Responses of suprachiasmatic nucleus neurons to light and dark adaptation: relative contributions of melanopsin and rod-cone inputs.

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Responses of SCN neurons to light and dark adaptation: relative contributions of melanopsin and rod-cone inputs

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Abstract

The circadian oscillator in the SCN is entrained to the environmental light/dark cycle through photic information conveyed from the retina. The vast majority of projections to the SCN arise from melanopsin expressing ganglion cells that are intrinsically light sensitive and that receive inputs from both rods and cones. In order to investigate the relative contributions of the different photoreceptive systems in shaping the photic signal influencing the circadian clock, we analyzed neuronal responses of single SCN neurons using extra-cellular electrophysiological recordings under different conditions of light adaptation. In the majority of neurons (78%) the spike rate is increased by light stimulation while the remainder are light-inhibited. The neuronal response to light is composed of several components distinguished by their temporal dynamics and degree of alteration following prior light exposure. SCN neurons display a sustained response to light followed by persistence of the response after light offset. These responses are sluggish and relatively unaffected by prior light exposures. Neurons also respond with a brisk, excitatory ON response and often an OFF response that is either excitatory or inhibitory. ON-OFF responses are transient and strongly reduced by prior bright white light exposure. Furthermore, two types of neuronal response patterns can be distinguished by the presence or absence of a slow-transient component that follows the transient ON response. The transient ON-OFF components express light adaptation properties characteristic of retinal channels involving cones, whereas the sustained and persistent components are consistent with *in vitro* response properties reported for melanopsin expressing ganglion cells.

Key Words : retina, circadian, entrainment, phase shift, vision, electrophysiology

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Introduction

In mammals, the suprachiasmatic nucleus (SCN) of hypothalamus contains an endogenous circadian pacemaker that controls most physiological and behavioral rhythms. Entrainment of circadian rhythms and other non-visual responses to light involve photoreceptors located in both the inner and outer layers of the retina (Hattar et al., 2003; Panda et al., 2003). The main projection to the SCN arises from a subpopulation of intrinsically photosensitive retinal ganglion cells (***ipRGCs***; Berson et al., 2002; Warren et al., 2003; Dacey et al., 2005) that also receive synaptic inputs from rods and cones via amacrine and cone bipolar cell synapses (Belenky et al., 2003; Sollars et al., 2003).

While the absence of all three classes of photoreceptors abolishes circadian entrainment (Hattar et al., 2003; Panda et al., 2003), their relative contributions in photic responses remain unclear. Studies in retinally deficient mice lacking one or more functional photoreceptors show that alteration of behavioral responses to light depends on the photoreceptor deletion and the type of light exposure. Mice lacking melanopsin (Panda et al., 2002; Hattar et al., 2003; Panda et al., 2003), cones, or both rods and cones (Freedman et al., 1999; Semo et al., 2003; Dkhissi-Benyahya et al., 2007) entrain normally to a bright white light:dark cycle, but exhibit diminished phase shifts to monochromatic stimulations depending on the wavelength and duration of the light pulse. Mice that predominantly lack rods (*rd/rd*; *rds*) have normal phase shifts but fail to entrain at low (<1 lux) light levels (Ebihara and Tsuji, 1980; Mrosovsky, 2003)

These different behavioural consequences could be interpreted to reflect a simple complementarity, with the functional loss of one photopigment compensated by the remaining photopigment(s). While this view is consistent with the coding of different light stimulus attributes by each photoreceptor type (Dacey et al., 2005; Dkhissi-Benyahya et al., 2007), recent *in vitro* recordings of ipRGCs reveal a greater range of complexity. The melanopsin driven response to light onset is typically sluggish, persists following light offset and is relatively resistant to bleaching (Berson et al., 2002; Warren et al., 2003; Dacey et al., 2005; Wong et al., 2005). This contrasts with phasic cone driven ON-OFF responses present in ipRGCs, consisting of transient increases or decreases in activity that are highly reduced by light bleaching exposures (Dacey et al., 2005). In addition, a surprising degree of heterogeneity within the melanopsin-driven neuronal responses has recently been demonstrated by Tu et al. (2005).

A potentially diverse range of stimulus responses emanating from different photoreceptor streams thus converges to shape the profile of photic information impinging on individual pacemaker neurons in the SCN. While photoreceptor deficient animals provide invaluable tools for the study of photoreceptor inputs, genetic alterations are not devoid of developmental consequences (for review see Gingrich and Hen, 2000) that can range from subtle remodelling to major reorganization of the retina (Marc and Jones, 2003). As specific examples, the absence of one photoreceptor can alter expression of the conserved photopigments (Sakamoto et al., 2004; Barnard et al., 2006; Dkhissi-Benyahya et al., 2007) or of ganglion cell responses to light (Pu et al., 2006). Information from intact animals endowed with a complete set of retinal photoreceptors is thus necessary to fully understand photoreceptor contributions and interactions.

Materials and Methods

Animals

Male Wistar rats (n=18, 4-6 months) from Charles River Laboratories (Charbonnières, France) were maintained on a 12hr light/12 hr dark cycle. Food and water were available *ad libitum* and all experiments were in accordance with current international regulations on animal care.

Electrophysiology

Animals were anaesthetized with an intraperitoneal injection of 25% urethane (1.2g/kg body weight). Heart rate and temperature were continuously recorded to monitor a stable level of anesthesia. Animals were mounted in a stereotaxic instrument and a dorsal craniotomy was made to allow placement of microelectrodes into the suprachiasmatic

region of the hypothalamus. Stereotaxic coordinates of the SCN were determined as: 73 µm anterior to bregma, 936 µm ventral to the cortical surface and 100 µm lateral to the midline. The eyelids were retracted and pupils were pharmacologically dilated by application of 1% atropine sulfate to the cornea. Eyes and brain surface were maintained hydrated using 0.9% saline. Extra-cellular single neuron activity was recorded using glass insulated, tungsten microelectrodes (Ainsworth Inc. UK). The exposed tips of the electrode (10 µm length, 1 µm at tip) were platinum–gold plated with an impedance of 0.5-1 MΩ to effectively isolate single neurons. Recordings and stimulations were made using a TDT (Tucker Davis Technology, USA) digital recording system. A multi-spike waveform discriminator (MSD, Alpha Omega Co., Israel) was used to track on-line the activity of individual neurons based on spike amplitude and shape.

Light stimulation protocols

Animals were exposed to a 500 nm monochromatic stimulations. Stimulation profiles and irradiance were controlled by a computer and monochromatic light was produced using tungsten halogen light source, IR absorbing filters, collimating lenses and a Schott interference filter (10 nm band-width at half peak transmission). Light was projected through an opal diffuser into a stimulation sphere centered with reference to the animals head, thereby providing a uniform illumination that encompassed the entire visual field. Using an irradiance of $2x10^{14}$ photons/cm^2/sec, neurons were recorded under two conditions of dark adaptation (DA) and light adaptation (LA, Figure 1). DA consisted of 30 min of total darkness (allowing the rods and cones to recover full sensitivity, Fain et al., 2001) preceding the monochromatic test stimulation, while in the LA condition, the monochromatic stimulation is preceded by a bright white light stimulation (56 W/m^2/sec @400-700 nm (>10^{17} photons/cm^2/sec; 3000 photopic lux) lasting one minute and ending one minute before the test stimulation, during which the animal is exposed to complete darkness in order to allow the neuron to return to a stable baseline.

Light adaptation is a classical strategy used to study photoreceptive channels, from single photoreceptors to higher visual processes, by selectively reduce specific response components (Knoblauch and Shevell, 2001; Dacey et al., 2005). Light adaptation involves mechanisms of both chromophore depletion and a neural process (producing a maintained decrease of intracellular Ca^{2+} concentration affecting the transduction cascade) leading to desensitization of the photoreceptor (for review see: Fain et al., 2001). Although ipRGCs, like rods and cones, have been shown to adapt to light they appear more highly resistant to light bleaching (Wong et al., 2005). Recordings in intact retina indicate that following high light exposures ipRGCs continue to respond to light whereas non-melanopsin expressing cells fail to respond, presumably resulting from extensively bleaching of rods and cones (Berson et al., 2002; Warren et al., 2003). This desensitizing effect is analogous to the use of synaptic blockers to prevent rod and cone synaptic input to ipRGCs employed during in vitro retinal studies. An advantage of this approach is that by alternating the light adaptation exposures with appropriate dark adaptation recovery periods, repeated evoked responses can be assayed from the same neuron.

Stimulations were repeated 3 times for each condition and for each neuron. A full protocol lasted 2 hours. Five neurons could be recorded for longer periods (up to 5 hrs) and were further investigated at lower irradiances under DA. Stimulations were repeated three times at $2x10^{13}$ photons/cm^2/sec and two times at $2x10^{12}$ photons/cm^2/sec and $2x10^{11}$ photons/cm^2/sec for each neuron.

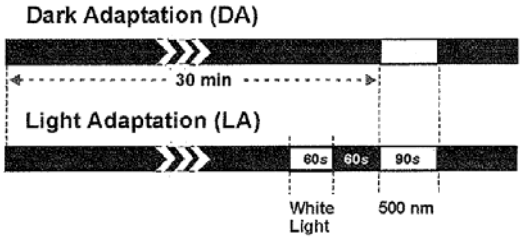

Figure 1. Schematic Illustration of the light stimulation protocol in dark adaptation (DA) and light adaptation (LA). Animals were exposed to 30 minutes of darkness prior to 90 seconds of 500 nm light stimulation. In the LA condition the monochromatic light stimulation occurred following a 60 seconds exposure with bright white light.

Selection of light responsive neurons

The following criteria were used to select light responsive neurons. Once the neuronal

signal was isolated, the capacity to respond to light was tested using a stimulation protocol consisting of 3 successive monochromatic light pulses alternating with darkness lasting 10 sec each, at an irradiance of $2x10^{14}$ photons/cm²/s. Neurons that displayed a detectable alteration in firing rate during the light pulses were classified as light responsive and retained for analysis. Neurons that expressed a baseline firing rate but were unresponsive to light stimulation, or neurons that were unable to maintain a clear response to light were not conserved for further study. Some neurons that were initially classified as light responsive subsequently decreased or ceased light responsiveness, became unstable or erratic in their responses. This prevented the recording of a complete data set though the duration of the 3 response cycles. These neurons were thus excluded from the analysis.

Out of a total of 287 neurons encountered during the descents, 132 neurons (46%) were clearly light responsive. A total of 29 of these light responsive neurons could be fully characterized according to the stringent criteria. Recordings from an individual animal generally lasted 18-30 hours, usually beginning at ZT4. We did not measure any significant nycthemeral variations of the basal firing rate of neurons in the dark or in their response amplitude to light stimulation.

Analysis

Recordings of neuronal spike frequencies were analyzed using software developed under Matlab 7.0 (The Mathworks, Inc., MA). The bin size used for averaging responses was 0.1sec. For purposes of illustration a bin size of 0.2 sec is used unless otherwise indicated. Parameters measured included latency of the response, average firing rate and the amplitude of the response. Several different response components could be differentiated according to these parameters and their time of occurrence in relation to the light stimulation (see results).

Statistical analysis was computed using SigmaStat software (Systat Software Inc., Point Richmond, CA). The non-parametric Mann-Whitney test was used for comparisons between groups and the non-parametric Wilcoxon test for repeated measures. The results are expressed as means ±SEM.

Histology

At the end of the experiment the final electrode track was marked with 1-3 electrolytic lesions. The animal received a lethal dose of sodium pentobarbital, the brain removed and fixed with by immersion in Zamboni fixative (4% phosphate buffered paraformaldehyde, picric acid) during 24 hours. Brains were then cryoprotected by immersion in 30% sucrose in phosphate buffer (0.01 M; pH 7.4) for 24 hours. Serial coronal sections were made at 50 µm on a freezing microtome. The positions of the recording sites in the SCN were confirmed by comparing the stereotaxic coordinates of recorded neurons to the positions of the electrode tracks locations of the marked lesions.

Results

Response patterns in dark adapted conditions: evidence for two groups of components with different kinetics

A total of 132 light responsive neurons were encountered in the SCN of which the majority of cells (78%) were light excitatory and responded to photic stimulation by an increase in their firing rate. Figure 2 (A-G) illustrates several examples of light response patterns of individual SCN neurons after a dark adaptation period of 30 min. Under these conditions, rods and cones are fully responsive to light during the whole field exposure of both eyes to monochromatic light stimulation. However at the high irradiance used (500 nm; $2x10^{14}$ photons/cm^2/sec) rods are probably not responsive to the 90 sec light pulse (Aggelopoulos and Meissl, 2000;Nathan et al., 2006). A total of 29 neurons were fully characterized under DA conditions. The average spontaneous firing rate of these SCN neurons in the dark is 1.93±0.56 Hz. Despite a certain degree of diversity among individual SCN neurons the response to light can be characterized by several temporally defined transient and sustained components (shown in Fig. 2).

The onset of light stimulation leads to a rapid, brisk onset response of short latency (0.13±0.01 sec) and of high spike frequency (20.80±2.03 Hz). This initial fast component is always excitatory, transient, and generally lasts

less than 500 ms (Fig. 2A-F), although in certain neurons the firing rate shows a gradual decrease following the initial transient response (Fig. 2D). This rapid phasic component is referred as ***fast-transient ON response***.

Following the initial fast-transient response, SCN neurons show a tonic ***sustained response*** throughout the entire period of light exposure. Quantified as the average firing rate during the entire period of light stimulation (excluding the initial 500 ms transient) this sustained response equals 8.23±1.01 Hz for all neurons (n=29). However, based on the profile of the tonic firing rate discharge pattern two groups of neurons can be distinguished.

Type I neurons (15 of 29) display a ***slow-transient*** component expressed as a slow initial increase in firing rate following the fast transient ON response that attains a maximum on average within 10.00±0.46 seconds after light onset, and then declines gradually to a stable sustained level. The slow-transient is defined by an increase in firing rate ≥25% than the averaged sustained response rate, lasting at least 3 seconds and occurring within the first third portion of the light exposure. Figures 2A and 2B represent two examples of this type I response pattern and figure 2C shows the average response pattern.

Type II neurons lack the ***slow-transient*** response (14/29) and only express the stable sustained response that sharply increases in firing rate following the initial transient and subsequently maintains a stable response rate throughout the duration of light exposure. Figures 2D and 2E represent two examples of this type II response pattern and figure 2F shows the average response profile.

Type I and type II neurons also significantly differ in their basal firing rate and in the firing rate of the sustained response to light (type I basal rate = 0.81±0.19Hz; type II basal rate = 3.11±1.06Hz; Mann-Whitney $p<0.05$) whereas both the ON and sustained responses are similar in neurons type I and II (Mann-Whitney, p=0.116 and p=0.111 respectively)

At light offset, about half of the neurons (13/29) display a third component consisting of a ***fast-transient OFF*** response. An excitatory OFF response is present in both type I (5/15) and type II (5/14) neurons (Fig. 2A, B, D) whereas an inhibitory OFF response is less common and only observed in type II neurons (3/14; Fig. 2E). The OFF excitatory response is of short latency (0.17±0.02 sec), occurs within first 500 ms following light offset and is of relatively high amplitude (16.61±1.70Hz). The firing rate of the transient excitatory OFF response is typically less that of the ON transient response. The OFF inhibitory response was difficult to quantify given the small number of neurons and was not analyzed further.

Following the end of the light stimulation, neuronal activity slowly returns to base-line in an average time of 44.95±2.34 sec (Fig. 2). The duration of this ***post-stimulus persistence*** response is defined as the time required to return to a response level equal to 50% of the sustained response following light offset. The firing rate of the response is quantified as the averaging firing rate within this duration. On the average, the return to baseline firing rate is similar in type I (46.29±2.86sec, Fig.2C) and type II neurons (43.27±3.97sec; Mann-Whitney test, p=0.369; Fig.2F).

In the dark adapted state, all the recorded neurons display the fast-transient ON, sustained and post-stimulus response components. No pure fast-transient ON or OFF or isolated sustained-only responses were observed.

All the neurons discussed above show an increase in firing rate in response to light stimulation. However, we estimate that in 22% of the light responsive neurons in the SCN, neuronal activity is inhibited by light (Fig. 2G, type III). The relative instability of light responsiveness in these neurons did not allow a complete characterization over the entire experimental protocol. Most of these light inhibited neurons either rapidly ceased to respond to light stimulation or showed a progressive decrease in the response after a series of light stimulations. This also occurred in some of the light excited neurons that accordingly were not included in the analysis. In some rare cases, the neuron's initial inhibitory response to light spontaneously reversed into an excitatory response during a subsequent bout of light stimulation. Despite the difficulty to perform a full recording protocol from light inhibited neurons, observation of the firing rate profiles of light-inhibited neurons during the light stimulation tests shows that the response patterns were basically similar to those of light activated neurons. In general, the responses display a transient suppression of activity at light onset that continues during stimulation followed

by a subsequent persistence of suppression after light offset. Since this inhibitory response pattern has not been observed in ipRGCs, these cells may be post-synaptic targets of SCN neurons that receive direct contacts from retinal fibers since retinal inputs involve the excitatory neurotransmitter glutamate (for reviews, see Ebling, 1996; Rea, 1998).

Effect of light adaptation on neuronal response pattern: response components are differentially affected

Of the 29 neurons recorded under the dark-adapted condition (DA), 27 (13 of type I and 14 of type II) neurons were also fully characterized following light adaptation (LA). In this condition, the test stimulation was preceded by a full field bright white light stimulation (pre-adaptation 'bleaching' stimulation) known to induce a prolonged desensitization of the rods and cones (Baylor and Hodgkin, 1974; Fain et al., 2001). Figure 3 shows several examples of individual and average recordings in both the DA and LA conditions for type I and type II neurons. The bright light exposure that precedes the test stimulation obviously affects the response pattern, thereby showing that the immediate prior light history has a profound impact on the photic responses of SCN neurons. All the neurons show lower average spike frequencies during the light stimulation in the LA condition compared to the DA condition, although the fast-transient ON and the sustained responses are differentially affected by prior light exposure.

After light adaptation, the fast-transient ON component observed in DA is either totally abolished or when detectable is strongly diminished in amplitude for both type I-II neurons. The average reduction is 67.13±7.94% (Fig. 3, 4). The transient excitatory OFF response is similarly affected and on average is reduced by 39.49±5.75%. Only two neurons (type I) retain a detectable OFF response. The reductions in responsiveness of the transient ON-OFF components are consistent with and typical of rod/cone driven retinal mechanisms (Berson et al., 2002; Dacey et al., 2005).

In contrast, the firing rate of the sustained response is less affected by light adaptation than the transient responses (Fig 3). On the average, for all the neurons, the sustained response is decreased by only 21.81±6.28% in LA although the degree of reduction of the response differs between type I and type II neurons. In type I neurons, the firing rate amplitude is significantly reduced for both the slow-transient (38.73±8.54%) and the sustained responses (31.20±10.06%, Fig. 3A, 4A). In contrast, type II neurons do not display a significant reduction of the sustained component (13.64±7.72%) after bright light exposure (Fig. 3B-C, 4B). Four of the neurons actually showed a slight increase in firing rate (see example in Fig. 3C).

The firing rate of the post-stimulation persistence after light offset also remains unaltered by the prior light exposure in both type I and II neurons (average decrease= 2.82±7.27%; Fig. 3). The greater resistance of the sustained and post-stimulation persistence in the LA condition is consistent with the idea that both rely on the same response mechanism. These two response components are similar to the response properties previously described in melanopsin ipRGCs (Berson et al., 2002; Dacey et al., 2005; Tu et al., 2005).

Since the various light responsive components are differentially reduced by prior light exposure, we used a subtractive method to derive the pattern of response profiles of the components that are most affected by light adaptation. Figure 3D-E (upper panels) shows the averaged responses for type I and type II neurons in LA (in gray) superimposed on the averaged responses in DA (in black). The difference in shading allows a distinction between the two response patterns and overall consequences of prior light adaptation in both type I and type II neurons. Subtracting the responses of LA from DA unmasks the response amplitudes attenuated or abolished by light adaptation (Fig. 3D-E, lower panels). These histograms reveal light adapted responses that closely resemble cone-mediated responses of melanopsin ganglion cells illustrated by Dacey et al. (2005). These typical light adaptation properties convincingly suggest that this contribution is derived from cone (and/or rod) photoreceptors.

Effect of irradiance on the response pattern

Five out of the 29 neurons that were recorded in DA at a high irradiance ($2x10^{14}$

photons/cm^2/sec) were also tested at a lower irradiance (2x10^{13} photons/cm^2/sec) in DA conditions. The other 24 neurons could not be investigated further following the long DA-LA protocols since either the neuronal response became inconsistent or the electrophysiological signal was lost. Due to the small sample size, statistical analysis of the quantitative alterations was not carried out.

An example of an individual type I neuron is shown in figure 5A, and the average responses of the 4 type I neurons are displayed in figure 5B. Recordings of the single type II neuron are shown in figure 5C. Both types show a similar pattern of reduction in firing rate with the reduction in irradiance and the average change in individual parameters were calculated for the 4 type I and single type II neurons. A reduction in the irradiance by one log unit within the photopic range leads to a significant overall decrease of the firing rate of the SCN neuronal response (Fig. 5) as would expected from luminance coding units. The average overall reduction in firing rate (all components) during the light stimulation at 2x10^{13} photons/cm^2/sec is 47.9% that of the rate at 2x10^{14} photons/cm^2/sec. The firing rate of the fast-transient ON response is strongly affected by the reduction in irradiance and all five neurons show a substantial decrease in amplitude (67.4±3.53%; Fig. 5). Three neurons displayed an excitatory OFF response at 2x10^{14} photons/cm^2/sec that was undetectable at the lower irradiance (Fig. 5). One neuron displayed an inhibitory OFF response at 2x10^{14} photons/cm^2/sec but was not detectable at 2x10^{13} photons/cm^2/sec.

The slow-transient component (4 type I neurons) is totally absent at the lower irradiance of 2x10^{13} photons/cm2/sec (Fig. 5A-B). The sustained response is decreased in all neurons (average reduction= 27.51±0.59%). Four of the five neurons show a slight decrease in the post-stimulus persistence (average reduction = 8.53±0.52%). Based on response amplitude, both the sustained and the fast-transient components of the light response express properties of irradiance encoding, although the fast-transient ON and OFF components are more susceptible to alteration for the decrease in irradiance.

Examination of the temporal aspects of the response reveals that the latency of the fast-transient ON component is unaffected by the decrease in irradiance (average latency = 0.14±0.02 sec at 2x10^{13} photons/cm^2/sec). The only excitatory OFF response detectable at 2x10^{13} photons/cm^2/sec showed no change in latency compared to the higher irradiance (0.2sec at both irradiances, not shown). In contrast, at 1 log unit lower irradiance the time required for the sustained component to attain the plateau level increases to 36.78±2.65 seconds. Finally, the duration of the response persistence after light offset remains relatively unchanged at lower irradiance and is only reduced by 6.32±3.44%.

The amplitude and kinetics of the sustained and persistent components compared to the transient components are differentially affected by the modulation of irradiance. This reinforces the idea that these two sets of components rely on different channels with distinct photoreceptive properties.

The responses of these five neurons were also recorded at 1-2 log unit lower irradiances. At 2x10^{12} photons/cm²/sec only two of the neurons displayed a clear response to light (Fig. 5D). Response latency was very high and the firing rate extremely sluggish. At the lowest irradiance tested of 2x10^{11} photons/cm²/sec we did not observe any detectable neuronal response in the present light exposure conditions (Fig. 5E).

Discussion

The SCN neuronal response to light is complex and systematically includes two response components that are distinguished by different temporal kinetics, resistance to bleaching and responses to irradiance. Fast-transient ON (excitatory) and transient OFF (excitatory or inhibitory) responses are of short latency, strongly influenced by prior light exposure and rapidly decrease in amplitude at lower irradiance levels. The sustained components, including the slow-transient component and the persistence of the response after light offset, show longer latencies, higher resistant to bleaching and are less affected by decreases in irradiance. The properties of these two groups of response components are consistent with retinal channels involving outer retinal cone photoreceptors and inner retinal melanopsin ipRGCs that both encode irradiance at photopic levels but are differentially modulated.

The fast-transient ON-OFF components are derived from outer retinal photoreceptors

Excitatory transient ON responses are observed in all SCN neurons, while transient OFF responses (mainly excitatory) are present in half the neuronal population. Both transients are more prominent in the dark adapted condition and modulated by irradiance. These properties are strikingly different from the sustained responses reported for melanopsin ganglion cells but are fully consistent with the response properties of retinal channels involving cones and rods (Volgyi et al., 2004; Wassle, 2004). However, while both cones and rods display transient ON-OFF responses to light, it is unlikely that rods contribute significantly to the responses recorded in the present study. Rod transients have longer latencies (>200 ms) and the intensities used in our study correspond to the cone dominated photopic domain (Aggelopoulos and Meissl , 2000; Nathan et al., 2006) that preclude rod influences at the cone-bipolar interface (Bloomfield and Dacheux, 2001). It is nevertheless premature to discount a potential contribution from rods at lower light levels. At scotopic levels, Aggelopoulos and Meissl (2000) report a rod dominated response in SCN neurons with a peak spectral sensitivity at 505 nm that shifts to 510 nm (MW opsin) in photopic conditions, and rods appear to be required for entrainment at low light levels (Ebihara and Tsuji, 1980; Mrosovsky, 2003)

Cone (and rod) signals from the outer retina can be relayed to the SCN via pathways from melanopsin ipRGCs or non-melanopsin RGCs (Morin et al., 2003; Sollars et al., 2003; Hattar et al., 2006). The ON-OFF responses are derived from the well known anatomical and physiological stratification of the inner plexiform layer (Wassle, 2004). ipRGCs can be mono-stratified with synaptic arbors in either the ON or the OFF sublamina or bi-stratified with arborisations in both sublamina (Pu, 1999; Provencio et al., 2002; Belenky et al., 2003; Warren et al., 2003). This is consistent with the report that ipRGCs display primarily excitatory-glutamatergic, primarily inhibitory-GABA-ergic, or both types of synaptic events (Perez-Leon et al., 2006). Transient ON-OFF responses to light are typical of ipRGCs (rodents: Berson et al., 2002;Warren et al., 2003; Perez-Leon et al., 2006; primate: Dacey et al., 2005) and in the SCN (rat: Aggelopoulos and Meissl, 2000; Pu, 2000; mouse: Mure et al., 2007).

In addition to the temporal properties of the transient response, the vulnerability to light adaptation confirms that this component emerges from retinal channels involving outer retinal photoreceptors. Extrinsic responses from rods and cones recorded in both rat and primate ipRGCs *in vitro* are eliminated under synaptic blockade or maintained light exposure without affecting the slow sustained intrinsic depolarisation (Berson et al., 2002; Warren et al., 2003; Dacey et al., 2005). Bleaching adaptation by a light bright enough to photoconvert a significant fraction of the photopigment produces a prolonged desensitization of photoreceptors that slowly recover in the dark (Fain et al., 2001). Aggelopoulos and Meissl (2000) showed that sensitivity of SCN neuronal response to short light pulse continuously improves with extended periods of darkness. These response dynamics may represent a cellular correlate of the effects of prior photic history on melatonin suppression by light in humans (Hebert et al., 2002; Smith et al., 2004) or phase shifting responses in the rodent (Khammanivong and Nelson, 2000).

The sustained, slow-transient and persistent components to the SCN derive from melanopsin photoresponses

The slow response kinetics of the sustained response to constant light are well-documented features of SCN neurons dating from .the early electrophysiological recordings by Groos and Meijer, who originally described the gradual increase of the response to light as well as the subsequent slow decay to baseline at the end of stimulation (Groos and Mason, 1978; Meijer et al., 1986). The temporal response of ipRGCs, including the sustained response to continuous illumination, encoding of stimulus energy in the photopic range and sluggish responses to light onsets and offsets (Berson et al., 2002; Warren et al., 2003; Dacey et al., 2005; Tu et al., 2005) are in agreement with our SCN recordings.

In addition to response kinetics, the observation that the sustained and persistent components are weakly affected by the prior bright white light exposure is consistent with melanopsin photoresponse properties. The capacity of ipRGCs to resist bleaching by light has been proposed to derive from the invertebrate-like phototransduction mechanisms of the melanopsin photopigment (Nayak et al., 2007. Isoldi et al., 2005; Berson, 2007). This

maintenance of photic responsiveness is attributed to the bistable nature of melanopsin and it's ability to use light to regenerate the chromophore as suggested in several recent *in vitro* (Melyan et al., 2005; Panda et al., 2005) *in vivo* (Mure et al., 2007) studies.

Neuronal Response Classes in the SCN

Sekaran et al. (2003) using calcium imaging and more recently Tu et al. (2005) have described a surprising heterogeneity in the intrinsic light response properties of the adult retina, suggesting the existence of distinct melanopsin ipRGC cell classes. Tu et al., (2005) describe a type III ipRGC, characterized by a rapid onset, high light sensitivity and very slow offset that corresponds to our type II SCN neuronal response. The authors also describe a type II ipRGC with a slow onset, lower light sensitivity and slow offset. For decreasing irradiances, the latency increases while amplitude significantly decreases in type II ipRGCs, whereas the type III ipRGCs are relatively less affected. Based on the analogous photic response properties, it is likely that the type II melanopsin ipRGCs provide the slow-transient component we observe in type I SCN neurons, while the type III ipRGCs supply the sustained response typical of type II SCN neurons.

However, it appears plausible that the slow-transient and sustained components are combined in different proportions in individual SCN neurons and reflect a continuum of convergent responses originating from the different types of melanopsin ipRGCs. This convergence is consistent with previous observations that SCN-projecting RGCs have receptive fields of only 2-5 degrees (Pu, 2000), whereas light-responsive SCN neurons have large receptive fields often exceeding 20 degrees in diameter (Groos and Mason, 1980).

Signals from inner and outer retinal channels combine to shape the photic response of the SCN

Our results show that SCN neurons receive a complex photic signal in which the response kinetics, response to irradiance, and resistance to bleaching are typical for channels involving both classical photoreceptors and melanopsin ipRGCs (Fig. 6). The melanopsin-based photoresponse provides the SCN with a signal that is sustained and only weakly affected by light adaptation in line with the canonical ability of the circadian system to integrate irradiance over long time periods (Nelson and Takahashi, 1991; Dkhissi-Benyahya et al., 2007). Studies in $Opn_4^{-/-}$ mice clearly demonstrate the contribution of melanopsin in photic entrainment and light induced phase shifts (Panda et al., 2002; Ruby et al., 2002; Hattar et al., 2003; Dkhissi-Benyahya et al., 2007). From these same studies, the retained ability of melanopsin knockout mice to entrain and phase shift locomotor activity must necessarily rely on input from outer retinal photoreceptors.

The precise function of the transient input originating from the outer retina photoreceptors to the SCN is less clear. The transient input may compensate for the slow kinetics of the melanopsin cell response thereby increasing sensitivity of circadian system to short light pulses (Gronfier et al., 2007). This is explicitly evoked by Dacey et al. (2005) as a "priming" effect of cones to elicit the ipRGC response of melanopsin at threshold levels, in the absence of which the sustained component would not normally occur. This may alo explain the behavioral deficit observed in MW-coneless mice for duration light pulses (Dkhissi-Benyahya et al., 2007). The cone contribution could play a significant role in natural conditions where eye blinks and periodic light sampling behaviors (Boulos et al., 1996) could produce pulses of light on the retinal surface resulting in robust transient ON-OFF responses.

Our results suggest a hierarchy of several photoreceptive streams that converge on SCN neurons (Fig. 6). A first stage of convergence combines inner retinal melanopsin ipRGCs with inputs from outer retinal cone and rod photoreceptors. Outer retinal channels carry photic information linked to rapid changes in irradiance in either photopic (cones) or scotopic (rods) domains, in relation to the spectral tuning of the involved opsins. Melanopsin ipRGCs also show heterogeneity in their responses to light (Tu et al., 2005) that appear to come together in individual SCN neurons. Several response properties still remain uncharacterized, including the response from SW cones, the contribution from rods, possible inputs from non-melanopsin ipRGCs and the role of melanopsin bistabilty (Mure et al,, 2007). Elucidating these response properties may require devising novel strategies of light stimulation. The convergence of these photoreceptive streams, differentially encoding irradiance, wavelength and duration may serve

several functions including extension of the irradiance operating range of the photic system, adaptation to temporal changes in light and coding of dawn and dusk according to the direction of change in light quality and the adaptive state of the retina.

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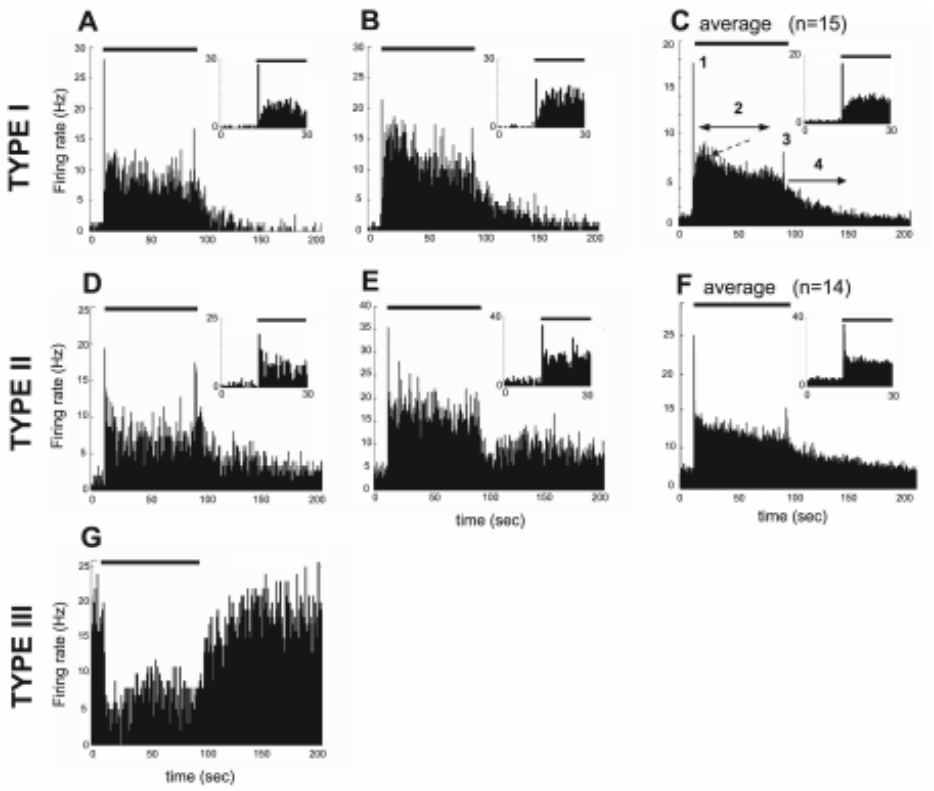

Figure 2. Single unit response of SCN neurons to light in dark adapted (**DA**) condition. After 30 minutes of dark adaptation the animal is exposed to a 90 sec duration pulse (black bar) of monochromatic light at 500 nm ($2x10^{14}$ photons/cm^2/s). The inset in each panel depicts the first 30 seconds of the recording (bin size = 0.1 sec). Three types of light responses were found: light-excited neurons with a slow-transient component (**type I, A-C**) or without a slow-transient component (**type II**, **D-F**) and light inhibited neurons **(type III, G)**.

The response to light includes several temporally distinct components **(**shown in **C)**: fast-transient ON response (1), sustained response (2), transient OFF (3), response, post-stimulus persistence (4). The slow transient component is indicated by the gray dashed arrow. Individual **(A-B)** and average (**C,** n=15) recordings of type I neurons showing the initial transient-ON response followed by a slow-transient component that reaches the maximum firing rate within about 10 seconds (arrow). Recordings of individual type II neurons **(D-E)** that lack the slow-transient. Note the presence in these neurons of a transient-OFF response that is excitatory **(D)** or inhibitory **(E)**. Example of a light inhibited neuron **(G)**, estimated to represent 22% of the light responsive cells in the SCN.

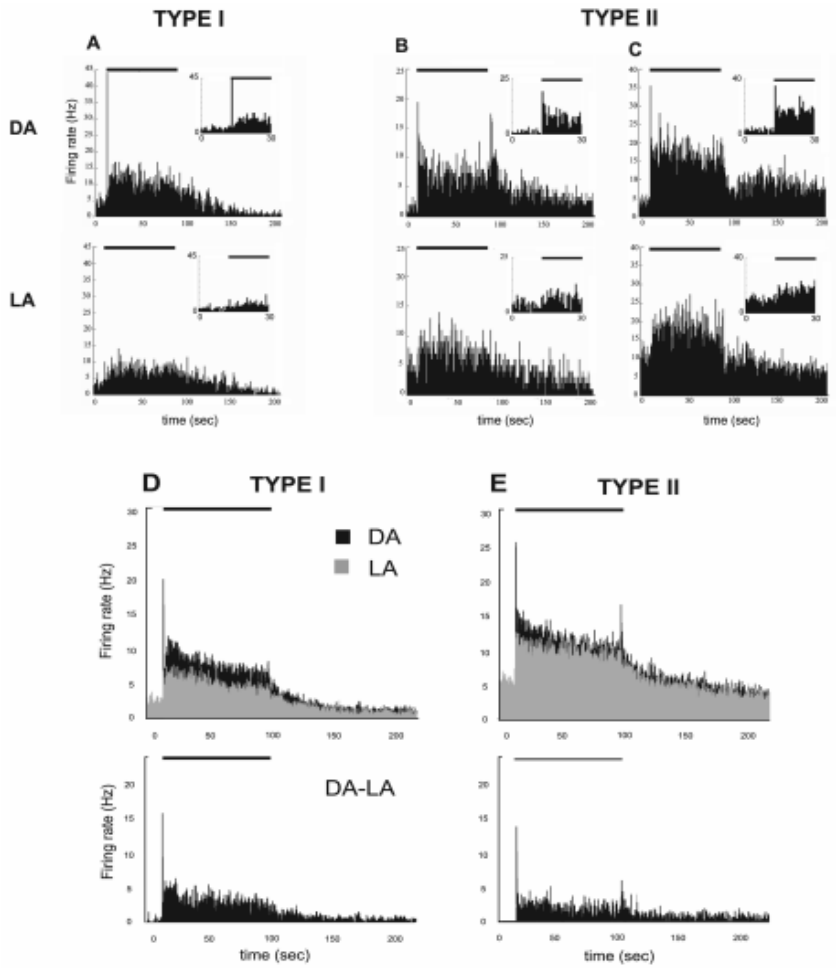

Figure 3. Single unit light responses of individual SCN neurons in the dark adapted (**DA**) and the light adapted (**LA**) conditions. For light adaptation, the animal was exposed to 1 min bright white light (56 W/m^2/sec) followed by 90 sec stimulation (black bar) of monochromatic light at 500 nm ($2x10^{14}$ photons/cm^2/s). Insets represent the first 30 seconds of the recordings (bin size = 0.1 sec). **(A)** Example of a type I neuron that exhibits an initial slow-transient component associated with the sustained response in DA. **(B-C)** Examples of two type II neurons that do not exhibit an initial slow-transient component in DA. Note that for both types of neurons, light adaptation dramatically affects the fast-transient ON and OFF responses to light.The averaged responses of type I **(D)** and II **(E)** neurons are shown the upper panels with average LA response (grey), superimposed on the average DA response (black). The lower panels in **D** and **E** show the subtracted averaged LA from DA responses to light for both types of neurons, revealing the response pattern of the light-attenuated components. The transient-ON and -OFF responses are clearly visible, indicating that these components are most affected by prior light exposure. This response pattern is typical of rod-cone responses. A part of the sustained response and of the post stimulus persistence of the response is also affected by the light pre-adaptation.

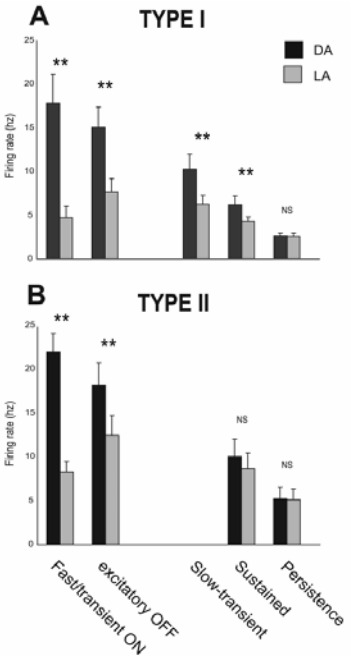

Figure 4. Effect of light adaptation on the firing rate of different components of the response in type I **(A)** and type II **(B)** neurons shown for DA and LA conditions. The transient-ON and transient-OFF responses are the most affected by light adaptation (*OFF responses, n=10, pooled for statistical analysis*). In type I neurons, both the slow-transient and sustained responses are significantly decreased by light adaptation, whereas in type II neurons the sustained response remains unchanged. The post-stimulus persistence of the response is unaffected by prior light adaptation. Recall that since type II neurons do not exhibit a slow-transient component this value is not shown in the histogram in **B**. Values are expressed as mean+SEM (n=27), ** p=<0.01 (Wilcoxon test).

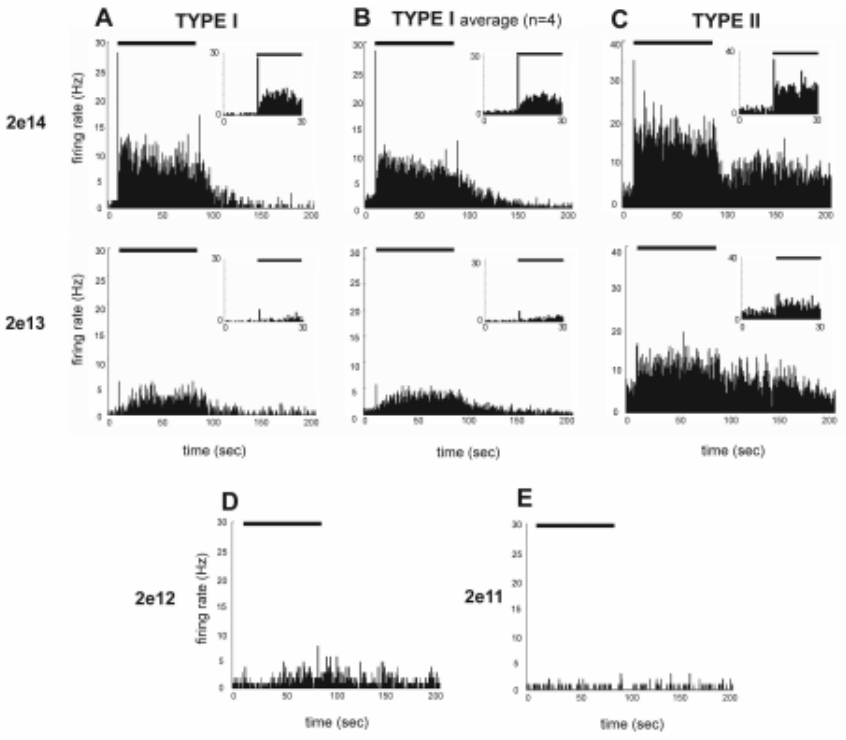

Figure 5. Single unit responses of SCN neurons at different irradiances in the dark adapted condition. After 30 min of dark adaptation the animal is exposed to 500 nm monochromatic light at irradiances from $2x10^{11}$ to $2x10^{14}$ photons/cm^2/sec during 90 seconds (black bar). Example of a single type I neuron **(A)** and averaged responses of type I neurons **(B)** at $2x10^{14}$ and $2x10^{13}$ photons/cm^2/sec. **(C)** A Type II neuronal response to light at $2x10^{14}$ and $2x10^{13}$ photons/cm^2/sec. Example of type I neuronal responses to lower irradiances at $2x10^{12}$ photons/cm^2/sec **(D)** and at $2x10^{11}$ photons/cm^2/sec **(E)** where no further light responses were detected.

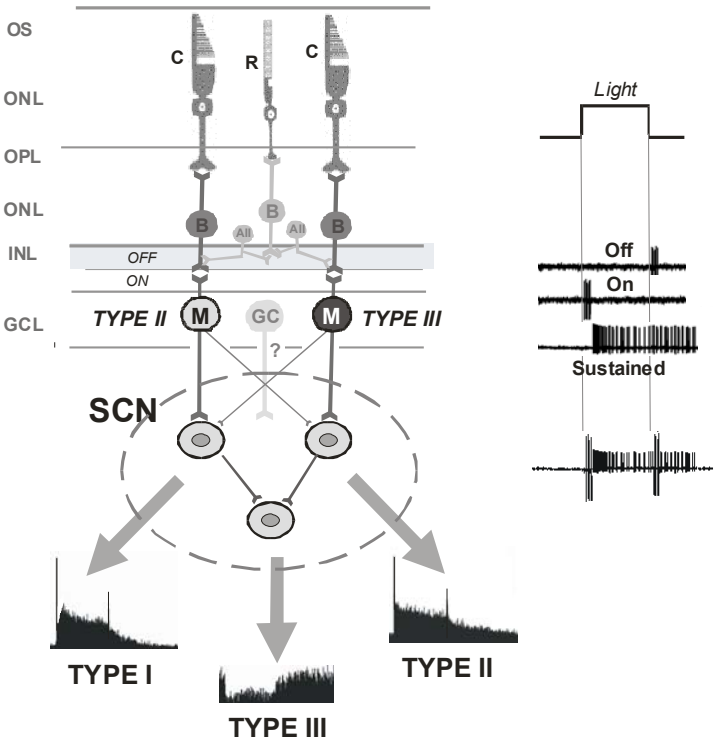

Figure 6. Schematic illustration of the retina and diversity of photic inputs to the SCN. Cones (C) and possibly (R) rods provide transient-ON and OFF responses to light that converge on melanopsin ipRGCs (M) and other ganglion cells (GC) via bipolar (CB, RB) and amacrine cell synapses (AII). Two known types of ipRGCs express sustained responses to light (type II and and type II ipRGCs; Tu et al., 2005). Inner and outer retinal photic inputs converge on the SCN to shape the photic response profiles of individual cells. Spike patterns to a light pulse at different levels are shown on the right side. All SCN neurons show both transient, sustained and post-stimulus persistence of the response. The individual response profiles of light-excitatory Type I and type II SCN neurons are derived from varied proportions of input from the different ipRGC types. It is unknown whether non melanopsin ganglion cells contribute to SCN responses. Light-inhibited neurons (type III) are likely post-synaptic to direct retino-recipient SCN cells since no this type inhibitory response is not observed in ipRGCs.