Orexin A excites the rat olivary pretectal nucleus via OX_2 receptor in a daily manner

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ABSTRACT

Pronounced environmental changes between the day and night led to evolution of specialised mechanisms organising their daily physiology, named circadian clocks. Currently, it has become clear that the master clock in the suprachiasmatic nuclei of the hypothalamus is not an exclusive brain site to generate daily rhythms. Indeed, several brain areas, including the subcortical visual system have been recently shown to change their neuronal activity across the daily cycle. Here we focus our investigation on the olivary pretectal nucleus (OPN) – a retinorecipient structure primarily involved in the pupillary light reflex. Using the multi-electrode array technology *ex vivo* we provide evidence for OPN neurons to elevate their firing during the behaviourally quiescent light phase. Additionally, we report the robust responsivity to orexin A via the identified OX_2 receptor in this pretectal centre, with higher responsiveness noted during the night. Interestingly, we likewise report a daily variation in the response to PAC1 receptor activation, with implications for the convergence of orexinergic and visual input on the same OPN neurons. Altogether, our report is first to suggest a daily modulation of the OPN activity via intrinsic and extrinsic mechanisms, organising its temporal physiology.

1. Introduction

Biological clocks adapt animals to rhythmically occurring changes in the environment, with the cyclic changes in the ambient light levels being the most robust and prominent rhythm of the outside world. In mammals, the master clock is localised in the suprachiasmatic nucleus (SCN) of the hypothalamus (Hastings et al., 2019; Hastings et al., 2018; Takahashi, 2017). However, recent advances show several other brain areas and peripheral tissues to exhibit autonomous or semi-autonomous circadian rhythms (Begemann et al., 2020; Guinding and Piggins, 2007; Paul et al., 2020). The orexinergic system delivers arousal for a plethora of brain sites in a circadian manner including the master clock, with orexinergic fibres surrounding the SCN. Moreover, the orexinergic system of the lateral hypothalamus has been schematised to act as the hands of the clock (Azeez et al., 2018; Belle et al., 2014; Belle et al., 2017; Marston et al., 2008; Nixon and Smale, 2007).

The olivary pretectal nucleus (OPN) is a small retinorecipient area which constitutes a key element of the pupillary light reflex (Szabadi, 2018; Young and Lund, 1998). Similarly to the master clock, the majority of retinal fibres that reach OPN belong to intrinsically

photosensitive retinal ganglion cells (ipRGCs) (Gamlin, 2006; Hattar et al., 2006; Klooster et al., 1995). Recently, orexin has been found to modulate pupillary light by a direct action upon ipRGCs (Zhou et al., 2021). Together with glutamate, ipRGCs co-utilise pituitary adenylate cyclase activating peptide (PACAP) acting via PAC1 receptors to excite its targets (Hannibal et al., 2017; Hannibal et al., 2000). It has been previously hypothesised that the OPN is involved in circadian time-keeping, due to its reciprocal connections with both the intergeniculate leaflet and the SCN (Gamlin, 2006; Klooster et al., 1995). It was also demonstrated that orexinergic fibres reach the OPN and display daily variation in orexin content (Chrobok et al., 2021b). However, daily changes in the spontaneous activity of OPN neurons or in their responsiveness to neuromodulators like orexin or PACAP has not been studied thus far.

Here, with the use of multi-electrode array (MEA) technology we report that OPN neurons increase their firing during the light phase. Additionally, we report for the first time the excitatory action of orexin A (OXA) upon the OPN neuronal activity, with the OX_2 receptor mediating the effect. Interestingly, the responsiveness to OXA and the activation of PAC1 receptor by its selective agonist evoked stronger excitations

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during the night, when the spontaneous firing was lower. Altogether this report suggests intrinsic (spontaneous firing) and extrinsic mechanisms (responses to peptides) to shape OPN neuronal activity in a daily fashion.

2. Results

2.1. OPN neurons exhibit daily changes in spontaneous neuronal activity *ex vivo*

Neurons of the master clock and these localised in extra-SCN oscillators modulate their firing rates across the daily cycle to transmit information on their circadian phase (Hastings et al., 2018; Paul et al., 2020). Thus, we first aimed to establish, if OPN neurons similarly organise their neuronal activity in regards to the light–dark cycle. We recorded spontaneous firing from nine pretectal slices *ex vivo* using a MEA technology, with four slices obtained from three rats culled at the middle of a day (ZT6), and five from three other rats culled at the opposite daily time point during the night (ZT18). Evaluation of neuronal activity from the recording locations in the OPN revealed higher firing rates during the day (4.17 ± 0.5 Hz, $n = 74$), compared to the night (2.50 ± 0.3 Hz, $n = 102$; $p = 0.0007$, Mann-Whitney test; Fig. 1A, B). These results show that OPN neurons are more spontaneously active *ex vivo* during the light phase.

2.2. OXA potently excites OPN neurons, with higher efficacy during the dark phase

The OPN has been demonstrated to be a target of the orexinergic system, with orexinergic afferents showing a nocturnal rise in orexin content (Chrobok et al., 2021b). Hence, we next evaluated if the orexinergic system may modulate neuronal activity in the OPN, by exogenous applications of OXA (200 nM) at day and night upon pretectal slices, which we previously tested for spontaneous firing levels. Notably, OXA robustly activated OPN neurons, with higher amplitude seen during the night ($\Delta\text{FR}: 5.38 \pm 3.3$ Hz, $n = 37$), compared to the day ($\Delta\text{FR}: 3.56 \pm 2.3$ Hz, $n = 72$; $p = 0.0017$, Mann-Whitney test; Fig. 1A, C).

Additionally, excitations were seen significantly more often at ZT18 compared to ZT6 (70.6% vs 50.0%, $p = 0.0074$, Fisher's test; Fig. 1D). However, the comparison between absolute levels of firing rate during the maximum of OXA-evoked responses did not show significant daily changes (6.52 ± 0.5 Hz vs. 7.03 ± 0.5 Hz; $p = 0.8683$, Mann-Whitney test), suggesting the daily change in response amplitude may be assigned to baseline variation. This dataset suggests that the orexinergic system influences OPN neurons more effectively during the behaviourally active night, what is a result of daily changes in baseline firing.

2.3. OXA acts in the OPN predominately via OX_2 receptor

Orexins bind to two metabotropic receptors: OX_1 and OX_2 receptor, which display a distinct expression pattern across the central nervous system (Li and de Lecea, 2020). To elucidate which of these two receptors mediates neuronal responses to orexins in the OPN, we applied OXA for the second time in the presence of a specific OX_2 receptor antagonist – TCS- OX_2 -29 (10 μM) upon four pretectal slices previously treated with OXA in the standard ACSF. Then, OXA was re-applied for the third time, after the thorough washout of the antagonist. Two slices were recorded during the day and two during the night, and results were pooled together. The application of TCS- OX_2 -29 itself resulted in significant decline in the spontaneous activity of OPN neurons ($p < 0.0001$, Wilcoxon's test; Fig. 2A). Evidently, the presence of TCS- OX_2 -29 blocked the entirety of the response to OXA in the OPN ($p < 0.0001$, $n = 49$; Friedman test, Fig. 2A–D), with the response completely re-established after the antagonist washout (OXA1 vs OXA2 + TCS: $p < 0.0001$; OXA2 + TCS vs OXA3: $p < 0.0001$, Dunn's multiple comparison test, Fig. 2D). This effect was also true when data were analysed for ZT6 and ZT18 separately (both $p < 0.0001$, Friedman tests). This experiment shows the predominant contribution of OX_2 receptor in the response of OPN neurons to OXA.

2.4. Most of the OXA-sensitive neurons in the OPN are excited by the activation of PAC1 receptor

Retinal PACAP is released from the terminals of ipRGCs to excite

Fig. 1. Neurons in the olivary pretectal nucleus (OPN) are more active during the day, but respond to orexin A (OXA) more robustly during the night. (Aa) Single unit activity (SUA) heatmaps showing the neuronal excitation following the application of OXA (200 nM) during the day (ZT6) and night (ZT18). Units were segregated from top to bottom, starting from these exhibiting the highest firing rates during the response to OXA. Warm colours code the highest, while cold – the lowest activity levels. Red bars represent the time of OXA application. (Ab) Average plots showing the mean $\pm$ SEM response to OXA at ZT6 and ZT18. Dotted line marks the baseline activity level at ZT18. (B) Spontaneous firing of individual OPN neurons. $***p = 0.0007$, Mann-Whitney test. (C) The amplitude of individual responses to OXA. $**p = 0.0017$, Mann-Whitney test. (D) Frequency of the response to OXA. $**p = 0.0074$, Fisher's test. ZT – Zeitgeber time. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. Orexin A (OXA) excites neurons in the olivary pretectal nucleus (OPN) via the OX_2 receptor. (Aa) Single unit activity (SUA) heatmap normalised for each unit separately, showing the change in neuronal firing following the application of OXA (200 nM, red bar). Second application of OXA was performed in the presence of OX_2 receptor antagonist – TCS-OX2-29 (TCS, blue bar). Finally, OXA was applied for the third time after the complete washout of the antagonist. Single units were segregated from top to bottom, with these showing the highest relative response to the first OXA application at the top. (Ab) Average plot of the mean $\pm$ SEM relative response to OXA in these three conditions. Note the lack of response to OXA in the presence of TCS. (B) Photomicrograph showing the positioning of the OPN (outlined) on the multi-electrode array (grey circles), with the reconstruction below. Red circles code recording locations in the OPN. Bars show 100 μ m. (C) Example spatial heatmaps of the multi-unit activity (MUA) showing the amplitude of the first, second (with TCS) and third (after TCS washout) response to OXA. (D) Changes in the response to OXA of individual OPN neurons in these three conditions. **** $p < 0.0001$, Dunn's multiple comparison test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

neurons in the subcortical visual system via the PAC1 receptor (Hannibal et al., 2017; Hannibal et al., 2000). To test the possibility if orexinergic system targets the same OPN neurons that respond to the activation of PAC1 receptor, we applied maxadilan (MAX) – a highly specific PAC1 receptor agonist (Moro and Lerner, 1997), following the application of OXA on five pretectal slices (two at ZT6 and three at

ZT18). The application of MAX evoked an irreversible excitation of OPN neurons, with a higher amplitude during the night (Δ FR: 5.11 ± 0.5 Hz, $n = 21$), compared to the day (Δ FR: 3.37 ± 0.4 Hz, $n = 26$; $p = 0.0218$, Mann-Whitney test; Fig. 3A, B). The proportion of cells sensitive to the treatment was however similar between phases ($p = 0.5286$, Fisher's test; Fig. 3C). Interestingly, 39.6% of neurons tested were activated by

Fig. 3. Most of the orexin A (OXA)-sensitive neurons in the olivary pretectal nucleus (OPN) are excited by the selective activation of PAC1 receptor with maxadilan (MAX). (Aa) Single unit activity (SUA) heatmaps showing the irreversible neuronal excitation following the application of MAX (100 nM) during the day (ZT6) and night (ZT18). Units were segregated from top to bottom, starting from these exhibiting the highest firing rates during the response to OXA. Green bars represent the time of MAX application. (Ab) Average plots showing the mean $\pm$ SEM response to MAX at ZT6 and ZT18. Dotted line marks the baseline activity level at ZT18. (B) The amplitude of individual responses to MAX. * $p = 0.0218$, Mann-Whitney test. (C) Frequency of the response to MAX. ZT – Zeitgeber time. (D) The proportion of neurons responsive to MAX and OXA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

both neuropeptides, while 27.1% responded to OXA alone, and only 9.4% were exclusively excited by MAX (Fig. 3D). These results suggest that OPN neurons sensitive to OXA also respond to retinal PACAP, with higher amplitudes of responses to PAC1 receptor activation observed during the dark phase.

3. Discussion

In this report we suggest that the orexinergic system of the lateral hypothalamus exerts excitatory actions upon OPN neurons predominantly via OX_2 receptor. Additionally, we identify a daytime rise in the spontaneous firing rate of OPN neurons, oppositely phased to the enhanced responsivity to OXA and the activation of PAC1 receptor, occurring at night. Finally, we propose a coupling of orexinergic and retinal information on the same OPN neurons, which respond to both OXA and PAC1 receptor agonist.

The modulation of neuronal activity in the subcortical visual system by orexins has been proposed before, for such areas as the lateral geniculate nucleus (LGN; all three parts) (Chrobok et al., 2021b; Chrobok et al., 2017; Chrobok et al., 2016; Orlowska-Feuer et al., 2019; Palus et al., 2015; Pekala et al., 2011), the SCN (Belle et al., 2014; Belle et al., 2017; Brown et al., 2008), or recently for the superior colliculus (Chrobok et al., 2021a). The functional connection between these two systems has also been strengthened by reports on orexin action in the retina (Qiao et al., 2017; Zhang et al., 2018) and the primary visual cortex (Bayer, 2004). Here, we provide evidence for OXA to robustly excite the majority of OPN neurons. With the OPN being primarily responsible for the generation of a pupillary light reflex (Young and Lund, 1998), we suspect that orexins mediate direct effects of arousal on pupil dilation. This hypothesis is reinforced by our observation, that OXA in the OPN acts through the OX_2 receptor, suggested to transmit arousal-related actions of this peptide (Xu et al., 2004) and by the higher responsiveness of OPN neurons to OXA during the rats' active phase.

Historically, the SCN was believed to exclusively generate circadian rhythmicity, at the level of clock gene expression and regulation of neuronal activity across the daily cycle (Takahashi, 2017). However, recent evidence strongly suggests that circadian control of many physiological processes must be devolved to local clocks localised in several brain structures and peripheral tissues (Abe et al., 2002; Begemann et al., 2020; Paul et al., 2020). Recently, we have reported circadian timekeeping properties in the subcortical visual system outside of the SCN (Chrobok et al., 2021a; Chrobok et al., 2021b). Here, we demonstrate that the OPN changes its firing rate between the light and dark phase, with higher activity levels recorded *ex vivo* during the behaviourally quiescent day; a time of high retinal input *in vivo*. Interestingly, we reported higher nocturnal responsiveness to OXA and PAC1 receptor activation in the OPN. The first may be explained by improved sensitivity to orexins at night, when they are actually released *in vivo* due to an intensified behavioural activity and circadian regulation (Azeez et al., 2018). The higher night-time responsiveness to PAC1 receptor activation is however more surprising, due to higher day-time activity of the retina, higher retinal PACAP release would be suspected during the light phase. This suggests an active mechanism promoting neuropeptidergic input from ipRGCs during the behaviourally active phase, when the retinal information is sparse but critically needed. To our knowledge, this is the first report on the daily variation of OPN neurophysiology, but further detailed circadian studies are critically needed to resolve the intrinsic vs extrinsic source of this daily change.

4. Experimental procedure

4.1. Animals and ethical approval

The study described in this report was performed on six 6–7 week old male Sprague Dawley rats. Rats were bred and housed in the Animal Facility at the Institute of Zoology and Biomedical Research,

Jagiellonian University in Krakow under standard 12:12 h light–dark cycle at $23 \pm 2^\circ\text{C}$ and $67 \pm 3\%$ relative humidity. All experiments were approved by Local Ethics Committee in Krakow and performed according to Polish regulations and the European Communities Council Directive (86/609/EEC). All possible efforts were made to minimise the number of animals used and their suffering.

4.2. Electrophysiology

4.2.1. Tissue preparation

Animals were anaesthetised with isoflurane (2 ml per anaesthetic chamber) and culled at two daily time points: in the middle of the day (ZT6), or in the middle of the night (ZT18). Then, 250 μm thick acute coronal pretectal slices containing the OPN were obtained in the same way as described previously for the LGN (Chrobok et al., 2021b). In brief, slices were cut on a vibroslicer (Leica VT1000S, Germany) in an ice-cold preparation artificial cerebro-spinal fluid (ACSF), composed of: NaHCO_3 25, KCl 3, Na_2HPO_4 1.2, CaCl_2 2, MgCl_2 10, glucose 10, sucrose 125 and phenol red 0.01 mg/l. Slices were transferred to carbogenated recording ACSF (32°C , cooled down to room temperature), composed of (in mM): NaCl 125, NaHCO_3 25, KCl 3, Na_2HPO_4 1.2, CaCl_2 2, MgCl_2 2, glucose 5, phenol red 0.01 mg/l. Sections were transferred to the recording chamber of the multi-electrode array (MEA) after a one hour incubation period.

4.2.2. Recording

Neuronal activity of the OPN was evaluated with the use of the two-well perforated MEA *ex vivo* technology (Belle et al., 2021), according to a previously described procedure (Chrobok et al., 2021b, Chrobok et al., 2021c). Briefly, slices containing the OPN were transferred to the recording wells of the MEA2100-System (Multichannel Systems GmbH, Germany) and positioned upon the 6×10 recording array of the perforated MEA (60pMEA100/30iR-Ti, Multichannel Systems). Slices were constantly perfused with fresh recording ACSF (32°C , 2 ml/min) and sucked down into the array. Data were continuously collected with Multi Channel Experimenter software (sampling frequency = 20 kHz; Multichannel Systems).

4.2.3. Drugs

Orexin A (OXA, 200 nM; Bachem, Bubendorf, Switzerland), maxadilan (MAX; PAC1 receptor agonist; 100 nM; Bachem) and TCS- OX_2 -29 (TCS; OX_2 receptor antagonist; 10 μM Tocris, Bristol, UK) were stored as $100 \times$ concentrates at -20°C . All drugs were diluted in fresh recording ACSF prior to application and delivered by bath perfusion. Approximately 4 min was needed for the drug to reach the recording chamber via the MEA tubing system.

4.2.4. Spike-sorting and analysis

Data recorded with Multichannel Systems hardware and software were prepared for spike-sorting with the use of custom-made tools, described in detail in Chrobok et al., 2021b; Chrobok et al., 2021c. Then, files were automatically spike-sorted with KiloSort programme (Pachitariu et al., 2016) in MatLab environment (R2018a version, MathWorks). Spike-sorting results were transferred into previously prepared CED-64 files (Spike2 8.11; Cambridge Electronic Design Ltd.) for further visualisation. Finally, spike-sorting results were manually revised in Spike2 8.11 with the aid of autocorrelation, principal component analysis and spike shape inspection to refine automatic sorting.

Further analyses were performed in Spike2 8.11. First, single unit activity (SUA) was 1 s binned. Then, mean spontaneous activity was explored in the 30 min epoch, starting 30 min after the initiation of recording. For examination of drug response, binned data were analysed in two epochs: 10 min before the drug application (baseline) and 30 min following the application (response). If after drug application the change in single unit activity (SUA) exceeded three standard deviations from baseline mean, a unit was classified as responsive. Response amplitudes

were further calculated as a difference between 10 min baseline mean and the maximal value during the response to a drug.

4.2.5. Data visualisation

Plots and pie charts were created in Prism 7 (GraphPad Software, USA). Heatmaps and average plots were generated in MatLab with custom-made scripts. For temporal heatmaps single unit data were first 10 s binned in NeuroExplorer 6 (Nex Technologies, USA) and then visualised as colour-coded firing rates or normalised SUA. Data normalisation was performed for each unit separately, with 1 coding the maximal and 0 – the minimal firing rate. Units were segregated from top to bottom with regards to the strength of the first response to a drug. Spatial heatmaps were generated from 100 s binned multi-unit activity (MUA) which was prepared in NeuroExplorer 6 for each recording location. The amplitude of response in MUA was calculated as the difference between a 100 s bin preceding the drug application and a 100 s bin during the maximal response.

4.3. Statistics

Statistical testing was performed in Prism 7. Differences between two groups were assessed with Mann-Whitney test (unpaired) or Wilcoxon test (paired), whereas Friedman test followed by Dunn's multiple comparison test were used to test changes amongst three groups of paired data. Fisher's test was used to study differences in group proportions.

5. Ethics approval

Experiments were approved by the Local (Krakow) Ethical Commission and performed in accordance with the European Community Council Directive of 24 November 1986 (86/0609/EEC) and the Polish Animal Welfare Act of 23 May 2012 (82/2012).

6. Data availability

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Author contributions

LC conceived the study and provided financial support. LC and MHL supervised the study. LC designed protocols and interpreted results of the study. AA performed electrophysiological recordings and analysed them with help of JDK. KP provided custom-made tools for spike-sorting, data analysis and visualisation. LC wrote the paper and all authors agreed to the final version.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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