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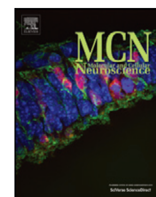

Polysialic acid enters the cell nucleus attached to a fragment of the neural cell adhesion molecule NCAM to regulate the circadian rhythm in mouse brain

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ABSTRACT

In the mammalian nervous system, the neural cell adhesion molecule NCAM is the major carrier of the glycan polymer polysialic acid (PSA) which confers important functions to NCAM's protein backbone. PSA attached to NCAM contributes not only to cell migration, neuritogenesis, synaptic plasticity, and behavior, but also to regulation of the circadian rhythm by yet unknown molecular mechanisms. Here, we show that a PSA-carrying transmembrane NCAM fragment enters the nucleus after stimulation of cultured neurons with surrogate NCAM ligands, a phenomenon that depends on the circadian rhythm. Enhanced nuclear import of the PSA-carrying NCAM fragment is associated with altered expression of clock-related genes, as shown by analysis of cultured neuronal cells deprived of PSA by specific enzymatic removal. *In vivo*, levels of nuclear PSA in different mouse brain regions depend on the circadian rhythm and clock-related gene expression in suprachiasmatic nucleus and cerebellum is affected by the presence of PSA-carrying NCAM in the cell nucleus. Our conceptually novel observations reveal that PSA attached to a transmembrane proteolytic NCAM fragment containing part of the extracellular domain enters the cell nucleus, where PSA-carrying NCAM contributes to the regulation of clock-related gene expression and of the circadian rhythm.

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1. Introduction

In the nervous system, polysialic acid (PSA), a polymer of α2,8-linked sialic acids (Finne, 1982), is predominantly carried by NCAM in its extracellular domain. PSA contributes to regulation of cell interactions during ontogenetic development and affects synaptic activities and regeneration after trauma in the adult nervous system (Eckhardt et al., 2000; Angata et al., 2004; Dityatev et al., 2004; Kleene and Schachner, 2004; Rutishauser, 2008; Bonfanti and Theodosis, 2009; Mühlenhoff et al., 2009; El Maarouf and Rutishauser, 2010; Gascon et al., 2010; Hildebrandt et al., 2010; Kochlamazashvili et al., 2010; Nacher et al., 2010; Schiff et al., 2011). PSA not only modulates the functions of NCAM indirectly but also directly influences cellular features by binding to histone H1, brain-derived neurotrophic factor, fibroblast growth factor 2 and myristoylated alanine-rich C kinase substrate (Muller et al., 2000; Kanato et al., 2008; Mishra et al., 2010; Ono et al., 2012; Theis et al., 2013). In addition to its multiple cellular functions, PSA has been implicated in regulating circadian networks (Glass et al., 1994, 2000; Shen et al., 1997, 1999, 2001; Fedorkova et al., 2002; Prosser et al., 2003). PSA is expressed in the suprachiasmatic nucleus (SCN) (Glass et al., 1994; Shen et al., 1999), the principal anatomical structure responsible for generation and entrainment of circadian rhythmicity that is regulated by the daily light-dark cycle and nonphotic inputs, such as locomotor activity, social interaction and sleep patterns, which are superimposed on an organism-endogenous clock (for review, see Golombek and Rosenstein, 2010). In this superimposition, PSA directs photic entrainment and the nonphotic circadian phase by resetting the SCN circadian clock (Shen et al., 1997, 2001; Glass et al., 2000; Fedorkova et al., 2002; Prosser et al., 2003). Mice deficient in PSA-NCAM lack circadian rhythmicity (Shen et al., 1997, 2001). Furthermore, removal of PSA by microinjection of endoneuraminidase N (EndoN) into the murine SCN impairs the circadian rhythm (Glass et al., 1994, 2000; Shen et al., 1997, 2001; Fedorkova et al., 2002; Prosser et al., 2003). The mechanisms by which PSA regulates rhythmicity have remained largely unexplored.

Abbreviations: CLOCK, Circadian Locomotor Output Cycles Kaput; EndoN, endoneuraminidase N; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NCAM, neural cell adhesion molecule; NCAM-Fc, NCAM fused to Fc; Per-1, Period-1; PNGase F, peptide-*N*-glycosidase F; PSA, polysialic acid; SCN, suprachiasmatic nucleus; SDS, sodium dodecyl sulfate.

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Because of PSA's ability to modify the functions of NCAM at the cell surface, little interest had been focused on PSA in other cellular compartments. Since proteolytic NCAM fragments comprising the extracellular parts influence cell interactions at the cell surface, the functions of these fragments, which are generated by different metalloproteases and serine proteases, have received increasing attention (Endo et al., 1998, 1999; Hubschmann et al., 2005; Pillai-Nair et al., 2005; Kalus et al., 2006; Dean and Overall, 2007; Brennaman et al., 2011; Shichi et al., 2011). However, little is known about the functions of NCAM fragments containing the intracellular domain, the transmembrane portion and part of the extracellular domain carrying PSA. Cell surface glycoproteins and fragments thereof have been detected in cell nuclei (Hart and West, 2009), raising the barely examined question whether the glycan moieties of glycoproteins affect cellular functions in the nucleus. Full-length glycosylated transmembrane cell surface receptors have been detected in the nucleus after translocation from the plasma membrane, induced by cell surface ligands (Planque, 2006; Oppizzi et al., 2008; Lee et al., 2009; Wang and Hung, 2012). Having shown that proteolytic fragments of the transmembrane adhesion molecules L1 and NCAM can enter the cell nucleus upon application of function-triggering antibodies as surrogate ligands in cell culture (Kleene et al., 2010; Lutz et al., 2012), we addressed the question whether PSA could reach the nucleus when attached to a transmembrane NCAM fragment. The two N-glycosylation sites for the attachment of PSA to the NCAM protein backbone are localized in its fifth immunoglobulin-like domain (von der Ohe et al., 2002; Wuhrer et al., 2003) and it deemed therefore likely that PSA reaches the nucleus only when covalently bound to the transmembrane NCAM fragment. In search for a functional consequence following the entrance of PSA into the nucleus, we chose circadian rhythmicity as a convenient read-out and asked whether the nuclear PSA-carrying NCAM fragment contributes to PSA-dependent functions in circadian rhythm. We here show that nuclear PSA alters clock-related gene expression *in vitro* and that nuclear PSA levels depend on diurnal activity *in vivo*, suggesting that nuclear PSA is a molecular trigger that regulates circadian rhythmicity.

2. Material and methods

2.1. Animals

Mice were bred and maintained at the animal facility of the Universitätsklinikum Hamburg-Eppendorf. Animals were housed on a 12 h light/12 h dark cycle (lights on either at 7 am or at 7 pm) at 25 °C with *ad libitum* access to food and water. NCAM-deficient mice (Cremer et al., 1994) had been backcrossed onto the C57BL/6J background for more than eight generations. C57BL/6J mice, NCAM-deficient mice and their wild-type littermates of both sexes were used. All animal experiments were approved by the local authorities of the State of Hamburg (animal permit numbers ORG 535), comply with the ARRIVE guidelines and conformed to the guidelines set by the European Union.

At different time points of the 12 h light/12 h dark cycle (Zeitgeber time (ZT) 2, 6, 10, 14, 18 and 22 or 2, 5, 8, 11, 14, 17, 20 and 23 relative to ZT0 which is defined as lights on at 7 pm), cerebellum, cerebral cortex, hippocampus, striatum, midbrain and SCN were removed from the 3-month-old adult males and females and subjected either to subcellular fractionation using the Subcellular Protein Fractionation Kit for Tissues (Thermo Scientific, Darmstadt, Germany) or to isolation of RNA using Qiashredder and RNAeasy Plus Kit (Qiagen, Hilden, Germany). Similar results were obtained for males and females.

2.2. Antibodies and reagents

Production of NCAM-Fc comprising the extracellular domain of mouse NCAM fused to the Fc part of human IgG, the function-triggering polyclonal rabbit antibody 1β2 against mouse NCAM-Fc and the monoclonal rat antibody P61 against the intracellular mouse NCAM domain have been described (Kleene et al., 2010). The polyclonal chicken function triggering antibody against mouse NCAM-Fc was produced by Pineda (Berlin, Germany). The rabbit and chicken NCAM antibodies were used interchangeably with the same results. The monoclonal mouse PSA antibody 735 (Frosch et al., 1985) and endoneuraminidase N (EndoN) (Gerardy-Schahn et al., 1995) were kind gifts from Rita Gerardy-Schahn (Zentrum Biochemie, Zelluläre Chemie, Medizinische Hochschule, Hannover, Germany). The goat NCAM antibody (C-20; sc-1507) and antibodies against actin (I-9; sc-1616), heterogeneous nuclear ribonucleoprotein A (hnRNPA) (pan hnRNPA; H-200; sc-15385), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (FL-335; sc-25778), calreticulin (C-17; sc-6467) and histone H3 (C-16; sc-8654) were from Santa Cruz Biotechnology (Heidelberg, Germany) and histone H1 antibody (17510-1-AP) was from Acris (Herford, Germany). All secondary antibodies and Fc fragment of human IgG were from Dianova (Hamburg, Germany). Peptide-*N*-glycosidase F (PNGase F) was from New England BioLabs (Frankfurt, Germany).

2.3. Culture of cerebellar granule neurons

At 8 am (lights on at 7 am) cerebellar granule neurons were prepared from 6- to 8-day-old mice (6 males and 6 females were taken for each experiment) (Kleene et al., 2010), maintained in 6-well plates in serum-free medium for 30 h or for the indicated time periods, incubated for 30 min with or without EndoN (25 units per well) followed by treatment for 30 min without (mock-treatment) or with rabbit NCAM antibody (50 μg of purified IgG), chicken NCAM antibody (400 μg of antibody precipitated from egg yolk by polyethylene glycol), NCAM-Fc (20 μg) or Fc (40 μg) in phosphate-buffered saline, pH 7.4 (PBS). The treated neurons were subjected to subcellular fractionation using the Subcellular Protein Fractionation Kit for Cultured Cells (Thermo Scientific) or processed for isolation of RNA using Qiashredder and RNAeasy Plus Kit (Qiagen). For immunoprecipitation and PNGase F or EndoN treatment, soluble nuclear and membrane fractions were subjected to immunoprecipitation using the PSA antibody 735 and Protein A/G beads followed by EndoN treatment of the immunoprecipitates or by PNGase F treatment of the immunoprecipitates according to the manufacturer's instructions. Cell surface biotinylation of the neurons before the treatments was performed as described (Kleene et al., 2010).

2.4. Immunoblot analysis

SDS-PAGE using 4–20% Criterion™ Tris-HCl 26 well gels (Bio-Rad, Munich, Germany) and immunoblot analysis using chemiluminescent substrates were as described (Kleene et al., 2010). Chemiluminescence was monitored using ImageQuant™ LAS 4000mini (GE Healthcare, Freiburg, Germany) and band intensities were quantified using ImageJ software. Intensities relative to loading controls were calculated. For statistical analysis of the relative intensities, data were examined by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Significance of difference between the groups was estimated relative to PSA levels after a 24 h culture time period or relative to the ZT2 group.

2.5. qPCR

For reverse transcription, oligoT_{18} primer and SuperScript® II reverse transcriptase (Thermo Scientific) was used. qPCR was performed in triplicates using reverse transcribed mRNA, the 7900HT Fast Real-Time PCR System (Thermo Scientific), the qPCR kit SYBR® Green I, ROX (Eurogentec, Cologne, Germany) and primers for determination of the mRNA levels of Circadian Locomotor Output Cycles Kaput (CLOCK), Period-1 (Per-1) or the reference genes actin, tubulin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The SDS 2.4 software was used for analysis of the qPCR data. The mRNA levels of

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