Daily coordination of orexinergic gating in the rat superior colliculus—Implications for intrinsic clock activities in the visual system

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Abstract
The orexinergic system delivers excitation for multiple brain centers to facilitate behavioral arousal, with its malfunction resulting in narcolepsy, somnolence, and notably, visual hallucinations. Since the circadian clock underlies the daily arousal, a timed coordination is expected between the orexin system and its target subcortical visual system, including the superior colliculus (SC). Here, we use a combination of electrophysiological, immunohistochemical, and molecular approaches across 24 h, together with the neuronal tract-tracing methods to investigate the daily coordination between the orexin system and the rodent SC. Higher orexinergic input was found to occur nocturnally in the superficial layers of the SC, in time for nocturnal silencing of spontaneous firing in this visual brain area. We identify autonomous daily and circadian expression of clock genes in the SC, which may underlie these day–night changes. Additionally, we establish the lateral hypothalamic origin of the orexin innervation to the SC and that the

Abbreviations: ACSF, artificial cerebro-spinal fluid; AP, anteroposterior; CT, circadian time; CtB, cholera toxin B subunit; DD, constant darkness; DORAs, double orexin receptor antagonists; DV, dorsoventral; FFT, fast Fourier transformation; GABA, γ-aminobutyric acid; InG, intermediate gray layer; InWh, intermediate white layer; LD, light–dark cycle; LH, lateral hypothalamus; LM, lateromedial; MEA, multi-electrode array; MUA, multi-unit activity; NDS, normal donkey serum; Op, optic tract layer; Ox1, receptor, orexin receptor 1; Ox2, receptor, orexin receptor 2; OXA, orexin A; OXB, orexin B; PBS, phosphate-buffered saline; PER2::LUC, PERIOD2::LUCIFERASE; PFA, paraformaldehyde; PVA, parvalbumin; ROI, regions of interest; RQ, relative gene expression; RT-qPCR, real-time quantitative reverse transcription polymerase chain reaction; SC, superior colliculus; SCN, suprachiasmatic nucleus of the hypothalamus; SUA, single-unit activity; SuG, superficial gray layer; Zo, the zona layer; ZT, Zeitgeber time.
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1 | INTRODUCTION

The orexinergic system originating in the lateral hypothalamus (LH) consists of two evolutionary conserved peptides: orexin A (OXA) and orexin B (OXB), which bind to two metabotropic receptors named OX₁ and OX₂ receptors.1,2 Neurons that synthesize orexins provide extensive innervation to multiple brain centers, delivering arousal-related information in a daily (under light-dark conditions, LD) and circadian time-dependent manner (under constant darkness, DD). The activity of orexinergic system confers wakefulness and promotes feeding.3,4 The suprachiasmatic nucleus of the hypothalamus (SCN) reciprocally connects with the orexinergic system and directs the profound day-to-night changes in arousal, where orexins have been hypothesized to act as hands of the clock.5–8 However, new findings challenge the omnipotent role of the SCN in the circadian control of the brain physiology and function. Thus, independent roles of autonomous peripheral clocks are now being considered. In the brain, several neuronal centers were found to rhythmically express clock genes independently from the central clock.9–11 The extent to which these local circadian clocks interact and collectively shape the brain physiology is an active area of research. Although some of the clock interactions have been known in the regulatory areas of the brain such as the hypothalamus,12 epiphysis,13 lateral hypothalamus (habenula: Refs. [13,14]), brainstem,15 forebrain circumventricular organs,16 and even the choroid plexus,17 it remains largely unexplored as to how the clock interactions extend to higher brain areas such as the visual system.

SC neurons robustly respond to orexin A via OX₂ receptor in both excitatory and GABA_A receptor-dependent inhibitory manners. Together, our evidence elucidates the combination of intrinsic and extrinsic clock mechanisms that shape the daily function of the visual layers of the SC.

KEYWORDS

circadian clock, multi-electrode array, orexin, superior colliculus, visual system

Growing evidence supports the functional link between the orexinergic and visual systems.18–24 However, whether orexins influence the neurophysiology of the extra-geniculate pathway, including the superior colliculus (SC), has not been demonstrated so far. The SC is a particularly important area since it connects heavily to the cortex while serving as a subcortical center of multisensory integration. In rodents, the SC is a layered midbrain structure that orients the animal towards the stimuli of the outside world during the wake phase. It consists of the retinorecipient superficial layers, the deep layers implicated in motor control, and the intermediate layers associated with both motor functions and integration of the multisensory input.25–29 In humans, impaired activity of the superficial layers of the SC has been shown to diminish saccadic eye movement, cause attention deficits, and promote visual hallucinations.30–32

Last two decades were marked by the emergence of new drugs targeting the orexinergic system, with double orexin receptor antagonists (DORAs) showing benefits in improving sleep onset and sleep maintenance.33 Thus, the discovery and description of orexin binding sites in the brain stands as both basic scientific and clinical achievement. Despite a distinct safety profile of DORAs, the most frequently reported side effects include somnolence, headache and fatigue; in particular, the side effects also include visual hallucinations and nightmares, as if to suggest an internal misalignment of wake and sleep.34

The functional wiring from orexin neurons has been proposed to occur with daily rhythmicity.35 While the circadian clock provides a predictive cue for this rhythmicity, orexin can provide a feedback cue from sleep.36 We
evaluated if the two cues coordinate in the orexinergic modulation of neuronal activity in the SC visual layer, and sought to identify the source (extrinsic vs. intrinsic) of the daily variation. Here, we present compelling evidence for the robust modulatory action of orexins upon the neuronal activity in the retinorecipient superficial layers of the SC. These neurophysiological effects were not exclusively excitatory but also inhibitory, mediated by the GABA_A-dependent network mechanisms. Importantly, orexinergic input assessed by the ligand presence in the axonal terminals to the SC increased during the night time, together with the augmented response towards OXA. This higher orexinergic drive during the behaviourally active night was accompanied by lowered spontaneous neuronal activity in the SC, compared to the behaviourally quiescent day. The daily timing of these neuronal activities coincided with the timing of local rhythmic clock gene expression ex vivo and in vivo, both in the daily (under light–dark cycles) and circadian (constant dark) fashion. Additionally, we identify the OX2 receptor to be predominately expressed and functional in the SC, suggesting that the orexinergic pathway in the SC is arousal dependent. Together, these findings point to the broader circadian modulation at work in the SC.

2 | MATERIALS AND METHODS

2.1 | Animals and ethical approval

This study was performed on 106 adult male Sprague-Dawley (10–12 weeks old) rats kept under a standard 12:12 light–dark cycle, unless stated otherwise, at 23 ± 2°C and 67% ± 5% relative humidity. Animals were fed ad libitum and had free access to water. Rats were bred in-house by the Animal Facility of the Institute of Zoology and Biomedical Research at the Jagiellonian University in Krakow. All procedures were approved by the Local Ethics Committee and were performed in accordance with Polish and the European Parliament and Council Directive (2010/63/EU). Procedures performed in darkness (including culls) were carried out with the use of night vision infrared goggles (Pulsar, Vilnius, Lithuania).

PERIOD2::LUCIFERASE (PER2::LUC) mice (RRID: IMSR_JAX:006852) were kept under a 12:12 light–dark cycle at 21 ± 2°C and 59% ± 4% relative humidity in Taipei Medical University Laboratory Animal Centre. The animals and protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Taipei Medical University (IACUC Approval No: LAC-2019-0118). All experiments were designed to minimize the number of animals used and their sufferings.

2.2 | Intraocular injections of cholera toxin B subunit

2.2.1 | Surgery

Surgery for the intraocular injections of the cholera toxin B subunit (CtxB) was performed as described previously. In brief, seven rats were deeply anesthetized with isoflurane (3% v/v air mixture; Baxter, Deerfield, IL, USA) and placed in the gas free-flow mask throughout the procedure. Intraocular injections of 2 μl of CtxB (0.5% m/v in saline; Sigma, Darmstadt, Germany) were performed into the vitreous chamber of one eye. Animals were intramuscularly injected with Torbugesic (0.2 mg kg−1 body weight; Zoetis, Parsippany, NJ, USA) and Tolfedine 4% (4 mg kg−1 body weight; Biowet, Pulawy, Poland) and returned to the Animal Facility for 3 days with free access to drinking water containing the antibiotic Sul-Tridin 24% (1:300; sulfadiazine 200 mg ml−1 + trimethoprim 40 mg ml−1; ScanVet, Poland).

2.2.2 | Tissue preparation and immunostaining

Next, rats were deeply anesthetized with sodium pento-barbital (2 ml kg−1 body weight; Biowet) and transcardially perfused with 4% paraformaldehyde (PFA) solution in 0.1 M phosphate-buffered saline (PBS) near the transition between light–dark phases: at the beginning of the day (Zeitgeber time, ZT1, n = 3) or beginning of the night (ZT13, n = 4). Subsequently, the brains removed from the skulls were post-fixed in the same solution overnight. Blocks of tissue containing midbrain were cut on the vibroslicer (VT1000S; Leica Microsystems, Wetzlar, Germany) on 40 μm thick coronal slices. Slices were rinsed in PBS and permeabilized with 0.5% Triton-X100 (Sigma) solution in PBS, additionally containing normal donkey serum (NDS; Abcam, Cambridge, UK) at room temperature for 35 min. Then, slices were transferred to a PBS solution composed of 0.1% Triton-X100, 0.5% NDS, and primary antiserum: goat anti-α-OXB (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse anti-CtxB (1:250, Abcam). After 24 h of incubation in 4°C, sections were rinsed in fresh PBS and kept in the secondary antibodies solution (donkey anti-goat Alexa Fluor 467 and donkey anti-mouse Cy3, 1:300; Jackson ImmunoResearch, West Grove, PA, USA) overnight at 4°C. Last, slices were washed off in PBS, mounted on glass slides, and coverslipped with Fluoroshield with DAPI (Sigma). Images were collected using the A1Si confocal laser scanning system (Nikon, Tokyo, Japan) built on an inverted Ti-E microscope (Nikon).
2.3 | Daily immunohistochemical studies

2.3.1 | Tissue preparation and immunostaining

A cohort of 22 Sprague–Dawley rats was anesthetized and transcardially perfused with 4% PFA in PBS (see Section 2.2.2) at four time points throughout 24 h: ZT0 (n = 5), ZT6 (n = 5), ZT12 (n = 6), and ZT18 (n = 6). Brains were extracted from the skull, post-fixed, and cut on the vibroslicer in 40 µm thick sections. Slices containing the SC were immunostained against OXB and parvalbumin (PVA) with primary: goat-anti OXB and rabbit-anti PVA (1:1000; Abcam), and secondary antisera: donkey anti-goat Alexa Fluor 637 and donkey anti-rabbit Cy3 (1:300; Jackson ImmunoResearch). We used OXB antisera to visualize orexnergic cells and fibers at general (which yield better signal/background ratio in comparison to these against OXA), as OXA and OXB derive from a common precursor preproorexin and are co-stored.4 Mounted sections were imaged under the confocal microscope under a 20x objective in 3 µm Z-stack steps.

2.3.2 | Quantification of OXB-ir fibers

Regions of interest (ROIs) were identified with marker staining for PVA, and the OXB-ir was analyzed for the superficial and intermediate layers separately. Images were binary converted and measured in Fiji software (NIH, USA) with the custom-made macro. First, Bernsen’s adaptive thresholding method was used to define regions of the high local contrast. Next, immunoreactive pixels representing OXB-ir fibers were counted in the six ROIs outlined by a 40 x 40-pixel oval for each SC layer, and then divided by the area for the measurement to represent the area fraction. Then, results from two slices for each rat were averaged. All images were analyzed with the same settings. Statistical analysis was performed in Prism 7 (GraphPad Software, San Diego, CA, USA) with ordinary one-way ANOVAs.

2.4 | Retrograde tract tracing

2.4.1 | Surgery

Neuronal tract tracing was performed on three Sprague–Dawley rats (250–350 g) according to a procedure described previously.18 In brief, isoflurane-anesthetized rats were mounted in the Small Animal Stereotaxic System (SAS-4100; ASI Instruments, Warren, MI, USA) and the same anesthetic agent was continuously infused through the mask. Retrograde tracers: FluoroRed and FluoroGreen (20 nl; Tombow Pencil Co., Tokyo, Japan) were injected bilaterally into the superficial layers of the SC with 1 µl Hamilton syringe connected to borosilicate glass micropipette made at a vertical puller (Narishige, Tokyo, Japan). FluoroRed injection was aimed at left and FluoroGreen at right hemisphere: anteroposterior (AP) = −7.0, lateromedial (LM) = ±1.0, dorsoventral (DV) = −3.5 mm from Bregma. After 7 days, rats were subjected to the same surgical procedure, during which the colchicine (0.1 mg in 5 µl saline; Sigma) was injected into the lateral ventricle (AP = −0.7, LM = +1.8, DV = −4 mm from Bregma). Subsequently, 24 h after the procedure rats were re-anesthetized with sodium pentobarbital and perfused with 4% PFA in PBS.

2.4.2 | Immunostaining and imaging

Brains were extracted from the skull, post-fixed, and cut on the vibroslicer into 40-µm-thick coronal sections containing the lateral hypothalamus (every third slice was collected from approximately −3.5 to −1.7 mm from Bregma in the anteroposterior dimension) and into 100-µm-thick coronal midbrain slices containing the injection site. Next, the LH sections were immunostained against OXB (see Section 2.2.2) and imaged on the epifluorescence microscope under 20x magnification (Axio Imager M2; Carl Zeiss, Oberkochen, Germany).

2.4.3 | Cell counting

The LH slices were sorted from the most caudal to rostral and 14 slices of the same stereotactic coordinates were taken for further analysis from each brain. Cell counting was performed manually in ZEN software (ZEN 2.3. black edition; Zeiss, Germany). OXB-ir cells and these co-localizing the retrograde dye were counted for each hemisphere. As every third slice was collected during the cutting procedure, all data were extrapolated by multiplying the results of cell counting by three. Results of overall cell counting are presented as average data from independent animals. The lateralization of retrograde dye-filled OXB-ir cells relative to the injection side was statistically analyzed in Prism 7 (GraphPad Software) with the paired t-test.

2.5 | Electrophysiological recordings ex vivo

2.5.1 | Tissue preparation

In total, 20 Sprague–Dawley rats were culled at four time points across the day and night, namely at ZT0, 6, 12 and 18 (n = 5 each). Brains were immediately removed from the skull and immersed in the ice-cold preparation...
artificial cerebro-spinal fluid (ACSF), composed of (in mM): 5 NaHCO₃, 3 KCl, 1.2 Na₂HPO₄, 2 CaCl₂, 10 MgCl₂·6H₂O, 10 glucose, 125 sucrose, and 0.01 g L⁻¹ phenol red (Sigma); constantly oxygenated with carbogen (95% oxygen, 5% CO₂). Subsequently, midbrain sections were cut into 250 µm thick acute slices in a chamber of a vibriscoler filled with the same solution. Next, slices containing the SC were incubated for 1 h before being transferred to the recording chamber of the multi-electrode array (MEA) system (Multi Channel Systems GmbH, Reutlingen, Germany).

2.5.2 | Recording

Slices were placed above the electrodes, anchored, and positioned such that recording electrodes were in contact with the superficial layers of the SC near the midline of the brain. In all experiments, 6 × 10 perforated MEAs (100 µm spacing; 60pMEA100/30iR-Ti; Multi Channel Systems GmbH) were used. During the recording, gentle suction was applied to ensure proper contact with the recording electrodes and signal stability. Prior to the start of the recording, always 2 h after the cull, slices were allowed to settle for half an hour. Slices were continuously rinsed with fresh, carbogenated ACSF (2 ml min⁻¹) heated to 32°C. All drugs were applied by bath perfusion. Signal was sampled at 20 kHz and acquired with the Multi Channel Experimenter software (Multi Channel Systems GmbH).

2.5.3 | Drugs

All drugs were stored as 100× concentrated stocks and were diluted in fresh ACSF prior to each application to their working concentration: OXA (200 nM, Bachem, Bubendorf, Germany), OXA17-37 (1 µM, Bachem), TCS-OX2-29 (20 µM, Tocris, Bristol, UK), and bicuculline (20 µM, Tocris).

2.5.4 | Spike sorting

Data were automatically spike-sorted in KiloSort program working in the MatLab R2018a environment (MathWorks, Natick, MA, USA) as described before. First, raw data were exported with Multi Channel DataManager (Multichannel Systems GmbH) to HDF5 files. These were further processed with a custom-made script in MatLab, for data re-mapping and conversion to DAT format. DAT files were first automatically spike-sorted with KiloSort, with a GPU was used to boost spike-sorting speed (NVIDIA GeForce GTX 1050Ti GPU; CUDA 9.0 for Windows). In parallel, raw data were exported with Multi Channel DataManager to CED-64 files. Following, they were filtered from 0.3 to 7.5 kHz (Butterworth band pass filter, fourth order) and remapped with custom-made Spike2 script (Spike2 8.11 software, Cambridge Electronic Design Ltd., Cambridge, UK). Finally, results of an automatic spike-sorting were transferred into the prepared CED-64 files. Then, manual refinement of spike-sorted putative single units was conducted in Spike2 8.11 with the use of autocorrelation, principal component analysis, and evaluation of spike shapes.

2.5.5 | Analysis of firing rate and responses to drugs

Single unit data were next binned into firing rate histograms and further analyzed in NeuroExplorer 6 (Nex Technologies, Colorado Springs, CO, USA). For the spontaneous firing rate analysis, data were 1800 s binned and compared in Prism 7 (GraphPad Software) with Kruskal–Wallis test. For the examination of drug response, data were 30 s binned. A unit was classified as activated by the treatment if the peak firing rate after the drug application exceeded three standard deviations (SDs) from the baseline mean. Similarly, if the single-unit activity (SUA) was lowered by three SDs, the unit was deemed inhibited. Response amplitudes were calculated by extracting the 600 s baseline mean from the maximal/minimal firing rate value during the response. These were statistically compared in Prism 7 (GraphPad Software) with Friedman tests (for paired comparisons) or Kruskal–Wallis tests (for unpaired comparisons). Post hoc testing of paired data was performed with Dunn’s multiple comparison test. Outliers were identified with ROUT test (Q = 0.01%). Pie charts were also generated in Prism 7 (GraphPad Software) and compared with Chi-square test.

2.5.6 | Heatmaps, average, and bubble density plots

Heatmaps, average, and bubble density plots were generated with the use of custom-made scripts in MatLab (MathWorks, Natick, MA, USA). For temporal heatmaps, data were 30 s binned and smoothed with the Gaussian filter (width: 5 bins). Next, the firing rate of each single unit was normalized from 0 to 1 to represent the minimal and maximal value throughout the recording. These were then sorted according to the relative SUA during the first response to OXA. Spatial heatmaps representing the amplitude of the response to OXA were calculated for each recording location by extracting the 400 s baseline multi-unit
distinct SC layer using the stereotaxic atlas of the rat brain. Bubble density plots and spatial density heatmaps were based on the location of single units, which position (a stereotactic coordinate in relation to Bregma) was extracted and classified into the distinct SC layer using the stereotaxic atlas of the rat brain. Bubble density plots were color-coded for excitation, inhibition, and lack of response, whereas distinct special density heatmaps were generated for each condition. For the purpose of bubble density plot preparation, the surface containing positions of all units recorded was divided into small square subregions. Next, the number of units within each square was counted; for each square a bubble was drawn, with the size based on the number of units counted. Last, each bubble was repositioned to the center of mass of all neurons located within the corresponding square.

2.6 Hybridization in situ

2.6.1 Tissue preparation

Six adult Sprague–Dawley rats were deeply anesthetized with isoflurane and culled by decapitation (n = 3 at ZT6, n = 3 at ZT18). Brains were then quickly removed from the skull, flash frozen on dry ice, and stored at −80°C. Then, they were cryo-sectioned into 16-µm-thick slices at −20°C on a cryostat (Leica CM1950), thaw-mounted on Superfrost-Plus slides (Fisher Scientific, USA) and stored at −80°C overnight. At the next day, sections were fixed in cold (4°C) 4% PFA solution in PBS for 15 min, rinsed in fresh PBS and dehydrated in increasing ethanol concentrations (50%, 70%, 100%, and 100%). Following, slides were let to air-dry and each slice was drawn around with a hydrophobic barrier pen.

2.6.2 RNAscope assay and imaging

Immediately following the tissue preparation, slices were processed with the RNAscope multiplex in situ hybridization protocol (Advanced Cell Diagnostics—ACD, USA). First, slices were pre-treated with protease IV for 20 mins, rinsed in PBS and incubated for 2 h at 40°C with probes targeting Hcrtr2, Per2, and Penk. Following, sections were rinsed with wash buffer and transferred to a four-step signal amplification protocol, terminating with the fluorophore tagging (Hcrtr2 with Alexa 488, Per2 with Atto 550, and Penk with Atto 647). Last, sections were rinsed twice with the wash buffer, stained with DAPI and coverslipped with the fluorescent mounting medium (ProLong™ Gold antifade reagent, Invitrogen, USA). Two SCs per animal were imaged under 20x magnification with the epifluorescence microscope (Axio Imager. M2, Zeiss, Germany) and images were inspected in ZEN software (ZEN 2.3. blue edition, Zeiss).

2.7 Real-time quantitative reverse transcription polymerase chain reaction

2.7.1 Animals and tissue preparation

Two cohorts of Sprague–Dawley rats (24 each) were used in this study. First, one was kept under standard 12:12 light–dark cycle (LD), and another was moved to the constant darkness (DD) 48 h before cul. Rats were culled in four daily/circadian time points across 24 h: ZT/CT0, 6, 12, and 18 (n = 6/time point/light conditions). Brains were quickly extracted from the skull and placed in the cold preparation ACSF. Subsequently, they followed the same slicing procedure into 250-µm-thick sections as described in Section 2.5.1. The superficial layers of the bilateral SC were then dissected from these midbrain slices with a surgical scalpel and immediately flash frozen upon the dry ice. These fragments of tissue were stored at −80°C up to 1 week. All instruments used for the preparation were surface treated with RNaseZap (Sigma), to minimize ribonuclease activity.

2.7.2 RNA isolation and real-time quantitative reverse transcription polymerase chain reaction

RNA was extracted from the dissected fragments of tissue with the use of ReliaPrep RNA Tissue Miniprep System (Promega, Madison, WI, USA). The obtained RNA was stored in RNase-free water at −80°C. Next, reverse transcription was performed with the High-Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA), for the same amount of RNA from each sample. Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) was carried out with the PowerUp SYBR Green Mastermix (ThermoFisher Scientific, Vilnius, Lithuania) at the StepOnePlus Real-Time PCR System (Applied Biosystems). For transcript amplification, the QuantiTect primer assay was used (Qiagen, Hilden, Germany). The studied genes included: Hcrtr1, Hcrtr2 (for OX1 and OX2 receptor, respectively), Arr1 (for Bmal1), Nr1d1 (for Rev-Erbα), Per1 and Clock, whereas Gapdh served as a housekeeping gene.

2.7.3 Relative quantification of gene expression

Data were analyzed using the ΔΔCT method using Gapdh as a reference gen. The next relative target gene expression
(RQ) was calculated with ZT/CT0 mean value as 1. For the comparison of Hcrtr2 and Hcrtr1 gene expression, RQ was calculated from the mean ΔΔCT for the Hcrtr2, which level served as a reference for both transcripts. Data were statistically tested in Prism 7 (GraphPad Software) with the use of ordinary one-way ANOVAs (for daily/circadian comparisons) or paired t-test (for relative receptor levels).

2.8 | PER2::LUC bioluminescence monitoring of slice explant

Four 11-week-old male heterozygous PER2::LUC mice (RRID: IMSR_JAX:006852) were used for bioluminescence monitoring experiments. Coronal brain slices containing anterior and posterior sections of the SC were prepared at 300 μm thickness in ice-cold HBSS (Gibco/Thermo Fisher Scientific, Waltham, MA) on a vibratome (Leica VT1000S, Heidelberg, Germany). The two slices, respectively, corresponded to the 26th and the 28th sections in the OX2 receptor expression data of the Allen Brain Atlas (ISH data: Hcrtr2—RP_080424_01_A11—coronal). The SC explants were quickly cut on a stereomicroscope and transferred to culture membranes (Millipore Millicell-CM, Bedford, MA) and cultured in a phenol-red-free Dulbecco’s modified eagle medium (DMEM) (Sigma) containing 2% B-27 supplement (Gibco), 4.2 mM sodium bicarbonate (Gibco), 10 mM HEPES (Gibco), 1% penicillin-streptomycin (Gibco), and 300 μM beetle luciferin (Promega, Madison, WI) in 35 mm dish sealed with silicone vacuum grease (Dow Corning, Midland, MI).

Bioluminescence from the PER2::LUC reporter was continuously recorded for one week from the culture with the use of ordinary one-way ANOVAs (for daily/circadian comparisons) or paired t-test (for relative receptor levels).

3 | RESULTS

3.1 | Orexinergic fibers are present in the SC with a higher density of OXB-ir during the night

Despite a limited area populated by somas of orexinergic neurons, their axons can be found throughout the whole brain. Early studies locate orexin-ir fibers at the area of the rat SC, but the exact spatio-temporal distribution of orexinergic innervation of distinct SC layers has not been studied before. Thus, we first performed an immunohistochemical staining against OXB on the tissue obtained from seven rats intra-ocularly injected with CtB, culled either at the beginning of light (ZT1, n = 3) or dark phase (ZT13, n = 4). This enabled us to observe orexinergic fibers at the retinorecipient areas of the SC, which were immunostained against CtB (Figure 1A,B). In detail, the CtB immunostaining was found in the superficial layers, namely in the zona layer (Zo) and the full depth of superficial gray layer (SuG), contralateral to the injected eye. In all seven rats tested, OXB-ir fibers were present throughout the SC, including the superficial (Zo, SuG, and optic tract layer—Op) and intermediate layers (the intermediate gray—InG and intermediate white layer—InWh). The orexinergic signal in the CtB-positive layers was found in both daily timepoints, with these two signals clearly originating from different fibers (different morphology, no evident co-localization).

As the orexinergic system of the hypothalamus exhibits profound daily and circadian rhythmicity and day-to-night differences in the orexin immunoreactivity were previously shown in their axons innervating the thalamus18,19 we next tested the possibility of daily changes in the OXB-ir in the SC. To quantify the density of OXB-ir fibers in the superficial and subjacent intermediate layers of the SC, we transcardially perfused 22 rats at four time points across the day and night (ZT0 n = 5, ZT6 n = 5, ZT12 n = 6, and ZT18 n = 6). A significant variation in the density of OXB-ir fibers with a prominent night-time rise was present in the superficial layers of the SC (Zo/SuG/Op: p = .0112), but this did not reach the significance threshold for the intermediate layers (InG/InWh: p = .2300, one-way ANOVAs, Figure 1C). The immunostaining against PVA helped us to reliably distinguish between the distinct SC layers (Figure 1D). This dataset shows the orexinergic innervation of the entirety of the SC, including the visual superficial layers, with the latter exhibiting evident day-to-night changes in the OXB immunoreactivity.

3.2 | The orexinergic system of the lateral hypothalamus innervates the superficial layers of the SC

The lateral hypothalamus area (LH) is established to be the primary, if not exclusive source of orexins in the brain.1–3 Therefore, we next aimed to confirm that the LH orexinergic neurons project to the SC. Three rats were successfully injected with retrograde dyes: FluoroRed (into the left) and FluoroGreen (into the right hemisphere), targeting the superficial layers of the SC. At 1 week post-injection,
rats were treated with colchicine 24 h before cull, to improve somatic OXB staining and minimize false-negative errors. Subsequently, every third slice containing the LH was immunostained against OXB and analyzed. Despite the focal and limited injection site in the medial part of the SC (Figure 2A), we found the OXB-ir cells containing tracer granules scattered across the LH. Overall, our analysis revealed 8727 ± 188 OXB-ir cells in the hypothalamus, with 81 ± 11 filled with FluoroRed and 78 ± 5 with FluoroGreen (Figure 2B,C). No lateralization of OXB-ir cells filled with the retrograde dye was found in relation to the side of injection \( (p = .0931, \text{paired } t\text{-test, Figure 2B})\). Numerous cells containing tracer granules but lacking immunoreactivity against OXB were present above the LH, thus were not further quantified. These results show that the bilateral LH is a source of the orexinergic innervation of the SC.

### 3.3 Orexin A modulates neuronal activity of the superficial layers of the SC in a daily fashion

The orexinergic system modulates neuronal activity of the subcortical visual system, including the lateral...
geniculate nucleus of the thalamus\textsuperscript{18–22,24,44} and the SCN.\textsuperscript{45–47} Orexins were also found to potently excite neurons in the visual cortex\textsuperscript{48} and act at the level of the retina.\textsuperscript{49,50} Therefore, we investigated whether the orexinergic system innervating the SC influences its spontaneous neuronal activity. To resolve this, 20 rats were culled at four time points across the daily cycle (ZT0, 6, 12, and 18; \( n = 5 \) each) and the firing rates of neurons localized in the superficial layers of the SC were examined through the multi-electrode array (MEA) registrations ex vivo. On the whole, 1740 single units were spike-sorted from 20 recordings (20 brain slices), with 1002 neurons located in the Zo/SuG and 738 in the Op. Subsequently, their activities were evaluated following the application of OXA (200 nM), which significantly affected firing rates of the majority of neurons recorded. Both OXA-evoked activations and inhibitions of single-unit activity (SUA) were found in the Zo/SuG and Op (Figure 3A,C,D), with the first layer being significantly more responsive to the treatment than the latter (\( p < .0001 \), Chisquare test, Figure 3E,G). However, when SUA was averaged, the application of OXA evoked the overall activation of the SC (Figure 3B). This implies that on the level of a whole structure, these OXA-evoked excitations prevail inhibitions.

Both the spontaneous firing rate and the amplitude of response to OXA were subject to a daily change, with distinct temporal patterns. Neurons localized in both the Zo/SuG and Op exhibited a pronounced drop in spontaneous SUA at early night, compared to the day, which rebounded at ZT18 (\( p < .0001 \), Kruskal–Wallis tests, Figure 3F). The amplitude of OXA-evoked activations exhibited daily changes reaching its peak at ZT12, staying in close to anti-phasic relation to the spontaneous firing (Zo/SuG: \( p = .0157 \), Op: \( p = .0029 \), Kruskal–Wallis tests, Figure 3F). No day-to-night changes in the amplitude of OXA-evoked inhibitions were noted (Zo/SuG: \( p = .1124 \), Op: \( p = .5011 \), Kruskal–Wallis tests, Figure 3F). However, the proportion of OXA-evoked activations, inhibitions, and no effects was subject to daily change, with the highest percent of responsive cells at the day–night and night–day transitions. In the SuG, this was significant between ZT0 and 6 (\( p = .0030 \)), ZT0 and 18 (\( p = .0010 \)), and ZT0 and 18 (\( p = .0387 \)). In the Op, the
A significant difference in this ratio was observed between ZT6 and 12 only (p = .0119; Chi2 tests, Figure 3D). These data demonstrate a prominent modulatory action of OXA on the neuronal activity of the superficial layers of the SC. This modulatory action occurs with daily variation such that the spontaneous activity of SC neurons and their responsiveness to OXA are oppositely timed around the daily cycle.

3.4 | Orexin A-evoked inhibitions of the SC neuronal activity depend on GABAergic mechanisms

Direct action of orexins upon a variety of neuronal subpopulations in the central nervous system is predominately excitatory, with the postsynaptic hyperpolarization of clock cells in the SCN by OXA remaining...
an exception. The SC contains multiple subpopulations of GABAergic neurons, and the SuG in particular is populated by GABAergic interneurons that do not project outside the SC, but instead provide horizontal inhibitory tone inside this brain structure. To evaluate if OXA-evoked inhibitions of SUA in the SC are mediated by these GABAergic connections, we performed subsequent applications of OXA (200 nM) in control conditions and in the presence of GABA_A receptor antagonist, bicuculline (20 µM) on three brain slices recorded on the MEA ex vivo. Pre-treatment with bicuculline temporarily disinhibited neuronal activity in the SC, what proves the preservation of GABAergic network in the studied brain slices (Figure 4A, B). In the presence of GABA_A receptor blockage, OXA failed to evoke inhibitions in SUA, or the amplitude of these inhibitions was significantly reduced (p < .0001, Friedman test followed by Dunn’s multiple comparison, Figure 4C). On the contrary, the amplitude of OXA-evoked activations was unaffected by the antagonist (p = .7124). Results of this experiment provide evidence on the GABAergic network-dependent nature of this OXA-evoked inhibition of the SC neuronal activity.

3.5 | Orexin A modulates neuronal activity in the SC by the activation of OX2 receptor

Orexins act on two metabotropic G-protein-coupled receptors: OX1 and OX2 receptor, with OXA binding to these two with similar affinity. As the expression of orexin receptors varies across brain structures, we next set up to establish the predominant receptor functional in the SC. First, we tested if repetitive OXA applications cause orexin receptors to desensitize, by applying OXA three times without any drugs added on one SC slice. The amplitude of OXA-evoked activations (n = 44, p = .3284) and inhibitions (n = 13, p = .3679, Friedman tests, Figure 5A) did not significantly change over time. Second, we performed triple OXA (200 nM) applications on five SC slices: in control conditions, in the presence of a specific OX1 receptor antagonist OXA17-33 (1 µM), and after its washout. The application of the antagonist upon two SC slices failed to reduce both activations (n = 48, p = .0742) and inhibitions in the response to OXA (n = 14, p > .9999, Dunn’s multiple comparisons, Figure 5B). Finally, we tested whether the OX2 receptor antagonist TCS-OX2-29 (10 µM) modulates the amplitude of OXA-evoked response of SC neurons. Both OXA-evoked activations (n = 212) and inhibitions (n = 29) were eliminated by TCS-OX2-29, and restored after the antagonist washout (p < .0001, Friedman tests followed by Dunn’s multiple comparisons, Figure 5C). These data provide evidence for the functional expression of OX2 receptors in the superficial layers of the SC.

3.6 | The superficial layers of the SC possess molecular clock mechanisms which are sustained in constant darkness

Neurons of the central clock but also at an emerging number of extra-SCN brain centers are being shown to express clock genes with 24 h period, to organize their circadian physiology. This includes daily rhythms in the firing rate or in the expression of different receptors, leading to day-to-night changes in the responsiveness to a variety of neurotransmitters and neuromodulators. Therefore, we next aimed to establish whether the superficial layers of the SC express the molecular clock in vivo, which enables SC neurons to regulate their circadian physiology, including their neuronal activity and responsiveness to orexins. First, 24 rats were maintained under the standard 12:12 light–dark cycle (LD) and culled in four daily time points: ZT0, 6, 12, and 18 (n = 6 each). Both Per1 and Bmal1 expression in the SC varied significantly over these time points (Per1: p = .0005; Bmal1: p = .0295, one-way ANOVA, Figure 6A). Daily pattern of expression for these two clock genes remained close to the antiphase relationship, with Per1 peaking at ZT12 and Bmal1 at ZT0. Next, another cohort of 24 rats was moved from these standard LD conditions to the constant darkness (DD) for 48 h before being culled in four circadian time points: circadian time (CT) 0, 6, 12, and 18 (n = 6 each). Under these conditions, the expression of Per1 and Bmal1 continued to change across 24 h (Per1: p = .0008; Bmal1: p < .0001, one-way ANOVA), with a peak in Per1 coinciding with a nadir in Bmal1 expression level. To gain more insights into the circadian expression of clock genes in the SC, we additionally measured the expression level of Rev-erba and Clock, which also significantly varied across CT (Rev-erba: p < .0001; Clock: p < .0001, one-way ANOVA, Figure 6B). Similar to Bmal1, the expression of both clock genes was the lowest at CT12, the time of Per1 acrophase. Thus, the rhythmic expression of clock genes in the SC follows a daily and circadian pattern in vivo.

Under both LD and DD conditions, we additionally measured the level of orexin receptor gene expression. In these SC samples, the expression of OX2 receptor gene (Hcrtr2) was approximately 50- to 100-fold higher than that of OX1 receptor gene (Hcrtr1) (p < .0001, paired t-tests, Figure 6A, B). Moreover, the expression of the prevalent Hcrtr2 varied significantly under DD (p < .0001, Figure 6B) but not LD conditions (p = .1939, one-way ANOVAs, Figure 6A). This dataset is in keeping with our
electrophysiological results pinpointing the functional OX₂ receptors express dominantly in the SC.

3.7 | Clock cells in the superficial layers of the SC express OX₂ receptors

The SC stands a powerful computational network of different neuronal subpopulations creating organized connections within this structure.⁵² Thus, we next aimed to unravel if OX₂ receptors are expressed by the same SC neurons which organize their temporal physiology due to clock gene expression. To address this, we utilized RNAscope technology—the fluorescent in situ hybridization, on SC slices obtained from six rats. For transcript detection we used three different probes: against Per₂ (a selected clock gene), Hcrtr₂ (OX₂ receptor gene), and Penk (the proenkephalin gene, used for delineation of distinct SC layers). Clusters of Hcrtr₂ and Per₂ around DAPI-stained nuclei were present in the whole extent of the superficial layers of the SC, particularly in the SuG and Op. Further visual inspection revealed high co-localization of Per₂ and Hcrtr₂ around the same nuclei of SC cells (Figure 7). Altogether, the RNAscope data strongly support that clock cells in the visual SC express OX₂ receptors.

3.8 | Intrinsic circadian oscillations in core clock gene expression in the mouse SC

Finally, we aimed to resolve whether rhythmic clock gene expression in the SC in vivo arises from local, intrinsic...
FIGURE 5  OX2 receptor is predominant in the superficial layers of the SC. (A) Control, triple applications of OXA (200 nM, duration: 3 min, red bar). (B) OX1 receptor antagonist OXA17-33 (1 µM, duration: 30 min, gray bar) failed to reduce the effects of OXA on the SUA. (C) The effects of OX1 receptor antagonist TCS-OX2-29 (TCS, 20 µM, duration: 30 min, gray bar) on the response amplitude to OXA. (a) Spatial heatmaps showing the change in multi-unit activity (MUA) after OXA application. The superficial layers of the SC were outlined according to the localization of the multi-electrode array. (b) Temporal heatmaps displaying normalized SUA throughout the recording (bin: 30 s). Units were sorted from top to bottom according to relative activity during the first response to OXA. (c) Average plots of the normalized SUA. Data were drawn as mean ± SEM. (d,e) Statistical analyses of the amplitudes of OXA-evoked activations (d) and inhibitions (e): before, during, and after the treatment with the antagonist, or during the triple control application of OXA. *p < .05; ****p < .0001
clock mechanisms in this structure, or is a consequence of the phase communication from another brain clock.\textsuperscript{54} Therefore, we used a PERIOD2::LUCIFERASE mouse model, where the Per2 expression is monitored through PER2::LUC bioluminescence.\textsuperscript{55} Under isolated culture conditions, the SC continued to maintain the molecular circadian clock for up to 6 days (Figure 8). Interestingly, the posterior SC in our preparation, corresponding to the

**FIGURE 6** The superficial layers of the rat SC express core clock genes in the daily and circadian fashion. (Aa,b) Expression of core clock genes Per1 and Bmal1 under standard 12:12 LD cycle. (Ac) The quantitative paired comparison of orexin receptor genes expression under LD, with all time points pooled together. (Ad) Expression of the prevalent Hcrtr2 under LD. (Ba–d) Expression of clock genes Per1, Bmal1, Rev-erba, and Clock under DD. (Bc) Paired comparison of Hcrtr1 and Hcrtr2 expression under DD, with all time points pooled together. (Bf) Expression pattern of the dominant Hcrtr2 under DD. Blue shading codes the darkness. CT, circadian time; RQ, relative gene expression; ZT, Zeitgeber time; *p < .05; ***p < .001; ****p < .0001

**FIGURE 7** Co-expression of OX2 receptor gene Hcrtr2 with a core clock gene Per2 in the superficial layers of the SC. (left panel) Low magnification photomicrography with superficial layers of the SC outlined: Op, the optic tract layer; SuG, the superficial gray layer; Zo, the zona layer. (right panel) High magnification of two inserts from the SuG (a,b) and one from the Op (c). Nuclei counterstained with DAPI are presented in cyan, Hcrtr2 in green, Per2 in red, and Penk (used as a marker for SC layers distinction) in yellow. The merged signal shows high co-localization of Hcrtr2 and Per2, particularly in the SuG.
area used in our electrophysiological protocols carried out on rats, was seen to maintain a higher amplitude bioluminescence oscillation than the anterior SC. These results indicate that SC harbors intrinsic circadian clock mechanisms independent from a daily input from other brain areas.

4 | DISCUSSION

We observed temporal changes in the modulatory actions of orexinergic system on the superficial layers of the SC and in the intrinsic SC physiology that occur in the daily timescale. The orexinergic innervation originating most likely from the lateral hypothalamus increases past the day–night transition point (ZT12), together with a higher amplitude night-time activation of SC neurons by OXA. We also found spontaneous neuronal activity in the SC to vary across the daily cycle, with the lowest firing rates at early night corresponding to an increased response to OXA. These temporal events are highly likely to be coordinated by local circadian clocks. Indeed, we verified the presence of the molecular circadian clock in the SC, exhibiting daily and circadian rhythmicity both in vivo and ex vivo.

We first show the presence of OXB-ir fibers at the whole extent of SC layers, including the retinorecipient ones, confirming early anatomical studies. The number of immunoreactive axons could be classified as sparse (in the superficial layers) to moderate (in the intermediate and deep layers). However, the low number of fibers does not necessarily indicate orexins to act on a limited number of SC cells; the volume rather than synaptic transmission is widely reported for peptides including orexins. The robust release of these neuropeptides into the SC is supported by the report showing the highest concentration of OXA in the SC tissue homogenate amongst all the brain areas studied outside the hypothalamus. Moreover, using retrograde neuronal tract-tracing methods, we recognize the LH as a source of the orexinergic innervation of the SC. The moderate number of cells scattered throughout the extent of the hypothalamus corresponds both to the limited site of tracer injection and the density of orexinergic axons found in superficial layers of the SC.

In keeping with the observation that the orexin release to the SC is relatively high despite a moderate number of fibers, we found that OXA is a potent modulator of the neuronal activity of most neurons localized in the superficial layers of the SC. This was true for both the Op and Zo/SuG, with the latter significantly more affected by OXA. Of note, these effects were not only excitatory, but 12.7% of the recorded single units were notably inhibited by the application of OXA. Since a direct orexin-evoked
phasic interrelations follow the transcriptional logic of PLEX18–20 or the olivary pretectal nucleus.63 Functionally, expression governed by E-box.10 This indicates that the SCN or other extra-SCN clocks: the phasing of core genes.65–67 However, accumulating evidence describes synchronization enough by local intrinsic mechanisms to produce a well-structured phasic output at the level of a whole-brain area; a mismatch seen before for different timekeeping brain areas.54,55 Thus, evidence for the existence of local circadian clock in the SC was strengthened by real-time PER2::LUC expression monitoring of the SC explant culture, where the bioluminescence reporting Per2 expression was rhythmic up to 6 days. Thus, the SC molecular clock operating independently of remote brain clocks may result in the autonomous circadian modulation of electrophysiological activities in the SC brain slice.

Our MEA recordings unraveled the daily variation in the spontaneous neuronal activity in the superficial layers of the SC, exhibiting a pronounced decrease at the beginning of the dark phase, compared to the high levels of SUA throughout the day. It is unlikely for this change to be caused by reduced excitatory retinal input in the darkness, as these rats were culled exactly at ZT12, thereby not being affected by dark conditions for longer than a few minutes. Moreover, the SC SUA rebounded in the middle of the night, which suggests this daily variation comes endogenously, and is not a simple fingerprint of the environmental light conditions before the cull. The daily fluctuation in the SC firing rate can be thus attributed to the local clock gene expression in situ, which may reorganize in vivo when it receives rhythmic inputs from the central clock. Intriguingly, the phase relation between clock gene expression and electrical activity in the SC does not follow the SCN logic. In the SCN, the peak in Per1 expression is in phase with its spontaneous neuronal activity (both during the day), whereas in the SC, they are in the anti-phasic relation (Per1 peaks at ZT12, when the SC firing is the lowest). This observation suggests different cellular mechanisms linking molecular and membrane clocks in the SCN and SC, which remain poorly understood in both oscillators.65,68

The orexinergic system of the hypothalamus also expresses daily and circadian changes, as reflected in the rhythmic neuronal activities and responsiveness to environmental light conditions.6 The arousal-promoting orexinergic neurons are more active during the behaviourally active phase, which is paralleled by the increase in OXB-ir in the SC, shown by our study. Additionally, at the beginning of the night, when the orexinergic input to the SC significantly rises compared to the day, neurons in its superficial layers exhibit the peak response amplitude to OXA. The daily/circadian change in the response to a peptide may stem from an intrinsic regulation of the receptor expression or alternatively, it may be a result of indirect neurophysiological changes, including the variation in membrane resistance or resting potential.15,46,69 Our study shows the same SC neurons to co-express OX2 receptors and clock gene Per2. However, the variation in the transcriptional expression level of HCRTR2 did not exhibit a standard daily pattern (rather a bimodal distribution) and this variation was only significant for DD conditions. This
suggests, that light may influence the expression of orexin receptors in the SC, while in the absence of light cues the SC neurons rely on their intrinsic clock abilities to tune the reception of orexergic signal.

Importantly, the daily change in responsiveness to OXA in the SC should not be attributed to changes in the orexin receptor genes expression alone. Furthermore, we find the spontaneous firing rate of SC neurons to change across the light–dark cycle, what most likely stems from changes in the membrane potential. This suggests that the daily change in OXA response amplitude is not an effect of differences in maximal firing rates during the response, but rather the daily changing baseline. Thus, as we believe that this rhythm in the response amplitude would be relevant in vivo, unraveling its mechanism needs further investigation with patch clamp recordings.

From the physiological perspective, the subcortical visual system experiences extreme day-to-night changes in its main excitatory input, which originates from the retina. Therefore, not only the detection of light, but also the anticipation of the transition to light phase, creates a huge evolutionary advantage for the animal. Our results show that the early-night increase in both the strength of response to orexin and the orexergic input is temporally aligned to a drop in neuronal activity in the retinoreceptive SC layers expressing clock genes. Thus, the higher orexergic excitatory tone during the behaviourally active night (in vivo) may compensate a drop in the spontaneous neuronal activity of SC neurons. We theorize that these enable the nocturnal animal to predict and detect limited light intensity in the environment during this acute behavioral arousal phase of the night. As the main function of the SC is the coordination of movements toward the visual stimulus,26,30 the facilitation of a sparse visual information under low ambient lighting must be crucial.

In conclusion, the results of our study describe and characterize visual layers of the SC to undergo daily modulation by the orexergic system of the hypothalamus and identify that the SC neuronal activities are shaped by both the external and intrinsic timekeeping mechanisms. Moreover, we provide physiological and pharmacological perspective for the orexergic excitation of SC neurons via the OX2 receptor to support arousal during the behaviourally active dark phase, when the detection of sparse ambient light is critically needed.

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DISCLOSURES
The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS
Łukasz Chrobok, Jagoda Stanisława Jeczmien-Lazur, and Marian Henryk Lewandowski conceived the study. Łukasz Chrobok designed experimental protocols, supervised the study, and provided financial support. Łukasz Chrobok, and Jagoda Stanisława Jeczmien-Lazur carried out multielectrode array recordings. Łukasz Chrobok analyzed and interpreted electrophysiological studies with the use of tools provided by Kamil Pradel. Kamil Pradel wrote custom Spike2 and MatLab scripts and optimized automated spike-sorting for the MultiChannel data. Jihwan Myung wrote a custom Mathematica script for spectral analysis. Łukasz Chrobok performed intraocular injections with the help from Jasmin Daniela Klich. Łukasz Chrobok and Mariusz Kepczynski performed confocal imaging. Jasmin Daniela Klich, Jagoda Stanisława Jeczmien-Lazur, and Łukasz Chrobok obtained tissue for RT-qPCR and Łukasz Chrobok performed RNA isolation. Monika Bubka designed, performed, and analyzed RT-qPCR measurements. Łukasz Chrobok, Jagoda Stanisława Jeczmien-Lazur, and Jasmin Daniela Klich performed and analyzed daily immunohistochemical studies. Jagoda Stanisława Jeczmien-Lazur carried out tract-tracing surgeries. Aleksandra Klekocinska performed epifluorescence imaging, analyzed, and interpreted tract tracing data. Amalia Ridla Rahim and Jihwan Myung performed bioluminescence monitoring experiment. Łukasz Chrobok performed in situ hybridization. Łukasz Chrobok and Jihwan Myung wrote the manuscript and all authors agreed to the final version.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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