NSP-C contributes to the upregulation of CLOCK/BMAL1-mediated transcription

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Abstract The bHLH-PAS transcription factors clock circadian regulator (CLOCK) and brain and muscle ARNT-like protein 1 (BMAL1) play essential roles in the generation of circadian gene expression rhythms through the activation of E-box-mediated transcription. Importantly, circadian transcriptional rhythms mediated by CLOCK/BMAL1 are observed in peripheral tissues as well as in the suprachiasmatic nucleus and contribute to tissue-specific functions. These findings suggest that CLOCK/BMAL1 have roles in many biological phenomena by interacting with various cellular regulators. In the present study, to understand the mechanisms underlying the multiple functional roles of CLOCK, we tried to identify new proteins that interact with CLOCK using a yeast two-hybrid system. We identified neuroendocrine-specific protein (NSP)-C, which is highly expressed in the brain, as a positive regulator of CLOCK/BMAL1-mediated transcription. We found that NSP-C interacted with CLOCK in mammalian cells. Co-expression of NSP-C with CLOCK/BMAL1 enhanced the

transcriptional activation by CLOCK/BMAL1. Furthermore, knockdown of endogenous NSP-C by small interfering RNA (siRNA) suppressed E-box-mediated transcription, while this reduction of transcription was rescued by the expression of NSP-C protected from the action of siRNA. These observations suggest that NSP-C contributes to the upregulation of CLOCK/BMAL1-mediated transcription.

Keywords CLOCK · NSP-C · Interaction · Transcription activation · Yeast-two hybrid

Introduction

Circadian rhythms regulate many physiological, biological, and behavioral processes in mammals (Dunlap 1999; Reppert and Weaver 2002; Panda et al. 2002). The circadian transcription factors clock circadian regulator (CLOCK) and brain and muscle ARNT-like protein 1 (BMAL1, also called MOP3) play essential roles in the generation of circadian rhythms (King et al. 1997; Bunger et al. 2000). CLOCK and BMAL1 form a heterodimer and activate the transcription of their target genes, including period (*Per1* and *Per2*) and cryptochrome (*Cry1* and *Cry2*) genes, by binding to a specific DNA sequence, the E-box (CACGTG) (Shearman et al. 1997; Balsalobre et al. 1998; Gekakis et al. 1998; Hogenesch et al. 1998; Cermakian and Sassone-Corsi 2000; King and Takahashi 2000; Bunger et al. 2000; Takahashi 2015; Trott and Menet

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2018). Conversely, the resulting gene products inhibit CLOCK/BMAL1-mediated transcription in a negative feedback loop. These positive and negative transcription regulatory mechanisms generate circadian transcriptional rhythms at the cellular level (Cermakian and Sassone-Corsi 2000; King and Takahashi 2000; Reppert and Weaver 2002).

CLOCK/BMAL1-mediated transcriptional rhythms are observed not only in the suprachiasmatic nucleus, the master circadian clock in the brain, but also in peripheral tissues (Bunger et al. 2000; Balsalobre et al. 1998; Albrecht et al. 1997). Importantly, peripheral local clocks contribute to circadian regulation of tissue-specific functions in the liver, muscle, brain, and so on (Cermakian and Sassone-Corsi 2000; McDearmon et al. 2006; Liu et al. 2007; Fuller et al. 2008; Bass and Takahashi 2010; Dibner et al. 2010; Marcheva et al. 2010; Cho et al. 2012; Christiansen et al. 2016; Mishra et al. 2016; Shimizu et al. 2016; Verwey et al. 2016). Accordingly, CLOCK/BMAL1-mediated transcription undergoes cell-type specific regulation by interacting with tissue-specific transcriptional regulators (Hosoda et al. 2009).

CLOCK contains functional domains such as a histone acetyl transferase (HAT) domain, glutamine-rich region (Q-rich region), and PAS domain (King et al. 1997; Hirayama et al. 2007). Furthermore, CLOCK undergoes covalent modifications such as phosphorylation by GSK3 β and AKT (Spengler et al. 2009; Luciano et al. 2018). CLOCK is thought to regulate peripheral tissue-specific functions as well as the circadian clock by interacting with multiple transcription factors and signal transduction regulators through these domains. However, fewer CLOCK-interacting proteins have been identified than expected considering its multiple biological functions (see Hosoda et al. 2009). In this study, we tried to find new proteins interacting with CLOCK to understand the molecular mechanisms underlying the multiple functions of CLOCK in peripheral tissues.

Materials and methods

Reagents

Anti-myc and anti-HA antibodies were purchased from Clontech (Palo Alto, CA, USA) and Santa Cruz (Santa Cruz, CA, USA), respectively. The expression

vectors pcDNA3, pCMV-myc, and pCMV-HA, were purchased from Clontech. The β -galactosidase expression plasmids pCH110 and pCMV β were purchased from Pharmacia (Whitehouse Station, NJ, USA) and Clontech, respectively.

Plasmid construction

As a bait for the yeast two-hybrid system, cDNA encoding the PAS region of human CLOCK (amino acids 109–377) was amplified by PCR from a human brain cDNA library (Clontech) as a template and inserted into the pGBKT7 DNA-BD cloning vector (Clontech) (pGal4-CLOCK-PAS). The expression plasmid of CLOCK and BMAL1 was constructed as described previously (Hosoda et al. 2009). cDNA encoding full-length mouse neuroendocrine-specific protein (NSP)-C was amplified by PCR from mouse brain cDNA as a template and inserted into the pBluescript II (SK-) vector (Stratagene, La Jolla, CA, USA). PCR was carried out using the following primers: forward, 5'-GGGGAATTCCTC-GAGGCCGCCACCATGGTGCGCTCCTCCAA-GAAC-3'; and reverse, 5'-GGGAAGCTTTCCGGACAGGAA-CAGGTGGTGGCGGC-3'. To generate pcDNA3-NSP-C and pHA-NSP-C, the resulting PCR product was inserted into the pcDNA3 and pCMV-HA vectors, respectively. The sequences of the PCR products were confirmed by DNA sequencing.

Yeast two-hybrid screening

Saccharomyces cerevisiae Y190 cells were transformed with pGal4-CLOCK-PAS and then mated with Y187 cells containing a pre-transformed human brain MATCHMAKER cDNA Library (Clontech). An interaction between CLOCK-PAS and the proteins expressed from the cDNA library increases the expression of the reporter genes HIS3 and lacZ. Screening of approximately 1.0×10^6 colonies was carried out in two stages: (1) incubation in the presence and absence of histidine in the medium and (2) a β -galactosidase assay. Colonies with high β -galactosidase activity were selected and regrown, after which plasmid cDNAs were extracted from the yeast and sequenced.

Cell culture and transfection

NIH3T3 and COS-1 cells were maintained as described previously (Hosoda et al. 2009), and were transiently transfected using the Lipofectamine-PLUS reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. PC12 cells were maintained as described previously (Iwamoto et al. 2005), and were transiently transfected using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

Immunoprecipitation and western blotting

COS-1 cells grown in 60-mm dishes were transiently transfected with expression vectors as indicated in the figure legends. Immunoprecipitation and western blotting were performed as described previously (Hosoda et al. 2009).

Reporter assay

NIH3T3 cells and COS-1 cells were grown in 24-well plates and 12-well plates, respectively; at 48 h following transfection, luciferase and β -galactosidase activity was measured as described previously (Hosoda et al. 2009). PC12 cells were grown in 6-well plates; at 72 h following transfection, luciferase and β -galactosidase activity was measured as described previously (Iwamoto et al. 2005). Luciferase activity was normalized to β -galactosidase activity. Each value represents the mean $\pm$ standard deviation (SD). Statistical analyses were performed using the unpaired Student's *t* test. $P < 0.05$ was considered to be statistically significant.

RNA interference (RNAi)

To silence the expression of NSP-C, the middle of the open reading frame of mouse NSP-C (AAGTCCGTTCTACAAGCTGTG, nucleotides 357–377 correspond to NSP-C mRNA sequence of accession number BK001693.1 in the GenBank database) was targeted as a sequence for small interfering RNA (siRNA). To prepare a plasmid to express the siRNA against NSP-C, psiNSP-C, PCR was carried out using the pSUPER vector as a template and with the following primers: forward, GAGACA-

CAGCTTGTAGAACGGACTTTTTGGAAAAGCT-TATCGATACCG; and reverse, TTGAACA-CAGCTTGTAGAACGGACGGGATCTGTGGTCT-CATACAG (the underlined text indicates the target sequences for NSP-C). RNAi vectors were transiently transfected into PC12 cells, which were analyzed 72 h later.

For rescue experiments, a plasmid expressing a mutant NSP-C protein (ProN) protected from siNSP-C was constructed as described below. The ProN sequence was generated by PCR with a silent mutation method (ProN, AAG AGC GTC CTG CAG GCT GTT; the underlined text indicates the mutated sequences) using pHA-NSP-C cDNA (AAG TCC GTT CTA CAA GCT GTG corresponds to KSVLQAV of amino acids 74–80) as a template. The sequences of the PCR products were confirmed by DNA sequencing. To identify cells expressing siNSP-C, cDNA encoding green fluorescent protein (GFP) was inserted into psiNSP-C (pcGFP-siNSP-C). To express siNSP-C, ProN, and GFP in the same cell, cDNA encoding ProN followed by the internal ribosome entry site (IRES) sequence (Hosoda et al. 2011) was inserted between the promoter region and the cDNA encoding GFP into pcGFP-siNSP-C, generating pcProN-IRES-GFP-siNSP-C.

Results and discussion

CLOCK interacts with NSP-C in mammalian cells

To identify CLOCK-interacting proteins in the brain, we performed a yeast two-hybrid screen of a human brain cDNA library using the PAS domain of CLOCK (amino acids 109–377) as bait. We screened approximately 1.0×10^6 transformants and identified several positive clones. Sequence analysis revealed that one of them encoded the N-terminus of NSP-C protein (amino acids 1–60) including the putative cytoplasmic domain (amino acids 1–33). We next examined whether NSP-C binds to CLOCK in mammalian cells. COS-1 cells were transfected with a vector expressing myc-tagged CLOCK together with a vector expressing HA-tagged BMAL1 (positive control) or NSP-C. Proteins in cell lysates prepared from the transfected cells were immunoprecipitated with an anti-myc antibody, and the resulting immunoprecipitates were subjected to western blot analysis using anti-myc or

anti-HA antibodies, respectively. Expression of the tagged proteins was detected in the cell lysates. Importantly, HA-NSP-C and HA-BMAL1, respectively, were detected in the anti-myc immunoprecipitates in the presence of myc-CLOCK co-expression, whereas neither HA-protein was observed in its absence (Fig. 1). These results suggested that NSP-C interacts with CLOCK in COS-1 cells.

NSPs, members of the reticulon and nogo family, are expressed in neural and neuroendocrine tissues (Hens et al. 1998; Goldberg and Barres 2000; GrandPré et al. 2000). NSP-A, NSP-B, and NSP-C, the three major subtypes of NSP, are expressed using an alternative promoter from the *NSP* gene (Hens et al. 1998; Goldberg and Barres 2000; GrandPré et al. 2000; Kim et al. 2000). Consequently, these NSP variants share the C-terminus, although their N-termini are different (Hens et al. 1998; Goldberg and Barres 2000; Kim et al. 2000). The *NSP-C* gene encodes a protein of 208 amino acid residues that is thought to localize to the endoplasmic reticulum (ER) membrane via two hydrophobic amino acid regions (Goldberg and Barres 2000; GrandPré et al. 2000; Kim et al. 2000). The N-terminal region of NSP-C has been shown to interact with Bcl-X, which is associated with apoptosis, and SNARE complex proteins, which have a role in the exocytosis of synaptic vesicles (Tagami

et al. 2000; Steiner et al. 2004). Interestingly, NSP-C has been used as a differentiation marker of neurons to analyze Down's syndrome and Alzheimer's disease (Hens et al. 1998; Goldberg and Barres 2000; Kim et al. 2000). However, the cellular function of NSP-C has not been well clarified.

NSP-C enhances CLOCK/BMAL1-mediated transcription in NIH3T3 and COS-1 cells

To clarify the role of the interaction between NSP-C and CLOCK, we examined the effects of the forced expression of NSP-C on CLOCK/BMAL1-mediated transcription in NIH3T3 cells. The cells were transiently transfected with an E-box reporter construct together with expression plasmids encoding CLOCK and BMAL1, in the presence or absence of an expression plasmid encoding NSP-C. CLOCK and BMAL1 expression activated transcription from a synthetic E-box-dependent promoter containing three copies of the E-box sequence harbored in the vasopressin promoter (Hosoda et al. 2009) (Fig. 2a). Importantly, co-expression of NSP-C with CLOCK and BMAL1 significantly enhanced the activation of CLOCK/BMAL1-mediated transcription ($P < 0.05$). In contrast, NSP-C did not affect the transcription from the E-box dependent promoter in the absence of CLOCK/BMAL1 co-expression (Fig. 2a).

It is possible that the enhancement of CLOCK/BMAL1-mediated transcription by NSP-C is specific for the synthetic E-box-dependent promoter used in this study. Therefore, we next examined the effects of NSP-C expression on a natural promoter containing an E-box (the mouse period 1 [*Per1*] promoter; −1772 to +1692). CLOCK and BMAL1 expression activated transcription from the *Per1* promoter ($P < 0.05$) (Fig. 2b). Consistent with the result shown in Fig. 2a, co-expression of NSP-C with CLOCK and BMAL1 significantly enhanced CLOCK/BMAL1-mediated transcription ($P < 0.05$), although NSP-C alone did not affect transcription from the *Per1* promoter (Fig. 2b).

We previously reported that CLOCK/BMAL1-mediated transcription is positively or negatively regulated by the universal transcriptional coactivator CBP/p300 in a cell type-specific manner; CBP/p300 enhances CLOCK/BMAL1-mediated transcription in COS-1 cells, while CBP/p300 represses it in NIH3T3 cells (Hosoda et al. 2009). Therefore, we next

Fig. 1 NSP-C interacts with CLOCK in mammalian cells. COS-1 cells in 60-mm dishes were transiently transfected with pHA-NSP-C (500 ng), pmyc-CLOCK (2000 ng), or pHA-BMAL1 (5500 ng). Empty vector (pCMV-myc) was used to standardize the total amount of transfected DNA (8000 ng). Immunoprecipitation (IP) was performed using anti-myc antibodies, and then western blot analysis was performed using anti-myc or anti-HA antibodies, as indicated. The input lanes represent 10% of total cell lysate in the binding reaction

Fig. 2 NSP-C enhances CLOCK/BMAL1-mediated transcription. **a** and **b** NIH3T3 cells in 24 well plate were transiently transfected with either **a** pE-box (2 ng) or **b** pPer1-Luc (10 ng) and with or without pcDNA3-CLOCK (100 ng) plus pcDNA3-BMAL1 (100 ng) in combination with pcDNA3-NSP-C (100 ng), as indicated. Empty vector (pcDNA3) was used to standardize the total amount of transfected DNA (**a** 402 ng or **b** 410 ng). pCH110 (100 ng) was co-transfected as a control for transfection efficiency. **c** COS-1 cells in 12-well plates were

transiently transfected with pPer1-Luc (10 ng) and with or without pcDNA3-CLOCK (10 ng) plus pcDNA3-BMAL1 (10 ng) in combination with pcDNA3-NSP-C (100 ng), as indicated. Empty vector (pcDNA3) was used to standardize the total amount of transfected DNA (410 ng). pCMV-β (100 ng) was co-transfected as a control for transfection efficiency. Each value represents the mean $\pm$ SD. An asterisk indicates $P < 0.05$ (Student's t test, $n = 3$)

examined the effects of NSP-C expression on CLOCK/BMAL1-mediated transcription in a different cell line, COS-1 cells. We performed similar experiments as shown in Fig. 2b using COS-1 cells. Similarly with our observations in NIH3T3 cells, CLOCK/BMAL1 expression activated transcription from the *Per1* promoter in COS-1 cells (Fig. 2b). Importantly, NSP-C significantly enhanced CLOCK/BMAL1-mediated transcriptional activation in COS-1 cells ($P < 0.05$) (Fig. 2c), although NSP-C alone did not affect transcription from the *Per1* promoter (Fig. 2c). Collectively, our observations suggest that NSP-C positively regulates transcriptional activation by CLOCK/BMAL1, but NSP-C does not display cell-specific effects on CLOCK/BMAL1-mediated transcription.

Endogenous NSP-C enhances E-box-mediated transcription in PC12 cells

The enhancement of E-box-mediated transcription by NSP-C was observed only when CLOCK and BMAL1 were co-expressed (Fig. 2). Therefore, we attempted to investigate the roles of endogenous NSP-C in E-box-mediated transcription by examining the

effects of knocking down NSP-C. To do this, we used PC12 cells that express NSP-C endogenously.

We first examined whether endogenous NSP-C expression could be knocked down by RNAi. Endogenous expression of NSP-C mRNA was observed in PC12 cells transfected with the RNAi backbone control vector (pSUPER) (Fig. 3a, lane 1). Importantly, NSP-C mRNA was not detected when pGFP-siNSP-C was transfected (Fig. 3a, lane 2), suggesting that siNSP-C led to a knockdown of NSP-C expression. However, NSP-C mRNA was observed again when a mutant of NSP-C (ProN) protected from RNAi-mediated knockdown was co-expressed with siNSP-C by transfection of pProN-IRES-GFP-siNSP-C. It is important to note that GFP mRNAs were observed in each group as a positive control of effective transfection (Fig. 3a, lanes 1–3). These observations showed that (1) PC12 cells express NSP-C, (2) siNSP-C knocks down NSP-C mRNA expression, and (3) ProN blocks siNSP-C-mediated knockdown of NSP-C.

To clarify the roles of endogenous NSP-C in E-box-mediated transcription in PC12 cells, we examined the effects of endogenous NSP-C knockdown by RNAi using the E-box reporter construct (Fig. 2a) and a similar reporter construct lacking the E-box sequences

Fig. 3 Endogenous NSP-C enhances E-box-mediated transcription. **a** PC12 cells in 6-well plates were transiently transfected with pSUPER (500 ng) and pEGFP-C1 (500 ng), pcIRES-GFP-siNSP-C (1000 ng), or pcProN-IRES-GFP-siNSP-C (1000 ng) as indicated. At 72 h after transfection, the cells were collected, total RNA was prepared, and cDNA was synthesized. NSP-C, GFP, and β-actin mRNAs were amplified by a semi-quantitative PCR method. The PCR products were subjected to agarose gel electrophoresis and the bands were detected with ethidium bromide. NSP-C and GFP mRNAs were

detected as amplification products of 27 cycles. β-Actin mRNA was detected as an amplification product of 22 cycles. **b** PC12 cells in 24-well plates were transfected with either pE-box (100 ng) or pE-less (100 ng), with or without pSUPER (100 ng) and pE-EGFP-C1 (100 ng), pGFP-siNSP-C (200 ng), or pProN-IRES-GFP-siNSP-C (200 ng) as indicated. pCMV-β (100 ng) was co-transfected as a control for transfection efficiency. Each value represents the mean ± SD. An asterisk represents $P < 0.05$ (Student's test, $n = 4$)

(E-less promoter) as a negative control. The E-box-dependent promoter showed significantly higher transcription activity compared to the E-less promoter when PC12 cells were transfected with control pSUPER ($P < 0.05$) (Fig. 3b, lane 1 vs. lane 4), indicating that E-box-mediated transcription was activated in PC12 cells. In contrast, siNSP-C expression significantly suppressed transcription from the E-box-dependent promoter without affecting transcription from the E-less promoter ($P < 0.05$) (Fig. 3b, lane 4 vs. lane 5). However, co-expression of ProN with siNSP-C rescued this reduction of E-box-mediated transcription ($P < 0.05$) (Fig. 3b, lane 5 vs. lane 6). It is important to note that knockdown of NSP-C by siNSP-C or forced expression of ProN with siNSP-C did not affect transcription from the E-less promoter (Fig. 3b, lanes 1–3), suggesting that NSP-C showed a specific role in E-box-dependent transcription without affecting the basal level of transcription from the E-less promoter. These observations suggested that NSP-C knockdown blocks transcription from the E-box-dependent promoter, but ProN expression recovered this impaired transcription. Collectively, our observations suggest that endogenous NSP-C functions as a positive regulator of E-box-dependent transcription mediated by CLOCK/BMAL1.

NSP-C shows limited but strong expression in neural and neuroendocrine tissues and is suggested to be a two-transmembrane protein localized in the ER of neural cell (Hens et al. 1998; Goldberg and Barres 2000; GrandPré et al. 2000; Kim et al. 2000). Conversely, the nuclear localization of BMAL1 is involved in the transcriptional activation by CLOCK/BMAL1 (Kwon et al. 2006). Moreover, phosphorylation of CLOCK and BMAL1 triggers their translocation into the nucleus, activation of transcription, and protein degradation (Spengler et al. 2009; Tamaru et al. 2009; Sahar et al. 2010; Luciano et al. 2018). Interestingly, BMAL1 participates in translational regulation in the cytoplasm (Ikeda and Ikeda 2014). From these findings, the translocalization of CLOCK and BMAL1 seems to be a critical step for the regulation of the functions of CLOCK and BMAL1. In this study, the interaction of CLOCK and NSP-C was observed in cell lysates; however, it is unknown how CLOCK interacts with NSP-C in the cell since they localize to the nucleus and ER, respectively (GrandPré et al. 2000; Kim et al. 2000; Spengler et al. 2009; Luciano et al. 2018). It is possible that NSP-C plays a role in the translocation of CLOCK/BMAL1 into the nucleus by interacting with them in the ER. Further studies are required to examine the mechanisms underlying the

interaction of CLOCK and NSP-C in living cells using in vivo imaging techniques.

In summary, we identified NSP-C as a new CLOCK-interacting protein in mammalian cells (Fig. 1). The forced expression of NSP-C enhanced CLOCK/BMAL1-mediated transcription in NIH3T3 and COS-1 cells (Fig. 2). More importantly, we found that endogenous NSP-C is required for the upregulation of E-box-mediated transcription in PC12 cells (Fig. 3). These results suggested that NSP-C contributes to the activation of CLOCK/BMAL1-mediated transcription. Our findings suggest a novel mechanism for the upregulation of CLOCK/BMAL1-mediated transcription by NSP-C.

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Authors' contributions SK is responsible for the hypothesis development and overall design of the research and experiment, and supervised the experimental analyses and wrote the paper. HH performed and analyzed experiments and wrote the paper.

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