu R-M (2010) Multiple LRRK2 variants modulate risk of Parkinson Disease: a Chinese multicenter study human. *Hum Mutat* **31**, 561–568.

[23] Paisán-Ruíz C, Evans EW, Jain S, Xiromerisiou G, Gibbs JR, Eerola J, Gourbali V, Hellström O, Duckworth J, Papadimitriou A, Tienari PJ, Hadjigeorgiou GM, B SA (2006) Testing association between LRRK2 and Parkinson's disease and investigating linkage disequilibrium. *J Med Genet* **43**, 1–6.

[24] de Silva R, Hardy J, Crook J, Khan N, Graham EA, Morris CM, Wood NW, Lees AJ (2002) The tau locus is not significantly associated with pathologically confirmed sporadic Parkinson's disease. *Neuroscielne Lett* **330**, 201–203.

[25] Johansson A, Zetterberg H, Hakansson A, Nissbrandt H, Blennow K (2005) TAU haplotype and the Saitohin Q7R gene polymorphism do not influence CSF Tau in Alzheimer's disease and are not associated with frontotemporal dementia or Parkinson's disease. *Neurodegener Dis* **2**, 28–35.

[26] Fung HC, Xiromerisiou G, Gibbs JR, Wu Y, Eerola J, Gourbali V, Hellström O, Chen CM, Duckworth J, Papadimitriou A, Tienari PJ, Hadjigeorgiou GM, Hardy J, Singleton AB (2006) Association of Tau haplotype-tagging polymorphisms with Parkinson's disease in diverse ethnic Parkinson's disease cohorts. *Neurodegener Dis* **3**, 327–333.

[27] Beecham GW, Dickson DW, Scott WK, Martin ER, Schellenberg G, Nuytemans K, Larson EB, Buxbaum JD, Trojanowski JQ, Deerlin VM Van, Hurtig HI, Mash DC, Beach TG, Troncoso JC, Pletnikova O, Frosch MP, Foroud TM, Ghetti B, Honig LS, Marder K, Vonsattel JP, Goldman SM, Vinters H V, Ross OA, Wszolek ZK, Wang L, Dykxhoorn DM, Pericak-Vance MA, Montine TJ, Leverenz JB, Dawson TM, Vance JM (2015) PARK10 is a major locus for sporadic neuropathologically confirmed Parkinson disease. *Am Acad Neurol* **84**, 972–980.

[28] Guo Y, Tan T, Deng X, Song Z, Yang Z, Yang Y, Deng H (2015) TCEANC2 rs10788972 and rs12046178 variants in the PARK10 region in Chinese Han patients with sporadic Parkinson's disease. *Neurobiol Aging* **36**, 3335.e1-3335.e2.

[29] Tian S, Yang X, Zhao Q, Zheng J, Huang H, Chen Y, An R, Xu Y (2017) No association of PARK10 polymorphism with Parkinson's disease in Han Chinese population. *Park Relat Disord* **42**, 106–106.

[30] Bond CS, Fox AH (2009) Paraspeckles : nuclear bodies built on long noncoding RNA. *J Cell Biol* **186**, 637–644.

[31] Yan W, Chen ZY, Chen JQ, Chen HM (2018) LncRNA NEAT1 promotes autophagy in MPTP-induced Parkinson's disease through stabilizing PINK1 protein. *Biochem Biophys Res Commun* **496**, 1019–1024.

[32] Stetler AR, Leak KR, Gan Y, Li P, Hu X, Jing Z, Chen J, Zigmond MJ, Gao Y (2014) Preconditioning provides neuroprotection in models of CNS disease: paradigms and clinical significance. *Prog Neurobiol* **114**, 58–83.

[33] Aslantürk ÖS (2018) In vitro cytotoxicity and cell viability assays: principles, advantages, and disadvantages. In *Genotoxicity - A predictable risk to our actual world*, Larramendy ML, Soloneski S, eds., pp. 1–18.

[34] Strober W (2015) Trypan blue exclusion test of cell viability. *Curr Protoc Immunol* **111**, A3.B.1.-A3.B.3.