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LIGHT EVOKED C-FOS EXPRESSION IN THE SCN IS DIFFERENT UNDER ON/OFF AND TWILIGHT CONDITIONS

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1. INTRODUCTION

Circadian rhythms in mammals are entrained to the external daily light cycle and allow adaptation of physiology and behavior to the environment. Whereas in nature changes in irradiance include gradual variations at dawn and dusk, most laboratory investigations of light entrainment use a square wave cycle of artificial light, characterized by abrupt shifts from light to dark periods. Twilight periods are characterized by rapid modifications in light intensity and composition, and often correspond to the time of alteration of the activity patterns of diurnal and nocturnal animals. Irradiance levels span 8 log units with the greatest variation in amplitude occurring during dawn and dusk and may thus be critical for the entrainment of daily rhythms. An increasing body of evidence suggests that entrainment by natural sinusoidal light cycles including dawn and dusk transitions differs from entrainment by square wave light regimes with lack these transition periods. For example, acitivity onsets and offsets in many species are correlated with irradiance levels during twilight (1-8). There is also some evidence indicating that the strength of the LD zeitgeber increases if twilight transitions are included in the light dark cycle (1, 8-9). In addition, many nocturnal rodents spend most of the day in burrows, and are only exposed to light for a short duration at dawn or dusk (6-8). Thus the animal's light sampling behavior may be important in fine tuning daily shifts of the circadian pacemaker (7-8).

In contrast to an increasing number of behavioral observations which stress the importance of twilight in light entrainment, little is known of the effects of twilight on the pacemaker itself at the cellular level. The effects of light on the expression in the SCN of the proto-oncogene *c-fos*, and it's protein product c-Fos, have been extensively studied. Levels of *c-fos* mRNA or immunoreactive c-Fos protein are elevated following the administration of a light pulse during the dark phase of the LD cycle, or during subjective night (10-15). This photic activation is characterized by a phase dependence similar to that for light induced

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phase shifts of locomotor activity (10-13,15). According to the time of administration and the intensity of light stimulation, the degree of c-Fos expression in the SCN is generally proportional to the amplitude of the induced behavioral phase shift (12, 14). Although c-Fos activation can occur in the SCN without a concomitant shift of locomotor activity (for example certain pharmacological treatments), under most nautral entrainment conditions light alters the level of protein expression (16-17).

Since behavioral observations support the idea that entrainment of locomotor activity differs depending on whether the light cycle includes or does not include twilight transitions, the objective of the present study was to examine c-Fos induction in a nocturnal rodent, the gerbil, under different lighting conditions. Each series included animals sampled at numerous time points over a complete 24 hour period. The first series (CTcontrol) examined c-Fos induction under conditions of continous darkness, in order to determine if there is an endogenous rhythm of c-Fos expression in the SCN in the absence of light. The second series (CTpulse) included animals exposed to a 30 minute light pulse administered at different times of the circadian cycle, to obtain a phase response curve for c-Fos induction in the SCN. The third and forth series used 12lL/12D cycles providing a light zeitgeber. However, in series 3 (ZTsquare), a square wave artifical light stimulation was used, whereas in series 4 (ZTtwilight), lighting consisted of natural environmental light including dawn and dusk transitions. We also conducted some preliminary obervations of the animals activity under the different light regimes, to determine if entrainment differed under these two latter conditions..

2. METHODS

Animal maintenaince and light exposure. Animals (*Taterillus petteri*) were captured in the field in Mali and maintained in the laboratory in individual cages on a diet of rodent pellets and apples. All animals (except those of the ZTtwilight series) were maintained for at least 4 weeks on an artifical square wave light cycle using flourescent lights (approximately 500 lux) prior to the experiments. For the CTcontrol and CTpulse series, the lights remained off on the day of testing, such that ZT0 was used as the begining of the subjective day (CT0). Animals in the CTpulse series were exposed at different circadian times to a 30 minute pulse of light (500W tungsten-halogen bulb, approximately 1000 lux). and perfused 60 minutes after the beginning of the light pulse. CT control animals were handled identically, but were not exposed to light. Animals in the ZTsquare series were maintained under the 12:L:12D light regime and perfused at various times of the 24 hour cycle. Animals in the ZTtwilight series were exposed to naural light conditions through a large window in the room with no artificial lights. Perfusion of animals in this series was also performed at various times of the 24 hour cycle. For each time point of each series, 3-4 animals were examined for the presence of c-Fos protien in the SCN (106 animals in total) using immunohistochemistry.

c-Fos immunohistochemistry. Animals were deeply anesthetized with nembutal (100 mg/kg i.p.) and perfused intracardially with heparinized saline followed by 300 ml of 4% paraformaldehyde in phosphate buffer (pH 7.4, 0.1 M) at 4°C. The brains of all animals were post-fixed for 24 hrs. at 4°C, and coronal sections were made at 40 microns on a freezing microtome. For immunohistochemistry, all the brain sections of each series were processed

simultaneously to avoid variations in the density of tissue staining. Endogenous peroxidase was suppressed using a solution of 50% ethanol in saline with 0.03% H_2O_2. Free floating sections were briefly rinsed in phosphate buffered saline (0.01M, pH 7.2) containing 0.3% triton and 0.1% sodium azide (PBSTA) and blocked with 1.5% normal goat serum (1 hour). Sections were rinsed twice in PBSTA and incubated in the primary antibody (rabbit polyclonal anti-c-Fos, concentration 1/2500 in PBSTA; Oncogene Science, #PC05) for 3 days at 4°C. Sections were then rinsed in PBSTA, and the presence of c-Fos-like protein demonstrated with the avidin-biotin technique using a secondary biotinylated anti-rabbit antibody (Vectastain, BA-1000), followed by avidin-biotin peroxidase complex (Vectastain, PK6100). Peroxidase was demonstrated by incubation for 10 min. in 0.2% DAB with 0.5% ammonium nickel sulfate and 0.003% H_2O_2 in tris buffer (0.04M, pH 7.6). The sections then were rinsed, mounted on glass slides, dehydrated, and coverslipped in Depex.

c-Fos-like immunopositive nuclei were counted by direct visual observation and by computer assisted image analysis (Biocom, Les Ulis, France) for detection of nuclear profiles according to density. The measure of total density measured in the SCN was obtained by subtracting the background density from that of the nucleus. Both methods gave identical results. The identity of the animals were unknown to the observer during the analysis and every other section of the SCN of each series was analysed.

Behavioral observations. In a separate group of animals maintained in individual cages under ZTsquare and ZT twilight conditions, records of the the animals behavior were made by direct visual observations. Observers were placed behind an opaque sheet, with a transparent window, and scored the individuals behavior during 5 minute bins. Activities recorded were (1) active locomotor activity (moving, digging or feeding), (2) awake but immobile (grooming), (3) alseep. Observations were made continually over 24 hours for 6 consequtive days. Both males and females were observed, but only the behavior of males will be discussed.

3. RESULTS

The first series studied (CTcontrols) was designed to examine whether there is an endogenous circadian rhythm of c-Fos expression in the SCN in the absence of light. As shown in figure 1A, animals sacrificed at different times during the circadian cycle in constant darkness show consistently low levels of c-Fos in the SCN. The total number of cells per SCN in these dark controls was 16 ±12.0, and the total density was 601 ±237.1.

In the CTpulse series, animals were exposed to a 30 minute light pulse at the same circadian times as above, in order to measure c-Fos induction to light administered at different points of circadian phase. Light induced c-Fos expression in the SCN only during the early part of subjective day, and during subjective night (figure 1A). Light exposure during the initial part of the subjective day (CT0-CT1) resulted in significant expression of c-Fos, whereas exposure after CT2 through to CT12 did not induce c-Fos levels different from that of the dark controls. During the subjective night, c-Fos levels are increased from CT13 through CT22 with a peak at about CT18.

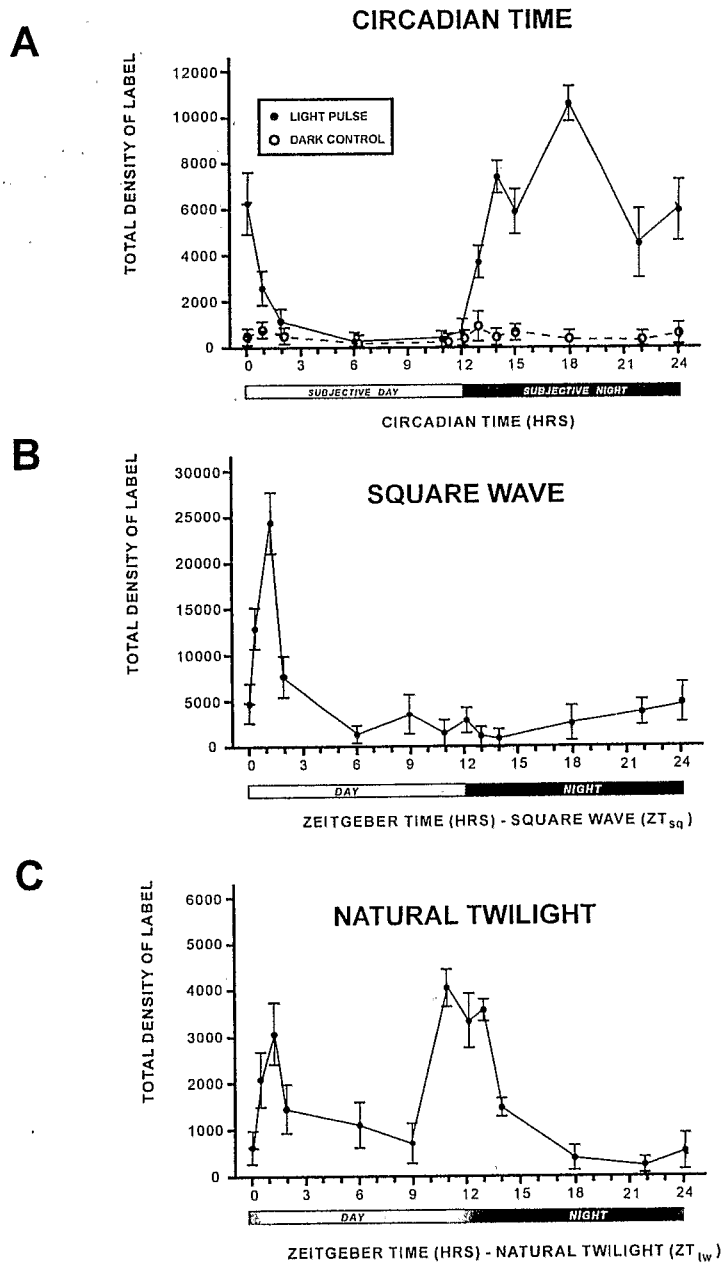

Figure 1. Levels of c-Fos expression in the SCN under different lighting conditions.
(A) Light pulsed (CTpulse) and control (CTcontrol) animals under circadian times.
(B) Animals exposed to a 12L:12D square wave artificial light cycle (ZT square).
(C) Animals exposed to a 12L:12D natural twilight cycle (ZTtwilight).

In animals exposed to an artificial square wave light regime, with abrupt transitions between lights on and lights off at ZT0 and ZT12, a single peak of c-Fos induction is observed only at the time of light onset between ZT0.5 and ZT2 (figure 1B). At all other times of the ZT cycle, c-Fos levels remain low or absent.

In animals that were exposed to natural 12L:12D light conditions including dawn and dusk transitions (figure 1C), c-Fos expression shows a pattern different from that of animals exposed to the same 12L:12D light cycle but lacking twilight transitions.. In animals sacrificed immediately before the beginning of the light phase (ZT0) c-Fos expression is low, but rapidly rises between ZT 0.5 -ZT2, during the night-day transition. During the middle of the light period, c-Fos levels decrease at ZT6, and then increase significantly at ZT11-ZT13 at the time of dusk. During the night, (ZT14-ZT24) c-Fos levels decrease to that of the control animals. This series thus shows two peaks of c-Fos expression, occurring at both the night-day transition and the day-night transition. The first peak occurs after light has increased, whereas the second peak occurs at the initial changes of light decrease.

Preliminary observations comparing activity and behavior during the ZTsquare and ZTtwilihgt light cycles show that the gerbils nocturnal activity is bimodal, with two bouts of locomotor activity occurring at the beginning and at the end of the night time period (figure 2). In between the two periods of night-time locomotor activity, a mid-night rest period is observed during which the animals are not actively moving, but are awake and engaged in behaviors such as grooming. Animals are inactive during the day, except for a short period at mid-day, during which the animals may move about or groom.

In both light regimes the onset of activity is fairly abrupt, but the temporal pattern differs between the two groups. The first difference concerns the times of onset and offset of activity. In animals exposed to natural light cycles, activity starts earlier, during the dusk transition period (ZT11.57) and ends earlier, prior to complete daylight (ZT23:28). Under square light cycles, activity begins later (ZT13.44) after light extinction and terminates some time after lights on (ZT0.55). The total duration during which the animals were active is longer under the square wave light regime (8 hours, 51 minutes) than under natural light conditions (8 hours 24 minutes). As a consequence, the mid-night rest period is shorter in ZTsquare wave (2 hours 11 minutes) as compared to ZTtwilight condiitions (3 hours 6 minutes). Finally there is much less variation in the time of onset of activity in ZT twilight as compared to ZTsquare conditions.

The above observations refer to behavior of male animals. Observations in females show similar patterns of behavior. The time of the activity onset and offset of behavior is similar in both males and females. However, the mid-night rest period is of longer duration in females (approximately 1 hour) and thus the second bout of locomotor activity is accordingly of shorter duration.

4. DISCUSSION

The results show that c-Fos induction in the SCN depends on the presence of light stimulation, on the phase of the circadian pacemaker, and also on the presence or absence of twilight transition periods. Under conditions of constant darkness (CTcontrol), c-Fos levels remain low during both subjective day and subjective night, illustrating that there is no

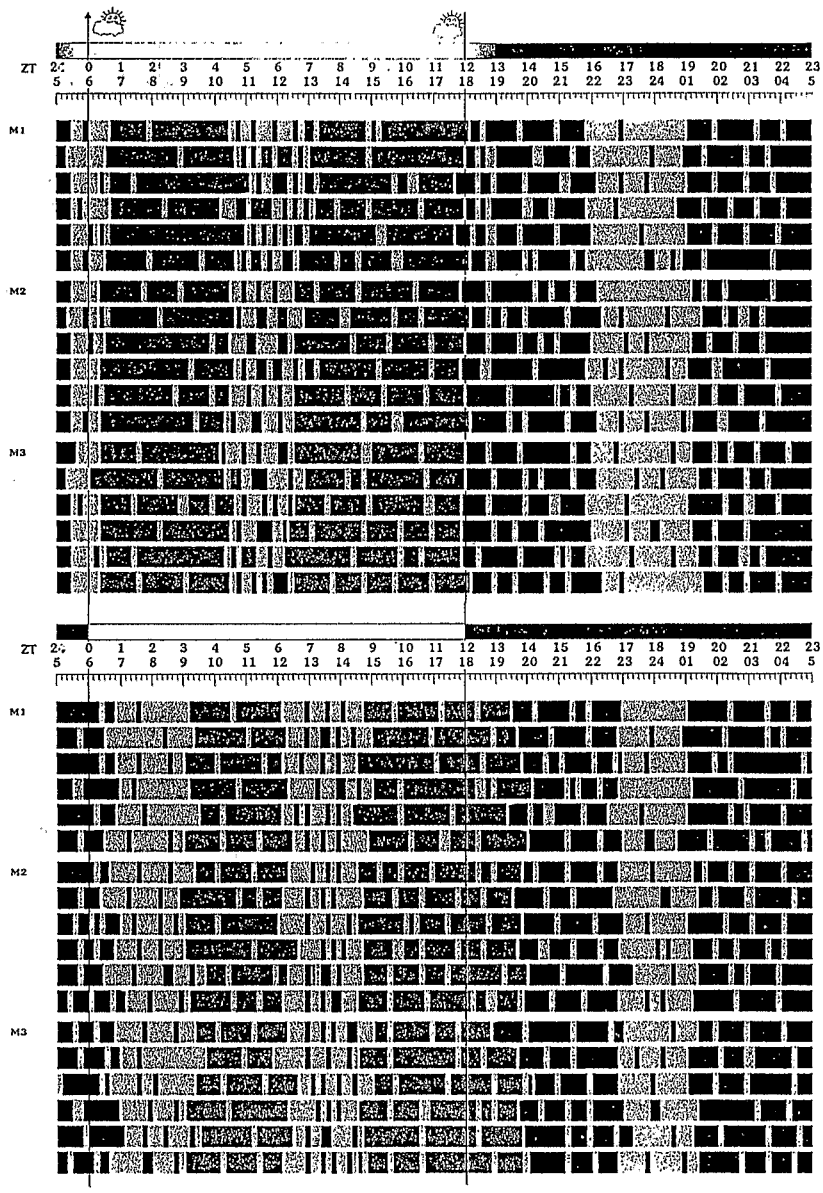

Figure 2. Activity patterns in gerbils under 12L: 12D natural light conditions including twilight transitions (ZTtwilight, above) and square wave light conditions (ZTsquare, below). Different activities were recorded during 5 minute periods :

- active locomotor movements
- awake but immobile
- asleep

endogenous oscillation of c-Fos in the absence of light. When animals are exposed to a pulse of light during free-running conditions, c-Fos is expressed only during the subjective night and early part of the subjective day. The phase response curve shows that c-Fos expression increases after CT12, reaches a peak at about CT18, and then gradually decreases towards the end of the subjective night; falling to control levels at CT2. These results are similar to the phase dependence of light induced c-Fos induction found in other nocturnal rodents (10-15), which parallels the effect of light on phase shifts of locomotor behavior.

In zeitgeber conditions under a 12L:12D light cycle, c-Fos induction is also dependent on the phase of the circadian pacemaker, but the results differ according to whether twilight transitions are included or not. Under an artificial square wave light regime, c-Fos is expressed only during the early part of the day at the time of the dark-light transition. The peak in c-Fos induction occurs at ZT0.5-ZT2, decreasing thereafter to the levels of the dark controls, despite the continuous presence of light. This peak in c-Fos expression corresponds to the peak which occurs in the CTpulse animals in that both groups are exposed to an increase in irradiance at the dawn period, following 12 hours of darkness and at a time when the circadian system is susceptible to a phase shift. The low levels of c-Fos during the remainder of the daytime period, despite the presence of light, shows that c-Fos expression depends on the phase of the circadian clock, and is not sustained under continuous exposure to light. Althought the present results differ from the effect of sustained light exposure in the rat (10), this result is not surprising since in the CTpulse series, a pulse of light during the latter part of the subjactive day also fails to induce c-Fos. Similarly, c-Fos levels remain low during the night period in the ZTsquare group, since although the phase of the circadian pacemeker gates expression to light during this time, no light stimulation is available.

In the animals maintained under a 12L:12D light cycle which includes gradual changes in irradiance at dawn and dusk, two peaks of c-Fos expression are observed. The first peak at ZT0.5-ZT2 corresponds to dawn peak observed in the ZTsquare group, and to animals receiving a light pulse at a similar time in the CTpulse group. C-Fos levels then decrease during the remainder of the day in both the ZTsquare and the ZTtwilight groups. However, a second peak of increased c-Fos levels is observed starting at ZT11 through ZT13, which is not observed in the ZTsquare group. This dusk peak of c-Fos preceeds complete darkness and also occurs prior to the rise in c-Fos observed in animals of the CTpulse group.

The observation of a second peak of c-Fos at dusk, in addition to the peak observed at dawn is a novel finding, although fos expression at both dawn and dusk might be expected since in other nocturnal rodents light causes phase delays in the late day-early night, and phase advances in the late night-early day (10, 13, 17), . However, a rise of c-Fos levels at dusk has not previously been observed in conditions of square wave light regimes (10, 13-14). Even under skeleton photoperiods with a one hour pulse of light at both dawn and dusk, only the dawn pulse was found to elicit c-Fos induction in rats (10). This would suggest that the peak of c-Fos observed under the present conditions including twilight transitions, may be due to either the decrease in light intensity at dusk, or the change in the spectral composition of light during twilight. However, more recent experiments by Schwartz et al. (10) in mice using skeleton photoperiods, showed that c-Fos could be induced at both early and late ZT times approximating dawn and dusk. Although the dawn and dusk light pulses induced equivalent levels of c-Fos, the behavioral phase shift was not equal. However, c-Fos induction was not completely independent of phase, and depended on whether the animals were active during

the short or long interval of darkness, stressing the importance of the phase of the circadian pacemaker in addition to the presence of light for c-Fos induction.

It is thus interesting to compare the differences in c-Fos induction and behavior in the gerbils under the square wave and the natural light regimes. Under twilight conditions, the animals onset of behavior preceeds that of the ZTsquare group by more than one hour. This implies that the circadian phase of the ZTtwilight group is advanced compared to that of the ZTsquare group. In this case, the presence of a peak of c-Fos levels at dusk may correspond to the advance of the sensitive period of the circadian pacemaker to c-Fos induction by light. This further suggests that in nature, light exposure which causes phase shifts at dawn and dusk can also influence early gene expression in the SCN, for which the animals own light sampling behavior may prove to be an important variable (8).

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