NEAT1 is overexpressed in Parkinson’s disease substantia nigra and confers drug-inducible neuroprotection from oxidative stress

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ABSTRACT: Recent reports attribute numerous regulatory functions to the nuclear paraspeckle-forming long non-coding RNA, nuclear enriched assembly transcript 1 (NEAT1), but the implications of its involvement in Parkinson’s disease (PD) remain controversial. To address this issue, we assessed NEAT1 expression levels and cell type patterns in the substantia nigra (SN) from 53 donors with and without PD, as well as in interference tissue culture tests followed by multiple in-house and web-available models of PD. PCR quantification identified elevated levels of NEAT1 expression in the PD SN compared with control brains, an elevation that was reproducible across a multitude of disease models. In situ RNA hybridization supported neuron-specific formation of NEAT1-based paraspeckles at the SN and demonstrated coincidences of NEAT1 and paraspeckles in cultured cells under paraquat (PQ)-induced oxidative stress. Furthermore, neuroprotective agents, including fenofibrate and simvastatin, induced NEAT1 up-regulation, whereas RNA interference-mediated depletion of NEAT1 exacerbated death of PQ-exposed cells in a leucine-rich repeat kinase 2-mediated manner. Our findings highlight a novel protective role for NEAT1 in PD and suggest a previously unknown mechanism for the neuroprotective traits of widely used preventive therapeutics.—Simchovitz, A., Hanan, M., Niederhoffer, N., Madrer, N., Yayon, N., Bennett, E. R., Greenberg, D. S., Madrer, N., Yayon, N., Bennett, E. R., Greenberg, D. S., Kadener, S., Soreq, H. NEAT1 is overexpressed in Parkinson’s disease substantia nigra and confers drug-inducible neuroprotection from oxidative stress. FASEB J. 33, 11223–11234 (2019). www.fasebj.org

KEY WORDS: lncRNA · neurodegeneration · paraspeckles

NEAT1 expression in the PD SN compared with control brains, an elevation that was reproducible across a multitude of disease models. In situ RNA hybridization supported neuron-specific formation of NEAT1-based paraspeckles at the SN and demonstrated coincidences of NEAT1 and paraspeckles in cultured cells under paraquat (PQ)-induced oxidative stress. Furthermore, neuroprotective agents, including fenofibrate and simvastatin, induced NEAT1 up-regulation, whereas RNA interference-mediated depletion of NEAT1 exacerbated death of PQ-exposed cells in a leucine-rich repeat kinase 2-mediated manner. Our findings highlight a novel protective role for NEAT1 in PD and suggest a previously unknown mechanism for the neuroprotective traits of widely used preventive therapeutics.

Parkinson’s disease (PD) is the second most common neurodegenerative disorder, affecting ~2% of adults over the age of 70 worldwide (1). PD involves the progressive and selective death of dopaminergic neurons in the substantia nigra (SN) pars compacta, affecting the nigrostriatal pathway and inducing bradykinesia, gait abnormalities, resting tremor, and additional symptoms (2). PD is largely considered a sporadic disease, with some patient communities suffering from different versions of its familial forms. Autosomal dominant or recessive mutations in several genes have been identified as being involved in familial PD (3), including α-synuclein (4) and the leucine-rich repeat kinase 2 (LRRK2) (5), a PD-associated mutation of which is prevalent in Ashkenazi Jews (6). Other risk factors associated with increased PD prevalence include certain genetic loci (7), male sex (8), and environmental exposure to oxidative stress–inducing toxins (9) such as the herbicide paraquat (PQ) and the fungicide maneb (10). Nevertheless, current therapeutics, as well as most efforts toward development of novel preventive and therapeutic measurements, are similar and indiscriminative for all patients with PD.
Clinical diagnosis of PD is based solely on the aforementioned hallmark symptoms of the disease, which appear after a majority of the dopaminergic neurons have died (11). However, this state follows a long prodromal phase of PD that is associated with nonspecific symptoms such as anosmia and gastrointestinal disturbances. Current PD therapeutics are symptomatic, aimed at pharmacologic up-regulation of dopaminergic signaling or electrical modification of dopaminergic pathways rather than mitigating the progressive neurodegenerative process (12, 13). Also, preventive strategies are quite limited and rely mainly on increasing physical activity (14). These facts raise a pressing need for therapeutics that could ameliorate symptoms, delay disease initiation, or stop its progression. Several such agents are currently in clinical trials, including the tyrosine kinase inhibitor nilotinib (15) and 3-hydroxy-3-methylglutaryl–coenzyme A inhibitors such as simvastatin (16), but their underlying mechanisms of action are incompletely understood.

One class of RNAs whose role in PD initiation and progression is still unclear is long noncoding RNAs (lncRNAs), which are a diverse subset of transcripts longer than 200 nt that do not encode for proteins (17). lncRNAs participate in key cellular functions such as modification of transcriptional and translational processes by various molecular mechanisms, including scaffolding of RNA-protein structures, competition with endogenous mRNAs over microRNA binding (18), and epigenetic regulation (19).

Nuclear enriched assembly transcript 1 (NEAT1) is a highly expressed, mammal-specific lncRNA that forms the scaffold of nuclear paraspeckles (20), membraneless organelles that are causally involved in the regulation of transcription via protein sequestration and RNA retention (21). Although the sequence of NEAT1 is not highly conserved across species, its functions as the paraspeckle assembly scaffold are well established (22). Notably, NEAT1 also affects mitochondrial abundance and function (23). Nuclear paraspeckles have been identified as causally involved in immune responses (24) and cancer (25), and recent research on NEAT1 functions in the context of the CNS has revealed its involvement in Huntington’s disease (26) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)–induced neurotoxicity (27, 28). However, although the involvement of NEAT1 in PD has been suggested in animal models of neurodegeneration, they have not yet been thoroughly examined in human disease. To study NEAT1 involvement in human PD and investigate its nuclear-mitochondrial links in PD pathophysiology, we combined immunostaining and quantitative PCR (qPCR) quantification in human brain tissues and various cell culture models with NEAT1 knockdown in cultured cells under oxidative stress.

**MATERIALS AND METHODS**

**Brain samples**

Frozen human brain tissues were obtained from the Netherlands Brain Bank (Amsterdam, The Netherlands) and approved for use by the Netherlands Brain Bank and by the Ethics Committee of the Hebrew University (for full list of samples, see Supplemental Table S1). For RNA extraction, tissue pieces were cut on dry ice and were snap-frozen in liquid nitrogen to maintain RNA integrity. Lysis and homogenization (using a pellet pestle) were performed with 700 µl Qiazol Lysis Reagent, after which homogenates were snap-frozen again, before RNA extraction with the miRNeasy Kit (both from Qiagen, Germantown, MD, USA).

**Cell culture**

Human embryonic kidney 293T (HEK-293T) and SH-SY5Y cells were both grown in standard conditions, according to the American Type Culture Collection (Manassas, VA, USA) guidelines, using reagents from Biologic Industries (Beit Haemek, Israel). Growth medium for HEK-293T cells was DMEM supplemented with 10% fetal calf serum, 1% L-glutamine, and 1% prostate-specific antigen. Growth medium for SH-SY5Y cells was a 1:1 mixture of Eagle’s minimum essential medium and Ham’s F12 nutrient mixture, supplemented with 10% fetal calf serum, 1% L-glutamine, and 1% prostate-specific antigen. Cells were grown at 37°C and in 5% CO₂.

**RNA extraction from cell culture samples**

Medium was aspirated, and wells were washed once with PBS, followed by cell lysis and homogenization in 700 µl Qiazol. RNA was extracted using the miRNeasy Kit according to the manufacturer’s instructions and was treated with Ambion DNase (Thermo Fisher Scientific, Waltham, MA, USA).

**qPCR**

cDNA was prepared using the Quanta qScript mRNA cDNA Synthesis Kit (Quantabio, Beverly, MA, USA) according to the manufacturer’s instructions and diluted 1:10 in double-distilled water prior to qPCR plate preparation. qPCR was performed in either 384-well or 96-well plates, using Perfecta Sybr Green FastMix with low or No Rox (Quantabio) at a final well volume of 5 or 15 µl, respectively. As housekeeping genes, tubulin-β3 class III was used for human brain samples and for SH-SY5Y cells, and glyceraldehyde 3-phosphate dehydrogenase was used for HEK-293T cells. Expression was calculated as ΔΔCt, values (primer sequences are detailed in Supplemental Table S2).

**Knockdown experiments**

Twenty-four hours prior to transfection, 96- or 12-well plates were plated with 20,000 or 300,000 HEK-293T cells per well or with 50,000 or 500,000 SH-SY5Y cells per well, respectively. Transfection of HEK-293T cells was performed with small interfering RNA (siRNA) pools consisting of 20 different siRNAs (siPools, siTools Biotech, Planegg, Germany) at a total final concentration of 10 nM siRNA using Lipofectamine RNAiMax Transfection Reagent (Thermo Fisher Scientific) according to the manufacturer’s protocol. Transfection of SH-SY5Y cells was performed with locked nucleic acid (LNA) GapmeRs, at a final concentration of 75 nM, with Hiperfect transfection reagent (Qiagen). Lovastatin, simvastatin, and PF-06447475 (all from MilliporeSigma, Burlington, MA, USA), dissolved in DMSO or a DMSO-only control, was added 24 h post-transfection. PQ or tert-butyl hydroperoxide (tBHP) (both from MilliporeSigma), dissolved in double-distilled water, or double-distilled water-only control, was added 48 h post-transfection.
Thiazolyl blue tetrazolium bromide cell viability assay

The assay was carried out in 96-well plates 24 h after PQ administration. Cell medium was supplemented with 20 μl of 5 mg/ml thiazolyl blue tetrazolium bromide (MTT; MilliporeSigma) in PBS, followed by 4 h of incubation at 37°C and 5% CO₂. Salt crystals were then dissolved by adding 100 μl 10% SDS containing 0.01 N HCl and incubating overnight at 37°C. Absorptions were read at 570 nm for MTT and 690 nm for reference using a Spark 10M microplate reader (Tecan, Männendorf, Switzerland). After subtraction of reference from MTT reads and further subtraction of no-cell controls from test wells, each well was normalized to the mean value of non-PQ-treated cells in the same siRNA plus simvastatin-PF-06447475 conditions. This allowed cross-condition and cross-plated comparisons while accounting for condition-specific cell proliferation differences.

RNA FISH in cells

HEK-293T cells were plated on 18-mm round microscope cover glasses treated with poly-L-lysine, were exposed to 1 mM PQ, and 24 h later were fixed in 4% paraformaldehyde for 1 h. FISH was performed using the NEAT1.5 or NEAT1_Mid ready-made probe sets, targeting either both variants or selectively the long variant of NEAT1, respectively (Stellaris, Biosearch Technologies, London, United Kingdom), according to the manufacturer’s instructions. Images were obtained using a Fluoview FV10i confocal microscope (Olympus, Tokyo, Japan) and were analyzed using ImageJ (National Institutes of Health, Bethesda, MD, USA). No staining was detected in controls exposed to negative control solutions.

Brain tissue immunohistochemistry and FISH

Samples were embedded in optimal cutting temperature medium (SciGen, Singapore) in Tissue-Tek cryomolds (Sakura Finetek, Tokyo, Japan) and were stored at −80°C until use. Frozen 20-μm thick sections were prepared with a Leica CM-1850 cryostat (Leica Biosystems, Wetzlar, Germany) and were mounted on glass slides. Sections were fixed in 4% paraformaldehyde in PBS for 1 h at 4°C and were permeabilized in PBS-0.2% Triton X-100 for 30 min, followed by blocking in PBS-0.2% Triton X-100 and 5% normal donkey serum for 1 h. Incubation with the primary antibodies was overnight at 4°C in PBS-0.2% Tween-20 with 10 μg/ml heparin; the primary antibodies employed targeted tyrosine hydroxylase (TH; Merck AB152, rabbit polyclonal; Merck, Darmstadt, Germany) at a 1:1000 dilution and paraspokele component 1 (PSPC1; C-3, 374181, mouse monoclonal; Santa Cruz Biotechnology, Dallas, TX, USA) at a 1:500 dilution. AlexaFluor-647-conjugated donkey-anti-rabbit (11-605-152) and cyanine 3-conjugated goat anti-mouse (115-165-072; both from The Jackson Laboratory, Bar Harbor, ME, USA) were used as secondary antibodies at dilutions of 1:500. Nuclei were stained with DAPI at a 1:1000 dilution. Fluorescent images were taken using confocal microscopy (Olympus Fluoview FV10i; Olympus) at a ×60 magnification. Quantification and image analysis were performed using ImageJ. Supplemental Movie S1 was composed using the 3-dimensional viewer Fiji plugin based on a typical TH-positive cell as the corresponding analysis.

For FISH analysis, immunohistochemistry for TH was performed as detailed above. Subsequently, FISH was performed using the NEAT1_Mid ready-made probe set according to the manufacturer’s instructions (Biosearch Technologies); the Stellaris RNA FISH protocol for sequential immunofluorescence and FISH in adherent cells was adapted for use in fresh frozen human brain tissue based on the Stellaris RNA FISH protocol for fresh frozen mouse brain tissue. Slides were sealed using Immu-Mount (Thermo Fisher Scientific), and images obtained using a confocal microscope (Olympus Fluoview FV10i; Olympus) and subsequently analyzed using ImageJ.

For image analysis, a median filter of 2.0 pixels was applied to each DAPI channel image, followed by the setting of a default threshold. For each NEAT-1 image, an intermodes threshold was applied. For the paraspeckle analysis, the Speckle Inspector plugin from the BioVoxxel toolbox (J. Brocher and T. Wagner; https://imagej.net/BioVoxxel_Toolbox) was used, which identifies bigger features by the number of contained smaller speckles. The DAPI image was set as the big objects, with the object size set to match the expected size of DAPI stained neurons and support cells. The NEAT-1 image was set as the small speckles, with the speckle size set to match the expected range of paraspeckles based on the literature (20) and the exclusion of objects on edges. The few DAPI-positive conglomerates of multiple overlaid cells were excluded from the analysis. Paraspeckles were quantified by measuring the number of NEAT1 aggregates per nucleus.

Western blotting

HEK-293T cells were harvested 48 h after treatment with NEAT1 or control siRNA by lysis (20 min on ice) in standard RIPA buffer containing protease and phosphatase inhibitors (5871 and 5870, both diluted 1:100; Cell Signaling Technology, Danvers, MA, USA), 150 μl per 6-well plate. Cells were then collected into Eppendorf test tubes and centrifuged (10 min, 20,000 relative centrifugal force, 4°C) and then transferred to a fresh Eppendorf tube and stored at −20°C. Protein concentrations were determined using the Lowry method (DC Protein Assay; Bio-Rad, Hercules, CA, USA). Samples were separated using standard SDS-PAGE protocols, running 20 μg total protein per lane on 4–15% Mini-Protean TGX Precast Protein Gels (4561083; Bio-Rad). Transfer was done using nitrocellulose Trans-Blot Turbo Transfer Packs (1704158; Bio-Rad) and the Trans-Blot Turbo Transfer System (Bio-Rad) set at 1.3 A, 25 V, 11 min. After blocking (1 h at room temperature in standard Tris-buffered saline containing 0.1% Tween-20 and 5% bovine serum albumin) blots were exposed to anti-LRRK2 antibody (ab133474 [M1FF2 (c41-2)], 1:5000; Abcam, Cambridge, MA, USA) for 20 h at 4°C, then peroxidase AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch 111-035-114; The Jackson Laboratory), and the protein visualized with chemiluminescence (SignalFire Elite ECL Reagent, 12757; Cell Signaling Technology) using a myECL Imager (Thermo Fisher Scientific). To normalize to a loading control blots were stripped and exposed to anti-vinculin antibody (VIN-54; ab130007, 1:5000; Abcam) for 20 h at 4°C and peroxidase AffiniPure goat anti-mouse IgG (Jackson ImmunoResearch 115-035-062; The Jackson Laboratory) with visualization as above. Quantification was performed using myImageAnalysis Software (Thermo Fisher Scientific).

Mitochondrial quantification

All measurements were performed 48 h after NEAT1 knockdown. For Mitotracker-based quantification, HEK-293T cells were incubated for 10 min with 1 μM MitoTracker Red CMXRos (Thermo Fisher Scientific) in Eagle’s minimum essential medium. Bright field cyanine 3 fields were imaged using a Retiga 2000 camera (Teledyne QImaging, Surrey, BC, Canada) and a Nikon Eclipse Ti Microscope. Image analysis was performed using ImageJ. For
Neural stem cells (NSCs) were obtained from 3-month-old male C57BL/6J mice. NSCs were detached using Trypsin (Biologic Industries) and centrifuged at 1000 g. After addition of 50 μl QuickExtract buffer, samples were vortexed for 15 s, incubated for 6 min at 65°C, vortexed for 15 s more, and incubated at 98°C for 2 min. DNA concentration was measured, and all samples were brought to a concentration of 1 ng/μl for the qPCR reaction, performed as previously described. The NEAT1 5’ primer, which is not intron spanning and amplifies genomic DNA, was used for normalization to genomic DNA. Primer sequences are detailed in Supplemental Table S2.

Web-available Gene Expression Omnibus data sets

Human data were acquired from the Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/). Analysis was performed using Galaxy. For murine samples, we used the M15 version of the GeneCode (https://www.genecodes.org/) annotation. Data analysis was performed using the edgeR tool (http://bioconductor.org/)(29). A full list of data sets used, including their description, origin, and group definitions, is detailed in Supplemental Table S3.

Statistics

Statistical analysis was performed using R (https://www.r-project.org/). For 2-group comparisons, we first used the Kolmogorov-Smirnov test to determine whether the data showed a normal distribution. If they did, we performed Welch’s f test, and if not, we used the Mann-Whitney U test. Normally distributed data were presented in bar plots, with error bars marking the SE. Other data were displayed as either a cumulative frequency plot or a boxplot, in which the line represents the median, box limits represent the 25th and 75th percentiles, and the whiskers extend to the farthest data point, which is at a maximum of the 25th to 75th percentile distance from the edge of the boxplot.

RESULTS

NEAT1 promotes nuclear paraspeckle formation in PD SN dopaminergic neurons

Prior research has identified NEAT1 as a component of nuclear paraspeckles along with several protein-coding genes, including PSPC1, non-POU domain containing octamer binding (NONO), and splicing factor proline and glutamine rich (SFPQ). Furthermore, NEAT1, NONO, and SFPQ are essential for paraspeckle formation (20). To investigate the characteristics of cells containing paraspeckles in vivo, we costained sections of human SN from control donors without PD with antibodies to PSPC1 and to the dopaminergic cell marker TH. We also performed FISH for NEAT1, which we costained with TH. Image reconstruction supported by 3-dimensional analysis (30) from these brain samples identified PSPC1 aggregates inside the nucleus of TH-expressing cells (3-dimensional reconstruction in Fig. 1A and Supplemental Movie S1). Furthermore, quantification of intranuclear PSPC1 and NEAT1 aggregates revealed a large difference between cell types. Although the majority of TH-positive cells—i.e., dopaminergic neurons—were costained with PSPC1 or NEAT1 (86 and 80%, respectively; Fig. 1B), only a minority of glial cells, characterized by small and dense nuclei and lack of cytoplasmic TH staining, were costained with PSPC1 or NEAT1 (1.5 and 20%, respectively; Fig. 1C). This difference was highly statistically significant (Fig. 1D, E). Furthermore, counting the number of paraspeckles per cell revealed that dopaminergic neurons formed 15-fold more paraspeckles than glial cells: 4 nuclear paraspeckles per average in each dopaminergic cell nucleus (Fig. 1F). We have thus identified dopaminergic neurons as the major paraspeckle-forming, NEAT1-expressing cell type in the human SN.

To seek NEAT1 in additional brain neurons, we analyzed 3 GEO data sets. Translating ribosomal affinity purification (TRAP) sequencing of mouse dopaminergic and serotonergic neurons revealed an enrichment for PSPC1 (4- and 5-fold, respectively) and NONO (2.1- and 2.3-fold, respectively) in both cell types (Supplemental Fig. S1; GSE108371 and GSE108372) (31), supporting our findings of paraspeckle formation in SN dopaminergic neurons and providing data on paraspeckle formation in serotonergic neurons at large. Single-nuclei droplet sequencing (Drop-Seq) (GSE97930) (32) of 3 regions in the human brain—cerebellum, frontal cortex, and visual cortex—enabled comparing the number of nuclei expressing both of the neuronal markers neuronal nuclear antigen and microtubule-associated protein 2 with those not expressing either of these markers. In all 3 regions, PSPC1 was expressed in a 2.3–3.1-fold higher fraction of neuronal marker–positive cells compared with neuronal marker–negative cells (Supplemental Fig. S1). Together, these findings suggest that the formation of paraspeckles is preferentially neuronal but not SN-specific and occurs in additional areas of the brain.

Elevated NEAT1 transcript and paraspeckle abundance in PD brains, cell culture, and murine models of oxidative stress

To further explore the relevance of NEAT1 for human PD, we measured NEAT1 expression in a large cohort of human SN samples (control: n = 24; PD: n = 29). Two splice variants of NEAT1 are expressed in the human brain, a 27.7 kb variant (NEAT1_2, long variant), essential for paraspeckle formation (33), and a shorter 3.7 kb variant (NEAT1_1, short variant) that is included in the longer variant (34) (scheme in Fig. 2A). qPCR tests demonstrated a 2.3-fold increase of NEAT1 in the PD SN using the long variant–specific primers vs. a 1.7-fold increase using the nonspecific primers (Fig. 2B). Because oxidative stress is of high relevance for PD (35), we further tested for NEAT1 elevation in conditions of oxidative stress. For that purpose, we exposed 2 cell lines to the herbicide PQ, known to trigger oxidative stress and PD-related pathways (9). We found robust up-regulation of NEAT1 RNA upon PQ exposure in both the embryonic kidney cell line HEK-293T and the human-derived neuroblastoma cell line SH-SY5Y. This elevation was more pronounced when using a primer pair specific to the long splice variant (2.5- and 7-fold, respectively, for the long variant; Fig. 2C, D). To more selectively seek for links between oxidative stress and NEAT1 up-regulation, we exposed SH-SY5Y cells to the...
stable peroxide tBHP, which specifically creates free radicals and induces oxidative stress (36). Similar to PQ, tBHP exposure up-regulated the levels of NEAT1 and more prominently of its long variant (1.8- and 2-fold elevation, respectively; Fig. 2D). 

Alternation between the short and long splice variants of NEAT1 is associated with alternative polyadenylation regulated by the heterogeneous nuclear RNP K (HNRNPK) and cleavage and polyadenylation–specific factor 6 (CPSF6) proteins (34). Therefore, we quantified both these transcripts in HEK-293T cells but did not detect modified mRNA expression levels of either of them (Supplemental Fig. S2).

To test for NEAT1 relevance in models of PD that are not based on oxidative stress, we also explored its levels in data sets available in the GEO. In induced pluripotent stem (iPS) cell–derived neurons cultured from donors with and without PD (GSE62642), we found 2.1-fold elevated levels of NEAT1 (Supplemental Fig. S3A). Next, we used models not based on a single cell type, which better reflect whole-tissue changes. In murine neuronal primary cultures (GSE70368), we identified a 1.8-fold increase of Neat1 levels in cells overexpressing α-synuclein compared with cells overexpressing red fluorescent protein (Supplemental Fig. S3B). Finally, interrogating a web-available RNA sequencing (RNA-Seq) data set of the ventro-medial mouse midbrain coexposed to PQ and the PD-associated fungicide maneb (GSE36232) (37) revealed a 1.9-fold increase in Neat1 levels in the exposed mice (Supplemental Fig. S3C); moreover, long variant–specific reads were increased 2.9-fold compared with 1.6-fold in the common region (Supplemental Fig. S3D), suggesting that oxidative stress elevates NEAT1 levels at both the tissue and the single cell level and that this elevation was more prominently reflected in the long NEAT1 variant in both mice and human brains.

Figure 1. Nuclear paraspeckles are formed in SN dopaminergic neurons. A) Intranuclear PSPC1 aggregates in TH-expressing cells (see Supplemental Movie S1). Scale bar, 10 μm. B) Human SN brain slices costained for TH and PSPC1 or NEAT1 show nuclear PSPC1 and NEAT1 staining of paraspeckles in dopaminergic neurons (arrows). Scale bar, 10 μm. C) Costaining of these slices as above reveals lack of TH and paraspeckle staining in support cells. Scale bar, 10 μm. D–F) Quantification of paraspeckle staining, showing a higher proportion of paraspeckle-forming nuclei in TH-positive compared with TH-negative cells for both PSPC1 (D), NEAT1 (E), and a higher number of paraspeckles per nucleus in TH-positive compared with TH-negative cells (F). ****P < 0.0001 (Fisher’s exact test or Mann-Whitney U test).
Recent reports demonstrated that NEAT1 overexpression increases the number of paraspeckles (22, 38). In comparison, up-regulation of protein-coding genes coexpressed with NEAT1 does not affect paraspeckle abundance, although their knockdown abolishes nuclear paraspeckles (39). To test if paraspeckle abundance is influenced by oxidative stress, we exposed HEK-293T cells to PQ and performed RNA FISH with a probe specific to the long NEAT1 variant. Although PQ exposure increased the mean number of paraspeckles per nucleus by 60% and the median by 33% (Fig. 2E), it did not affect the fraction of paraspeckle-forming cells (Fig. 2F), nor did it affect the nuclear localization of the long NEAT1 variant (Fig. 2G). Thus, PQ-induced elevation of NEAT1 expression was correlated with the paraspeckle content in our cell culture model. In view of the human brain expression data and the immunolocalization of PSPC1 and NEAT1 to dopaminergic neurons of the SN, these findings suggest that NEAT1 changes in the SN could reflect a neuron-specific increase of paraspeckle formation in response to neurotoxicity. The robust elevation of NEAT1 in PD, which is more prominent for the long variant and is reflected across multiple models including neuron-specific ones, thus predicted elevation of the long, nuclear paraspeckle–essential NEAT1 variant in SN neurons, promoting paraspeckle formation in these stressed neurons.
NEAT1 knockdown exacerbates LRRK2-mediated oxidative stress-induced damage

The elevation of NEAT1 observed under PQ and tBHP exposure reflected its overexpression under oxidative stress. This might precipitate disease pathophysiology, inversely attenuate disease progression and assist cellular survival, or be a mere side effect of the cellular response to oxidative stress with no impact on cell survival. To understand the functional role of NEAT1 and thereby distinguish between these options, we used siRNA Pools (siTools Biotech; https://www.sitoolsbiotech.com/) and LNA GapmeRs to knock down the expression of NEAT1 in HEK-293T and SH-SY5Y cells, respectively (scheme in Fig. 3A). RNA FISH in HEK-293T cells using a probe identifying both NEAT1 variants demonstrated that NEAT1 knockdown using siRNA targeting of both variants reduced the proportion of paraspeckle-forming cells as well as the median paraspeckle number per nucleus (by 20 and 50%, respectively) (Fig. 3B–D). In addition, qPCR analysis revealed a reduction of 75% in NEAT1 levels compared with treatment with control siRNA (Fig. 3E). Because NEAT1 has recently been implicated in the regulation of mitochondrial function and formation (23), we also quantified mitochondria under conditions of NEAT1 knockdown in HEK-293T cells. Microscopic quantification of the mitochondrial-selective fluorescent probe MitoTracker (40) and comparative qPCR using 2 mitochondrial DNA–specific primers demonstrated decreased mitochondrial abundance (Supplemental Fig. S4). In SH-SY5Y cells, GapmeRs targeting the long NEAT1 variant reduced its levels by 45–55% (Fig. 3F, left) while not affecting the common region, suggesting variant specificity in knockdown efficiency (Fig. 3F, right). We conclude that NEAT1 depletion affects both the abundance and function of paraspeckles and the abundance of mitochondria.

NEAT1 knockdown also affected cell viability; using the MTT assay (experimental scheme shown in Fig. 3A), we found that NEAT1 depletion in HEK-293T cells decreased cell viability following exposure to PQ by ~15% (Fig. 3G). The GapmeR targeting the long NEAT1 variant led to a similar ~20% decline in SH-SY5Y cells (Fig. 3H), in which we also observed a ~30% decrease in cell viability under exposure to the more specific oxidative stress agent tBHP (Fig. 3I). Taken together, these results indicate that NEAT1 up-regulation could support cellular survival under conditions of oxidative stress.

Web-available mass spectrometry data sets have shown that LRRK2 interacts with the paraspeckle proteins NONO and SFPQ (41, 42), suggesting that the LRRK2 protein may also be sequestered in nuclear paraspeckles, in a manner similar to that of other proteins (21). Although LRRK2 mRNA and protein levels did not change significantly in NEAT1-depleted HEK-293T cells (see qPCR and Western blot in Supplemental Fig. S5), pretreating both HEK-293T and SH-SY5Y cells with the small molecule LRRK2 kinase inhibitor PF-0647475 (43) abolished the effect of NEAT1 depletion on PQ-induced cell death (Fig. 3G–I). Thus, NEAT1 may modulate LRRK2 physiologic activity, as has been reported in ref. 21 for other paraspeckle-bound proteins, while not directly affecting LRRK2 levels.

Screening for drug modulators of NEAT1 reveals possible candidates for exogenous up-regulation of NEAT1 as a disease-modifying strategy

Directly modulating the expression of brain transcripts recently emerged as a therapeutic approach in several diseases (44). However, because of the complicated means of administration and the high price of such treatments, this approach is still reserved for very distinct conditions in which specific transcripts are exclusively responsible for disease progression (45). A promising alternative that could be suitable in PD involves the indirect reinforcement of NEAT1 increases via non-specific agents, thereby potentiating its neuroprotective effect. To test this hypothesis, we used a recently published data set of RNA-Seq data from a drug screen performed on murine cerebrocortical cultures (GSE110256; obtained from http://bigbear.med.uottawa.ca:1000/) (46). This search revealed various degrees of NEAT1 up-regulation by nearly 40 agents (Fig. 4A). We further found GEO data sets demonstrating up-regulation of NEAT1 upon treatment with 2 of these agents, which are both lipid-lowering drugs: the peroxisome proliferator-activated receptor (PPAR)-α agonist fenofibrate in murine livers (Fig. 4B, GSE68480) (47) and the 3-hydroxy-3-methylglutaryl–coenzyme A inhibitor simvastatin in HUVECs (Fig. 4C, GSE85799, unpublished results). Data from chromatin immunoprecipitation sequencing experiments revealed binding of PPAR-α to the NEAT1 promoter region (48), compatible with the up-regulation of NEAT1 in cerebrocortical cultures treated with various PPAR-α agonists including fenofibrate, cipofibrate, and clofibrate by 3.6–12 fold (Fig. 4A). Moreover, treatment of SH-SY5Y cells with fenofibrate and simvastatin demonstrated significant increases in NEAT1 levels (Fig. 4D), particularly of its long variant (2.3- vs. 4.4-fold for fenofibrate; 2.7- vs. 3.4-fold for simvastatin). Intriguingly, treatment with statins elevated cell viability after PQ treatment in HEK-293T by 5% and after both PQ and tBHP treatment by 10–35% in SH-SY5Y cells. Notably, the former effect was abolished by LRRK2 inhibition (Supplemental Fig. S6), in line with a NEAT1-mediated effect. We conclude that preventive medicine with these and parallel agents may enable prolonged neuroprotection of disease-vulnerable neurons via NEAT1 elevation.

DISCUSSION

To explore the implications of NEAT1 involvement in PD, we measured its expression and the formation of nuclear paraspeckles in the SN of human brains, interrupted its function by depleting its levels using siRNA agents in several cellular models, and pursued its changes in diverse web-available data sets. Displaying both robustness and clinical relevance of NEAT1 to human PD, we further found robustly elevated NEAT1 in several non-MPTP models of PD, as well as in the SN of patients with PD.
Figure 3. NEAT1 knockdown (KD) abolishes paraspeckles and exposes HEK-293T and SH-SY5Y cells to PQ damage mediated by LRRK2 kinase activity. A) Scheme outlining the experimental procedure of the MTT assays. B) Confocal microscopy imaging of RNA FISH in HEK-293T cells, targeting both variants of NEAT1, and costained with DAPI for nuclear localization. Note nuclear paraspeckles (marked by arrows) in both control and knockdown cells, yet less prominent staining after knockdown. Scale bar, 10 μm. C) Quantification of nuclei that contain paraspeckles, showing a smaller proportion of paraspeckle-forming nuclei after NEAT1 knockdown. D) Quantification (ImageJ) of paraspeckles per nucleus in control and NEAT1 knockdown cells shows a decrease in the number of paraspeckles per nucleus. E) qPCR quantification of NEAT1 knockdown in HEK-293T cells; siRNA pools target and down-regulate both NEAT1 variants. F) qPCR quantification of NEAT1 knockdown in SH-SY5Y cells; 3 different long variant–specific LNA GapmeRs (G1−G3) target and down-regulate the long but not the short NEAT1 variant. G) Cell viability under NEAT1 knockdown was determined by MTT assay and shows exacerbated death of HEK-293T cells exposed to 2 mM PQ, which is abolished under treatment with the LRRK2 inhibitor PF-06447475 [n.s., not significant (P = 0.07), for NEAT1-induced change in cell mortality]. H) Cell viability under NEAT1 knockdown, determined by MTT assay, and showing exacerbated death of SH-SY5Y cells treated with 1 mM PQ, which is abolished under treatment with the LRRK2 inhibitor PF-06447475 (n.s., P > 0.49). I) Cell viability as above for SH-SY5Y cells exposed to 20 μM tBHP. *P < 0.05 (for long variant quantification, Welch’s t test), **P < 0.01 (Welch’s t test or Mann-Whitney U test), ***P < 0.001 (χ² test).
Others have shown NEAT1 involvement in a plethora of cellular processes through its protein and RNA sequestration activity (21) and identified it in Huntington’s disease (26) and in MPTP models of PD (27, 28). However, the SN undergoes extensive histologic changes in PD, including depletion of dopaminergic neurons and up-regulation of other cell types (49). Therefore, we also explored PD models that only reflect a single cell type, including in-house PQ and tBHP-exposed cell culture and web-available data sets of in vitro differentiated dopaminergic cells. The observed elevation of NEAT1 in all of these models suggests that NEAT1 elevation in the SN may reflect its up-regulation in the remaining dopaminergic neurons. Variant-specific analysis using long variant–specific primers further revealed that this increase is more dominant when examining the longer, paraspeckle-forming splice variant of NEAT1. Thus, we present the first in vivo identification of NEAT1 modulation in the human SN and suggest that the impact of NEAT1 accumulation reflects its long variant.

The long variant of NEAT1 functions as the scaffold of nuclear paraspeckles. Hence, we performed immune labeling and RNA FISH of human SN sections, seeking paraspeckles, and analyzed web-available data sets of RNA-Seq data from human and murine brains. This identified SN nuclear paraspeckle formation as occurring primarily in dopaminergic neurons. Notably, this cell-specific dominance is not unique to the SN, because parallel changes emerged in web-available data sets from TRAP experiments of both murine serotonergic and dopaminergic neurons, as well as in Drop-Seq data from 3 human brain regions. The TRAP data set further showed enrichment for the mRNA of the paraspeckle markers PSPC1 and NONO in both serotonergic and dopaminergic neurons, and the Drop-Seq data set demonstrated a larger percentage of PSPC1-expressing nuclei among those nuclei that express the neuronal markers neuronal nuclear antigen and microtubule-associated protein 2. This indicates neuronal specificity of nuclear paraspeckles in the human brain, which we have identified in several regions and neuronal populations. Also, combining our immunofluorescence and qPCR data from an oxidative stress model with that of the human SN supports the hypothesis that NEAT1’s elevation in this region reflects its accumulation and the excessive formation of paraspeckles in the dying neurons of the SN and is a hallmark of the human PD neurodegenerative process, adding this role to NEAT1’s involvement in immune functions (24), cancer (25), and Huntington’s disease (26).

The membraneless nuclear paraspeckles can modulate the activity of transcription factors and other proteins (38) by retaining them in a separate liquid phase, thus distancing them from their targets (21, 24). The functions of nuclear paraspeckles also include microRNA processing (50), suggesting that NEAT1 changes may underlie some of the substantial changes observed in the microRNA content of PD brains and blood cells (51–53). Thus, NEAT1 may regulate both the transcriptional and post-transcriptional aspects of gene expression, with substantial effects on the transcriptome (54). That those effects preferably relate to the transcription factors that reside within paraspeckles (34, 55) may be of high importance in PD.

Generation of the long NEAT1 variant is induced by overall elevation of transcription in the NEAT1 locus or by avoiding a polyadenylation site at the 3’ end of the

Figure 4. Various drugs induce up-regulation of the paraspeckle-forming long NEAT1 variant in multiple cell types. A) Bar plots depicting the expression profile of NEAT1 in cerebrocortical cultures under treatment with various drugs; data presented as z score for the difference between a single exposure to each drug and the average of 8 control samples (GSE1109256); note the PPARα agonists marked in green and simvastatin in brown. B) Boxplot depicting microarray data of fenofibrate-induced up-regulation of NEAT1 in the murine liver (GSE68480, significance according to GEO microarray analysis algorithm). C) Boxplot depicting RNA-Seq data showing simvastatin-induced up-regulation of NEAT1 in HUVECs (HUVEC; GSE85799). CPM, counts per million. D) NEAT1 levels in SH-SY5Y cells treated with 80 μM fenofibrate or 10 μM simvastatin and compared with DMSO control, evaluated by qPCR using primers specific to the long variant (left) and for both variants (right) *P < 0.05 (Welch’s t test; n = 3/group), ****P < 0.0001 (significance according to edgeR statistical analysis).
short variant, which is regulated by the proteins encoded by the HNRNPK and CPSF6 genes (34). Our qPCR measurements in PQ-treated cells and analysis of RNA-Seq data from additional models do not support differential expression of the mRNA of these genes, suggesting independent up-regulation of the NEAT1 locus rather than alternative splicing regulation by the activities of HNRNPK and CPSF6 in SN neurons of the PD brain. Importantly, although our data do not suggest up-regulation of the short NEAT1 variant, they cannot refute its involvement, because its levels were not measured directly. Conversely, although PQ caused an increase in the number of paraspeckles in some nuclei, the proportion of paraspeckle-forming nuclei was not altered. This may suggest that the reaction to oxidative stress involves post-transcriptional increase of the long NEAT1 isoform in cells that already express NEAT1, rather than reflecting an overall up-regulation of the NEAT1 locus. This apparent incongruence may be reconciled by a recently identified, bidirectional regulation of mitochondria and nuclear paraspeckles.

The proposed regulation by mitochondria over NEAT1 expression and paraspeckle formation is complex and multilayered; although mitochondrial stressors change the levels of both variants, knockdown of specific mitochondrial genes affects the intervariant balance as well (23). An interaction between mitochondria abundance and function and the bioavailability of mitochondrial proteins may thus explain the unique up-regulation of NEAT1 and nuclear paraspeckles in PD and Huntington’s disease. Both these neurodegenerative conditions involve pervasive mitochondrial dysfunction (56, 57). Likewise, our model of oxidative stress triggers changes in mitochondrial abundance and function through modulation of mitophagy (58).

To gain a view into the role of NEAT1 up-regulation in PD brains, we exposed cultured cells of human origin to PQ-induced oxidative stress while using siRNA pools or LNA GapmeRs to knock down NEAT1 expression. This predictably resulted in reduced paraspeckle formation in both human neuroblastoma SH-SY5Y and embryonic kidney HEK-293T cells, in which NEAT1 deprivation exacerbated cell death upon induction of oxidative stress by either PQ or tBHP. Importantly, this phenotype was mediated by LRRK2 activity and was abolished by treatment with an inhibitor of LRRK2 kinase activity. Because mass spectrometry data show LRRK2 binding to the paraspeckle proteins NONO and SFPQ, it is possible that NEAT1 protects cells by binding to the LRRK2 protein in paraspeckles, functioning as a bona fide LRRK2 inhibitor; other proteins have been reported to be suppressed by paraspeckles in a similar manner (21, 24). Thus, up-regulation of NEAT1 and the consequent formation of nuclear paraspeckles may act as a general cellular protective mechanism against oxidative stress. We conclude that in PD, dopaminergic neurons may up-regulate NEAT1 transcription to enhance nuclear paraspeckle formation, thus protecting cells from LRRK2-mediated damage. Intriguingly, gain-of-function mutations in LRRK2 cause an autosomal dominant, early-onset familial PD, especially in Ashkenazy Jews (6); LRRK2 sequestration by NEAT1 may be of higher importance in carriers of this mutation.

NEAT1 showed highly variable expression levels in the SN, which may reflect its reaction to a wide variety of stimuli, including estrogen (25), hepatic fibrosis (59), oxidative stress, and treatment with various therapeutics including simvastatin and fenofibrate (this study)—possibly through the effects of these drugs on the activity of transcription factors of the PPAR family (60, 61), binding sites for which and for their interactor retinoid X receptor-α lie upstream to the NEAT1 transcription start site (47, 62). This offers another mechanism to explain the neuroprotective traits of these drugs (16, 60, 63, 64) and calls for investigating the effects of additional blood-brain barrier–permeable agents on NEAT1 levels. However, although simvastatin and fenofibrate elevate NEAT1 expression, other widely used drugs may suppress its expression, which could be harmful in particular patients with PD, such as carriers of the LRRK2 mutation. Interestingly, SN dopaminergic neurons in women may be protected against cumulative damages because of the potentiation of NEAT1 expression by estrogen (25), possibly contributing to the lower frequency of PD in women compared with men (8). However, estrogen may also affect drug interactions with NEAT1 expression and the prospects of consequent neuroprotection.

In summary, the impact of PD on the one hand and prescription drug use on the other hand on NEAT1 expression and nuclear paraspeckle formation in the PD SN opens new venues for prevention and treatment of specific subgroups of patients with PD, based on attributes such as patients’ sex or LRRK2 mutations, and introduces an angle of precision medicine to the treatment of patients with PD with different underlying medical conditions. Investigating this option requires further studies in large clinical cohorts for acquiring population-level understanding and may lead to clinical recommendations indicating early treatment or otherwise tailored use of NEAT1-modulating drugs in patients who stand to benefit from such treatment at a stage when their SN neurons can still be protected.

ACKNOWLEDGMENTS

The authors acknowledge support by the Michael J. Fox Foundation for Parkinson’s Research (Grant 11183 to S.K. and H.S.); the Edmond and Lily Safra Center of Brain Sciences (ELSC) Postdoctoral Fellowship (to M.H.); the Israeli Ministry of Science, Technology, and Space (Grant 53140 to H.S.); and The Clore Foundation Scholars Fellowship (to A.S.). The authors appreciate the Netherlands Brain Bank contribution of brain tissues. The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

A. Simchovitz planned and performed most experiments; M. Hanan analyzed expression levels; N. Niederhoffer performed brain tissue immunohistochemistry; N. Yayon cooperated with N. Niederhoffer and produced Supplemen
tal Movie S1; N. Madrer performed SH-SY5Y experiments; E. R. Bennett performed Western blotting experiments; S. Kadener and H. Soreq provided scientific guidance; A. Simchovitz and H. Soreq wrote the paper;
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Received for publication March 31, 2019. Accepted for publication June 17, 2019.