Iranian Journal of Basic Medical Sciences
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CRISPR/Cas9-mediated knockout of NEAT1 attenuates neurotoxicity in 6-hydroxydopamine model of Parkinson's disease

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ARTICLE INFO

Article type:
Original

Article history:
Received: Nov 25, 2025
Accepted: Feb 24, 2026

Keywords:
Alpha-synuclein
Apoptosis
CRISPR/Cas9
Long non-coding RNA
MicroRNAs
NEAT1
Oxidative stress
Parkinson's disease

ABSTRACT

Objective(s): Parkinson's disease (PD), a progressive neurodegenerative condition, involves the degeneration of dopaminergic neurons and the aggregation of α-synuclein (SNCA); however, the molecular mechanisms of this disorder remain incompletely understood.

Materials and Methods: This study explored the role of the long non-coding RNA NEAT1 in PD by employing CRISPR/Cas9-mediated knockout in SH-SY5Y neuroblastoma cells treated with 6-hydroxydopamine (6-OHDA).

Results: Deletion of NEAT1 was associated with increased cellular viability, attenuated both cytotoxicity and apoptosis, and elevated total antioxidant capacity (TAC), alongside a marked down-regulation of SNCA expression. Mechanistically, NEAT1 knockout was accompanied by increased hsa-let-7a-5p and decreased miR-5[illegible] expression, suggesting its function as a competing endogenous RNA (ceRNA) in miRNA mediated stress pathways. Tyrosine hydroxylase (TH) levels remained unchanged, indicating that NEAT1 may influence neurotoxicity through post-transcriptional mechanisms.

Conclusion: These results suggest NEAT1 is a crucial modulator of neurotoxicity in PD, with its inhibition offering therapeutic promise. Despite the in vitro nature of this study, our findings provide foundational insight into NEAT1's dualistic roles in neurodegeneration and underscore its potential as a therapeutic target in PD.

▶ Please cite this article as
Samareh A, Nematollahi MH, Pourghadamyari H, Rastegar S, Ebrahimi H, Hadizadeh M, Wang H, Tao L, Sasan H, Asadikaram Gh. CRISPR/Cas9-mediated knockout of NEAT1 attenuates neurotoxicity in 6-hydroxydopamine model of Parkinson's disease. Iran J Basic Med Sci 2026; 29:

Introduction

Parkinson's disease (PD), the second most prevalent neurodegenerative disorder, involves the gradual degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc). This neuronal loss leads to motor dysfunctions, including bradykinesia, rigidity, tremors, and postural instability, as well as non-motor symptoms, such as cognitive decline, depression, and autonomic dysfunction. Pathologically, PD is characterized by dopamine depletion in the SNpc and intracellular accumulation of misfolded α-synuclein (SNCA) in the form of Lewy bodies (LBs), which are strongly associated with disease progression (1).

The global prevalence of PD has risen over the past decades. In 2019, an estimated 8.5 million individuals were affected by the disease. PD accounted for approximately 5.8 million disability-adjusted life years (DALYs), marking an 81% increase since 2000, and was responsible for 329,000 deaths, more than doubling since 2000 (2). While the exact etiology of PD remains elusive, accumulating evidence highlights a complex interplay between genetic susceptibilities and environmental exposures in the pathogenesis of the disease. Despite extensive research, the molecular mechanisms underlying PD remain incompletely

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understood, emphasizing the urgent need for novel therapeutic targets and treatment strategies (3).

Given the complexity of PD pathogenesis and the limited efficacy of current therapies, recent research has focused on non-coding RNAs, including long non-coding RNAs (lncRNAs), as key regulators in neurodevelopment, neuroprotection, and neurodegeneration (4). LncRNAs, typically exceeding 200 nucleotides in length, serve as molecular scaffolds, microRNA (miRNA) sponges, transcriptional regulators, and modulators of protein interactions (5). MiRNAs regulate gene expression by binding to the 3′ untranslated regions (3′UTRs) of messenger RNAs (mRNAs), leading to gene silencing at the post-transcriptional level. As competing endogenous RNAs (ceRNAs), lncRNAs sequester miRNAs, preventing their interaction with target mRNAs and thereby modulating gene expression. The interaction between lncRNAs and miRNAs forms a regulatory network that influences numerous biological processes, including cell growth, metabolism, differentiation, proliferation, and angiogenesis (6, 7). Dysregulation of lncRNAs has been implicated in multiple neurodegenerative diseases, including Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), and Huntington's disease (4). In the context of PD, several lncRNAs have been identified as key modulators of disease progression, influencing inflammation, oxidative stress, and mitochondrial function (4). Among these, nuclear paraspeckle assembly transcript 1 (NEAT1) has garnered significant attention due to its involvement in paraspeckle formation, RNA processing, and cellular stress responses (8).

NEAT1, a highly conserved lncRNA, plays a crucial role in the formation of paraspeckles, subnuclear structures that regulate gene expression and cellular homeostasis under stress conditions (8). NEAT1 is implicated in several cellular processes, including apoptosis, inflammation, and autophagy, all of which are relevant to PD pathology (9, 10). Furthermore, NEAT1 interacts with critical signaling pathways, including NF-κB and p53, which regulate inflammation and apoptosis, respectively (10, 11). Recent studies suggest that NEAT1 influences neuroinflammation through interactions with RNA-binding proteins and transcription factors responsible for cytokine expression (12). Elevated NEAT1 levels have been observed in neurodegenerative disease models, indicating its role in cellular stress responses (13). Additionally, NEAT1 has been associated with mitochondrial dysfunction and energy metabolism, both of which are known contributors to PD pathogenesis (14). Some studies indicate that NEAT1 may exacerbate neurodegeneration by modulating SNCA expression and interacting with PTEN-induced kinase 1 (PINK1), while others propose that it serves a protective function through the inhibition of leucine-rich repeat kinase 2 (LRRK2)-mediated toxicity (12, 15). However, despite these findings, the precise role of NEAT1 in PD remains unclear, and whether it exerts neuroprotective or neurotoxic effects is still under debate.

Our previous research demonstrated elevated NEAT1 expression and reduced levels of hsa-let-7a-5p and miR-506-3p in PD patients, suggesting their involvement in PD pathogenesis (16). To elucidate the role of NEAT1 in PD, this study aimed to investigate the effects of NEAT1 knockout in SH-SY5Y cells using CRISPR/Cas9 genome-editing technology. Subsequently, the impact of NEAT1 deletion was evaluated by analyzing gene expression, cell viability, oxidative stress, apoptosis, and cytotoxicity. These findings could provide valuable insights into the molecular mechanisms underlying PD and contribute to the development of novel therapeutic strategies.

Materials and Methods

Cell lines and culture

SH-SY5Y human neuroblastoma cell line (ATCC® CRL-2266™) was maintained in Dulbecco's Modified Eagle Medium (DMEM; Bioidea, Iran), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Bioidea, Iran) and 1% (v/v) penicillin-streptomycin (containing 100 IU/mL penicillin and 100 µg/ml streptomycin; Bioidea, Iran). Cultures were incubated at 37 ˚C in a humidified atmosphere with 5% CO_2.

Establishment of the Parkinson's disease in vitro model

To generate a PD-like neurotoxic model, SH-SY5Y cells were seeded at 3×10^4 cells/well in 48-well plates. After 24 hr, cells were treated with 6-hydroxydopamine (6-OHDA)(0-100 µM) dissolved in 15% vitamin C in phosphate-buffered saline (PBS) to maintain compound stability. Following 24 hours of exposure, cell viability was assessed using the MTT assay, and the half-maximal inhibitory concentration (IC_{50}) was determined. It is important to note that control cells were treated with 15% vitamin C alone to ensure experimental reliability.

6-OHDA is widely utilized in PD research due to its capacity to induce oxidative stress and endoplasmic reticulum (ER) stress, ultimately leading to neuronal cell death.

CRISPR/Cas9-based NEAT1 gene knockout

The Homo sapiens NEAT1 gene reference sequence was obtained from the GenBank database at the National Center for Biotechnology Information (NCBI). Specific single-guide RNAs (sgRNAs) were designed to target NEAT1 in SH-SY5Y cells for efficient knockout. Two distinct sgRNAs (sgRNA1 and sgRNA2) were chosen to induce double-strand breaks (DSBs) within the NEAT1 gene. The design of the sgRNAs was carried out using the CHOPCHOP (https://chopchop.cbu.uib.no) and CRISPOR (https://crispor.gi.ucsc.edu) online tools. sgRNAs were selected based on efficiency scores and minimal off-target effects (predicted via Cas-OFFinder).

The selected sgRNA target sequences were subsequently incorporated into an expression vector. For the construction of recombinant plasmids, the CRISPR/Cas9 expression vector pSpCas9(BB)-2A-Puro (PX459) V2.0 (Plasmid #62988; Addgene) was utilized to generate pX459-NEAT1-sgRNA. Additionally, the pSpCas9(BB)-2A-GFP (PX458) plasmid (Plasmid #48138; Addgene), which encodes the GFP gene, was employed as a transfection control.

The sequences of the designed sgRNAs are provided in Table 1.

Construction of recombinant sgRNA-expressing plasmids

The sgRNAs were annealed into double-stranded DNA via incubation at 95 ˚C for 5 min, followed by a gradual cooling from 85 ˚C to 30 ˚C at 0.6 ˚C/min using a thermocycler.

To linearize circular plasmids, the BbsI enzyme was

Table 1. Genetic sequences used in the current study

Primers	sgRNA1	FWD	5′-CACCCTGAGTTAGATGAGACGAGG-3′
		REV	5′-AAACCCTCGTCTCATCTAACTCAG-3′
	sgRNA2	FWD	5′-CACCGAACTGAACTTAGCTCGACG-3′
		REV	5′-AAACCGTCGAGCTAAGTTCAGTTC-3′
	hU6	FWD	5′-GAGGGCCTATTTCCCATGATT-3′
		REV	5′-TAGATGTACTGCCAAGTAGG-3′
	NEAT1	FWD	5′-GACCTCTCACCTACCCACCT-3′
		REV	5′-ATGCCCAAACTAGACCTGCC-3′
	TH	FWD	5′-CTCAGGAGCTATGCCTCACG-3′
		REV	5′-CACCTAGCCAATGGCACTCA-3′
	SNCA	FWD	5′-GTGCATGGTGTGGCAACAGT-3′
		REV	5′-CACTGTCTTCTGGGCTACTGC-3′
	GAPDH	FWD	5′-TGACTTCAACAGCGACACCCA-3′
		REV	5′-CACCCTGTTGCTGTAGCCAAA-3′
	hsa-let-7a-5p	FWD	5′-UGAGGUAGUAGGUUGUAUAGUU-3′
		REV	5′-GCTGCTACCTCGGACCCT-3′
	miR-506-3p	FWD	5′-AGAGGCACCCTTCTGAGTAGA-3′
		REV	5′-GCTGCTACCTCGGACCCT-3′
	U6	FWD	5′-ACAAGGATGACACGCAAATTCG-3′
		REV	5′-GCTGCTACCTCGGACCCT-3′
Stem-loops	hsa-let-7a-5p	5′-GTATGCTGCTACCTCGGACCCTGCTTAGTGCCATGCCT GCCATCGAGCAGCATACAACTAT-3′	
	miR-506-3p	5′-GTATGCTGCTACCTCGGACCCTGCTTAGTGCCATGCCT GCCATCGAGCAGCATACTCTACT-3′	
	U6	5′-GTATGCTGCTACCTCGGACCCTGCTTAGTGCCATGCCT GCCATCGAGCAGCATACCGAATT-3′	
Probe		5′-AGTGCCATGCCTGCCATCGAGC-3′	

employed at 37 ˚C for 24 hr, followed by inactivation at 90 ˚C. The annealed sgRNA was then ligated into the linearized plasmid using T4 ligase, with incubation carried out at 16 ˚C and 22 ˚C. The reaction was subsequently inactivated at 65 ˚C. The recombinant plasmid was purified through ethanol precipitation.

Bacterial transformation and plasmid verification

Recombinant plasmids were introduced into chemically competent *Escherichia coli DH5α* (prepared using $CaCl_2$ treatment) through heat shock, and transformants were selected based on ampicillin resistance. Plasmid extraction was performed using the FavorPrep Mini Kit, and the purity of the plasmid preparations was determined using a NanoDrop spectrophotometer.

The recombinant plasmids PX459-NEAT1-sgRNA1 and PX459-NEAT1-sgRNA2 were further validated via PCR. Colony PCR was conducted using a forward primer targeting the hU6 vector promoter and a reverse primer, as detailed in Table 1. Furthermore, the sequence of positive clones was confirmed by Sanger sequencing.

Transfection and selection of knockout cells

Cells were seeded onto six-well plates at a density of 3×10^5 and incubated for 24 hr. To evaluate whether the CRISPR/Cas9 system can target the NEAT1 gene for cleavage, SH-SY5Y cells were transfected with Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's protocol. The PX458 plasmid was utilized to monitor transfection efficiency.

After 48 hr, cells were subjected to puromycin selection (2 μg/ml) for 7 days. After puromycin selection, single-cell clones were isolated via limiting dilution in 96-well plates. Following transfection for the designated duration, cells were subsequently collected for downstream assays. The study investigated three distinct cell groups: (1) the scrambled control group, consisting of cells transfected with PX459 without inserted sgRNA; (2) the Knockout group (NEAT1-KO), comprising cells transfected with the PX459-sgRNA1 and PX459-sgRNA2 plasmids; and (3) the PX458 group, which included cells transfected with the PX458-GFP plasmid.

Confirmation of NEAT1 knockout

NEAT1 knockout in SH-SY5Y cells was confirmed at the transcript level using quantitative real-time polymerase chain reaction (RT-qPCR). Total RNA was extracted using TRIzol reagent, followed by phase separation with chloroform and RNA precipitation with isopropanol. The RNA was purified through ethanol washes and resuspended in nuclease-free water. RNA integrity was assessed via agarose gel electrophoresis, while purity and concentration were determined using spectrophotometric absorbance measurements. For cDNA synthesis, 500 ng of total RNA was reverse-transcribed using a high-capacity reverse transcription kit. RT-qPCR reactions were performed using SYBR Green dye, with GAPDH serving as the internal reference gene. The relative gene expression levels were quantified using the $2^{-\Delta\Delta CT}$ method.

After confirming the NEAT1 gene knockout and establishing the optimal 6-OHDA concentration, subsequent experiments examined its role in PD pathology. For this purpose, experimental groups were treated with the optimal concentration of 6-OHDA, and further analyses were conducted to assess the effects of NEAT1 Knockout in the PD model.

Gene expression analysis

This study investigated the expression profiles of tyrosine hydroxylase (TH), Alpha-Synuclein (SNCA), hsa-let-7a-5p, and miR-506-3p in scrambled control and NEAT1-KO groups following the induction of PD.

Total RNA was isolated using the TRIzol reagent, as described previously. For the expression analysis of TH and SNCA, cDNA was synthesized from 500 ng of total RNA. RT-qPCR was then performed using SYBR Green dye, with GAPDH serving as the internal reference gene. Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method.

For hsa-let-7a-5p and miR-506-3p expression analysis, cDNA synthesis was carried out using stem-loop primers and Moloney murine leukemia virus (MLV) reverse transcriptase. The RT-qPCR reaction followed a standardized protocol, incorporating a qPCR Probe Master Mix along with gene-specific primers. U6 was utilized as the reference gene. Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. Primer sequences for all analyzed genes are provided in Table 1.

Cell viability assay

The MTT assay, a widely used method for evaluating cell viability, was employed to quantify surviving cells in scrambled control and NEAT1-KO groups in a PD cell model. This analysis aimed to assess cell viability and

cytotoxic effects following treatment. To determine cell viability after exposure to 6-OHDA, 30 µl of MTT solution (5 mg/ml) was added to each well. The cells were incubated at 37 ˚C for 2 hr, after which the supernatant was carefully and thoroughly removed. Subsequently, 200 µl of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. After an additional 60-minute incubation period, absorbance was measured at 570 nm. Control groups included untreated cells and a well containing no cells to ensure reliable comparisons.

Cytotoxicity assessment via LDH release

Cytotoxicity levels in scrambled control and NEAT1-KO groups was assessed by measuring lactate dehydrogenase (LDH) release. Cellular damage was evaluated based on the LDH activity detected in the culture medium. Twenty-four hours post-treatment, LDH activity was quantified using a commercially available detection kit (Kiazist), following the manufacturer's protocol. Absorbance at 490 nm was recorded using a microplate reader to determine the extent of cellular injury.

Annexin V/PI apoptosis assay

Apoptosis levels were determined using an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) assay. SH-SY5Y cells (1×10^5 cells) were cultured in a 12-well plate and incubated for 24 hr. After 24 hr of treatment with 6-OHDA, the cells were suspended in 100 µl of binding buffer containing 10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, and 2.5 mM $CaCl_2$. To stain apoptotic and necrotic cells, FITC Annexin V (2 µg/ml) and PI (2.5 µg/ml) were added, and the cell suspension was gently mixed and incubated for 15 min at room temperature in the dark. Following this incubation, 400 µl of binding buffer was added to each tube, and flow cytometric analysis was performed within one hour. FITC and PI fluorescence signals were analyzed using a flow cytometer. Cells negative for both PI and Annexin V were classified as viable, cells positive for Annexin V but negative for PI were considered apoptotic, and cells positive for both Annexin V and PI were categorized as necrotic. Each experimental condition was tested in triplicate to ensure reproducibility.

Oxidative stress and antioxidant analysis

To evaluate oxidative stress levels, the supernatant of SH-SY5Y cells was collected 24 hr post-treatment for biochemical analysis. The total antioxidant capacity (TAC) was quantified using the FRAP method, which measures the reduction of ferric-tripyridyltriazine (Fe(III)-TPTZ) to its ferrous form (Fe(II)-TPTZ), with absorbance recorded at 593 nm. Superoxide dismutase (SOD) activity was assessed utilizing the Ransod kit, based on the reduction of nitro blue tetrazolium (NBT) to diformazan, monitored at 560 nm. Glutathione peroxidase (GPx) activity was determined through a coupled reaction with glutathione reductase, wherein NADPH oxidation was measured at 340 nm. Catalase (CAT) activity was analyzed via the reduction of dichromate in acetic acid upon reaction with hydrogen peroxide (H_2O_2), with absorbance measured at 570 nm. Malondialdehyde (MDA) levels were quantified using a modified Yagi method (17, 18), which involves a thiobarbituric acid reaction, subsequent extraction with n-butanol, and absorbance detection at 532 nm.

Statistical analysis

All experiments were performed using at least three independent biological replicates (n=3). Data are presented as mean±SEM. Normality was assessed using the Shapiro-Wilk test. Two-group comparisons were analyzed using an unpaired two-tailed Student's *t*-test, while comparisons among multiple groups were performed using one- or two-way ANOVA with appropriate *post hoc* corrections. Non-parametric tests were applied when normality assumptions were not met. Statistical analyses were conducted using SPSS software (version 22; IBM), with $P<0.05$ considered statistically significant.

Results

In silico sgRNA design

In this study, the NEAT1 sequence (NCBI Reference Sequence NR_028272.1, NR_131012.1) was retrieved, and two pairs of sgRNAs were designed to facilitate the knockout of the NEAT1 gene. Dual sgRNAs were selected to enhance knockout efficiency and minimize off-target effects. The target DNA sequences and their respective sgRNAs, along with the vector design strategy, including the cleavage site, length of the knockout fragment, and sgRNA binding regions, are illustrated in Figure 1A.

Following the construction of the sgRNA vector and bacterial transformation, individual colonies were screened to confirm the presence or absence of sgRNA inserts. Specifically, a single isolated colony was selected and subjected to PCR analysis to verify the incorporation of the gRNA sequence. Three distinct primer sets were employed, yielding amplicons of 700 bp, 457 bp, and 269 bp, thereby

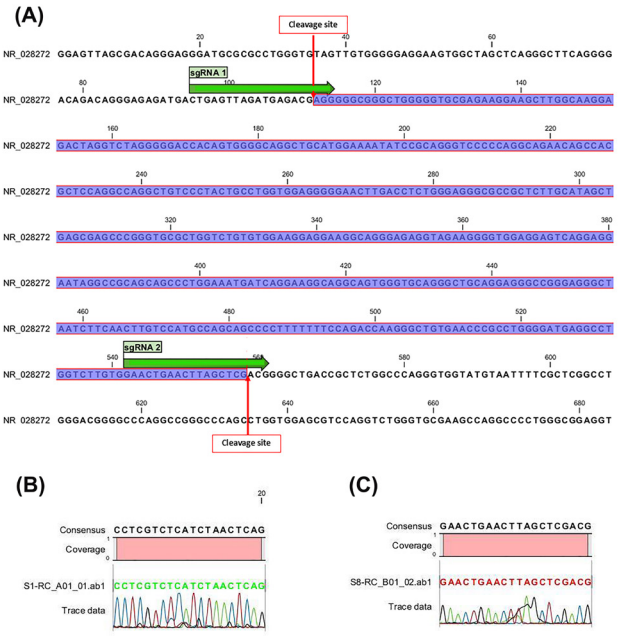

Figure 1. Design and validation of CRISPR/Cas9–mediated target cleavage and sgRNA integration

(A) Schematic representation of the target DNA loci, the corresponding single guide RNAs (sgRNA1, sgRNA2), their predicted Cas9 cleavage sites, the length of the excised knockout fragment, and the precise sgRNA binding regions. (B) Sequencing alignment of cloned sgRNA1 PCR product against the vector reference, demonstrating a 100% nucleotide match and correct integration. (C) Sequencing alignment of cloned sgRNA2 PCR product against the vector reference, also showing complete concordance with the intended construct

CRISPR; Clustered regularly interspaced short palindromic repeats, Cas9; CRISPR-associated protein 9, sgRNA; Single-guide RNA, PCR; Polymerase chain reaction

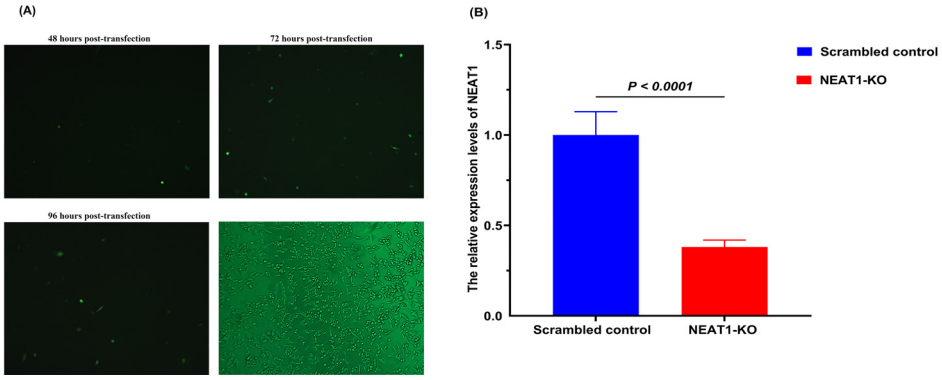

Figure 2. Validation of CRISPR/Cas9-mediated NEAT1 knockout

(A) Fluorescence microscopy images of SH-SY5Y cells transfected with PX458-GFP plasmid at 48, 72, and 96 hr post-transfection, showing optimal GFP expression at 72 hr. (B) Quantitative analysis of NEAT1 transcript levels in SH SY5Y cells following CRISPR–Cas9–mediated knockout. Results are shown as mean±SEM from n=3 independent biological replicates. Statistical analysis: unpaired two-tailed Student's t-test; $P<0.05$ is considered significant. The NEAT1 KO group exhibited a marked reduction in expression, confirming successful gene disruption at the mRNA level

CRISPR; Clustered regularly interspaced short palindromic repeats, Cas9; CRISPR-associated protein 9, SH-SY5Y; Human neuroblastoma cell line, PX458; Cas9-GFP expression plasmid, GFP; Green fluorescent protein, NEAT1; Nuclear paraspeckle assembly transcript 1, NEAT1-KO; NEAT1 Knockout

confirming the successful integration of the gRNA fragment into the plasmids and its amplification in *E. coli* DH5-Alpha. Subsequent sequencing and analysis demonstrated a 100% match between the cloned gRNA sequences and the intended vector constructs, ensuring precise and accurate integration (Figures 1B and 1C).

In vitro knockout of the NEAT1 gene

To assess the efficiency of transfection, a PX458-GFP-containing plasmid was introduced into the SH-SY5Y cell line. Following plasmid delivery, fluorescence microscopy was employed to assess the cells at 48, 72, and 96 hr post-transfection. The results indicated that the highest fluorescence intensity was observed at 72 hr after plasmid transfer (Figure 2A).

Plasmid-containing cells were selectively isolated by applying a concentration of 2 µg/ml puromycin, which effectively eliminated cells lacking the PX459 plasmid.

To evaluate the efficiency of NEAT1 knockout by CRISPR-Cas9 in SH-SY5Y cells, NEAT1 transcript levels were quantified by RT-qPCR. Analysis revealed a robust ~60% reduction in NEAT1 expression in the knockout group (0.40±0.038) compared to the scrambled control (1.00±0.13), which was statistically significant ($P<0.001$, Figure 2B). These data confirm that CRISPR-mediated deletion led to a substantial decrease in NEAT1 transcript levels, consistent with efficient gene disruption.

Determination of 6-OHDA IC50

The 7.5 µM optimal concentration of 6-OHDA was determined based on preliminary MTT assays showing ~50% cell viability reduction (IC50), consistent with prior studies (19). Consequently, this concentration was utilized in subsequent experiments to induce a PD model in the human neuroblastoma cell line SH-SY5Y.

Gene and miRNA expression profiles

TH enzyme gene expression demonstrated a modest increase in the NEAT1-KO group (1.82±0.41) relative to the scrambled control group (0.95±0.27); however, this difference was not statistically significant ($P>0.05$, Figure 3A). In addition, NEAT1 knockout was associated

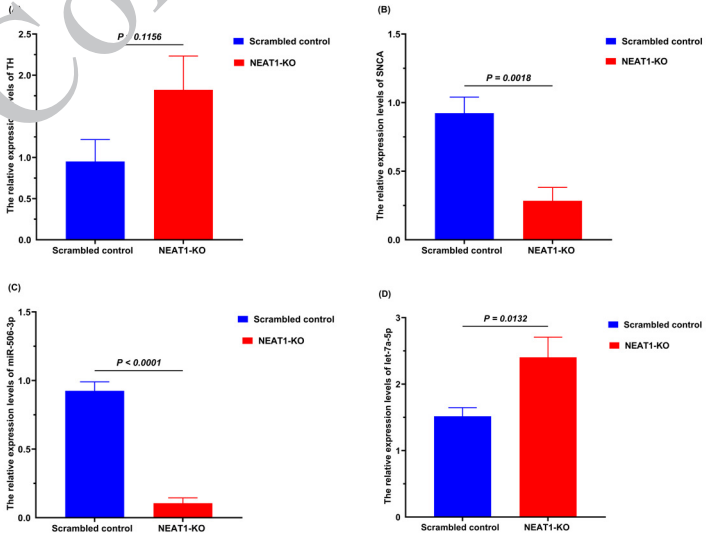

Figure 3. Expression levels of TH (A), SNCA (B), miR-506-3p (C), and hsa-let-7a-5p (D) following NEAT1 Knockout

Data are shown as mean±SEM from n=3 independent biological replicates (each qPCR was run in technical triplicate). Statistical analysis: one-way ANOVA followed by Tukey's *post hoc* test. $P<0.05$ is considered significant. The findings indicate that the knockout of NEAT1 resulted in a decreased expression of SNCA. Furthermore, in the knockout group, the expression level of miR-506-3p was markedly down-regulated, whereas the expression level of hsa-let-7a-5p exhibited an increase

TH: Tyrosine Hydroxylase; SNCA: Alpha-synuclein, NEAT1; Nuclear paraspeckle assembly transcript 1, SEM;Standard error of the mean, qPCR; Quantitative polymerase chain reaction

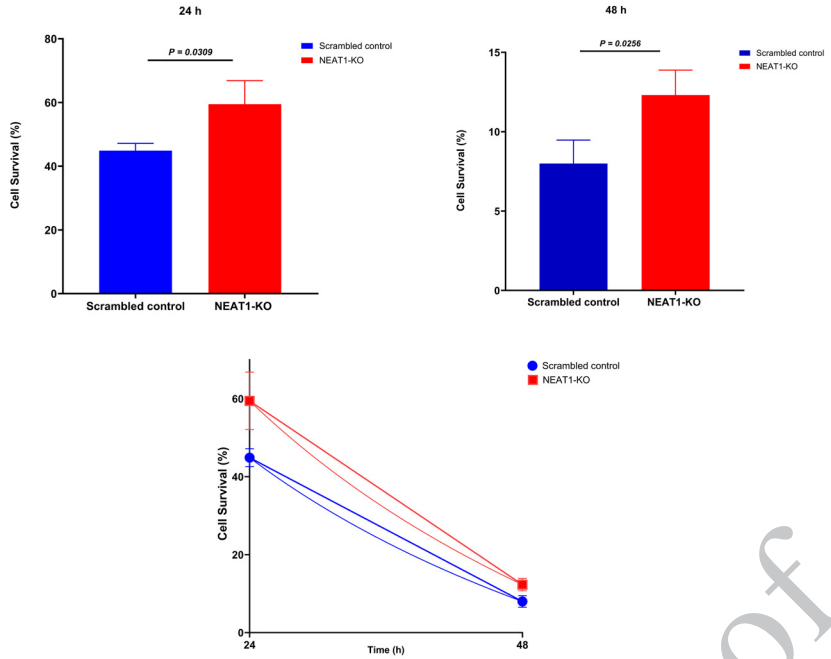

Figure 4. ENEAT1 knockout enhances cell viability following 6-OHDA treatment
Quantitative 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) analysis of cell viability at 24 and 48 h post-treatment. NEAT1-KO cells exhibit significantly increased survival compared to scrambled controls, suggesting a protective effect against 6-OHDA-induced cytotoxicity. Data represent mean±SEM from n=3 independent biological replicates; each biological replicate included triplicate wells. Statistical analysis: two-way ANOVA (treatment × time) with Bonferroni *post hoc* test; $P<0.05$ is considered significant
NEAT1; Nuclear paraspeckle assembly transcript 1, 6-OHDA; 6-hydroxydopamine, NEAT1-KO; NEAT1 knockout, SEM; Standard error of the mean

with reduced SNCA expression in the NEAT1-KO (0.28±0.1) compared to the scrambled control group (0.92±0.12, $P<0.05$, Figure 3B).

Furthermore, miR-506-3p expression exhibited a significant reduction in the knockout group (0.11±0.04) relative to the scrambled control group (0.93±0.07, $P<0.0001$, Figure 3C). Conversely, the results indicated a significant up-regulation of hsa-let-7a-5p in knockout cells (2.40±0.30) compared to the scrambled control group (1.52±0.12, $P<0.05$, Figure 3D).

Effect of NEAT1 knockout on cell viability

To assess NEAT1's effect on neuronal viability, the MTT assay was performed on NEAT1-KO and scrambled control plasmid (scrambled control). Cell survival was measured at 24 and 48 hr post-treatment.

The MTT assay demonstrated significantly higher viability in NEAT1-KO cells compared to scrambled controls at both 24 hr (59.46±4.26 vs. 44.89±1.31; $P<0.05$) and 48 hr (12.31±0.9 vs. 7.99±0.85; $P<0.05$) post-treatment (Figure 4).

Impact of NEAT1 gene deficiency on cytotoxicity: LDH assay

LDH is a stable cytoplasmic enzyme present in most cells, and it is rapidly released upon damage to the plasma membrane. Consequently, an increase in membrane damage or cell death leads to elevated LDH activity in the surrounding culture medium. As shown in Figure 5, a marked reduction in LDH activity was observed in NEAT1-deficient cells (32.89±5.58) relative to the scrambled control group (57.93±7.21; $P<0.05$).

Assessment of apoptosis by flow cytometry

To investigate the effect of NEAT1 knockout on apoptosis in the PD model, a flow cytometry-based analysis of living, apoptotic, and necrotic cells was performed. The experimental groups included control cells (NEAT1+/6-OHDA-), scrambled control cells (NEAT1+/6-OHDA+), and NEAT1 knockout cells (NEAT1-/6-OHDA+).

In the control group, where no 6-OHDA treatment was applied, the majority of cells remained viable, with minimal apoptosis and necrosis observed. However, upon 6-OHDA treatment, scrambled control cells showed a significant increase in apoptosis, as evidenced by a higher percentage of apoptotic cells. Notably, NEAT1 knockout cells (NEAT1-

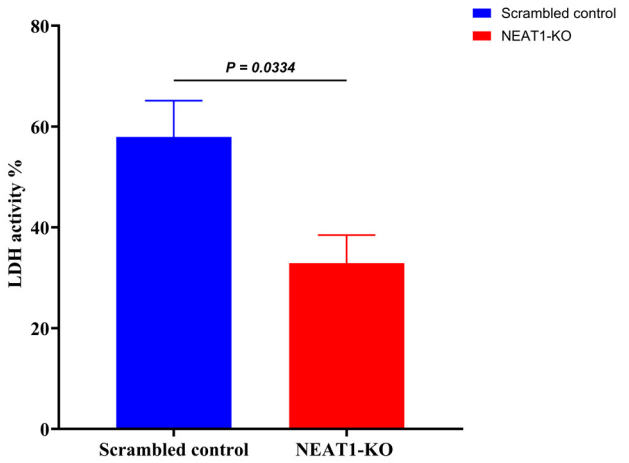

Figure 5. Reduced cytotoxicity in NEAT1-KO cells assessed by LDH release
LDH activity was significantly decreased in NEAT1-KO cells compared to scrambled controls, indicating improved membrane integrity and reduced cytotoxicity. Results are shown as mean±SEM from n=3 independent biological replicates (technical triplicates per experiment). Statistical analysis: one-way ANOVA with Tukey's *post hoc*; $P<0.05$ is considered significant; LDH: Lactate dehydrogenase
NEAT1; Nuclear paraspeckle assembly transcript 1, NEAT1-KO;NEAT1 Knockout, LDH; Lactate dehydrogenase, SEM; Standard error of the mean

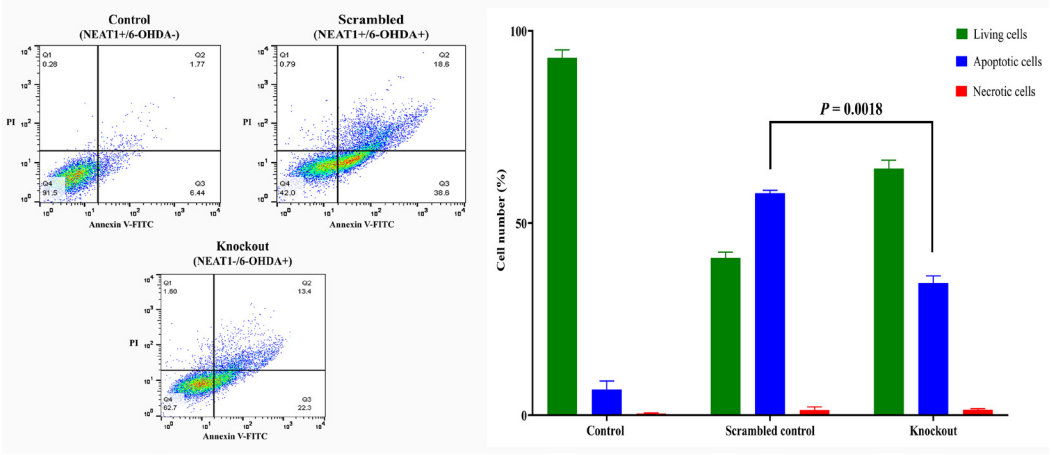

Figure 6. Flow cytometry analysis of apoptosis in treated SH-SY5Y cells
The experimental groups were categorized as follows: Control: untreated cells; Scrambled: cells subjected to treatment with 6-OHDA but lacking genetic modification (transduced with a vector lacking sgRNA); and knockout cells transduced with a vector containing sgRNA and treated with 6-OHDA. Results are shown as mean±SEM from n=3 independent experiments. Statistical analysis: one-way ANOVA with Tukey's *post hoc*; P<0.05 is considered significant. NEAT1 knockout markedly increased viable cells while reducing apoptotic populations relative to scrambled controls. The flow cytometry results were interpreted based on quadrant distribution: Q1: necrotic cells; Q2: late-stage apoptosis; Q3: early-stage apoptosis; Q4: viable cells
SH-SY5Y; Human neuroblastoma cell line, 6-OHDA; 6-hydroxydopamine, sgRNA; Single-guide RNA, SEM; Standard error of the mean

/6-OHDA+) demonstrated a 1.5-fold reduction in apoptosis compared to scrambled control cells (P<0.05). The proportion of viable cells was significantly higher in the knockout group, while apoptotic cell death was reduced (Figure 6).

Oxidative stress profile

To investigate the effects of NEAT1 knockout on oxidative stress-related parameters, the activities of CAT, SOD, and GPx, as well as the levels of TAC and MDA, were analyzed in knockout and scrambled control groups.

The measurement of oxidative stress factors revealed a significant increase in TAC in the NEAT1-KO group (200.1±24.16) compared to the scrambled control group (149.5±22.04), with the difference being statistically significant (P<0.05). However, no significant differences were observed between the two groups regarding the activities of CAT, SOD, GPx, and MDA levels (P>0.05, Figure 7).

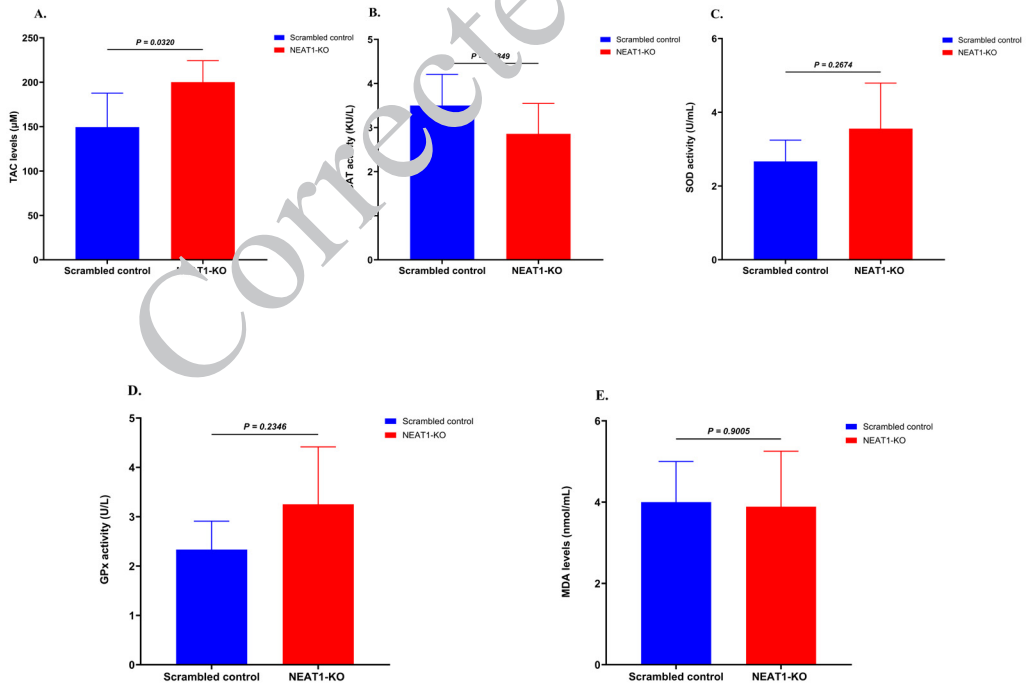

Figure 7. Comparison of oxidative stress markers between NEAT1-KO and scrambled control groups
The findings indicate that the TAC significantly increased following the knockout of NEAT1. However, no substantial differences were observed in other oxidative stress parameters between the compared groups. Data are shown as mean±SEM from n=3 independent biological replicates. Statistical analysis: one-way ANOVA with Tukey's post hoc test; P<0.05 is considered significant. A: TAC, B: CAT activity, C: SOD, D: GPx, E: MDA level; TAC: Total antioxidant capacity; SOD: Superoxide dismutase; GPx: Glutathione peroxidase; MDA: Malondialdehyde
NEAT1; Nuclear paraspeckle assembly transcript 1, NEAT1-KO; NEAT1 Knockout, TAC; Total antioxidant capacity, SOD; Superoxide dismutase, GPx; Glutathione peroxidase, MDA; Malondialdehyde, SEM; Standard error of the mean

Discussion

PD is a chronic and progressive neurodegenerative disorder primarily characterized by the degeneration of dopaminergic neurons within the SNpc and the pathological accumulation of SNCA protein in the brain. Although the precise etiology underlying neuronal loss remains unclear, both genetic predispositions and environmental influences are considered significant contributors to its onset and progression (1). At present, PD remains incurable, and current therapeutic strategies are predominantly focused on alleviating symptoms and enhancing the quality of life for affected individuals. Common treatment modalities include pharmacological approaches, such as levodopa and other oral medications, as well as surgical interventions, such as deep-brain stimulation. Despite their benefits, these interventions often lead to adverse effects, including cognitive impairments, levodopa-induced dyskinesias, and motor fluctuations characterized by on-off phenomena (20). Therefore, a better understanding of the cellular and molecular pathways implicated in PD pathogenesis is essential for the development of earlier diagnostic tools and more effective, targeted therapeutic interventions.

In recent years, emerging data suggest that both lncRNAs and miRNAs are pivotal intracellular molecular regulators whose dysregulation contributes significantly to the onset and progression of neurodegenerative diseases, including PD (21, 22).

Among these regulatory non-coding RNAs, NEAT1 has garnered particular interest due to its multifaceted roles in cellular homeostasis and neurodegeneration. Initially associated with multiple endocrine neoplasia (MEN) type 1, NEAT1 is now recognized as a key modulator of PD pathology (23). Functionally, NEAT1 plays a crucial role in the formation of nuclear paraspeckles and regulates gene expression under conditions of cellular stress (24). Numerous studies have demonstrated NEAT1's involvement in a variety of cellular processes such as proliferation, apoptosis, inflammation, migration, invasion, and neuronal function. These effects are mediated through its interaction with several key signaling pathways, including AKT, NF-κB, Notch, p53, and MAPK (25-27). Although NEAT1 has been extensively studied, its precise mechanisms in PD, particularly in human models, are not yet fully understood. NEAT1 expression has been found to be up-regulated in various PD models, implicating it in disease progression. However, its exact role, whether protective or pathogenic, remains controversial. Research findings suggest that NEAT1 can trigger multiple forms of programmed cell death (including autophagy, apoptosis, and ferroptosis) while also modulating inflammation and cellular viability via interactions with proteins such as PINK1 and BAX (14, 28, 29). Intriguingly, mass spectrometry analyses have revealed associations between LRRK2 and the paraspeckle-associated proteins, NONO and SFPQ, suggesting a potential interplay between NEAT1 and LRRK2 within paraspeckles. This interaction raises the possibility that NEAT1 may inhibit LRRK2 activity. Such inhibition could exert a neuroprotective function, particularly by mitigating oxidative stress (15). NEAT1 has emerged as a key regulator in various neurodegenerative disorders due to its involvement in RNA metabolism, the formation of stress granules, and the modulation of gene expression (30). As an essential structural component of paraspeckles, nuclear bodies that regulate gene expression during stress, NEAT1 may function as a molecular sponge, sequestering specific RNAs and proteins and thereby altering their bioavailability and downstream signaling. Dysregulation of NEAT1, therefore, can disrupt cellular homeostasis and contribute to the progression of neurodegeneration (30).

The let-7 family of microRNAs is well-documented for its roles in neuronal differentiation and inflammatory regulation. hsa-let-7a-5p, in particular, targets mRNAs involved in apoptotic and inflammatory signaling cascades. For example, a study demonstrates that let-7a can suppress α-Synuclein-induced inflammation in microglia by targeting the STAT3 signaling pathway in PD (31). Moreover, let-7a-5p has been associated with reduced cellular senescence in hippocampal neural stem cells (H-NSCs) by inhibiting the mechanistic target of rapamycin complex 1 (mTORC1) signaling, enhancing nuclear translocation of transcription factor EB (TFEB), and promoting lysosomal resumption (32). Furthermore, the secretion of let-7a-5p via mesenchymal stem cell-derived extracellular vesicles (MSC-EVs) underscores its therapeutic potential in enhancing neural regeneration and repair, primarily through modulation of the HMGA2/SMAD2 signaling axis (33). The findings of the present study offer novel insights into the molecular mechanisms underpinning NEAT1's role in PD. Specifically, the knockout of NEAT1 in SH-SY5Y neuroblastoma cell lines resulted in a significant up-regulation of the miRNA hsa-let-7a-5p. This suggests that NEAT1 may act as a ceRNA, modulating the activity of hsa-let-7a-5p through a sponge-like mechanism. The observed increase in hsa-let-7a-5p expression following NEAT1 knockdown may imply its involvement in pathways related to PD pathogenesis. These findings reinforce the hypothesis that let-7a-5p is a pivotal regulatory molecule in PD and may serve as a promising target for novel therapeutic strategies aimed at mitigating neurodegenerative progression.

Conversely, the present study shows a significant down-regulation of miR-506-3p expression in NEAT1 knockout cells relative to scrambled controls. Although the role of miR-506-3p in PD has not been extensively characterized, existing evidence indicates that this miRNA is implicated in cellular stress responses and synaptic plasticity. Notably, miR-506-3p targets key components of the Wnt/β-catenin signaling pathway, which plays a vital role in neuronal survival and function (34, 35). Its up-regulation has been associated with the modulation of oxidative stress and mitochondrial function, processes crucial to maintaining neuronal integrity (36). Furthermore, miR-506-3p has been identified as a potent promoter of neuroblastoma cell differentiation and is known to exert neuroprotective and anti-inflammatory effects through regulation of the CCL2/CCR2/NF-κB signaling axis (36, 37). Prior studies have also highlighted miR-506-3p as a key regulator of neural stem cell behavior via its targeting of TCF3, a transcription factor integral to NSC proliferation and differentiation. Notably, miR-506-3p expression is up-regulated during NSC differentiation, and its overexpression not only facilitates this process but also concurrently inhibits cell proliferation, indicating its dual role in regulating NSC fate. Furthermore, the exogenous introduction of miR-506-3p has been shown to suppress Wnt signaling, a downstream pathway modulated by TCF3, thereby emphasizing its role in the coordinated regulation of NSC proliferation

and differentiation. Collectively, these findings underscore the significance of miR-506-3p as a crucial molecular modulator in neural development, suggesting its potential utility in exploring the underlying mechanisms of nervous system formation (38).

These insights further support the notion that NEAT1 may engage in complex regulatory interactions with miRNAs such as miR-506-3p and hsa-let-7a-5p. Such interactions are likely components of broader regulatory networks regulated by lncRNAs, which govern a wide array of cellular processes. A deeper understanding of these molecular interplays may provide novel therapeutic avenues for the management and treatment of neurological disorders.

Previous studies have consistently reported a reduction in TH expression in experimental models of PD. For instance, a study demonstrated that the administration of MPTP, a neurotoxin that mimics PD-like symptoms, led to decreased TH-positive neurons in the mouse midbrain. Interestingly, in previous models, NEAT1 silencing was shown to mitigate this effect by increasing the number of TH-positive neurons, thereby implicating NEAT1 in the pathogenesis of dopaminergic neurodegeneration (12, 39). However, the current investigation found contrasting results, showing no significant alterations in TH expression following NEAT1 knockout. These findings suggest that NEAT1 knockout may influence TH protein stability through post-transcriptional modification and degradation pathways, revealing the multifaceted nature of NEAT1's function. Emerging evidence reveals that NEAT1 modulates cellular processes beyond transcriptional regulation. Specifically, NEAT1 functions as a molecular sponge for miRNAs, like miR-212-5p, which plays a pivotal role in regulating protein stability and degradation. Additionally, NEAT1 modulates autophagic pathways, potentially influencing protein turnover rates without affecting expression levels (40). The disparity between present findings and previous silencing studies underscores the intricate nature of NEAT1's functional role in dopaminergic neurons. While silencing approaches may temporarily reduce NEAT1 levels, complete knockout could activate compensatory mechanisms that maintain TH expression levels. This observation suggests that NEAT1's influence on PD pathology extends beyond simple transcriptional regulation, encompassing complex networks of post-transcriptional control and protein quality maintenance.

Also, the present study suggests that NEAT1 knockout in SH-SY5Y cells results in a significant down-regulation of SNCA expression compared to scrambled control cells, aligning with emerging evidence regarding the multifaceted role of NEAT1 in neurodegenerative disorders, particularly PD. Previous studies have reported elevated NEAT1 expression in PD models and post-mortem brain tissues of PD patients, implicating its involvement in disease pathogenesis (13, 15). However, the functional impact of NEAT1 appears to be context-dependent, with both up-regulation and down-regulation exhibiting neuroprotective effects in various experimental settings (12). Notably, NEAT1 has been implicated in the regulation of SNCA expression and aggregation, which are key pathological features of PD. The observed reduction in SNCA levels following NEAT1 knockout in this study corroborates prior findings suggesting that NEAT1 may influence disease progression through modulation of this protein (41). Furthermore, other lncRNAs, including MALAT1 and SNHG1, have similarly been shown to regulate SNCA dynamics, underscoring the broader regulatory role of lncRNAs in PD pathophysiology (12).

Experimental data from the current study further revealed the role of NEAT1 in neurodegeneration. In SH-SY5Y cells exposed to 6-OHDA, NEAT1 knockout resulted in significantly higher cell survival at both 24 and 48 hr compared to scrambled control cells, suggesting that NEAT1 may facilitate neurotoxicity under specific stress conditions. These data reinforce the notion that NEAT1 functions as a key regulator in PD models. Previous studies have also reported conflicting roles for NEAT1 in neurodegenerative contexts. For example, Simchovitz *et al.* demonstrated elevated NEAT1 expression in the substantia nigra of PD patients. Their study found that NEAT1 knockdown increased susceptibility to oxidative stress-induced cell death in SH-SY5Y cells exposed to paraquat and tert-butyl hydroperoxide, indicating a protective role for NEAT1 under certain oxidative conditions (15). Conversely, other investigations revealed that silencing NEAT1 in SH-SY5Y and SK-N-SH cells treated with amyloid-beta (Aβ) enhanced cell viability and reduced apoptosis, suggesting a deleterious effect of NEAT1 in the context of Aβ-induced neurotoxicity (42). Such inconsistencies may stem from differences in experimental models, stressor types, or time-dependent activation of cellular signaling cascades. It is plausible that NEAT1 exerts context-dependent functions, acting protectively under specific stress conditions while contributing to neuronal damage under others. Further research is imperative to unravel the molecular mechanisms underlying NEAT1's dualistic role in neuronal survival and degeneration.

The result of the present study demonstrated that CRISPR/Cas9-mediated knockout of NEAT1 in SH-SY5Y cells resulted in a significant reduction in cytotoxicity, as indicated by decreased LDH release following treatment with 6-OHDA. This suggests that NEAT1 knockout enhances membrane integrity and confers cellular protection under oxidative stress, potentially offering a neuroprotective effect in PD models. NEAT1, up-regulated in multiple neurodegenerative disorders, regulates stress responses by modulating inflammation, apoptosis, and mitochondrial dysfunction. Its ablation may reduce pro-death signaling and improve neuronal resilience by modulating apoptotic pathways (such as Bcl-2/Bax regulation), preserving mitochondrial function, or attenuating neuroinflammatory responses (12, 39, 43).

In this regard, the data of the current investigation indicate that NEAT1 knockout in SH-SY5Y cell lines leads to a significant reduction in apoptosis compared to the scrambled control group. Consistent with previous research, elevated NEAT1 expression has been linked to deleterious outcomes in PD models, whereas its silencing has been shown to enhance cell viability and diminish apoptosis in MPP^+-treated neuronal cells (41). The neuroprotective effect observed following NEAT1 knockout appears to involve multiple molecular pathways. Notably, several studies have reported that NEAT1 knockdown leads to reduced expression of SNCA, a protein frequently up-regulated in PD pathology (44). In addition, NEAT1 knockout has been associated with a decreased Bax/Bcl-2 ratio and reduced caspase activity, suggesting attenuated cellular stress and

suppression of apoptotic signaling (44). Moreover, its knockout suppresses pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α, thereby promoting neuronal survival (45). Interestingly, elevated NEAT1 levels have also been found to stabilize PINK1, a protein involved in mitochondrial dysfunction and autophagy. While autophagy is generally considered protective, excessive activation through NEAT1 overexpression may exacerbate neurodegenerative processes (14). Collectively, these observations suggest that targeting lncRNAs such as NEAT1 could be explored further as a strategy to modulate neurodegenerative processes, pending rigorous mechanistic and *in vivo* validation.

Additionally, our results demonstrate that NEAT1 knockout was associated with enhanced TAC in SH-SY5Y cells compared to scrambled controls. Interestingly, this elevation occurs in the absence of notable changes in the extracellular activity of canonical antioxidant enzymes, including GPx, SOD, CAT, or the lipid peroxidation marker MDA. These findings suggest a nuanced cellular response in which the knockout of NEAT1 modulates antioxidant defense through non-canonical pathways, potentially involving membrane integrity, molecular transport, or intracellular stress responses, rather than direct modulation of enzymatic activity. Furthermore, even with an elevated TAC, extracellular markers of oxidative stress remained constant. This, along with reduced cytotoxicity (as shown by lower LDH activity), suggests that the NEAT1 knockout may protect cells by stabilizing cellular membranes and controlling the passive efflux of antioxidants, or by activating efflux transporters like MRP1, which can export glutathione without altering extracellular enzyme activities (46).

The unchanged MDA further confirms that oxidative stress is not exacerbated, while the elevated FRAP signal highlight enhanced non-enzymatic antioxidant systems. Although analyses focused on extracellular media, intracellular antioxidant dynamics likely contribute indirectly to the observed TAC increase. The findings are consistent with NEAT1's critical role in regulating redox homeostasis, aligning with broader evidence that lncRNAs fine-tune cellular stress responses through integrated enzymatic and non-enzymatic mechanisms (47). The preservation of extracellular antioxidant enzyme levels, despite increased total antioxidant capacity, underscores the involvement of complex, finely tuned regulatory mechanisms encompassing both enzymatic and non-enzymatic antioxidant systems (47, 48). Considering NEAT1's significant role in stress responses and inflammatory processes, targeting NEAT1 represents a promising therapeutic strategy for diseases characterized by excessive cell death or OS.

Strengths and limitations

This study has several strengths that support the interpretation of the findings. A CRISPR/Cas9-based genetic loss-of-function approach was employed to disrupt *NEAT1* in a human neuronal cell model, enabling stable investigation of *NEAT1*-associated molecular and cellular responses in an established *in vitro* PD paradigm. The integration of gene expression analysis with multiple functional and oxidative stress assays allowed for a multidimensional assessment of cellular resilience to 6-OHDA-induced neurotoxicity, thereby strengthening the biological relevance of the observed associations. Collectively, this approach provides initial evidence linking *NEAT1* modulation to altered transcriptional profiles and neuroprotective cellular phenotypes.

This investigation also has the following limitations that should be considered when interpreting the findings. First, the work was conducted entirely in vitro using the SH-SY5Y neuroblastoma cell line and the neurotoxin 6-OHDA. While this is a well-established model for investigating Parkinson's disease-related pathways, it cannot fully recapitulate the complexity of the human brain or the systemic aspects of the disease. Therefore, the neuroprotective effects associated with NEAT1 knockout warrant further validation in in vivo models. Second, a key limitation is the absence of protein-level confirmation for critical targets. Although TH transcript levels were measured and no significant change following NEAT1 disruption was observed, mRNA abundance does not always correlate directly with protein abundance or enzymatic function due to post-transcriptional regulation. Thus, protein-level analyses, such as Western blotting or immunocytochemistry for TH and other key targets, including SNCA, are necessary to evaluate effects on dopaminergic integrity fully and to validate the reported transcriptional changes. Notwithstanding these limitations, the present study establishes a rationale for future in vivo and protein-level investigations as essential follow-up work.

Conclusion

In conclusion, our findings indicate that CRISPR/Cas9-mediated NEAT1 deletion is associated with cellular changes consistent with reduced neurotoxicity in a 6-OHDA *in vitro* model of PD. Specifically, the knockout of NEAT1 resulted in improved cell viability, reduced cytotoxicity and apoptosis, and an enhanced total antioxidant capacity. These functional changes were accompanied by a marked down-regulation of SNCA expression and differential modulation of key miRNAs, with an up-regulation of hsa-let-7a-5p and suppression of miR-506-3p, suggesting its role as a ceRNA in miRNA-mediated stress pathways. Although TH expression levels remained largely unaffected, the data suggest that NEAT1 may regulate post-transcriptional regulatory mechanisms that contribute to both neurodegenerative processes and cellular stress responses. Taken together, these findings identify NEAT1 as a candidate for future RNA-based therapeutic strategies and warrant further *in vivo* validation.

Acknowledgment

The results presented in this paper were part of a PhD student thesis

Funding

The current study was financially supported by the Kerman University of Medical Sciences (Grant No. KMU. AC.IR. 400001168) and Iran National Science Foundation (INSF)(Grant No. 4001872).

Availability of data and material

Data are available upon reasonable request from the corresponding authors, Dr Hossein Pourghadamyari and Dr Gholamreza Asadikaram.

Ethics Approval

The present study was approved by the Ethics Committee

of Kerman University of Medical Sciences (Ethics code: IR.KMU.REC.1401.508). All procedures were conducted in accordance with the ethical standards of the Declaration of Helsinki.

Authors' Contributions

A S provided methodology, performed all experiments, investigation, data collection and analysis, and drafted the manuscript. H P conceived the study, provided methodology, interpreted the data, and contributed to review and editing. G A performed supervision, conceived the study, provided continuous guidance throughout the study, and interpreted the data. MH N provided scientific support and contributed to review and editing. S R contributed to investigation and methodology. H E provided scientific support. M H contributed to investigation. H W and L T contributed to methodology. H S provided scientific support and contributed to methodology. All authors have approved the final version of the manuscript.

Conflicts of Interest

The authors have no relevant financial or non-financial interests to disclose.

Declaration

We have not used any AI tools or technologies to prepare this manuscript.

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