

Transcriptomics reveals the effects of NTRK1 on endoplasmic reticulum stress response-associated genes in human neuronal cell lines

Bo Jiao^{1,*}, Mi Zhang^{1,2,*}, Caixia Zhang¹, Xueqin Cao¹, Baowen Liu¹, Ningbo Li¹, Jiaoli Sun¹ and Xianwei Zhang¹

¹ Department of Anesthesiology, Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei Province, China

² Department of Anesthesiology, Zhongnan Hospital, Wuhan University, Wuhan, Hubei Province, China

* These authors contributed equally to this work.

ABSTRACT

Background: *NTRK1* gene, encoding TrkA, is essential for the nervous system and drives a variety of biological processes, including pain. Given the unsatisfied analgesic effects of some new drugs targeting *NTRK1* in clinic, a deeper understanding for the mechanism of *NTRK1* in neurons is crucial.

Methods: We assessed the transcriptional responses in SH-SY5Y cells with *NTRK1* overexpression using bioinformatics analysis. GO and KEGG analyses were performed, PPI networks were constructed, and the functional modules and top 10 genes were screened. Subsequently, hub genes were validated using RT-qPCR.

Results: A total of 419 DEGs were identified, including 193 upregulated and 226 downregulated genes. GO showed that upregulated genes were mainly enriched in response to endoplasmic reticulum (ER) stress, protein folding in ER, etc., and downregulated genes were highly enriched in a series of cellular parts and cellular processes. KEGG showed DEGs were enriched in protein processing in ER and pathways associated with cell proliferation and migration. The finest module was dramatically enriched in the ER stress response-related biological process.

The verified seven hub genes consisted of five upregulated genes (COL1A1, P4HB, HSPA5, THBS1, and XBP1) and two downregulated genes (CCND1 and COL3A1), and almost all were correlated with response to ER stress.

Conclusion: Our data demonstrated that *NTRK1* significantly influenced the gene transcription of ER stress response in SH-SY5Y cells. It indicated that ER stress response could contribute to various functions of *NTRK1*-dependent neurons, and therefore, ER stress response-associated genes need further study for neurological dysfunction implicated in *NTRK1*.

Submitted 27 December 2022

Accepted 21 March 2023

Published 12 April 2023

Corresponding author

Xianwei Zhang, ourpain@163.com

Academic editor

Nagendran Tharmalingam

Additional Information and
Declarations can be found on
page 14

DOI 10.7717/peerj.15219

© Copyright

2023 Jiao et al.

Distributed under

Creative Commons CC-BY 4.0

OPEN ACCESS

Subjects Genomics, Neuroscience

Keywords *NTRK1*, SH-SY5Y cells, Neurons, RNA sequencing, Endoplasmic reticulum stress, Unfolded protein response

INTRODUCTION

Neurotrophic tyrosine kinase receptor type 1 (*NTRK1*) gene encodes tropomyosin receptor kinase A (TrkA), a member of the neurotrophic tyrosine receptor kinase (NTRK) family, and is expressed in various tissues and organs, such as the nervous system, and it is located in the cytosol and vesicles in cells. TrkA, acted by nerve growth factor (NGF), plays a major part in embryonic neuronal development, and in several biological processes in adults, including pain, homeostasis, inflammation, and emotions and feelings (*Indo, 2018*). Encouragingly, the *NTRK* gene has been precisely targeted for anticancer treatment recently (*Klink et al., 2022*). These data suggested the powerful enchantment of the *NTRK1* gene. Although there are several mechanisms that were identified previously, the underlying mechanism of the *NTRK1* is still not fully understood.

Congenital insensitivity to pain with anhidrosis (CIPA) is caused by loss-of-function mutations in the *NTRK1* gene. It is characterized by insensitivity to pain, anhidrosis, mental retardation, and characteristic behaviors (*Li et al., 2019; Swanson, 1963*). Approximately one in 125 million people worldwide suffer from this rare syndrome. Only a few hundred cases have been described (*Cascella et al., 2022*). To date, about 128 *NTRK1* mutations have been reported in CIPA patients (*Lv et al., 2017*). We have previously reported a series of clinical phenotypes and mutation characteristics of CIPA patients, further expanding our knowledge about the function of the protein encoded by the *NTRK1* gene (*Li et al., 2019, 2018; Wang et al., 2015, 2016*). However, the neural mechanism behind various symptoms remains elusive. Given the fact that defects in *NTRK1* lead to CIPA, the *NTRK1* is crucial to nociceptive development. On the other hand, the excessive signaling of *NTRK1* results in chronic pain by hypersensitizing pain-mediating neurons (*Hefti et al., 2006*). Remarkably, a growing body of pre-clinical evidence showed that blocking NGF and/or TrkA can alleviate pain and hyperalgesia (*Hsieh et al., 2018; Tabata et al., 2012; Ugolini et al., 2007; Wu et al., 2016*). Clinically, Tanezumab and Fasinumab are the most advanced analgesics targeting the NGF-TrkA signaling pathway in recent years. While, NGF/TrkA inhibitors provided less pain relief than was initially anticipated. Despite a series of setbacks, including side effects and the FDA halting the study at one point, the agency reopened the NGF/TrkA antibody lately. Due to the great value of TrkA/*NTRK1* for pain treatment, and the dilemma in the new drug development, the specific mechanism of *NTRK1* in neurons needs to be explored urgently.

At present, the specific mechanism of *NTRK1* in neurons are unclear. *NTRK1* is essential for nociceptive sensory and sympathetic neurons to survive, and programmed cell death occurs when neurons lack *NTRK1* (*Levi-Montalcini, 1987*), which hampered studies in depth to a certain degree. In this study, we expressed exogenous *NTRK1* in human SH-SY5Y cells, a neuronal model that does not express TrkA receptors, to study the specific *NTRK1* signal transduction pathway. We obtained a series of differentially expressed genes (DEGs) with *NTRK1* overexpression compared with the control by high-throughput RNA sequencing (RNA-seq). Subsequently, the functionalities of DEGs were predicted using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses; then, the hub genes in the protein interaction network (PPI) were

obtained. The results may provide an important clue to elucidate various functions of *NTRK1*-dependent neurons and neurological dysfunction implicated in *NTRK1* including CIPA and chronic pain.

MATERIALS AND METHODS

Cloning and plasmid construction

Overexpressed *NTRK1* lentiviruses were purchased from GenePharma Co., Ltd. (Suzhou, China). Transcript information included [NM_002529.4](#), and the lentiviral vector was LV5 (EF-1a/GFP/Puro/Amp).

Cell culture and transfection

The SH-SY5Y cell lines (Procell Life Science & Technology Co., Ltd., Shanghai, China) were cultured at 37 °C with 5% CO₂ in a minimum essential medium (MEM)/F12 (Procell Life Science & Technology Co., Ltd., Wuhan, China) supplemented with 10% fetal bovine serum (FBS; Gibco, New York, NY, USA), 100 µg/mL streptomycin, and 100 U/mL penicillin. SH-SY5Y cells were infected with lentiviruses (multiplicity of infection (MOI) = 35). Infected cells harvested for 48 h were subjected to quantitative reverse transcription-polymerase chain reaction (RT-qPCR), Western blot, and Cell Counting Kit-8 (CCK-8) assays.

Assessment of gene overexpression

NTRK1 overexpression was evaluated using Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control gene. In the present study, cDNA synthesis was performed by standard procedures, and RT-qPCR was carried out on the Bio-Rad S1000 with Hieff® qPCR SYBR® Green Master Mix kit (Low Rox Plus; YEASEN, Shanghai, China). As shown in [Table S1](#), the primer sequences were listed. Using the $2^{-\Delta\Delta CT}$ method, the transcript concentration was normalized to the GAPDH mRNA level ([Livak & Schmittgen, 2001](#)). By using GraphPad Prism, we conducted comparisons *via* the paired Student's t-test (GraphPad Software Inc., San Diego, CA, USA).

Western blotting

In ice-cold lysis buffer (1× phosphate-buffered saline (PBS), 0.1% sodium dodecyl-sulfate (SDS), 0.5% NP-40, and 0.5% sodium deoxycholate) supplemented with a protease inhibitor cocktail (Roche, Basel, Switzerland), SH-SY5Y cells were lysed and incubated on ice for 30 min. Afterward, we boiled samples with 1× SDS sample buffer for 10 min, separated them by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred them to membranes. With TBST buffer (20 mM Tris-buffered saline and 0.1% Tween-20) containing 5% non-fat milk powder, membranes were incubated for 1 h at room temperature with FLAG (1:1,000; Cat. No. 2368S; Cell Signaling Technology Inc., Danvers, MA, USA), GAPDH (1:5,000; Cat. No. 60004-1-Ig; Proteintech, Rosemont, IL, USA), GRP78 (1:3,000; Cat. No. 11587-1-AP; Proteintech, Rosemont, IL, USA), p-IRE1 (1:500; Cat. No. AF7150; Affinity, West Bridgford, UK), XBP1s (1:2,000; Cat. No. 24868-1-AP; Proteintech, Rosemont, IL, USA), and ATF6 (1:1,000; Cat. No. DF6009; Affinity, West

Bridgford, UK) primary antibodies, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody. Enhanced chemiluminescence reagent (ECL; Cat. No. 170506; Bio-Rad Laboratories Inc., Hercules, CA, USA) was used to visualize the immunoblots.

RNA extraction and sequencing

In SH-SY5Y cells, total RNA was extracted with TRIzol reagent (Cat. No. 15596026; Invitrogen, Carlsbad, CA, USA), as described by [Chomczynski & Sacchi \(1987\)](#). After RNA extraction, DNaseI was used to digest DNA. With the Nanodrop™ One Cspectrophotometer system (Thermo Fisher Scientific Inc., Waltham, MA, USA), RNA quality was assessed using A260/A280. Electrophoresis of 1.5% agarose gel was used to confirm RNA integrity. Final quantification of qualified RNAs was performed with the Qubit™ RNA Broad Range Assay kit (Cat. No. Q10210; Life Technologies Corp., Carlsbad, CA, USA) using the Qubit 3.0 Fluorometer.

In addition, KCTM Stranded mRNA Library Prep kit for Illumina (Cat. No. DR08402; Wuhan Seqhealth Co., Ltd., Wuhan, China) was used to prepare stranded RNA sequencing libraries. On the Novaseq 6000 sequencer (Illumina; Chicago, IL, USA) with PE150 model, 200–500 bps PCR products were enriched, quantified, and finally sequenced.

RNA-seq data processing

First, raw reads with more than 2-N bases were discarded. Then, FASTX-Toolkit (Ver. 0.0.13) was used to trim adaptors and low-quality bases from raw sequencing read. The short reads <16-nt were also dropped. After that, An alignment of clean reads to the GRCh38 genome was performed by HISAT2 ([Kim, Langmead & Salzberg, 2015](#)) with four mismatches allowed. Reads that were uniquely mapped were counted and fragments per kilobase of transcript per million fragments mapped (FPKM) were calculated ([Trapnell et al., 2010](#)).

CCK-8 assay

Cell proliferation was measured using a CCK-8 assay kit (MCE Co., Ltd., Beijing, China). SH-SY5Y cells were seeded into 96-well plates in triplicate at a density of 2×10^4 cells/well and cultured for 72 h. At 37 °C for 2 h, the cells were then incubated in 10% CCK-8 solution diluted in the MEM/F12. An enzyme-labeling instrument (Bio-Rad Laboratories Inc., Hercules, CA, USA) was used to measure the absorbance (optical density (OD) value) at 450 nm wavelength.

Analysis of DEGs

DEGs were screened with DESeq2 from R Bioconductor ([Love, Huber & Anders, 2014](#)). To identify DEGs, we used a *P* value of 0.05 and a fold-change (FC) of 1.5 or 0.67 as cut-off criteria.

Functional enrichment analysis

With KOBAS 2.0, GO and KEGG pathway enrichment analyses were conducted to sort DEGs into functional categories ([Xie et al., 2011](#)). To determine the enrichment of each

term, the hypergeometric test and Benjamini-Hochberg false discovery rate (FDR) controlling procedure were applied.

PPI network construction, modules, and identification of top 10 genes

Search tool for the retrieval of interacting genes (STRING) database (<http://stringdb.org/>) (Szklarczyk *et al.*, 2019) was employed to construct PPI networks by mapping the DEGs into PPI data. The symbols of the DEGs were imported into the database, and high-resolution bitmaps were generated. The bitmap only included interactors with a combined confidence score of 0.4 or higher. The hotspot module was obtained in large PPI networks by the Molecular Complex Detection (MCODE) which is a Cytoscape plugin. In this study, MCODE parameters were as follows: the degree of cut-off = 2; cluster finding, haircut; node score cut-off = 0.2; k-core = 2; and the maximum depth = 100 (Bader & Hogue, 2003). The modules with established score >9 and the number of nodes >9 were screened out. The GO enrichment analysis was used to analyze genes in the modules by DAVID. CytoHubba, a plugin for Cytoscape 3.9.1, was used to calculate the degree of connectivity and identify the top 10 genes in the PPI networks (Shannon *et al.*, 2003). The PPI networks, modules, as well as the top 10 genes were visualized based on their node degree using Cytoscape software.

RT-qPCR for validation of the hub genes

An RT-qPCR analysis was performed and normalized with GAPDH to clarify the validity of the top 10 hub genes. RT-qPCR was performed using the same cell lines as RNA-seq. For the amplification program, denaturing was carried out for 30 s at 95 °C, followed by 40 cycles of denaturing at 95 °C for 10 s and 60 °C for 30 s, then annealing and extension at 95 °C for 15 s, 60 °C for 60 s, and 95 °C for 15 s. For each sample, the experiment was conducted in triplicate. Table S1 illustrates the list of the primer sequences used for RT-qPCR analysis.

Functional annotation of the hub genes

The ClueGO (ver. 2.5.8) and CluePedia (ver. 1.5.8) Cytoscape plugins were performed to decipher the enrichment analysis of the hub genes. GO-biological process (GO-BP) and pathways were considered statistically significant at $P < 0.05$.

Statistical analysis

According to RNASeqPower, this experimental design has a statistical power of 0.96. The cell biology and RT-qPCR data between the two groups were compared using an unpaired two-tailed t-test. All values were presented as the mean \pm standard deviation (SD). Except for the RNA-seq experiment, each experiment was performed at least three times. There was a statistically significant difference at $P < 0.05$.

RESULTS

***NTRK1* overexpression promoted the proliferation of SH-SY5Y cells**

A lentiviral vector expressing the *NTRK1* gene (*NTRK1*-OE) or an empty lentiviral vector (not expressing the *NTRK1* gene) (NC) was used to transfect SH-SY5Y cells. The results of

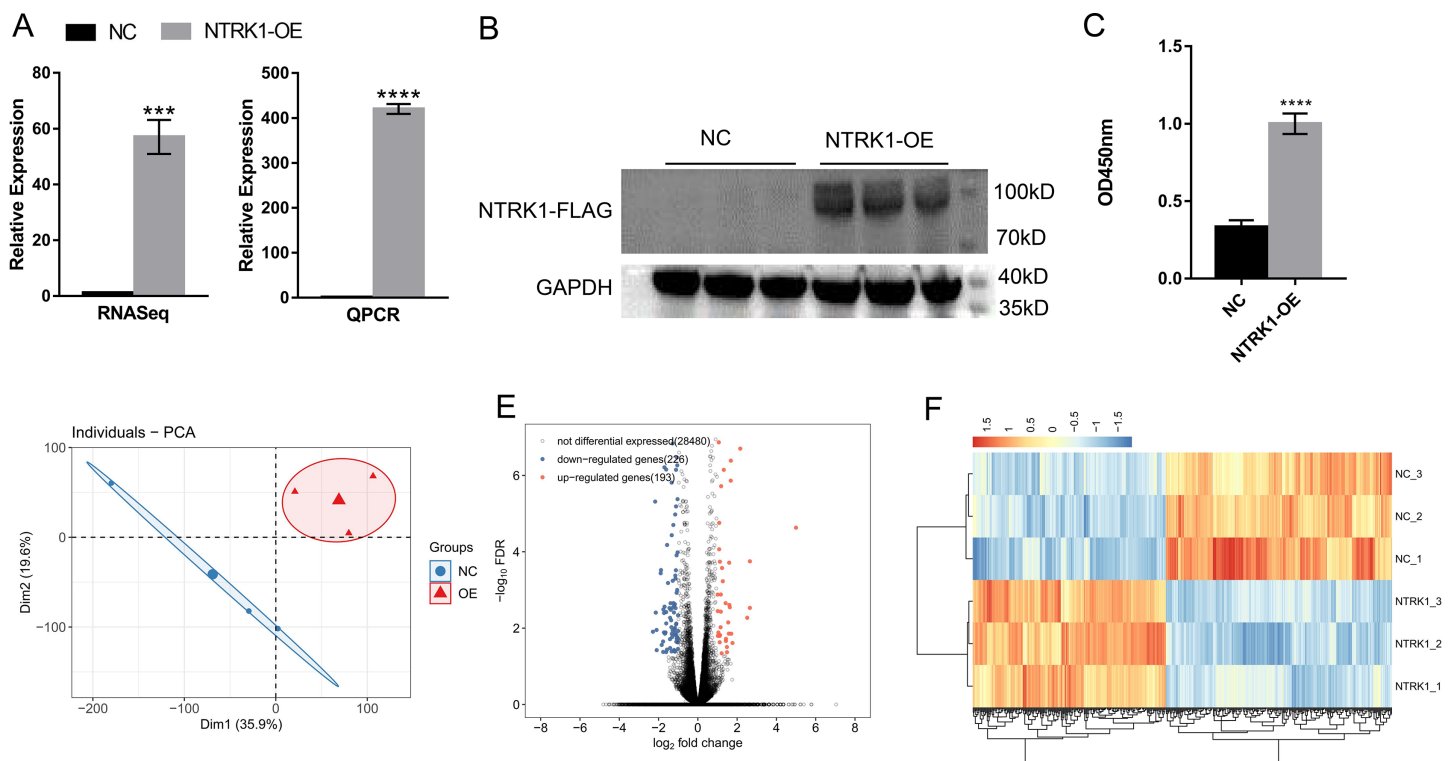


Figure 1 Analysis of the change in gene expression in response to *NTRK1* overexpression in SH-SY5Y cells. (A) Bar plot showing the expression levels of *NTRK1* in RNA-seq data and RT-qPCR results. (B) *NTRK1* was overexpressed and verified by Western blotting. Full-length blots/gels are presented in Figs. SB1 and SB2. (C) *NTRK1* overexpression increases the proliferation of SH-SY5Y cells. (D) PCA base on FPKM value of all detected genes. The ellipse for each group is the confidence ellipse. (E) Volcano plot showing all differentially expressed genes (DEGs) between OE and NC samples with DESeq2. FDR < 0.01 and FC (fold change) ≥ 1.5 or ≤ 0.67 . (F) Hierarchical clustering heat map showing expression levels of all DEGs. Error bars represent mean \pm SEM. ***P-value < 0.001. ****P-value < 0.0001. [Full-size DOI: 10.7717/peerj.15219/fig-1](https://doi.org/10.7717/peerj.15219/fig-1)

the RNAseq, RT-qPCR, and Western blotting showed that *NTRK1* was significantly overexpressed in SH-SY5Y cells (Figs. 1A and 1B). Moreover, the overexpression of *NTRK1* significantly promoted SH-SY5Y cell proliferation (Fig. 1C). The results validated that *NTRK1*-OE was successfully constructed and *NTRK1* favored the survival of the neurons.

NTRK1 overexpression altered the gene expression profiles in SH-SY5Y cells

RNA-seq was performed to examine the *NTRK1*-mediated transcriptional regulation in SH-SY5Y cells. In total, six RNA-seq libraries were prepared from *NTRK1*-OE and NC SH-SY5Y cells, with three biological replicates per group (*NTRK1_1*, *NTRK1_2*, and *NTRK1_3*; NC_1, NC_2, and NC_3). After removing low-quality reads and sequence adaptors, a clean pair-end read was obtained for 69.1 million samples on average. Approximately 62.8 million read pairs were uniquely mapped per sample to the human genome (Table S2).

Furthermore, gene expression was calculated using these uniquely mapped reads. A proprietary pipeline was used to calculate FPKM, which represents gene expression levels.

There were 22,154 genes expressed (FPKM > 0), and 10,364 genes expressed at FPKM > 1 in at least one sample (Table S3). A correlation matrix using FPKM values for all 22,154 genes was calculated using Pearson's correlation value (more than 0.99) between *NTRK1*-OE and the control, which indicated the similar expression of most genes. Moreover, *NTRK1*-OE and NC samples were clearly differentiated by principal component analysis (PCA) based on FPKM values for all detected genes (Fig. 1D), and revealed that SH-SY5Y cells overexpressing *NTRK1* showed altered gene expression profiles.

For further analysis of *NTRK1*'s transcriptional regulation, DESeq2 was performed to identify the genes that are differentially expressed between *NTRK1*-OE and NC cells, with a cut-off FC ≥ 1.5 or ≤ 0.67 and an FDR of 5%. The gene expression level in SH-SY5Y cells was extensively regulated by *NTRK1* overexpression (Fig. 1E), as shown by 193 upregulated and 226 downregulated genes (Table S4). Additionally, the *NTRK1*-OE and NC samples were clearly distinct in the hierarchical clustering analysis of normalized FPKM values of DEGs, and the three replicate datasets also showed a high level of consistency (Fig. 1F). These results indicated that *NTRK1* overexpression significantly changed the transcript expression level of a series of genes in neurons.

***NTRK1* overexpression affected the biological process in SH-SY5Y cells**

The GO and KEGG pathway enrichment analyses of all 419 DEGs were carried out to determine their potential roles. A significant increase in the expression levels of 193 genes was observed. According to the GO enrichment analysis, these genes were mostly enriched in response to endoplasmic reticulum (ER) stress, protein folding in ER, IRE1-mediated unfolded protein response (UPR), collagen fibril organization, ATF6-mediated UPR, cellular protein metabolic process, response to unfolded protein, ER unfolded protein response, folic acid metabolic process, and extracellular matrix (ECM) organization (Fig. 2A). The KEGG pathway analysis further confirmed that the upregulated genes were highly enriched in protein processing in ER (Fig. 2C). Moreover, upregulated genes were also highly enriched in a range of biochemical processes and p53 signaling pathway. A total of 226 genes downregulated by *NTRK1* were mainly enriched in a series of cellular parts and cellular processes, and pathways associated with cell proliferation and migration, such as Dopaminergic synapses, ECM-receptor interactions, transforming growth factor- β (TGF- β) signaling pathway, and Wnt signaling pathway (Figs. 2B and 2D). Overall, these data suggest that a range of biological processes are affected by *NTRK1* overexpression in human neuronal cell lines, in which response to ER stress occupies great importance.

PPI network analysis and hot modules

A PPI network based on the DEGs was constructed on STRING analysis for functional association and sequentially visualized on Cytoscape, and the results are shown in Fig. 3A. Furthermore, there are 294 nodes and 1,062 edges in the network.

Using the Cytoscape plugin "MCODE", two functional modules (modules 1 and 2) were detected with scores >9 and nodes >9. Module 1 covered 143 edges and 23 nodes, with a

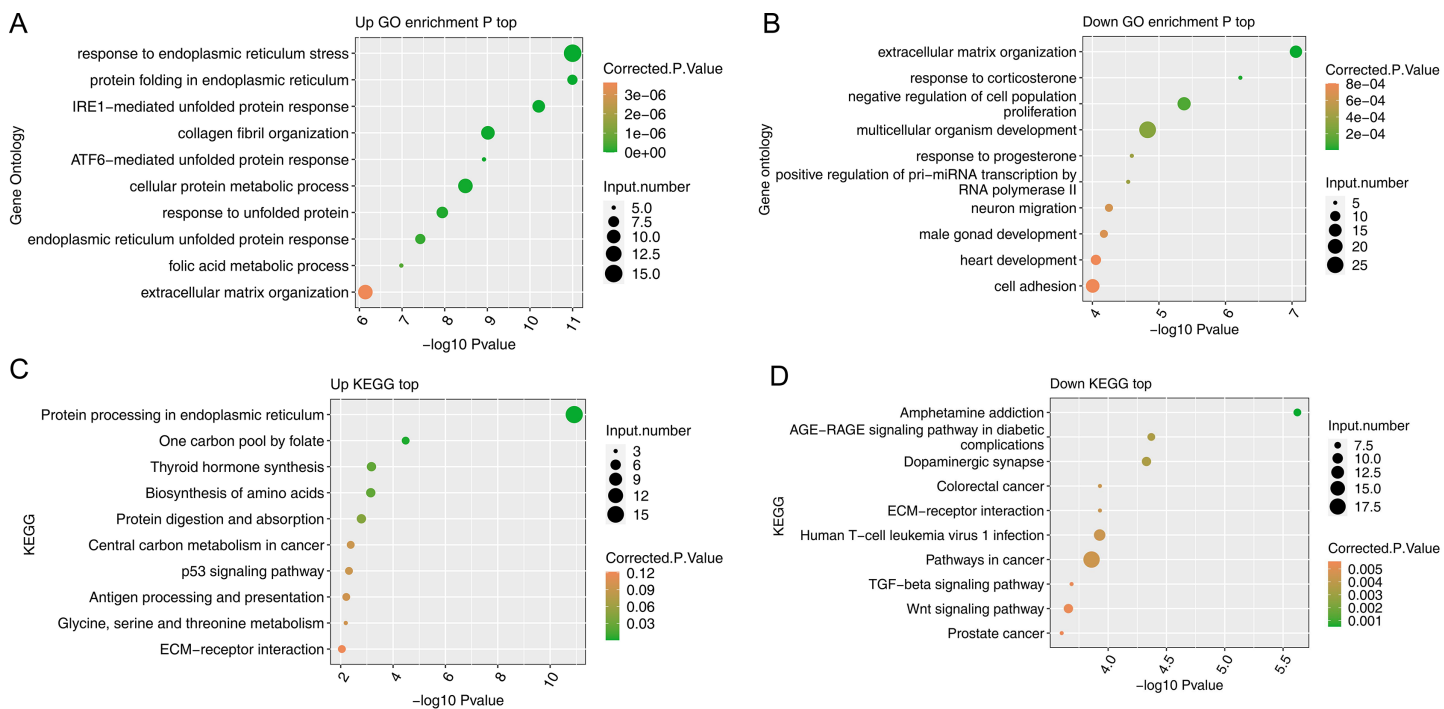


Figure 2 Functional analysis of DEGs after *NTRK1* overexpression in SHSY-5Y cells. (A–D) Bubble diagram exhibiting the most enriched GO biological process and KEGG pathways results of the up or down-regulated DEGs. [Full-size !\[\]\(5f471a71b78d7676bc356df190b88ab4_img.jpg\) DOI: 10.7717/peerj.15219/fig-2](https://doi.org/10.7717/peerj.15219/fig-2)

score of 13, containing 21 upregulated and two downregulated genes (Fig. 3B). Module 1 functional enrichment analysis revealed that ER stress-related functions dominated the most significant enrichment results in GO-BP (Table 1). Having 42 edges and 10 nodes, module 2 scored 9.33, with 10 genes downregulated (Fig. 3C), and these genes were mainly enriched in mitotic sister chromatid segregation (Table 1). The results of hot modules in the PPI network suggest that ER stress-related functions exert vital roles in neurons with *NTRK1* overexpression.

Identification of the top 10 genes and validation of hub genes

Using the cytoHubba plugin, the top 10 target genes were identified based on scores, and interactors of the top 10 genes were reconstructed (Fig. 3D). These top 10 genes with detailed information are presented in Table 2.

The expression levels of the top 10 genes were quantified using RT-qPCR to verify the effects of *NTRK1*-OE on them. Overall, seven hub genes were verified, including five upregulated and two downregulated DEGs (Fig. 4A). The upregulated DEGs included COL1A1 ($|\log_2FC| = 0.63$), P4HB ($|\log_2FC| = 0.65$), HSPA5 ($|\log_2FC| = 1.27$), THBS1 ($|\log_2FC| = 0.73$), and XBP1 ($|\log_2FC| = 0.90$); the downregulated DEGs included CCND1 ($|\log_2FC| = 0.73$) and COL3A1 ($|\log_2FC| = 1.31$). Hub genes tended to be enriched in terms of ‘response to endoplasmic reticulum stress’, ‘cellular response to transforming growth factor beta stimulus’, and ‘collagen fibril organization’ as the biological process

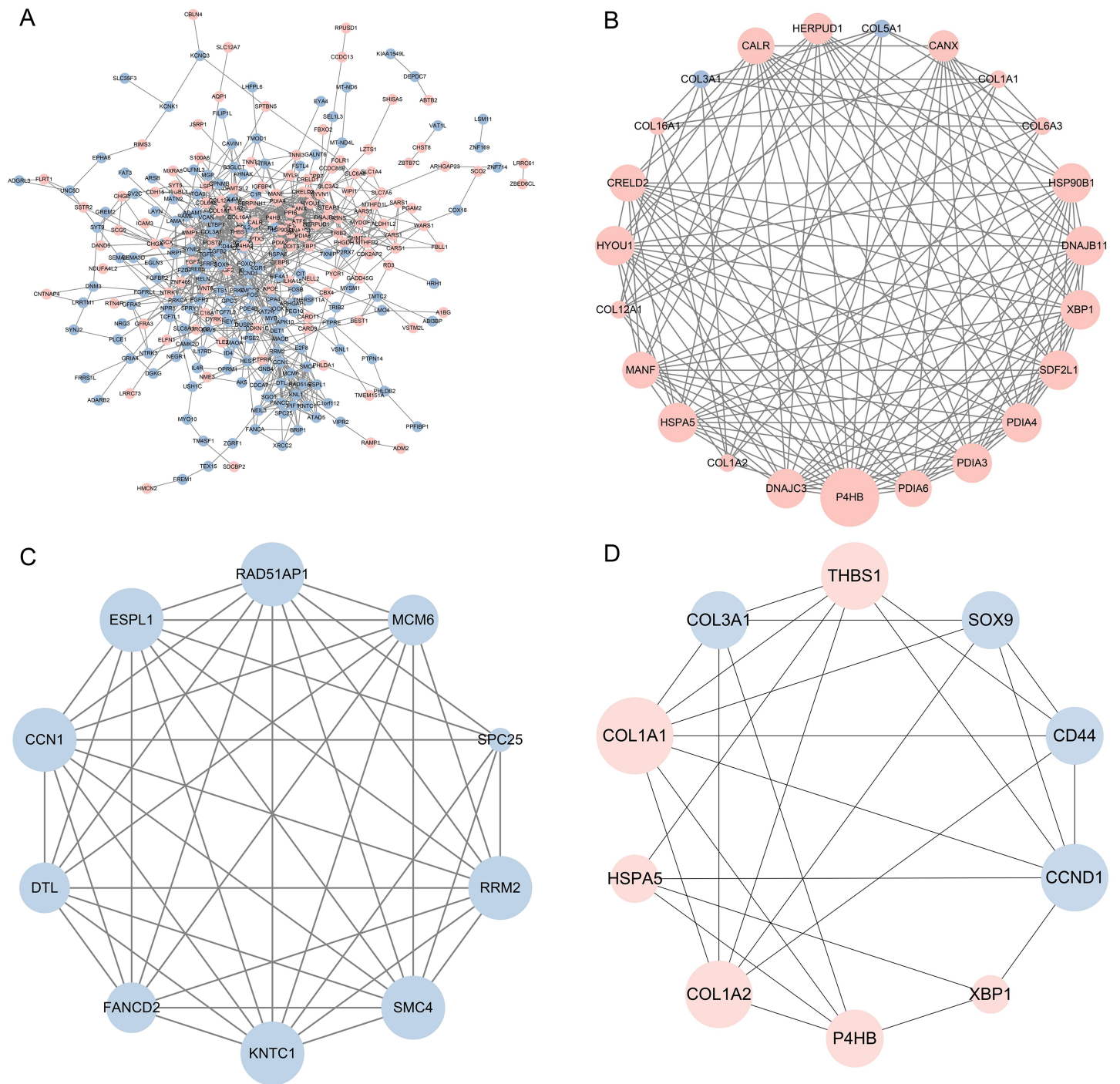


Figure 3 The PPI network of DEGs were constructed, two significant modules and top 10 genes were identified by Cytoscape. (A) PPI network of differentially expressed genes. Circles represent genes, and lines represent protein interactions between genes. Data with interaction score >0.4 were selected from protein-protein interactions to construct a PPI network. (B) Module 1, MCODE score = 13. (C) Module 2, MCODE score = 9.33. (D) Top 10 genes of differentially expressed mRNAs. The circle size indicates the degree of association of the gene in the network. Upregulated genes were marked in pink; downregulated genes were marked in blue. [Full-size !\[\]\(b345a1c4255362eec3746050dd71ccac_img.jpg\) DOI: 10.7717/peerj.15219-fig-3](https://doi.org/10.7717/peerj.15219-fig-3)

Table 1 The biological processes associated with modules 1 and 2.

Term	P value	Count	Genes
Module 1			
Protein folding in endoplasmic reticulum	3.43E-16	7	DNAJC3, PDIA3, HSPA5, CANX, P4HB, CALR, HSP90B1
Collagen fibril organization	1.24E-11	8	COL1A1, COL3A1, COL16A1, COL1A2, COL5A1, COL12A1, COL6A3, P4HB
Response to endoplasmic reticulum stress	2.48E-10	7	PDIA3, XBP1, HYOU1, P4HB, HSP90B1, PDIA4, HERPUD1
Protein folding	6.77E-10	8	PDIA3, CANX, DNAJB11, P4HB, CALR, PDIA6, HSP90B1, PDIA4
IRE1-mediated unfolded protein response	3.03E-09	6	DNAJC3, XBP1, HSPA5, DNAJB11, HYOU1, PDIA6
ATF6-mediated unfolded protein response	9.22E-08	4	XBP1, HSPA5, CALR, HSP90B1
Endoplasmic reticulum unfolded protein response	3.13E-07	5	XBP1, HSPA5, CANX, CALR, HERPUD1
Cellular response to amino acid stimulus	3.37E-07	5	COL1A1, COL3A1, XBP1, COL16A1, COL1A2
Ubiquitin-dependent ERAD pathway	1.62E-06	5	HSPA5, CANX, CALR, HSP90B1, HERPUD1
Extracellular matrix organization	7.19E-06	6	COL1A1, COL3A1, COL16A1, COL1A2, COL5A1, COL6A3
Antigen processing and presentation of peptide antigen <i>via</i> MHC class I	3.87E-04	3	PDIA3, CANX, CALR
Cellular protein metabolic process	5.48E-04	4	DNAJC3, P4HB, PDIA6, HSP90B1
Skin development	8.97E-04	3	COL1A1, COL3A1, COL5A1
Blood vessel development	0.001079868	3	COL1A1, COL1A2, COL5A1
Response to unfolded protein	0.00160925	3	DNAJC3, MANF, HERPUD1
Cell adhesion	0.002647269	5	COL1A1, COL16A1, COL5A1, COL12A1, COL6A3
Module 2			
Mitotic sister chromatid segregation	1.07E-04	3	ESPL1, KNTC1, SMC4

Table 2 Top 10 genes identified by cytoHubba.

Name	Description	Score	Log2 fold change	P value	Regulation
COL1A1	Collagen type I alpha 1 chain	38	0.630823936	0.000139094	Up
CCND1	Cyclin D1	36	-0.726736457	1.15E-15	Down
P4HB	Prolyl 4-hydroxylase subunit beta	33	0.647655166	1.40E-09	Up
HSPA5	Heat shock protein family A (Hsp70) member 5	33	1.271269024	1.97E-42	Up
THBS1	Thrombospondin 1	30	0.733834514	0.000724405	Up
COL3A1	Collagen type III alpha 1 chain	29	-1.315967605	0.000	Down
SOX9	SRY-box transcription factor 9	28	-0.862402503	1.76E-23	Down
COL1A2	Collagen type I alpha 2 chain	27	4.199548198	1.01E-38	Up
XBP1	X-box binding protein 1	25	0.901297817	6.24E-18	Up
CD44	CD44 molecule	25	-0.95212	2.17E-05	Down

(Fig. 4B). Furthermore, the western blot results showed *NTRK1* over-expression increased expression of ER stress markers in neurons, including GRP78, p-IRE, XBP1s, and ATF6 (Fig. S2). Hence, these data further confirm that ER stress response is dominant in neurons with *NTRK1* overexpression.

A ■ NC ■ NTRK1-OE

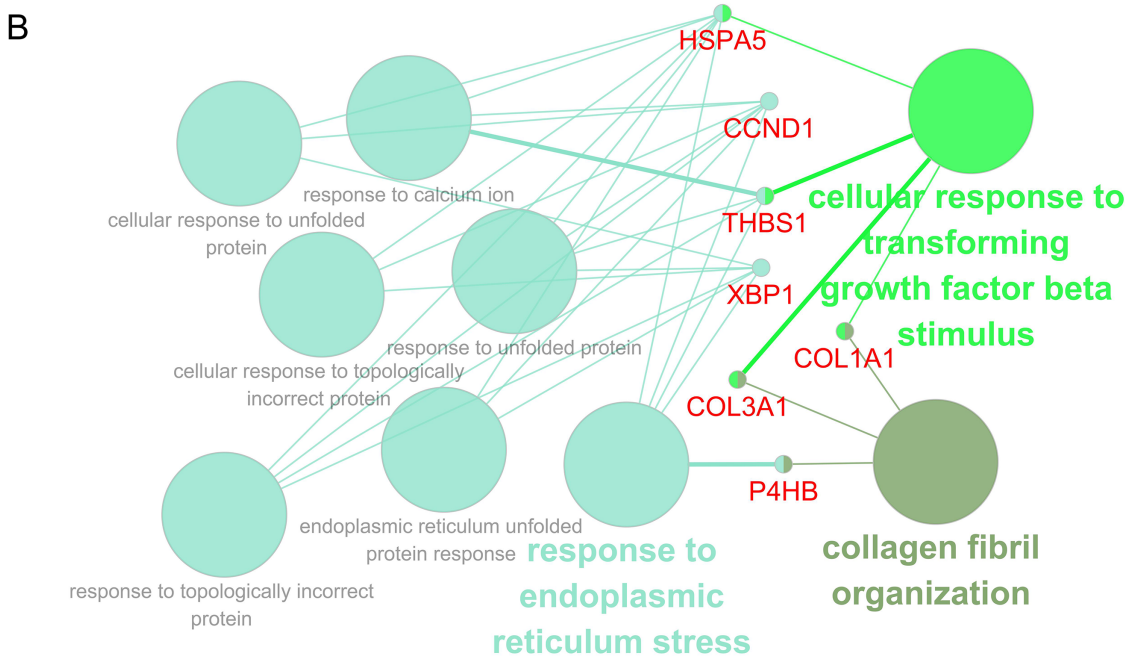
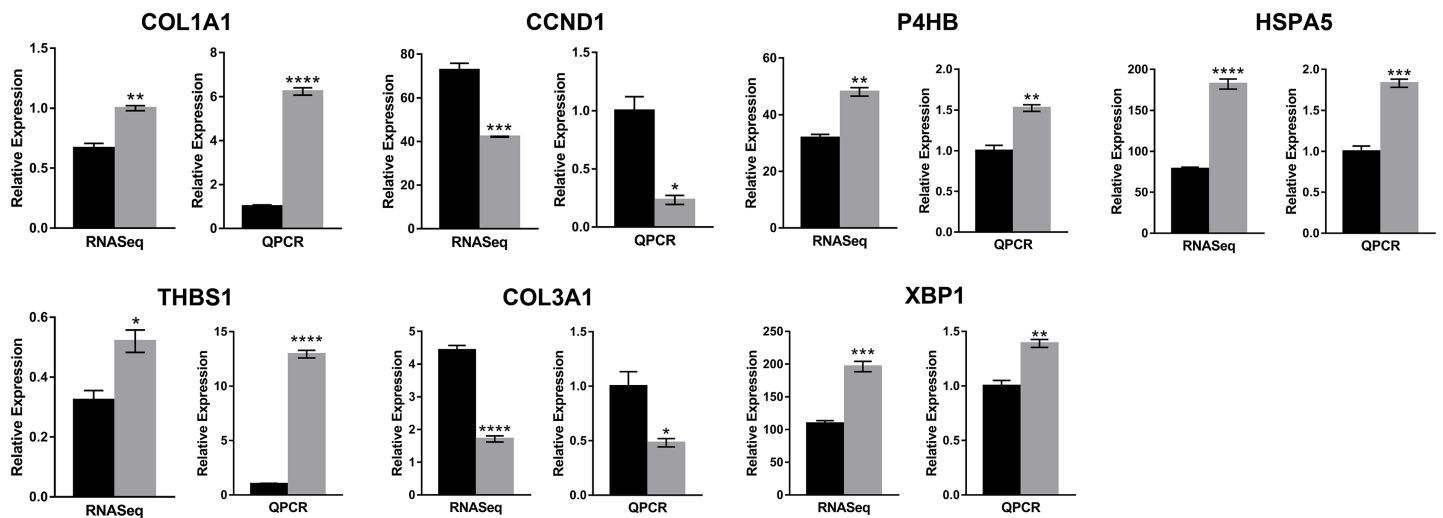


Figure 4 Identification and functional annotation of hub genes. (A) Bar plot showing the expression pattern and statistical difference of DEGs. Error bars represent mean \pm SEM. **P*-value < 0.05, ***P*-value < 0.01, ****P*-value < 0.001, *****P*-value < 0.0001. (B) The GO biological processes functional annotation analysis of hub genes. Different colors of nodes refer to the functional annotation of ontologies. The nodes with red font represent the hub genes. [Full-size !\[\]\(9d188a796ceef961be962a3cd4b57b68_img.jpg\) DOI: 10.7717/peerj.15219/fig-4](https://doi.org/10.7717/peerj.15219/fig-4)

DISCUSSION

NTRK1 is a protein-coding gene that encodes TrkA, a member of the neurotrophic tyrosine kinase receptor family. TrkA, a receptor for NGF, transmits NGF-induced signaling cascades necessary for sympathetic and nervous neurons' proliferation, differentiation, and survival. NGF-TrkA contributes to many biological processes in

adults, such as pain, homeostasis, and inflammation (Indo, 2018). In the present study, 419 DEGs were obtained in human neuronal cell lines (SH-SY5Y cells) with *NTRK1* overexpression compared with the control by RNA-seq. These DEGs consisted of 193 upregulated and 226 downregulated DEGs. DEGs enriched in GO and KEGG pathways showed a range of biological processes and pathways that were affected by *NTRK1* overexpression, in which response to ER stress occupied great importance. Furthermore, the enrichment analysis of hotspot modules of DEGs revealed that response to ER stress was dramatically enriched in the finest hot modules with MCODE scores >9. Additionally, ER stress was also a key biological process involved in hub genes in the PPI network of DEGs. Taken together, our results demonstrated that *NTRK1* significantly influenced the gene transcription of response to ER stress in neurons.

Previous studies have indicated that the p53 signaling pathway, ECM organization, and TGF- β signaling pathway are affected by *NTRK1*, which is reminiscent of our discovery. It was reported that p53 was regulated through the TrkA and p75 neurotrophin receptors in neonatal sympathetic neurons (Aloyz *et al.*, 1998). As for ECM organization in the previous study, the researchers developed a high-throughput screening platform using fibroblast-derived matrices imaged with automated confocal microscopy in 384-well plates to explore mechanisms controlling matrix organization (Jones *et al.*, 2022). It was identified that *NTRK1* was one of the modulators of matrix alignment. Moreover, a previous study showed that genetically inhibiting the neural innervation of the TrkA sensory nerve causes premature closure of the calvarial suture with altered TGF- β signaling pathway transcription levels (Tower *et al.*, 2021). In our study, in addition to the above pathways, we also found responses to ER stress, protein folding in ER, response to unfolded protein, Dopaminergic synapses, Wnt signaling pathway, and so on were significantly enriched in neurons with *NTRK1* overexpression, especially ER stress response.

The accumulation of misfolded/unfolded proteins that deviates from the norm causes ER stress, which triggers an adaptive response. This response involves activating PERK, IRE1 α (inositol-requiring transmembrane kinase/endoribonuclease 1 α), and ATF6 (activating transcription factor 6) pathways as part of the UPR/ER stress response (Hayashi *et al.*, 2007). The involvement of UPR/ER stress response contributes to neuronal differentiation, neurogenesis, and neurite outgrowth (Godin *et al.*, 2016; Hayashi *et al.*, 2007; Hetz & Saxena, 2017). Thus, we speculated that *NTRK1* may influence various functions of neurons by modulating the expression of genes involved in response to ER stress.

To date, the relationship among ER, UPR, and neuropathology has been well established (Ghemrawi & Khair, 2020). The build-up of misfolded proteins within the ER of neurons is a common pathological characteristic of various neurological dysfunctions. In response to ER stress, the UPR minimizes stress, reduces protein misfolding, and maintains cellular homeostasis (Hetz & Saxena, 2017). The crosslink between neuroinflammation and ER stress has been recognized in various diseases, including neurodegenerative diseases and pain (Lei *et al.*, 2021; Mao *et al.*, 2020; Sprengle *et al.*, 2017). Therefore, it can be inferred

that UPR/ER stress response-associated genes may be novel potential targets for neurological dysfunction implicated in *NTRK1*.

In our study, five hub genes implicated in ER stress response were validated, including four upregulated genes (P4HB, HSPA5, THBS1, and XBP1) and one downregulated gene (CCND1). HSPA5 encodes the binding immunoglobulin protein, initiates the UPR, and decreases unfolded/misfolded protein load (Wang *et al.*, 2017). XBP1, a key transcription factor, is spliced by IRE1 in response to ER stress to active UPR (Yoshida *et al.*, 2001). P4HB is an essential redox-sensitive activator of PERK during the UPR (Brewer & Diehl, 2000; Kranz *et al.*, 2017), and THBS1 was also shown to activate PERK that mediates the ER stress response (Vanhoutte *et al.*, 2021). Moreover, it was reported that CCND1 expression directly targets the UPR (Bustany *et al.*, 2015). Therefore, the UPR, especially the IRE1 α -XBP1 and PERK signaling pathways, might play key roles in *NTRK1* affecting neurons, which provides insights for the future research directions on the mechanisms of neurological dysfunction implicated in *NTRK1*.

Of greatest significance, however, is the fact *NTRK1* participates in the growth and survival of both sympathetic and sensory neurons, there is very likely a significant limitation in dissecting the exact mechanism of *NTRK1*. To date, few studies have attempted to examine selectively the mechanism of *NTRK1* in neurons. Our study provides more meaningful results in assessing the direct actions of *NTRK1* in neurons. Further, molecular transcriptional responses of *NTRK1* in human neuronal cell lines will offer distinctive opportunities to investigate the crucial roles of *NTRK1* in neurons. Further insight into *NTRK1* signaling may reveal unexpected roles of *NTRK1*-dependent neurons in human physiology, and suggest new options for the treatment of neurological dysfunction implicated in *NTRK1*, including CIPA and chronic pain.

Nevertheless, it should be noted that this study is only taking its first steps, and it has several limitations that must be addressed in future studies. First, our study used SH-SY5Y cells, human neuronal cell lines, rather than native cells. Furthermore, our study does not include animal experiments or research regarding the possible mechanism linking ER stress response to *NTRK1*. Therefore, further studies are needed with animal experiments and the use of native cells to explore the potential causal mechanisms between the ER stress response and *NTRK1* to facilitate future scientific studies and provide guidance for the neurological dysfunction implicated in *NTRK1*.

CONCLUSIONS

In summary, the present bioinformatics analysis revealed that various ER stress response-related genes were regulated by *NTRK1* overexpression in human neuronal cell lines, although further *in vivo* and *in vitro* validation is required.

ACKNOWLEDGEMENTS

We are grateful to the team members from ABLife Inc., in guidance and suggestions for the design of the manuscript.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

The authors received no funding for this work.

Competing Interests

The authors declare that they have no competing interests.

Author Contributions

- Bo Jiao performed the experiments, analyzed the data, authored or reviewed drafts of the article, and approved the final draft.
- Mi Zhang performed the experiments, analyzed the data, authored or reviewed drafts of the article, and approved the final draft.
- Caixia Zhang performed the experiments, analyzed the data, prepared figures and/or tables, and approved the final draft.
- Xueqin Cao performed the experiments, analyzed the data, prepared figures and/or tables, and approved the final draft.
- Baowen Liu performed the experiments, prepared figures and/or tables, and approved the final draft.
- Ningbo Li performed the experiments, prepared figures and/or tables, and approved the final draft.
- Jiaoli Sun performed the experiments, prepared figures and/or tables, and approved the final draft.
- Xianwei Zhang conceived and designed the experiments, authored or reviewed drafts of the article, and approved the final draft.

Data Availability

The following information was supplied regarding data availability:

The data is available at NCBI GEO: [GSE221028](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE221028).

Supplemental Information

Supplemental information for this article can be found online at <http://dx.doi.org/10.7717/peerj.15219#supplemental-information>.

REFERENCES

- Aloyz RS, Bamji SX, Pozniak CD, Toma JG, Atwal J, Kaplan DR, Miller FD. 1998. p53 is essential for developmental neuron death as regulated by the TrkA and p75 neurotrophin receptors. *Journal of Cell Biology* **143**(6):1691–1703 DOI [10.1083/jcb.143.6.1691](https://doi.org/10.1083/jcb.143.6.1691).
- Bader GD, Hogue CW. 2003. An automated method for finding molecular complexes in large protein interaction networks. *BMC Bioinformatics* **4**(1):2 DOI [10.1186/1471-2105-4-2](https://doi.org/10.1186/1471-2105-4-2).
- Brewer JW, Diehl JA. 2000. PERK mediates cell-cycle exit during the mammalian unfolded protein response. *Proceedings of the National Academy of Sciences of the United States of America* **97**(23):12625–12630 DOI [10.1073/pnas.220247197](https://doi.org/10.1073/pnas.220247197).

- Bustany S, Cahu J, Guardiola P, Sola B. 2015.** Cyclin D1 sensitizes myeloma cells to endoplasmic reticulum stress-mediated apoptosis by activating the unfolded protein response pathway. *BMC Cancer* **15**(1):262 DOI [10.1186/s12885-015-1240-y](https://doi.org/10.1186/s12885-015-1240-y).
- Cascella M, Muzio MR, Monaco F, Nocerino D, Ottaiano A, Perri F, Innamorato MA. 2022.** Pathophysiology of nociception and rare genetic disorders with increased pain threshold or pain insensitivity. *Pathophysiology* **29**(3):435–452 DOI [10.3390/pathophysiology29030035](https://doi.org/10.3390/pathophysiology29030035).
- Chomczynski P, Sacchi N. 1987.** Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* **162**(1):156–159 DOI [10.1016/0003-2697\(87\)90021-2](https://doi.org/10.1016/0003-2697(87)90021-2).
- Ghemrawi R, Khair M. 2020.** Endoplasmic reticulum stress and unfolded protein response in neurodegenerative diseases. *International Journal of Molecular Sciences* **21**(17):6127 DOI [10.3390/ijms21176127](https://doi.org/10.3390/ijms21176127).
- Godin JD, Creppe C, Laguesse S, Nguyen L. 2016.** Emerging roles for the unfolded protein response in the developing nervous system. *Trends in Neurosciences* **39**(6):394–404 DOI [10.1016/j.tins.2016.04.002](https://doi.org/10.1016/j.tins.2016.04.002).
- Hayashi A, Kasahara T, Iwamoto K, Ishiwata M, Kametani M, Kakiuchi C, Furuichi T, Kato T. 2007.** The role of brain-derived neurotrophic factor (BDNF)-induced XBP1 splicing during brain development. *Journal of Biological Chemistry* **282**(47):34525–34534 DOI [10.1074/jbc.M704300200](https://doi.org/10.1074/jbc.M704300200).
- Hefti FF, Rosenthal A, Walicke PA, Wyatt S, Vergara G, Shelton DL, Davies AM. 2006.** Novel class of pain drugs based on antagonism of NGF. *Trends in Pharmacological Sciences* **27**(2):85–91 DOI [10.1016/j.tips.2005.12.001](https://doi.org/10.1016/j.tips.2005.12.001).
- Hetz C, Saxena S. 2017.** ER stress and the unfolded protein response in neurodegeneration. *Nature Reviews Neurology* **13**(8):477–491 DOI [10.1038/nrneurol.2017.99](https://doi.org/10.1038/nrneurol.2017.99).
- Hsieh YL, Kan HW, Chiang H, Lee YC, Hsieh ST. 2018.** Distinct TrkA and Ret modulated negative and positive neuropathic behaviors in a mouse model of resiniferatoxin-induced small fiber neuropathy. *Experimental Neurology* **300**(5):87–99 DOI [10.1016/j.expneurol.2017.10.026](https://doi.org/10.1016/j.expneurol.2017.10.026).
- Indo Y. 2018.** NGF-dependent neurons and neurobiology of emotions and feelings: lessons from congenital insensitivity to pain with anhidrosis. *Neuroscience & Biobehavioral Reviews* **87**(Suppl. 1):1–16 DOI [10.1016/j.neubiorev.2018.01.013](https://doi.org/10.1016/j.neubiorev.2018.01.013).
- Jones CE, Sharick JT, Sizemore ST, Cukierman E, Strohecker AM, Leight JL. 2022.** A miniaturized screening platform to identify novel regulators of extracellular matrix alignment. *Cancer Research Communications* **2**(11):1471–1486 DOI [10.1158/2767-9764.CRC-22-0157](https://doi.org/10.1158/2767-9764.CRC-22-0157).
- Kim D, Langmead B, Salzberg SL. 2015.** HISAT: a fast spliced aligner with low memory requirements. *Nature Methods* **12**(4):357–360 DOI [10.1038/nmeth.3317](https://doi.org/10.1038/nmeth.3317).
- Klink AJ, Kavati A, Gassama AT, Kozlek T, Gajra A, Antoine R. 2022.** Timing of NTRK gene fusion testing and treatment modifications following TRK fusion status among US oncologists treating TRK fusion cancer. *Targeted Oncology* **17**(3):321–328 DOI [10.1007/s11523-022-00887-w](https://doi.org/10.1007/s11523-022-00887-w).
- Kranz P, Neumann F, Wolf A, Classen F, Pomsch M, Ocklenburg T, Baumann J, Janke K, Baumann M, Goepelt K, Riffkin H, Metzen E, Brockmeier U. 2017.** PDI is an essential redox-sensitive activator of PERK during the unfolded protein response (UPR). *Cell Death & Disease* **8**(8):e2986 DOI [10.1038/cddis.2017.369](https://doi.org/10.1038/cddis.2017.369).
- Lei J, Paul J, Wang Y, Gupta M, Vang D, Thompson S, Jha R, Nguyen J, Valverde Y, Lamarre Y, Jones MK, Gupta K. 2021.** Heme causes pain in sickle mice via toll-like receptor 4-mediated reactive oxygen species- and endoplasmic reticulum stress-induced glial activation. *Antioxidants & Redox Signaling* **34**(4):279–293 DOI [10.1089/ars.2019.7913](https://doi.org/10.1089/ars.2019.7913).

- Levi-Montalcini R. 1987.** The nerve growth factor 35 years later. *Science* **237(4819)**:1154–1162 DOI [10.1126/science.3306916](https://doi.org/10.1126/science.3306916).
- Li N, Guo S, Wang Q, Duan G, Sun J, Liu Y, Zhang J, Wang C, Zhu C, Liu J, Zhang X. 2019.** Heterogeneity of clinical features and mutation analysis of *NTRK1* in Han Chinese patients with congenital insensitivity to pain with anhidrosis. *Journal of Pain Research* **12**:453–465 DOI [10.2147/JPR.S188566](https://doi.org/10.2147/JPR.S188566).
- Li N, Sun J, Guo S, Liu Y, Wang C, Zhu C, Zhang X. 2018.** Phenotypic and genotypic features of a pair of Chinese identical twins with congenital insensitivity to pain and anhidrosis: a case report. *Medicine* **97(47)**:e13209 DOI [10.1097/MD.00000000000013209](https://doi.org/10.1097/MD.00000000000013209).
- Livak KJ, Schmittgen TD. 2001.** Analysis of relative gene expression data using real-time quantitative PCR and the 2^{(-Delta Delta C(T))} method. *Methods* **25(4)**:402–408 DOI [10.1006/meth.2001.1262](https://doi.org/10.1006/meth.2001.1262).
- Love MI, Huber W, Anders S. 2014.** Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology* **15(12)**:550 DOI [10.1186/s13059-014-0550-8](https://doi.org/10.1186/s13059-014-0550-8).
- Lv F, Xu XJ, Song YW, Li LJ, Wang O, Jiang Y, Xia WB, Xing XP, Gao P, Li M. 2017.** Recurrent and novel mutations in the *NTRK1* gene lead to rare congenital insensitivity to pain with anhidrosis in two Chinese patients. *Clinica Chimica Acta* **468**:39–45 DOI [10.1016/j.cca.2017.02.007](https://doi.org/10.1016/j.cca.2017.02.007).
- Mao Y, Wang C, Tian X, Huang Y, Zhang Y, Wu H, Yang S, Xu K, Liu Y, Zhang W, Gu X, Ma Z. 2020.** Endoplasmic reticulum stress contributes to nociception via neuroinflammation in a murine bone cancer pain model. *Anesthesiology* **132(2)**:357–372 DOI [10.1097/ALN.0000000000003078](https://doi.org/10.1097/ALN.0000000000003078).
- Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T. 2003.** Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Research* **13(11)**:2498–2504 DOI [10.1101/gr.1239303](https://doi.org/10.1101/gr.1239303).
- Sprenkle NT, Sims SG, Sanchez CL, Meares GP. 2017.** Endoplasmic reticulum stress and inflammation in the central nervous system. *Molecular Neurodegeneration* **12(1)**:42 DOI [10.1186/s13024-017-0183-y](https://doi.org/10.1186/s13024-017-0183-y).
- Swanson AG. 1963.** Congenital insensitivity to pain with anhidrosis. A unique syndrome in two male siblings. *Archives of Neurology* **8(3)**:299–306 DOI [10.1001/archneur.1963.00460030083008](https://doi.org/10.1001/archneur.1963.00460030083008).
- Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, Simonovic M, Doncheva NT, Morris JH, Bork P, Jensen LJ, Mering CV. 2019.** STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Research* **47(D1)**:D607–D613 DOI [10.1093/nar/gky1131](https://doi.org/10.1093/nar/gky1131).
- Tabata M, Murata E, Ueda K, Kato-Kogoe N, Kuroda Y, Hirose M. 2012.** Effects of TrkA inhibitory peptide on cancer-induced pain in a mouse melanoma model. *Journal of Anesthesia* **26(4)**:545–551 DOI [10.1007/s00540-012-1377-7](https://doi.org/10.1007/s00540-012-1377-7).
- Tower RJ, Li Z, Cheng YH, Wang XW, Rajbhandari L, Zhang Q, Negri S, Uytingco CR, Venkatesan A, Zhou FQ, Cahan P, James AW, Clemens TL. 2021.** Spatial transcriptomics reveals a role for sensory nerves in preserving cranial suture patency through modulation of BMP/TGF-beta signaling. *Proceedings of the National Academy of Sciences of the United States of America* **118(42)**:e2103087118 DOI [10.1073/pnas.2103087118](https://doi.org/10.1073/pnas.2103087118).
- Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, Salzberg SL, Wold BJ, Pachter L. 2010.** Transcript assembly and quantification by RNA-seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nature Biotechnology* **28(5)**:511–515 DOI [10.1038/nbt.1621](https://doi.org/10.1038/nbt.1621).

- Ugolini G, Marinelli S, Covaceuszach S, Cattaneo A, Pavone F. 2007.** The function neutralizing anti-TrkA antibody MNAC13 reduces inflammatory and neuropathic pain. *Proceedings of the National Academy of Sciences of the United States of America* **104(8)**:2985–2990 DOI [10.1073/pnas.0611253104](https://doi.org/10.1073/pnas.0611253104).
- Vanhoutte D, Schips TG, Vo A, Grimes KM, Baldwin TA, Brody MJ, Accornero F, Sargent MA, Molkentin JD. 2021.** Thbs1 induces lethal cardiac atrophy through PERK-ATF4 regulated autophagy. *Nature Communications* **12(1)**:3928 DOI [10.1038/s41467-021-24215-4](https://doi.org/10.1038/s41467-021-24215-4).
- Wang Q, Guo S, Duan G, Xiang G, Ying Y, Zhang Y, Zhang X. 2015.** Novel and novel de novo mutations in *NTRK1* associated with congenital insensitivity to pain with anhidrosis: a case report. *Medicine* **94(19)**:e871 DOI [10.1097/MD.0000000000000871](https://doi.org/10.1097/MD.0000000000000871).
- Wang QL, Guo S, Duan G, Ying Y, Huang P, Liu JY, Zhang X. 2016.** Phenotypes and genotypes in five children with congenital insensitivity to pain with anhidrosis. *Pediatric Neurology* **61**:63–69 DOI [10.1016/j.pediatrneurol.2016.04.006](https://doi.org/10.1016/j.pediatrneurol.2016.04.006).
- Wang J, Lee J, Liem D, Ping P. 2017.** HSPA5 gene encoding Hsp70 chaperone BiP in the endoplasmic reticulum. *Gene* **618(3)**:14–23 DOI [10.1016/j.gene.2017.03.005](https://doi.org/10.1016/j.gene.2017.03.005).
- Wu CH, Ho WY, Lee YC, Lin CL, Hsieh YL. 2016.** EXPRESS: NGF-trkA signaling modulates the analgesic effects of prostatic acid phosphatase in resiniferatoxin-induced neuropathy. *Molecular Pain* **12**:174480691665684 DOI [10.1177/1744806916656846](https://doi.org/10.1177/1744806916656846).
- Xie C, Mao X, Huang J, Ding Y, Wu J, Dong S, Kong L, Gao G, Li CY, Wei L. 2011.** KOBAS 2.0: a web server for annotation and identification of enriched pathways and diseases. *Nucleic Acids Research* **39(suppl_2)**:W316–W322 DOI [10.1093/nar/gkr483](https://doi.org/10.1093/nar/gkr483).
- Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K. 2001.** XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell* **107(7)**:881–891 DOI [10.1016/S0092-8674\(01\)00611-0](https://doi.org/10.1016/S0092-8674(01)00611-0).