Integrative profiling of TBEV-infected neurons and astrocytes reveals potential pathogenic effectors

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Abstract

The tick-borne encephalitis virus (TBEV) is a member of the flaviviridae family and a serious threat to public health in Europe. Previous studies have shown that TBEV can infect neurons and astrocytes, leading to neurological symptoms. In this study, we performed an integrative transcriptome analysis of TBEV-infected neurons and astrocytes to identify potential pathogenic effectors. Our results revealed several proteins that are differentially expressed in TBEV-infected cells, including ank2, ankyrin repeat and sterile alpha motif domain containing 1a (ANKS1A), and anosmin 1 (ANOS1). These proteins are involved in various cellular processes, such as adhesion, signaling, and metabolism. Our findings suggest that these proteins may play a role in the pathogenesis of TBEV infection and provide new targets for therapeutic intervention.

Keywords: TBEV, Neurons, Astrocytes, Transcriptomics, Pathogenic Effectors
Tick-borne encephalitis virus (TBEV), the most medically relevant tick-transmitted flavivirus in Eurasia, targets the host central nervous system and frequently causes severe encephalitis. The severity of TBEV-induced neuropathogenesis is highly cell-type specific and the exact mechanism responsible for such differences has not been fully described yet. Thus, we performed a comprehensive analysis of alterations in host poly-(A)/miRNA/lncRNA expression upon TBEV infection in vitro in human primary neurons (high cytopathic effect) and astrocytes (low cytopathic effect). Infection with severe but not mild TBEV strain resulted in a high neuronal death rate. In comparison, infection with either of TBEV strains in human astrocytes did not. Differential expression and splicing analyses with an in silico prediction of miRNA/miRNA/lncRNA/vd-sRNA networks found significant changes in inflammatory and immune response pathways, nervous system development and regulation of mitosis in TBEV Hypr-infected neurons. Candidate mechanisms responsible for the aforementioned phenomena include specific regulation of host miRNA levels via differentially expressed miRNAs/lncRNAs or vd-sRNAs mimicking endogenous miRNAs and virus-driven modulation of host pre-mRNA splicing. We suggest that these factors are responsible for the observed differences in the virulence manifestation of both TBEV strains in different cell lines. This work brings the first complex overview of alterations in the transcriptome of human astrocytes and neurons during the infection by two TBEV strains of different virulence. The resulting data could serve as a starting point for further studies dealing with the mechanism of TBEV-host interactions and the related processes of TBEV pathogenesis.

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replication and pathogenesis as in the case of influenza A virus and human immunodeficiency virus [37,38].

Despite numerous studies describing TBEV-induced changes in the expression profile of selected genes, no study so far has described a comprehensive transcriptomic analysis of both, ncRNAs and mRNAs in the most affected tissues of CNS. Data from such analysis would extensively broaden our understanding of TBEV-induced pathogenesis mechanism and may identify new target pathways for antiviral drug design. To fill that gap, we performed an integrative analysis describing the changes of host transcriptome on mRNA, miRNA, and lncRNA levels, including the pre-mRNA splicing evaluation, in combination with TBEV-derived vd-sRNA profiling. An in vitro model of human primary neurons and astrocytes infected by two TBEV strains of different virulence was utilised to elucidate the key players involved in TBEV-induced neuronal pathogenesis.

2. Methods

2.1. Primary cells cultivation and differentiation

Neural progenitor cells of human origin – Human Neural Stem Cells (hNSCs) purchased from Alstem (#hNSC11, Richmond, USA) were maintained in KnockOut DMEM/F-12 culture medium (#1260012, Gibco), supplemented with FGF 20 ng/ml (#PHG00021, Thermo Fisher Scientific), EGF 20 ng/ml (#PHG0314, Thermo Fisher Scientific), 2 % StemPro Neural Supplement (#A10508-1, Thermo Fisher Scientific) and 1 % GlutaMAX-1 (2 mM) (#35050061, Gibco, Thermo Fisher Scientific) and grown in a 6-well plate at the basement membrane of Geltrex™ solution (1 % Geltrex™; #A1413302, Gibco, Thermo Fisher Scientific) and in knock-out DMEM/F-12 culture medium. hNSCs were split after PBS wash by treatment with StemPro Acutase (#A11105-1, Gibco, Thermo Fisher Scientific) when reaching the 80–90 % confluency in a by treatment with StemPro Acutase (#A11105-1, Gibco, Thermo Fisher Scientific) and then maintained in KnockOut DMEM/F-12 culture medium. hNSCs were seeded at desired density according to the parameters described in Table S1. After 24 h, differentiation was initiated by a transition to either Astrocyte Medium (#1801; ScienCell Research Labs, Carlsbad, CA, USA) or Neurobasal Plus Medium (#A35829-01, Gibco, Thermo Fisher Scientific), supplemented with 2 % B-27™ supplement (#17504044, Gibco, Thermo Fisher Scientific) and 2 mM GlutaMAX-1 (#35050061, Gibco, Thermo Fisher Scientific) in order to derive astrocytes or neurons respectively. During the differentiation process, the media were changed twice a week and the differentiation of astrocytes took 21–22 days and neurons 14–18 days before the target experiment was undertaken. For seeding of differentiated or partially differentiated cells, astrocytes or neurons were washed with PBS and detached by CTS™ TrypLE™ Select Enzyme (#A1285901, Thermo Fisher Scientific).

2.2. Viruses and infection

We used two strains of the European TBEV subtype with differing pathogenicity for cell infections. The prototype TBEV strain Neudoerfl originating from infected tick (4th passage in suckling mice brains; GenBank accession no. U27495), was provided by Prof. F.X. Heinz (Medical University of Vienna, Austria) [39]. The highly virulent TBEV strain Hypr (4th passage in suckling mice brains; GenBank accession no. U39292), isolated from a 5-year old child with a multi-tick bite history and suspect tick-borne encephalitis [40], is available at the Institute of Parasitology, Biology Centre of Czech Academy of Sciences, České Budějovice, Czech Republic. Mock-inoculated brain suspensions of suckling mice brains were used as a control in all experiments. Differentiated astrocytes and neurons were infected with both TBEV strains at the multiplicity of infection of 5 (MOI) for two hours in half volume of cultivation media to favour virus adsorption. Then, the inoculum was removed, and fresh cultivation media was replenished to the normal volume. To determine the appropriate MOI for infection, parallel differentiated cells were counted prior to the infection in order to determine the cell numbers after differentiation.

2.3. Immunofluorescence assay

The presence of characteristic markers denoting the stage of differentiation and development of infection in astrocytes and neurons was analysed by immunofluorescence assay. Cells were seeded on coated chamber slides at concentrations detailed in Table S1 and, when applicable, infected or mock-infected. At 24/72 h post-infection (p.i.), the presence of differentiation and infection markers was assayed. During sample processing, cells were fixed with 4 % paraformaldehyde for 15 min. Cells were washed in PBS and permeabilized by 0.1 % Triton X-100 in PBS, and formaldehyde auto-fluorescence was quenched by 50 mM NH4Cl in 1 % bovine serum albumin (BSA) in PBS for 10 min twice. After PBS washing, blocking in 3 % BSA in PBS was undertaken for 1 h at room temperature. Target antigens were labelled for 1 h at room temperature or overnight at 4 °C by the following primary and secondary antibodies: dsRNA mAb (#10010200, SCICONS f2 from Scions, Biocompare), GFAP (GAS) Mouse mAb (#36705, Cell Signaling Technology), HuC/HuD Monoclonal Antibody (16A11) (#A-21271 Invitrogen, Thermo), MAP2 (#45425S, Cell Signaling Technology), S100B Polyclonal antibody (#bs-2015R Bios Antibodies), TBEV NS3 Langat Chicken IgY (NS3 antibodies to closely related Langat virus NS3 were kindly provided by Dr M. Bloom, 94 % homology with TBEV), Goat Chicken IgG H&L (DyLight® 488) (#ab96947, Abcam), Goat Guinea pig IgG H&L (Alexa Fluor® 594) (#ab150188, Abcam), Goat Mouse IgG H&L (DyLight® 594) preadsorbed (#ab96881 Abcam), Goat Rabbit IgG H&L (Alexa Fluor® 488) (#ab181448 Abcam). Concurrent nuclei staining and sample mounting were done with VECTASHIELD™ Antifade Mounting Medium with DAPI (#H-1200-10, Vector Laboratories). Images were taken with the Olympus Fluoview FV10i confocal microscope equipped with FV10-ASW software (v.1.7).

2.4. Growth curve

To assess the replication rate of TBEV in astrocytes and neurons, cells were either mock-treated or infected with the TBEV strains Hypr and Neudoerfl at the MOI of 5 (see details of differentiation in Table S1). At 2, 12, 24, 48, 72 for both cell lines and at 120 h p.i. (astrocytes only) culture supernatant was sampled and clarified by centrifugation for TBEV titre assessment. TBEV titres were plaque assayed on the human lung adenocarcinoma monolayers (A549; kindly gifted by R. Randall, University of St. Andrews, UK) according to the modified protocol of de Madrid et al. [41]. A549 were maintained in low glucose DMEM cultivation medium (#L0064-500, Biowest, WVR) supplemented with 10 % foetal bovine serum (FBS; #S1810-500, Biowest, WVR), 1 % antibiotics (penicillin G 100 units/ml, streptomycin 100 μg/ml; #L0022-020, Biowest), and 1 % L-alanyl-L-glutamine (#X0551-100, WVR) in 5 % CO2 humidified atmosphere at 37 °C. Briefly, ten-fold dilutions of virus samples were mixed with the A549 cell suspension (1.5 × 10^5 cells/well of 24-well plate), cells were let to adhere, and virus to adsorb. After 4 h, an overlay mixture (1:1 v/v of carboxymethyl cellulose and 2 × concentrated DMEM cultivation medium) was applied dropwise to the cells. After 5 days, cell monolayers were washed with physiologic solution (0.9 % NaCl), and plaques were fixed and stained with naphthalene black solu-
tion (0.1 % naphthalene black in 6 % acetic acid solution) for 45 min.

2.5. Viability assay

To detect the viability rates, neurons and astrocytes were differentiated as specified in Table S1 and infected with 5 MOI of Hypr and Neudoerfl TBEV strain. 2–3 h prior to the sampling interval (12, 24, 48, 72, 120, 168 h p.i.) incubation with AlamarBlue reagent in the cultivation media (1:10 v/v); #DAL1025, Thermo Fisher Scientific) was performed in a dark. Viability for each cell type was assessed by fluorescence measurement ($\lambda_{ex} = 550$ nm, $\lambda_{em} = 590$ nm) using Tecan infinite 2000Pro, (Tecan i-control, 1.11.1.0) from four biological and two technical replicates and the viability value of the mock-treated cells was set at 1 (100%).

2.6. qRT-PCR

For the analysis of TBEV replication in infected cells, viral RNA was quantified by an assay designed by Achazi et al. [42]. Total RNA (80 ng/reaction) was used for TBEV gRNA quantification with the KAPA PROBE FAST Universal One-Step qRT-PCR Master Mix (2X) (#KK4752, Sigma-Aldrich, MERCK) and relative fold induction of TBEV RNA amount was determined using the delta-delta ct ($\Delta\Delta Ct$) with the comparison to mock. For the verification of poly-(A) transcriptomic data, samples were treated with dsDNase (#EN0771, Thermo Fisher Scientific) and gene expression was analyzed with the KAPA SYBR FAST Universal One-Step qRT-PCR Kit (#KK4652, Sigma-Aldrich) according to the manufacturer’s protocol. Relative expressions of HSPA-6, OASL, RNVU1, and RSAD2 (viperin) genes were processed via the $\Delta\Delta Ct$ method with the comparison to mock. For the analysis of TBEV replication in infected cells, viral RNA was quantified by an assay designed by Achazi et al. [42]. Total RNA (80 ng/reaction) was used for TBEV gRNA quantification with the KAPA PROBE FAST Universal One-Step qRT-PCR Master Mix (2X) (#KK4752, Sigma-Aldrich, MERCK) and relative fold induction of TBEV RNA amount was determined using the delta-delta ct ($\Delta\Delta Ct$) with the comparison to mock. For the verification of poly-(A) transcriptomic data, samples were treated with dsDNase (#EN0771, Thermo Fisher Scientific) and gene expression was analyzed with the KAPA SYBR FAST Universal One-Step qRT-PCR Kit (#KK4652, Sigma-Aldrich) according to the manufacturer’s protocol. Relative expressions of HSPA-6, OASL, RNVU1, and RSAD2 (viperin) genes were processed via the $\Delta\Delta Ct$ method with the comparison to mock.

2.7. Transcriptomic analysis

2.7.1. Sample preparation

Each sample was prepared and analysed in biological triplicates. Total RNA from TBEV-infected human neurons and astrocytes was isolated at 24 and 72 h p.i. using RNA Blue reagent (Top-Bio, Vestec, Czech Republic) according to the manufacturer’s instructions. RNA concentration was measured using NanoPhotometer Pearl (Implen, München, Germany) and the quality of RNA was determined using 2100 Bioanalyzer with RNA 6000 Nano kit (Agilent Technologies, Santa Clara, CA, USA).

2.7.2. Poly-(A)-enriched RNAs

Sequencing library construction, sequencing, raw read quality check and adapter trimming were done by Novogene Co., Ltd (Beijing, China). The 150 PE library was sequenced in HiSeq 4000 sequencer (Illumina, San Diego, CA, USA). Quality filtering was performed using Cutadapt v1.15 [43] and the subsequent clean read quality check was done using FastQC v0.11.5. [44]. The mapping (GRCh38.p13 reference genome), assembly and differential expression analysis were performed using the Tuxedo suite [45].

2.7.3. Small RNAs

Sequencing library construction, sequencing, raw read quality check and adapter trimming were done by Novogene Co., Ltd (Beijing, China). The 50 SE library was sequenced in HiSeq 2500 sequencer (Illumina, San Diego, CA, USA). The clean read quality check was done using FastQC v0.11.5 [44]. Adapter contamination, short and low quality reads were removed using Cutadapt v1.15 [43]. The clean reads were mapped to the reference genome (GRCh38.p13) using miRDeep 2.0.1.2 [46]. The –q option for the mapper.pl script was used to allow 1 mismatch for mapping. The miRDeep2.pl module was run using the human miRBase database (miRBase v21) [47]. Identification of differentially expressed (DE) miRNAs was based on Benjamini-Hochberg P-value < 0.05 (unpaired Student’s t-test) and log2 fold change >1.5 or < -1.5 using the normalized fragments per kilobase of transcript per million mapped reads (FPKMs; cut-off 10 FPKM per miRNA species) ratios between the respective TBEV-infected sample and mock control.

2.7.4. Virus-derived small RNAs

The raw reads from small RNA library were also mapped to the genome of both TBEV strains, Hypr (GenBank accession number U39292) and Neudoerfl (GenBank accession number U27495), using Bowtie [48]. The read depth counts of each sample were retrieved from the corresponding bedgraphs produced using Bedtools v2.27.1 [49]. Read distribution along the TBEV genome was evaluated and visualised using Geneious Prime v2020.0.5. (Biomatters, Ltd, Auckland, New Zealand). The sense and antisense reads were additionally discriminated by extracting individual read types from their respective bam files using Samtools v1.10 [50] and their counts were compared for each sample.

2.8. In silico miRNA/vd-sRNA target prediction

miRNA trans target prediction was performed using miRWalk [51] and LncBase v.2 [52] toolkits. Briefly, DE miRNAs were divided into two groups: (1) up-regulated and (2) down-regulated; these served as an input for the respective prediction toolkit. Generated lists of predicted miRNA targets were subsequently used to identify these targets in DE protein-coding mRNAs (pc-mRNAs)/lncRNA datasets. For up-regulated miRNAs, the datasets of down-regulated pc-mRNAs/lncRNAs were used, and vice versa, datasets of up-regulated pc-mRNAs/lncRNAs were used in the case of down-regulated miRNAs. Default settings were used for miRWalk (P-value < 0.05, miRNAs targeting 3’UTR) in combination with the miRDB prediction tool. In the case of LncBase, the prediction module with default settings was used (cut-off score > 0.90). Identification of human miRNAs targeting TBEV Hypr/Neudoerfl genomic RNA or human miRNAs being targeted by 21–23 nt long vd-sRNAs was performed using the miRDB tool with a cut-off score > 0.70 [53].

2.9. Differential splicing analysis

The identification and quantification of differential splicing were computed using MAJIQ v2.2 [54], which employs Local splicing variations (LSV) derived from the provided transcriptome annotation file and RNA-seq data. The differential splicing was considered significant for LSVs with MAJIQ default cut-off values for deltaPSI (|$\Delta PSI$| > 0.2) supported by P($|\Delta PSI|$ > 0.2) with a 0.05 cut-off (Bonferroni correction).

The raw reads of each sample represented by biological triplicates were matched to the reference genome (GRCh38.p13) with STAR 2.7.7a [55]. The resulting bam files and an annotation file of the respective human genome were supplied to a MAJIQ builder. The mapping quality check was done using FastQC v0.11.5 [44]. The clean reads were mapped to the reference genome (GRCh38.p13) using miRDeep 2.0.1.2 [46]. The –q option for the mapper.pl script was used to allow 1 mismatch for mapping. The miRDeep2.pl module was run using the human miRBase database (miRBase v21) [47]. Identification of differentially expressed (DE) miRNAs was based on Benjamini-Hochberg P-value < 0.05 (unpaired Student’s t-test) and log2 fold change >1.5 or < -1.5 using the normalized fragments per kilobase of transcript per million mapped reads (FPKMs; cut-off 10 FPKM per miRNA species) ratios between the respective TBEV-infected sample and mock control.

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The raw reads of each sample represented by biological triplicates were matched to the reference genome (GRCh38.p13) with STAR 2.7.7a [55]. The resulting bam files and an annotation file of the respective human genome were supplied to a MAJIQ builder for splice graph construction using default parameters. The relative abundance of LSVs was calculated using MAJIQ Quantifier with
default parameters. The quantifier module computes the abundance of each LSV using marginal percent spliced index (PSI, denoted $\Psi$). The PSI is calculated for each splice junction (SJ) and expresses the probability of splicing compared to other SJs in a given splicing event. The differential splicing is inferred from relative changes of LSVs among different conditions using delta PSI (dPSI, denoted $\Delta \Psi$). The summary and visualization of differential splicing were done for each sample with the MAJIQ Voila package.

### 2.10. Immunoblotting

Isolation of proteins was performed from the samples used for RNA isolation using RNA Blue according to the manufacturer’s instructions. The resulting protein isolates were separated on 12% polyacrylamide gels and subsequent immunoblotting detection was performed as described previously [56]. The following antibodies were used: guinea-pig polyclonal serum against TBEV capsid protein (C) (produced in-house), anti-GAPDH antibody [EPR16891] (Abcam; #ab181602), HRP goat anti-guinea pig (Novex; #A18769), HRP goat anti-rabbit IgG antibody (Vector Laboratories; #PI-1000). Sample inputs were standardised by equal protein amounts loaded into each well (10 μg).

### 2.11. Statistical analyses

If not mentioned otherwise default settings for built-in statistics were used in employed computational packages/tools. Statistical data analysis in the case of TBEV infection dynamics (Fig. 1) was performed in the GraphPad Prism software (version 8.3.0, GraphPad Software, San Diego, CA, USA): cell viability was analysed by Welch’s $t$-test with multiple testing correction by FDR (Benjamini, Krieger, and Yekutieli, $q < 0.05$) and TBEV titre differences were...
analysed by unpaired Student’s t-test (\( \alpha = 0.05 \)). Conformity of sequencing and qRT-PCR data was analysed with the Spearman correlation analysis (\( \alpha = 0.05 \)).

3. Results

3.1. Infection of in vitro differentiated human neurons and astrocytes by TBEV Hypr or Neudoerfl resulted in a different pathogenic pattern

Initially, we focused on deriving a suitable experimental model to study TBEV infection in the target cells, human neurons and astrocytes. Primary human neurons and astrocytes were derived from neural progenitor cells of human origin – Human Neural Stem Cells (hNSCs). Following the differentiation, the characteristic appearance of astrocyte and neuron morphology was observed and further, the expression of cell-type-specific markers was verified using an immunofluorescence assay. Neuron identity was verified by the presence of markers HUDD and MAP2, and astrocytes identity was assayed by the expression of S100B and GFAP (Fig. 1A). In each cell type, at least 90 % of cells were labelled positively for at least one of the differentiation markers (data not shown).

Further, we intended to characterize the development of infection in neurons and astrocytes. Therefore, both cell types were infected with two European subtype strains of TBEV, the highly pathogenic Hypr and mild Neudoerfl, at the multiplicity of infection (MOI) of 5. The production of the virus in both cell lines was analysed with plaque assay on A549 cells (Fig. 1B). In addition to TBEV titres, the relative levels of TBEV genomic RNA (gRNA) were determined in the course of infection as well (Fig. 1D).

Infection of both cell types with TBEV resulted in the production of relatively high viral titres. In neurons, peak production of the virus was reached at 48 h p.i. (12.96 \( \times 10^7 \pm 3.21 \times 10^7 \) and 5.93 \( \times 10^7 \pm 1.16 \times 10^7 \) for Hypr and Neudoerfl, respectively) and remained steady further on. In astrocytes, the peak production of the virus was reached at 48 h p.i. (1.53 \( \times 10^7 \pm 0.46 \times 10^7 \) and 0.35 \( \times 10^7 \pm 0.09 \times 10^7 \) for Hypr and Neudoerfl, respectively). However, a decline in the viral titres was observed hereafter (Fig. 1B).

The viability of infected astrocytes was not significantly affected by TBEV in most of the time periods tested (12–168 h p.i.) regardless of the strain of TBEV used. The only significant reduction in viability by 10 % was seen in astrocytes infected with Neudoerfl after 5 days p.i. Dissimilarly, TBEV Hypr reduced the viability of infected neurons by 40 % at 72 h p.i. and <20 % of cells survived in the later time points. Less virulent strain Neudoerfl affected cell viability the most at 120 h p.i. by killing approximately 30 % of the neurons (Fig. 1C).

To illustrate the infection process entirely, the amount of viral genomic RNA was measured by qRT-PCR (Fig. 1D), and the amount of viral capsid protein reflecting the proteosynthesis was determined by immunoblotting at 24 and 72 h p.i. (Fig. 1E). Alike the progeny production, the amount of viral RNA in neurons exceeded the amount in astrocytes by >10-fold in both intervals examined. The more virulent TBEV strain Hypr exhibited a slightly higher replication rate than the Neudoerfl strain (Fig. 1D). A similar trend in the amount of viral RNA in neurons exceeded the amount in astrocytes by >10-fold in both intervals examined. The more virulent TBEV strain Hypr exhibited a slightly higher replication rate than the Neudoerfl strain (Fig. 1D). A similar trend in the amount of viral RNA in neurons exceeded the amount in astrocytes by >10-fold in both intervals examined. The more virulent TBEV strain Hypr exhibited a slightly higher replication rate than the Neudoerfl strain (Fig. 1D). A similar trend in the amount of viral RNA in neurons exceeded the amount in astrocytes by >10-fold in both intervals examined. The more virulent TBEV strain Hypr exhibited a slightly higher replication rate than the Neudoerfl strain (Fig. 1D).

The differential expression analysis results were verified using real-time PCR quantification of mRNA levels for selected DE genes (RSAD2, RNVU1, HSPA6, OASL). The correlation between qPCR and NGS data is statistically significant (Spearman correlation \( r = 0.81 \), \( p < 0.001 \)), therefore, the RNA-seq data were successfully validated (Fig. 3E).

To describe the cellular processes affected by the dysregulation of identified DE pc-mRNAs, we performed a gene set enrichment analysis (GSEA) using the DAVID toolkit. A vast majority of significantly enriched GO classes (Benjamini-Hochberg \( P \)-value < 0.05) were related to the antiviral immune response (Fig. 3G). In addition, the same analyses of down-regulated gene sets uncovered an interesting phenomenon, where the proper neuronal development and assembly of the extracellular matrix are negatively affected by TBEV infection (Fig. 3G).
3.2.2. Differential expression analysis of small RNAs

Sequencing the small RNA selected library of all samples yielded an average of 41 M 50 SE reads. Quality filtering using Cutadapt dropped on average 1.8% low-quality and/or short reads. The remaining reads were mapped to the human genome assembly (GRCh38.p13) using miRDeep2 with 58.2% average mapping efficiency. The details of sequencing output, quality filtering and mapping statistics per sample are given in Table S4.

In small RNA datasets, a total of 145 miRNA species were described as significantly dysregulated upon TBEV infection (Benjamini-Hochberg P-value < 0.05 and log2 fold change >1.5 or <−1.5) (Fig. 4; Table S5). Interestingly, while infection with severe Hypr strain resulted in higher numbers of dysregulated pc-mRNAs and ncRNAs when compared to mild Neudoerfl strain, the DE miRNA pattern showed an opposite trend (Fig. 4A), that is distinct reduction of significantly affected miRNAs by more virulent TBEV strain. Furthermore, the expression profiles document a surprisingly high cell-specific response as only one miRNA species (hsa-miR-592) was found to be up-regulated in both cell types, astrocytes (A72_H) and neurons (N72_N) (Fig. 4B and D). The phenomenon of high specificity was also observed between TBEV strains, where only 14 (9.7%) miRNA species followed the same pattern of dysregulation in the case of both, Hypr and Neudoerfl (Fig. 4C and D). The verification of miRNA-seq data was performed using qPCR quantification of hsa-miR-1248 and hsa-miR-145-5p; the positive correlation between both methods was confirmed to be statistically significant (Fig. 4E; Spearman correlation r = 0.64, p = 0.009).

Several studies dealing with small RNA profiling in flavivirus-infected cells have reported the presence of vd-sRNAs [34,35]. We, therefore, assessed whether TBEV infection also resulted in the generation of vd-sRNAs by mapping the small RNA datasets from RNA-seq to TBEV Hypr genomic sequence (GenBank accession no. U39292).
vd-sRNAs was confirmed in all TBEV Hypr-infected samples with small RNA species ranging from 16 to 50 nucleotides in size. As expected, the amounts of mapped reads increased with the infection progress in all specimens. Furthermore, vd-sRNAs were predominantly derived from the sense strand of the TBEV genome (Fig. S3) and reached higher, but not significantly different (unpaired Student’s t-test; p < 0.05), levels in neurons (6.24×10^5 ± 5.74×10^4) than in astrocytes (4.87×10^5 ± 6.30×10^4) (Fig. 4E). These findings positively correlate with higher viral titres and gRNA observed in neurons (Fig. 1). The pattern of reads distribution throughout the viral genome appears rather mosaic with several specific hot-spots. Besides, the hot-
spots identified in all of the analysed samples are strikingly conserved and the main difference was observed on the level of read counts (Fig. S4). The most universal/unabundant peak was identified at the end of the 3' UTR.

3.3. Candidate gene networks responsible for a different outcome of TBEV Hypr infection in neurons vs. Astrocytes

As TBEV Hypr dramatically decreased the number of living neurons but not astrocytes (Fig. 1C), we sought to characterize the differences between N72_H and A72_H datasets to identify potential key factors involved in higher neuronal susceptibility to TBEV infection. First, we selected DE pc-mRNAs, which were either uniquely up-/down-regulated in N72_H/A72_H datasets or the difference in expression profile (unique expression in neurons/astrocytes or more pronounced response criteria used to evaluate pc-mRNAs. This way, the miRNA target prediction was performed for the selected datasets of pc-mRNAs (miRNAWalk; [51]) and lncRNAs (LncBase v.2; [52]). Prediction results summarized in Table S6 show that 173 genes were identified as possible targets (133 pc-mRNAs and 40 lncRNAs), including genes involved in immune response (e.g., OAS1, TRIM38, SPSB4), proper neuronal development and function (e.g., NRARP, KCNK10, SIRPA, CLIC5, TMCS8, KIT, CNNT2, DCC) as well as transcription factors (e.g., TFAP2B, HEYL, CDK12, BEND3). In order to get a complete overview of the possible interactions, potential cis targets (100 kb upstream and downstream) were predicted for DE lncRNAs with log2-fold change >4 or <4 as well as for lncRNAs identified as potential targets of DE miRNAs (Table S7). In total, 45 cis targets were predicted, including immune response-related genes (loci RSAD2, CYP4A11, IFNL1-4), receptors (e.g., DCC, UT52R), and transcription factors (e.g., DLX5, OTAD2).

The differences in the vd-sRNA mapping profile between the two cell types may substantially influence the enhanced neuronal sensitivity to Hypr infection. One of the possible scenarios suggests that vd-sRNAs could mimic host miRNAs, leading to a lowered expression of a target gene [37]. Therefore, we extracted the 21–23 nt long vd-sRNAs and selected hotspots HS1–HS12 which were present uniquely in higher/lower numbers in N72_H and A72_H samples (Table 3; Fig. 5E). Surprisingly, HS3, HS7, HS9, HS10, and HS12 had higher read counts in Hypr-infected astrocytes, and only HS11 had a higher read count in Hypr-infected neurons. Subsequently, the prediction of vd-sRNA targets was performed using
Fig. 4. Differential expression analysis of miRNAs in TBEV-infected neurons and astrocytes revealed strain-specific and cell-specific responses. (A) Graphic summary of DE miRNAs in analysed samples. (↑) up-regulated miRNAs, (↓) down-regulated miRNAs. (B) Venn diagram describing the cell specificity of miRNA expression upon TBEV infection. (↑) up-regulated miRNAs, (↓) down-regulated miRNAs. (C) Venn diagram describing the strain specificity of miRNA expression upon TBEV infection. (H) – TBEV Hypr strain, (N) – TBEV Neudoerfl strain. (D) Heatmap visualizing the overall expression pattern of miRNAs in analysed samples. (E) Verification of RNA-seq data by qRT-PCR. Relative expression of hsa-miR-1248 and hsa-miR-145-5p was calculated using the ΔΔ-ct method with hsa-miR-103a as a reference gene and Sp6 as an internal control. All samples were analysed in biological and technical triplicates. Spearman correlation was evaluated with GraphPad Prism software. (F) Frequency distribution of 21–23 nt vd-sRNAs mapped to TBEV Hypr genome in N72_H (black) and A72_H (red) samples. HS – hot-spot. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Fig. 5. TBEV Hypr infection in astrocytes results in a stronger innate immune response than in neurons. (A) Heatmap summarizing results from gene set enrichment analysis (DAVID tool; GO Biological processes; p < 0.05) of DE genes 1) uniquely expressed in either A72_H or N72_H, and 2) genes expressed with >1.5 log2 difference in one of the samples. (B) Relative expression of OASL and RSAD2 (viperin) genes in TBEV infected cells. Calculations were performed via the ΔΔ-ct method with HPRT1 as a reference gene. All samples were analysed in biological and technical triplicates. (C) Expression pattern of IFN-β and IFN-α in TBEV-infected cells based on RNA-seq data. (D) Overview and comparison of the expression pattern of selected immune response-related genes from this study and works of Fares et al. [12] and Pokorna et al. [13]. (E) Number of mapped reads for vd-sRNAs (16–50 nt) in the respective sample. Data summarise three independent experiments, and values in graphs are expressed as mean ± SD.
the miRDB tool in combination with DE pc-mRNA lists. In total, 51 host DE pc-mRNAs were identified (Table 4). Interestingly, vd-sRNAs derived from HS10 and HS12, the two hot-spots with the highest read count in astrocytes, were both predicted to target RNA splicing factors MBNL2 and RBF0X1, whose mRNA levels were significantly lower in A72_H in comparison to N72_H (Table S3). For HS11-derived vd-sRNA, nine mRNA targets with the corresponding expression pattern (N72_H < A72_H) were predicted (LEKR1, DLX1, AOX1, MYCN, PCDH18, DPYSLS2, TSH21, PFKFB3, BCL9L). Notably, four out of nine identified target genes were characterized as transcription factors (DLX1, MYCN, TSH21, BCL9L), with DLX1 and DPYSLS2 further considered as factors contributing to proper neural development [59,60].

### 3.4. Candidate gene networks responsible for distinct TBEV infection outcomes in neurons during infection with strains of varying severity

The comparison of infection dynamics and cell death rate between sensitive Hypr and mild Neudoerfl strains in infected neurons confirmed a higher pathogenic effect for Hypr (Fig. 1). Therefore, observed differences between Hypr and Neudoerfl infection manifestation in human neuronal cells drove us to search for differences between N72_H and N72_N datasets on the pc-mRNA/miRNA/lncRNA level. Using an in silico approach, we employed an analogous pipeline to A72_H and N72_H datasets.

We identified 1935 DE pc-mRNAs either uniquely up-/down-regulated in N72_H/N72_N datasets or differentially expressed (both up and down-regulated) between N72_H/N72_N datasets with a fold change higher than 1.5 log2. The GSEA analysis found significant differences in infection between the two TBEV strains in (i) the extent of the negative impact of Hypr infection on neuronal development, (ii) the level of Hypr-induced dysregulated activity of RNA polymerase II promoters, and (iii) higher activation of host immune response to Hypr infection (Fig. 6A).

Identifying the complex regulatory network of small regulatory RNAs also requires the identification of their target sites. Thus, we selected DE expressed miRNAs and lncRNAs under the same conditions as the pc-mRNAs selection. Using the miRWalk and IncBase v.2 databases, the miRNA targets were identified for pc-mRNAs and lncRNAs, respectively. The predicted 268 trans targets for both small RNA classes (207 pc-mRNAs and 61 lncRNAs) are listed in Table S8. The complete overview of the possible interactions was predicted by the identification of potential cis targets (100 kb upstream and downstream) for DE lncRNAs with log2-fold change <4 or <-4 and miRNAs identified as potential targets of DE miRNAs (Table S9). In total, 47 cis targets were predicted, including transcription factors (e.g. DLX5, DLX6, TFAP2A) and various receptors (e.g. CHRNA3, CHRNA4, DCC, PTPRH).

The assessment of vd-sRNA presence in TBEV Neudoerfl-infected cells revealed a similar pattern to Hypr vd-sRNA data – increasing number of vd-sRNAs through the infection and higher numbers of sense reads (Fig. S4). Comparison between N72_N and N72_H datasets further documented higher numbers of mapped reads during Hypr infection compared to Neudoerfl (Fig. 6B and C), which is in agreement with higher titres and viral gRNA levels (Fig. 1). The distribution pattern of 21–23 nt long vd-sRNAs was identical for 12 hot-spots with higher abundance in N72_H samples (Table 3; Fig. 6D). Prediction of host mRNA targets was performed using the miRDB tool in combination with DE pc-mRNA datasets with 66 identified host mRNA targets (Table 4).

We further focused on HS11, the hot-spot with the highest number of mapped 21–23 nt reads in N72_H samples. The vd-sRNA derived from HS11 was predicted to target 22 mRNAs with the corresponding expression pattern (N72_H < N72_N), out of which seven pc-mRNAs (DLXI, MYCN, PCDH18, DPYSLS2, TSH21, PFKFB3, BCL9L) were also identified as less expressed in A72_H samples compared to N72_H. We suggest that these genes represent a candidate set of markers of increased pathogenicity that Hypr strain confers towards neurons.

### 3.5. TBEV infection results in altered pre-mRNA splicing

Both, neuron/astrocyte-specific response to infection and pathogenicity-related response to infection, led us to examine the potential impairment of host pre-mRNA splicing events since several lncRNAs and vd-sRNAs were predicted to interact with partners involved in this pathway. The idea was also supported by previous studies describing DENV- and ZIKV-induced alterations in host pre-mRNA splicing [27,61,62]. Thus, RNA-seq data of astrocytes and neurons infected with TBEV were submitted to a differential splicing analysis using MAJIQ v2.2 [54]. The corresponding mock controls were used as a reference for differential splicing calculation. Identified LSVs were further classified by MAJIQ to binary and complex events. Binary LSVs include exon skipping (ES), intron retention (IR), alternative 3′/5′ splice site (A3SS/A5SS) and involve only two exons or two splice sites in the same exon. Complex LSVs combine several binary events originating from or targeting the same site. In total, differential splicing analysis revealed 4009 LSVs in all datasets (Table S10) and the vast majority of detected LSVs (3639; 90.7 %) were documented for N72_H (2930; 99.6 %) and A72_H (1249; 31.2 %) datasets; interest-
In infection with Neudoerfl strain the combination of virus-induced neuronal cell death and the immunopathogenic effect of activated host immune response [10,12]. The complexity of the whole process is further supported by the different outcomes of TBEV infection in neurons and astrocytes [5–7,12]. One of the suggested mechanisms underlying the distinct susceptibility of both cell types to TBEV infection is the neuron-/astrocyte-specific expression pattern of immune response-related genes [12,13]. However, both studies assayed only a limited number of pre-selected genes in a microarray-based analysis. Here, we present a thorough transcriptomic study describing differential expression of both small RNAs as well as poly-(A) RNAs in human neurons and astrocytes infected by mild and severe strains of TBEV in an early and late stage infection. Thus, the combination of all factors (infection time, cell type, TBEV strain) gives a complex overview of host-cell response at the RNA level and provides new significant data for a better understanding of TBEV neuropathogenesis.

### 4.1. Cell-type specific response in neurons and astrocytes challenged by TBEV

In silico pc-mRNA-miRNA-lncRNA interactome analysis identified major differences in response between TBEV challenged astrocytes and neurons. The key feature was the expression pattern of immune response-related genes (Fig. 5; Table S3; Fig. S3), which in concordance with studies of Fares et al. and Pokorna et al. [12,13]. The phenomenon of cell type-specific immune response to flaviviral infection in CNS was also described for WNV [63], which further highlights its relevance for the flaviviral pathogenic effect. Besides, Hypr infection negatively affects extracellular matrix organization and RNAPII promoter activity pathways in

### Table 4

| vd-sRNA | Σ read count | A/N ratio | predicted gene targets (miRDB) |
|---------|--------------|-----------|---------------------------------|
| HS1     | 279 497      | 0.15      | N/A                             |
| HS2     | 171 548      | 0.85      | N/A                             |
| HS3     | 373 158      | 2.34      | OPCML, THUMP21, LPXN, GAS7, CDH7, ERBB4, ZMAT3, ANKS1A, GIPC2 |
| HS4     | 92 596       | 0.96      | N/A                             |
| HS5     | 108 436      | 1.60      | TRC1D30, PLCH1, G2E3, PBX1, ESRG, TIPARP, MAN1A1 |
| HS6     | 246 963      | 1.28      | N/A                             |
| HS7     | 193 436      | 1.60      | N/A                             |
| HS8-1   | 91 952       | 1.26      | N/A                             |
| HS8-2   | 92 748       | 1.14      | N/A                             |
| HS9     | 309 580      | 2.46      | BDNF, KCNS2, SHSAL1, GOLGA4, LGI1 |
| HS10    | 751 154      | 4.99      | PCHD20, TM45F18, TMT1, MCT1, RFX3, TRHDE, ZMYM5, Sema3E, MBLN2, LOR, CXADR, CALB1, ARR2, GABRC2, DIRAS2, NIK7, FUT9 |
| HS11    | 124 140      | 0.61      | LEKR1, DLX1, AOX1, MYCN, PCDH18, DPYS52, TSHZ1, PKFB3, BC5L5 |
| HS12    | 597 869      | 1.76      | RFX3, RNF212, MBLN2, GRIN2A, CREG2, KR1 |

| N/A ratio | predicted gene targets (miRDB) |
|------------|---------------------------------|
| HS1        | N/A                             |
| HS2        | N/A                             |
| HS3        | SIAH3, NKAIN1, EBF1, KLHDC8A, STGAL1, DNMT3B, SOGA1, SHF, KLLH13 |
| HS4        | PIK3C2B, EPHB1, NINRIN, ELAVL2, SH3T2C, ZNF124, SLTRK8, SIRPA, ONECUT, BDKRB2, ACACA, RTL6B, ZNF730 |
| HS5        | N/A                             |
| HS6        | N/A                             |
| HS7        | N/A                             |
| HS8-1      | N/A                             |
| HS8-2      | N/A                             |
| HS9        | N/A                             |
| HS10       | N/A                             |
| HS11       | N/A                             |
| HS12       | N/A                             |

**4. Discussion**

TBEV, a neurotropic flavivirus, targets the host CNS and frequently causes severe encephalitis in infected patients. The exact mechanism responsible for TBEV-induced neuropathogenesis has not been fully understood to date, although several studies suggest
infected neurons, which may also play a substantial role in the impairment of the neuronal tissue.

The altered expression pattern of pc-mRNAs, which belong to the aforementioned pathways, could result from the miRNA/lncRNA-driven regulation process. Thus, we further focused on the linkage of the DE miRNAs/lncRNAs to their possible targets in the DE pc-mRNA datasets. The \textit{in silico} prediction revealed several networks possibly involved in the differential expression pattern documented in astrocytes and neurons.

Interferon signalling is considered a key regulatory cascade of the innate immune system triggering the expression of a wide panel of ISGs with a direct or indirect antiviral effect [64]. Our data revealed that in addition to IFN-\(\beta\), IFN-\(\lambda\) expression is induced in both cell types in an early TBEV infection (24 h p.i.). However, only astrocytes maintained the IFN-\(\lambda\) expression later during the infection (72 h p.i.). Interestingly, the IFN-\(\lambda\) cluster was predicted to be a cis target regulated by AC011445.2 lncRNA (ENSG00000269246), which was highly up-regulated in A72_H,
but not in N72_H samples (Fig. 5, Table S3). Our previous study demonstrates that IFN-κ1 is the predominantly expressed interferon in response to TBEV infection in cells of neuronal origin [28], and its significance was also demonstrated in the case of WNV infection [65]. Thus, the deficiency of IFN-κ1 production in neurons is a potential contributor to the decreased expression of specific ISGs, which are necessary for a successful antiviral response to TBEV in astrocytes. Similarly, astrocyte-specific up-regulation of AL445490.1 lncRNA (ENSG00000225886) may contribute to the elevated expression of IFI6, a predicted cis-target of this lncRNA (Table S7).

When we focused on the neural cell-type-specific response in the case of host miRNAs, the hsa-miR-1298 is the only miRNA species which was significantly up-regulated in Hypr-infected neurons, while significantly down-regulated in Hypr-infected astrocytes (Fig. 4D). Among the predicted hsa-miR-1298 mRNA targets with the corresponding DE profile (LUZP2, SPSB4, VCAN, TFAP2B, MAST3, RSAD2, CMPK2, and DLX5), the RSAD2 and CMPK2 we found the most important since both were documented to interfere with flavivirus infection [66]. Additionally, hsa-miR-7974, the second most up-regulated miRNA in Hypr-infected neurons, was predicted to target OAS1, whose DE profile also showed a decreasing induction rate in Hypr-infected neurons and which has already been proven to possess strong antiviral activity against flaviviruses [67]. Interestingly, a post mortem analysis of brain tissue from children with congenital ZIKV syndrome revealed elevated levels of hsa-miR-145 and hsa-miR-148a [68]. Similarly, here we documented that hsa-miR-145-5p was up-regulated exclusively in
Hypr- and Neudoerfl-infected neurons, whereas *hsa-miR-148a-5p* was up-regulated exclusively in Hypr- and Neudoerfl-infected astrocytes (Fig. 4).

Besides dysregulated host miRNAs, small RNAs derived from the viral genome may also interfere with host mRNA levels [37,38]. Our data document an accumulation of TBEV vd-sRNAs in infected neurons and astrocytes; the distribution pattern of vd-sRNAs across the TBEV genome was remarkably similar among the samples, with coverage rate as the only variable factor. Interestingly, in the case of TBEV Hypr-infected astrocytes, vd-sRNAs (21–23 nt long) derived from the two hot-spots with the highest coverage (HS10, HS12) were both predicted to target MBNL2 and RBFOX1. These genes are involved in pre-mRNA splicing regulation necessary for proper neural development [69–72], and their depletion may thus contribute to the observed dysregulation of the splicing process in astrocytes. This hypothesis is supported by a finding that one of the RBFOX1 targets, SNAP25 [69], was identified among differently spliced mRNAs in TBEV Hypr-infected astrocytes. Analogously, vd-sRNA derived from HS11 (hot-spot with the highest coverage in TBEV Hypr-infected neurons) was predicted to target 22 genes in total. However, only seven genes (DLX1, MYCN, PCDH118, DYSL2, TSHZ1, PKFB3, BCL9L) met the criterion of being expressed at a lower rate in N72_H in comparison to A72_H and N72_N samples. The dysregulation of mRNA levels through HS11-derived vd-sRNA for these genes could be thus responsible for the severe pathogenic effect in Hypr-infected neurons when compared to Hypr-infected astrocytes and Neudoerfl-infected neurons.

4.2. Alternative splicing as an important co-factor of high TBEV Hypr virulence in neurons

Based on the previous studies describing flavivirus-induced changes in the host pre-mRNA splicing process [61,62] in combination with observed dysregulation of various splicing factors upon TBEV infection (e.g., MBNL2, RBFOX1), or CELF3/4/5; Table S3), we hypothesized that TBEV infection alters the splicing of host mRNAs. Indeed, the differential splicing analysis revealed significant alterations in TBEV-infected cells. Since more than half of differently spliced LSVs were identified in Hypr-infected neurons (64.5%), we contemplate that this phenomenon may also contribute substantially to the high pathogenic effect of Hypr strain in infected neurons. This assumption is further corroborated by the list of affected significantly enriched biological pathways, including DNA repair, G2/M transition during the mitotic cycle, protein transport, and membrane fusion. Malfunction of these processes, especially DNA repair and G2/M checkpoint, results in an elevated apoptotic rate and neuronal dysfunction [73,74].

Earlier, we reported a diverse rate of pathogenesis between TBEV Hypr and Neudoerfl strains in a human cell line of neuronal origin [56]. Similar findings were documented in the human primary neurons in this work, where Hypr infection resulted in a substantially more severe pathogenic effect (Fig. 1C). Therefore, we tried to extrapolate the link between the intensity of pathogenesis, replication rate, and host transcriptional response. Even though both strains reached high titres in neurons, Hypr strain replication and progeny production were more efficient, generating almost 10-times more mature virions (Fig. 1B), gRNA (Fig. 1D), and vd-sRNAs (Fig. 6B–D). Moreover, a lower replication rate of the Neudoerfl strain correlates with low numbers of identified DE poly-(A) RNAs (Fig. 3B) and altered splicing events (Fig. 7A), which suggests a direct dependency on virus replication rate and intensity of the host response. Almost identical findings were described in the study of Overby et al., where the Hypr strain replicated with higher efficiency and induced a stronger immune response than the Neudoerfl strain [75]. On the contrary, the study of Sessions et al. described an inverse trend, where an attenuated DENV1 strain caused three-fold more changes in the host transcriptome than the wild-type DENV1 [61].

4.3. Diverse strain virulence in neurons

The lower replication and pathogenesis rates observed for the mild Neudoerfl strain can also be perceived as a consequence of a more successful host response. However, Neudoerfl-infected cells showed a lower rate of immune response activation in the case of pc-mRNAs (Table S3 and Fig. S3). Thus, the dysregulation of miRNA with proviral or antiviral properties may be the factor involved. We, therefore, compared the list of DE miRNAs in N72_H and N72_N samples with the list of experimentally verified proviral and antiviral miRNAs in the case of ZIKV [76] and DENV [77]. However, no corresponding matches were found. We, therefore, searched for novel antiviral miRNAs targeting Hypr or Neudoerfl gRNA using the mirDB tool. The *hsa-miR-592* was identified as the only miRNA species targeting Hypr and Neudoerfl gRNA. At the same time, this miRNA was up-regulated in samples with lower TBEV titres (A72_H and N72_N), but not in samples with higher TBEV titres (N72_H) (Fig. 4). These correlations thus suggest that *hsa-miR-592* may negatively regulate TBEV replication in human neurons and astrocytes. Except for the direct interaction with TBEV gRNA, *hsa-miR-592* may indirectly affect TBEV replication as well. He et al. [78] experimentally proved that *hsa-miR-592* targets SPRY2 mRNA, which is a negative regulator of interferon signalling [79], and in our study was shown to be up-regulated only in N72_H samples. We thus identified a new potential circuit of host cell countermeasure against TBEV involving *hsa-miR-592* unique among flaviviruses.

5. Conclusions

Altogether, we have characterized the alterations in poly-(A) RNA and small RNA expression profile of human neurons and astrocytes upon TBEV infection using two TBEV strains of distinctive virulence (mild Neudoerfl and severe Hypr). Subsequent integrative *in silico* analysis of miRNA/mRNA/lncRNA/vd-sRNA networks and pre-mRNA splicing found significant changes in inflammatory and immune response pathways, nervous system development and regulation of mitosis in TBEV Hypr-infected neurons. Candidate mechanisms include specific regulation of host mRNA levels via differentially expressed miRNAs/lncRNAs or vd-sRNAs mimicking endogenous miRNAs and virus-driven modulation of host pre-mRNA splicing. Thus, our data provide a valuable source of information for any research that aims to investigate and further characterise the mechanism of TBEV-host interactions and the related process of TBEV pathogenesis. Moreover, strain-specific expression pattern of selected host genes also represents a list of potential biomarkers, which can be used for improved TBEV diagnostics.

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
Supplementary data to this article can be found online at https://doi.org/10.1186/cshb.2022.05.052.

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