



# Paradoxical sleep suppresses immediate early gene expression in the rodent suprachiasmatic nuclei

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Light stimulates neuronal activity with subsequent expression of the protein product of the immediate early gene, *c-fos*, in the Suprachiasmatic Nuclei (SCN). Non-photic stimuli is also thought to modulate activity within the SCN. Here, we sought to determine the effects intrinsic stimuli, specifically, the states of sleep upon *c-fos* protein expression in the SCN. In 16 rats, *c-fos* protein expression was evaluated at a fixed time of 1600 h (subjective night), following 1 h of electroencephalographically defined sleep. During sleep, as the state of paradoxical sleep (PS) increased, *c-fos* protein expression decreased ( $r = -0.41$ ,  $p < 0.033$ ). The PS-associated reduction of *c-fos* positive cells occurred equally between animals asleep in the light and those asleep in the dark. We propose a model whereby PS duration might function as a homeostatic-entraining mechanism to reduce neuronal activity within the SCN, and thereby modulate circadian rhythms during sleep.

**Keywords:** suprachiasmatic nucleus, sleep, *c-fos*

## INTRODUCTION

The Suprachiasmatic Nuclei (SCN) are the neuroanatomic sites of the mammalian biological clock (Miller et al., 1996). Subpopulations of SCN neurons exhibit spontaneous patterns of discharge, and accordingly, are described as self-sustaining neural oscillators, or pacemakers (Welsh et al., 1995; Prosser, 1998; Scheper et al., 1999). When maintained in culture and devoid of afferent input, these neural pacemakers follow a “free-running” discharge pattern lasting 24–25 h (Liu et al., 1997).

Activity of SCN pacemaker cells is significantly influenced by both extrinsic and intrinsic stimuli, which are conveyed through afferent neural pathways. Ambient illumination, the SCN’s primary extrinsic stimuli, increases the duration and intensity of neuronal activity with concomitant expression of the protein product of the immediate early gene, *c-fos* (Groos and Meijer, 1985; Rea, 1989; Amir and Robinson, 1995; Meijer et al., 1998). Extrinsic non-photic stimuli also modulate neuronal activity and *c-fos* protein expression within the SCN (Amir and Stewart, 1998). For example, following Pavlovian conditioning techniques in which non-photic and photic stimuli are coupled and presented, non-photic stimuli alone are able to induce *c-fos* protein expression (Amir and Stewart, 1996).

In addition to extrinsic stimuli, intrinsic stimuli such as behavioral state can modulate activity within the circadian pacemaker. Fear, for example, attenuates light-induced *c-fos* protein expression (Amir and Stewart, 1998; Amir and Stewart, 1999). The state of sleep also influences activity within the SCN (Deboer et al., 2003; Lee et al., 2009).

The SCN itself does not generate the states of wakefulness nor sleep (Mistlberger et al., 1983). Rather, it entrains the appearance of these states to coincide with the diurnal light-dark cycle (Kas

and Edgar, 1999). This suggests that the SCN is a primary component within the neuroanatomic circuits modulating the onset and duration of wakefulness and sleep. Therefore, we hypothesized that sleep states might influence patterns of activity within the circadian pacemaker. To evaluate this hypothesis we assessed *c-fos* protein expression within the rodent SCN following electroencephalographically (EEG)-defined wakefulness and sleep, while controlling for the presence and absence of environmental illumination.

## MATERIALS AND METHODS

### STUDY GROUPS

All protocols were reviewed and approved by the Institutional Animal Care and Use Committee at Case Western Reserve University, where this project was developed and performed. To account for known differences in retinal innervation of the SCN that exist between albino and pigmented rat strains (Steininger et al., 1993), both Sprague Dawley (SD) and Brown Norway (BN) rats were studied. All animals were male, 4–6 months of age, and were obtained from a single vendor (Harlan). The average weight of the SD was  $377 \pm 60$  grams while that of the BN was  $294 \pm 35$  grams. While in the animal facility the animals were housed under a 12:12 h light–dark cycle with lights-on beginning at 7:00 AM.

**Figure 1** graphically portrays the study protocol. As illustrated, following characterization of sleep/wake architecture, each rat was randomized to one of two separate experimental conditions, then sacrificed and the brain immunohistochemically processed for *c-fos* protein expression. Our preliminary studies suggested that in order to detect any strain-related differences of at least  $45 \pm 15$  *c-fos* positive cells in the SCN (statistical power of 0.90,  $\alpha = 0.05$ ) each of the experimental conditions required four animals from each strain. Consequently, 8 SD and 8 BN rats were used for this study.

## SURGICAL PREPARATION

Animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (45 mg/kg). Four holes were drilled at coordinates which would permit recordings of both cortical EEG and hippocampal theta wave activity (Bergmann et al., 1989). One 0.80 × 3/32 inch stainless steel screw (Vintage Machine, Medina, OH, USA) was placed into each hole and attached to a micro-connector (Continental Connector Corp, Trenton, NJ, USA). For the purpose of measuring and recording EMG activity, two wires were inserted into the dorsal neck muscles and also attached to the micro-connector. After placement of the EEG recording screws and EMG fine wire electrodes, the entire matrix was encased in a dental acrylic skullcap that was molded to the shape of the rat's head. The incision around the skullcap was then sutured closed.

## PROTOCOL FOR RECORDING SLEEP IN THE LIGHT AND IN THE DARK

On the seventh day of surgical recovery each animal was acclimated to a sound attenuated, ventilated, and light-regulated environmental cubicle (BRSLVE Davis, Maryland). The acclimatization protocol consisted of placing the animal into the cubicle 2 h after light onset (approximately at 0900 h), then connecting a cable from a polygraph (Grass model 78 D) to the micro-connector attached to the animal's head. Inside the cubicle the light intensity was 3,600 lx (General Electric dichroic bulb), the ambient temperature was maintained between 76° and 79°F and food and water were provided *ad libitum*. With the exception of the small cable attached to the micro-connector mounted on its head, the animal was unrestrained inside of the cubicle.

Each animal was studied on the day following its acclimatization to the environmental cubicle. As illustrated in **Figure 1**, sleep-wake behavior was characterized between 1130 and 1500 h on day 8. Then, at 1500 h, animals were randomly assigned to either remain asleep in the light or else asleep in the dark. Following the conclusion of this hour, at 1600 h, each animal was sacrificed by a lethal intraperitoneal dose of pentobarbital sodium. Each animal was permitted to maintain its state of wakefulness or sleep up until the moment of injection. Following perfusion (described below), the brain was removed for *c-fos* immunohistochemistry.

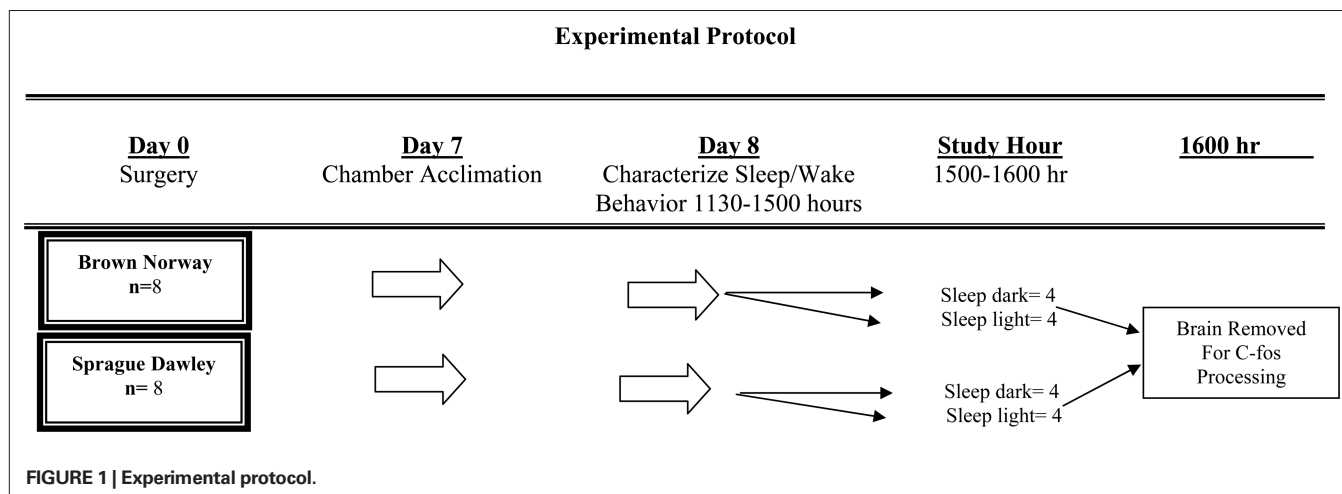
## TISSUE COLLECTION AND PREPARATION

Following the lethal dose of pentobarbital sodium, the animal was transcardially perfused with 100 ml of ice-cold heparinized 0.9% saline solution, followed by 250 ml of 0.4% paraformaldehyde (PF) in 0.1 molar (m) weight phosphate buffer (PB), followed by 100 ml of 10% sucrose solution in 0.1 m PB. Brains were removed and post-fixed overnight in 4.0% PF in 0.1 m PB. The following day the brain was placed in a cryoprotectant solution of ethylene glycol and glycerol in 0.4 m PB and stored at -20°C.

After all 16 brains had been acquired, they were removed from the freezer, rinsed four times (12 h per rinse) in 0.1 m PB to remove the cryoprotectant. The brains were then soaked in 30% sucrose solution made in 0.1 m PB until sinking (approximately 48 h). Individual brains were placed onto the platform of a freezing microtome and the entire brain sectioned, in the coronal plane, at 50 µm per section. All sections from each brain were collected as a 1:4 series and placed into six-well culture dishes containing 0.1 m tris PB. After all brains had been sectioned, one of the 1:4 series of sections was selected from each rat brain for *c-fos* immunohistochemistry. Individual series of tissue sections were batch-processed in parallel groups of eight brains, in a free-floating fashion, using a plexiglass-divided staining net system (Brain Research).

## IMMUNOHISTOCHEMISTRY PROTOCOL

Endogenous peroxidase activity was eliminated by incubating sections for 60 min on a rocker platform at room temperature in 100 ml of 0.1 m tris buffered saline (TBS) with 3.0% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and 1.0% Triton-X100. Following incubation, sections were rinsed three times in TBS. Sections were then incubated at +4° centigrade for 24 h in a TBS based solution containing 4.0% goat serum, 0.3% Triton-X100, and rabbit anti *c-fos* antibody (Cal Biochem) at a 1:10,000 dilution. Following this initial incubation, the sections were rinsed with TBS and then incubated for 2 h in biotinylated anti-rabbit IgG (Vector Laboratories Inc, Burlingame, CA, USA). Subsequently, sections were again rinsed in TBS and then incubated for 1 h in an ABC reagent (ABC peroxidase kit, Vector Laboratories Inc, Burlingame, CA, USA). After incubation in the ABC reagent, tissue sections were rinsed in TBS before a 2- to 5-min incubation in a filtered TBS solution containing H<sub>2</sub>O<sub>2</sub>, and



as a chromogen, diaminobenzidine (Sigma) and nickel chloride. Following these steps all sections were mounted on gelatin-coated glass slides, dehydrated, defatted, counter-stained with methyl green and cover-slipped.

## DATA ANALYSIS AND STATISTICAL METHODS

### Scoring of EEG defined wakefulness and sleep

At the conclusion of the study, each animal's EEG recording was manually scored. Electroencephalographic waveforms were characterized by established frequency and amplitude criteria (Bergmann et al., 1989). Epochs were classified as either awake (W), NREM slow-wave sleep (SWS), or paradoxical sleep (PS) (Decker et al., 2003).

### Quantification of *c-fos* positive cells within the SCN

An Olympus bright-field microscope (20× objective), equipped with a high-resolution color video camera, was used to magnify and project coronal sections of the SCN to a hi-resolution color monitor (Sony Trinitron). To reliably count *c-fos* positive cells within the SCN, it was necessary that the boundaries of the nucleus were well-defined. As a distinct nuclear border does not exist in the most caudal portion of SCN, counting of *c-fos* positive neurons was performed only in the rostral and mid portion of the nucleus. Consequently, two 50 μm coronal sections in which the nuclear boundary of the SCN was evident, were selected from each animal. Because the SCN occurs bilaterally, and given hemispheric asymmetries intrinsic to tissue sectioning, this strategy enabled us to count *c-fos* positive cells in four distinct antero-posterior levels per animal. Counting of *c-fos* positive cells was performed by an observer blinded to both the identification of each specimen, as well as the study design. The glass slides containing SCN sections were presented to the scorer in a randomized manner. Cells expressing the *c-fos* protein product were identified as those with a rounded purple-black nucleus.

### Statistical approaches to the data

After each animal's EEG record was scored, values of W, SWS, or PS were entered, epoch by epoch, into a database for statistical evaluation (SPSS, Chicago, IL, USA). The values for numbers of *c-fos* positive neurons from individual SCN cross sections for each animal were also entered into the database. The data were then coded to allow inter- and intra- strain comparisons between the effects of ambient lighting and sleep state architecture with numbers of *c-fos* positive cells within the SCN. The associations between these variables were assessed by bivariate correlation analysis and

relationships between variables explored with *t*-tests, multivariate ANOVA, and linear regression analysis modeling. In all approaches a two tailed significance level of  $p < 0.05$  was used to determine statistical significance.

## RESULTS

### EFFECTS OF ENVIRONMENTAL ILLUMINATION UPON SLEEP

#### ARCHITECTURE AND *c-fos* EXPRESSION

During the hour prior to sacrifice (1500–1600), no difference existed between the sleep architecture of either SD or BN rats asleep in the light when compared with those asleep in the dark (Table 1). No strain-related differences existed in values of *c-fos* positive cells in the SCN between SD rats and BN rats (data not shown) randomized to the same experimental condition (i.e., asleep in the light, asleep in the dark). Therefore, values of sleep-wake architecture and *c-fos* positive cells in the SCN from both SD rats and BN rats were pooled according to experimental condition.

#### ASSOCIATIONS BETWEEN PARADOXICAL SLEEP AND *c-fos* EXPRESSION IN THE SCN

Table 2 summarizes correlations between individual sleep stages with *c-fos* protein expression in the SCN. Bivariate analyses reveal a significant inverse correlation between percent of time spent in PS with numbers of *c-fos* positive cells. Figure 2 illustrates that relationship and reveals a significant  $r^2$  of 0.41 ( $p = 0.033$ ). Regression analyses assessing predictors of *c-fos* positive cells in the SCN (percent time awake, percent time in SWS, percent time in PS, presence or absence of light, rat strain) confirm that duration of PS was associated with reduced numbers of *c-fos* positive cells ( $p = 0.028$ ).

Figure 3 demonstrates differences between *c-fos* protein expression within the SCN of two sleeping animals. The rodent sample shown on the left had no PS during the hour prior to sacrifice while the other shown on the right spent 11% of the hour in PS. That animal, which spent 11% of the hour in PS, demonstrated a large reduction in *c-fos* positive cells in the ventral portion of the nucleus, a region containing potential pacemakers of the circadian clock (Miller et al., 1996). This pattern of reduction was even more pronounced in those animals which spent a greater amount of time in PS.

## DISCUSSION

We demonstrate that the occurrence and duration of PS is associated with pronounced suppression of *c-fos* protein expression within the SCN. These observations compliment and extend prior studies demonstrating that neuronal firing (Deboer et al., 2003) and gene expression (Lee et al., 2009) within the SCN are also influenced

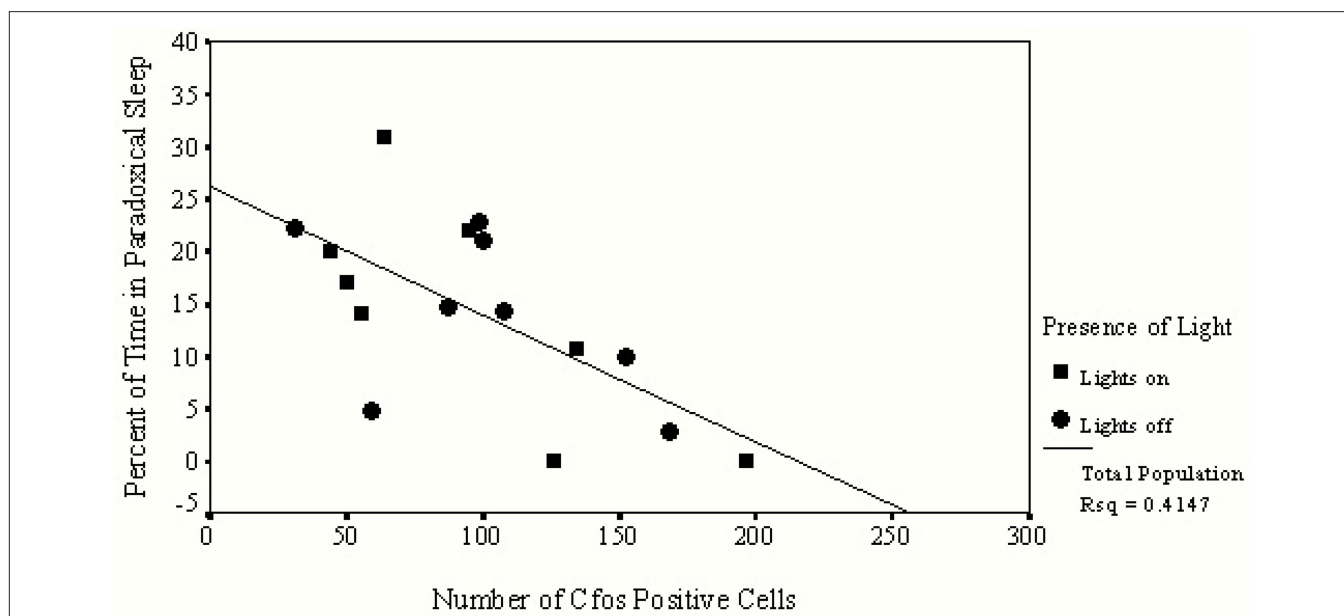
**Table 1 | Sleep architecture is unaffected by the presence of light during the final hour of sleep.**

SPRAGUE DAWLEY			
Sleep stage	Lights on	Lights off	2-tailed significance
% Wake	12% ± 3%	12% ± 5%	0.952
% NREM (NSW + SWS)	82% ± 3%	71% ± 4%	0.710
% PS	13% ± 8%	17% ± 5%	0.183
BROWN NORWAY			
Sleep stage	Lights on	Lights off	2-tailed significance
% Wake	6% ± 2%	14% ± 4%	0.153
% NREM (NSW + SWS)	78% ± 1%	75% ± 3%	0.356
% PS	18% ± 2%	11% ± 2%	0.186

**Table 2 | Bivariate correlations between percent of time spent in each state with numbers of *c-fos* positive cells in the SCN.**

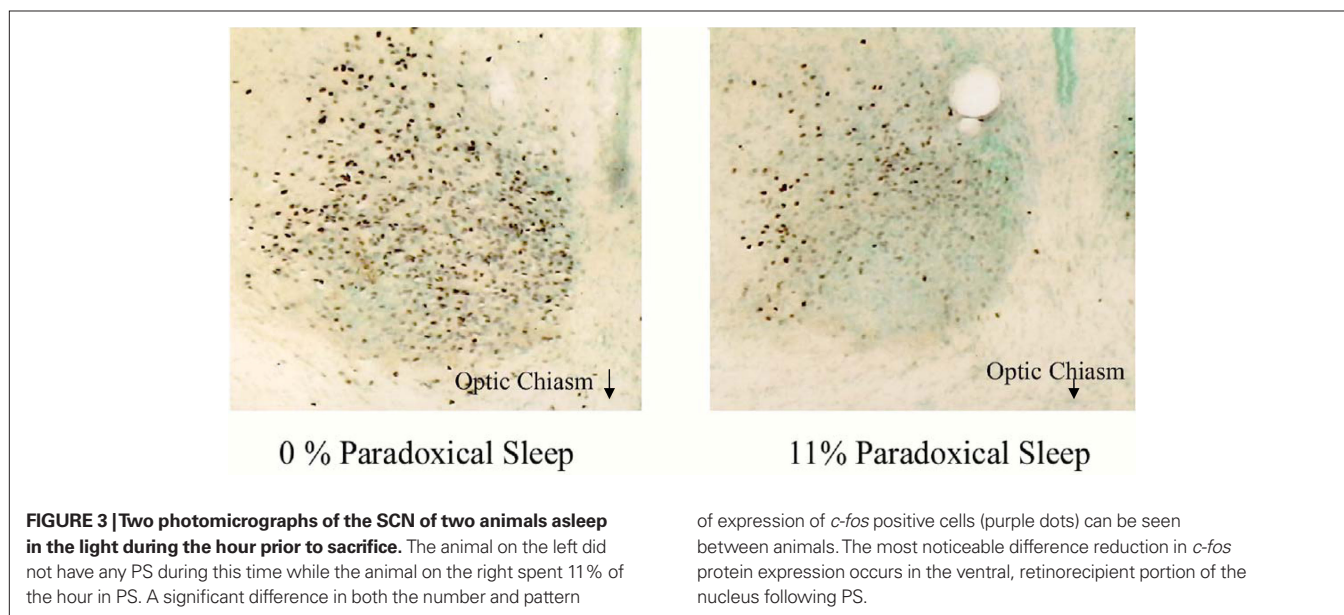
BN and SD combined $n = 16$	% Wake	% NREM (NSW + SWS)	% PS
Pearson Correlation coefficient	0.148	-0.11	-0.38
2 tail significance	0.418 <i>ns</i>	0.536 <i>ns</i>	0.033*

\*Significant at  $p < 0.05$ .



**FIGURE 2 |** This figure provides data from only those animals permitted to sleep during the hour prior to sacrifice. Values of *c-fos* positive cells in the SCN of each animal are indicated on the horizontal axis and the amount of time spent in PS is indicated on the vertical axis.

Animals asleep in the light are represented by squares, those asleep in the dark are represented by circles. A significant inverse relationship exists between the number of *c-fos* positive cells found in the SCN and the duration of time spent in PS.



**FIGURE 3 |** Two photomicrographs of the SCN of two animals asleep in the light during the hour prior to sacrifice. The animal on the left did not have any PS during this time while the animal on the right spent 11% of the hour in PS. A significant difference in both the number and pattern

of expression of *c-fos* positive cells (purple dots) can be seen between animals. The most noticeable difference reduction in *c-fos* protein expression occurs in the ventral, retinorecipient portion of the nucleus following PS.

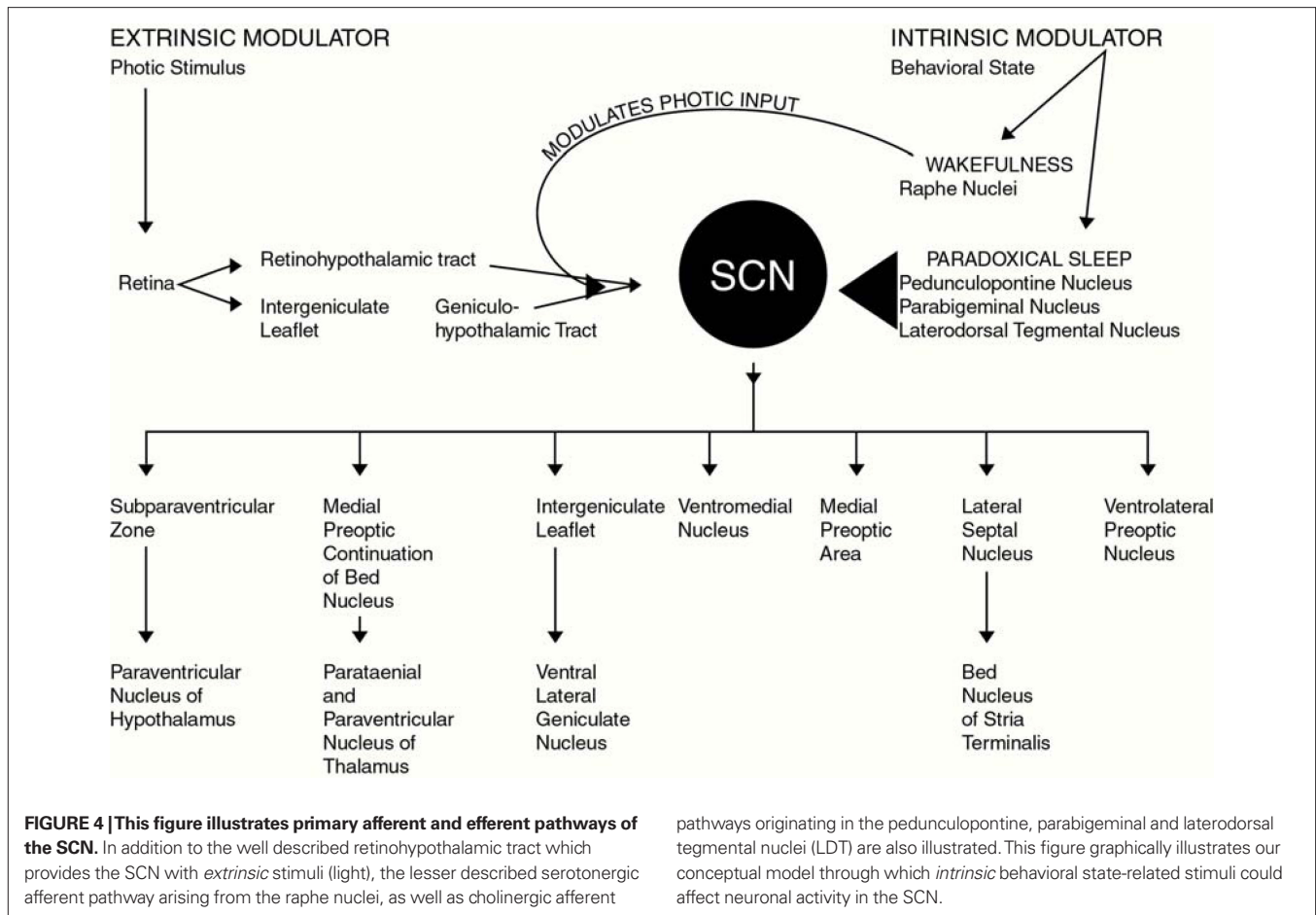
by the occurrence of PS. In addition, our findings converge with those of Torterolo et al. (2009) who reported (in Table 1 of their publication) that *c-fos* positive cells in the rostral SCN totaled  $83.4 \pm 25.6$  during NREM sleep. But, during carbachol-induced REM, that number was reduced to  $9.6 \pm 2.7$ .

Photomicrographs presented in Figure 3 demonstrate the topographical specificity of PS suppression of *c-fos* protein expression within the SCN. As seen in this figure, the ventral area exhibits the greatest reduction in *c-fos* protein expression following PS.

When considering the neural substrates that may contribute to this finding, it is useful to consider those brain regions that: (1) project to and innervate the SCN and; (2) exhibit behavioral state-dependent neural activity, and also; (3) are implicated in behavioral state, particularly PS, control. Those regions and their projections to the SCN are illustrated in Figure 4.

The SCN receives the densest serotonergic input of any hypothalamic nucleus (Bosler and Beaudet, 1985). Release of serotonin into the SCN may be modulated by sleep-wake state to either facilitate or





disfacilitate overall neuronal activity levels, and subsequently *c-fos* protein expression (Pickard et al., 1996). A reduction of serotonergic activity that accompanies the onset of PS may lead to a coincident reduction of *c-fos* activity. This mechanism is purely speculative, however, since we did not measure or quantify serotonin activity within the SCN during PS.

Acetylcholine would appear to have a critical role in the PS-specific reduction in *c-fos* protein expression that we observed. Projections originating within the pedunculo-pontine (PPN) and laterodorsal tegmental nuclei (LDT), which terminate within the SCN (Bina et al., 1993; Rye, 1997) may provide a pathway through which cholinergic inhibition of neuronal activity could occur (Liu et al., 1997). Cholinergic activity in the PPN and LDT increases during PS (Shiromani et al., 1995; Thakkar et al., 1998) and those PPN and LDT neurons involved with the onset and propagation of PS may also be the same which project to the SCN. There, they may potentially suppress neuronal activity and subsequent *c-fos* protein expression. Consistent with this, *in vitro* SCN slice preparations demonstrate acetylcholine's inhibitory effect on neuronal discharge (Liu and Gillette, 1996). While PS-specific cholinergic inhibition of the circadian pacemaker is only conjectural, it could explain one role of muscarinic receptors in the SCN, particularly since they appear not to participate in transmission of photic stimuli (Takeuchi et al., 1993; Challet et al., 1997).

Our findings further confirm speculation that *c-fos* protein product within the SCN is not only also modulated by extrinsic stimuli such as ambient illumination, but also by intrinsic stimuli, specifically related to PS. Whereas ambient illumination increases neuronal activity and subsequently *c-fos* protein expression, PS appears to dampen neuronal activity and *c-fos* protein expression. Accordingly, we speculate that PS might function as a mechanism to reduce neuronal activity within the SCN, and thereby influence the expression of circadian rhythms (i.e., body temperature and blood pressure (Sano et al., 1995) during sleep. Although plausible substrates through which wakefulness and sleep affect circadian pacemaker do exist (Figure 4), the cellular mechanisms through which this could occur are in need of further elucidation.

#### LIMITATIONS

While our findings concur with other those from other investigators who have employed *c-fos* protein expression as a marker of neuronal activity during wakefulness and sleep (Tortorolo et al., 2009), this technique is not without procedural and interpretative limitations. First, during immunohistochemical processing, variations in primary, secondary, or tertiary antibody concentrations could lead to differences in staining of *c-fos* positive cells. To minimize potential confounds associated antibody concentrations; immunohistochemical processing was performed by the same

individual who “batch-processed” groups of eight animal brains to reduce the potential for variations in antibody concentrations and incubation times.

In addition to the immunohistochemical components of this study, potential issues surrounding data analysis and interpretation were also considered. Specifically, to ensure consistency in counting of *c-fos* positive cells, all scoring was performed by the same individual. As an independent measure of quality control, a subset of slides were presented to the scorer on at least two separate occasions, occurring on separate days. Repeat scoring of the same tissue sections typically yielded values within 8.0–10.0% of previously scored values (data not shown). Finally, an emphasis was placed on selecting sections of SCN with comparable rostral-to-caudal coordinates. This was achieved by noting the presence and shape of the optic chiasm, third ventricle, as well as the shape and size of nearby hypothalamic nuclei such as the paraventricular nucleus.

Despite procedural and interpretative limitations associated with measuring *c-fos* protein expression, we felt that this approach outweighed limitations inherent with techniques such as single cell recordings. We appreciate that measuring the synchronicity between the onset and duration of PS with subsequent *c-fos* expression is not as precise as measuring changes in single cell firing patterns during PS. However, an inherent problem with single cell recordings is adjacent neurons can behave quite differently from the cell being recorded, and those differences will be remain undetected. For example, Lee describes that during the daytime, the clock gene *Per1* is active in the ventrolateral portion of the SCN but it is quiescent within the dorsomedial portion of the nucleus (Lee et al., 2009). Single cell recording methodology may not have had the ability to demonstrate those differential levels of activity between neurons within the same discrete nucleus (Lee et al., 2009).

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