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CONTRIBUTION OF CONES TO THE LIGHT RESPONSE OF THE MAMMALIAN BIOLOGICAL CLOCK

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ABSTRACT

The detection of ambient light is important for the circadian clock to synchronize to the external solar cycle. Light is detected by rods, cones and photosensitive retinal ganglion cells in the retina which send activating signals to the circadian clock. Here we address the role of cone photoreceptors in the transmission of photic information to the SCN by testing the ability of cone-only mice to entrain to light:dark (LD) cycles and performing *in vivo* electrophysiological recordings in mice having cones as the only functional photoreceptors in their retina. The cone-only mice were exposed to UV (λ_{max} 365 nm), blue (λ_{max} 467 nm) and green (λ_{max} 505 nm) light to determine entrainment to various wavelength of light. The majority of the mice entrained to a LD cycle with a positive phase angle of entrainment. The phase angle of entrainment was significantly smaller during UV and blue light exposure compared to during green light exposure, indicating reduced capacity to entrain to longer wavelengths. *In vivo* electrophysiological recordings show that the SCN neurons of cone-only mice respond to all three wavelengths of light. The light-induced increases in SCN neuronal activity decayed with a half-time of 50 seconds. Together, our data provide evidence for the ability of cone photoreceptors to transmit photic information to the circadian clock during the initial phase of light exposure.

INTRODUCTION

Light is sensed in the retina by classical rod- and cone photoreceptors and by a specialized subset of photosensitive retinal ganglion cells (pRGCs) containing the photopigment melanopsin. The classical rod- and cone photoreceptors are required for the generation of visual images. pRGCs project to non-image forming brain areas, such as the biological clock which is located in the suprachiasmatic nucleus (SCN) of the hypothalamus. The ablation of pRGCs results in the loss of photoentrainment [1]. Although classical rod- and cone photoreceptors are not essential for entrainment of the biological clock to the external light-dark (LD) cycle, both rod- and cone photoreceptors can influence the SCN. This is evidenced by the experimental finding that mice can entrain to a LD cycle in the absence of melanopsin [2, 3]. In addition, recordings in the SCN of melanopsin-deficient mice show preservation of sustained light responses in the SCN, which seem unaffected by the absence of melanopsin [4, 5].

The question is whether rods are the primary photoreceptors that mediate these responses or whether cones play an additional role. Whereas rods are capable of driving photoentrainment at a wide range of light intensities [6], mice with cones as the only photoreceptors in their retina show surprisingly large inter-individual differences in their ability to entrain but in general they show strongly reduced entrainment. The majority of these mice are not able to entrain to low intensity white light and some of them exhibit a positive phase angle of entrainment suggesting no functional role for cones [7, 8]. However, phase shifting responses in mice lacking mid-wavelength sensitive cones (M-cones) are attenuated [9, 10], which is difficult to reconcile with the reduced ability of cone-only mice to entrain to a LD cycle.

Photoentrainment is dependent on light-induced changes in SCN neuronal activity [11, 12]. Typically, SCN neurons respond to light with a transient increase in SCN electrical activity followed by a sustained component throughout light exposure. Rod- and cone photoreceptors together can mediate light responses at the level of the SCN including both the fast and the sustained component [4, 5, 13]. These findings are consistent with responses of pRGCs [14-16]. In this study we determined the specific contribution of the cone photoreceptors to circadian photoreception. We performed behavioral and *in vivo* electrophysiological recordings in cone-only mice to determine the effects of UV (λ_{max} 365 nm), blue (λ_{max} 467 nm) and green (λ_{max} 505 nm) light on photoentrainment and light-induced response characteristics of SCN neurons. We measured responses to these wavelengths to determine the role of the short-wavelength sensitive and mid-wavelength sensitive cones.

METHODS

Animals

Experiments were approved by the ethical committee of the Leiden University Medical Center and were carried out according to their guidelines. *Opn4-/-Gnat1-/-* mice originated from the lab of Prof. Samer Hattar (Johns Hopkins University, Baltimore) and were backcrossed on a C57/Bl6 background at the Leiden University Medical Center. Experiments were carried out with male and female mice homozygous for the *Opn4* and *Gnat1* gene.

Behavioral activity recordings

Behavioral activity rhythms were assessed using running wheels. Mice (n=21) were housed individually in plastic cages which were equipped with running wheels to stimulate active behavior of the mice in a measurable way. The amount of rotations of the wheel was recorded using a ClockLab data acquisition system (Actimetrics, Wilmette, IL, US) and stored on a computer in 1-minute bins. Food and water were available *ad libitum* during the experiment. Mice were housed in a 12:12 LD schedule. During the light phase mice were exposed to either UV light (365 nm, NCSU033B, Nichia), blue light (470 nm, LXML-PB01-0023, RS company) or green light (505 nm, LXML-PE01-0070, RS company). After at least 7 days in a LD cycle, mice were released in continuous darkness.

In vivo electrophysiological experiments

Extracellular activity of SCN neurons was recorded in freely moving mice. Tripolar stainless steel electrodes (Plastics One, Roanoke, VA, USA) were implanted in an anaesthetized mouse. The mouse was fixed in a stereotactic frame (Stoelting, Wood Dale, IL, USA) and the electrodes were implanted in the brain targeting the SCN. Two polyimide insulated twisted electrodes were aimed at the SCN under a 5 degree angle, 0.61 mm lateral from bregma and 5.38 mm ventral to the dura and used for differential recording. The third uncoated electrode was placed in the cortex as a reference. The electrodes were fixed to the skull of the mouse with the use of additional screws and dental cement. After a week of recovery mice were connected to a custom designed recording chamber to measure extracellular activity of SCN neurons while the animal was able to move freely. The electrical signal was amplified and bandwidth filtered between 0.5 and 5 kHz. The electrical SCN signal was digitized at 25 kHz using Spike2 hardware and software (Cambridge Electronic Design Cambridge, UK) and stored for offline analysis.

A custom-designed sphere was introduced in the recording setup to expose the animal to specific monochromatic wavelengths of light. The diameter of the sphere was 30 cm and it was coated with high-reflectance paint (barium sulphate; WRC-680; Labsphere Inc., North Sutton, NH, USA) to ensure uniform illumination levels. At the top of the sphere high power UV (365 nm, NCSU033B, Nichia), blue

(470 nm, LXML-PB01-0023, RS company) or green (505 nm, LXML-PE01-0070, RS company) LEDs were positioned at the top of the sphere with a baffle to prevent that the animal directly looks into the light source. The wavelength and intensity of light were measured with a calibrated spectrometer (AvaSpec2048, Avantes, Apeldoorn, the Netherlands). Animals were exposed to three wavelengths of light of various light intensities between CT12 and CT16, which corresponds to the beginning of the subjective day.

For detailed analysis of the response characteristics to light the digitized recordings were imported in MATLAB as waveform data using parts of the sigTOOL SON Library, including data from light sensors. Imported waveform data were triggered at fixed voltage amplitude settings. Time and amplitude of the action potentials were used for analysis. Digitized action potentials were counted in 1 sec bins. For a detailed analysis of population activity, a baseline recording was used to create spike amplitude histograms. On the basis of this amplitude histogram, thresholds were set in such a way that the average number of counts within each threshold window was equal. Threshold windows were non-overlapping and started above noise level. Actions potentials were counted within each step of a set threshold for the remainder of the recording.

Histology

At the end of each recording mice were sacrificed in a CO_2 chamber. A small electrolytic current was passed through the electrodes to mark the recording site. Brains were taken out and kept in 4% paraformaldehyde solution containing potassium ferrocyanide. The brains were sectioned coronally and checked with a microscope for reconstruction of the recording site.

RESULTS

Photoentrainment

Photoentrainment was tested in *Opn4-/- Gnat1-/-* mice lacking rod signaling elements and melanopsin, thereby leaving cones as the only functional photoreceptors in the retina. The ability to entrain was tested under 12:12 LD cycles using either UV, blue or green light during the light phase at relatively high irradiances (log13 photons/cm^2/s). Seven out of 21 mice (33%) showed normal entrainment under a 12:12 hour LD cycle (Figure 1A). Twelve out of 21 mice (57%) were able to entrain to a 12:12 hour LD cycle, but with a difference in phase angle of entrainment (Figure 1B). Two out of 21 mice (9.5%) were not able to entrain to the LD cycle. The phase angle of entrainment under a LD cycle with green light during the light phase was significantly larger (204±56min) compared to blue (36±34min) and UV light (49±17min) (1-way ANOVA, P=0.007). No differences were detected in phase angle of entrainment between blue and UV light (Student's t-test, P=0.7)(Figure 1C).

In vivo electrophysiology

In vivo electrophysiological recordings were performed in *Opn4-/- Gnat1-/-* mice (n=5) to determine the light-induced effects on extracellular activity of SCN neurons. We investigated the effects of UV (λ_{max} 365 nm), blue (λ_{max} 467 nm) and green (λ_{max} 505 nm) light on the response properties of a population of SCN

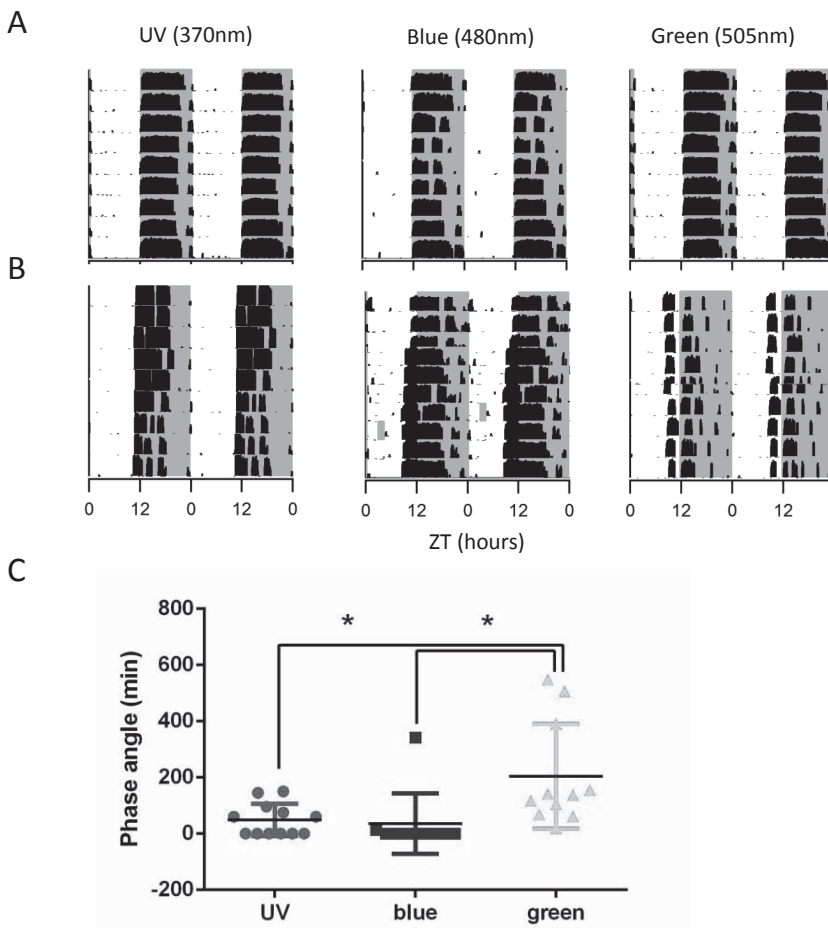

Figure 1. Double plotted behavioral activity recordings of *Opn4-/-Gnat1-/-* mice mice kept on a 12:12 LD cycle. The gray shading indicates the dark phase, and the white background indicates the light phase. During the light phase the mice were exposed to UV (λ_{max} 365nm, left), blue (λ_{max} 467nm, middle) or green (λ_{max} 505nm, right) light. Behavioral activity was measured using running wheel activity; the black bars indicate when the mouse runs in a wheel, and the bin size is 1 minute. Examples of *Opn4-/-Gnat1-/-* mice showing normal entrainment (A) or entrainment with a different phase angle (B). C. Phase angle differences during 12:12 LD cycles compared to lights off in *Opn4-/-Gnat1-/-* mice during UV (λ_{max} 365nm, left), blue (λ_{max} 467nm, middle) or green (λ_{max} 505nm, right) light exposure. *, $P<0.05$.

neurons of these mice. Exposure to 100 seconds of monochromatic light of all three wavelengths led to a robust increase in SCN neuronal activity with a typical transient overshoot in electrical discharge rate followed by a sustained component for the duration of lights on (Figure 2A). When the light was switched off a fast decrease in SCN neuronal activity was observed before electrical discharge levels returned to baseline.

To investigate the sensitivity to the different wavelengths of light, we determined the light-induced increases in SCN neuronal activity in *Opn4-/-Gnat1-/-* mice in response to a range of light intensities (Figure 2A). Mice were

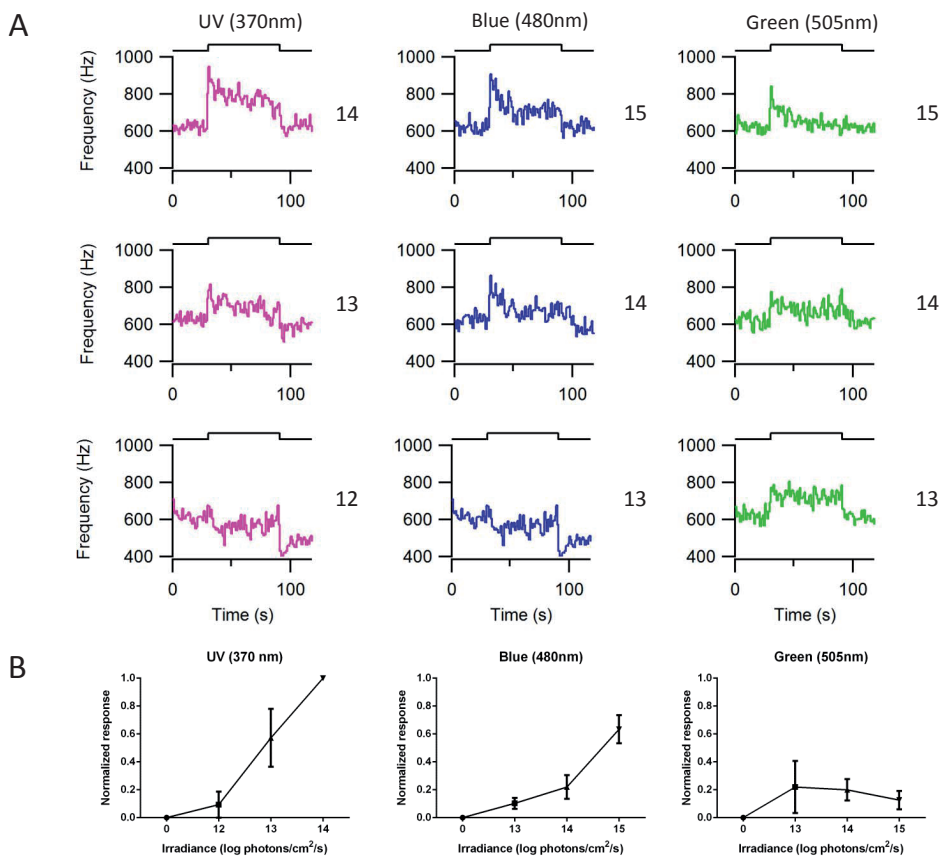

Figure 2. A. SCN *in vivo* electrophysiological responses to UV (λ_{max} 365nm, left), blue (λ_{max} 467nm, middle) or green (λ_{max} 505nm, right) light exposure at three different irradiance levels. *Opn4-/-Gnat1-/-* mice were exposed to 1-minute light pulses indicated in a step diagram above each graph. The photon flux is indicated next to each graph as log photons/cm^2/s. Bin size is 1 second. B. Mean (±SEM) normalized responses in SCN electrical activity. Graphs show the difference in spike frequency between sustained and baseline levels for each irradiance level and for each wavelength of light.

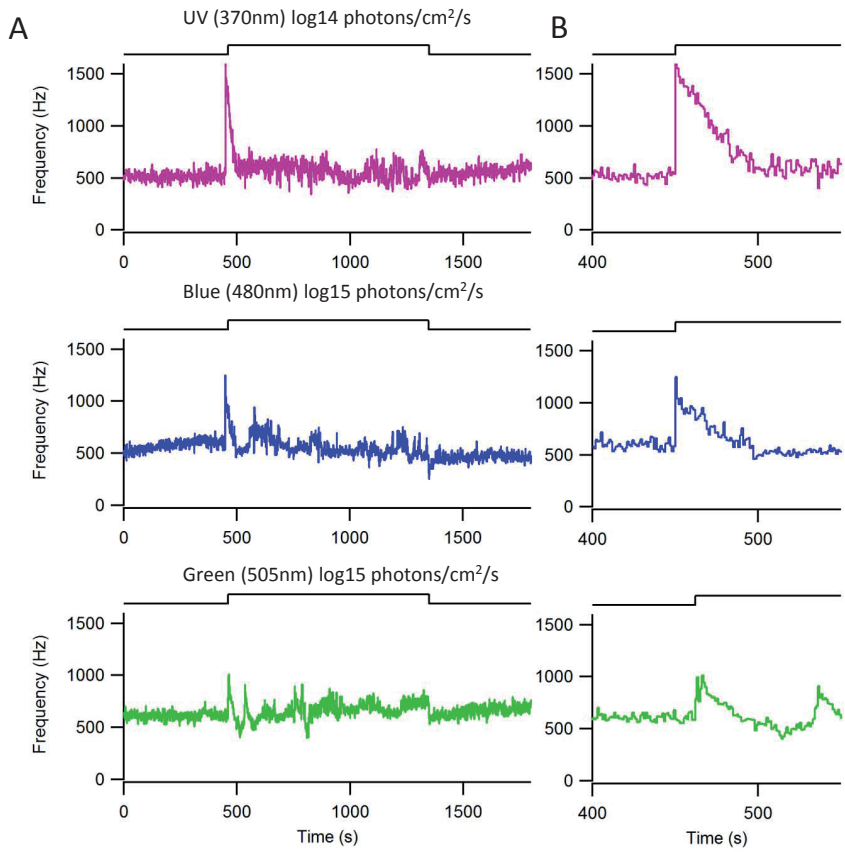

Figure 3. A. SCN *in vivo* electrophysiological responses to UV (λ_{max} 365 nm), blue (λ_{max} 467 nm) or green (λ_{max} 505 nm) light exposure of 15 minutes. Light pulse duration is shown in a step diagram above each graph. Photon flux and wavelength of light are indicated above each graph. Bin size is 1 second. B. SCN *in vivo* electrophysiological responses to UV (λ_{max} 365 nm), blue (λ_{max} 467 nm) or green (λ_{max} 505 nm) light exposure during the initial phase of light pulses of 15 minutes. Lights on are indicated in the step diagram above the graph.

exposed to 100 seconds of monochromatic light of various wavelengths ranging from low to high irradiances (log12-15 photons/cm^2/s). Exposure to UV and blue light of low light intensities induced a relatively small increase in SCN electrical discharge rate, higher light intensities led to an irradiance-dependent enhancement in SCN neuronal activity. The sustained component of the UV and blue light-induced increase was larger at high irradiances (log14-15 photons/cm^2/s) compared to the lowest irradiances (log 12-13 photons/cm^2/s). UV light induced a higher increase in SCN neuronal activity compared to blue and green light (1-way ANOVA, P=0.012)(Figure 2B). Green light exposure triggered responses in the SCN that were smaller than those evoked by UV and blue light.

Interestingly, the response to green light increased when light intensity was lowered (Figure 2A and B).

To determine the contribution of cone photoreceptors to light exposure of longer durations, *Opn4-/- Gnat1-/-* mice were exposed to light pulses ranging from 15 minutes to 1 hour. Typically, light exposure induced a transient overshoot in SCN electrical discharge rates followed by a sustained component. The duration of the increase in SCN neuronal activity was quantified by determination of half maximum values compared to average SCN neuronal activity levels 4-10 seconds after lights on. This epoch was selected as in wild type mice sustained response levels are obtained in this time window. The light-induced increase in SCN neuronal activity slowly decayed to baseline levels during light exposure and half maximum values were obtained after 52±13 seconds. No differences in half maximum values were determined between UV, blue and green light. Switching off the lights after 15 minutes of light exposure induced small changes in SCN neuronal activity. UV light exposure induced larger increases in SCN neuronal activity compared to blue and green light (Figure 3).

DISCUSSION

We determined entrainment in 'cone-only mice', lacking melanopsin and rod photoreceptors in their retina and show that cone photoreceptors are able to contribute to phototransduction to the SCN. Entrainment to UV, blue or green LD cycles was normal in 33% of the mice, while 9.5% of the mice could not entrain to any wavelength of light and 57% of the mice entrained with a large phase angle of entrainment, especially in response to green light. These findings provide evidence that cones are able to contribute to photoentrainment, but with a reduced capacity compared to wild type mice. To investigate the light response at the level of the SCN we performed *in vivo* extracellular recordings of SCN neurons. We observed light-induced increases in SCN electrical activity in response to UV, blue and green light. These responses exhibited fast initial increases in SCN electrical activity lasting on average 52 seconds. These results indicate that cone photoreceptors transmit photic information to the SCN during the initial phase of light exposure. After the initial response, the sustained component decays to baseline firing rates. Light-induced increases in SCN electrical discharge rates were larger in magnitude in response to short wavelength light (UV and blue) compared to green light. Both the behavioral and the electrophysiological recordings show a larger sensitivity for short wavelength light (UV and blue) compared to long wavelength light (green). The findings provide evidence for a contribution of UV cones to phototransduction to the SCN.

Our data reveal a contribution of cones during the first phase of light detection. Previous studies showed that mice lacking the mid wavelength sensitive cones

exhibit reduced phase shifting capacity in response to long wavelength light (λ_{max} 530 nm), while the response to short wavelength light (λ_{max} 480 nm and λ_{max} 360 nm) was not altered [9, 10]. The differences in phase shifting responses were only present in response to short light pulses (1 and 5 min), which, in correspondence with our study, indicates a role for cones in the initial part of light detection. While previous studies focused on the role of mid wavelength sensitive (M)-cones, we now show a significant contribution of the short wavelength sensitive (S)-cone.

It was long thought that melanopsin plays a crucial role in regulation of the sustained response to light. More recently it became evident that classical photoreceptors also contribute to sustained light signaling to the SCN [4, 5, 13, 17]. Our findings show that cones can influence SCN electrical activity in the absence of both melanopsin and rod photoreceptor signaling.

The majority of cone-photoreceptors co-express M- and S-opsins. The large response in SCN discharge rates to UV light could be caused by the higher levels of S-opsin compared to M-opsin expression in the mouse retina. The ratio of M-opsin versus S-opsin expression in the mouse retina is 1:3 [18]. Furthermore, the distribution of S-cones and M-cones over the retina is different, with higher levels of M-cones in the dorsal retina and more S-opsins in the ventral retina [19]. Environmental light is mainly detected by the ventral retina, the abundant presence of S-cones in the ventral retina may therefore be of importance for photoentrainment under natural conditions.

Several studies reported responses to steady light stimuli during recordings directly from the membrane currents of M- and S-cone photoreceptors [20-22]. Furthermore, activation of classical photoreceptors can lead to sustained responses at the level of pRGCs in the absence of melanopsin [14, 16]. Both M- as well as S- cone activation in the outer retina can provide sustained synaptic input to the pRGCs in the inner retina [15, 16]. Other studies revealed an inhibitory role of S-cones on primate pRGCs [23] and the pupil response in humans [24]. Together these studies indicate a significant contribution for cones in non-image forming functions.

A role for cones in the regulation of light effects on the human circadian system was also elucidated. The level of melatonin suppression is similar in response to long and short wavelength light, which suggests the ability for cone photoreceptors to play a role in the regulation of melatonin suppression [25, 26]. The study shows that cones provide a substantial contribution to melatonin suppression during the first phase of light exposure (<90 min), whereas the cone contribution decayed during prolonged light exposure [25].

There is evidence that cones contribute significantly to phase shifting responses during exposure to discontinuous light stimuli. This was tested in red cone knockin mice by presenting long wavelength red light as series of 1 minute pulses compared to a continuous light stimulus of 15 minutes. Discontinuous light stimuli lead to

significant larger phase shifts in behavioral activity [7]. In our study we made use of uniform illumination levels to make sure the mice perceived a specific photon flux per wavelength of light. In nature animals are expected to be exposed to large fluctuations in illumination levels due to the movement of the animal in and out the shade or burrow. Therefore, cone photoreceptors may have a larger contribution to phototransduction in nature than in the lab, where illumination levels are artificially constant.

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