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研究課題名（和文）Long Non-Coding RNAs in Viral Infection

研究課題名（英文）Long Non-Coding RNAs in Viral Infection

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研究成果の概要（和文）：日本脳炎ウイルス(JEV)による神経傷害機構の検索を目的とし、JEVに感染した神経細胞のトランスクリプトームを実施し、ER ストレス関与因子（SESN2）、アミノ酸輸送体（ xCT） の発現が増強していることを見出した。JEV感染ではパーキンソン病様症状が後遺症となること、SESN2の発現上昇とパーキンソン病が関与することが報告されており、JEV感染脳でのSESN2の発現を調べること、および薬物によりxCTを低下させたJEV感染モデルマウスのウイルス複製、生存率を調べることは重要であることが示唆された。

研究成果の学術的意義や社会的意義
The academic significance rests on a publicly available database of a JEV-infected neuronal transcriptome for researchers studying flavivirus-induced neuropathogenesis. The social significance derives from allowing a deepening of collaboration between scientists in Japan with colleagues overseas.

研究成果の概要（英文）：We made publicly available the first JEV-infected neuronal transcriptome for research on flavivirus-associated neuropathology. We focused on previously unrecognised responses to virus infection: upregulation of novel ER stress signalling transcripts (Sestrin2 and TRIB3) and of the light and heavy chains of the xCT amino acid antiporter. The prior link between elevated SESN2 and Parkinson's disease is intriguing in the context of JEV infection as one of the distinct neurological sequelae associated with JEV is a Parkinsonian-like phenotype. SESN2 levels in brains of flavivirus models should be examined. Our findings raise the possibility that xCT activation and efflux of the amino acid glutamate (activator of excitatory neurotransmitter receptors) is potentially implicated in the pathophysiology of JEV-induced neurodegeneration. Perturbation of xCT by drugs in vivo should be performed to see the impact on viral replication and survival rates in mouse models of flavivirus encephalitis.

研究分野：ウイルス学

キーワード：Flavivirus　RNA-seq　transcriptomics　JEV　Zika virus　SH-SY5Y　neuron　Endoplasmic reticulum

様　式　C－１９、F－１９－1、Z－１９、CK－１９（共通）

１．研究開始当初の背景

The innate immune response to viral infection is essential to limit viral replication of pathogens and to affect their elimination from host tissues. Despite the existence of safe and well-tolerated vaccines, the flavivirus Japanese encephalitis virus (JEV) still exerts a profound burden of viral encephalitis with the underlying mechanisms of neuropathogenesis imperfectly understood. Traditionally, neurons were thought of as immunologically quiescent, which may explain the paucity of JEV infection studies in human neuronal cells; however, there is growing evidence for effective neuronal antiviral responses. Previous studies have employed cDNA microarrays of JEV-infected murine neuroblastoma cell lines which identified significantly altered mRNA expression, including genes involved in host antiviral responses and apoptotic cell death. Based on findings of the mechanism of JEV neuropathogenesis in rhesus macaques, a model has been proposed for apoptosis of neurons and activation of glia and cytokine release as important steps in JEV-mediated neuronal death. Macaque studies corroborate that neurons are the principal targets of JEV, as has also been previously indicated by post-mortem human studies, where neurons in the cortex and brainstem were positive for viral antigen. A detailed neuronal transcriptome from JEV-infected human cells was lacking before the present study.

２．研究の目的

We have investigated the differentially expressed transcripts in the neuronal transcriptome during JEV infection by RNA sequencing (RNA-Seq) of virus-infected SH-SY5Y human neuroblastoma cells to better understand the early transcriptomic responses of neurons to flavivirus infection. The deregulated targets identified in the neuronal transcriptome provide a basis for future work to study neuronal pathogenesis induced by flaviviruses.

３．研究の方法

Total RNA was extracted from SH-SY5Y neuroblastoma cells grown in 6-well plates following mock infection or infection with a high neurovirulence JEV strain (Sw/Mie/40/2004) 24 hours post-infection (hpi) with a multiplicity of infection (MOI) of 25 with four biological replicates for each experimental condition and DNaseI treated and ribosomal RNA (rRNA) depleted. Library construction was performed and sequenced on the NovaSeq 6000 platform (Illumina) with 150 base-length read chemistry in a paired-end mode. Targets were validated by qPCR and immunoblotting. The raw data of sequence reads from this study have been submitted to the Sequence Read Archive (SRA) public database (https://www.ncbi.nlm.nih.gov/sra) under accession number DRA007709.

４．研究成果

We have investigated the differentially expressed transcripts in the neuronal transcriptome during JEV infection by RNA sequencing (RNA-Seq) of virus-infected SH-SY5Y human neuroblastoma cells. We have shown for the first time that a number of transcripts in the ER stress response, including *sestrin2* (*SESN2*) and the *tribbles pseudokinase* (*TRIB3*), are deregulated upon JEV (Fig. 1 & 2; Table) and also Zika virus (Fig. 3) infection of neuroblastoma cells *in vitro*. We have also demonstrated for the first time that upon JEV infection of neuroblastoma cells that the mRNAs encoding the two constituent light- and heavy-chain subunits, *SLC3A2* and *SLC7A11* respectively, of the amino acid antiporter xCT are each upregulated (see heat map in Fig. 1a and Fig. 4). Gene ontology analysis revealed significant enrichment from two main pathways: endoplasmic reticulum (ER)-nucleus signaling (*P* value: 5.75E-18; false discovery rate [FDR] 3.11E-15) and the ER unfolded protein response (*P* value: 7.58E-18; FDR 3.11E-15). qPCR validation showed significant upregulation and differential expression ($P < 0.01$) of ER stress-signaling transcripts (*SESN2*, *TRIB3*, *DDIT3*, *DDIT4*, *XBP1* and *ATF4*) at 24 hours post-infection for both low (LN) and high (HN) neurovirulence JEV strains. Immunoblot analysis following JEV infection of SH-SY5Y cells showed an increase in levels of SESN2 protein following JEV infection. Similarly, Zika virus (MR-776) infection of SH-SY5Y showed a titre-dependent increase in ER stress-signaling transcripts; however, this was absent or diminished for *DDIT4* and *ATF4*, respectively, suggestive of differences in the induction of stress response transcripts between flaviviruses. Interestingly, *SLC7A11* and *SLC3A2* mRNA were also both deregulated in JEV-infected SH-SY5Y cells and encode the two constituent subunits of the plasma membrane xCT amino acid antiporter that relieves oxidative stress by export of glutamate and import of cystine. Infection of SH-SY5Y and HEK293T cells by the JEV HN strain Sw/Mie/40/2004 lead to significant upregulation of the *SLC7A11* mRNA to levels comparable to *DDIT3.*

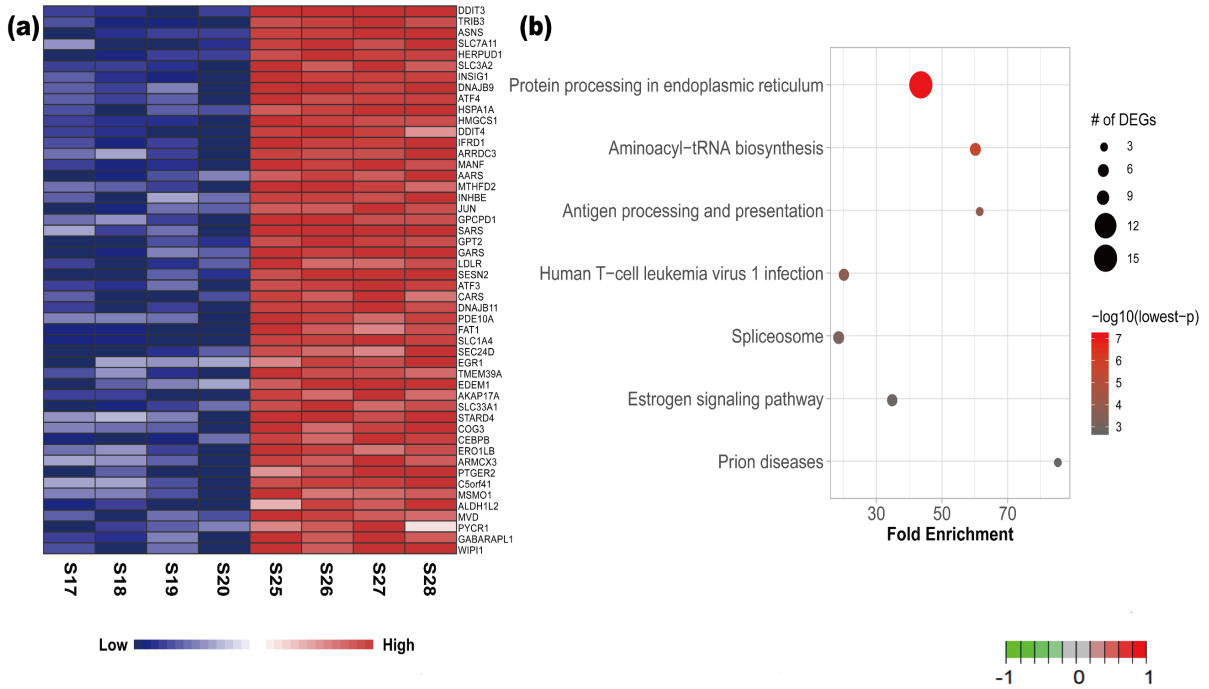

Figure 1. JEV-infected neuronal transcriptome. (a) Heat map analysis of the JEV-infected neuronal transcriptome. The samples S17-S20 represent four biological replicates derived from mock-infected SH-SY5Y neuroblastoma cells and the samples S25-S28 represent four biological replicates derived from JEV high neurovirulence (Sw/Mie/40/2004)-infected (MOI 25) SH-SY5Y cells 24 hpi. The upper and bottom heat maps show the up- and downregulated genes in the infected cells, respectively. (b) Pathways inferred as enriched by deregulated genes. The size of the circles is proportional to the number of genes identified to be significantly deregulated and the colour reflects the significance of the number of deregulated genes. The vertical axis shows the pathways in descending order by the number of deregulated genes and the horizontal axis represents the fold enrichment.

Neurodegenerative diseases are typified by both excitotoxicity and neuroinflammatory processes that ultimately leads to neuronal damage and subsequent death. An emerging hypothesis in the study of glial, and other brain tumors, is that glutamate release is a self-preservation mechanism elicited by cancerous cells. Upregulating the xCT antiporter (system x_c^-) leads to an increase in the uptake and the intracellular concentration of cystine, an essential amino acid, required for GSH synthesis, an antioxidant required for tumor proliferation and survival. Whether an analogous scenario exists in flavivirus-infected neuronal cells with increased reactive oxygen species and ER stress necessitating increased GSH biosynthesis but coupled to the deleterious effects associated with increased glutamate excitotoxicity is suggested by the present work as we determined significantly increased *SLC7A11* and *SLC3A2* mRNA transcripts in JEV-infected neuroblastoma cells by RNA-Seq. Indeed, *SLC7A11* upregulation in HEK293T cells, while relative levels were decreased compared to the upregulation determined in SH-SY5Y cells, showed that this preceded the upregulation of the DNA damage response transcription factor *DDIT3* which was the most significantly deregulated neuronal transcript in the RNA-Seq analysis. Several transcription factors that regulate xCT expression have been identified. The PI3K/Akt/GSK3β/eIF2α/ATF4 pathway has been recently shown to be implicated in increased xCT expression in the hippocampal region in neurological disorder. ATF4 can bind to a number of amino acid response elements in the promoter of the specific light chain subunit of xCT (i.e. SLC7A11) resulting in elevated transcription of xCT. Our transcriptomic analysis and qPCR analysis showed ATF4 was also significantly upregulated in JEV-infected neuronal cells *in vitro* suggesting this may be the mechanism whereby increased *SLC7A11* expression occurs. Whether some of the neurological manifestations of JEV and other flaviviral-associated encephalitides are attributable to glutamate excitotoxicity arising from increased system x_c^- expression and glutamate excitotoxicity is unknown and warrants further study.

Table. Gene ontology (GO) term analysis of the deregulated pathways following JEV HN high neurovirulence (Sw/Mie/40/2004)-infected SH-SY5Y cells 24 hpi.

Deregulated Biological Pathway	Total	Expected	Hits	*P* Value	FDR†
ER unfolded protein response	93	1.01	18	5.75E-18	3.11E-15
ER-nucleus signaling pathway	111	1.2	19	7.58E-18	3.11E-15
Homophilic cell adhesion	139	1.51	11	3.25E-07	8.89E-05
Positive regulation of hydrolase activity	497	5.39	18	7.42E-06	0.00152
Response to organic substance	2500	27.1	47	5.88E-05	0.00964
Neutral amino acid transport	23	0.25	4	1.00E-04	0.0137
Steroid biosynthetic process	183	1.99	9	0.000172	0.0202
Regulation of neuron apoptotic process	150	1.63	8	0.000229	0.0235
Positive regulation of transferase activity	510	5.53	15	0.000437	0.0398
ER to Golgi vesicle-mediated transport	62	0.673	5	0.000554	0.0423
Cellular response to stress	1620	17.6	32	0.000568	0.0423

†FDR: False discovery rate.

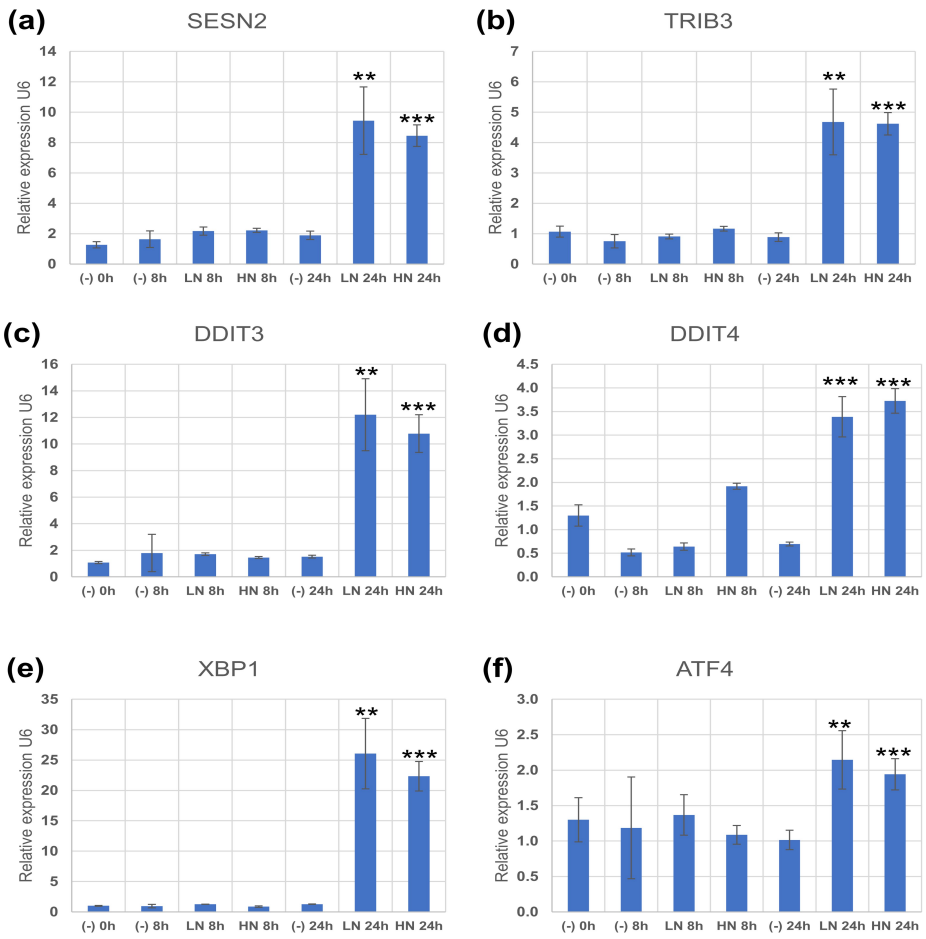

Figure 2. Sestrin 2, TRIB3 and ER-stress signaling transcripts are significantly deregulated in JEV-infected neurons. The endoplasmic reticulum stress signaling transcripts, *SESN2*, *TRIB3*, *DDIT3*, *DDIT4*, *XBP1* and *ATF4* (a-f), were examined by specific SYBR green qPCR assays following mock or JEV infection with low or high neurovirulence strains (MOI 25) of SH-SY5Y neuroblastoma cells at the indicated time points. The relative mRNA levels to the endogenous control *U6* were measured by qPCR and presented as the fold change

compared to mock-infected control cells at 0 h. The standard deviation was calculated from four biological replicates at each indicated time point. (-) 0h: mock-infected 0 h; (-) 8h: mock-infected 8 h; LN 8h: JEV LN-infected 8 h; HN 8 h: JEV HN-infected 8 h; (-) 24h: mock-infected 24 h; LN 24h: JEV LN-infected 24 h; HN 24h: JEV HN-infected 24 h. Statistical significance was evaluated at each time point relative to transcripts levels of uninfected SH-SY5Y cells at 0h. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

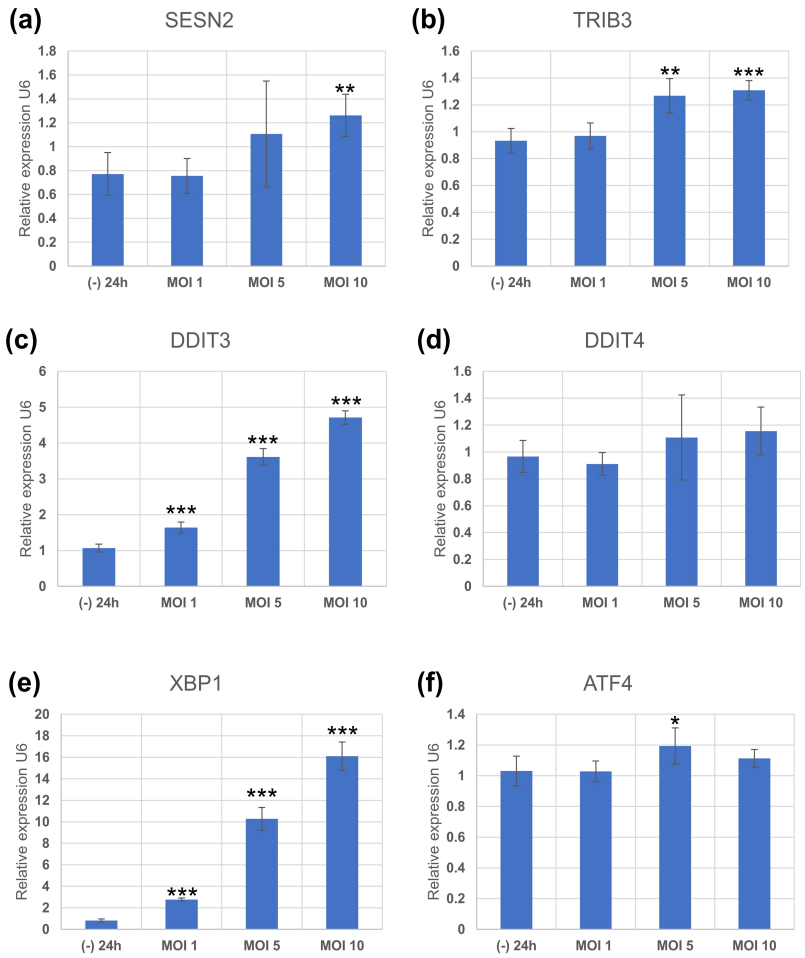

Figure 3. ER-stress signaling transcripts are significantly deregulated in ZIKV-infected neurons. The endoplasmic reticulum stress signaling transcripts, *SESN2*, *TRIB3*, *DDIT3*, *DDIT4*, *XBP1* and *ATF4* (a-f), were examined by specific SYBR green qPCR assays following mock or ZIKV (prototype strain MR-766) infection of SH-SY5Y neuroblastoma cells at 24 h with increasing MOIs (1, 5 and 10). The relative mRNA levels to the endogenous control *U6* were measured by qPCR and are presented as the fold change compared to mock-infected control cells at 24 h. The standard deviation was calculated from four biological replicates at each indicated time point. (-) 0h: mock-infected 0 h; (-) 8h: mock-infected 8 h; LN 8h: JEV LN-infected 8 h; HN 8 h: JEV HN-infected 8 h; (-) 24h: mock-infected 24 h; LN 24h: JEV LN-infected 24 h; HN 24h: JEV HN-infected 24 h. Statistical significance was evaluated relative to transcripts levels of uninfected SH-SY5Y cells at 24 h.*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

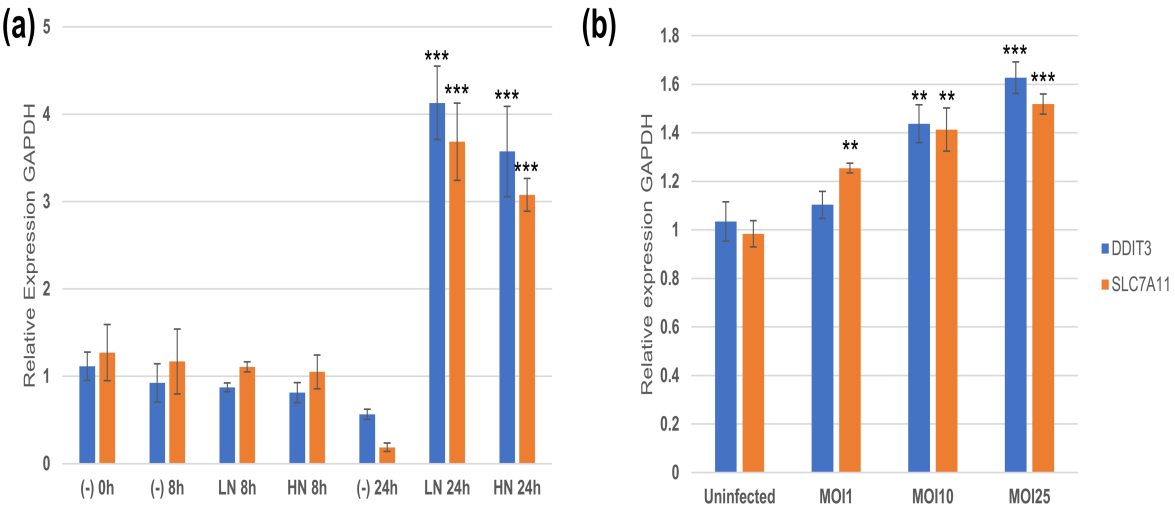

Figure 4. The *SLC7A11* mRNA encoding the light chain of the xCT antiporter (system x_c^-) is deregulated in neuronal cells. (a) Relative expression of *SLC7A11* and *DDIT3* mRNA (the top ranked hit on the heat map in Figure 1a) to GAPDH was examined by TaqMan qPCR following mock or JEV infection (MOI 25) with low or high neurovirulence strains of SH-SY5Y neuroblastoma cells at the indicated time points. Statistical significance was evaluated relative to transcripts levels of uninfected SH-SY5Y cells at 0 h.*: P < 0.05; **: P < 0.01; ***: P < 0.001. (b) Relative expression of *SLC7A11* and *DDIT3* was examined by TaqMan qPCR following mock or JEV infection with low or high neurovirulence strains of HEK293T cells at 24 hpi. Statistical significance was evaluated relative to transcripts levels of uninfected SH-SY5Y cells at 24 h.*: *P* < 0.05; **: *P* < 0.01; ***: *P* < 0.001.

５．主な発表論文等

〔雑誌論文〕（計　1　件）

Michael Carr, Gabriel Gonzalez, Axel Martinelli, Christida E. Wastika, Kimihito Ito, Yasuko Orba, Michihito Sasaki, William W. Hall and Hirofumi Sawa. Upregulated Expression of the Antioxidant Sestrin 2 identified by Transcriptomic Analysis of Japanese Encephalitis Virus-Infected SH-SY5Y Neuroblastoma Cells. Virus Genes (2019) responding to reviewers.

〔学会発表〕（計　3　件）

1. July 2018 –*Transcriptomic Analysis of Viral Infection in Neurons*, Sixth Symposium of the Consortium for the Control of Zoonoses, Hokkaido University, Sapporo, Japan
2. May 2018 – *Investigation of the Transcriptomic Response in Neuronal Cells to Japanese Encephalitis Virus Infection*, CZC Research Meeting, Hokkaido University, Japan
3. September 2017 – *Long Non-Coding RNAs in Viral Infection*, European Molecular Biology Laboratory (EMBL), Heidelberg, Germany

６．研究組織

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