The Retina-Attached SCN Slice Preparation: An In Vitro Mammalian Circadian Visual System

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Abstract The suprachiasmatic nucleus (SCN), the mammalian circadian pacemaker, receives information about ambient light levels through the retinohypothalamic tract. This information resets the molecular clock of SCN neurons, thereby entraining overt animal behavior and physiology to the solar cycle. Progress toward functional characterization of retinal influences on the SCN has been hampered by limitations of established experimental paradigms. To overcome this hurdle, the authors have developed a novel in vitro preparation of the rat retinohypothalamic circuit that maintains functional connectivity between the retinas and the SCN. This method permits whole-cell patch-clamp recordings from visually identified, light-responsive SCN neurons. Using this preparation, the authors have found that in the SCN, light-evoked responses are partly driven by the melanopsin photosensory system of the intrinsically photosensitive retinal ganglion cells and that SCN neurons exhibit light adaptation. The authors have also been able to generate this preparation from mice, demonstrating the feasibility of applying this method to transgenic mice.

Key words photoentrainment, melanopsin, electrophysiology, rat, mouse, SCN, retina, brain slice

Neurons of the hypothalamic SCN exhibit intrinsic circadian rhythmicity and coordinate daily cycles in behavior and physiology (Antle and Silver, 2005). Photoentrainment is the process by which light synchronizes these circadian rhythms to the external light/dark cycle. This process requires ocular photoreception and signal transmission through the retinohypothalamic tract (RHT), a direct axonal projection from the eye to the SCN (Moore and Lenn, 1972; Nelson and Zucker, 1981). The RHT arises predominantly from intrinsically photosensitive retinal ganglion cells (ipRGCs), novel retinal photoreceptors that use the photopigment melanopsin (Gooley et al., 2001; Berson et al., 2002; Hattar et al., 2002). Rods and cones also influence the SCN, at least partly by way of polysynaptic links to ipRGCs (Hattar et al., 2003; Dacey et al., 2005; Lupi et al., 2006; Dkhissi-Benyahya et al., 2007; Wong et al., 2007).

Despite impressive progress in dissecting synaptic and cellular mechanisms of photoentrainment (Antle and Silver, 2005), the limitations of available experimental preparations have made it difficult to assess the relative contributions of rods, cones, and ipRGCs to SCN light responses and to determine which subregions and cell types of the SCN receive direct retinal input (Hattar et al., 2006, and discussion therein). Studies of SCN photoresponses have until now relied on extracellular recordings in vivo (Groos and Mason, 1980; Meijer et al., 1986; Aggelopoulos and Meissl, 2000). Such recordings cannot be readily directed to

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particular SCN subdivisions and do not permit study of synaptic currents or potentials. Furthermore, it is difficult to do well-controlled pharmacological manipulations of the SCN or retina in vivo, and the anesthesia typically used during such studies (but see Meijer et al., 1998) may alter neuronal activity.

Immunohistochemical methods for detecting expression of cFos, an immediate early gene, have proven very useful for assessing the location and neurochemistry of photoresponsive SCN cells (e.g., Schwartz et al., 1994; Karatsoreos et al., 2004). However, this approach has far less sensitivity and temporal resolution than electrophysiological measures. Furthermore, because it does not permit repeated observations in the same cells, it is less suitable for pharmacological studies of retinohypothalamic transmission.

Retinohypothalamic communication has also been studied in brain slices by shocking the optic chiasm and recording intracellularly the responses of SCN neurons. Although very informative about the intrinsic electrical properties of SCN neurons and the synaptic basis of retinohypothalamic transmission (Shibata et al., 1984; Cahill and Menaker, 1989; Jiang et al., 1997; Jiao and Rusak, 2003), this approach sheds little light on the nature of the retinal afferent signal. Also, the afferent volleys are synchronous and do not reproduce the natural temporal structure of the spike trains. Applied current could also spread from the chiasm to the SCN itself, where activation of intrinsic intranuclear circuits (Jiang et al., 1997) could distort the findings.

To overcome these technical obstacles, we have developed a new slice preparation of the SCN that has complete functional connectivity with both retinas and is responsive to light. We describe in detail how this preparation is generated and illustrate how it can be exploited to advance our knowledge of the properties of the circuits mediating photoentrainment of mammalian circadian rhythms.

MATERIALS AND METHODS

All procedures conformed to National Institutes of Health (NIH) guidelines for work with laboratory animals and were approved by the Institutional Animal Care and Use Committee at Brown University.

Subjects

Sprague-Dawley rats 22 to 27 days of age and Swiss Webster mice about 2 months of age were housed in a 12-hour light:dark cycle with free access to food and water. Most experiments were conducted during the dark phase, when SCN cells are more sensitive to light (Meijer et al., 1998), although a few were performed during the light phase. Light-evoked responses were detected in SCN neurons in both cases.

Retina-Attached SCN Slice Preparation

The entire procedure was performed under white room light or bright red light and took about 45 min. Animals were euthanized with carbon dioxide. The scalp was cut away with scissors and the lower jaw removed by making a horizontal cut with a razor blade just below eye level ("1" in Fig. 1A). Most of the nasal bones and some of the frontal bones were cut away with a razor blade using 2 vertical cuts ("2" and "3" in Fig. 1A). A pair of scissors was then used to cut along the base of the calvarium bilaterally ("4" in Fig. 1A), and the calvarium was peeled back and removed with a pair of forceps. The head was removed from the body with a razor blade ("5" in Fig. 1A) and submerged in an ice-cold bath of bicarbonate-based Ames's medium constantly bubbled with 95% O_2 5% CO_2. The cornea and lens of both eyes were removed through hemisection to improve the access of the retina to the oxygenated bath. The brain and eyes were isolated as a unit by carefully cutting away all remaining surrounding bones and connective tissue with microscissors. To maximize retinal access to the bathing solution, most sclera, choroids, and pigment epithelium were removed with forceps and microscissors. The optic nerves were bent backward and the retinas placed on the base of the brain to expose the frontal lobe. The brain and connected eyecups were lifted out of solution and the brain mounted, dorsal side down, on a piece of filter paper. The frontal lobe was blocked in the coronal plane about 2 mm rostral to the chiasm with a razor blade. The blocked surface of the brain was mounted with cyanoacrylate glue onto the stage of a vibrating microtome (Leica VT-1000S vibratome; Bannockburn, IL) with the ventral side of the brain and connected retinas facing away from the blade (Valet brand, World Precision Instruments, Sarasota, FL). The preparation was then covered with ice-cold bubbled Ames's medium in a slicing chamber, and coronal slices were made initially 1 mm thick and gradually reduced in thickness to 50 μm until the caudal limit of the SCN was reached, as monitored through a dissecting scope. A final slice 900 μm in thickness for rats and 700 μm for mice was made while using a paintbrush to gently lift the optic nerves clear of the approaching blade. These proved to be the minimum thicknesses

at which the connections between the optic nerves and the SCN could be kept largely intact. The resulting preparation (Fig. 1B) was then transferred to a custom-made recording chamber and the brain slice anchored by a weighted nylon mesh. Ames's medium gassed with 95% O_2 5% CO_2 and maintained at 32 °C with a temperature controller (ATR, Quest Scientific, Canada) was gravity fed into the recording chamber at 4 mL min^{-1}. To permit selective pharmacological manipulation of the retinas, in some experiments, a ribbon of petroleum jelly (Vaseline) was applied over the optic nerves and extended to the margins of the chamber, dividing the chamber into 2 baths, one containing the retinas and the other containing the brain slice. The retina chamber had a volume of ~1 mL, whereas the SCN chamber had a volume of ~3 mL. Each compartment was equipped with its own perfusion line, which delivered Ames's medium as above. Drug application to the retina was carried out by addition of agents to the superfusate. Inlet and outlet ports for the retinal compartment directed flow away from the slice chamber.

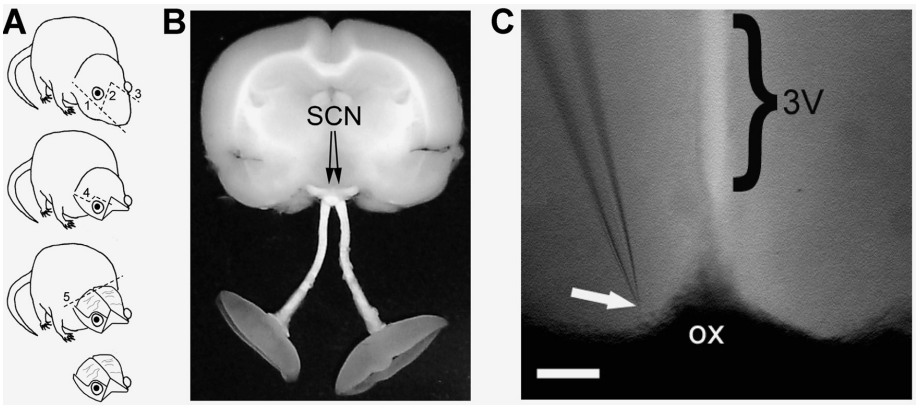

Figure 1. Generation of the retina-attached SCN slice preparation. (A) Sequential steps (numbered) in the dissection. See text for the details. (B) Low-power photomicrograph of the completed preparation. Intact isolated retinas are apparent at the bottom of the image. (C) The position of a recorded cell (*arrow*) relative to the optic chiasm. 3V, third ventricle; OX, optic chiasm. Scale bar = 200 μm.

Electrophysiology and Light Stimulation

The recording chamber was mounted on a fixed-stage upright microscope (Nikon E600FN; Melville, NY) and left in constant darkness for 40 to 60 min before initiating recordings to permit the tissue to dark adapt and to equilibrate to physiological temperature. Thereafter, darkness was maintained between occasional light stimuli. The ventral SCN was viewed with infrared transillumination through a 40× water immersion lens. Neurons roughly 2 to 4 cell layers beneath the surface of the slice and within about 100 μm of the chiasm were targeted for recording using standard whole-cell patch-clamp recording techniques (Fig. 1C). Pipettes were pulled from thick-walled borosilicate tubing (tip resistances 4-7 MΩ) on a Flaming/Brown P-97 puller (Sutter Instruments, Novato, CA). Recordings were made with a Multiclamp 700A amplifier (Molecular Devices, Sunnyvale, CA). Electrode capacitance and series resistance were partially compensated, and the remaining series resistance was typically around 15 to 25 MΩ. pCLAMP 9 software (Molecular Devices) was used for data acquisition. Signals were low-pass filtered (200 Hz to 2.4 kHz), and the sampling frequency was at least 4 times higher than the low-pass filter cutoff. Liquid junction potentials (14 mV for K^+-based and 10 mV for the Cs^+-based internal solutions; see below) were corrected for all recordings.

The light stimuli were 2 blue-green light-emitting diodes (Nichia Corp., #NSPE590S, Japan), positioned about 1 to 2 cm from the retinas. Their emission spectrum, which peaked near 505 nm and had a width at half height of about 40 nm, overlapped significantly with the absorption spectrum for melanopsin in ipRGCs (Berson et al., 2002). Light stimuli were of high irradiance (1.7 to 4.1 × 10^{16} quanta cm^{-2} sec^{-1}) so as to maximally activate as many ipRGCs as possible. Stimulus timing was controlled through a Digidata 1322A (Molecular Devices) using pCLAMP 9 software. Relatively long interstimulus intervals (5-8 min) were used to permit full poststimulus recovery of membrane potential, which is extremely sluggish in ipRGCs (Berson et al., 2002).

Microcal Origin 6 software (OriginLab Corporation, Northampton, MA) was used for statistical calculations and for generating data plots.

Bathing and Intracellular Solutions

Intracellular solutions contained (in mM) 120 K-gluconate or Cs-methanesulfonate, 5 NaCl, 4 KCl or

CsCl, 10 HEPES, 2 EGTA, 4 Mg-ATP, 0.3 Na-GTP, and 7 $Tris_2$-phosphocreatine and were adjusted to pH 7.3 with KOH or CsOH. The calculated Nernst potential for chloride is around –67 mV. The potassium-based solution was usually used in current-clamp experiments, whereas the cesium-based solution was usually used in voltage-clamp experiments to improve space clamp by blocking potassium channels. Chemicals were dissolved in distilled water to generate stock solutions. L-AP4 and DNQX were purchased from Tocris (Ellisville, MO). All other chemicals were purchased from Sigma (St. Louis, MO).

RESULTS

The Rat Retina-Attached SCN Slice Preparation Is Technically Feasible

We successfully generated a functionally viable retina-attached SCN slice preparation from rats. During development of the method, it quickly became apparent that optimizing the age of the animals would be crucial. In animals younger than 3 weeks of age, the RHT was very fragile and vulnerable to interruption by detachment of the chiasm from the hypothalamus and of the retinas from the optic nerves. In rats more than 4 weeks old, the increased thickness and hardness of the bone impeded the timely isolation of the brain and attached retinas. We obtained optimal results at intermediate ages (22-27 days old). Circadian rhythmicity, gene expression, firing rate of individual neurons, and light responsiveness in the rat SCN are fully mature by postnatal day 21 (Fuchs and Moore, 1980; Shibata et al., 1983; Shibata and Moore, 1987; Isobe et al., 1995; Ban et al., 1997; Lundkvist et al., 1999).

With practice, about 3 out of every 4 attempts resulted in preparations that were without obvious physical damage to the retinohypothalamic connections. When visualized with infrared transillumination under a 40× water immersion objective lens, cells in the top 4 layers of the slice were clearly visible, and the great majority of these cells appeared spherical and smooth, with no signs of vacuolization. When these cells were targeted for recording, seals exceeding 4 GΩ could be readily achieved. Resting potentials ranged from –55 to –70 mV and input resistances from 500 MΩ to 2 GΩ. All cells spontaneously generated action potentials > 50 mV in amplitude. We cannot attest to the health of cells deeper in the slice because these could not be visualized clearly or readily patched.

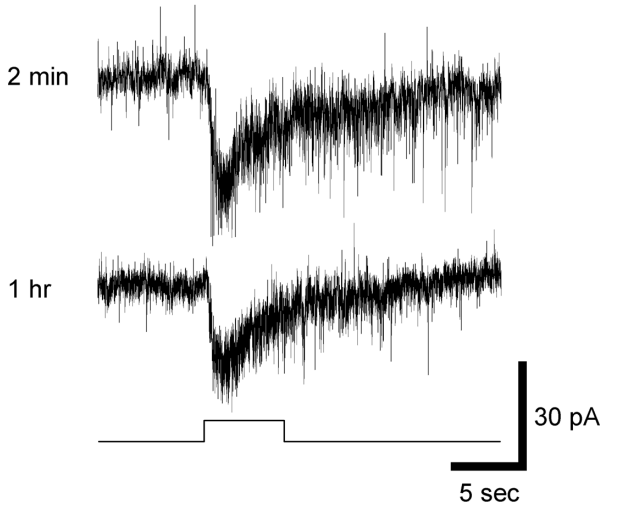

Figure 2. Robust light-evoked responses could be obtained from individual rat SCN cells for at least an hour. Shown here are 2 light-evoked responses recorded from the same cell, first about 2 min after whole-cell recording was established (*top trace*) and then about 1 h later (*bottom trace*). Holding potential = –40 mV.

Each preparation remained healthy as reflected by cellular morphology and electrical behavior for at least 6 h. In all experiments presented in this communication, we recorded exclusively from neurons in the ventral SCN (see Materials and Methods), a region of the SCN known to receive dense afferents from the retinas (Moore and Lenn, 1972; Shibata et al., 1984; Johnson et al., 1988; Schwartz et al., 1994; Morin et al., 2006; Hattar et al., 2006). We typically recorded from 5 cells in each preparation, and typically 3 of those were repeatably responsive to light. Stable recordings could be made for at least an hour in most cells (Fig. 2). Thus, it is possible to generate and maintain a functionally viable retinohypothalamic circuit in a reduced, in vitro preparation.

To further confirm that the functional properties of the retinohypothalamic connections are well preserved in this preparation, we investigated the ionic bases of the light-evoked responses of ventral SCN neurons. We performed whole-cell patch recordings on a total of 24 cells and encountered 3 types of responses to a 5-sec light flash. In the most common type of response (17 of 24; 71%), the evoked current was inward when the cell was held at negative membrane potentials and outward at positive holding potentials ($n = 9$). Light thus appears to trigger an increase in cationic conductance in these cells (Fig. 3A, *top*). Under current clamp, light evoked a depolarization and an increase in spike rate, confirming that light excites these cells ($n = 4$; Fig. 3A, *bottom*).

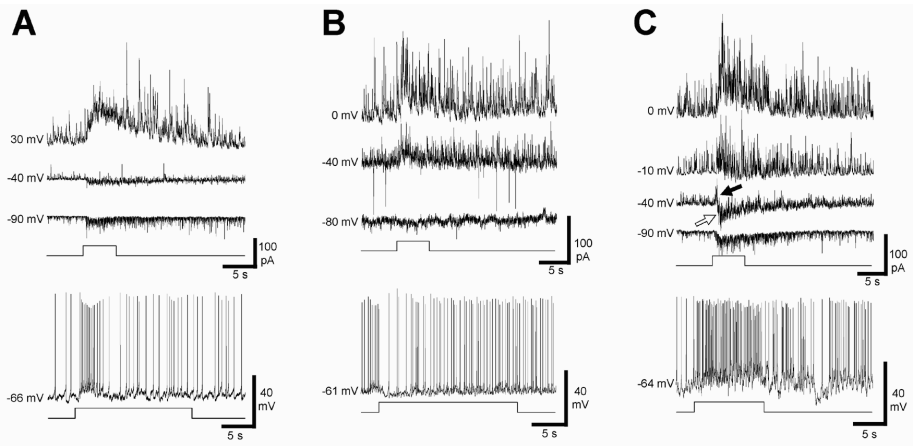

Figure 3. Three types of light-evoked responses of rat SCN neurons recorded by whole-cell patch method in the eye-attached SCN slice preparation, as illustrated in these responses from 6 different cells. Upper 3 to 4 traces in each panel are voltage-clamp recordings at several holding potentials, and the lower trace is current-clamp recording. (A) Typical predominantly excitatory response. Light, indicated by a step below physiological traces, triggered an increase in cationic conductance (*top*), leading to depolarization and an increase in spike rate (*bottom*). (B) Rarer, predominantly inhibitory response. Light triggered an apparent chloride conductance (*top*), which caused hyperpolarization and a reduction in spiking in current clamp (*bottom*). (C) Very rare mixed excitatory (cationic; *white arrow*) and inhibitory (chloride; *black arrow*) conductances, with the inhibition preceding the excitation (*top*). Under current clamp at the resting potential, the excitatory component appeared to dominate (*bottom*).

The second most common type of light response (5 of 24 cells; 21%) consisted of an outward current at –40 mV, which became larger at more positive holding potentials but smaller or absent when the membrane was held near the reversal potential for chloride ($n = 3$; Fig. 3B, *top*). Under current clamp, light hyperpolarized these cells 5 to 10 mV from their resting potentials (approximately –60 mV) and reduced their spike rates, confirming that light has a net inhibitory effect on these cells ($n = 2$; Fig. 3B, *bottom*).

The third type of light response was rare (2 of 24 cells; 8%) and was a hybrid of the first 2 response types. When voltage-clamped at –40 mV, these cells first generated a brief outward current (Fig. 3C, *top, black arrow*), followed by a longer lasting inward current (Fig. 3C, *top, white arrow*). The early transient component reversed near the chloride reversal potential, whereas the later sustained component reversed near 0 mV (Fig. 3C, *top*), implying that they are mediated by chloride and cations, respectively. Under current clamp at the resting potential, the cationic component appeared to dominate because light evoked a purely depolarizing response ($n = 1$; Fig. 3C, *bottom*).

Two Simple Applications of the Preparation

We next performed 2 relatively simple experiments using this preparation to illustrate its scientific utility.

Evidence for Melanopsin Contribution to SCN Light Responses

Behavioral evidence indicates that both classical (rod and cone) and ganglion-cell photoreceptors contribute to non-image-forming visual responses in rodents, including those mediated by the SCN (Hattar et al., 2003; Panda et al., 2003). However, while prior in vivo recordings have implicated rods and cones in the generation of SCN light responses (Sawaki, 1979; Aggelopoulos and Meissl, 2000), direct electrophysiological evidence for melanopsin-driven responses has been lacking (but see Colwell and Foster, 1992, and Lupi et al., 2006, for cFos immunohistochemical evidence). Several observations imply that the light responses shown in Figures 2 and 3 were mediated primarily by melanopsin rather than by rods or cones. First, prolonged exposure of the isolated retinas to moderate to bright light during tissue preparation presumably severely disrupted rod phototransduction through the bleaching of rhodopsin, which requires the retinal pigment epithelium (RPE) for the regeneration of its retinaldehyde chromophore (Pepperberg et al., 1978; Dowling, 1987; Lamb and Pugh, 2004). By contrast, melanopsin-based phototransduction in ipRGCs is not dependent on the RPE and thus should have been largely unaffected (Berson et al., 2002). Second, the sluggish kinetics of the light responses were consistent with those of phototransduction in ipRGCs but not in rods and cones (Dowling, 1987; Berson et al., 2002). Latency to peak was typically > 1 sec, and responses persisted for many seconds after lights-off (Figs. 2-5), strikingly similar to the melanopsin-based light responses recorded directly from rat ipRGCs in response to relatively bright light (Berson et al., 2002; Wong et al., 2005).

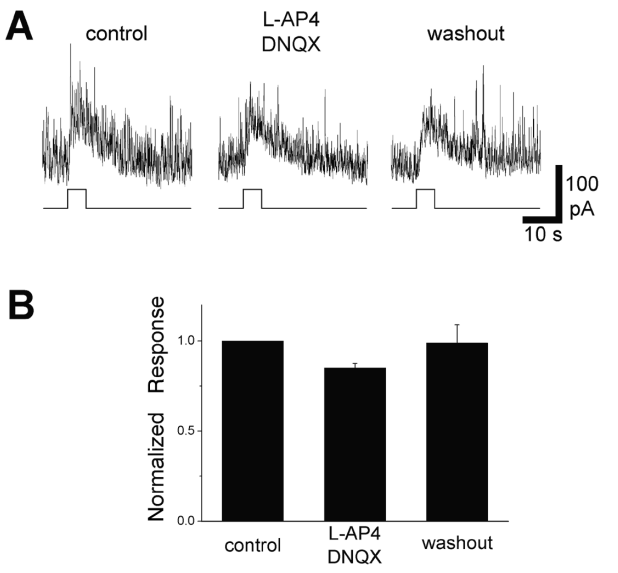

Figure 4. Evidence for contribution to rat SCN light responses from melanopsin-based phototransduction in ipRGCs. (A) Three voltage-clamp recordings from the same cell illustrating light-evoked currents obtained under control conditions (*left*), during retinal application of antagonists completely blocking intraretinal transmission of rod and cone signals (50 μM L-AP4, 20 μM DNQX; *middle*) and again after washout (*right*). Holding potential = 30 mV. (B) A summary of all 5 cells tested. The light response amplitude measured under control conditions is normalized to 1. Error bars = *SE*.

We further implicated melanopsin as the primary source of photic influence on the recorded SCN cells by showing that such influence persisted after blocking intraretinal rod and cone circuits pharmacologically (Fig. 4). Specifically, we bath applied L-AP4 (50 μM; L(+)-2-amino-4-phosphonobutyrate) to block rod and cone signals to ON bipolar cells and DNQX (20 μM; 6,7-dinitroquinoxaline2,3-dione) to block such signals to OFF bipolar cells (Slaughter and Miller, 1981; Hensley et al., 1993; $n = 5$). These drugs completely abolished the electroretinogram b- and d-waves measured using eyecups in a separate experiment ($n = 3$; data not shown), confirming that they effectively prevented all rod/cone-driven light signals from propagating beyond the first synapse in the retina. The amplitude of light-activated synaptic currents was only attenuated slightly (Fig. 4), confirming that melanopsin generated the great majority of these responses. Nevertheless, the slight reduction (15%) in amplitude was statistically significant (paired t test p value = 0.005), which could reflect a small input from the retinal cone photoreceptors. Cones have been shown to remain light responsive in the absence of the RPE (Hood and Hock, 1973; Mata et al., 2002; Trevino et al., 2005) and thus might be functional in our preparation.

Evidence for Light Adaptation in SCN Cells

In the second set of experiments, we explored whether ventral SCN cells have the ability to adapt to lighting conditions. Earlier work demonstrated a lack of light adaptation in the photoentrainment pathway when evaluated behaviorally (Nelson and Takahashi, 1999), but rods and cones clearly exhibit such adaptation (Dowling, 1987; Perlman and Normann, 1998), and we recently showed that melanopsin-driven responses in ipRGCs do so as well (Wong et al., 2005). In addition, in vivo extracellular recordings show that many (but not all) SCN neurons display phasic light responses, consistent with their being adaptive to light stimulation (Groos and Mason, 1980; Meijer et al., 1992, 1998; Aggelopoulos and Meissl, 2000).

We found 2 lines of evidence that SCN cells do adjust their response to light according to the recent history of photic stimulation. First, their responses during steady illumination nearly always decayed substantially from the peak response within a few seconds of stimulus onset, whether measured under current clamp ($n = 5$; Fig. 3A,B, *bottom traces*) or voltage clamp ($n = 4$; Figs. 2 and 5A). Second, when probed with a series of shorter flashes, response amplitudes were largest when the cell had spent at least several minutes in continuous darkness, whereas they could be attenuated by as much as 60% from their control values by recent prior stimulation. Similar results were obtained in all 5 cells tested in this manner, of which 4 were excited by light and 1 was inhibited by light (Fig. 5B). As illustrated in the summary diagram in Figure 5B (*bottom*), the response amplitude increases as the time spent in darkness after the previous flash increases, indicating recovery from previous light adaptation (i.e., "dark adaptation"). In conclusion, the gain of the photoresponse of cells in the ventral SCN is reduced by prior light exposure, much as it is in ipRGCs and in rods and cones (Dowling, 1987; Perlman and Normann, 1998).

Applying the Preparation to Mice

A wide variety of transgenic mouse lines with relevance to circadian rhythm research are currently available (e.g., Wilsbacher et al., 2002; Hattar et al., 2003; Karatsoreos et al., 2004), and an ability to apply the

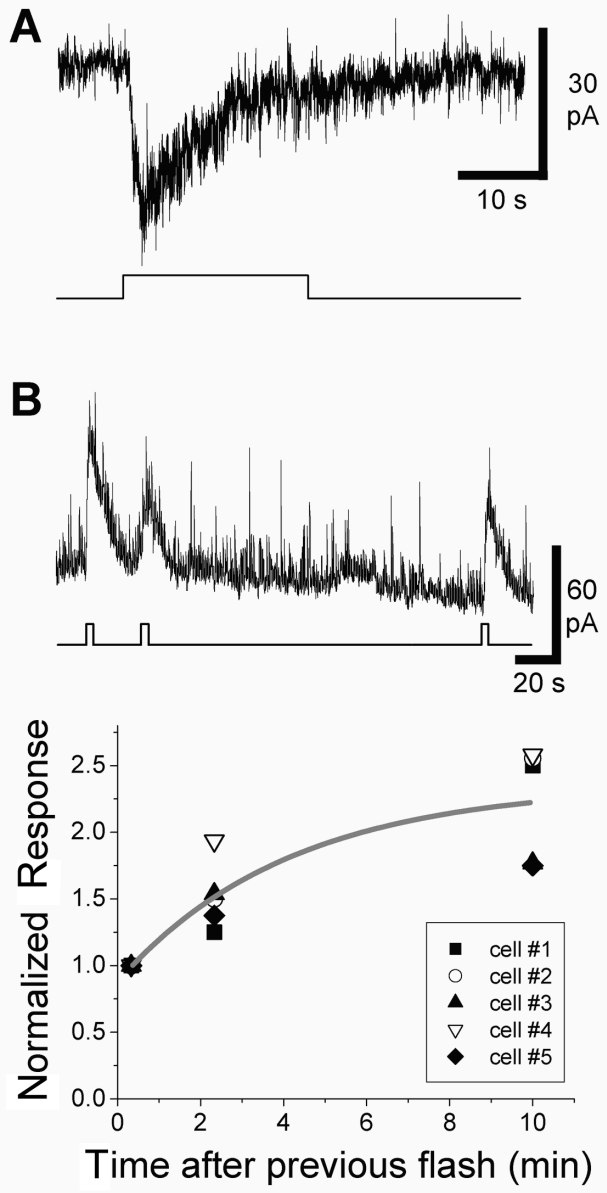

Figure 5. Evidence for the ability of rat SCN neurons to adapt to light. (A) Photoresponse of this SCN neuron peaked within several seconds, then gradually decayed during the prolonged (20-sec) stimulus, indicating adaptation to the light pulse. Holding potential = –40 mV. (B) *Top:* **Voltage-clamp recording from another cell showing prior light exposure desensitizes SCN neurons. Of 3 identical light pulses presented to this neuron, the first, applied after 10 min of dark adaptation, produced the largest current response. The second, which followed on the heels of the first (20-sec interstimulus interval [i.s.i.]) evoked a smaller response. Response amplitude recovered somewhat at longer i.s.i. (140 sec). Holding potential = 30 mV.** *Bottom:* **A summary plot of the data from all 5 cells tested, showing response amplitude as a function of time spent in darkness after the previous flash. The amplitude of the response measured 20 sec after the previous flash (i.e., the response to the second light pulse in the raw data trace above) is normalized to 1. The gray line is a first-order exponential decay fit to the data, with a time constant of 4.2 min. Cell 4 corresponds to the response shown in the trace above.**

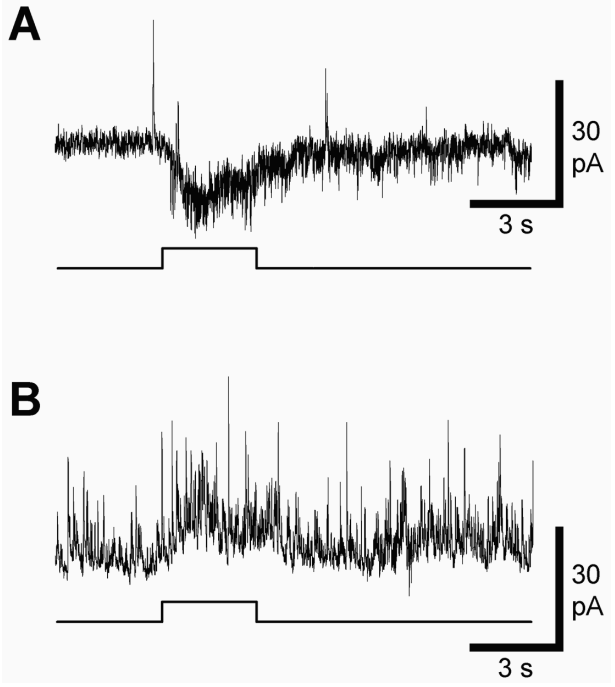

Figure 6. Light-evoked responses from mouse SCN neurons. (A) An excitatory light response. (B) An inhibitory light response from another cell. The holding potential was –40 mV for both cells.

retina-attached SCN preparation to these mice would obviously be advantageous. We have found it possible to generate functionally viable preparations from young adult mice about 2 months of age. While the smaller size of the brain makes the procedure technically more challenging, the percentage of attempted preparations that yielded light-responsive cells remained fairly high, about 50%. This percentage will likely improve with further practice. Two examples of light-evoked responses recorded from mouse SCN neurons are shown in Figure 6.

DISCUSSION

We have developed a novel in vitro preparation of the complete retinohypothalamic circuit, from retinal photoreceptors to target neurons in the SCN. The preparation combines the strengths of existing in vitro and in vivo methods for studying this circuit. It shares with established SCN slice methods the opportunity for straightforward pharmacological manipulation, the mechanical stability required for stable intracellular recording and dye filling, and the ability to direct

recordings to visually identified neurons. As in other in vitro SCN preparations, afferents from the intergeniculate leaflet, as well as raphe and other nuclei (Moga and Moore, 1997; Morin and Allen, 2006), are severed during slicing, thus eliminating any photic and nonphotic influences they may mediate in vivo. However, this loss can be advantageous when the goal is to characterize direct retinohypothalamic influences. A particular benefit of the new method over conventional SCN slices is that retinal afferents can be activated by physiologically relevant light stimulation. In this, it more closely resembles methods for in vivo extracellular recordings of light-evoked activity in SCN neurons.

This novel preparation can be exploited and adapted to explore retinohypothalamic signaling in ways that are beyond the reach of established methods. For example, the receptive fields of individual SCN neurons can be mapped with focal photic stimulation of the retina. The preparation can be used to assess the relative contributions of rods, cones, and melanopsin to SCN light responses by generating it under infrared illumination to preserve rod and cone visual pigments and subjecting it to appropriate pharmacological manipulations. It should be possible to adopt calcium imaging techniques already developed for SCN slices (van den Pol et al., 1992; Dziema and Obrietan, 2002; Ikeda et al., 2003) to monitor the light responses of many SCN cells simultaneously. This would be an efficient means to compare SCN subregions for possible differences in the kinetics or circadian phase dependence of light responses. Retinal influences can be studied for subtypes of SCN neurons targeted for recording on the basis of their transgenic expression of fluorescent proteins (Karatsoreos et al., 2004). Finally, used in animals with fluorescent reporters of clock gene expression (Yamazaki et al., 2000; Wilsbacher et al., 2002), the preparation could be used to monitor in real time the effect of retinal illumination on the molecular clock.

Our data are in broad agreement with earlier studies of the polarity of responses evoked in SCN neurons by light or by electrical activation of optic afferents. In 70% of the cells we recorded, light triggered an excitatory cationic conductance. In another 20%, the dominant response was an inhibitory increase in chloride conductance. The remaining 10% had biphasic responses in which light evoked a fast inhibitory current followed by a delayed excitatory current, although excitation appeared to dominate under current clamp. Both in vivo extracellular recordings (Sawaki, 1979; Groos and Mason, 1980; Meijer et al., 1986) and in vitro intracellular SCN slice recordings (Shibata et al., 1984; Cahill and Menaker, 1989; Jiang et al., 1997; Jiao and Rusak, 2003) similarly report the presence of both excitatory and inhibitory effects of retinal afferents on SCN neurons as well as the dominance of excitatory effects. Biphasic effects have been seen in the in vitro studies but not in vivo, presumably because inhibition cannot be detected directly in extracellular spike recordings.

The present findings provide the most direct evidence to date that melanopsin makes a significant contribution to light-evoked responses in the SCN. Surprisingly, this question has never been tested directly in electrophysiological studies, although such an influence has been widely assumed on the basis of behavioral data cited below. Earlier in vivo studies of SCN light-evoked responses (Groos and Mason, 1980; Aggelopoulos and Meissl, 2000) implied a dominant input from rods and/or cones. Those studies predated the discovery of ipRGCs, and reexamination of those data in the context of present knowledge reveals no obvious influence of melanopsin. For example, the kinetics of the light responses at both light onset and offset were much faster than typical for melanopsin-dependent intrinsic photoresponses in ipRGCs (Groos and Mason, 1980; Aggelopoulos and Meissl, 2000). Furthermore, the action spectra for these SCN responses showed sensitivity peaks at around 375 nm and 505 nm (Aggelopoulos and Meissl, 2000), consistent with the spectra for ultraviolet-sensitive cones and for rods and/or mid-wavelength cones but not with that for melanopsin (peak sensitivity ~480 nm; Berson et al., 2002; Panda et al., 2005; Qiu et al., 2005). Finally, the SCN photoresponses in the earlier studies were so sensitive as to be almost certainly mediated by rods (Sawaki, 1979; Aggelopoulos and Meissl, 2000), whereas melanopsin-based ipRGC photoresponses have a much higher threshold (Berson et al., 2002).

The absence of overt melanopsin contributions to the light responses in these earlier reports is surprising. Anatomical and electrophysiological evidence indicates that the great majority of ganglion cells innervating the SCN express melanopsin and are intrinsically photosensitive (Gooley et al., 2001; Berson et al., 2002; Morin et al., 2003; Sollars et al., 2003; Hattar et al., 2006). In addition, mice lacking rods and cones show robust photoentrainment responses (Freedman et al., 1999; Panda et al., 2003) and exhibit light-induced cFos expression in the SCN (Colwell and Foster, 1992). Finally, the action spectrum for circadian photoentrainment of rodless-coneless mice peaks at 480 nm, closely

matching the spectral behavior of melanopsin in the ipRGCs (Yoshimura and Ebihara, 1996; Berson et al., 2002; Hattar et al., 2003).

The preparation described here enabled us to provide direct electrophysiological evidence that melanopsin-based phototransduction can activate SCN neurons. Robust light-evoked responses could be measured from SCN neurons even when rods and cones were extensively bleached by bright light exposure during the dissection and when intraretinal rod/cone signaling was blocked pharmacologically. The SCN light responses recorded under these conditions resembled the melanopsin-based intrinsic light responses of ipRGCs, including a relatively long latency to response peak (typically > 1 sec) and a response that outlasted the stimulus by many seconds (Berson et al., 2002; Warren et al., 2003; Tu et al., 2005). Except for some inhibitory responses (see Groos and Mason, 1980; Meijer et al., 1986, 1998), such kinetics have not been seen previously, probably because the relatively dim and/or brief light flashes used failed to drive sufficient melanopsin-based transduction. Conversely, even without pharmacological blockade, we never observed the brisk onset and termination of light responses reported in vivo, presumably because rod and cone function was compromised by photobleaching during the dissection and/or because the slicing disrupted the geniculohypothalamic tract and other polysynaptic circuits by which light might influence the SCN (Moga and Moore, 1997; Morin and Allen, 2006). Taken together, the electrophysiological evidence now closely parallels the behavioral evidence in indicating that both classical photoreceptors and melanopsin-based ganglion-cell photoreceptors contribute to the photic influences on the circadian pacemaker.

We have also exploited the new preparation to provide preliminary evidence for light adaptation in SCN neurons. There has been conflicting evidence on whether such adaptation occurs. We had previously shown that melanopsin-driven light responses in the ipRGCs can adapt to light, just like the rod and cone photoreceptors (Wong et al., 2005). Furthermore, others had shown that all SCN cells exhibit spike accommodation to sustained current injection, which should further allow these cells to adapt to steady retinal illumination (Kim and Dudek, 1993). Indeed, in several in vivo studies, many (but not all) SCN cells were found to generate transient light responses, suggestive of adaptation (Groos and Mason, 1980; Meijer et al., 1992, 1998; Aggelopoulos and Meissl, 2000). But surprisingly, behavioral studies had found a lack of light adaptation in the photoentrainment pathway (Nelson and Takahashi, 1999). In the present study, we found that SCN neurons are desensitized by exposure to light and that this desensitization persisted for at least several minutes after lights-off. These phenomena are hallmarks of light adaptation. We also observed that SCN neurons gradually regain sensitivity after prior light exposure, indicative of dark adaptation. Thus, we have obtained further proof that the ability of ipRGCs to adapt to light is propagated to at least the ventral SCN, and the lack of obvious adaptation at the behavioral level must occur downstream of the initial retinohypothalamic synapse. This conclusion appears consonant with the dissociation observed by Meijer et al. (1992) between the effects of light on SCN firing rates and on behavioral phase shifting.

To our knowledge, this is the first report of light-evoked responses recorded from the mammalian brain in vitro. It seems likely that other retinal targets could be studied by variations on the preparation described here. Even in its present form, the preparation should make it possible to probe other hypothalamic targets of retinal input such as the supraoptic nucleus (Hattar et al., 2006). Other favorable targets for study would be visual nuclei fairly near the brain surface, such as the accessory optic system and pretectal nuclei. With appropriate modification of the level and angle of slicing, it may even be possible to apply this approach to the lateral geniculate nucleus or superior colliculus.

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