NEURAL CONTROL OF CIRCADIAN RHYTHMS

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OC optic chiasm

PVN paraventricular nucleus of the hypothalamus

SCN suprachiasmatic nucleus

SON supraoptic nucleus

3V third ventricle

τ period length

arrows on the photomicrographs indicate glial scar tissue

arrows on the event records indicate day of surgery

calibration bars on the photomicrographs represent 100 μm

ABSTRACT

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The suprachiasmatic nucleus (SCN) of the hypothalamus and its efferent projections are necessary for the generation of circadian rhythms. To further investigate the role of SCN connections in the generation of behavioral rhythms, male Long-Evans rats were housed in constant conditions and given horizontal knife cuts aimed dorsal to the SCN, bilateral parasagittal cuts lateral to the SCN, or sham surgery. Rhythms in locomotor activity and drinking behavior were monitored using a microcomputer. Horizontal cuts that spared the SCN failed to abolish rhythms. Effects of horizontal cuts that damaged the SCN ranged from changes in period length of the rhythms to abolition of drinking rhythms. Parasagittal cuts did not damage the SCN or abolish rhythms. These and previous results indicate a functional redundancy in SCN efferent connections and that neural circuits within the SCN may be more important than SCN efferent connections in the generation of behavioral circadian rhythms.

INTRODUCTION

Animals display daily fluctuations in a wide variety of behavioral and physiological functions. These daily variations have been called circadian rhythms (from the Latin words circa--about and dies--day) and persist even under constant environmental conditions. The persistence of "free-running" ryhthms in the absence of external time cues implies that the animal has an endogenous oscillator or "biological clock". Normally the timekeeping mechanism is synchronized by the light/dark cycle and the rhythm shows a period (average time between activity onsets on consecutive days) of 24 hours. However, in the absence of external time cues, the period of the rhythm is close to but usually not exactly 24 hours.

Total or nearly total lesions of the suprachiasmatic nucleus of the hypothalamus (SCN) in rodents abolish free-running circadian rhythms in a variety of behavioral and physiological functions (Moore & Eichler, 1972; Stephan & Zucker, 1972; Moore & Klein, 1974). Anatomical studies have revealed that SCN neurons send efferent projections in caudal, lateral, dorsal, and rostral directions (Stephan et al., 1981; Berk & Finkelstein, 1981; Swanson & Cowan, 1975). Hypothalamic knife cuts which cut all of these projections

(i.e. surgical isolations of the SCN) are equivalent to SCN lesions in that they abolish rhythms in drinking, activity, sleep, and brain temperature (Stephan & Nunez, 1977). Furthermore, Inouye and Kawamura (1979) found that after isolation of the SCN from the rest of the brain, circadian rhythms in multiple unit activity persisted within an "island" of tissue containing the SCN but not in other areas of the hypothalamus.

More selective hypothalamic cuts have also been used to elucidate the functional significance of different SCN efferent projections. Retrochiasmatic cuts (placed caudal to the SCN) in a coronal plane disrupt rhythms in adrenal corticosterone secretion and pineal serotonin N-acetyltransferase activity (Moore & Eichler, 1972; 1976; Moore & Klein, 1974) but not drinking (Nunez & Stephan, 1977), feeding (Nishio et al., 1979), or locomotor activity (Nunez & Casati, 1979). It has also been shown that coronal cuts anterior to the nucleus and bilateral parasagittal cuts do not abolish the nocturnal pattern of drinking in rats kept on a light/dark cycle (Nunez & Stephan, 1977). However, persistence of the nocturnal pattern of drinking in animals with anterior or parasagittal cuts could have been due to a "masking" effect of light, as these animals were never observed under constant conditions. Additionally, the parasagittal cuts used in previous experiments were shallow and fibers projecting dorso-laterally from the SCN may have survived the cuts. Nunez and Stephan (1977) reported that

knife cuts which partially isolated the nucleus, severing fibers projecting dorsally, laterally, and caudally, but not rostrally abolished rhythms in drinking and activity in rats housed under a light/dark cycle or in constant dim light. Dark (1980), however, reported that similar partial isolation of the SCN did not abolish entrainment of drinking to a light/dark cycle or phase shifting when the light/dark cycle was phase delayed by four hours but did abolish free-running drinking rhythms when the rats were placed in constant dim light. From these results it can be concluded that: (i) the SCN is necessary for the generation of normal circadian rhythms, (ii) rhythms are mediated by SCN efferent projections, (iii) rostral projections from the SCN alone are not sufficient to maintain behavioral rhythms in constant environmental conditions.

The evidence indirectly suggests that either there is functional redundancy in the circuitry mediating behavioral rhythms (i.e. the information is carried by more than one set of fibers and it is not necessary for all of them to be intact to maintain rhythms) or behavioral rhythms are mediated by dorsal or dorso-lateral projections from the SCN. These conclusions, however, are tentative as no one has yet reported observations on animals with cuts that interrupt only dorsal projections from the SCN or on animals housed in constant conditions after receiving bilateral parasagittal cuts. The goal of the research presented here was to determine the effects of deep parasagittal cuts and

dorsal cuts on free-running behavioral rhythms in order to provide evidence to evaluate these tentative conclusions, and clarify the hypothalamic circuitry involved in the endogenous generation of behavioral rhythms.

EXPERIMENT 1

To determine whether fibers projecting dorsally out of the SCN are critical for the generation of activity and drinking rhythms, knife cuts in a horizontal plane aimed dorsal to the SCN were made in rats housed in constant dim light and rhythms of the animals were monitored before and after surgery. In additional animals, bilateral parasagittal cuts were made to determine whether laterally or dorsolaterally projecting efferents of the SCN are critical for the generation of free-running rhythms.

Method

Subjects and Housing

Male Long-Evans rats (Blue Spruce Farms) were individually housed in cages with access to activity wheels, and given ad lib access to food and water. These animals were housed under constant dim illumination. The light intensity, as measured by reflectance off of a white card using a Pentax Spotmeter, was 7 foot-candles. After 17-28 days of adaptation to these conditions, 16-48 days of baseline data were collected. After baseline data collection, ryhthms of the animals were monitored for 20-25 consecutive days following the different surgical manipulations.

Data Collection and Analysis

Drinking spout licks were measured using an optoelectronic drinkometer composed of an infrared light emitting diode (LED) aimed at a phototransistor. The phototransistor and LED were placed beside the end of the drinking spout such that the tongue of the animal interrupted the light beam during each spout lick. Activity was monitored by a similar LED/phototransistor pair placed in a position such that a cam on the axle of the wheel interrupted the light beam once during each wheel revolution.

The phototransistors were connected to optoisolated controller boards (Mullen Computer Products) inside a 64K Northstar Horizon computer. The computer also contained a real time clock board (Mountain Hardware, Inc.). A program written in BASIC, with assembly language subroutines for time-critical functions such as accessing the clock and reading the input ports, was used to count responses per 12 minute interval. Each day 119 intervals (23 hours, 48 minutes) of data were collected and then written on 5.25 inch floppy disks (Verbatim Corporation) for permanent storage. Data for interval 120 of each day were not collected but were estimated by averaging interval 119 with interval 1 of the next day.

For visual inspection of the records, the data were "doubleplotted". This procedure involves printing the data for a 48-hour period on each line and repeating the second

half of the line on the first half of the next line. Therefore, consecutive days follow each other vertically with each day repeated. Responses were added over 3 consecutive 12 minute intervals and total responses for each 36 minute interval were represented by one printed character. For activity records, a dark rectangle was printed for each 36 minute interval in which there were 3 or more responses while a blank space was printed if there were fewer than 3 responses. For drinking records, a threshold of 15 responses/36 minutes was used.

For further analysis, individual records were divided into blocks of 10 days each. A chi-square periodogram (Sokolove & Bushell, 1978) was used to test for the presence of rhythms in the circadian range (23-26 hours). For each test period, P, within the range tested, the periodogram program yields a statistic, Q_p, which is related to the root mean square amplitude of oscillations in the data set at a period of P. To aid in interpretation of the data, plots of Q_p versus P are made. In a "monte carlo" study, Sokolove and Bushell determined that for rhythmic data such a plot shows a peak Q_p value at a test period at or near the period of the cyclicity in the data while for data that are random with respect to time, Q_p distributes approximately as a chi-square with P-1 degrees of freedom. This provides a convenient significance test for the presence of rhythms. An interplolation procedure (Enright, 1965) was used to obtain Q_p values at a resolution of 0.1 hours. In many cases

the animals showed rhythms that were readily distinguishable visually and appeared to have clear onset and termination of activity with a stable period length. When this occured the periodograms generally yielded clearly distinguishable significant peaks at test periods that seemed to correspond well with the "visually apparent" period. An example of a doubleplot and corresponding periodogram of this type of data is shown in Figure 1A. In other cases, blocks of the data appeared to be arhythmic or to have low amplitude rhythms with high amplitude "noise" (i.e. noncircadian) components. As illustrated in Figure 1B., periodograms computed on "noisy" data showed a reduced peak or no peak at all. In a few cases the data visually appeared to have clear rhythms but with multiple components such as different periods for onset and termination of activity. When this occured, periodogram analysis often resulted in multiple peaks as shown in Figure 1C. In these cases a second estimate of period length was obtained by visually obtaining and drawing a best fitting straight line across the onsets and terminations of activity. In these cases the period of the rhythm was defined as the test period of the periodogram peak that most closely corresponded with the visual estimate based on onset of activity. For each 10 day block of data, the average number of responses per 12 minute interval was also computed by the periodogram program, and postsurgical activity and drinking levels were computed as per cent of baseline.

Figure 1: Representative doubleplotted event records and periodograms for data sets containing: A, high amplitude rhythms with low amplitude "noise" components; B, low or zero amplitude ryhthms; and C, multiple circadian periodicities.

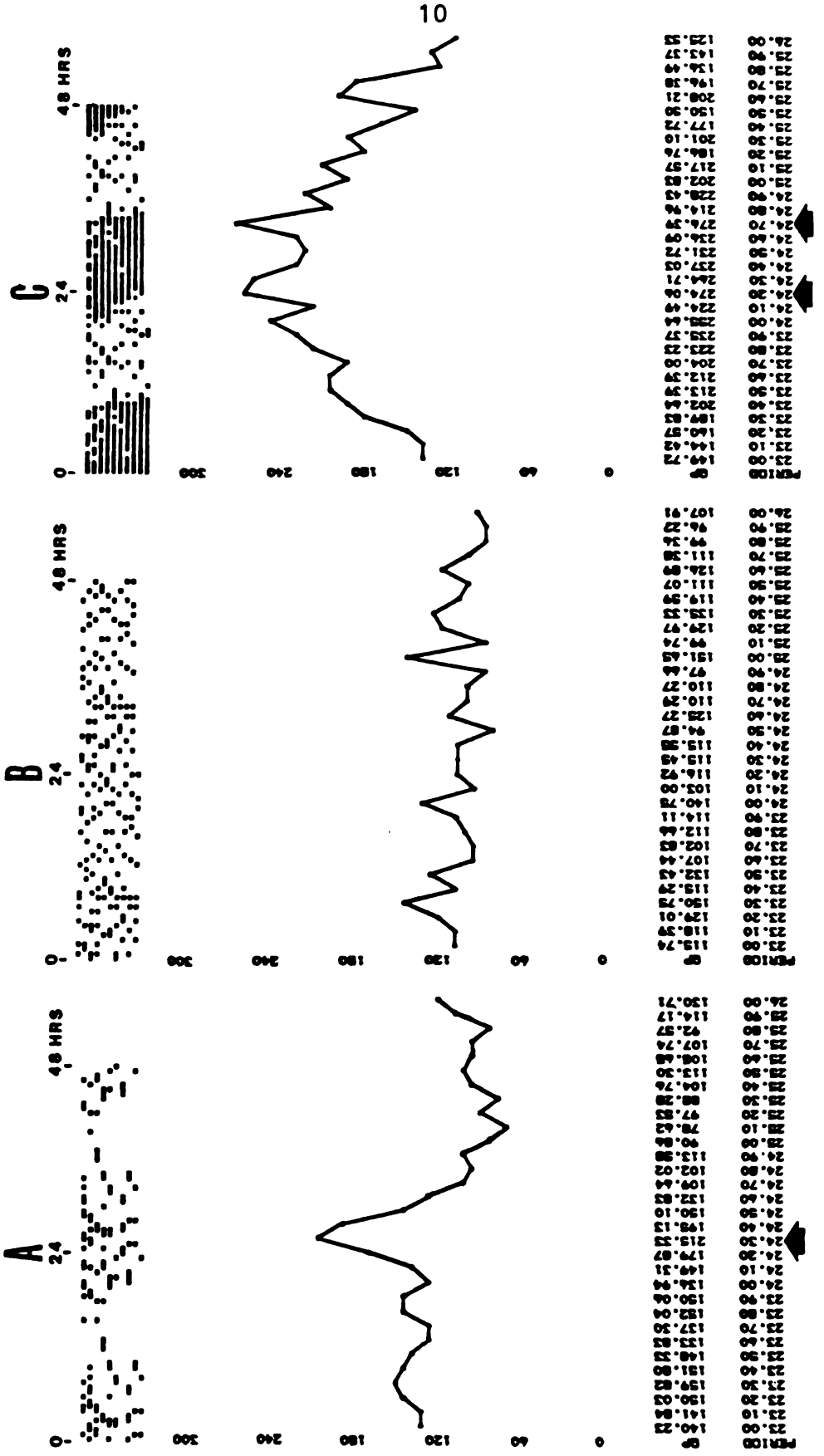


Figure 1.

Surgery

Each animal was randomly assigned to one of the following surgical conditions.

Horizontal knife cuts. After baseline data collection, surgery was performed under sodium pentobarbital (Nembutal, Abbott Laboratories) anesthesia (40 mg/kg body weight, injected intraperitoneally) The surgery involved placing the rats in a stereotaxic apparatus (Kopff Instruments) then removing a flap of skull, retracting the superior sagittal sinus and lowering a Scouten (Scouten et al., 1981) retractable wire microknife through the brain to a point aimed midway between the SCN and the paraventricular nucleus of the hypothalamus (PVN). The stereotaxic coordinates were 2 or 1.5 mm posterior to the bregma and 7 or 7.1 mm ventral to the dura at the midline of the animal with the incisor bar 7.5 mm below ear bar zero. The wire was then extended 2 mm from the barrel and the whole assembly rotated 360^{o} in each direction to make a circular cut aimed at severing all of the dorsal projections from the SCN. Animals were then observed for a minimum of two weeks to determine whether the cut disrupted rhythms in drinking and activity.

Bilateral parasagittal knife cuts. After baseline data collection, surgery aimed at cutting lateral projections was performed on a second group of animals. The knife was lowered to the base of the brain 3 mm posterior to the bregma and 1 mm lateral to the midline on each side of the

animal. The blade was then extended 2 mm in the rostral direction and raised 3 mm to make a deep cut in a parasagittal plane. Animals were then observed for a minimum of two weeks to determine whether this type of cut had any effect on free-running rhythms.

Sham surgery. Sham surgery consisted of making an incision through the skin and tissue on top of the skull and suturing this wound.

Histology

At the end of the experiment, all lesioned animals were sacrificed by overdose of sodium pentobarbitol and perfused transcardially with physiological saline followed by 10% (w/v) formaldehyde. The brains were removed and stored in 10% formaldehyde/30% (w/v) sucrose. Frozen 50 μm coronal sections were then made of the preoptic area and anterior hypothalamus. Every other section was mounted and stained with cresyl violet acetate and the slides coverslipped. A Zeiss microscope equiped with a camera system was used to verify the location of the lesions. For presentation of histological results, selected sections were photographed using Kodak Technical Pan Film 2415. Prints of these sections were made on Kodak Polycontrast Rapid II paper.

Results

With the exception of 2 animals (see below), the rats exhibited clear circadian rhythms in drinking and activity before surgery. The results presented here are for animals that recovered from the brain surgery and for which

histological evaluation of the damage was possible. After surgery, activity levels were reduced in 11 out of these 12 animals. Postsurgical activity and drinking responses expressed as per cent of baseline are presented in Table 1. Results of the periodogram analysis for selected 10 day blocks of activity and drinking records are presented in Table 2.

Sham Surgery (n=3)

Activity and drinking data for a representative sham-operated control rat are presented in Figure 2. All of the sham-operated control animals exhibited clear rhythms in drinking and activity before surgery. Two of the animals became hypoactive after surgery and statistical verification of postsurgical activity rhythms was not possible. Visual inspection of the activity records however, revealed bouts of activity with circadian periodicities and in phase with the presurgical rhythms. While the third sham-operated rat showed a relatively low activity level, it displayed an activity rhythm that was readily apparent visually although the periodogram peak for the presurgical data sample was not statistically significant. Periodogram peaks for the postsurgical activity record of this animal were statistically significant. Drinking records for all of these animals showed rhythms with circadian periodicities that were evident both visually and by significant periodogram peaks. In all of these cases the drinking rhythms persisted after surgery with no changes in period length.

TABLE 1: POSTSURGICAL LEVELS OF ACTIVITY* AND DRINKING EXPRESSED AS PER CENT OF BASELINE

TYPE OF SURGERY	ANIMAL NUMBER	ACTIVITY FIRST 10 DAYS	ACTIVITY SECOND 10 DAYS	DRINKING FIRST 10 DAYS	DRINKING SECOND 10 DAYS
HZ	101	14.64*	11.46*	188.27	159.98
HZ	102	28.65	18.56*	450.51	164.72
HZ	110	64.62*	56.00	132.92	28.31
HZ	112	15.59*	3.97*	101.93	113.78
HZ	113	43.02	48.04	127.25	151.44
HZ	115	119.55	27.82	109.47	129.49
PS	108	38.56*	22.72*	106.31	29.94
PS	109	26.93	19.77	209.87	117.74
PS	111	20.91*	-----	104.86	97.23
PS	113**	103.45	-----	120.66	-----
PS	115**	121.30	-----	57.05	-----
SH	105	41.49*	-----	122.49	95.33
SH	106	11.77*	0.47*	232.37	351.66
SH	114	62.58	56.13	119.45	84.96

* Activity levels were weighted according to the number of days that the animals ran.

** A 10 day sample of data after horizontal cuts were made was used in calculating post-parasagittal cut data.

TABLE 2: RESULTS OF CHI-SQUARE PERIODOGRAM ANALYSIS OF SELECTED 10 DAY BLOCKS OF ACTIVITY AND DRINKING RECORDS

TYPE OF SURGERY	ANIMAL NUMBER	ACTIVITY						DRINKING					
		DAYS 1-10		DAYS 18-27		DAYS 29-37		DAYS 1-10		DAYS 18-27		DAYS 28-37	
		τ	Q_p	τ	Q_p	τ	Q_p	τ	Q_p	τ	Q_p	τ	Q_p
HZ	101	24.6	223.25	24.4	205.44	----	------	24.6	242.29	24.6	137.10*	24.5	153.28*
HZ	102	24.3	270.43	23.6	142.29*	23.0	130.01*	24.0	202.87	23.9	129.22*	23.1	174.48
HZ	110	24.1	258.81	25.7	188.39	24.1	163.32*	24.1	186.48	----	**	----	**
HZ	112	24.7	233.07	24.8	203.62	25.8	196.82	24.8	236.61	24.7	137.95*	24.6	176.51
HZ	113†	24.9	235.01	24.1	276.25	24.2	185.58	----	**	24.6	144.96*	24.5	165.13
HZ	115†	25.4	208.48	----	**	25.1	177.22	25.3	172.23	24.2	160.91*	24.6	186.84
SH	105	24.7	173.97	----	------	----	------	24.0	196.68	24.0	166.38	24.0	213.73
SH	106	24.8	490.94	----	------	----	------	24.7	234.32	24.7	189.70	24.5	241.63
SH	114†	----	------	24.4	229.79	24.4	260.53	24.7	191.92	24.9	178.33	24.5	185.73
PS	108	24.3	341.98	24.3	337.81	24.7	289.53	24.3	216.8	24.6	157.85*	24.4	223.93
PS	109	24.0	385.18	24.9	205.08	24.7	243.17	24.3	223.93	----	**	24.3	162.59*
PS	111	----	**	----	------	----	------	24.5	280.57	24.7	212.39	24.8	214.30
PS	113§	24.2	185.58	24.6	190.14	----	------	24.5	165.13	24.8	161.89*	----	------
PS	115§	25.1	177.22	24.9	112.47*	----	------	24.6	186.84	24.8	152.83*	----	------

Q_p values were significant at $P < 0.05$ except as otherwise noted.
Surgery was performed on days 16-17 except as otherwise noted.

* Q_p not significant at $P = 0.05$

** No salient peaks were found in the periodgram.

† Baseline data were taken from days 37-46. Surgery was performed on day 48 Data from days 50-59 and 60-69 were used as the first and second postsurgical samples.

§ A 10 day sample (days 60-69) of data collected after horizontal cuts were made was used as baseline for bilateral parasagittal cuts. Surgery was performed on day 72 and a sample of data from days 74-83 was used for postsurgical analysis.

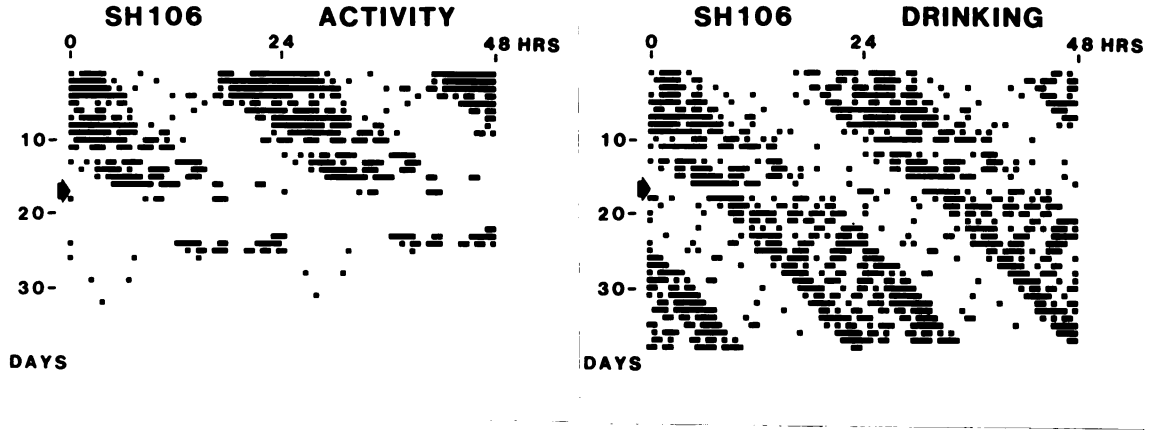


Figure 2: Doubleplotted activity and drinking records for a sham-operated control rat (arrows on these and subsequent event records indicate day of surgery).

Bilateral Parasagittal Knife Cuts (n=3)

Histological results on all of these animals indicate that the knife cuts were bilateral and located lateral to the SCN but medial to the SON on each side of the animal. The glial scar tissue does not appear to be present in sections taken through the rostral pole of the SCN thus efferents projecting laterally from the rostral 1/3 of the SCN may have been spared. In at least one case there appears to be evidence of damage to the optic chiasm and optic tract as shown in Figure 3.

Activity data for one animal were omitted from the analysis as this animal did not run enough to permit statistical treatment of the data. In the other two cases, activity rhythms were clearly present after surgery as evidenced by both visual inspection and significant periodogram peaks. As illustrated in Figure 3, the animal which sustained damage to the optic chiasm and optic tract showed an increase in the period length of the rhythm after surgery. In two cases the drinking rhythms became noisy after surgery. Although visually the rhythms appear to have persisted, periodogram analysis did not reveal statistically significant circadian periodicities in the first 10 postsurgical days of these records, but did for the second 10 postsurgical days. The drinking rhythm of the third animal clearly persisted after surgery.

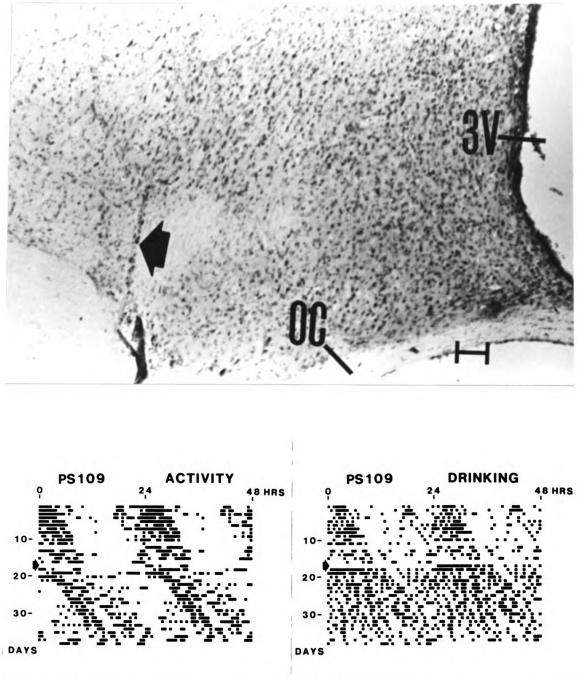


Figure 3: Photomicrographic representation of the location of a parasagittal knife cut that damaged the optic chiasm and activity and drinking records showing an increase in period length of the rhythms after surgery.

Horizontal Knife Cuts (n=6)

In three of these six cases (101, 102, 112) the cuts damaged the dorsal part of the SCN while in a fourth case (110) the cut was more ventral, lying approximately in the middle of the dorso-ventral extent of the SCN. Histological and behavioral data for a representative animal with damage to the dorsal part of the SCN are presented in Figure 4. Postsurgical activity levels were reduced in these animals making it difficult to evaluate activity rhythms. Periodogram analysis, however, resulted in significant peaks for the first 10 postsurgical days in 3 of these 4 cases. Visually, the drinking records of these animals appeared to be arhythmic for at least the first 10 days after surgery and periodograms for the first 10 postsurgical days of data did not contain significant Q_p values in any of these cases. One animal (112) showed a partial recovery of rhythmicity begining about 11 days after surgery. Periodogram analysis of this block of data (postsurgical days 11-20) yielded a significant Q_p value confirming the results of visual inspection. Two other cases (101, 102) showed less recovery of circadian rhythmicity begining about 11 days after surgery. Again periodogram analysis is in agreement with visual inspection of the drinking records. For rat number 101 the Q_p value increased for the second 10 postsurgical days but not enough to reach statistical significance while for rat number 102 a significant Q_p value was obtained but at a much shorter period than would be predicted on the

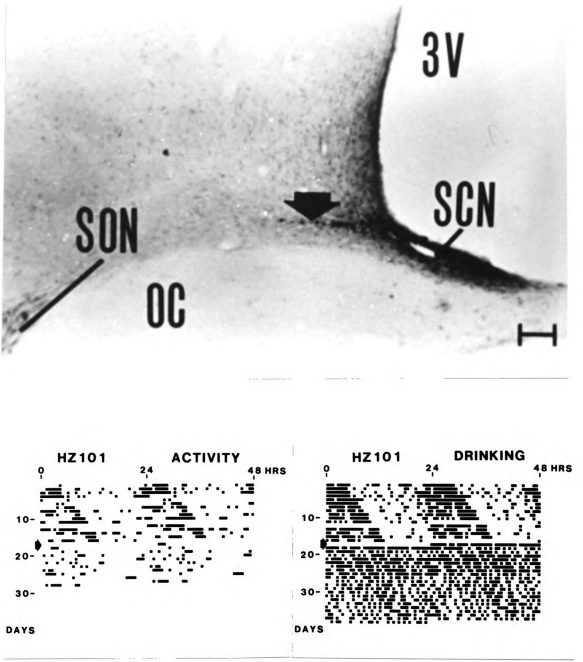


Figure 4: Photomicrograph of the glial scar resulting from a horizontal cut that damaged the SCN and activity and drinking records showing a loss of rhythmicity after surgery.

basis of visual inspection. The fourth case (110) visually appeared to be largely arhythmic throughout the postsurgical drinking record and significant periodogram peaks were not obtained for any portion of the postsurgical drinking record.

On the basis of these results two additional cases of horizontal cuts were added with the aim of avoiding direct damage to the SCN. Twenty-five days after receiving the horizontal cuts these two animals were given bilateral parasagittal cuts. In one of these animals, the horizontal cut was located dorsal to the SCN and did not damage the nucleus while in the other rat, the horizontal cut was through the ventral portion of the SCN. In both cases, the parasagittal cuts were located similarly to those in the animals that received parasagittal cuts alone. Visual inspection of activity and drinking records for these animals indicated that both of them showed activity and drinking rhythms before surgery and after both surgical manipulations although for portions of the postsurgical records, significant periodogram peaks were not obtained. Histological and behavioral data for the rat in which the horizontal cut spared the SCN are presented in Figure 5.

Figure 5: Schematic representation of the location of combined horizontal and bilateral parasagittal cuts and activity and drinking records showing that neither cut abolished the rhythms.

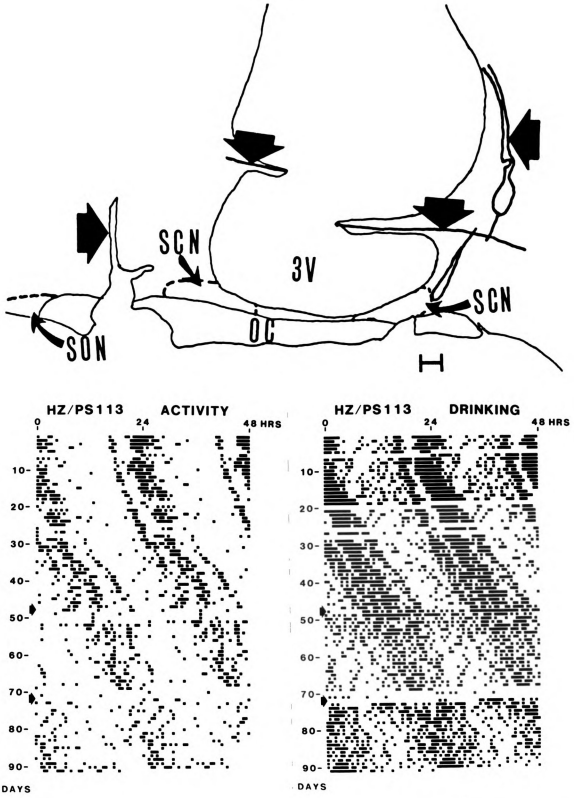

SCN
3V
SCN
SON
OC
H
HZ/PS113 ACTIVITY
0
24
48 HRS
10
20
30
40
50
60
70
80
90
DAYS
HZ/PS113 DRINKING
0
24
48 HRS
10
20
30
40
50
60
70
80
90
DAYS

Figure 5.

Discussion

In 11 out of 12 animals, activity levels were reduced after surgery. Activity levels are reduced in castrated male rats and can be restored by injections of estradiol benzoate or testosterone propionate (Roy & Wade, 1975). Thus, the reduction of activity after surgery may have been due to a decrease in testosterone levels. Reduced activity levels have also been reported in rats following SCN lesions (Stephan & Zucker, 1972) and isolation of the SCN (Stephan & Nunez, 1977). Since in the present experiment, postsurgical activity levels were also reduced in sham-operated control rats, it is unclear whether the effect was due to brain damage.

Parasagittal knife cuts did not damage the SCN or abolish circadian rhythms although in one rat the cut damaged the optic chiasm and resulted in increased period length of the rhythms after surgery. For most nocturnal species, the free-running period length of rhythms exhibited by animals housed in constant light is directly related to light intensity--i.e. period length increases with increasing light intensity (Aschoff, 1960). Thus, in the rat that showed an increased period length after surgery, the cuts mimicked the effect of increased light intensity. This may have been due to damage to retinal input to the SCN. While the drinking records of two of these rats showed an increase in noncircadian components after surgery, the rhythms clearly persisted after surgery.

In four cases the horizontal cuts damaged part of the SCN and abolished drinking rhythms for at least 10 days after surgery. In at least one case, partial recovery of rhythmicity occured by postsurgical day 11 and continued until postsurgical day 21.

In 2 animals bilateral parasagittal cuts that did not damage the SCN were made after horizontal cuts. In one of these rats the horizontal cut was dorsal to the SCN and did not damage the nucleus while in the other rat the horizontal cut was through the ventral part of the SCN. In neither case did the cuts abolish the ryhthms. In summary, the results suggest that direct damage to the SCN disrupts behavioral rhythms but that selective destruction of specific efferent projections from the nucleus is not sufficient to produce significant effects on these rhythms.

EXPERIMENT 2

To further examine the differential effects of knife cuts that damaged the SCN versus those that did not, additional cases were prepared. These additional animals were blinded to control the amount of retinal input reaching the SCN. To maintain constant, high levels of testosterone and thus facilitate assessment of the effects of knife cuts on activity rhythms, the animals were also castrated and given testosterone replacement.

Method

Housing, data collection and analysis, and histological procedures were the same as in Experiment 1.

Surgical Procedures

Under methoxyflurane (Metofane, Pitman-Moore, Inc.) anesthesia the eyes of the animals were removed. Castrations were then performed via a single scrotal incision. The animals were given testosterone replacement in the form of a 3 cm. long segment of 0.125 in. o.d., 0.062 in. i.d. Dow Corning Silastic medical grade tubing filled with crystalline testosterone propionate (4-androsten-17β-ol-3 one propionate, Stearaloids, Inc.) and sealed at each end with wooden plugs covered by Silastic silicone sealer. Incubation of the capsules was accomplished by implanting them subcutaneously in a rat for which behavioral data were

not collected. After a 24 hour incubation period, the capsules were removed and then were individually implanted in the animals for which behavioral data were to be collected. Each subcutaneous implantation was made through an incision on the dorsal surface of the animal. After a 24 hour recovery period the animals were placed in activity wheels and allowed to habituate to the housing conditions for 14-27 days before collection of 16-18 days of baseline data.

After baseline data collection, animals received horizontal or bilateral parasagittal cuts or sham surgery. The surgical procedures were similar to those in Experiment 1 except that the stereotaxic coordinates were adjusted in attempt to avoid damage to the SCN and SON and the horizontal cuts were made by projecting the knife perpendicular to the midline on one side of the animal to puncture the wall of the third ventricle then rotating it 90^{o} in one direction and then 180^{o} in the opposite direction. The knife was then retracted, rotated and the cut repeated on the opposite side of the animal. Stereotaxic coordinates for the horizontal cuts were 1.5 mm posterior to the bregma and 7.1-7.8 mm ventral to the dura at the midline of the animal. For the parasagittal cuts the coordinates were 2.5 mm posterior to the bregma and 0.7 mm lateral to the midline on each side of the animal. Postsurgical data were collected for 19-109 days before the animals were sacrificed and the brains prepared for histological

evaluation according to the procedure outlined in Experiment 1. To evaluate the efficacy of the testosterone implants, both seminal vessicles of each animal were removed immediately prior to perfusion and weighed to the nearest 0.0001 g on a Mettler analytical balance. Seminal vessicles were also removed from 3 of the animals from Experiment 1 for comparison.

Results

With the exception of 2 animals, the rats exhibited clear circadian rhythms in activity and drinking before surgery. The results presented here are for animals that recovered from surgery and for which histological evaluation of the damage was possible. Animals that were sacrificed within 98 days of testosterone replacement had relatively normal sized to slightly hypertrophied seminal vessicles (0.538 ± 0.0700 % of body weight, mean$\pm$S.E.M.) as compared to gonadally intact (0.292 ± 0.0250) animals. One animal that was sacrificed 177 days after castration and testosterone replacement had atrophied seminal vessicles (0.037 % body weight). While activity levels were reduced in 10 out of 14 animals after surgery, the reduction was not as pronounced as for the rats in Experiment 1. Seminal vessicle weights (expressed as per cent of body weight) and postsurgical activity and drinking responses (expressed as per cent of baseline) are presented in Table 3. Results of periodogram analysis on selected 10 day blocks of the activity and drinking records are presented in Table 4.

TABLE 3: SEMINAL VESSICLE WEIGHTS (EXPRESSED AS PER CENT OF BODY WEIGHT) AND POSTSURGICAL LEVELS* OF ACTIVITY AND DRINKING (EXPRESSED AS PER CENT OF BASELINE).

TYPE OF SURGERY	ANIMAL NUMBER	S.V.W.	ACTIVITY		DRINKING	
			FIRST 10 DAYS	SECOND 10 DAYS	FIRST 10 DAYS	SECOND 10 DAYS
HZ	202	0.678	49.64	41.29	115.14	136.02
HZ	204	0.642	48.79	66.24	93.66	111.79
HZ	208	0.982	77.54	82.38	139.15	111.84
HZ	212**	0.601	5.23	-----	30.22	------
HZ	213**	0.694	36.89	-----	79.76	------
HZ	216**	0.037	49.48	-----	132.06	------
HZ	219	-----	45.84	59.05	116.67*	80.30
HZ	220	0.284	7.78	4.15	72.29*	56.24*
HZ	221	0.267	25.69*	10.91	103.00*	86.42
HZ	225	-----	252.76*	313.43	74.42*	120.29
PS	205	0.373	50.41*	47.16*	675.96	134.01
PS	206	0.279	53.73	75.41	94.13	77.46
PS	210	0.584	82.83	101.98	117.35	191.06
SH	212	-----	201.16	215.85	242.02	309.60
SH	213	-----	88.88	74.71	96.04	97.03
SH	216	-----	143.25*	128.36	95.61	91.90
SH	226	-----	79.20	97.03	46.04	5.52*

* Weighted to account for missing data.

** A 10 day sample of data after sham surgery was used as baseline data in calculations of post-horizontal cut levels.

TABLE 4: RESULTS OF CHI-SQUARE PERIODOGRAM ANALYSIS OF SELECTED 10 DAY BLOCKS OF ACTIVITY AND DRINKING RECORDS OF BLINDED RATS

TYPE OF SURGERY	ANIMAL NUMBER	ACTIVITY						DRINKING					
		DAYS 1-10		DAYS 18-27		DAYS 29-37		DAYS 1-10		DAYS 18-27		DAYS 28-37	
		τ	Q_p	τ	Q_p	τ	Q_p	τ	Q_p	τ	Q_p	τ	Q_p
HZ	202	24.1	701.17	24.2	312.28	23.9	366.19	24.0	264.99	24.2	360.84	23.8	348.08
HZ	204	24.5	664.59	24.3	302.28	24.6	604.29	24.7	276.39	24.2	163.22*	24.2	206.31
HZ	208	24.1	572.38	23.8	343.15	23.5	463.88	24.1	243.24	23.7	170.97	23.0	228.54
HZ	212†	24.2	404.22	24.2	217.28	----	------	24.5	317.59	24.2	164.81*	----	------
HZ	213†	24.1	870.72	24.2	375.42	----	------	24.0	320.6	24.4	192.64	----	------
HZ	216†	24.6	425.97	24.3	170.62	----	------	24.7	227.17	----	**	----	------
HZ	219	24.5	354.24	24.1	185.88	24.5	268.68	24.9	231.39	----	------	----	------
HZ	220	24.3	845.56	24.3	215.33	25.1	204.85	24.2	291.51	----	**	----	**
HZ	221	24.6	325.56	24.4	209.73	24.1	185.62	24.5	311.89	----	**	24.2	139.49*
HZ	225	24.5	286.32	24.1	368.56	24.3	280.33	24.9	163.89*	25.0	141.13*	24.0	162.72*
PS	205	24.0	606.96	24.2	292.74	24.3	410.81	24.1	290.36	----	**	----	**
PS	206	24.3	525.84	24.5	228.76	24.5	650.09	24.3	311.20	24.4	321.02	24.4	361.71
PS	210	24.4	222.54	24.3	210.19	24.1	239.81	24.4	158.37*	24.7	175.78	----	**
SH	212	23.9	330.24	24.1	301.20	24.2	404.22	24.3	250.32	24.1	184.12	24.5	317.59
SH	213	24.1	705.45	24.2	710.28	24.1	870.72	24.0	264.91	24.2	356.56	24.0	320.6
SH	216	24.5	716.58	24.6	669.93	24.4	737.40	24.5	571.40	24.6	555.92	24.3	433.26

Q_p values were significant at $P < 0.05$ except as otherwise noted.
Surgery was performed on days 16-18 except as otherwise noted.

* Q_p not significant at $P = 0.05$.

** No salient peaks were found in the periodgram.

† A 10 day sample of data (days 29-38) collected after sham surgery was used as baseline for horizontal cuts. For rats 212 and 213, horizontal cuts were made on day 49 and data from days 56-65 were used in the postsurgical analysis. For rat 216, surgery was performed on day 124 and data from days 111-120, and 125-134 were used for presurgical and postsurgical analysis, respectively.

Sham Surgery (n=4)

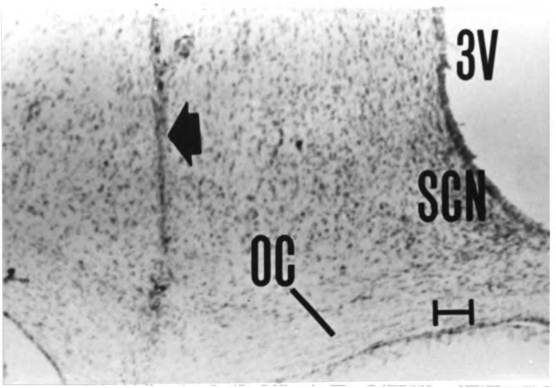
These animals showed significant rhythms in drinking and activity after surgery. Two of these (212, 213) received horizontal cuts 31 days after sham surgery and a third one (216) received a horizontal cut 107 days after sham surgery. The effects of brain surgery are presented below with the other cases of animals with horizontal cuts.

Bilateral Parasagittal Cuts (n=3)

Glial scars in the brains of these animals were very similar to those of the sighted animals (Experiment 1) that received parasagittal cuts, indicating that the cuts were bilaterally positioned medial to the SON and lateral to the SCN on each side of each animal and extending from the base of the brain about 3 mm dorsal. It appears that these cuts, like those of the previous experiment, may have spared laterally projecting efferents from the rostral 1/3 of the SCN. A photomicrograph of a brain section of an animal representative of this group is presented in Figure 6.

All of these animals showed activity rhythms after surgery with no changes in period length. Two of them (206, 210) also showed drinking rhythms after surgery. Activity and drinking records for a rat in which the cuts damaged the optic chiasm but did not result in changes in period length are presented in Figure 6. For the third animal, the presurgical drinking rhythm was not robust (i.e. lack of a significant peak on the periodogram analysis) and the effects of surgery on this measure could not be determined.

Figure 6: Photomicrograph showing the typical location of parasagittal cuts in blinded animals and activity and drinking records showing that this cut did not abolish the rhythms or change the period length of the rhythms.


3V
SCN
OC

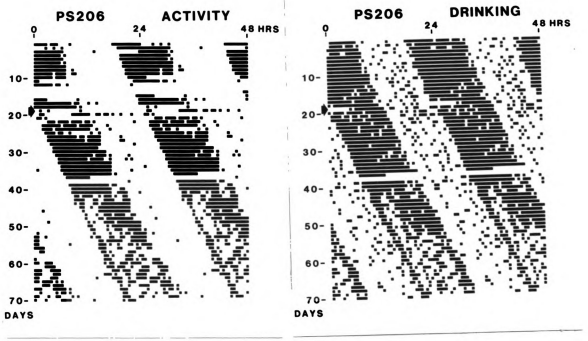

PS206
ACTIVITY
0
24
48 HRS
10-
20-
30-
40-
50-
60-
70-
DAYS
PS206
DRINKING
0
24
48 HRS
10-
20-
30-
40-
50-
60-
70-
DAYS

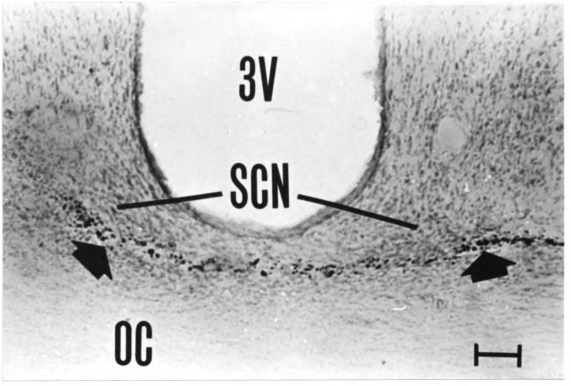
Figure 6.

Horizontal Cuts (n=8)

As previously mentioned, 3 animals received horizontal cuts at least 31 days after sham surgery. Five additional animals received horizontal cuts as their first or only surgical manipulation and survived long enough to permit assessment of postsurgical behavioral rhythms. These animals were divisible into two groups on the basis of whether the cuts damaged the SCN.

In one case (208) a glial scar was present extending bilaterally through the ventral portion of the SCN indicating that the cut separated the ventralmost part of the nucleus from more dorsal portions as shown in Figure 7. Visual inspection of the behavioral records (Figure 7) indicates that before surgery this animal had drinking and activity rhythms with a period greater than 24 hours. After surgery these free-running rhythms persisted but with a period less than 24 hours and disturbances in the records such as a possible 2-3 hour phase delay on day 30-31 of the drinking record. Periodogram analysis confirms these observations indicating significant 24.1 hour ryhthms in both behaviors before surgery and 23.0-23.8 hour cyclicities containing multiple components after surgery.

Bilateral damage to the SCN was also sustained in two other animals (220, 221). Histological results indicate that in rat 220 the damage was limited to the dorsal part of the SCN while in rat 221 the cut was more ventral passing through approximately the middle of the dorso-ventral extent



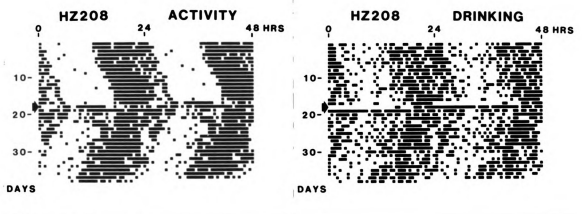


Figure 7: Photomicrograph depicting the scar tissue that resulted from a cut that damaged the ventral part of the SCN and activity and drinking records showing a decrease in period length of the rhythms after surgery.

of the nucleus. A photomicrograph of a brain section and activity and drinking records of one of these animals are presented in Figure 8. Both of these animals had statistically significant rhythms in drinking and activity before surgery. While the activity rhythms were not as pronounced after surgery, they were clearly present both visually and by periodogram analysis. Because of an equipment failure, drinking data for rat number 220 are not available for days 7-17 postsurgically. The drinking data that are available for these two animals however, did not display circadian rhythmicity either by visual inspection or by periodogram analysis for at least the first 10 days after surgery. After that time however, there appears to have been a partial recovery of rhythmicity in these records although no portion of the postsurgical drinking records showed a circadian pattern robust enough to reach statistical significance.

In four cases the glial scar tissue lies dorsal to the SCN and extends beyond the lateral edge of the SCN on both sides indicating that the nucleus was not damaged and that the cut probably severed most or all of the axons projecting dorsally from the SCN. A photomicrograph of a brain section and activity and drinking records of an animal that received sham surgery followed by a horizontal cut that did not damage the SCN are presented in Figure 9.

All of these animals showed very pronounced activity rhythms that persisted after surgery and yielded significant

Figure 8: Photomicrographic representation of the location of a horizontal cut that damaged the dorsal part of the SCN and event records showing that after surgery the activity rhythm persisted while the drinking rhythm was temporarily abolished.

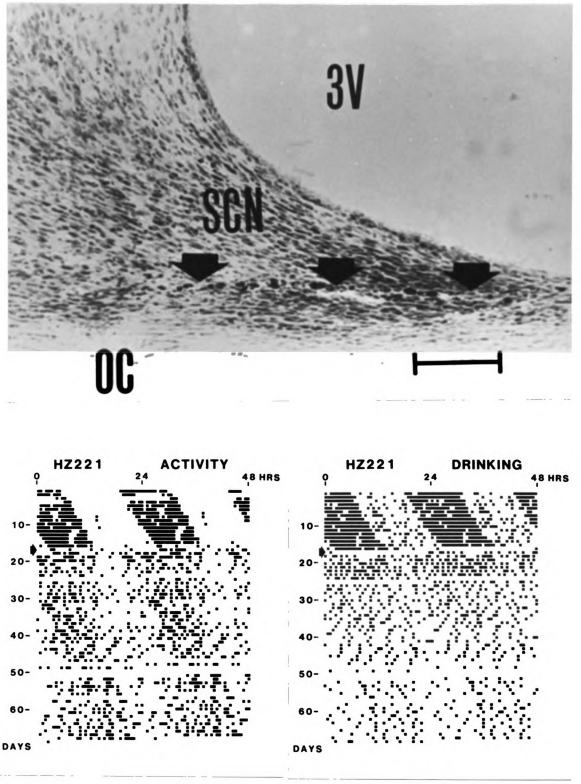
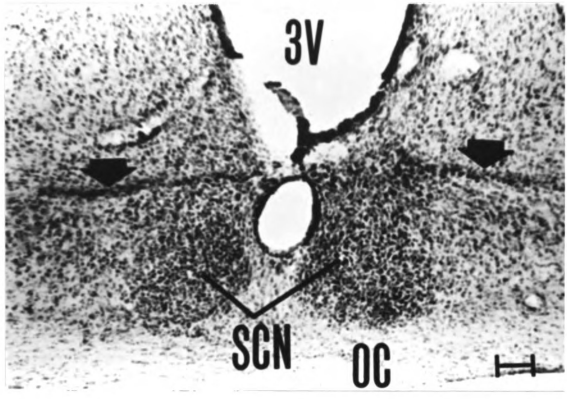

3V
SCN
OC
HZ221
ACTIVITY
0
24
48 HRS
10-
20-
30-
40-
50-
60-
DAYS
HZ221
DRINKING
0
24
48 HRS
10-
20-
30-
40-
50-
60-
DAYS

Figure 8.

Figure 9: Photomicrograph showing the location of a horizontal cut that did not damage the SCN and activity and drinking records showing that neither sham surgery (indicated by first arrow) nor the horizontal cut (indicated by second arrow) abolished the rhythms.


3V
SCN
OC

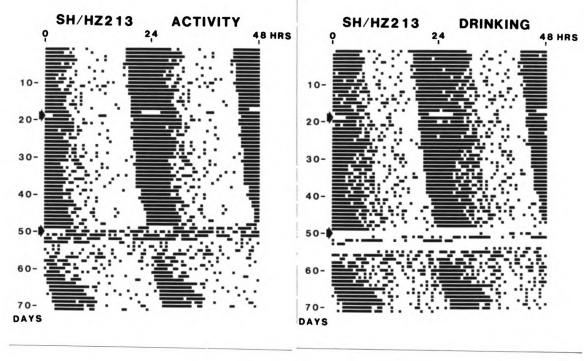

SH/HZ213 ACTIVITY
0
24
48 HRS
10-
20-
30-
40-
50-
60-
70-
DAYS
SH/HZ213 DRINKING
0
24
48 HRS
10-
20-
30-
40-
50-
60-
70-
DAYS

Figure 9.

periodogram peaks for all of the blocks of data sampled.

Visual inspection of the postsurgical records indicated that drinking rhythms persisted in all of these four cases although in 3 cases the periodogram peaks for portions of the records did not exceed the 0.05 level of significance. In all of these cases it appears that the lack of significant Q_p values is due to disturbances and/or "noise" in the records. In these cases, significant Q_p values were obtained by shifting the block of data analyzed by a few days.

In one rat the cut was ventral to the SCN. This animal showed statistically significant rhythms throughout the postsurgical activity and drinking records.

GENERAL DISCUSSION

While bilateral parasagittal cuts caused minor disruptions in the drinking rhythms of two animals in the first experiment, the rhythms appear to have persisted in these 2 rats and in the other cases with this type of knife cuts (Experiments 1 and 2). Five of the rats with bilateral parasagittal cuts showed postsurgical activity levels that were high enough to permit assessment of rhythmicity and in all of these rats the activity rhythms clearly persisted after surgery. These results are consistent with earlier observations on rats that were housed in cyclic light after receiving bilateral parasagittal cuts (Nunez & Stephan, 1977) and indicate that laterally-projecting efferents from the SCN are not critical for the generation or expression of behavioral rhythms.

Horizontal cuts that were placed dorsal to the SCN and ventral to the PVN failed to abolish or severely disrupt activity or drinking rhythms. Similar cuts have been shown to abolish gonadal regression as a result of short (i.e., 12 hr or less) photoperiod in hamsters without abolishing activity rhythms (Brown, Youngstrom, Nunez, unpublished observation; Eskes & Rusak, 1984). This suggests that while dorsally-projecting SCN efferents are involved in photoperiodic responses in seasonally breeding mammals, they

are not critical for the generation and expression of behavioral rhythms.

Nunez and Stephan (1977) had shown that knife cuts that isolated the SCN from structures that lie dorsal, lateral, and caudal to it abolished both entrainment of drinking to the light/dark cycle and free-running drinking rhythms while cuts placed lateral or caudal to the SCN failed to abolish entrainment of drinking to the light/dark cycle. Their results in combination with the present observations that bilateral parasagittal cuts placed lateral to the SCN and horizontal cuts placed dorsal to the SCN fail to abolish free-running rhythms indicate that there is a functional redundancy in the neural pathways that convey circadian information to the neural mechanisms that regulate drinking behavior. Thus, knife cuts that sever all (Stephan & Nunez, 1977) or nearly all (Nunez & Stephan, 1977) of the axons leaving the SCN eliminate behavioral circadian rhythms while more selective cuts that sever a smaller fraction of the efferent projections from the SCN generally fail to affect behavioral rhythms.

Further evidence for functional redundancy in the efferent projections of the SCN comes from studies of the vasopressin deficient Brattleboro rat. Cells in the dorsomedial part of the SCN show immunoreactivity for argininine vasopressin (Vandesande et al., 1975; Moore & Card, 1983) which, in the brain, may act as a neurotransmitter or neuromodulator. A portion of the

efferent projections of the SCN also show arginine vasopressin-like immunoreactivity (Sofroniew & Weindl, 1978; Hoorneman & Biujs, 1982). Brattleboro rats homozygous for diabetes insipidus (DI) fail to show normal arginine vasopressin in their brains (Valtin, 1967) but show circadian rhythms in activity and drinking behavior (Groblewski et al., 1981; Peterson et al., 1980), pineal serotonin N-acetyltransferase activity (Peterson et al., 1980) and sleep (Brown & Nunez, 1984). Thus, while a portion of the SCN cells may be unable to communicate with their efferent targets due to lack of neurotransmitter, the remaining "output cells" of the nucleus appear to be sufficient for the generation of normal rhythms.

Different from the lack of effect of selective knife cuts placed around the SCN on behavioral rhythms, selective cuts have been found to affect hormonal rhythms. For example, while retrochiasmatic cuts placed caudal to the SCN failed to abolish feeding (Nishio et al., 1979), drinking (Nunez & Stephan, 1977), or activity (Nunez & Casati, 1979) rhythms, they have been shown to abolish estrous cycles (Nunez & Casati, 1979), circadian rhythms in adrenal corticosterone secretion (Moore & Eichler, 1972), and prolactin surges during pseudopregnancy (Freeman et al., 1974) indicating that these hormonal cycles may be mediated by caudally-projecting SCN efferents. Pineal rhythms have also been abolished by "retrochiasmatic" cuts (Moore & Klein, 1974) that severed fibers projecting dorsally as well

as those projecting caudally out of the SCN. This result was originally interpreted as being due to interruption of caudal projections from the SCN but was later reinterpreted as being due to the severing of fibers projecting dorsally from the SCN to the PVN since lesions of the PVN yielded the same results (Klein et al., 1983). Further evidence implicating dorsally-projecting SCN efferents in the generation of pineal rhythms is provided by the observations that horizontal cuts dorsal to the SCN (Brown, Youngstrom, & Nunez, unpublished observations; Eskes & Rusak, 1984), lesions of the PVN (Pickard & Turek, 1983; Lehman et al., 1984), and pinealectomy (Reiter & Hester, 1966) all prevent short photoperiod induced gonadal regression in hamsters.

In contrast to the lack of effect of horizontal cuts that did not damage the SCN, horizontal cuts that passed through the SCN abolished or severely disrupted drinking rhythms in 6 cases and caused substantial decreases in the period length of activity and drinking rhythms of one rat. Effects of horizontal cuts through the SCN on activity rhythms are difficult to evaluate since most of these rats showed activity levels that were substantially reduced after surgery. In 2 cases, however, it is clear that activity rhythms persisted after cuts that damaged the SCN and severely disrupted drinking rhythms. Disruptions of behavioral rhythms after knife cuts through the SCN have been reported previously (Dark, 1980). Rosenwasser et al. (1984) found that midsagittal cuts that were placed between

the two suprachiasmatic nuclei and did not damage either SCN had little or no effect on activity rhythms while cuts of similar size and orientation that pased through one SCN caused disruptions (i.e. increased random components) in the rhythms. The observation that horizontal cuts through the SCN disrupt drinking rhythms while similar cuts placed outside the SCN do not suggests that neural circuits intrinsic to the SCN are more important in the generation of behavioral circadian rhythms than are specific sets of efferent projections of the SCN. Evidence for neural circuits intrinsic to the SCN has been provided by anatomical and immunohistochemical analysis of the nucleus. Van den Pol (1980) found short axons and axon collaterals that are confined to the SCN and make up to 100 or more synaptic contacts within the nucleus. Guldner & Wolff (1978) found dendro-dendritic "autapses" or synapses between a dendrite and one of its own branches. Additionally, Card et al. (1981) observed cells in the ventral portion of the SCN that show vasoactive intestinal polypeptide-like immunoreactivity and make both presynaptic and postsynaptic axodendritic synaptic contact with unlabelled cells within the SCN. The knife cuts through the SCN in the present experiment probably interrupted a portion of these intrinsic circuits and thus, may have disrupted drinking rhythms by interrupting communication between the SCN cells that make up the circadian oscillator and/or output region of the nucleus.

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