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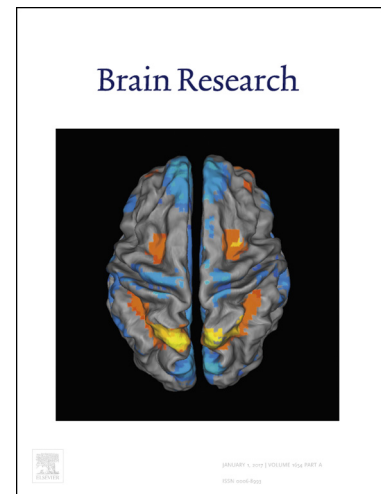
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Increased level of NEAT1 long non-coding RNA is detectable in peripheral blood cells of patients with Parkinson's disease

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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.

Keywords: Long non-coding RNA, Parkinson's disease, NEAT1, neurodegeneration

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.

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

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 their families and society as well. Although only a relatively small fraction of PD cases is familial, 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) (D. Q. Wang et al., 2018) 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 mitochondrial homeostasis, oxidative stress response, apoptosis. (Simchovitz et al., 2019; Y. Wang et al., 2018). 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 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 Santoro *et al.* and Kraus *et al.* (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., Figure 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., Figure 1B and C). Between patients with- and without DBS we did not find significant difference in NEAT1 level (Suppl. Table 1., Figure 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., Figure 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., Figure 2H 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., Figure 2J-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 (RP11-409K20.6 and RP11-124N14.3) 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 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 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 mitochondrion 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 (Y. Wang et al., 2018). 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 (Figure 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 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 tumoursuppressor 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-1 β , IL-6 and TNF α . 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 coincidentally 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, $DD: 4.9\pm 2.8$ years), while the 'long DD' - $DD \geq 10$ years - subgroup (LDD) consisted of 15 individuals (age: 63.7 ± 10.9 years, $DD: 14.6\pm 5.0$ 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 hours 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 RT² First Strand Kit (Qiagen) following the manufacturer's instructions. Real-timePCR was performed with the use of RT² SYBR Green Mastermix (Qiagen). Equal volumes of cDNA samples were aliquoted into the wells of a specifically designed Custom RT² 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

RT² 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 RT² PCR analysis web portal (<http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php>) and GraphPad Prism 6.01 statistics software using the $\Delta\Delta\text{Ct}$ method. In short, ΔCt was calculated as the difference between a gene of interest and the average of reference gene, $\Delta\Delta\text{Ct}$ was calculated as ΔCt (patient) – average ΔCt (control) and fold change was determined as $2^{-\Delta\Delta\text{Ct}}$ value (Livak and Schmittgen, 2001). For the identification of the outliers among $2^{-\Delta\Delta\text{Ct}}$ 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.

Data Availability

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

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Conflict of 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.

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Figure 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 upregulated 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.

Figure 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. (Y. Wang et al., 2018).

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.

Figure 1. Comparisons of NEAT1 lncRNA level between controls (n=36) and PD patients (n=43) and their subgroups.

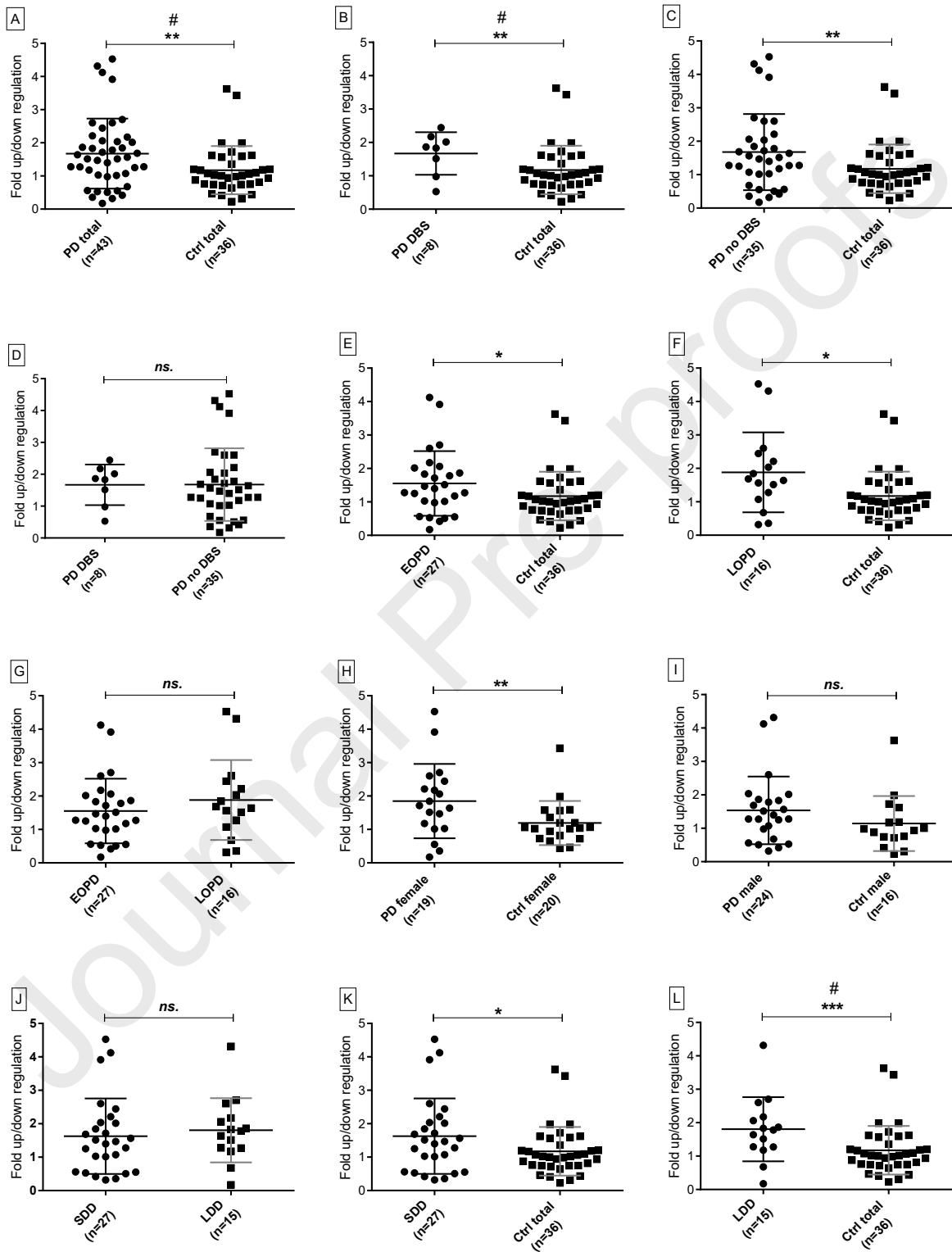


Figure 2. Mechanisms by which NEAT1 might effect cell viability and PD.

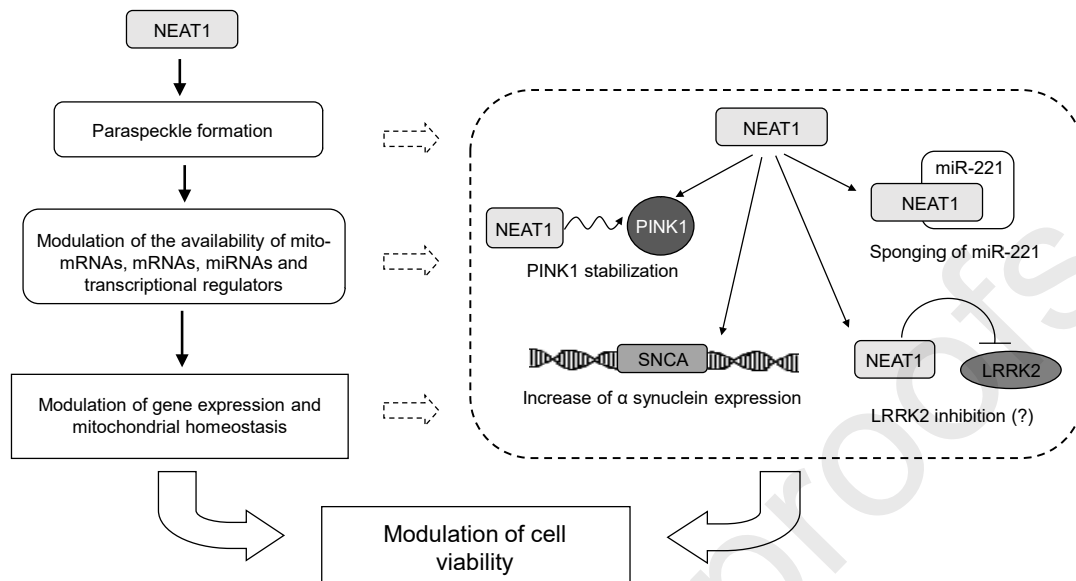


Table 1. Neurodegeneration implicated lncRNAs included in the preliminary study (control n=3, PD n=3).

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

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

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

Gene Symbol	Average Ct		Fold change (PD/ Ctrl)	P value
	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.

changes.

LncRNAs detected in Ct >35 were excluded from further analysis.

Abbreviations: PD: Parkinson's disease; Ctrl: control.

Supplementary Table 1.

Fold regulation of NEAT1 in validation study II.

NEAT1				
	Fold regulation	p	Statistical test	Bonferroni corr. (p< 0.0042)
PD total (n=43) vs. Ctrl total (n=34)	1.62	0.0019**	Mann-Whitney U test	significant
PD no DBS (n=35) vs. Ctrl total (n=34)	1.62	0.0072**	Mann-Whitney U test	ns.
PD DBS (n=8) vs. Ctrl total (n=34)	1.61	0.0021**	unpaired t test	significant
PD DBS (n=8) vs. PD no DBS (n=35)	0.99	0.53	Mann-Whitney U test	ns.
PD male (n=24) vs. Ctrl male (n= 16)	1.35	0.1	Mann-Whitney U test	ns.
PD female (n=19) vs. Ctrl female (n=19)	1.72	0.0073**	unpaired t test	ns.
EOPD (n=27) vs. Ctrl total (n=34)	1.5	0.0181*	Mann-Whitney U test	ns.
LOPD (n=16) vs. Ctrl total (n=34)	1.82	0.0113*	unpaired t test	ns.
EOPD (n=27) vs. LOPD (n=16)	0.83	0.32	Mann-Whitney U test	ns.
SDD (n=27) vs. LDD (n=15)	0.9	0.33	Mann-Whitney U test	ns.
SDD (n=27) vs. Ctrl total (n=34)	1.57	0.028*	Mann-Whitney U test	ns.
LDD (n=15) vs. Ctrl total (n=34)	1.74	0.0008***	Mann-Whitney U test	significant

Abbreviations: NEAT1: Nuclear Paraspeckle Assembly Transcript 1; 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; *: $p < 0,05$; **: $p < 0,01$; ***: $p < 0,001$.

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Supplementary Table 2.

Fold regulation of TUG1 in validation study II.

TUG1			
	Fold regulation	p	Statistical test
PD total (n=43) vs. Ctrl total (n=36)	0.98	0.84	unpaired t test
PD no DBS (n=35) vs. Ctrl total (n=36)	0.98	0.83	unpaired t test
PD DBS (n=8) vs. Ctrl total (n=36)	0.99	0.98	unpaired t test
PD DBS (n=8) vs. PD no DBS (n=35)	1.02	0.98	unpaired t test
PD male (n=24) vs. Ctrl male (n= 16)	0.92	0.44	unpaired t test
PD female (n=19) vs. Ctrl female (n=20)	1.05	0.71	unpaired t test
EOPD (n=27) vs. Ctrl total (n=36)	0.9	0.22	unpaired t test
LOPD (n=16) vs. Ctrl total (n=36)	1.12	0.26	unpaired t test
EOPD (n=27) vs. LOPD (n=16)	0.8	0.057	unpaired t test
SDD (n=27) vs. LDD (n=15)	0.91	0.42	unpaired t test
SDD (n=27) vs. Ctrl total (n=36)	0.95	0.57	unpaired t test
LDD (n=15) vs. Ctrl total (n=36)	1.05	0.63	unpaired t test

Abbreviations: TUG1: Taurine Up-Regulated Gene 1; 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.

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Supplementary Table 3.

Demographic data of participants involved in validation studies I. and II.

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.				
	n (male/female)	Age (mean ± SD; years)	Age at disease onset (mean ± SD; years)	Disease duration (mean ± SD; years)
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: Ctrl: control; PD: Parkinson's disease; DBS: deep brain stimulation; EOPD: early onset PD; LOPD: late onset PD; SDD: short disease duration; LDD: long disease duration.

Clinical characteristics of participants involved in validation study II.

Family history of PD	
yes/no	4/39
Presence of tremor	
yes/no	30/13
Modified Hoehn and Yahr scale	
PD total (n=43) (mean±SD)	1.9±0.8
1	10 (23.3%)
2	21 (48.8%)
2.5	9 (21%)
3	1 (2.3%)
4	1 (2.3%)
5	1 (2.3%)
UPDRS motor score	
PD total (n=43) (mean±SD)	12±8.9
<10	22 (51.2%)
10-20	16 (37.2%)
>20	5 (11.6%)
Applied medication at the time of sample collection	
Levodopa	36 (83.7%)
other (ropinirole, entacapone, razagiline)	3 (7%)
none	4 (9.3%)
Levodopa treatment duration at the time of sample collection	
PD total (n=36) (mean years±SD)	6.4 ±4.1
<5 years	13 (36.1%)
5-10 years	17 (47.2%)
>10 years	6 (16.7%)

Abbreviations: PD: Parkinson's disease.

Fanni Annamária Boros: methodology, perform the experiments, writing draft

Rita Török: methodology, perform the experiments, data validation, reviewing and editing

László Vécsei: Reviewing and editing, funding acquisition

Peter Klivényi: Conceptualization, patient selection, patient's data, reviewing and editing

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- 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.