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Photically responsive neurons in the hypothalamus of a diurnal ground squirrel

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The suprachiasmatic nuclei (SCN) function as a circadian pacemaker. Entrainment to the external light–dark cycle is mediated by the retina which gives rise to both direct and indirect projections to the SCN. The hypothalamic targets of the retinohypothalamic tract (RHT) were investigated in thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*) following WGA–HRP injections. The results indicate that retinal fibers project to the entire SCN. Extracellular single-unit activity was recorded in and near the SCN of thiopental sodium anesthetized squirrels while the eyes were photically stimulated. A small population of hypothalamic cells were responsive to retinal illumination. About half of these cells were activated by light while the others were light-suppressed. The majority of these cells responded in a sustained way to light pulses. Light intensities of at least 1000 lux appeared necessary to induce a sustained response to light. No differences in light responsiveness were observed between visual cells inside and outside the SCN. The visual properties of SCN cells have previously been investigated in hamsters and rats, both nocturnal species. Hypothalamic cells in all 3 species were similar in that they showed predominantly sustained responses to retinal illumination. The diurnal squirrel differed from the other two species in that there was a higher proportion of photically suppressed cells in the squirrel, and in that higher light intensities were required to stimulate photically responsive neurons.

INTRODUCTION

The suprachiasmatic nuclei (SCN) of the hypothalamus serve as a major pacemaker for daily (circadian) rhythms in many mammalian species, including both nocturnal and diurnal rodents[37,38]. Circadian rhythms are entrained to the environmental light–dark cycle via the retina[45]. From the retina, the retinohypothalamic tract projects directly to the SCN[13,23,24]. An indirect photic afferent arises from the geniculate nucleus and intergeniculate leaflet[5,12,27,35,43]. The visual response characteristics of SCN cells have been studied in the albino rat[7–9,16,26,39,40], hamster[21] and cat[8,9]. Visual SCN cells have large receptive fields; most cells show a sustained increase, while some show a tonic decrease of their discharge rate in response to sustained retinal illumination[8,9,11,21] or in response to electrical stimulation of the optic nerve[39,40]. Since the discharge rate is a monotonic function of light intensity,these cells can code for luminance[8,21].

The light responsiveness of the circadian system of the diurnal squirrel differs in several ways from the responsiveness of nocturnal rats and hamsters. The circadian rhythm of the golden hamster can be phase shifted 1 or 2 h by light pulses of several minutes[44]. In diurnal squirrels, much longer light pulses are required to induce a similar shift[32]. Another difference between these species is their response to constant illumination. The free-running period of most diurnal squirrels (*Eutamias sibiricus, Ammospermophilus leucorus, Tamiasciurus hudsonicus*) decreases[19] or does not vary systematically[32] with elevated light intensities. The free-running period of nocturnal rodents gets longer with higher intensities[2,6,29]. Splitting of unitary circadian rhythms into two components is observed when diurnal rodents are exposed to constant dim illumination while

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nocturnal rodents show splitting in constant bright illumination. The threshold light intensity that causes suppression of melatonin secretion is much higher in wild-caught squirrels than in laboratory-reared, nocturnal rodents[3,15,33,36]. Melatonin suppression is mediated by photic information reaching the SCN[18,25].

The several differences between nocturnal and diurnal rodents in their responsiveness to light suggest the possibility that the photic responsiveness of SCN neurons may differ in these groups. Thus far the electrophysiological properties of visual SCN cells have only been described for nocturnal rodents. It would therefore be worthwhile to extend these studies to diurnal rodents. In addition, we examined the retinal projections to the hypothalamus in the thirteen-lined ground squirrel (*Spermophilus tridecemlineatus*) to assess the degree to which retinal fibers reach the SCN and adjacent hypothalamic areas.

MATERIALS AND METHODS

Twenty-six male thirteen-lined ground squirrels weighing 208–305 g, were obtained from TLS Research (Chicago, IL). The animals had been trapped in the wild and were brought into the laboratory in November 1986. Upon arrival, they were immediately transferred to individual plastic-bottom cages with sawdust and shredded paper as bedding material. They had free access to Purina rat pellets and water supplemented occasionally with sunflower seeds. The room temperature was 23 °C. The squirrels were kept in an artificial photoperiod LD 12:12, with lights on at midnight (light intensity 30–100 lux or 8–12 $\mu W/cm^2$ inside the cage). The neurophysiological experiments were performed between the fourth and sixth month of captivity between 08.00 and 22.00 h.

To determine the stereotaxic coordinates of the SCN, 6 squirrels were sacrificed for mapping studies. Squirrels were anesthetized with 20% urethane anesthesia (0.65 ml initial dose and additional dosages of 0.15 ml every 10 min) or with sodium pentobarbital (12.5 mg/100 g). The animals were mounted in a stereotaxic apparatus with the toothbar 2 mm below the interaural line. Craniotomy was performed to allow lowering of a lesion electrode and 1–3 lesions were made in each animal. The squirrels were sacrificed with an anesthetic overdose, their brains perfused and prepared for histological analysis.

Twenty squirrels were used for the electrophysiological experiments. The anesthetic urethane which is commonly used in electrophysiological experiments[21], appeared to adversely affect the respiration of the squirrels. We therefore used thiopental sodium instead, which causes fewer respiratory problems (C. Michael, personal communication). The animals were anesthetized with 0.85 ml thiopental sodium (25 mg/ml, initial dose). Supplementary doses were delivered throughout the experiment. Extracellular single cell recordings in the suprachiasmatic region of the hypothalamus were made using tungsten parylene-coated microelectrodes with a 1–3 μm tip and an impedance of 1.0–1.7 MΩ at 1 kHz (Microprobe, Clarksburg, MD). The stereotaxic coordinates for recordings were 7.5–8.5 mm anterior to the interaural line, 7.5–10.0 mm ventral to the dura and starting 0.2–0.7 mm lateral to the midline at an angle of 2.0–3.5 degrees relative to the vertical axis. The electrodes were lowered using an electronic microdrive. Amplification and processing of the extracellularly recorded spikes was done as in previous experiments[21]. Action potentials were converted to electronic pulses with a window discriminator and the data were recorded by an Apple computer that also

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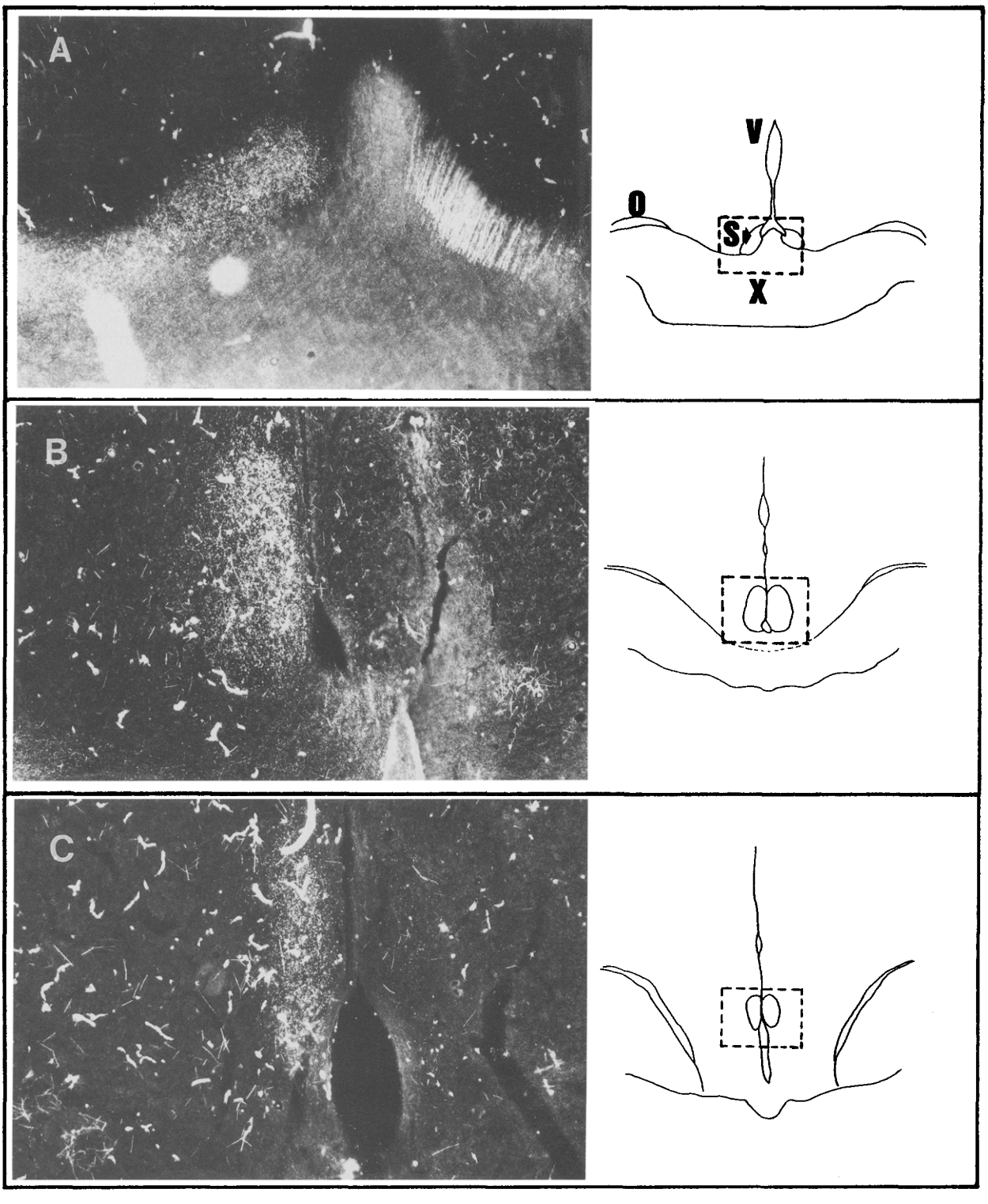
Fig. 1. The retinal projection to the suprachiasmatic nucleus of the thirteen-lined ground squirrel. The left portion of each panel is a dark-field photograph taken through the suprachiasmatic area, showing the grain-like distribution of WGA–HRP reaction product overlying the suprachiasmatic nucleus contralateral to the injected eye. Panels A, B and C represent, respectively, an anterior, middle and caudal section through the SCN. The line-drawing beside each photograph shows a lower-power camera lucida drawing of a Kluver–Barrera stained section immediately next to the section depicted in the photograph. The dashed outline encloses roughly the area shown in the photograph. Abbreviations: S, suprachiasmatic nucleus indicated by arrow; V, third ventricle; X, optic chiasm; O, supraoptic nucleus. The right side of the photograph in panel B shows damage caused by penetration of a recording electrode; this was not reproduced in the accompanying drawing. The staining outside the SCN (especially ventrally in the chiasm) is a result of the electrode damage and there is no similar staining where there is no electrode track.


A
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controlled the presentation of photic stimuli.

The cells were tested for visual responsiveness by presentation of light pulses (40 s duration at 3500 lux or 85 $\mu W/cm^2$) directed binocularly. Light from a tungsten–halogen lamp (Sylvania, EKE) was led to the eyes by glass fiber light guides. Infrared wavelengths were eliminated with the aid of an infrared stepfilter. Diffusing screens were placed close to the eyes to provide diffuse illumination of the whole retina. The pupils of the eyes were maximally dilated with a 1% atropine sulphate solution. The eyes were protected from drying by a coating of mineral oil. The pattern and intensity of the light stimuli were regulated by an electronic shutter and neutral density filters, except that the filters were not used in one case (see Results). Between light exposures the animal was in complete darkness. Long light pulses were occasionally applied to assess the time course of adaptation to the light stimulus. Cells were classified as visually reponsive when they showed a consistent response to light on at least 5 consecutive trials. Spike train statistics and peri-stimulus time histograms (PSTHs) were computed on-line and further processed off-line. The electrode track was marked with an electrolytic microlesion (10 μA, 10 s). The recording site was histologically verified by inspection of 40 μm thick, Cresyl violet-stained brain sections.

Five squirrels that were used for the electrophysiological experiments also received unilateral eye injections with the anterograde tracer wheatgerm agglutinin–horseradish peroxidase (WGA–HRP) one day before the recording sessions. Single injections of 8–10 μl 1% WGA–HRP (Sigma, Type IV) in saline were delivered unilaterally in the eye under 0.2 ml sodium pentobarbital anesthesia (65 mg/ml). The injections were delivered with a Hamilton syringe over a period of 8 min. Upon termination of the injection the needle was kept in the eye for an additional 2 min to prevent leakage of the WGA–HRP.

The animals were perfused transcardially 24–36 h after the WGA–HRP injection (at the end of the recording session) with 1% sodium nitrite in phosphate buffer, followed by 2% glutaraldehyde (15–30 min). The brain was stored for 4 h in 1.5% phosphate buffer with 20% sucrose and next in the same buffer with a 30% sucrose solution. Alternate 50 μm brain sections were stained with Cresyl violet. Intermediate sections were reacted with tetramethyl benzidine (TMB) to reveal the WGA–HRP reaction product.

RESULTS

The shape of the SCN of the thirteen-lined ground squirrel was determined using Cresyl violet to identify the densely packed somata that characterize

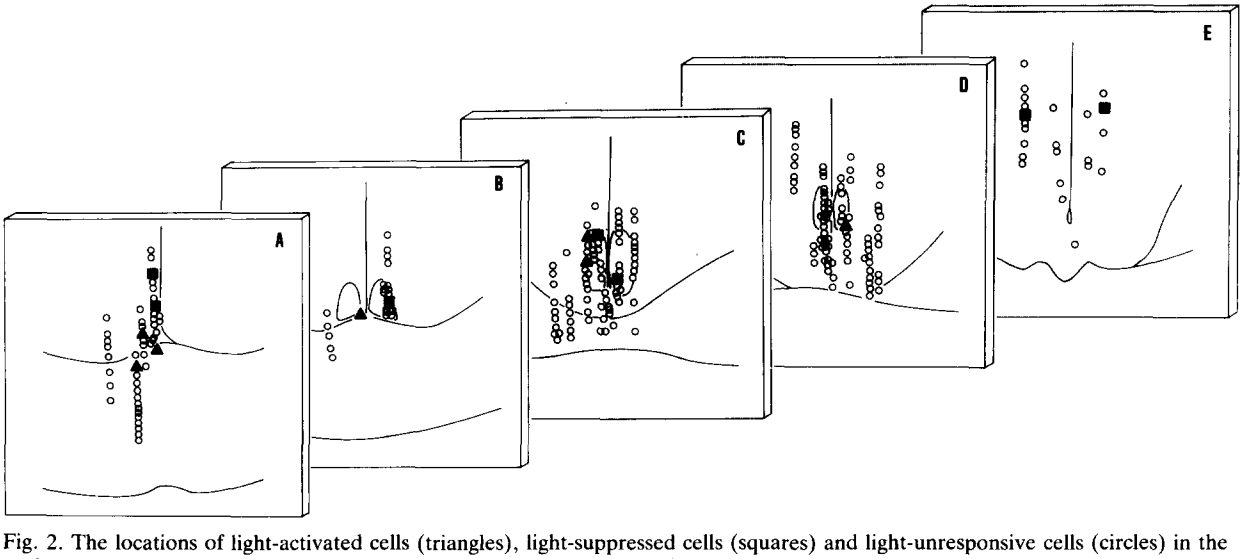

Fig. 2. The locations of light-activated cells (triangles), light-suppressed cells (squares) and light-unresponsive cells (circles) in the squirrel suprachiasmatic area are shown from rostral (A) to caudal (E) levels of the hypothalamus.

the mammalian SCN. On transverse slices the SCN appear ovoid with a maximal height of 550 μm and a maximal width (per nucleus) of 320 μm. The WGA–HRP reaction product closely overlapped the full extent of the Nissl-stained SCN (Fig. 1). The length of the SCN is 720 μm. Throughout the anterior–posterior axis the SCN are separated by the third ventricle.

In 19 animals, a total of 325 hypothalamic cells were recorded. Of these cells, 83 were located inside the SCN. A total of 15 cells were responsive to bilateral illumination of the eyes. These cells responded either with an increase (n = 7) or with a decrease (n = 8) of their discharge rate to increased levels of illumination. In accordance with previous experiments, these cells were classified as light-activated and light-suppressed cells, respectively[9]. The locations of both visual and non-visual cells were determined by reconstructions based on lesions made in each recording track (Fig. 2). A total of 7 visually responsive cells were located inside the SCN

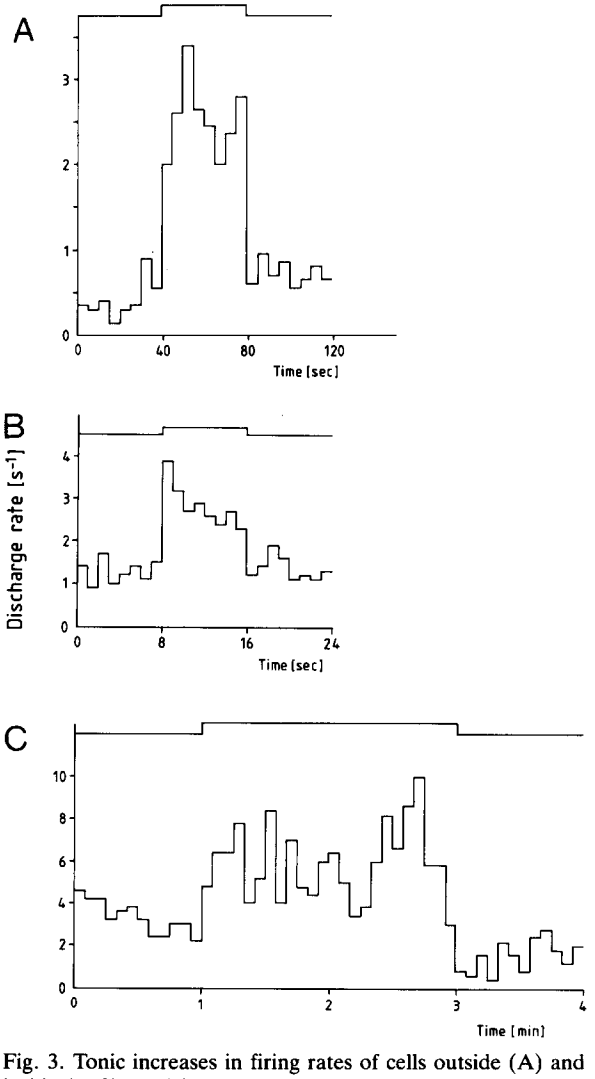

Fig. 3. Tonic increases in firing rates of cells outside (A) and inside (B,C) the SCN in response to retinal illumination. The timing of the photic stimulus is indicated above the record. A and B illustrate the similarity in visual responsiveness inside and outside the SCN. C shows the tonic response to a long (2 min) light stimulus.

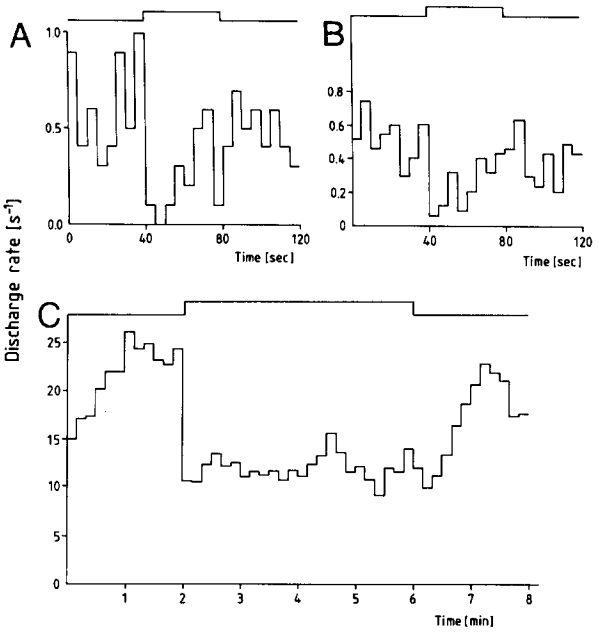

Fig. 4. Suppression of firing rates of cells outside (A,C) and inside (B) the SCN. Suppression by light was sometimes transient for cells both within and outside the SCN (see A and B).

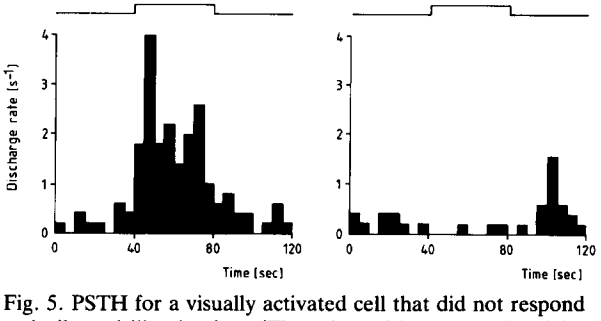

Fig. 5. PSTH for a visually activated cell that did not respond to ipsilateral illumination. (The rebound in neuronal activity following cessation of the stimulation of the ipsilateral eye was not reproducible).

and eight visual cells were found outside the SCN, at a distance of at most 1.3 mm from the SCN border. Thus, 8.4% of the suprachiasmatic cells recorded were light responsive while 3.3% of cells outside the SCN were responsive to light.

Light pulses of up to 2 min duration were used to stimulate light-activated cells. Light-activated cells always responded to these light stimuli with a sustained increase in discharge rate. Photically responsive cells outside the SCN had similar properties. Examples of light-activated cells inside and outside the SCN are presented in Fig. 3.

Light pulses up to 4 min in duration were used to stimulate visually suppressed cells. Some suppressed cells displayed a tonic suppression of firing rate in response to light (Fig. 4C). Other cells, however, were only transiently suppressed by long-lasting retinal illumination (Fig. 4A,B). The tonic or phasic response pattern did not appear to be related to the location of the cells.

Two activated and one suppressed cell were tested for the laterality of photic input by presenting light to each eye separately. In each of these cases, the visual response appeared to be driven by the contralateral eye only (Fig. 5). Light pulses with varying light intensities were presented to 3 visual cells. In Fig. 6 the responsiveness of a light-activated and a light-suppressed cell to stimuli at maximal and half-maximal light intensity are presented. Apparently, these cells did not respond to light pulses of 1750 lux. An intensity–response curve was obtained for a light-suppressed cell by plotting the mean steady-state discharge rate (after 20 s of light exposure) as a function of luminance. For this cell the discharge decreased monotonically as a function of light intensity (Fig. 7). The threshold for a tonic response was about 1000 lux. No saturation in the response to light was observed.

The spontaneous discharge rate of the hypothalamic cells only rarely exceeded 2 Hz, with discharge rates between 0.1 and 1 Hz being most common. The mean discharge rate (S.E.M.; n) inside the SCN was 0.51 Hz (0.099; $n = 83$) and outside the SCN 0.56 Hz (0.136; $n = 242$). The mean discharge for all visually responsive cells (excluding one) in complete darkness was 0.33 Hz; one cell which was suppressed by

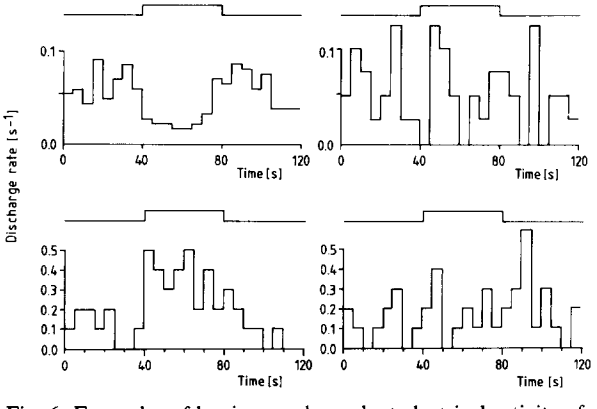

Fig. 6. Examples of luminance-dependent electrical activity of a light-suppressed and a light-activated cell in the squirrel. The light-suppressed cell is located inside the SCN, the light-activated cell outside the SCN. On the left, the applied light intensity is 3500 lux. On the right, intensity is decreased to 1750 lux by a neutral density filter.

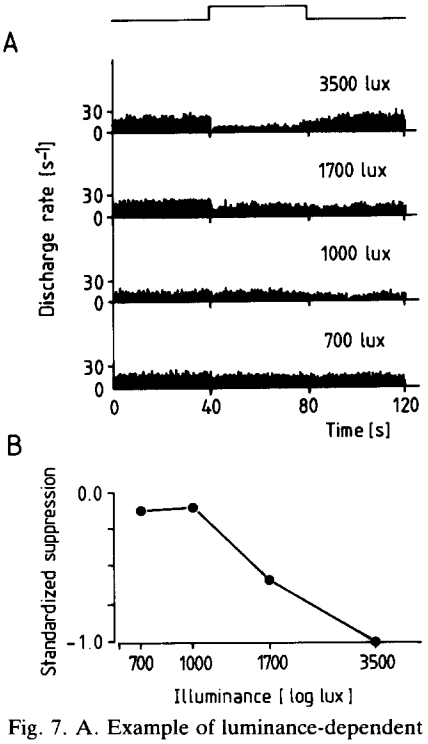

Fig. 7. A. Example of luminance-dependent electrical activity for a light-suppressed cell located outside the SCN. The various light intensities used are indicated in the figure. B. The relationship between the intensity of the light pulse and the steady state suppression. The steady state response to light was defined as the mean discharge rate 20–40 s after onset of the light pulse. Next, the steady state discharge rate was normalized with respect to the mean discharge rate before the light pulse and the maximum corrected response was normalized to −1.

photic input displayed an exceptionally high discharge rate of 25 Hz. A great variation in spike frequency was observed in these visual cells (up to 10-fold in the course of several hours). Whether the discharge rates of non-visual cells are equally variable is unknown since they were not recorded for equivalent lengths of time.

DISCUSSION

The several differences noted in the introduction between the responses of the circadian systems of diurnal and nocturnal species prompted our study of the photic responsiveness of hypothalamic cells in a diurnal squirrrel. Some of our results are consistent with earlier studies of nocturnal rodents and cats: a small proportion of cells were photically responsive; most of these showed sustained changes in firing rate during lengthy maintained light exposure; and both photically activated and suppressed cells were found. The finding of exclusively contralateral input to 3 cells is also consistent with earlier studies.

There were, however, also differences between our results and those from studies of nocturnal rodents. Light-suppressed cells often showed only transient responses during long stimulus presentations in squirrels, while these transient responses were seen only rarely in hamsters and never in rats. The proportions of suppressed and activated cells were also very different. In rats and hamsters, 73% and 84%, respectively, of responsive cells were activated by light presentation[21]. In the squirrels, only about 47% of cells were activated by light. Finally, the threshold intensities which caused sustained changes in firing rates were about 0.1 lux in rats, 10 lux in hamsters and 1000 lux in squirrels.

Differences in the way light was presented may account for small threshold differences, but they probably do not account for all of them, since the hamsters and squirrels at least were studied in the same apparatus using similar methods. A more likely artifactual source of these differences might be the different anesthetics used in these studies. The respiratory distress caused by urethane in squirrels prevented doing a study strictly comparable to the hamster study. Only future studies using unanesthetized animals could adequately assess the contribution of the type of anesthetic to these thresholds. A study[22] using thiopental anesthesia in the thirteen-lined ground squirrel found thresholds for responsiveness in the superior colliculus of less than 30 lux (2.0 log cd/m2). The high thresholds found in our study either are characteristic of the suprachiasmatic area or indicate that this area shows a special sensitivity to the anesthetic.

The possibility that these threshold differences are not artifactual is reinforced by the finding that very high light intensities were required to suppress pineal melatonin levels in wild-caught squirrels as compared to laboratory-reared animals[34,35]. The SCN presumably receive the photic information that regulates pineal function in squirrels as in other mammals[25]. Thus the high thresholds of wild-caught subjects may be related to their lighting history rather than to their diurnality[33]. The observation that these high thresholds are found in cells that may receive a monosynaptic input from the retina suggests that these differences might be inherent in the retina; alternatively, they could be a product of the characteristics of the hypothalamic cells themselves. In either of these cases high thresholds for phase shifting by light are to be expected in wild-caught animals.

Previous anatomical descriptions of the RHT projection to the hypothalamus have differed depending on the techniques and the species studied. The use of HRP as a tracer revealed a more extensive distribution of ganglion cell projections to the hypothalamus outside the SCN in hamsters[27] than was previously reported in other species using autoradiographic tracing methods[24]. Our anatomical results, obtained using WGA–HRP as a tracer, indicate a projection in ground squirrels that extends throughout the Nissl-stained SCN. This may not reflect the full extent of the visual projection to the hypothalamus, since recent studies using HRP conjugated to choleratoxin have demonstrated an even more extensive set of hypothalamic projections in hamsters[17,20].

The HRP labeling we observed corresponded with the locations of visual cells, indicating that these cells may have been driven by primary ganglion cell axons. Alternatively, visual cells may have been driven by the GST. Since the terminal field of the GST has not been described for squirrels, the location of visual cells does not indicate in which

cases the GST may have mediated the response.

Although the number of the visual cells recorded was small, there appeared to be a difference between hamsters and squirrels in the proportion of photically activated and suppressed cells found. In the hamster, 16 visual cells were activated by light while only 3 cells were light suppressed. In the squirrel, 7 light-activated and 8 light-suppressed cells were encountered. A chi-square test revealed a significant difference between squirrels and hamsters in their proportions of activated and suppressed cells ($P < 0.02$). This finding may be related to species differences in behavioral responsiveness of the circadian pacemaker to continuous light exposure. A good deal more information about the processing of light information by the circadian system will be needed before these differences in behavioral responses can be explained neurophysiologically. For the moment, one can only note that in two nocturnal species with a high proportion (73–84%) of light-activated cells in the suprachiasmatic area, continuous light exposure lengthens the free running period[2] and may lead to rhythm splitting[4,28]. The relatively low proportion (<50%) of light-activated cells in the SCN region in thirteen-lined ground squirrels may be related to the tendency of diurnal squirrels to shorten or preserve rather than lengthen free-running periods as light intensity increases[2,30], and to show splitting in continuous darkness rather than in bright illumination[14,28,30,42].

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