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Research article

Transcriptomic analysis of CNTF-treated mouse subventricular zone-derived neurosphere culture reveals key transcription factor genes related to adult neurogenesis

Bins Kathanadan Chackochan [a,b], Sinoy Johnson [a], Hilmi Jaufer Thameemul Ansari [a,b,d,1], Ajith Vengellur [a,b], Unnikrishnan Sivan [a,b,e,1], Sayuj Koyyappurath [a], Baby Chakrapani P S [a,b,c,*]

[a] Department of Biotechnology, Cochin University of Science and Technology, Cochin-682022, Kerala, India
[b] Centre for Neuroscience, Cochin University of Science and Technology, Cochin-682022, Kerala, India
[c] Centre for Excellence in Neurodegeneration and Brain Health, Kerala, India
[d] Luxembourg Centre for Systems Biomedicine (LCSB), University of Luxembourg, Esch-sur-Alzette, Luxembourg
[e] Kerala University of Fisheries and Ocean Studies, Cochin -682506, Kerala, India

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ABSTRACT

Neural Stem Progenitor Cells (NSPCs) maintenance and neuronal cell differentiation are the two key aspects of sustained neurogenesis in the adult mammalian brain. Transcription factors (TFs) are known to regulate these biological processes under the influence of various neurotrophic factors. Understanding the role of key TF genes in regulating adult neurogenesis is essential for determining the functional complexity and neuronal diversity seen in the adult mammalian brain. Although several molecular mechanisms leading to adult neurogenesis have been reported, details on its transcriptional regulation are still limited. Our initial results showed that Ciliary Neurotrophic Factor (CNTF) induced neuronal differentiation in SVZ-derived NSPC cultures. To investigate further the role of CNTF in inducing the expression of TF genes related to adult neurogenesis and the potential pathways involved, whole transcriptome RNA-sequencing (RNA-seq) analysis was done in CNTF-treated Sub-ventricular Zone derived neurosphere cultures from the mouse brain. The study revealed 483 differentially expressed genes (DEGs), among which 33 DEGs were identified as coding for transcription factors (TFs). Kyoto Encyclopedia of Gene and Genomes (KEGG) analysis revealed MAPK, PI3K-Akt, and FoxO as the significantly enriched signaling pathways. Gene co-expression network analysis identified five upregulated TF genes related to adult neurogenesis (Runx1, Hmga2, Fos, ID2, and Prrx1) in a single cluster, interacting with each other, and was also validated by quantitative PCR. Our data suggest several potential TFs that may act as critical regulators in the intrinsic transcriptional networks driving the adult neurogenesis process. Further investigation into these molecular regulators may yield a homogeneous population of neuronal progenitors for translational stem cell studies in the future.

* Corresponding author. Centre for Neuroscience & Assistant Professor Department of Biotechnology Cochin University of Science and Technology Kerala. Pin: 682022, India.

E-mail address: chakrapani@cusat.ac.in (B.C. P S).

[1] Current address.

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1. Introduction

Neurogenesis in the mammalian brain involves maintaining, differentiating, and integrating neural stem precursor cells (NSPCs) in the neurogenic niche [1]. NSPCs in the neurogenic niche can self-renew and differentiate into neurons, astrocytes, and oligodendrocytes [2]. In the rodent brain, these multipotent and heterogenous population of NSPCs consists of Neuronal Stem Cells (NSCs) and Neuronal Progenitors (NPs) [3] and are found in two prominent areas of the brain: the subventricular zone (SVZ) of the lateral ventricles and hippocampal Dentate gyrus. These NSPCs residing in the neurogenic environment regulate the development of the embryonic brain and the maintenance of neurogenesis in adulthood. They are also considered the main source of adult neurogenesis in the mammalian brain [2,4,5]. The gradual loss of neural precursors due to genetic alterations, aging, or injury may lead to several neurodegenerative diseases such as Alzheimer's disease (AD) [6] and Parkinson's disease (PD) [7,8].

Studies conducted on NSPCs have revealed that adult neurogenesis is sustained by a small number of NSCs undergoing symmetric self-renewal to maintain the stem cell pool. In contrast, a major fraction of these NSCs gets consumed by differentiative divisions to generate type C cells [9]. Transcription Factors can regulate the growth of NSPCs either at the level of the cell cycle or in early differentiation during neurogenesis [2]. Many TFs have been identified to serve as key regulatory molecules that could integrate the extrinsic and intrinsic transcriptional networks, leading to neurogenic processes [10]. Investigating the role of these TFs in the self-renewal of NSCs and the generation of specific cell types in the brain is important to understand the neurogenesis processes. However, TF activation is complex and involves several cell-signaling pathways to regulate the self-renewal and fate determination of NSCs.

Ciliary Neurotrophic Factor (CNTF), one of the important neurotrophic factors in the mammalian brain, has been known to promote the survival of neurons [11] as well as the activation of astrocytes to support neurons and oligodendrocytes [12,13]. CNTF was initially isolated in the 1980s from chick embryo ciliary ganglion neurons [14]. CNTF lacks a peptide signal, and therefore, it is secreted as a trauma factor in the brain by astrocytes in response to injury [15]. CNTF signals through its tripartite receptor complex composed of the CNTF receptor (CNTFRα), the LIF receptor (LIFR), and glycoprotein 130 (gp130), as a common signal transducer, in association with many other transcription factors [16].

The SVZ in the brain of young adult mice harbours radial glia-like NSPCs with astrocytic morphology, expresses glial fibrillary acidic protein (GFAP) marker, and produces CNTF to promote neurogenesis [17–19]. CNTF mediates its down-stream signalling through three major pathways: Janus Kinase (JAK)/signal transducers and activators of transcription (STAT) and mitogen-activated protein kinase (MAPK) pathways [20] and PI3K-Akt pathway [21]. CNTF can increase the embryonic forebrain neurogenesis through the self-renewal of epidermal growth factor (EGF) responsive neural stem cells *in vitro* [22] by activating the NOTCH1 pathway and by inhibiting the expression of MASH1 [23]. Previous studies have reported that CNTF/LIF/gp130 receptor signalling is necessary for the self-renewal of Fibroblast Growth Factor (FGF)-responsive Ventricular Zone precursors [24]. Nevertheless, the transcriptional regulation of adult neurogenesis via CNTF is largely unexplored.

Genome-wide transcriptome profiling studies have emerged as a powerful tool that can be used to elucidate the development and function of various cell types in the brain. Very little is known about how neurotrophic factors could influence the TFs to regulate adult neurogenesis. Therefore, a transcriptome analysis of NSPCs isolated from the adult mice brain may help identify new neurogenesis regulators. Here, we analyzed the transcriptome in CNTF-treated and Untreated SVZ-derived NSPCs to identify the differentially expressed transcription factor-related genes that can act as transcriptional regulators in various key molecular pathways associated with adult neurogenesis.

2. Materials and methods

2.1. Isolation of NSPCs and neurosphere culture

Eight to twelve-week-old male BALB/c mice were purchased (Institutional Animals Ethics Committee approval number 363/GO/Re/S/01/CPCSEA/2018/02) from Kerala Veterinary Animal Sciences University (KVASU), Mannuthy, Thrissur, Kerala and housed under standard conditions of 18–23°C temperature and 40–60 % humidity. The food and water were given *ad libitum,* and 12-h day and night cycles were maintained throughout the experiments. After two weeks of acclimatization, three mice were decapitated, and the brain was separated from the cranium. The whole brain was removed, and a coronal cut at the rostral side of the brain using a sterile scalpel was made to reveal the SVZ region. The SVZ region was micro-dissected, dissociated enzymatically, and passed the cell suspension through a 70 μm strainer (SPL, Korea). A total of three mice were sacrificed to generate primary neurosphere cultures for this study, and each experiment was carried out in triplicates.

Dissociated cells were cultured in Neurobasal A (Gibco, USA) media supplemented with 2 % B27 supplement (Gibco, USA), 20 ng/mL Fibroblast Growth Factor-2 (FGF; Peprotech, USA), 20 ng/mL Epidermal Growth Factor (EGF; Peprotech, USA), 2 mM Glutamax (Gibco, USA), 1 mM L-glutamine (Gibco, USA), and 1 % penicillin/streptomycin (Gibco, USA). Neural Stem/Progenitor cells were isolated from the SVZ [25] and were maintained as neurosphere primary cultures in a CO_2 incubator at 37°C. Neurospheres were passaged every five to seven days. Cell density in each passaging and for experimentation was maintained at 50,000 cells/5 mL in the T25 flask (S NunclonTM, Thermo Scientific, Denmark). Experiments were conducted on NSPCs maintained between passages six to eight. The NSPCs isolated from the SVZ region of adult mice brains were maintained as neurospheres in the complete Neurobasal A medium. A graphical representation of the experimental design is given in Supplementary Fig. S1.

2.2. Immunocytochemical analysis

To assess whether NSPCs differentiate into neurons in response to CNTF (Peprotech, USA), neurospheres at passage six were dissociated and seeded onto P-D-L (Gibco, USA) coated coverslips (8000 cells/well) in 24 well plates (S NunclonTM, Thermo Scientific, Denmark). Cells were treated with CNTF (20 ng/mL) on day zero and fixed using 4 % paraformaldehyde (Hi-Media, India) after three days of Incubation. Then, it was subjected to immunostaining using *Tuj-1* mouse monoclonal primary Ab (1:400, Abcam, USA). Fluorescent dye-conjugated secondary antibodies (1:400) were obtained from The Jackson Laboratory. Stained cells were then visualized using a Nikon Ti2 Eclipse Fluorescence Microscope, Japan.

2.3. RNA extraction and RNA-seq

For RNA isolation, neurospheres between passages P6 to P8 were used. Total RNA from control and CNTF-treated (20 ng/mL) neurospheres was extracted using TRIzol reagent (Invitrogen). The concentration and purity of total RNA were assessed by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, US) and NanoDrop (Thermo Fisher Scientific Inc. USA). RNA isolation and transcriptome sequencing using the Illumina HiSeq 2500 platform was done at OmicsGen Life Sciences Pvt Ltd, Kerala, India. RNA Seq pipeline is given in Supplementary Fig. S2.

The RNA integrity value of all mRNA was above seven and was used for the library preparation. Next-generation sequencing library preparation was constructed according to the manufacturer's protocol. (NEBNext® Ultra™ RNA Library Prep Kit for Illumina®). The poly(A) mRNA isolation was performed using the NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB). The mRNA fragmentation and priming were performed using NEBNext First Strand Synthesis Reaction Buffer and NEBNext Random Primers. First-strand cDNA was synthesized using ProtoScript II Reverse Transcriptase, and the second-strand cDNA was synthesized using Second Strand Synthesis Enzyme Mix. The purified double-stranded cDNA was then treated with End Prep Enzyme Mix to repair both ends and add a dA-tailing in one reaction, followed by a T-A ligation to add adaptors to both ends. Size selection of Adaptor-ligated DNA was then performed, and fragments of ~360 bp (with the approximate insert size of 300 bp) were recovered. Each sample was then amplified by PCR for 11 cycles using P5 and P7 primers, with both primers carrying sequences that can anneal with flow cell to perform bridge PCR and P7 primer carrying a six-base index allowing for multiplexing. The PCR products were cleaned and validated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and quantified by Qubit 4.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). The quality of the raw reads of all samples was checked using FastQC [26]. The average Q30 (Phred value) score was used as a cut-off to remove low-quality bases in the raw reads. The low-quality bases were removed using Trimmomatic Version 2 [27], and the adapters were removed using Cutadapt Version 2 [28]. For rRNA removal, the cleaned reads were aligned to the *Mus musculus* rRNA fasta sequences downloaded from the National Center for Biotechnology Information (NCBI) using Bowtie Version 2.4.2 [29], and only the unaligned reads were considered for further analysis. The pre-processed reads of CNTF-treated (A1-CNTF-SVZ, A2-CNTF-SVZ, and A3-CNTF-SVZ) and untreated samples (A1-Control- SVZ, A2-Control-SVZ, A3-Control-SVZ), were aligned to the reference *Mus musculus* genome using RNA-seq aligner HISAT2. All samples' normalized gene expression levels in FPKM were estimated, and the Cuffdiff algorithm in the Cufflinks version 2.2.1 with default parameters was used to identify the DEGs. Genes with FPKM ≥ 1 for both samples and p-value ≤ 0.05 were chosen as differentially expressed (DEGs) using a custom-made Perl script.

2.4. Identifying transcription factors involved in neurogenesis

To determine the Transcription factors (TFs) in all the DEGs, 33 TFs were selected from the Mouse Genome Informatics (MGI) database. For Comparative analysis, DEGs and TFs were represented as Volcano plots and heat maps using the "ggplot" package in R statistical software (R Core Team,2022). Gclust [30] was used for clustering analysis.

2.5. Functional enrichment analysis

Gene ontology (GO) enrichment analysis of DEGs was carried out by clusterProfiler (4.0) R package (Bioconductor) [31]. The Ensembl gene IDs were provided, and the "org.Mm.eg.db" package was used to obtain genome-wide annotations for mice. The GO enrichment and KEGG pathway enrichment analysis were performed by the gseGo and BrowseKEGG function respectively. To identify the significantly enriched GO terms and KEGG pathways, an adjusted p-value <0.05 was used as the threshold.

2.6. Gene Co-expression network

The significant pair-wise gene interactions of all the differentially expressed TFs and their first interacting targets (DEGs) were obtained, and a gene co-expression network was constructed using a significant partial correlation $|r| > 0.95$. The gene co-expression network was visualized with the expression correlation tool (Version 1.1.0) available in Cytoscape software (Version 3.10.2) [32]. The specific tool computes the Pearson correlation coefficient for each pair of genes in the dataset. The Network Construction is based on a specified correlation threshold (default cutoffs of "-0.95 & 0.95″), and edges are created between gene nodes. The strength of the correlation determines the edge weight. As the ExpressionCorrelation tool does not directly calculate p-values, we acknowledge the importance of assessing statistical significance.

2.7. qRT-PCR validation

To investigate the expression of transcription factor genes involved in adult neurogenesis under CNTF treatment and to validate the RNA-seq data, six TF genes were selected for qRT-PCR based on their involvement in NSPCs proliferation and differentiation. The primers of these genes were generated in NCBI primer blast software [33] (Supplementary Table S1), and validated. qRT-PCR reactions were run in reaction plates with Applied Biosystems, Real-Time PCR System, Singapore, according to the manufacturer's recommendations. HPRT Hypoxanthine-Guanine Phosphoribosyl Transferase (HPRT) was used as an endogenous control for the qRT-PCR, and the relative expression levels were determined by the $2^{-\triangle\triangle Ct}$ method [34].

3. Results

3.1. CNTF-induces differentiation of NSPCs into neurons

To investigate whether CNTF treatment promotes the generation of neurons, NSPCs grown as monolayer cultures were treated with 20 ng/mL CNTF and immunostained for *Tuj-1*, a marker for both mature [35] and immature neurons [36]. When dissociated neurospheres were dissociated and cultured in the presence of CNTF and stained on Day 3, CNTF-treated cultures contained more *Tuj-1*

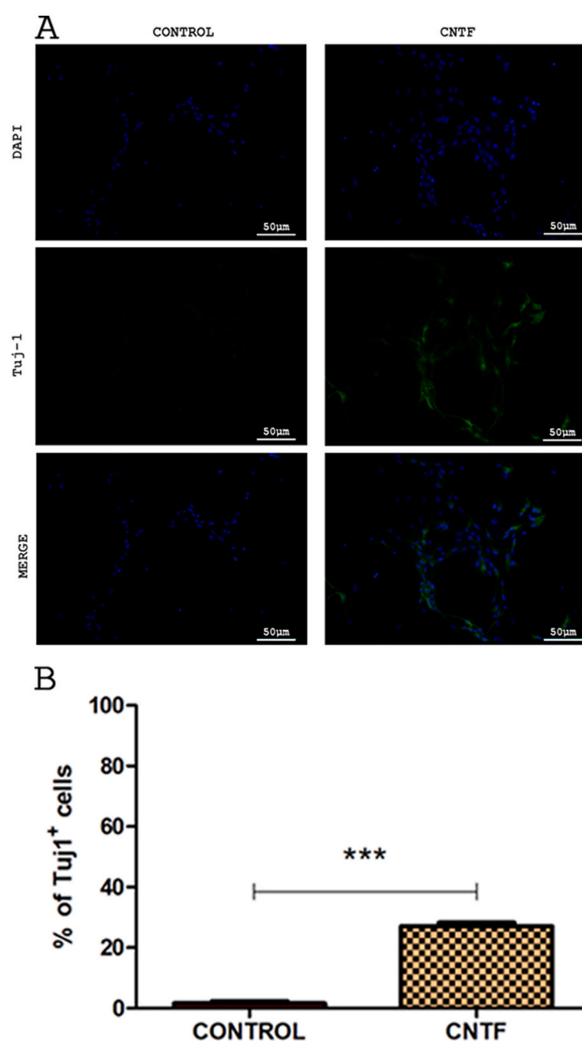

Fig. 1. Immunocytochemical Analysis
Fig. 1 (A) NSPCs grown in the presence of CNTF for 3 days were processed for *Tuj-1* immunocytochemistry, showing an increased number of *Tuj-1*$^+$ positive cells under CNTF treatment. Nuclei counterstained with Dapi (blue); stained with Tuj 1 (green); Scale bars = 50 μm. (**B**) Quantification of the effect of CNTF on NSPCs on Day 3 shows a significantly upregulated *Tuj-1*$^+$ neuronal population. Data represents mean ± SEM for data from three biological replicates; at least 120 cells were counted per replicate. ***$P < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

positive cells compared to the control cultures (Fig. 1A). A quantification of *Tuj-1* positive population revealed a significant increase in the number of neurons under CNTF treatment (Fig. 1B). A magnified *Tuj-1* staining and qRT-PCR quantification of *Tuj-1* expression shown in Supplementary Fig. S3.

3.2. RNA seq analysis reveals 483 differentially expressed genes

The dissociated NSPCs were cultured as neurospheres for three days for RNA sequence analysis. Later, the CNTF-treated and untreated neurospheres were harvested in RNAlater solution on Day 3 (72 h). In the RNA-Seq, the NGS QC Toolkit treatment filtered 122.2 million and 162.9 million high-quality paired-end reads from control, and CNTF-treated samples of BALB/c mice, respectively. The reads obtained in the libraries were of high quality, averaging 85 % of the total data passed $\geq$ 30 phred score. More than 92 % of the total pre-processed reads of all samples were mapped to the reference genome. The sample details and read alignment summary are given in Table 1. For gene annotation, all the DEGs were annotated based on the final *Mus musculus* annotation file. The differential gene expression analysis (p-value $\leq$ 0.05&|FPKM $\geq$ 1) of the comparison "Control vs CNTF" revealed a total of 483 differentially expressed genes with 214 and 269 upregulated and downregulated genes, respectively (Table 2).

3.3. GO analysis of DEGs

Gene Ontology enrichment of DEGs was done to check the distribution of significantly enriched functional terms (adjusted p-value <0.05) and identified 1791, 235, and 232 Biological Process (BP) terms, Cellular Component (CC) terms, and Molecular Function (MF) terms, respectively, among a total of 2258 terms. (Data given in Supplementary Table S2). Barplot (Fig. 2) shows the BP, CC, and MF terms that are significantly enriched. The mitotic cell cycle process (GO:1,903,047) was the highest significantly enriched term. All 10 terms in GO_BP terms were associated with the cell cycle. In the GO_CC terms, protein kinase, chromatin, and transcription factor binding were the most significantly enriched terms.

3.4. CNTF treatment shows significantly enriched KEGG pathways

KEGG enrichment analysis was done to identify the significant biological pathways enriched under CNTF treatment by mapping the DEGs onto reference pathways in the KEGG database. All the enriched pathways are listed in Supplementary Table S3. Since this study aims to understand the key signalling pathways involved in adult neurogenesis, we focussed on all the pathways under signalling and the nervous system from the KEGG *Mus musculus* genome database. A scatter plot representation of significantly enriched DEGs in 34 pathways (adjusted p-value <0.05) (Fig. 3). Results revealed that the MAPK Signalling pathway, PI3K-Akt signalling pathway, and FoxO signalling pathway are the most significantly enriched pathways. Dopaminergic and cholinergic synapses were also highly enriched, among other pathways, under CNTF treatment.

3.5. Key transcription factors involved in adult neurogenesis

To uncover the TFs that are differentially expressed, all the DEGs were compared against TFs present in the Ensemble database using local blast. 33 TFs were identified among 483 DEGs and are highlighted in the volcano plots (Fig. 4), which include *Tgif1*, *Runx1, Fos, Hmga2, ID2, and Prrx1*. The 12 upregulated and 21 downregulated genes among 33 TFs are represented as a heat map (Supplementary Fig. S4). TF genes with the highest (3.37) and lowest (−3.82) log2fold values were for *Tgif1* and *Tcf7l2,* respectively.

3.6. Gene Co-expression network analysis of TFs

A co-expression correlation analysis was performed to elucidate the potential interacting partners. All 32 TFs had interacting partners with DEGs, except for *csrnp2*. A total of 4 clusters of TF interactions were identified, and interestingly, 8 out of 33 TFs (*Runx1* [37], *Junb* [38], *Fos* [39], *Hmga2* [40], *Prrx1* [41], *Id2* [42], *Klf4* [43], *Nfe2l2* [44]); were in a single cluster (Fig. 5), indicating their involvement towards a common biological function of neural stem maintenance and/or neuronal differentiation. The other 3 clusters are listed in Supplementary Fig. S5. These 8 TFs were upregulated in CNTF-treated NSPCs. *Tgif1*, a key player in NSCs proliferation [45], and their first neighbours were identified in a different cluster.

Table 1
Sample Details and Read Alignment Summary
Table 1 shows the number of reads in each sample, and the corresponding alignment and Phred score.

Sl No	Sample Name	Total Reads	Total Pre-Processed Reads	Total Aligned Reads	Alignment %	Total Data ≥ Q30
1	A1-Control-SVZ	40,790,866	32,614,700	31,443,832	96.41 %	93.61 %
2	A2-Control-SVZ	23,366,940	20,141,456	19,418,377	96.40 %	86.80 %
3	A3-Control-SVZ	61,297,924	51,677,094	47,666,951	92.24 %	85.50 %
4	A1-CNTF-SVZ	57,424,910	45,831,664	44,012,146	96.03 %	92.34 %
5	A2-CNTF-SVZ	26,123,562	8,383,056	7,819,714	93.28 %	87.94 %
6	A3-CNTF-SVZ	97,136,084	79,381,900	76,055,798	95.81 %	92.21 %

Table 2
Total DEGs in Control VS Cntf comparison
Table 2 shows that RNA sequencing comparison of control vs CNTF revealed a total of 483 genes among which 214 genes were upregulated and 269 were downregulated.

Comparison	p-value cut off	Total genes Regulated	Up-regulated genes	Down-regulated genes
Control VS CNTF	≤0.05	483	214	269

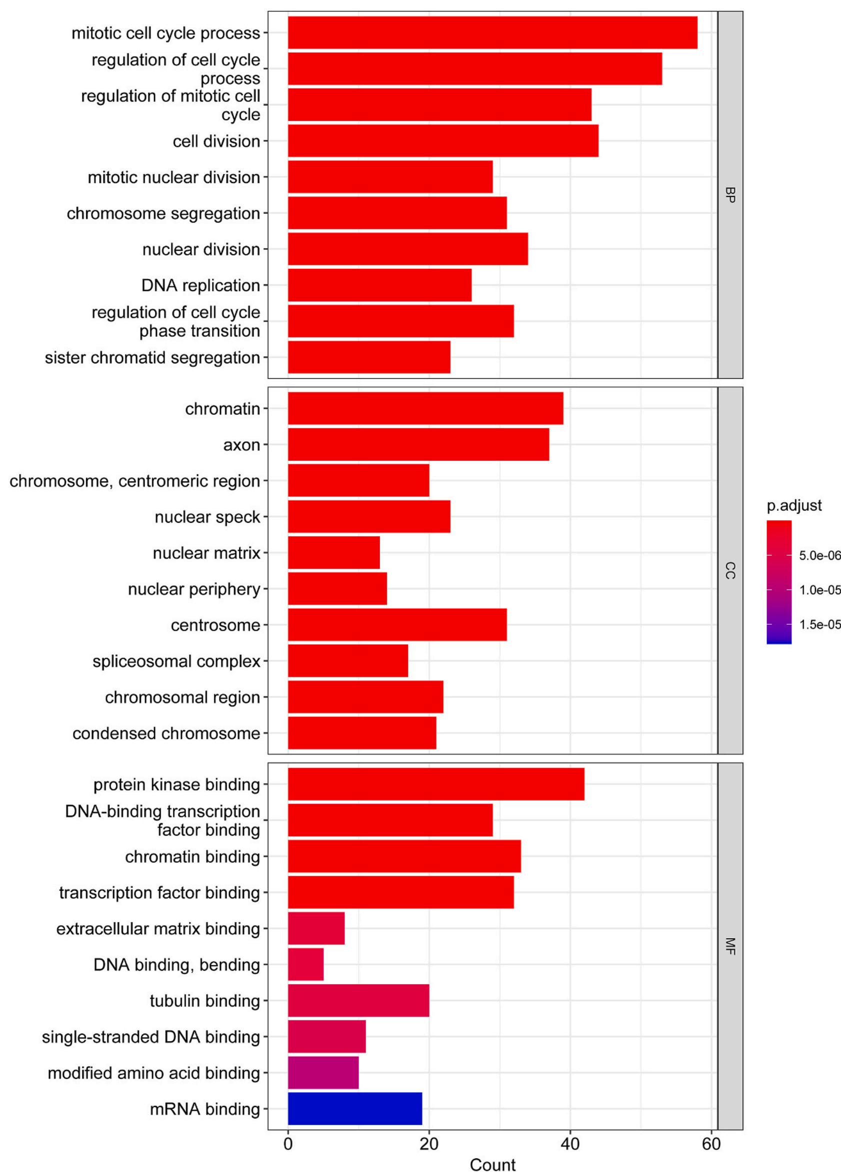

Fig: 2. Bar Plot
Fig. 2. GO enrichment analysis of the DEGs in the CNTF-treatment groups. The barplot displays the top 10 significantly enriched GO terms for three categories: BP, biological process, CC, cellular component, MF, molecular function on the y-axis, and x-axis represents the gene count, whereas the bar color represents the significance (adjusted p-value) of each GO term. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.7. qRT-PCR validation of selected TFs identified from RNA-seq

To study the role of TFs and further authenticate the RNA-seq analysis results, six TFs (*Tgif1*, *Runx1, Fos, Hmga2, ID2, and Prrx1*) were selected based on their involvement in NSPCs proliferation and differentiation. The qRT-PCR results show that the mRNA

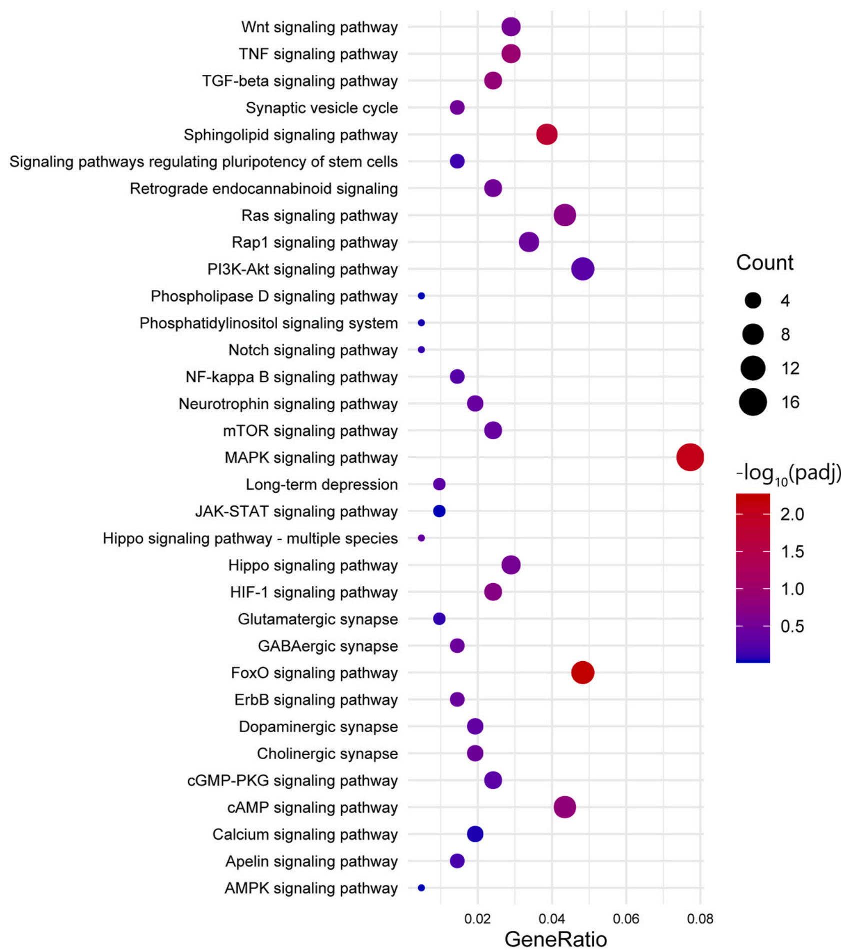

Fig: 3. Scatter Plot

Fig. 3. KEGG pathway enrichment analysis of the DEGs under CNTF treatment shows 34 significantly enriched pathways. The x-axis indicates the proportion of DEGs enriched in a KEGG pathway to the total number of DEGs enriched in all the KEGG signalling and nervous system pathways. The dot size represents the number of DEGs enriched in each KEGG pathways, whereas the colour of the dot represents the significance (adjusted *p*-value) of each KEGG pathway. KEGG, Kyoto Encyclopedia of Genes and Genomics. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

expression patterns of selected genes were similar to the expression profile obtained from RNA-seq data (Fig. 6).

4. Discussion

Understanding the transcriptional regulation of adult neurogenesis holds vital importance in devising a regenerative therapeutic strategy for brain repair using adult NSPCs. Although decades of research have contributed much to this field, little is known about the key genes and crucial pathways involved in the maintenance and/or fate commitment of NSPCs. TFs are key regulators of neuronal differentiation in the adult mammalian brain. The role of TFs in specifying the lineage commitment among NSPCs is largely unsolved, and this is being elucidated in the scientific community. Evidence shows that TFs play a crucial role in regulating neuronal commitment early in the progenitors residing in the ventricular regions of the mouse brain [46]. Although many TFs have been identified in mouse and their involvement in neurogenesis, how neurotrophic factors, such as CNTF, can regulate neurogenesis at the transcriptional level in the adult mouse brain lacks clarity.

Next-generation sequencing (NGS) technologies have expanded the research possibilities for studying the role of transcription factors in gene regulation. Gene expression profiling through mRNA deep sequencing (RNA Seq) can generate reads with higher accuracy and greater coverage of transcripts [47]. We show here that the transcriptome data generated by RNA sequencing of CNTF-treated and untreated mouse NSPCs will contribute to deciphering the important causal factors involved in regulating adult NSPCs' self-renewal and differentiation.

Our current research aims to uncover how transcription factors orchestrate gene expression during the differentiation of neurons from neurosphere cultures derived from SVZ, prominent neurogenic niche in adult mammalian brain. In our study, the

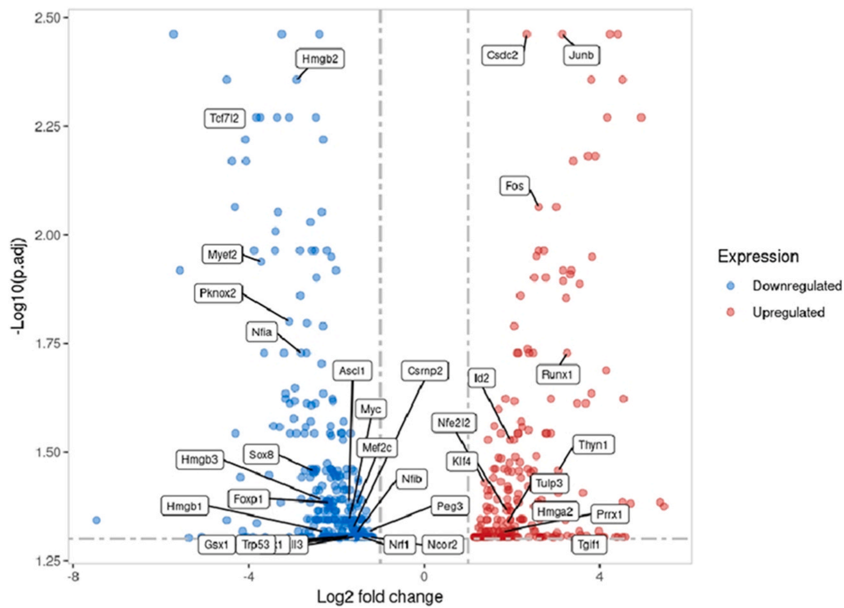

Fig: 4. Volcano Plot

Fig. 4. Volcano plot representing the total no of DEGs for control vs CNTF treatment comparison. The significance was calculated based on p ≤ 0.05| FC±1. The x-axis- and y-axis represent log 2(Fold change) and -log10 (adjusted p-value). Red dots represent upregulated, and blue dots represent down-regulated DEGs. 33 TFs are highlighted as squares in the image. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

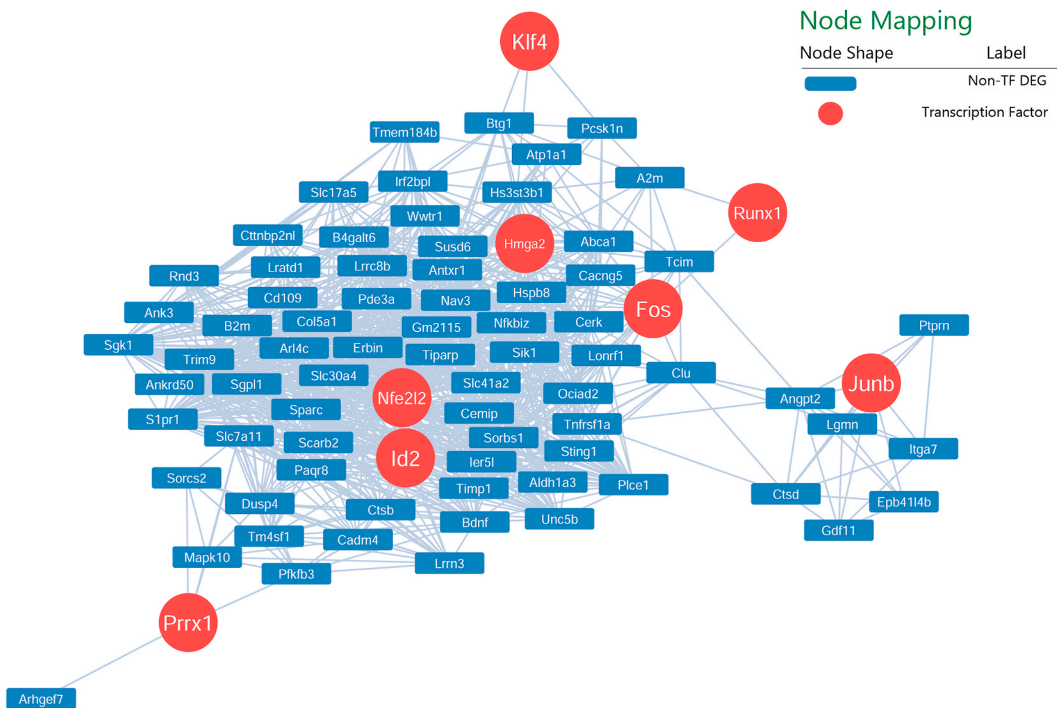

Fig: 5. Interactome

Fig. 5. Gene co-expression network of differentially expressed TFs and their respective first interacting partners with significant partial correlation |r|> 0.95. The correlation network is composed of 8 TFs and 73 non-TF DEGs. Nodes with ellipse shapes are TFs (red color-filled nodes), and those with rectangle shapes are non-TF DEGs (blue color-filled nodes). All the TFs in this cluster were upregulated in the study. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

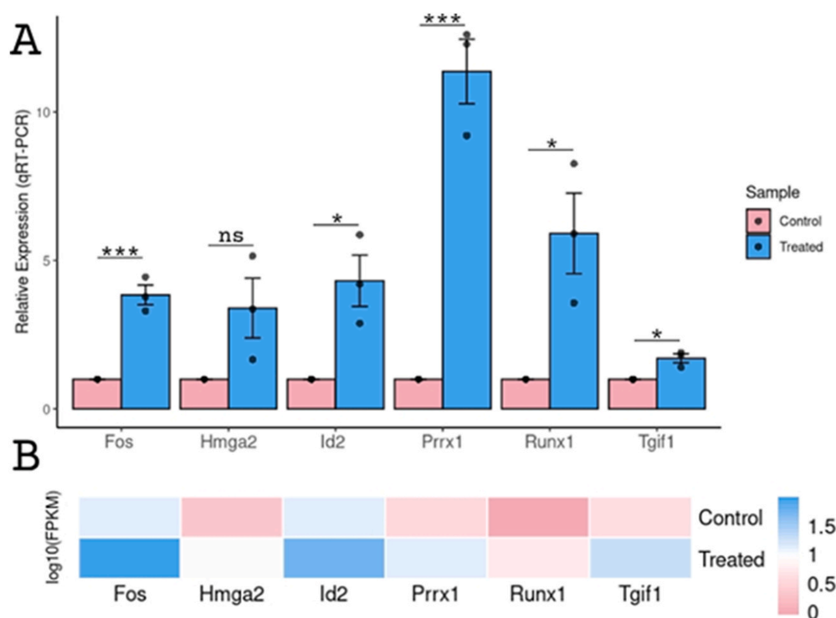

Fig: 6. qRT-PCR validation
Fig. 6. Validation of selected TF genes by qRT-PCR. **(A)** The relative expression levels of six TF genes (Fos, Hmga2, Id2, Prrx1, Runx1, Tgif1) by qRT-PCR. The x-axis represents the gene name, whereas the y-axis represents the relative expression levels of these DEGs. Unpaired *t*-test, two-tailed. *P < 0.05, ***P < 0.001. **(B)** A heat map of log10 transformed FPKM values of RNA Seq data between control and cntf-treated samples. FPKM, fragment per kilobase of transcript per million reads; RNA-seq, RNA sequencing; qRT-PCR, quantitative Reverse Transcription-polymerase chain reaction.

immunochemical analysis of CNTF-treated SVZ neurospheres revealed a higher number of *Tuj-1* positive cells compared to the control cultures. These findings aligns with Ding et al.'s work [48] and suggests that CNTF plays a pivotal role in driving the differentiation of adult NSPCs to neurons. (Although, *Tuj-1* is a well-known marker for both mature and immature neurons differentiated from NSPCs in the SVZ, it would be beneficial to use additional neuronal markers such as NeunN or MAP2B, to confirm the neuronal identity, especially during extended culturing periods). Therefore, based on the crucial role of CNTF in inducing neuronal differentiation, a transcriptome analysis of control and CNTF-treated NSPCs on day three was performed to identify the key molecular regulators involved in adult NSPCs proliferation and differentiation. As a result, several key TFs, including *Tgif1, Runx1, Fos, Hmga2, ID2, Prrx1,* and signalling pathways (MAPK Signalling pathway, PI3K-Akt signalling pathway, and FoxO signalling pathway) and Dopaminergic and Cholinergic synapses have been identified.

GO analysis of the 483 DEGs showed that the most impacted biological process is related to the cell cycle. This finding suggests that CNTF treatment affects gene expression in the cell cycle progression within NSPCs. Interestingly, these impacts on the cell cycle may direct NSPCs toward neuronal differentiation, as also evident from the *Tuj-1* immunostaining. Earlier reports have shown that CNTF promotes the self-renewal of embryonic neural stem cells [49] and neurogenesis in the adult hippocampus [50]. Nevertheless, research investigating the impact of CNTF within the subventricular zone (SVZ)—the largest stem cell niche in the adult mammalian brain—and its influence on genes related to adult neurogenesis remains insufficient for pinpointing the precise molecular regulators of neuronal differentiation. Our study bridges a critical gap by examining the transcriptome of CNTF-treated neural stem precursor cells (NSPCs) derived from the subventricular zone (SVZ). We propose that CNTF may drive NSPCs towards cell cycle exit in NSPCs, favouring a neuronal fate. Our transcriptome study provides intriguing leads, including the upregulation of transcription factor genes related to adult neurogenesis. However, further experiments are needed to validate these hypotheses. Additionally, it would be worthwhile to explore the impact of CNTF on the rest of the undifferentiated NSPC population and their commitment to non-neuronal fate. Notably, the enrichment of the 'Transcription Factor binding' gene ontology term suggests CNTF-induced upregulation of TF genes related to adult neurogenesis. Our study has identified five transcription factor genes related to neurogenesis: *Runx1, Fos, Hmga2, ID2, and Prrx1*. These findings are crucial in unraveling diverse neuronal populations within the SVZ niche. We speculate that CNTF activates these TF genes, individually or in combination, to regulate proneural gene expression. Proneural genes, in turn, govern the delicate balance between neuronal and glial fate commitment [51].

The analysis of DEGs using KEGG pathways revealed that CNTF treatment significantly enriches MAPK signalling, and PI3-Akt signalling pathways. Previous studies have highlighted the crucial roles of these maintaining, proliferating, and differentiating NSCs [52,53]. In mice, sustained MAPK activity is essential for the generating dorsal lateral ganglionic eminence and oligodendrocyte progenitors in the ventral telencephalon [54]. At the same time, PI3–Akt signalling supports the survival of new granule cells and synaptic plasticity in the dentate gyrus [55]. Additionally, the other enriched pathways related to neurogenesis include the Wnt signalling pathway [56], pluripotency-related pathways, the Calcium signalling pathway [57], JAK-STAT signalling [58], and the

FoxO signalling [59]. Given the above findings, these leads could be further explored to unravel the interplay between transcription factors and CNTF-enriched pathways in regulating neuronal differentiation in the adult mammalian Brain.

Among all the TFs studied, *Tgif1* exhibited the highest log.2-fold change (3.37) in the CNTF-treated cultures. *Tgif1* is a gene associated with proliferation and has a negative impact on - TGFβ signalling [60]. Exploring the function of *the Tgif1 gene could provide valuable insights into how CNTF influences* the proliferation of NSPCs by promoting their exit from the cell cycle toward a neuronal lineage. Another significantly upregulated TF in our study is *Runx1* (log.2-fold change of 3.25) which has been reported to increase the *Tuj-1*$^{+}$ population upon *Runx-1* over-expression in adult NSPCs [37]. The other upregulated TF genes identified in this study related to neurogenesis were *Hmga2*, *Fos*, *Id2*, and *Prrx1,* with log 2-fold values of 2.26, 2.60, 1.95, and 1.79, respectively. While Hmga2, a transcriptional regulator, has been associated with neural stem cell self-renewal in fetal and young adult mice [61], our qRT-PCR validation did not reveal significant upregulation under CNTF treatment. c-Fos, an immediate early response proto-oncogene, is a neuronal activity marker linked to various neural and behavioral responses triggered by acute stimuli. Studies indicate that c-Fos also play a crucial role in the generation of NSPCs during brain development [39]. Notably, our study observed significant upregulation of the Fos gene induced by CNTF. ID 2, an inhibitor of DNA binding 2, has been reported to regulate the proliferation of neural progenitors and their commitment to dopaminergic neurons in the adult SVZ [42]. Previous studies have indicated that the prrx1 protein primarily gives rise to astrocytic-like progenitors without impacting newborn neurons [41]. Surprisingly, our study observed a significant increase in prrx1 expression. However, the precise mechanism by which CNTF interacts with prrx1 to enhance neurogenesis in the adult SVZ remains largely unexplored. Our findings emphasize the role of CNTF in inducing the expression of transcription factor genes related to adult neurogenesis, paving the way for gene manipulation in several neurodegenerative studies. Our study highlights significant enrichment of dopaminergic and cholinergic synapses. This aligns with the earlier research showing that dopamine D2-receptor-dependant neurogenesis occurs in SVZ [62], and additionally, sympathetic neurons differentiate into cholinergic lineage [63] under the influence of CNTF. These results may be important in understanding the CNTF-induced synaptic plasticity in the adult mammalian brain. Besides, CNTF-induced Gene co-expression network analysis revealed that five of the six genes validated were part of a single cluster, indicating their involvement towards adult neurogenesis, whereas Tgif1, a proliferation-related gene, stood apart in a separate cluster.

5. Conclusion

This is the first to study the transcriptome of CNTF-treated NSPCs from the SVZ region in the adult mouse brain. Among the 483 differentially expressed genes, 33 were recognized as TF genes. Our results demonstrate increased expression of the *Tuj-1* marker in CNTF-treated SVZ NSPCs. Additionally, the upregulated TF genes are likely involved in both NSPC maintenance and neuronal differentiation in CNTF-treated SVZ NSPCs. As identified in the GO analysis, the enrichment of transcription factor binding suggests that CNTF may transcriptionally regulate differentiation processes among NSPCs.

This study indicates that CNTF-induced differentiation of SVZ NSPCs into neurons involves transcriptional regulation of cell cycle dynamics. It also highlights the key molecular players and signalling pathways, and synapses involved in determining the fate of NSCPs during this process. Moreover, our findings open up a window for designing additional experiments to validate the role of these key TF genes in adult neurogenesis. We acknowledge the importance of functional validation of these TFs by gene silencing or over-expression studies. Co-expression studies of TFs with neuronal markers are also essential to validate our claims.

In the future, the effect of CNTF in NSPCs can be further exploited by gene editing techniques such as CRISPR-Cas9 to engineer the potential transcription factors to regulate neuronal differentiation. Our findings may provide valuable insights into manipulating NSPCs to generate homogenous progenitor populations in brain regeneration therapies.

Data availability

The raw reads of six samples have been submitted to the NCBI Sequence Read Archive database (SRA) with the Bioproject accession number PRJNA901476 and SRA accession number SRR22348040 - SRR22348045.

Ethics statement

All the animal experimental procedures in this study met the guidelines of the Committee for Control and Supervision on Experiments on Animals (CPCSEA), India. All animal experiments were approved by the Institutional Animal Ethics Committee (IAEC), of Cochin University of Science and Technology (CUSAT), Kerala, India (approval number 363/GO/Re/S/01/CPCSEA/2018/02), and every effort was taken to minimize animal suffering.

Disclosure statement

The authors have expressed no conflict of interest.

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CRediT authorship contribution statement

Bins Kathanadan Chackochan: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sinoy Johnson:** Writing – original draft, Software, Data curation. **Hilmi Jaufer Thameemul Ansari:** Writing – review & editing, Dr. **Ajith Vengellur:** Writing – review & editing. **Unnikrishnan Sivan:** Writing – review & editing, Dr. **Sayuj Koyyappurath:** Writing – review & editing, Software. **Baby Chakrapani P S:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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