ACTIVITY AND THE CIRCADIAN SYSTEM IN THE C57BL/6J MOUSE

by

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ABSTRACT

This thesis first examined two methods of studying nonphotic effects on the phase and period of circadian rhythms in C57Bl/6j (C57) mice and, second, investigated the neural source of nonphotic input to the suprachiasmatic nucleus (SCN) circadian pacemaker. Experiment 1 examined the effects of the opioid agonist morphine on free-running circadian rhythms in C57 mice. Acute injections of morphine induced locomotor activity and wheel running for several hours, and phase shifted free-running rhythms. The phase-response curve (PRC) was similar to that for locomotor activity alone, and shifts were blocked by preventing activity. Experiment 2 established that forced treadmill (TM) running can reliably entrain free-running rhythms. This method provides better control over activity intensity and duration, and was adopted for further studies of neural mechanism. Prior evidence suggested that the intergeniculate leaflet (IGL), via neuropeptide Y (NPY), and the raphe nucleus (RN), via serotonin(5HT), may play a role in conveying non-photic inputs to the pacemaker. Experiment 3 examined the effects of serotonin depletion on entrainment to scheduled TM activity. Mice entrained to scheduled TM activity were fed a trytophan free-diet, which reduces 5HT synthesis. Circadian rhythmicity was strongly attenuated within 3 days, and independent effects on entrainment could thus not be determined with confidence. Experiment 5 examined the effects of radiofrequency IGL ablations and neurotoxin induced 5-HT depletion on entrainment to TM activity. Lesions were verified by NPY and 5-HT immunocytochemistry. Mice with complete IGL or 5HT ablations failed to entrain to regular scheduled TM running. Complete 5-HT ablated mice exhibited modulations of tau, whereas complete IGLx mice showed little or no modulation of tau in response to forced TM activity. Both the IGL and the RN inputs to the SCN appear to be necessary for entrainment to 24 h schedules of forced running, but the modulations of tau evident in the mice with complete 5HT lesions suggests that they might entrain if the period of the forced running schedule matched tau more closely.

DEDICATION

In loving memory of my father, Lowell Marchant.
Without whose support, I would never have dreamed of taking on such an endeavor.

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List of Terms & Acronyms [1]

5HT - Serotonin

5, 7- DHT - (5, 7-dihydroxytryptamine). Serotonin neurotoxin

Alpha (α) - Active period of the daily rest-activity cycle.

Amplitude - Difference between maximum (or minimum) and mean value in a sinusoidal oscillation.

ARC - Arcuate

DMI - Desipramine

Circadian Hour - Tau/24.

Circadian Rhythms - Self-sustained biological rhythms with a periodicity near 24 h in environments without time cues.

Circadian Time (CT) - Standardized measurement of time based on the individual's period. By convention, CT12 is defined by activity onset in nocturnal rodents.

Entrainment - Synchronization of a self-sustaining rhythm by a forced oscillation (zeitgeber). During entrainment the frequencies of the two oscillator are the same or integral multiples of each other.

Free-Run - State of a circadian rhythm in constant conditions, that is, in the absence of entraining agents.

IGL - Intergeniculate Leaflet

MPOA/AH - Medial preoptic/anterior hypothalamic area.

Period - see tau.

Phase - Instantaneous state of an oscillation.

Phase Angle - Value of the abscissa corresponding to a phase of the oscillation, usually given in degrees, where the whole period is defined as 360 degrees and the zero point is arbitary. It can be given in units of time if the length of the period is stated.

Phase Control - Control of the period and phase of a rhythm by a zeitgeber.

Phase Response Curve - Graphical plot indicating how the amount and the direction of a phase shift, induced by a single stimulus, depend on the phase at which the stimulus is applied.

Phase Shift - Single displacement of an oscillation along the time axis; may occur instantaneously or after several transient cycles.

PeVN - Periventricular nuclei

PVN - Paraventricle nucleus

Rho (ρ) - Inactive period of the daily rest-activity cycle.

SCN - Suprachiasmatic Nucleus

subPVZ - Subparaventricular zone

Tau (τ) - Time interval between recurrences of a defined phase of rhythms, also known as period.

VMH - Ventromedial hypothalamic nuclei

Zeitgeber - Forcing environmental oscillation which entrains a biological self-sustaining rhythm.

[1] Definitions taken from Moore-Ede, Sulzman & Fuller (1982)

Introduction

Almost all living organisms exhibit daily (24 h) rhythms of behavior and physiology. These rhythms may be imposed by environmental cycles of light and dark (LD), temperature or other stimuli, or they may be generated by internal biological clocks. Rhythms that are generated endogenously, and that "free-run" (i.e., persist) with a periodicity near 24 h in constant conditions (i.e., environments devoid of time cues), are known as circadian rhythms.

Converging lines of evidence suggest that the biological clock generating circadian rhythms in mammals resides in the SCN of the anterior hypothalamus. Destruction of the SCN disrupts or eliminates circadian rhythms in all mammalian species studied to date (Stephan & Zucker, 1972; van den Pol & Powley, 1979; Kittrell, 1991). Stimulation of the SCN, either electrically (Rusak & Groos, 1982) or chemically (many studies; see Meijer & Reitveld, 1989) can phase shift circadian rhythms. SCN neural firing rates and metabolic activity exhibit circadian rhythms in vivo and in vitro (e.g., Welsh et al., 1995; Prosser et al., 1992, 1993; Prosser & Gillette, 1989,1991; Schwartz & Gainer, 1977). SCN transplants can restore circadian rhythmicity in rodents bearing complete SCN ablations (e.g., Ralph et al., 1990). These observations constitute powerful evidence that the SCN is a master circadian pacemaker.

In normal environments, circadian rhythms do not free-run, but, rather, are entrained by environmental time cues (zeitgebers). The formal definition of entrainment is phase and period (τ) control of the endogenous circadian rhythm by a periodic stimulus. The dominant

zeitgeber for most species is the daily LD cycle. In adult mammals, entrainment of circadian rhythms by LD cycles is mediated exclusively by retinal photoreceptors, which influence the SCN pacemaker by a direct retinohypothalamic pathway (RHT; Moore, 1979; Johnson et al., 1988). This pathway probably utilizes an excitatory amino acid neurotransmitter (e.g., Rea et al., 1993).

A second, indirect pathway for photic stimulation of the SCN is the geniculohypothalamic tract (GHT), originating in the retinorecipient intergeniculate leaflet (IGL; Hickey & Spear, 1976; Card & Moore, 1982; Card & Moore, 1984). This pathway appears to utilize GABA and neuropeptide Y (NPY), and also contains immunoreactivity for enkephalon (ENK) in some species (reviewed in Morin, 1994). The GHT does not appear to be necessary for photic entrainment (e.g., Harrington & Rusak, 1986), although it may mediate some tonic effects of light on rhythms, such as the lengthening of τ observed as light intensity increases (Johnson et al., 1989).

Over the past two decades various non-photic stimuli have also been shown to be capable of influencing the phase and period of circadian rhythms. One of the most interesting developments has been the discovery that physical activity, such as wheel running, can affect circadian rhythms in rodents. As early as 1960, Aschoff noted a relationship between light intensity, τ and activity; in nocturnal rodents, greater light intensity is positively correlated with longer τ and reduced activity. The τ change is thus conceivably the result of changes in activity levels. Aschoff later reported that hamsters with access to running wheels exhibited longer free-running τ than

hamsters without wheels (Aschoff et al., 1973). Yamada et al. (1988) then showed that free-running τ in rats was also altered by manipulating access to running wheels, although in the opposite direction to that indicated by Aschoff's study (i.e., wheel access shortened τ). This effect has more recently been extended to mice (Edgar & Dement, 1991). These results suggest that behavior can affect the rate at which the circadian pacemaker cycles; since running activity is timed by the pacemaker, this can be conceptualized as feedback modulation of pacemaker motion.

In addition to this apparent feedback effect of spontaneous activity on τ, activity also appears to be capable of phase shifting and entraining circadian rhythms. Mrosovsky and colleagues (Mrosovsky, 1988; Reebs & Mrosovsky, 1989) reported that a discrete bout of wheel running induced by litter changing, exposure to a conspecific or transfer to a novel wheel could phase shift circadian rhythms in hamsters maintained in constant dark (DD). The PRC produced by this running stimulus was almost inverted relative to the photic PRC; activity caused phase advance shifts during the subjective day (the hamsters usual rest phase) and small phase delays in the latter half of the subjective night (the usual active phase), whereas light causes phase advances early in the subjective night, large delays late in the subjective night, and usually has little effect in the subjective day (Pittendrigh & Daan, 1976b). If repeated on a daily basis, a bout of induced running can entrain circadian rhythms free-running in constant lighting (Reebs & Mrosovsky, 1989). Others have demonstrated that daily schedules of voluntary wheel running in mice

can also entrain free-running rhythms (Edgar & Dement, 1992). This suggests that some physiological correlate of activity must have access to the circadian clock and can serve as a synchronizing cue.

In the past decade, other stimuli have been identified that shift rhythms in a manner consistent with the non-photic, activity PRC described by Mrosovsky. Many of these appear to cause shifts by inducing activity. For example, injections of triazolam (TZ) induce running in hamsters, and cause activity-like phase shifts, (Turek & Losee-Olson, 1986; Van Reeth & Turek, 1989), but fail to shift rhythms when activity is prevented (Mrosovsky & Salmon, 1990; Van Reeth & Turek, 1989). Similar results have been obtained for running stimulated by exposure to darkness (Van Reeth & Turek, 1989; Reebs et al., 1989), cold (Mistlberger et al., 1996) or refeeding after food deprivation (Mistlberger et al., in press). Phase shifts can also be induced by intraperitoneal saline injections, although these shifts may be dependent on stress and arousal and do not appear to require running activity (Hastings et al., 1995).

Understanding how the circadian system is altered by activity may have important applications. For example, appropriately timed activity (i.e., exercise) may provide relief from circadian disorders such as jetlag, shiftwork malaise and various sleep pathologies. A complete understanding of how non-photic information modulates the phase and period of the clock may also lead to the development of new classes of chronobiotics.

Two candidate neural pathways have been identified as potential mediators of non-photic effects on circadian rhythms. These include

the GHT-NPY input to the SCN and a serotonin (5HT) containing projection to the SCN from a subset of raphe nuclei (RN; Azmitia & Segal, 1978, Moore et al., 1978; Van de Kar & Lorens, 1979). The evidence for involvement of NPY is more direct; in hamsters, intraventricular injections of NPY cause activity-like phase shifts (Albers & Ferris, 1984), phase shifts to TZ (Johnson et al., 1988a) and novel wheels (Janik & Mrosovsky, 1994) can be blocked IGL lesions, and phase shifts to novel wheels can be blocked by injections of anti-NPY into the SCN area (Biello et al., 1994). The lesion studies are weak because damage to the IGL area is associated with a significant reduction in activity; failure to shift may be secondary to failure to run sufficiently in response to the experimental stimulus. Nonetheless, taken together, the results are consistent with a hypothesis that NPY release into the SCN is necessary for activity-induced phase shifting, at least in hamsters. These studies have not yet been extended to other species, or to other types of activity-inducing, non-photic zeitgebers.

The evidence for involvement of 5HT in activity-induced phase shifting is more circumstantial and less consistent; RN discharge and SCN 5HT levels correlate with motor activity (Jacobs & Azmitia, 1992; Shioiri et al., 1991; Dudley & Glass, 1996), 5HT agonists can cause phase shifts with a non-photic type PRC (e.g., Tominaga et al., 1992; Prosser et al., 1993; Edgar et al., 1993), and the 5HT2/7 antagonists ketanserin and ritanserin can block the small phase shifts caused by arousing saline injections in hamsters (Hastings et al., 1995). Lesions of 5HT inputs to the SCN produced by the neurotoxins 5,7-dihydroxytryptamine (DHT; Cutrera et al., 1994) or p-

chloroamphetamine (PCA; Penev et al., 1995) have been reported to block the phase shifting effects of TZ, although both studies may be confounded by a reduction in the activity-stimulating effects of TZ following the lesions. Finally, there are unpublished claims that 5HT lesions produced by DHT can eliminate entrainment to scheduled activity in mice (Edgar, personal communication). Taken together, these results are consistent with a hypothesis that activity-induced phase shifts of circadian rhythms are mediated by a serotonergic input to the SCN pacemaker. However, other studies have produced results inconsistent with this hypothesis. There is one report that DHT-induced 5HT lesions do not prevent phase shifts to TZ in hamsters (Meyer & Morin, 1995). In addition, unpublished studies indicate that general and specific 5HT antagonists, including metergoline, NAN-190, Way100-165 and ritanserin, at a range of doses, do not block or even attenuate phase shifts to novel wheel running in hamsters (Mistlberger, Marchant, Antle et al., unpublished). The absence of positive reports that serotonergic antagonists can block non-photic shifts induced by wheel running suggests that other labs have obtained similar negative results. Additional work will be necessary to clarify the roles of 5HT and NPY in non-photic shifting.

Aims

The general goal of the experiments described here was to gain insight into the neural mechanisms by which non-photic stimuli affect circadian rhythms in mice, a species garnering renewed interest in rhythms research, due to its amenability to molecular biological

analysis (reviewed in Takahashi et al., 1994). As discussed above, non-photic stimuli can phase shift and entrain circadian rhythms. However, most work has been confined to hamsters, and a PRC for activity-induced shifts is not yet available for mice. In Experiment 1, an attempt was made to develop such a PRC using intraperitoneal injections of morphine hydrochloride (MHCL), an ENK agonist that causes intense running activity in the C57 mouse (Moskowitz et al., 1985). ENK has recently been identified as a constituent of the GHT (Card & Moore, 1989; Morin et al., 1992) but its functional significance in this pathway is to date unknown. We report here that MHCL can induce phase shifts that are dependent on the expression of running activity. However, shifts were not consistently produced and large shifts, even at high doses, were uncommon.

Experiment 2 examined the effects of forced treadmill running (TM) on free-running rhythms in C57 mice. Edgar and Dement (1991) reported that scheduled opportunity to run can entrain rhythms in this strain of mice. However, voluntary wheel running is susceptible to disruption by physiological manipulations (e.g., IGL lesions greatly reduce spontaneous activity). In the experiment reported here, C57 mice received 3 hour bouts of forced, rather than voluntary running each day for approximately 6 weeks. Forced running proved to be as effective a zeitgeber as scheduled voluntary running. Forced TM running, however, provides better control over the duration and intensity of the running stimulus, and thus promised to be useful for investigating afferent pathways involved in mediating the effects of activity on the circadian system.

Experiment 3 examined the effects of reduced endogenous 5HT stores on TM-entrained mice. By eliminating tryptophan (TRY) from the diet, brain 5HT can be reduced by as much as 40% (Fernstrom & Wurtman, 1971a & b). However, this method failed to produce clear results, due to disruptions of rhythms during the TRY-free diet.

Experiment 4 examined the role of the SCN in both activity and food entrainment in the mouse. Previous studies have shown that rats and hamsters possess a circadian pacemaker outside the SCN that mediates entrainment to feeding schedules (entrainment is manifest as a rhythm of activity that anticipates daily feeding times, and that persists for several days during total food deprivation, even in animals with complete SCN ablations; reviewed in Mistlberger, 1994). Conceivably, such a pacemaker could mediate entrainment to other non-photic zeitgebers. However, while some mice with SCN ablations did exhibit food entrainment (i.e., they anticipated scheduled feedings), they did not exhibit evidence of circadian organization or entrainment during the forced TM running schedule.

Experiment 5 examined the role of the RN 5HT input and the IGL NPY input to the SCN in TM entrainment. Mice received either bilateral radio-frequency IGL lesions, or injections of the 5HT neurotoxin DHT into the SCN area. Both types of lesions reduced the incidence of TM entrainment, suggesting that both IGL NPY and RN 5HT may contribute to non-photic entrainment in the mouse.

CHAPTER 1

INTRODUCTION

To study phase shifting and entrainment by activity, a reliable method of inducing activity is needed. One method of inducing activity is by drug injection. This method has been used extensively in hamsters, which can be induced to run and phase shift to TZ injections. C57 mice do not run in response to TZ, but do exhibit marked hyperactivity in response to the opiate agonist MHCL (Moskowitz et al., 1985). We thus tested whether MHCL-induced activity can reliably induce phase shifts in these mice. The results of this experiment may also shed some light on the function of ENK-containing neurons known to project from the IGL to the SCN (Morin et al., 1992).

METHODS

Young, adult male C57 mice (15-16 grams, Charles River, Montreal; n=164) were housed in constant dim red light (DD, <1 lux) in separate plastic cages. Each cage was equipped with a 17 cm diameter running wheel which activated a microswitch monitored continuously by an IBM computer. Activity counts were summed and stored at 10 minute intervals and periodically transferred to a Macintosh computer for analysis and display using Circadia (Behavioral Cybernetics, MA) and Systat (Systat, Inc., Evanston, IL).

Fifty-one mice received intraperitoneal injections of MHCL (25 mg/kg, gift of Dr. Barry Beyerstein) or an equal volume of saline every 2-4 weeks at one of 8 circadian times (CT0, 3, 6, 9, 12, 15, 18, and 21). Individual mice received up to three injections at different circadian

times (CTs). CTs were calculated by dividing the injection day into 24 equal divisions (each of which is one "circadian" hour), beginning with the predicted onset of activity estimated by extrapolating a regression line fit to activity onsets on the previous 10-14 days. This activity onset time is designated CT12, by convention. Regression lines and activity onsets (defined as 50 wheel counts in a 10 minute bin following at least 240 minutes with fewer than 50 counts per 10 minute bin) were obtained by computer. Spurious onsets were deleted. Phase shifts were quantified by comparing regression lines before and after the injections. The τ of free-running rhythms was derived from the slope of the regression lines. The dependence of phase shifts on CT was evaluated by ANOVA and post hoc *t*-tests.

To assess whether phase shifts in response to MHCL were caused by changes in light exposure or steady-state retinal discharge, a second set of MHCL and saline injections were given to 113 enucleated C57 mice (15-16 grams). Under 30% vaporized halothane a .5 cc syringe with a 27 gauge needle was inserted behind the eye, and a 0.2 cc mixture of lidocaine, tetracycline and sterile saline was injected behind the eye. After 2 minutes, the eye was removed using tweezers and scissors. The socket was rinsed again with the solution, and the eyelids glued closed with 3M tissue glue.

To assess whether phase shifts in response to MHCL were due to drug-induced hyperactivity, MHCL (25 mg/kg) and saline injections were repeated at CT3, 6 and 9 in the enucleated mice. Following each injection, the mice were immediately covered by an inverted metal

food hopper (5 x 10 x 10 cm) to prevent wheel running and cage circling for 6 hours. Food and water were available ad lib.

RESULTS

MHCL injections induced phase shifts that, on average, differed in magnitude and direction as a function of circadian time ($F_{(7,116)}=3.651$, $p=.001$). A plot of this relation is illustrated in Figure 1a. Intact and enucleated mice did not show significant differences and their data were thus pooled. Significant phase advance shifts averaging 40 minutes (range = -20 to 350 minutes) were evident at CT6 and CT9 ($p<.001$; e.g., Figure 2a, b), while small delays averaging 20-30 minutes were observed at CT15, CT18, and CT21 (e.g., Figure 2c).

To evaluate the acute behavioral response to MHCL , five mice were videotaped for 6 hours following 25 mg/kg injections. The mice displayed immediate hyperactivity, first circling the perimeter of their cage, then eventually wheel running. Perimeter circling was interrupted by frequent pauses (<10 sec) for food, water or grooming, and persisted, on average, for 30-230 minutes, depending on the circadian phase of the injection ($p>0.05$, e.g., Figure 1b). Distance traveled during circling was estimated at about 200 $\pm$ 40 meters/hour. Circling was then replaced by wheel running, which persisted, on average, for 130-260 minutes, again depending on the circadian phase of the injection ($p>0.05$, e.g., Figure 1b). A criterion of 30 minutes without wheel running was used to define the end of the bout.

The latency and duration of wheel running after MHCL injections were combined and analyzed as a single hyperactivity duration measure. No difference as a function of circadian time was found ($F_{(7,112)}$=.396, p>.05). This is apparent when examining Figure 1b, as the two variables are near mirror images. The total duration of hyperactivity following MHCL injections thus did not appear to vary with circadian phase.

When mice were prevented from expressing wheel running and cage circling activity following injections at CT3, 6 and 9, no significant phase shifts were observed in response to MHCL ($F_{(2,46)}$=.751, p>.05; e.g., Figure 1d) or saline ($F_{(2,24)}$=2.96, p>.05)

Free-running τ was examined before and after the MHCL injections. Although clear τ changes were occasionally observed, the average magnitude and direction were not related to circadian time of injection ($F_{(7,98)}$=1.673, p>.05).

DISCUSSION

This study demonstrates that acute injections of MHCL can phase shift free-running circadian rhythms in mice. Phase shifts were not related to the injection procedure or to changes in retinal processing of light, since saline injections did not induce similar shifts and the MHCL PRC did not differ between intact and enucleated mice. However, phase shifts did appear to be dependent on the expression of cage-circling and/or wheel-running behavior, since significant shifts were not observed when these activities were prevented for 6 hour following drug administration. These data thus add to growing

evidence that induction of activity by drugs or other stimuli can have phase-resetting actions on circadian rhythms. This effect has so far been described for one other drug (TZ) and several environmental stimuli, including novel cages, social interactions and cold exposure in hamsters (Van Reeth & Turek, 1989; Mrosovsky et al., 1989). The PRCs to these stimuli are similar to the MHCL PRC described here for mice. Recent studies indicate that scheduled daily wheel access (Edgar & Dement, 1991) or TM running can entrain free-running rhythms in mice (e.g., Experiment 2), but the present study represents the first complete description of the phase-response characteristics of the mouse circadian system to a behavioral stimulus.

As phase shifts induced by MHCL were dependent on the expression of hyperactivity, it would appear that direct activation of endogenous opiate receptors in the mouse circadian system does not have phase-resetting consequences. Alternatively, such receptors in this system of this strain of mouse may be unaffected or insensitive to exogenously administered opiates at the doses utilized. These doses were, however, quite large.

Unlike phase shifts to MHCL , the duration of total behavioral activation, as defined by cage-circling and wheel running, did not vary with the circadian time of drug administration. However, the latency to wheel running did vary. This may be explained by a circadian rhythm of MHCL potency, as has been observed for opiate analgesia (Moskowitz et al., 1985). The latency may result from an inability to coordinate the presumably more difficult motor sequences required for wheel running immediately following a drug dose that is

effectively higher at some circadian phases. Whether variability of effective dose contributes in any substantive way to qualitative or quantitative features of the MHCL PRC would require additional study. This has been a generally neglected issue in prior pharmacological PRC work.

CONCLUSION

MHCL phase shifted circadian rhythms in free-running mice by acute induction of hyperactivity, and can thus be added to a short list of nonphotic stimuli that have recently been shown to shift rhythms by way of behavioral activation. However, it is not yet know whether each of these behavioral stimuli has an effect on a common activity/arousal input pathway to the circadian pacemaker, or whether there are multiple pathways. In addition, it is unclear whether these pathways are common across species. MHCL thus represents a tool for further study of the neural mechanisms and motivational or stimulus correlates of nonphotic phase shifting in this and possibly other species. The disadvantages of MHCL include, 1. the fact that phase shifts were often small or absent, and 2. the intensity and duration of the running response may be difficult to control.

CHAPTER 2

INTRODUCTION

The next approach that we tried for establishing reliable non-photic shifting and entrainment in mice was forced TM running. Edgar & Dement (1991) previously demonstrated that a daily schedule of restricted access to a home cage running wheel can entrain free-running rhythms mice. We substituted forced TM running for voluntary running. In addition, we also sought to test whether the TM could be used to produce phase shifts to discrete bouts of activity. In principle, TM running should provide the best experimental control of running intensity and duration, and may overcome deficits in the ability or motivation to run following physiological interventions by presenting a simpler locomotor task coupled with a strong incentive. However, entrainment by forced TM running has so far been demonstrated in only one study in rats, and, by comparison with some other methods for inducing activity in other species, its effects were characterized as weak (Mistlberger, 1991). Moreover, there have been unpublished reports that forced running is ineffective in mice (Edgar, personal communication) and hamsters (de Vries & Meijer, cited in Mrosovsky, 1995), two species that can be readily entrained by unforced scheduled wheel running. These reports of weak or no entrainment by forced running but strong entrainment by voluntary running have contributed to suggestions that the motivation to run may play an important role in determining the potency of behavioral activity as a Zeitgeber (Janik & Mrosovsky, 1993; Mistlberger, 1991). In light of this theoretical issue, and the potential advantages of forced

TM running as a behavioral tool for studying nonphotic entrainment, we compared the ability of forced and voluntary activity to entrain or acutely phase shift the circadian system of mice.

METHODS

A total of 114 male C57 mice (2-4 months age; Charles River, Montreal) were individually housed in plastic cages (47 x 26 x 20 cm) equipped with wire floors, 17 cm running wheels and contact drinkometers monitored continuously by computer. The mice were first habituated to their home recording cages in a 14:10 LD cycle before blinding by enucleation.

PROCEDURES

Experiment 1

This experiment examined the effects of daily schedules of forced TM running or voluntary home cage wheel running on circadian activity or drinking rhythms. Each mouse (n=74) was assigned to one of the following groups:

1a. Forced TM running (3 hour/day, 6 days/week, 48 days; n=11), with free access to the home cage wheel. The mice were removed from their home cages each day and transported to an automated TM in a separate test room. Each mouse was run in a separate lane (45 x 12 x 9 cm) at 16 m/minute, with one 10 minute rest provided each hour. Running speed was gradually increased to the target level within the first 5-10 minutes of each session. The mice were thus

required to run about 2.8 km in 3 hours. Each lane was equipped with a compressed air source triggered if the mouse rode the TM to the back of the lane. The mice proved to be avid TM runners and were able to run at 16 m/minute within the first 3 hour session without injury. When mice appeared entrained to TM running, access to the home wheel was blocked for 6 days/week.

1b. Forced TM running (3 hour/day, 42 days, N=16), with no access to a home cage wheel. This procedure controlled for possible interactions between home cage wheel running and scheduled TM running. Entrainment was assessed by monitoring drinking activity alone.

2a. Restricted access to the home cage wheel (3 hour/day, 6 days/week, 48 days, n=12). The home cage wheel was manually unlocked at a fixed time each day. The mice were not otherwise induced to run. Drinking activity was not recorded in this group, thus the home cage wheel was left unlocked for 45 consecutive hours (two circadian cycles) once each week as a "probe" test to assess circadian phase.

2b. Restricted access to the home cage wheel (3 hour/day, 42 days, n=8), with no weekly probe tests, and thus no access to the home wheel outside of the 3 hour daily session. Circadian phase was assessed by drinking activity alone. This condition replicates Edgar and Dement (1991).

3. Restricted access to water (3 hour/day, 53 days, n=5). Forced and voluntary running bouts were associated with increased drinking during or after the daily session. A water restriction condition was thus included to assess whether a scheduled bout of drinking (and associated food intake) can alone serve to entrain free-running rhythms in the mouse. Circadian phase was assessed by home cage wheel running activity.

4. Undisturbed controls (n=22, 60-80 days). These animals were not directly disturbed, but were exposed to noise in the recording room associated with removal of other animals for TM running.

Experiment 2

This experiment examined whether single 3 hour bouts of running scheduled at either CT6 or CT21 could phase shift free-running rhythms in blind mice (N=39). Circadian time was calculated with respect to the onset of the daily "active" period (measured here using drinking activity), which is designated CT12 by convention; CT6 thus represents 6 circadian hours ($6 \times \tau/24$) before activity onset. Running was induced by TM (16 m/minute) or by unlocking the home cage wheel. Mice in a TM "control" group were transferred to the TMs for 3 hour, but were not run. None of these groups had free-access to home cage wheels. Individual mice were used for up to three tests in different conditions or circadian test times.

DATA ANALYSIS

Data were visually inspected in the form of actograms and average waveforms. Mice were scored as entrained if drinking or home cage wheel-running rhythms assumed a stable phase relation to the imposed activity schedule during at least the last 7 days of the activity schedule, and free-ran from that phase when the schedule was terminated. The time required to achieve a stable entrained state was calculated by fitting a regression line to the beginning of the daily activity period (defined by either drinking activity or wheel running) during the last 7-10 days of the activity schedule and identifying the first day when activity onset occurred within 30 minute of this line. Free-running t before and after exposure to daily running schedules was quantified by regression lines fit to activity onset. The slope of the lines was calculated by computer. Phase shifts in response to single bouts of scheduled running were quantified by comparing regression lines fit to 7-10 day blocks immediately preceding or following the sessions. Lines were fit by two coders, independently, with at least one blind to the conditions. Averages of the two estimates were used. Significance of differences between the three conditions and two circadian times was assessed by ANOVA and Tukey HSD tests. All means are reported $\pm$SEM.

RESULTS

Experiment 1

Forced TM running entrained free-running rhythms in 3/11 mice that had continuous access to a running wheel in their home

cages (group 1a; e.g., Figure 3a). The latency to entrainment within this group of 3 mice was estimated at 2-10 days. In the entrained state, the onset of home cage activity (CT12) preceded the daily TM session by 7-8.5 hour (the difference between scheduled activity and CT12 in the entrained state will be referred to as the phase angle of entrainment). Among the 8 mice that did not entrain, most showed modulations of free-running rhythms. These included a 6 hour phase advance in one mouse when the TM session overlapped with CT5-8 (e.g., Figure 3b), a 3 hour phase delay in another mouse after the first TM. session (CT19-22), and τ changes in most of the other mice (e.g., Figure 3c). The mouse illustrated in Figure 3c showed a pattern reminiscent of "splitting", with a component of activity appearing to uncouple from the beginning of the subjective night and re-coupling with the end of the subjective night.

When the mice were returned to their home cages after TM sessions, they usually showed prominent bouts of home cage wheel running (e.g., Figure 3a). Conceivably, entrainment may have been mediated by this bout of voluntary running stimulated in response to forced running. To test this, a second group (1b) of mice that did not have access to home cage wheels were subjected to scheduled TM running. Of these, 14/16 showed stable entrainment (e.g., Figure 3d-f). The latency to entrainment was estimated at 23 ± 2 days, and the onset of the daily active period (measured by the drinking rhythm) during steady-state entrainment preceded the TM session by $11.2 \pm .6$ hour. One of the mice that did not clearly entrain within 42 days showed a slowing of τ, suggesting that stable entrainment would likely

have been achieved if the schedule had been continued. The other mouse showed modulations of τ indicative of relative coordination. The difference in percentage of animals entraining within groups 1a and 1b was significant ($p<.001$).

Scheduled access to the home cage wheel produced results very similar to 3 hour forced TM running. Among mice whose wheels were unlocked for 45 hour "probe" tests each week to assess circadian phase (group 2a), 5/12 entrained to the 3 hour daily wheel access (e.g., Figure 4a). Modulations of τ were common in mice that did not entrain (e.g., Figure 4b). Among mice that had access to home cage wheels for 3 hour/day without weekly probe tests, 6/8 entrained (Figure 4d-e), and one appeared likely to entrain if the schedule had been continued. The latency to and phase angle of entrainment were very similar in these two groups, and averaged 23.7 $\pm$.8 days and 12.0 $\pm$.4 hour, respectively (n=11, pooled). These values did not differ significantly from those obtained from mice that entrained to forced TM running.

Mice that did entrain to restricted home cage wheel access ran when the wheel was unlocked, averaging 2843 $\pm$ 530 wheel counts/3 hour (range of 1026-8092 counts/3 hour across animals), and were on most days active for at least the first hour of wheel access, and usually for the entire 3 hours. The amount of running in some of these mice varied widely from day to day. Among mice that did not entrain, some showed little or no running on most days, while others ran a great deal (over 5,000 counts/3 hour) at some circadian phases of wheel access (CT12-23), but not at other phases (usually CT0-11). In these cases,

failure to entrain may have been due to a failure to run sufficiently at the critical phase.

Mice that entrained to TM or home cage wheel access exhibited a group mean free-running τ of 23.53 $\pm$.04 hour during the week prior to entrainment (n=27, 1 case missing sufficient pre-entrainment data), and 23.80 $\pm$.03 hour during the first 7-10 days following entrainment (paired t = 6.56, p<.0009). Mice that did not entrain to scheduled TM or home cage running (n=20) exhibited pre- and post-schedule τ 's of 23.67 $\pm$.05 hour and 23.63 $\pm$.04 hour, respectively (paired t =.687, p>.5). Free-running τ of these mice prior to the activity schedules was, as a group, significantly longer (i.e. closer to 24 hours) than τ of mice that did entrain (t = 2.70, p<.01). The failure of these mice to entrain is thus not related to unusually short τ, although it is conceivable that observed τ was outside of the range of entrainment in some individual cases.

Among the 28 mice that entrained to scheduled activity, the latency to entrainment ranged from 2-33 days and the phase angle of entrainment ranged from 8-16 hours. The phase angle of entrainment was related to τ prior to entrainment, such that shorter τ was associated with a more advanced phase, i.e., a longer interval between CT12 and the beginning of scheduled running (Pearson r=.407, p=.04). The latency to entrainment was not related to τ, but was related to the circadian phase at which the first session of scheduled running began. The phase of the first session ranged from CT13 - CT22 across animals; the later the phase on day 1 of scheduled running, the shorter the latency (r=-.673, p<.0009). Differences in latency are thus at least in

part due to individual differences in the timing of the first (and thus subsequent) sessions. The group mean phase of the first session did not differ between mice that entrained and those that didn't.

During scheduled wheel access and following TM running sessions, visual inspection of actograms and average waveforms revealed considerable drinking activity. To test whether drinking mediated entrainment, five mice were subjected to a 3 hour/day water access schedule. None of these animals entrained, although modest τ changes were observed in two cases (e.g., Figure 4c). Free-running τ of these animals was similar to the other groups (23.68 $\pm$ .03 hour).

A total of 22 mice were allowed to free-run without direct physical disturbance. Free-running τ's measured for the weeks before and after the activity schedules carried out on neighboring mice were 23.74 $\pm$.07 hrs and 23.62 $\pm$.05 hrs, respectively (paired $t = 1.91$, $p<.07$). τ lengthened (i.e. moved toward 24 hour) from time 1 to time 2 in only 3/22 cases. No spontaneous phase shifts or rhythm dissociation's were apparent (e.g., Figure 4f).

Experiment 2

The effects of single, 3 hour bouts of TM running, TM transfer without running and home cage running at two circadian phases are summarized in Figure 5. From a total of 90 tests only a few very clear phase shifts were obtained (e.g., Figure 6a, b). Nonetheless, a significant effect of treatment and circadian phase was evident ($F_{(5,84)}=5.39$, $p<.0009$). The mean shift induced by forced TM running

at CT6 (21 ± 8 minute, i.e. a phase advance) differed significantly from that induced at CT21 (-12 ± 4 minute, i.e. a phase delay; $p<.001$), although it did not differ from the mean shift induced by transfer to the TM without running (6 ± 4 minute; $p=.2$). The mean shift induced by unlocking the home wheel at CT6 (15 ± 6 minute) differed marginally from the shift induced by home wheel access at CT21 (-3 ± 3 minute; $p=.04$). Due to technical limitations, the amount of wheel running expressed during wheel access was recorded in only 13/26 cases. Nonetheless, a significant relationship was evident between the amount of running and the absolute shift size, i.e. the greater the running response, the larger the phase advance or delay in this subsample ($r=.590$, $p=.03$)

DISCUSSION

This study demonstrates that daily schedules of either forced TM running or voluntary home cage wheel running can entrain circadian rhythms in blind mice, with the following characteristics under the conditions used: (1) Entrainment was generally preceded by an extended period of transients, as the free-running rhythm gradually slowed until a stable phase was attained. (2) The stable phase attained was related to τ prior to the activity schedule; shorter τ was associated with a more advanced phase angle of entrainment, suggesting that the pacemaker attains maximal phase delays late in the subjective night or early in the subjective day, about 10-14 h after CT12. (3) Following the schedules, drinking or wheel-running rhythms free-ran from the apparent phase of entrainment, with τ exhibiting

"aftereffects" (i.e., τ was closer to 24 hour after entrainment than before). These characteristics of entrainment, and the percentage of animals entraining, did not differ between the forced and voluntary running groups, suggesting that these two methods of inducing activity, at the parameters utilized, have a similar potency and common mechanism.

Scheduled activity appeared to be a more potent zeitgeber when mice did not have free-access to a home cage wheel, since the percentage of animals entraining was significantly greater in these groups (1b and 2b). Similar observations have been made previously with respect to the phase shifting effects of TZ in hamsters (Wickland & Turek, 1991) and the entraining effects of TM running in rats (Mistlberger, 1991). The mechanism for this is unclear. In the present study, it is not explained by a difference in τ prior to the running schedules, nor can it be attributed to differences in the amount of experimentally induced running, since all animals in the forced running groups had to run the same distance in the same time (2.8 kilometers/3 hour). Whatever the cause, the result provides confidence that entrainment to forced TM running is not dependent on bouts of voluntary, home-cage running triggered in mice after they have been returned from daily TM sessions. The failure of mice to entrain to daily schedules of water access also suggests that entrainment was not likely due to eating or drinking during or just after daily activity sessions.

Although daily 3 hour schedules of TM or home wheel activity were clearly effective zeitgebers for entraining free-running rhythms

(particularly in mice without free access to home cage wheels), single 3 hour bouts of TM or home wheel running had very modest phase shifting effects when applied at CT6 or CT21 in mice that did not have free access to home cage wheels. These CT's were selected on the basis of previous studies of nonphotic zeitgebers in hamsters and mice, which suggested that maximal advances to a bout of running should be obtained at about CT4-9, and maximal delays between CT21-CT3. Of particular relevance, in the experiment described in Chapter 1, C57 mice showed some large phase advances to injections of MHCL in the CT3-9 range, an effect which was apparently dependent on the expression of intense, drug-induced cage and wheel activity following the injections. Some results from the first TM group were consistent with these expectations; one very large advance was observed when TM activity overlapped with CT4-8, one large delay was observed following TM activity at CT18-21, and the first three mice entrained within 2-10 days, with TM activity producing the requisite phase delays at about CT21. Nonetheless, no similarly large phase shifts were observed in the 3 hour activity "pulse" experiment, regardless of the method used to induce activity.

However, in light of the general characteristics of entrainment observed in Experiment 1, this should not be surprising. Apart from the single large phase advance noted, there was virtually no other evidence of advancing shifts or transients when scheduled activity overlapped with CT3-12 (or any CT for that matter). Moreover, when TM sessions overlapped with CT13-21, a large number of small delaying transient cycles were evident before steady state

entrainment was achieved, suggesting that the pacemaker is subject to low amplitude (< 30 minute) phase delays through this portion of its circadian cycle, culminating in maximal phase delays (30-40 minute) in the CT21-CT3 range. Larger phase shifts may have been observed if the running stimulus was longer or more intense (although 16 m/minute is near maximal for a 3 hour session), or if other circadian phases had been assessed. For many animals, maximal phase delays may have been more readily obtained in the CT0-3 range, rather than CT21-0, although, again, we saw little evidence for large shifts in animals that ran at these circadian phases without entraining.

In summary, the results of this study indicate that both forced and voluntary running are effective non-photic zeitgebers for entraining free-running rhythms in mice. However, neither stimulus, at the intensity-duration parameters used, proved sufficient to reliably induce large, readily apparent phase shifts when applied as a single "pulse". Therefore, the entrainment paradigm would clearly seem to be the more useful for further study of the mechanisms by which non-drug induced behavioral activity affects the circadian system in mice. In addition, forced TM running may offer some special advantages, since it is at least the equivalent of voluntary running in efficacy (percentage of animals entrained), it can be used to generate activity at circadian phases when mice voluntarily may run very little (i.e. CT3-12), and it can potentially overcome deficits in running ability or motivation secondary to experimental interventions used to elucidate neural mechanisms. The drawbacks worth noting are the expense of TM equipment, the labor required for transferring

animals on a daily basis and the amount of time necessary to establish entrainment.

Finally, we note that the results do not support previous suggestions that forced running is in some way qualitatively different from voluntary activity in its effects on circadian rhythms (Janik & Mrosovsky, 1993; Mrosovsky, 1995). The results are instead more consistent with the idea that the stimulus to run is less important for the circadian system than is the intensity, duration or timing of running (see also Mistlberger, Marchant & Sinclair, 1996). Given the present data, this would seem to be a reasonable working hypothesis for mice. Previous observations of relatively weak entrainment by TM running in rats may be a function of the TM parameters used in that study (faster running rates are possible in highly trained rats) or could represent a true species difference (Mistlberger, 1991). The absence of reports (and anecdotal negative reports; see discussion in Mrosovsky, 1995) of entrainment or phase shifts to forced running in hamsters probably has more to do with the poor response of hamsters to coercion than to properties of forced running per se (unpublished observations). In any case, more comparative work is needed to evaluate how the circadian systems of different species respond to different methods of evoking behavioral arousal and activity before definitive statements can be made about the motivational and stimulus correlates of running that may be necessary and sufficient for entrainment.

CHAPTER 3

INTRODUCTION

As reviewed above, the SCN functions as a master pacemaker for the generation and photic entrainment of behavioral and physiological circadian rhythms that free-run (i.e., persist) in constant environmental conditions. Although the SCN is indispensable for normal circadian organization under most conditions, it is not the only circadian oscillator nor is it necessary for all circadian functions in mammals. Other tissues, including the retina (Reebs & Mrosovsky, 1989; Takahashi et al., 1994) and perhaps also the duodenum (Bunning, 1973; Comperatore & Stephan, 1987), exhibit some capacity for circadian oscillations in vivo and in vitro. In addition, a number of species can express circadian activity rhythms that are synchronized and anticipatory to a restricted daily feeding time (Boulos & Terman, 1980; Mistlberger, 1994). In two species, rats and hamsters, food anticipatory rhythms have been shown to persist after SCN (Abe & Rusak, 1992; Mistlberger, 1992; Stephan et al., 1979) and IGL ablations (Mistlberger, 1994). In intact and SCN-ablated rodents, these rhythms exhibit several properties, including limits to entrainment and persistence during food deprivation, that are consistent with regulation by a circadian pacemaker. Evidently, this pacemaker is outside of the SCN and is entrained by input pathways independent of the IGL. The site of this pacemaker and its entrainment pathway(s) remain to be determined (Mistlberger, 1994).

The free-running and photic entrainment properties of circadian rhythms in mice have been well characterized at the behavioral level

(Pittendrigh & Daan, 1976; Schwartz & Zimmerman, 1990), and two studies have confirmed a critical role for the SCN (Ibuka et al., 1980; Schwartz & Zimmerman, 1991). However, formal and physiological mechanisms of non-photic entrainment in mice are less well studied. Although one study has demonstrated that the C57 mouse strain can anticipate a daily feeding (Abe et al., 1989), it has not yet been established that this persists independent of the SCN. If it does, then it is conceivable that behavioral synchrony to other non-photic zeitgebers, such as TM running, might also be mediated by a non-SCN pacemaker. The objective of the present study, therefor, was to determine whether behavioral synchrony to daily feeding schedules and daily TM schedules in C57 mice can occur following SCN ablation.

METHODS

Procedures

Male C57BL/6j mice (18-20 grams) were obtained from Charles River (Montreal, Canada). After a week of acclimatization in a group colony room, the mice were transferred to individual plastic cages (45 X 25 X 20 cm) under a 12:12 LD cycle in a climate controlled room. Each cage was equipped with a 17 cm running wheel and contact drinkometers monitored continuously by computer.

After one week, 28 mice were anesthetized with a ketamine/xylazine cocktail and subjected to radio-frequency lesions (20-25 mA, 15 sec) of the SCN using a stereotaxic guided 00 insulated insect pin with a .3 mm blunted tip. Stereotaxic coordinates were .6 mm and .1 mm anterior to bregma, 0.0 mm lateral to midline and 5.8

mm and 5.7 mm ventral to dura. After 5-10 days for post-operative recovery the mice were transferred back to their home cages and wheel running and drinking data were collected for at least 15 days. Recordings were also taken from 7 additional, unoperated mice. All 35 mice were then acclimated to a restricted feeding cycle. Food availability was decreased from 12 to 6 to 4 h/day sequentially over 3 days and then fixed at 4 h/day for 5-9 weeks. On the last day of restricted feeding, food was delayed by 13 h, and then left freely available for a final 2-4 weeks. The mice were maintained in dim red light (< 1 lux) throughout the experiment.

In a second experiment, 12 mice from the first experiment were subjected to 3 weeks of 3 hours/day forced TM running. Procedures for TM running are as described in Chapter 2.

Following behavioral testing, all mice were sacrificed and perfused. Brains were fixed in sucrose-formaline, frozen sectioned at 50 mm and stained using cresyl violet with or without luxol fast blue.

Data Analysis

Wheel running and drinking data visually inspected in the form of actograms and average waveforms. Circadian periodicity during ad-lib food access was evaluated quantitatively using the periodogram method (Dorrscheidt & Beck, 1975) and regression lines fit to activity onsets. Food anticipatory activity was evaluated qualitatively by average waveforms and quantitatively by anticipation ratios described

below. Means were contrasted by t-tests, ANOVA and Tukey post hoc tests, and are reported ± SE.

RESULTS

Histology: general description.

Nine of 28 operated mice were judged to have unambiguously complete SCN ablations (e.g., Figure 7a). In these mice, large triangular or rectangular lesion cavities were evident above the optic chiasm, extending on average about 1.5 mm in length, 1 mm in width and as high as the top of the third ventricle. The mid-half of the optic chiasm was usually damaged. All of these lesions damaged the medial preoptic/anterior hypothalamic area (MPOA/AH) anterior to the SCN, the paraventricular (PVN) and periventricular nuclei (PeVN), the subparaventricular zone (subPVZ) between the SCN and PVN, most or all of the retrochiasmatic area, particularly medially, and portions of the arcuate (ARC) and ventromedial hypothalamic nuclei (VMH), particularly rostrally and medially.

Three additional mice were classified as sustaining "ambiguous" SCN ablations (e.g., Figure 7b). In these cases, sparing of a few cells or SCN fragments on one side could not be ruled out, due to a slight asymmetry. Damage to non-SCN areas was similar to that evident in mice with unambiguously complete SCN ablations.

Fourteen mice were judged to sustain incomplete SCN ablations. In 4 cases SCN damage was primarily unilateral. In the remaining cases, lesion cavities were similar in shape but smaller in size than

those evident in the mice with complete SCN ablations, and 5-95% of the SCN were spared ventrally (e.g., Figs. 7c). One partial SCN lesion appeared to destroy close to 100% of the retrochiasmatic area, median eminence (Me), ARC and VMH.

In one mouse damage was restricted to the striatum rostral and dorsal to the hypothalamus, and in another mouse no lesion was evident (Figure 7d).

Of the 12 mice used in the TM running schedule, 6 were determined to have unambiguously complete SCN lesions, 2 to have ambiguous SCN lesions and 4 to have partial SCN lesions. Areas damaged in complete and partial lesioned animals are listed in Table 1.

Activity rhythms during ad-lib food access

Nine mice failed to display circadian organization of wheel running and drinking during ad-lib food access both prior to and following food restriction (e.g., Figure 13a-d). Eight of these mice sustained unambiguous, complete SCN ablations. One sustained a lesion estimated at 95% complete, with SCN fragments clearly evident unilaterally (Figure 9b). Periodograms in the 22-25 h range were flat, with maximum Qp values averaging .29 $\pm$.04.

Two mice exhibited arrhythmic activity prior to food restriction, but a significant free-running rhythm during and after food restriction. One of these sustained an unambiguously complete SCN ablation, whereas the other was ambiguous, with SCN fragments possibly spared. Periodograms confirmed significant periodicity in the 23.5-23.83 h range.

All other operated mice displayed significant free-running rhythms prior to and after food restriction. Among these were two mice that sustained SCN ablations judged ambiguous, with fragments possibly spared (e.g., Figure 9a). The remaining mice sustained partial or no SCN damage. A number of these were remarkable in that the ablations appeared to destroy the dorsal and medial SCN bilaterally, with large lesion cavities framing the remaining SCN dorsally and caudally (e.g., Figure 9c). Average free-running τ among mice that sustained at least partial SCN damage was 23.59 $\pm$.06 h, with mean Qp values of .46 $\pm$.03.

All unoperated mice exhibited free-running rhythms, with a group mean τ of 23.76 $\pm$.16 h, which was not significantly different from mice with partial SCN ablations (e.g., Figure 10a-d). However, the mean periodogram Qp of .63 $\pm$.04 was significantly higher than mice with partial lesions ($p<.02$), indicating a more robust circadian signal.

Activity rhythms during food restriction.

Complete SCN ablations.

Two of 9 mice with complete SCN ablations exhibited wheel running in anticipation of daily feeding time (e.g., Figure 8a,b,e,f). One additional mouse showed some evidence of food anticipatory wheel running, but this was inconsistent during the last 2 weeks of food restriction. The other 7 SCN ablated mice showed no consistent food anticipatory running. In some of these cases activity was lowest in the hours immediately preceding food access (e.g., Figure 8c,g), whereas in

others activity was uniformly distributed throughout the day (e.g., Figure 8d,h).

All 3 mice with ambiguous SCN ablations showed food anticipatory wheel running. Two of these mice also exhibited clear free-running activity components that did not entrain to feeding time (e.g., Figure 9a,e).

Partial SCN ablations

All 14 mice with partial SCN ablations exhibited food anticipatory running during at least a portion of the restricted feeding schedule. In each case, anticipation was consistently evident when the feeding schedule overlapped with the active phase (α) of the free-running rhythm (e.g., Figure 9c,g), but was either less robust or absent when feeding time fell during the rest phase (ρ) (e.g., Figure 9d,h). In 2 cases, the free-running rhythm appeared to entrain to feeding time, although in neither case is it certain that entrainment would have remained stable (e.g., Figure 9d). In one case entrainment was preceded by advancing transients when feeding time fell during mid-ρ (e.g., Figure 9d). The phase angle of entrainment was such that activity onset preceded feeding time by 2-4 h. In at least 6 other cases, small τ or phase changes occurred during food restriction.

SCN Intact mice.

All 9 mice with intact SCN exhibited activity in anticipation of feeding, although this was generally much more robust in α than in ρ (e.g., Figure 10a-h). In 6 cases, free-running rhythms appeared to entrain to the restricted feeding schedules (e.g., Figure 10a,b), although it is again not clear if this would have remained stable in all cases. In one case, entrainment was preceded by advancing transients when feeding time overlapped with mid ρ (Figure 10a). In the other cases, feeding time overlapped with α, and entrainment was not preceded by rapid shifting (e.g., Figure 10b). In the entrained state, activity onset began at or just after feeding time in one mouse (Figure 10b) and preceded feeding time by 2-6 h in the others (e.g., Figure 10a).

Free-running rhythms in the 3 SCN-intact mice that clearly did not entrain to feeding time showed marked phase and τ changes. One mouse showed a phase advance and abrupt τ shortening when feeding time fell during late α - early ρ, another showed a 6 h phase advance when feeding time fell during mid-ρ (Figure 10c), and the third exhibited τ shortening as feeding time passed through late ρ (Figure 10d).

The proportion of SCN-intact mice exhibiting apparent entrainment (i.e., 6/9, 66%) was significantly greater than the corresponding proportion of partial SCN-ablated mice (i.e., 2/14,14%; $p<.001$).

Activity during delayed feeding

Previous studies have shown that food deprivation or delayed feeding can reveal mealtime associated activity in animals that fail to exhibit anticipatory activity (e.g., Mistlberger, 1991). Consequently, feeding time was delayed by 13 h on the last day of restricted feeding. Activity was quantified in six 4 h time blocks beginning 12 h before and ending 12 h after the usual feeding time and was expressed as ratios against total activity for that day. Means were obtained for 4 groups; SCN-ablated non-anticipators (n=7), SCN-ablated anticipators (n=5), partial SCN ablated (n=14), and SCN intact (n=9). Although the SCN-ablated non-anticipators showed little activity during the premeal time-block, all 3 lesion groups exhibited peak activity during the time-block corresponding to the usual feeding time (Figure 11). Activity then declined during the last two time blocks. The SCN-intact group showed low ratios during the premeal block, despite high absolute activity levels during that time, due to higher levels of activity sustained from the usual mealtime to the end of the deprivation (e.g., Figure 10b,c).

Relation of food anticipation to lesion size and placement

Lesion Size.

Lesion size was estimated by measurements of the length and width of the lesion cavities, and by the sum of damage to 5 prominent structures selected to represent rostral (median preoptic nucleus MnPO, MPO), middle (PVN) and caudal (VMH, ARC) levels with respect to the SCN. There was a trend for lesion length and width to be greater

in the 7 mice that failed to exhibit anticipatory activity, compared to the 20 mice that sustained observable lesions and that did anticipate feeding time (length: 1.55 $\pm$.18 mm Vs 1.12 $\pm$.1 mm, t =3.17, p=.08; width: 1.25 $\pm$.13 Vs .93 $\pm$.07, t =3.53, p=.07). Cumulative damage to the five representative structures, and to the MPO, ARC and VMH individually, was significantly greater in these mice (t =15.27, p<.0001).

Lesion placement

The preceding analysis suggested that damage to the MPO, ARC and VMH may be particularly associated with failure to anticipate feeding time. To further evaluate a possible relationship, activity counts during the 3 h prior to mealtime were quantified for the last 2 weeks of restricted feeding, and expressed as ratios with respect to total daily activity (excluding mealtime, i.e., 3h:20h ratio) and with respect to the same 3 h time of day during the 2 weeks prior to food restriction (i.e., 3h:3h ratio). The ratios were then correlated (Pearson) with percent damage to the MPO, MnPO, VMH, ARC and PVN. The 3h:20h ratio was not significantly correlated with damage to any of these structures. The 3h:3h ratio was significantly negatively correlated with damage to the MPO (r=-.36, p<.03) and MnPO (r=-.43, p<.01). However, damage to these structures was also negatively correlated with total daily activity and activity outside of the premeal window, indicating that this ratio may primarily reflect changes in overall activity levels, independent of time of day. In addition, mice with large rostral lesions also had large caudal lesions, suggesting that

associations between anticipation (or total activity) and MPO or MnPO damage may be secondary to total lesion size.

Additional evidence against a necessary role for the ARC or VMH is provided by individual cases in which these areas were heavily damaged, yet anticipation was clearly evident, e.g., one mouse with a partial SCN ablation showed clear anticipation, yet sustained nearly complete destruction of both the ARC and VMH (Figure 9b,f).

In addition to the SCN, the PVN, PeVN, subPVZ and retrochiasmatic area were completely destroyed in one or more mice that exhibited anticipation. Several other hypothalamic structures were partially damaged in most mice, but none of these sites were unique to those mice that failed to anticipate.

Relation of food anticipation to total activity levels

Average daily wheel running levels differed significantly across the following 4 groups; SCN-ablated non-anticipators (657 $\pm$ 1399 revs, n=7), SCN-ablated anticipators (3783 $\pm$ 1655 revs, n=5), partial SCN (anticipators, 4766 $\pm$ 955 revs, N=14), and SCN intact (anticipators, 6163 $\pm$ 1308 revs, n=9; $F(3,31) = 3.07$, $p=.04$). The SCN-ablated non-anticipators ran significantly less than the intact group ($p<.05$), but not significantly less than the SCN-ablated anticipators or the partial SCN groups. Nonetheless, the distributions in these groups overlapped (Figure 12a).

Relation of food anticipation to body weight

Body weights, measured after food restriction, differed significantly across the following 3 groups; SCN ablated non-anticipators (32 $\pm$ 2.3 gms, n=7), SCN ablated anticipators (30.1 $\pm$.4 gms, n=5) and partial SCN ablated plus intact (all anticipators, 28 $\pm$.5 gms, n=23; ($F_{(2,32)}$=3.43, p=.04). SCN-ablated non-anticipators were significantly heavier than the partial SCN and intact group (p<.04), but were not significantly heavier than the SCN-ablated anticipators. There was substantial overlap across distributions (Figure 12b).

Relation of entrainment to free-running period

By comparison with intact mice, free-running rhythms in mice with partial SCN ablations were less likely to entrain to the feeding schedule. This may be explained by group differences in free-running τ prior to food restriction. When all mice were considered, there were no significant differences in τ between mice that entrained and mice that didn't, or between intact and ablated mice. However, one intact mouse that entrained exhibited an unusually short τ of 22.16 h prior to food restriction (τ was slightly longer than 24 h after entrainment). When this extreme outlier was omitted, group differences were significant; mice that entrained had, on average, longer τ's (23.93 $\pm$.11 h) than mice that did not entrain (23.63 $\pm$.08 h; t=5.23, p=.03; Figure 17c), and intact mice had longer τ's (23.99 $\pm$.09 h) than mice with partial SCN ablations (23.59 $\pm$.06; t =11.34, p=.003). Free-running τ closer to 24 h was thus predictive of entrainment.

SCN lesions and forced TM running

TM running (3h/day, .8km/h, 21 days) had little or no organizing effects on drinking activity patterns in mice with complete or partial SCN lesions. Some weak 24 hour organization appeared in some of the mice, but this failed to persist in constant conditions.

DISCUSSION

This study provides two observations indicating that food anticipatory rhythms in mice are regulated independently of the SCN. First, some intact mice and mice with partial SCN ablations expressed two primary activity bouts each day, one free-running bout with τ different from 24 h, and a second food anticipatory bout coupled to the 24 h feeding schedule. Second, complete ablation of the SCN and loss of rhythmicity in constant conditions did not prevent the emergence of a food anticipatory rhythm during restricted feeding. These results confirm one previous report of food anticipatory rhythms in neurally intact C57 mice (Abe et al., 1989), and, to our knowledge, are the first to demonstrate persisting circadian function (i.e., food anticipatory rhythms) in mice in the absence of the SCN circadian pacemaker. Mice can thus be included with rats and hamsters as species in which the independence of food anticipatory rhythms from SCN mediation has been physiologically confirmed. Like these other rodent species, mice appear to possess physically separate circadian mechanisms for the generation of food-anticipatory and free-running, light-entrainable rhythms.

Food anticipatory rhythms in rats persist for several circadian cycles during complete food deprivation, and reappear during additional food deprivation tests after several weeks of ad-lib food access (Coleman et al., 1982, Reme et al., 1991). The persistence of these rhythms for several cycles in constant conditions is strong evidence that food anticipation is regulated by a self-sustaining circadian oscillator, rather than a simple hourglass process (e.g., activity triggered by a hunger threshold reached approximately 18-20 h after feeding). Due to their small size and high metabolic rate, mice with access to running wheels cannot be safely food deprived for much more than 24 h. Consequently, it was not possible to observe whether anticipatory rhythms in mice would persist for several days in the complete absence of food. However, an hourglass process can also be evaluated by analysis of activity when feeding time is delayed, a procedure that was used in this study. According to an hourglass model, activity should be dependent on time since last fed, and once activity is triggered, it should continue until food is obtained, or until exhaustion. However, mice with SCN-ablations did not show this pattern; rather, when food was delayed by 13 h on the last day of food restriction, mealtime-associated activity was maximal at the regular mealtime, and declined soon after the end of the usual mealtime. This suggests that activity was phase dependent, as would be predicted by an entrained oscillator model, and not merely hunger (or time-since-fed) dependent, as would be predicted by an hourglass model. In most of the SCN-intact mice, activity did continue until feeding time, but in all such cases, activity was coincident with α of the animals' free-

running or entrained rhythms, i.e., although activity was enhanced by hunger, its timing was nonetheless consistent with circadian phase. The very high levels of continuous activity that these mice displayed during the 13 h of delayed feeding provides confidence that the decline in activity evident in most of the other mice soon after the omitted mealtime was not due to exhaustion.

In contrast to previous studies of SCN-ablated rats and hamsters (Mistlberger, 1992b), a significant number of SCN-ablated mice in this study failed to exhibit food anticipatory wheel running. There are a number of possible explanations. One possibility is that the lesions destroyed a food-entrainable pacemaker, either contained within one hypothalamic nucleus or distributed across several. We were not able to identify any one nucleus as being critical for the expression of food anticipation. The SCN, PVN, PeV, SubPVZ and retrochiasmatic area were destroyed in one or more mice that anticipated feeding time, and the mouse with the largest caudal ablation, which destroyed most of the VMH and ARC, also showed good anticipation. Damage rostral to the SCN, in the MPO and MnPO areas, was particularly extensive in mice that failed to anticipate, but damage here was also associated with greater damage dorsal and caudal to the SCN. Other measures of lesion size, including length, width and cumulative damage to 5 selected structures, were generally consistent with this finding, i.e., mice that failed to anticipate, on average, had the largest lesions. This finding is consistent with the idea that food-entrainable oscillators may be distributed across several hypothalamic nuclei.

Alternatively, failure to anticipate by SCN-ablated mice may be specific to wheel running and drinking measures of activity. Previous studies have shown that rats may fail to anticipate feeding time in one measure of activity (e.g., horizontal locomotion detected by tilt-floors), while expressing robust anticipation in another (e.g., activity directed at a food bin, detected by photobeams). This was more commonly observed in rats with PVN ablations than in intact rats (Mistlberger & Rusak, 1988). Anticipatory wheel running, by contrast, has proven to be generally resistant to disruption by lesions in rats. Nonetheless, it is possible that large hypothalamic ablations in mice may disrupt the link between wheel running and the food entrainment mechanism, while sparing other anticipatory behaviors not measured.

A third possibility is that failure to anticipate was due to a change in the phase-angle at which food-entrainable oscillators couple to mealtime. Consistent with this hypothesis, mice that failed to anticipate did, nonetheless, tend to exhibit peak levels of activity at the usual feeding time when feeding was delayed by half a day, suggesting that an activity rhythm may have been synchronized with feeding time, albeit with a slightly negative phase angle with respect to usual meal-onset.

Diet-induced obesity in rats has been associated with reduced anticipatory wheel running (Persons et al., 1993). PVN, VMH and ARC lesions can induce obesity in rodents (Brooks et al., 1946; Gold et al., 1977; Olney, 1969) and a few mice in this study were clearly obese. However, body weight does not appear to explain the failure to anticipate, because half of the SCN-ablated mice that failed to

anticipate had body weights indistinguishable from SCN-ablated mice that did anticipate mealtime.

Consistent with two previous studies of mice, free-running rhythms were greatly disrupted or eliminated by SCN ablation (Ibuka et al., 1980; Schwartz & Zimmerman, 1991), but were retained if more than 5% of SCN tissue remained visible by Nissl stain. Similar observations have been made in rats (Eastman et al., 1984), hamsters (Davis & Gorski, 1974; Rusak, 1977) and squirrel monkeys (Edgar et al., 1994), and suggest that the SCN is necessary for the generation of free-running rhythms, and is highly redundant internally. However, we did observe one anomalous case in which a mouse with an apparently unambiguous, complete SCN ablation, was arrhythmic prior to restricted feeding, but showed a free-running rhythm after 2 weeks of restricted feeding that persisted for several weeks. The interpretation of these data is not clear. One possibility is that somehow a few SCN neurons survived even this very large ablation; behavioral circadian rhythms have been observed in hamsters retaining very small numbers of immunocytochemically identified SCN neurons (Harrington et al., 1993).

A number of mice in the present study sustained considerable damage to the SCN dorsally and medially, and yet expressed clear free-running rhythms. This suggests that in the mouse, the dorsomedial region of the SCN, which has anatomical features distinguishing it from the ventrolateral region (Van Den Pol, 1980), is not critical for either rhythm generation or for output of the circadian signal to effectors for locomotor activity. Some of the partial ablations

were surprisingly large dorsally and caudally, and probably represent complete dorsal-caudal deafferentations. In rats, large caudal ablations or knife cuts can eliminate circadian rhythms of sleep (Eastman et al., 1984) and feeding (Van den Pol & Powley, 1979), but this appears not to be true for locomotor rhythms in mice. Whether this represents a difference between species or between output pathways for different behaviors remains to be determined.

Although food anticipatory rhythms are not dependent on the SCN, they are subject to modulation by some aspect of SCN output. In the intact mice, food anticipatory activity was usually strongly attenuated during the rest phase of the free-running rhythm. Mice with partial SCN ablations generally showed less attenuation of activity. Attenuation presumably reflects some SCN-dependent inhibitory process, which is reduced in proportion to SCN ablation. During the "rest" phase, the SCN may directly inhibit output from behavioral effectors (cf., Vogelbaum & Menaker, 1992) or from food-entrainable oscillators. Alternatively, inhibition may be indirect, by way of competition with sleep homeostasis. The nature of the SCN's interaction with other oscillators and effector systems remains to be clarified at formal and physiological levels.

Modulation was also evident in the other direction; free-running rhythms were in many animals either entrained to feeding time, or abruptly altered in phase or period. Intact mice showed a higher incidence of entrainment by comparison with mice with partial SCN ablations, and this was probably because they expressed free-running τ 's closer to 24 h, and thus within the limits of entrainment for the

(probably weak) non-photic feeding zeitgeber. Period differences may also explain why a previous study did not observe entrainment of free-running rhythms by restricted feeding in C57 mice (Abe et al., 1989).

The phase-angle of entrainment to feeding time was somewhat variable. In most cases, activity onset in the entrained state occurred either at or a few hours before feeding time. This would appear to distinguish food entrainment from entrainment by exercise; as demonstrated in Chapter 1, intact C57 mice are readily entrained by 3 h daily scheduled activity, but the phase angle is much more negative, i.e., the onset of the subjective night (measured by drinking activity or home cage wheel running) begins 6-10 h prior to scheduled activity. This may be taken to suggest that feeding time and scheduled activity do not entrain the SCN circadian pacemaker by a common input pathway. However, additional study will be necessary to evaluate the role that τ differences may play in this apparent difference in phase angle of entrainment.

A few of the mice with partial SCN ablations showed very robust food anticipatory wheel running, yet their free-running rhythms did not entrain, even though anticipatory running fell at what has been previously shown to be the critical phase for entrainment by scheduled activity (Edgar & Dement, 1991; Chapter 1). Conceivably, the ablations in these mice may have cut the input pathway(s) carrying the non-photic signal to the SCN pacemaker. Consistent with this hypothesis, the two best cases of failure to entrain despite strong activity at the predicted sensitive phase both sustained large dorsal

and caudal ablations, which likely would interrupt SCN inputs from both the IGL and the raphe nuclei, two likely sources of non-photic input to the SCN pacemaker.

The direction of phase and period changes evident in free-running rhythms during restricted feeding can be used to infer a PRC for this non-photic event. A few phase advances, one as large as 6 h, were observed when feeding time occurred in a zone extending from the end of the subjective night to about the middle of the subjective day (inactive phase of the free-run). In a few cases, τ lengthened (moved closer to 24 h) as mealtime passed through the late subjective night. No large phase delays were observed. These effects suggest a PRC with a phase advance zone in the subjective day and a low amplitude phase delay zone in the subjective night. This general shape is consistent with the PRC described for several activity-inducing stimuli in hamsters (Mrosovosky, 1988) and mice (Chapter 1). However, the zeitgeber in this case is uncertain; it could be wheel running, feeding, arousal or coupling forces from a food-entrainable oscillator.

Feeding time is an obviously important nonphotic stimulus that can exert effects on behavior by entraining the entire circadian system or a subcomponent, the neural substrate of the which remains to be identified. The present study establishes the C57 mouse as a useful model organism for analysis of this form of non-photic synchronization, and suggests additional work is necessary to evaluate possible mediating or modulating roles for several hypothalamic areas beyond the SCN.

The failure of forced TM running to produce strong circadian organization of behavior suggests that entrainment by activity, unlike entrainment by food, is dependent on an intact SCN.

CHAPTER 4

INTRODUCTION

Given that the SCN is the pacemaker necessary for entrainment of circadian rhythms by activity, elucidation of the neural pathways responsible for this type of non-photic entrainment can reasonably proceed by analysis of known inputs to the SCN. As reviewed in the general introduction, there is considerable circumstantial evidence that the 5HT projection from the RN to the SCN may play a role in entrainment to activity schedules. SCN neurons respond to 5HT (e.g., Ying & Rusak, 1994), locomotor activity is associated with increases in 5HT levels in the brain generally (e.g., Chaouloff et al., 1985) and in the SCN specifically (Shioiri et al., 1991; Dudley & Glass, 1996), receptor agonists for 5HT, probably acting on a 5HT7 type receptor (Lovenberg et al., 1993), can induce phase shifts of circadian rhythms in vivo and in vitro (Medanic & Gillette, 1992; Prosser et al., 1990; Prosser et al., 1993) with an activity-like PRC, and there are reports that 5HT denervation or receptor blockade may attenuate or prevent some activity-induced phase shifts (reviewed in Hastings et al., 1995), although there may be interpretive problems with these methods, and negative results have also been reported. With the exception of the in vitro electrophysiology studies, some of which utilized rats, this work has been almost entirely limited to hamsters.

The goal of the present study was to further evaluate a potential role for 5HT in non-photic entrainment by utilizing dietary manipulation of endogenous 5HT activity in mice. Chronic ingestion of diets deficient in tryptophan (TRY), an essential amino acid necessary

for the production of 5HT, can decrease hypothalamic 5HT by as much as 30-40% (Fernstrom & Wurtman, 1971a; Zambotti et al., 1975). Conversely, a carbohydrate loaded meal can increase the amount of brain TRY and 5HT by as much as 19% within 2 hours following ingestion after a 15-18 hour food deprivation (Fernstrom & Wurtman, 1971a). Glass and colleagues (1995) demonstrated a 205 $\pm$ 30% increase in the extracellular concentration of 5HT, as assessed by in vivo microdialysis, when hamsters were treated with bolus intraperitoneal injections of TRY. These injections were also shown to attenuate the response of the SCN circadian pacemaker to light pulses. If 5HT is part of the pathway for entrainment of circadian rhythms by activity, then artificially manipulating TRY levels should affect entrainment. The following study assessed this is in two ways. One group of mice were first entrained to a TM schedule, and then placed on a TRY-free diet. According to the 5HT hypothesis, entrainment might be expected to fail as brain 5HT levels decrease. A second group of mice were placed on a TRY-free diet ad-lib, but received a 90 min TRY-rich meal at a fixed time each day. Increased 5HT activity after this meal might be expected to mimic locomotor activity, and result in entrainment of free-running rhythms.

METHODS

Thirty-two C57 mice (Charles River, Montreal) were divided into three groups. After one week of acclimatization to a 12:12 LD cycle the mice received bilateral enucleation (as described in Chapter 2) and were transferred to individual polyurethane cages (45x12x19 cm).

Each cage was equipped with a contact drinkometer and microswitch connected to a 17 cm running wheel, both of which were monitored continuously by computer.

The mice were divided into three groups. Group 1 (n=15) was first entrained to scheduled TM running (2 hours per day, approximately 1.9 kilometers) before being placed on a TRY-free diet. The mice were removed from their cages each day and transported to the TM in another room. Compressed air was released from the back of the TM if the mice slowed or stopped running. The mice were run for 2x50 minute sessions (18.6 meters/minute). During this period the home cage wheels were locked, so as to increase the likelihood of entrainment by TM activity (see Chapter 2). When an animal was determined to be entrained (at least 5 days of stable phase relationship with a τ of 24) the mice were placed on an ad lib TRY-free diet. Eventually, all animals were put on a TRY-free diet even if they did not entrain to the regular scheduled activity. The diet was composed of the following: 17.0% casein hydrolysate, .3% DL-methionine, 5.0% non-nutritive bulk, 3.5% AIN mineral mixture, 2.2% vitamin mixture, .2% choline (ICN Biochemicals, OH), 61.8% sucrose and 10.0% vegetable oil. Once on the TRY-free diet the weight of each mouse was taken every day following each TM session. At the point that the animals had reached 80% of starting body weight or 8 days, the mice were no longer run on the TM. The TRY-free diet was continued for 5 days before regular lab chow was reintroduced (pilot data suggested that 2 weeks of TRY-free diet was about maximal for maintaining health).

Group 2 mice (n=7) were maintained on a TRY-free diet for 22.5 hours per day. For 1.5 hours the mice were given access to a drinking tube containing Isomil baby formula (Ross Pediatrics). Baby formula has a high content of TRY and the Isomil formula contains one of the highest amounts of TRY of all commercially available baby formulas.

To provide a comparison with scheduled feeding of regular diet, Group 3 mice (n=9) were restricted to regular lab chow for 3 hours per day for 10 days. The weights of these mice were closely monitored to ensure that they did not drop below 80% of their ad-lib feeding weight.

RESULTS

In group 1, 10 mice entrained to the TM schedule, i.e., τ was 24 hours and phase was stable (e.g., Figure 13), and 5 mice did not entrain (e.g., Figure 14). Free-running τ prior to the TM schedule did not differ significantly between animals that entrained and those that did not ($t=.563$, $p<.583$). A lower percentage of mice entrained in this group (66%), compared to group 1b in Chapter 2 (88%), possibly because TM sessions were 2 h, rather than 3 h.

In most mice, the TRY-free diet resulted in a loss of definable phase and attenuation of circadian amplitude within about 2 days (e.g., Figure 13). When normal chow was resumed, the circadian pattern of drinking reappeared, again in about 2 days. In one mouse, the phase of the rhythm when it reappeared was shifted, suggesting that the TRY-free diet may have interfered with entrainment. However, in the remaining cases, there was no clear shift of phase, although the poor

precision of the rhythms during and immediately after TRY treatment make accurate phase assessments difficult (e.g., Figure 13). Among mice that did not entrain to the TM schedule, a few showed phase shifts when the TRY-free diet was terminated.

During the TRY-free diet, there was an 18% increase in average drinking activity/day (Figure 15). Drinking levels dropped a significant amount after the forced TM running was stopped (44%, $t=2.31$, $p<0.038$), and were further reduced (18%) following the cessation of the TRY-free diet. Drinking returned to normal levels within 1-2 days following the return of normal lab chow.

The mice receiving the TRY-free diet plus limited access to the milk formula (group 2) did not entrain to this daily window of TRY intake. However, some evidence of anticipation of milk access was evident in all mice (e.g., Figure 16a, b), and this bout of activity usually persisted for 1-2 days after the feeding schedule was terminated.

The mice maintained on restricted access to regular chow did not show entrainment of free-running rhythms (e.g., Figure 17b), although one mouse may have been close to entraining (Figure 17a). In some cases, the free-running rhythm was attenuated for periods of time, but reappeared without evidence of shifting (e.g., Figures 17a,b, days 37-47). Anticipatory drinking activity was minimal; most drinking occurred during or after the meal.

DISCUSSION

The TRY-free diet resulted in attenuation of drinking rhythmicity within 1-2 days, suggesting that reduced brain 5HT may disrupt functioning of the circadian clock. However, when the rhythms reappeared after the normal diet was restored, the phase of the rhythms appeared to be unshifted, indicating that a reduction of 5HT activity only masked the output of the circadian clock, at least as measured by drinking activity. There was minimal evidence that the TRY-free diet interfered with entrainment, but as noted, accurate phase assessments were difficult due to flattening of rhythm amplitude, and a reduction in the level of drinking when the TM schedule was terminated. A longer duration of TRY-free feeding may have been necessary for entrainment to fail, but this was not possible due to health concerns with prolonged TRY deprivation. In addition, drinking activity may not have been the optimal means of assessing rhythms, as 5HT is involved in the regulation of drinking and eating behavior (Jacobs & Azmitia, 1992). This study thus represents at best a weak test of the role of 5HT in non-photic entrainment in mice.

A previous study of long-term (weeks to months) TRY depletion by diet in rats reported that unusual patterns of activity emerged, suggestive of uncoupling of secondary circadian oscillators (Kawai et al., 1994), as if 5HT is necessary to maintain coupling among two or more oscillators in the circadian system. There was no evidence of this in the present study, but this may be due to the limited duration of TRY depletion.

In this study, 33% of the mice failed to entrain to the 2 h daily TM scheduled, as compared to only 12% that failed to entrain to a 3 h

daily TM schedule used in the comparable experiment (Group 1b) described in Chapter 2. This is consistent with the idea that a critical level or duration of activity is necessary for entrainment to occur, as suggested by Mrosovsky for hamsters (1995).

Group 2 mice fed a TRY-free diet, with 90 minutes of milk feeding each day (TRY enriched source), did not show entrainment of free-running rhythms to this schedule. This suggests that a daily increase in brain TRY and 5HT synthesis does not mimic activity as a circadian zeitgeber. However, as brain 5HT was not measured in this study, the significance of this finding is not clear; the feeding schedule may not have resulted in a significant increase in 5HT release, even if brain TRY and 5HT synthesis were enhanced for some hours after the milk intake.

The milk-fed mice did, however, show good anticipatory drinking activity to the daily milk access. This demonstrates that mice can anticipate a necessary dietary constituent (TRY). Anticipation of specific nutrients like salt and protein has previously been demonstrated in rats (reviewed in Mistlberger, 1994).

Unlike the milk-fed mice, the Group 3 mice on total food restriction did not show strong anticipatory drinking. This result is consistent with studies of other species (reviewed in Mistlberger, 1994). Food anticipation appears to be absent in drinking activity unless the food is actually provided by drinking spout, as was the case in the milk-fed mice of Group 2. Mice thus appear to learn where nutrients will occur, and sample that place (e.g., the water spout, if

that is how the nutrient is provided) in anticipation of a predictable feeding time.

CHAPTER 5

INTRODUCTION

A more direct approach to assessing the role of SCN input pathways for non-photic entrainment is experimental ablation of these pathways. As reviewed in the general introduction, two separate lines of work suggest that the IGL, RN or both may be necessary for non-photic entrainment, at least in the hamster. The following experiment was designed to explore the role of IGL-NPY and RN-5HT in activity entrainment in C57 mice.

One weakness of most of the experiments to date has been an inability to adequately control the duration and intensity of activity in response to various experimental treatments. Thus, failure to observe phase shifting to running induced by novel wheels (Janik & Mrosovsky, 1994) or TZ injections (Johnson et al., 1988; Wickland & Turek, 1994) after IGL ablations is confounded by the significant reduction in locomotor activity that occurs as a consequence of these lesions. Reductions in activity have also been apparent following DHT-induced 5HT lesions (e.g., Cutrera et al., 1994b). With the use of the TM, the same stimulus intensity can be applied in control and experimental animals. The use of TM running will also enable us to extend our understanding of the physiology of non-photic entrainment to include forced, as opposed to voluntary, activity, which may (or may not) be a better model of exercise in humans. By using mice, we extend the analysis to a species other than hamsters.

METHODS

Fifty-seven C57 mice were used in this experiment. The mice were housed in standard polyurethane rat cages with grid floors used in the prior experiments. Each cage was mounted with a 17cm running wheel and a contact drinkometer. All wheels were locked with a piece of wire with the exception of 9 IGL ablated (IGLx) mice which had wheel access throughout the experiment. Data where collected as described in earlier experiments.

The following groups of mice are evaluated;

1a. IGLx mice with home cage wheels (n=9)
1b. IGLx mice without wheels (n=6)
2a. Intact control mice with wheels (from Chapter 2, n=11)
2b. Intact control mice without wheels (from Chapter 2, n=16)
2c. Intact control mice starting TM running immediately after enucleation (n=14)
3. 5HT ablated (DHT) mice without wheels (n=19)

Following a 7 day period of LD Groups 1a-b received bilateral radiofrequency IGL ablations and Group 3 received 5HT ablations by DHT infusion within the SCN area. After an additional 7 days of LD, the IGLx mice were enucleated following the procedures detailed in Chapter 1. The DHT mice spent 1 day in DD prior to being enucleated. The animals were maintained on regularly scheduled activity until entrainment was demonstrated based on the criterion described in Chapter 2. The control Groups 2a,b were obtained from Chapter 2.

Surgeries

All surgeries were carried out on a Kopf stereotaxic with a Kopf mouse adapter. A xylamine/ketamine cocktail (wt<20gm =.2cc xylamine and .2cc ket / wt>20 grams=0.2cc xylamine/.3cc ketamine) was used as the general anesthetic. All mice received approximately .5 liters/hour of oxygen during and after surgery until consciousness was regained. The mice also received a 1.5 cc subcutaneous injection of lactated ringers solution prior to surgery and an additional 1 cc if bleeding from the sagittal sinus occurred. Following surgery the mice were placed in a standard sawdust cage with palatable mashed food and bedding material. Temperature of the recovery room was maintained around 27 degrees during recovery. Food and water intake was monitored hourly for 12 hours following surgery. Additional lactated ringers was given if little or no food was eaten within the first 6 hours. Four to seven days following surgery the mice were returned to their regular running wheel cages.

The IGLx mice all received 8 (4 per side) bilateral radio-frequency lesions using a stereotaxically guided "00" insect pin, insulated to .3 mm of its blunt tip. A 15 mA, 30 sec voltage was supplied by a Grass lesion maker.

The animals that received SCN injections of DHT were operated on in the same fashion as above with the exception of receiving 30 mg/kg of desipramine (Sigma) 30 minutes prior to anesthetic administration. Five microliters of saline containing 0.2% ascorbic acid and 80mg of DHT was administered to the SCN area over a period of 3

minutes through a 33 gauge injection syringe. The syringe was left in place for an additional 3 minutes to prevent backflow. Stereotaxic coordinates for the injections and lesions can be found in Table 2.

TM PROCEDURES

After recovery from surgery and at least 1 week of free-run following enucleation, the mice were subjected to daily TM running schedule. The animals were removed from their home cages and transferred to a procedure room where they were run for three 50 minute sessions, separated by 10 min breaks, at the approximate rate of 16 meters per minute (2800 meters/3 h). These parameters were used for all experimental groups. Compressed air, triggered by a photocell, was used to ensure that the mice continued to run during the experimental sessions. The compressed air was released from tubes at the back of the TM if the mouse slowed or ceased to run. Following the TM session the mice were returned to their home cages. The mice were left undisturbed except for standard care and maintenance. The mice were run for varying lengths of time, ranging from 3.5 - 9 weeks.

NPY IMMUNOCYTOCHEMISTRY

Following completion of behavioral testing all mice were sacrificed at CT12. The mice were killed using carbon dioxide and quickly perfused through the heart with ice-cold phosphate buffered saline (PBS) for approximately 5 minutes. This was followed by 5 minutes of 4% paraformalin. The brains were quickly removed and

post-fixed for 4.5 hours in the same solution. Following post-fixation, the brains were cryoprotected in a 20% sucrose PBS solution overnight.

Fifty micron slices were obtained using a sliding microtome and placed into free floating wells containing PBS. Sections from the anterior commissure to caudal SCN were used for ICC. Half of the sections were kept for longer term storage in a cryoprotectant solution. The sections caudal to the SCN, through the IGL, were sliced on a cryostat and stained with cresyl violet. The ICC protocol consisted of 3x10 minute rinse in PBS-gelatin triton (PBS-GT), 90 minute incubation in 10% normal goat serum (NGS) (Vector, CA) followed by a thirty-six hour incubation in anti-NPY (Peninsula, Ca) at a dilution of 1/15,000. The tissue was aggressively rinsed (6x30 minutes) followed by a biotin blocking step (Vector, CA). After rinsing (3x10 min.), the tissue was incubated in the secondary antibody (biotinylated goat-anti-rabbit, from Vector) in PBS-GT and NGS for one hour. The tissue was rinsed (3 x10 min.) in PBS-GT and then exposed to ABC material (Vector) for one hour. After rinsing (3x10 min.) the tissue was developed in .25% DAB with 60μl of nickel chloride in tris (pH 7.2).

The ICC protocol for 5HT-IR is essentially identical with the following exceptions: A mixture of picric acid (5%) and gluteraldhyde (3%) in 4% paraform was added as a additional perfusate, post-fixation was reduced to 2 hours, and a 1/35,000 dilution of anti-5HT (Incstar, Minnesota) was used. The rest of the protocol modeled the NPY protocol.

RESULTS

Histology

A 3 point rating scale was used in which a score of 1 signified the complete absence of NPY immunoreactivity (NPY-IR) in the SCN, 2 indicated IR equivalent to that found in the hypothalamus surrounding the SCN, and 3 indicated a darker immunoreaction in comparison to the surrounding hypothalamus. NPY-IR was assessed at rostral, middle, and caudal levels of the SCN, and a mean score was calculated based on all sections examined. Successful lesions were defined as those in which NPY-IR was completely absent from all regions of the SCN.

Five mice of the 15 in groups 1a & b were judged by both raters to have no NPY-IR in the SCN (Figure 18a-b). Sections caudal to the SCN were examined to confirm by Nissl stain that the IGL were ablated. In all 5 cases, there were large lesion cavities in the location of the IGL. Complete lesions typically also damaged the hippocampus (CA2 & CA3 regions), dorsal lateral geniculate (50%), reticular thalamic nucleus (80%), ventral posterolateral thalamic nucleus (70%), ventral posteromedial lateral thalamic nucleus (30%), fimbria hippocampus (40%), superior thalamic radiation (80%) and the ventral lateral geniculate nucleus (95%) (Figure 19). Damage extended down as far as CA3, laterally into the cortex and caudally just rostral to the superior colliculus.

Seven mice were rated as having partial IGL lesions, on the basis of having detectable NPY-IR in the SCN, but substantially lower mean density scores relative to unlesioned mice (mean density scores of 1.25 ± 0.08 Vs. $2.25 \pm .024$). In animals with partial IGL lesions,

damage tended to be focused lateral, dorsal and caudal to the IGL. Histological results for the remaining 3 mice were not adequate for analysis.

Twelve of 19 mice receiving DHT ablations were rated as having complete loss of SCN 5HT; the remaining mice showed some evidence of partial or normal innervation.

Behavioral Data

Entrainment: Control Groups

The numbers of intact control mice (summarized from Chapter 2) that entrained to scheduled TM running were as follows: Group 2a. (with access to home wheels) 3/11 entrained; Group 2b. (without access to home wheels) 14/16 entrained; 2c. (modified procedure, without access to home wheels)13/14 entrained (e.g., Figure 23a-b). In all groups, the phase angle of entrainment was negative; stable entrainment occurred when TM running fell late in the subjective night, usually between about CT20-24.

Entrainment: IGLx Group

All mice received TM sessions during the sensitive phases for entrainment, i.e., the mid subjective night to early subjective day, as determined from the control groups, and 7 mice received TM running at all circadian phases. However, only 2 of 15 mice that received IGL lesions entrained to the TM schedule (13%; Figure 19a-d). Both of these mice sustained histologically confirmed incomplete IGL lesions, with mean NPY-IR scores of 1.93, compared to the control mouse mean

rating of 2.25. The lesion that was visible was focused lateral and dorsal to the IGL. The phase angle of entrainment in these 2 mice was similar to the control mice, with the daily TM sessions occurring between CT21 and CT3 when stable entrainment was achieved. One of these mice displayed an unusually expanded α, with the inactive period being equivalent to only 1.6 circadian hours (Figure 20b).

Of the remaining 5 mice with histologically confirmed partial IGL ablations, none entrained, but one with τ near 24 h came close to entraining (Figure 21c), and the other 4 all showed τ changes, in some cases reminiscent of relative coordination. Of the 3 IGLx mice without histological confirmation, one showed τ modulations reminiscent of relative coordination, and a 5.5 h phase advance when the TM schedule occurred at about CT8-11.

Of the 5 mice with complete IGL lesions, there were no cases of entrainment to the TM schedule (e.g., Figure 21 a,b), no phase shifts, and only one case in which τ changed slightly during the TM schedule (Figure 20a).

Entrainment: 5HT Lesion Group.

All of the DHT lesioned mice received TM sessions during the sensitive phase for entrainment, and 6 received sessions at all circadian phases. However, of 19 mice, only 3 entrained to the TM schedule (e.g., Figure 22a,b). Each of these sustained partial 5HT lesions. Several additional mice showed transient periods of entrainment, but drifted out of entrainment after about 6 days (e.g., Figure 25a). No mouse with a complete lesion entrained. However, all

but one of the non-entraining DHT mice showed some evidence of τ changes over the course of the TM schedule (e.g., Figure 24a-b, Figure 25 b). In addition, 4 mice showed at least 1 phase shift (typically when TM running coincided with the beginning or end of α).

Relation of Entrainment to Tau

Free-running τ, measured prior to the TM schedules, did not differ significantly between complete IGLx (N=5, 23.54 $\pm$.05), partial IGLx (N=7, 23.65 $\pm$.212) and intact control mice run with (Group 2a (N=11, 23.663 $\pm$.268) or without (Group 2b (N=16, 23.628 $\pm$.210) home cage wheels ($F(3, 34)= .727$, $p<.727$).

Free-running τ also did not differ between the 5HT lesioned and intact animals, either before ($F(14,19)=2.123$, $p<.155$) or after ($F(14, 19)=3.016$, $p<.092$) the TM schedule. In addition, no significant difference in pre-TM τ was found between the mice that entrained and those that did not entrain. Further analysis by lesion category will be done when histological results are available.

The Relation of Entrainment to Wheel Availability.

Among mice with access to home cage wheels, and that ran in these wheels, 3/11 intact mice (Group 2a) entrained and 2/7 IGlx mice entrained (Group 1a, all with partial lesions). Among mice without wheel access or that didn't run in their wheels, 14/16 intact mice (Group 2b) entrained and 0/8 IGLx mice entrained (Groups 1a and 1b, 5 of which had complete lesions). Failure of IGLx mice to entrain is thus not due to home cage wheel running.

Changes in Activity (Drinking)

Statistical comparisons between the IGLx mice with wheels (1a) and intact mice with wheels (2c) revealed no significant differences in mean drinking activity. Comparisons between IGLx without wheels and control mice without wheels also showed no significant difference in mean drinking activity except for the pre-TM period when IGLx mice showed more activity than control mice (t =-4.786, p<.005; Figure 25).

When DHT lesioned mice were compared to controls a significant difference in drinking activity was found before, during and after the TM sessions. The 5HT animals showed 27, 37, and 46% more drinking activity during control mice in the pre-TM, TM and post-TM periods, respectively (Figure 25).

DISCUSSION

This study demonstrates that the IGL is necessary for entrainment by forced TM running in mice. The role of the IGL in activity induced changes to the circadian systems has been extensively studied in hamsters. (Biello et al., 1994; Biello et al., 1991; Janik & Mrosovsky, 1994; Johnson et al., 1988a, 1989; Mistlberger, 1995; Mrosovsky et al., 1989; Reebs & Mrosovsky, 1989b; Van Reeth & Turek, 1989; Wickland & Turek, 1994). Studies to date have not controlled for changes in activity following IGL ablations, although most have observed that these ablations strongly reduce activity in response to stimuli such as novel wheels and TZ. Despite this

shortcoming, most of the research has found that IGL lesions attenuate the response to activity. In the research conducted here, it is possible to conclude that when the independent variable, i.e., activity, is held constant between lesion and control groups, complete IGL ablations do block the effects of activity on the circadian system. Of the 5 mice determined to have complete IGL ablations, none showed clear τ modulations or phase shifts to forced TM running at any phase of the free-running rhythm. When taken with other research, this result strongly suggests that the IGL mediates the effects of activity on the SCN in mice.

One previous study that utilized a daily schedule of restricted, yet voluntary wheel running, reported that DHT lesions to the SCN prevented activity entrainment (Edgar, personal communication). In the present study, none of the mice with complete 5HT lesions entrained, suggesting that 5HT input to the SCN is also necessary for entrainment to forced activity schedules. However, several mice came close to entraining, and most showed τ changes during the TM schedule. In fact, 18 of 19 mice showed some type of τ or phase change. This indicates that non-photic stimuli can affect the circadian clock in the absence of 5HT innervation; conceivably, a forced running schedule with a periodicity that more closely matches τ might be capable of entraining these mice. Evidently, in the absence of 5HT input, the IGL-NPY pathway, or other pathways, are sufficient to mediate some effects of activity on the SCN clock.

Increased drinking activity in the DHT lesioned mice was apparent at all stages of the experiment (Figure 27). This is consistent

with other studies showing that hypothalamic 5HT plays a role in sodium and fluid homeostasis (Halliday et al., 1995).

SUMMARY AND CONCLUSIONS

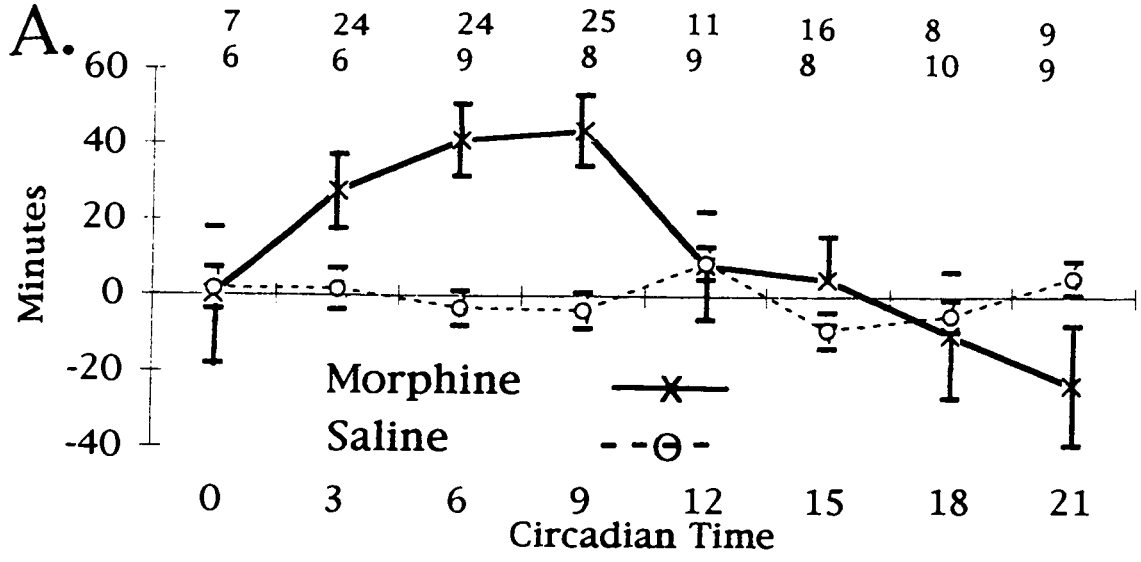
Experiment 1 established that the PRC to an activity-inducing stimulus in mice is quite similar to PRC's for such stimuli in hamsters. In experiment 2 forced running by TM was introduced as a means of precisely controlling the duration and intensity of activity. Activity pulses of 3 h duration induced by the TM failed to reliably induce clear phase shifts, but when administered on a daily basis, this activity stimulus was sufficient to entrain free-running rhythms. SCN-ablations demonstrated that the SCN is the site of the pacemaker that mediates entrainment to TM running schedules, although it is not the site of the pacemaker that times anticipatory activity to feeding schedules. To determine the pathway by which activity entrains the SCN pacemaker, brain TRY loading or depletion was attempted by dietary means, but little effect on TM entrainment or free-running pacemaker phase was evident. Radio frequency lesions of the IGL and DHT ablations of 5HT terminals within the SCN were conducted next to eliminate these 2 efferent pathways to the SCN. Complete IGL ablations blocked entrainment to TM running schedules, and largely eliminated τ-changes and phase shifts typically evident in partial lesioned or intact mice that fail to entrain. DHT lesions also greatly reduced the percentage of animals entraining. Those that entrained had partial lesions. Mice with complete lesions did show τ or phase

changes during the TM schedule. These results support a role for both the IGL and the RN in entrainment by forced running in mice. However, the precise role that these structures play is unclear. Both pathways seem to be necessary for this type of non-photic entrainment, but whether they contribute equally to non-photic effects induced by other types of stimuli remains to be determined. If both pathways are necessary, then presumably they interact at the circadian clock. At present, little information exists on the nature and effects of this interaction or coactivity at the cellular level.

APPENDIX A

Figure 1. (A) Phase-response curves showing the relationship between the size and direction of mean (± SE) phase shifts of wheel-running rhythms as a function of CT of MHCL (solid line) and saline (dashed line) injections in C57BL/6j mice. Positive values represent phase advances and negative values phase delays. The number of injections at each CT is indicated at the top of the figure (upper number = MHCL , lower = saline). (B) The latency (dashed line) and duration (solid line) of wheel running bouts following MHCL injections at eight circadian phases. Saline injections did not produce significant average phase shifts ($F(5,57)=1.663$, $p>.05$).

Figure 1.


A.
7 24 24 25 11 16 8 9
6 6 9 8 9 8 10 9
60
40
20
0
-20
-40
Minutes
Morphine
Saline
0 3 6 9 12 15 18 21
Circadian Time

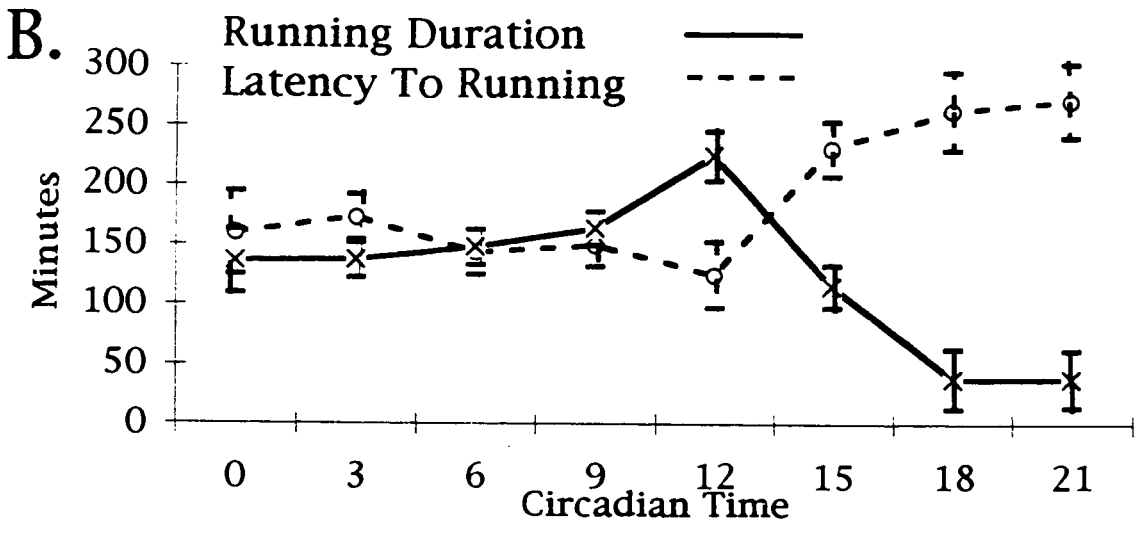

B.
Running Duration
Latency To Running
300
250
200
150
100
50
0
Minutes
0 3 6 9 12 15 18 21
Circadian Time

Figure 2. Activity records illustrating phase shifts induced by MHCL injections in individual mice. Each line represents 24 hours, plotted in 10 minute bins from left to right. Vertical deflections indicate bins during which wheel counts were recorded. Injection time is indicated by a circle. Regression lines were fit to activity onsets (opaque circles) by computer. (A) A 90 minute phase advance following injection at CT6. (B) The largest phase advance observed (350 minute, CT3 injection). (C) A 110 minute phase delay following injection at CT18. (D) Absence of phase shift when locomotor activity was prevented following an injection at CT3.

Figure 2.

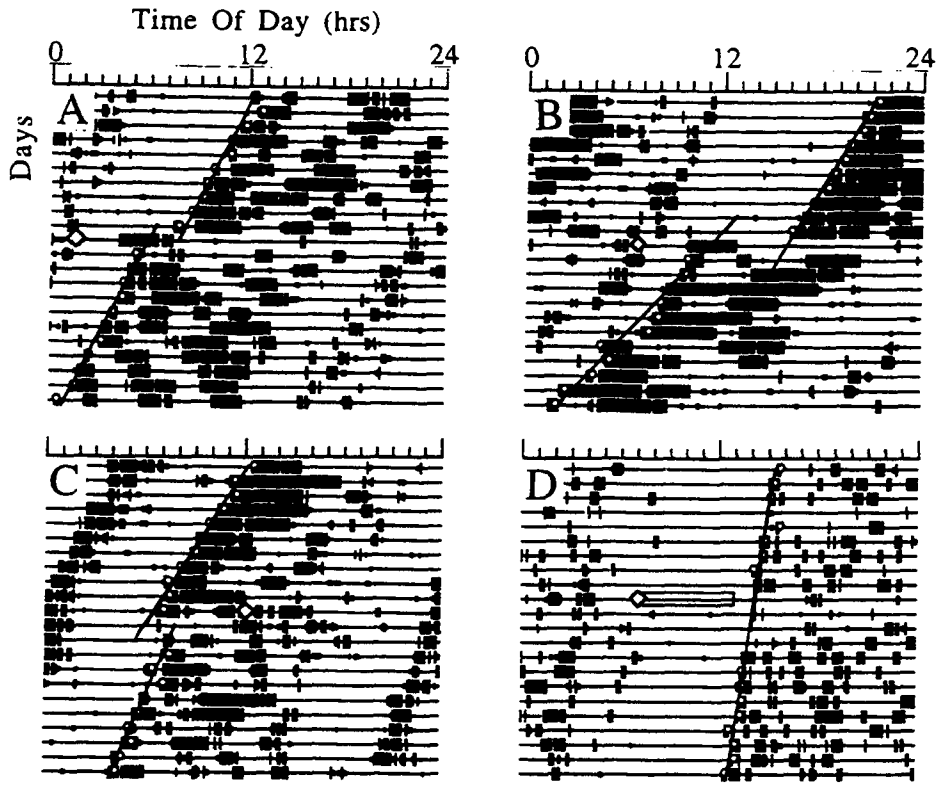


Figure 3. Double-plotted activity or drinking charts from mice subjected to forced TM running. Each line represents 48 consecutive hours, plotted in 10 minute bins from left to right. Consecutive days are also aligned vertically. Vertical deflections on each line represent bins in which wheel running (charts A-C) or drinking (charts D-F) were detected. Hours when mice were removed from their cages for TM running are bracketed and whited out on the second of each double-plot. (A) Mouse that entrained to forced TM running. During the last 3 weeks of TM running, the home cage running wheel was blocked for 6 days/week to ensure that entrainment was not due to wheel-running after TM sessions. (B) Mouse that exhibited a large phase advance when TM running occurred at about CT6. (C) Mouse that exhibited τ changes and apparent splitting. (D-F) Three mice from group 1b (no home cage wheel) that entrained to TM running.

Figure 3.

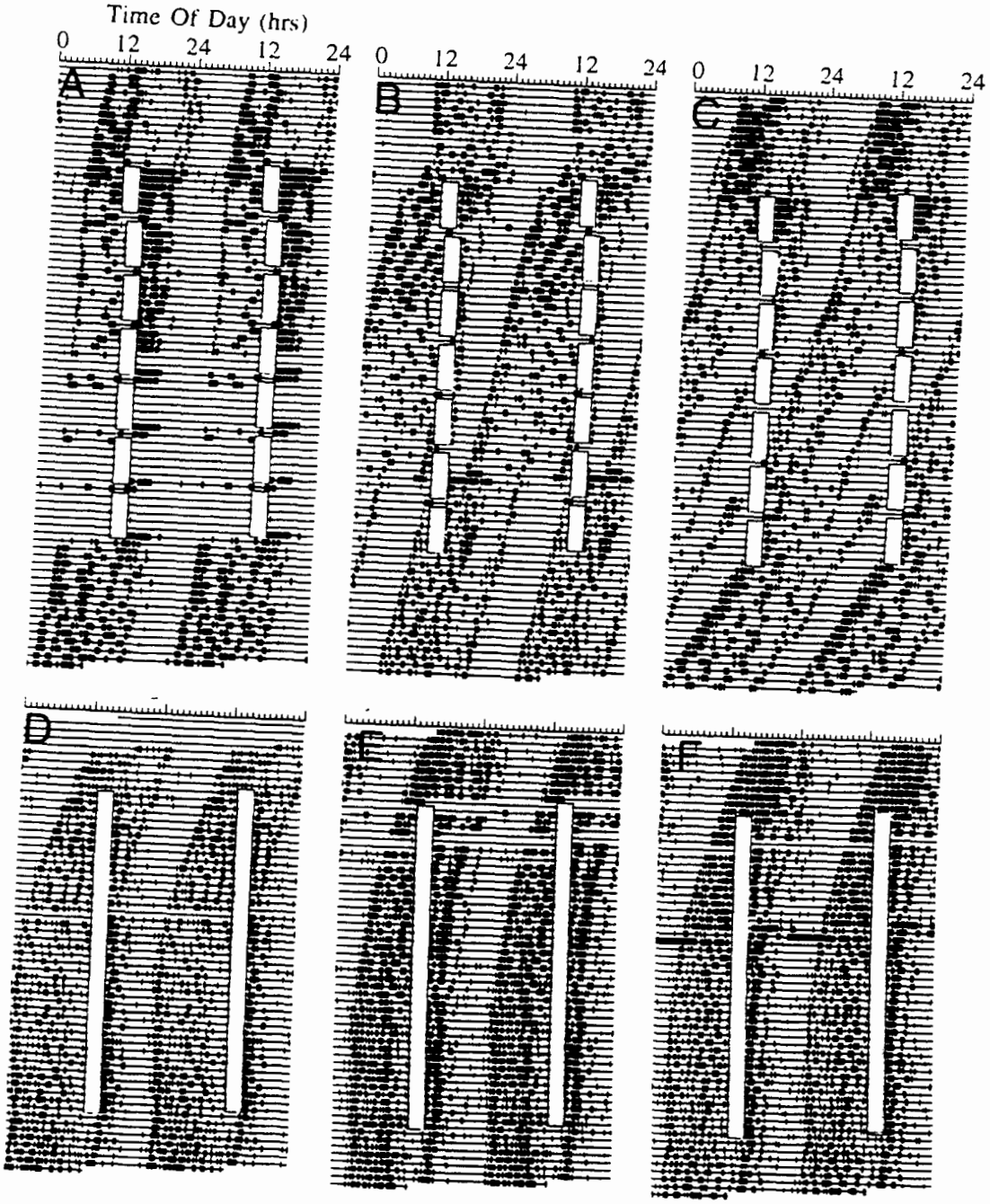


Figure 4. (A) Double-plotted wheel running chart of a mouse from group 2a that entrained to 3h/day access to home cage wheel. (B) Wheel-running rhythm of a mouse that did not entrain to wheel restriction, but that exhibited τ changes (illustrated by forward extrapolation of a regression line fit to activity onsets prior to wheel restriction). (C) Mouse that did not entrain to scheduled daily water access. (D-E) Drinking records from 2 mice from group 2b that did entrain to scheduled daily wheel access. (F) Wheel-running of control mouse. Plotting conventions as in Figure 3.

Figure 4.

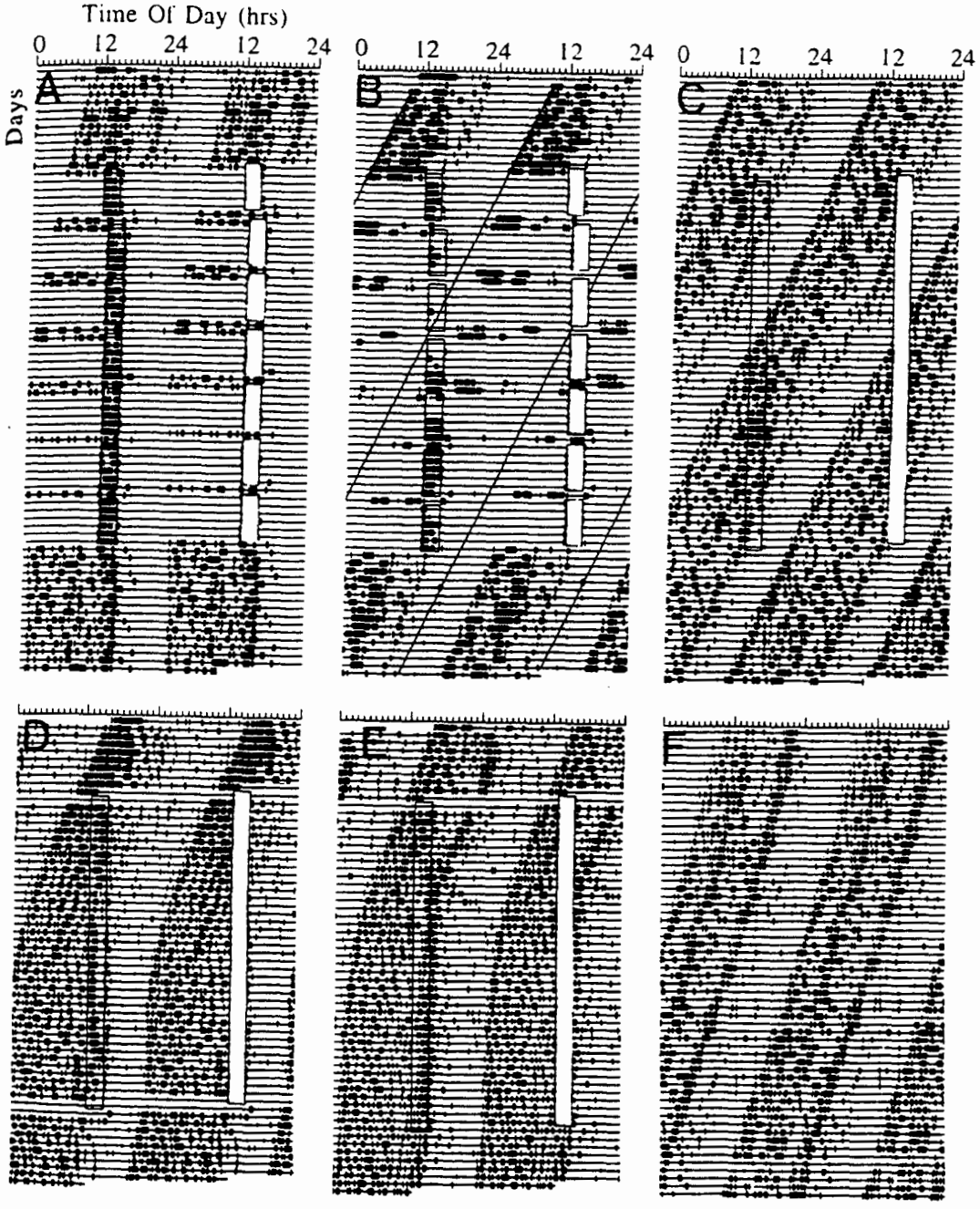


Figure 5. Mean (±SE) phase shifts induced by 3 hour TM running, TM transfer without running, or home cage wheel access at CT6 and CT21. **significantly different at $p<.001$, *$p<.05$.

Figure 5.

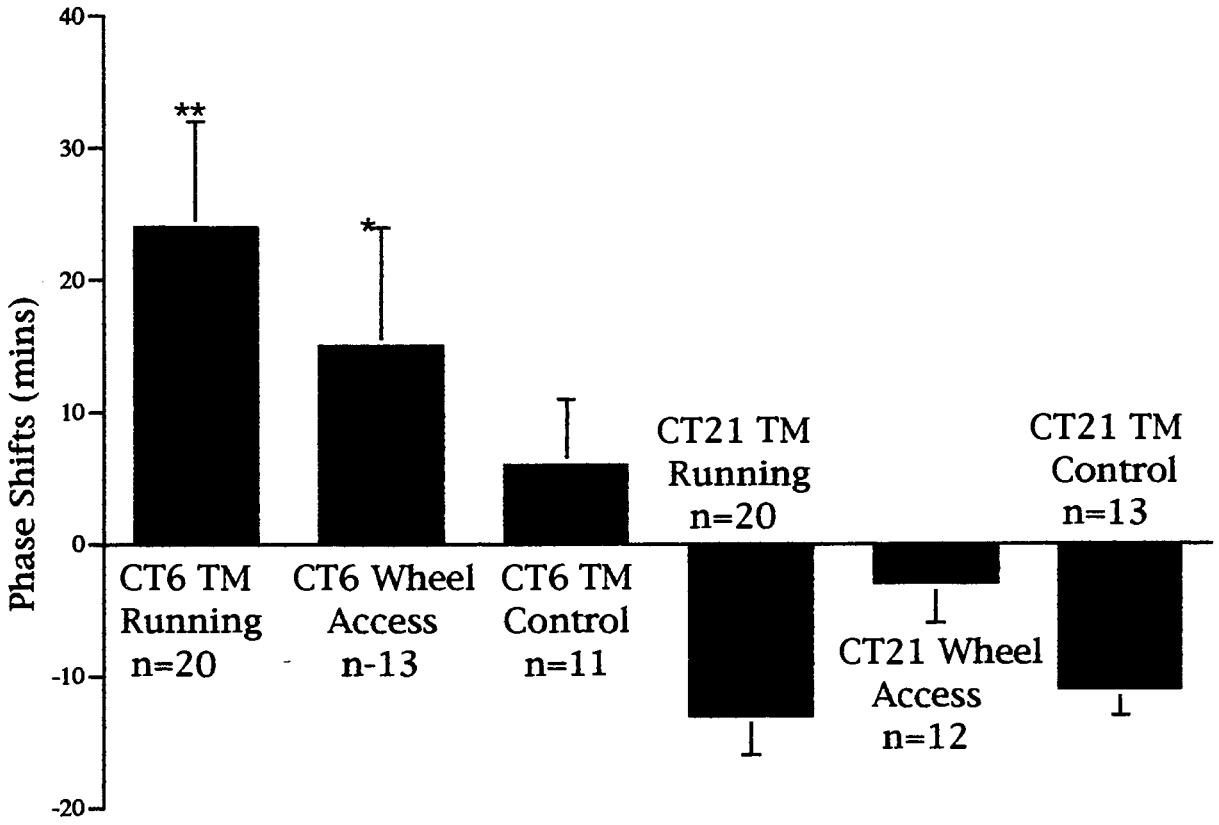

40
30
20
10
0
-10
-20
Phase Shifts (mins)
**
*
CT6 TM Running n=20
CT6 Wheel Access n-13
CT6 TM Control n=11
CT21 TM Running n=20
CT21 Wheel Access n=12
CT21 TM Control n=13

Figure 6. Drinking rhythms illustrating phase shifts induced by scheduled running (indicated by opaque rectangles). (A) 110 minute phase advance following 3 hour access to the home cage wheel (W; mouse ran 3,300 revolutions). (B) 50 minute phase advance following a TM session (T) at CT6. C. 20 minute phase delaying following "control" transfer to TM without running. (C) 30 minute phase delay following a TM session at CT21, and 20 minute phase delay following 3 hour access to the home cage wheel (mouse ran 6,000 half revolutions). Plotting conventions as in Figure 1.

Figure 6.

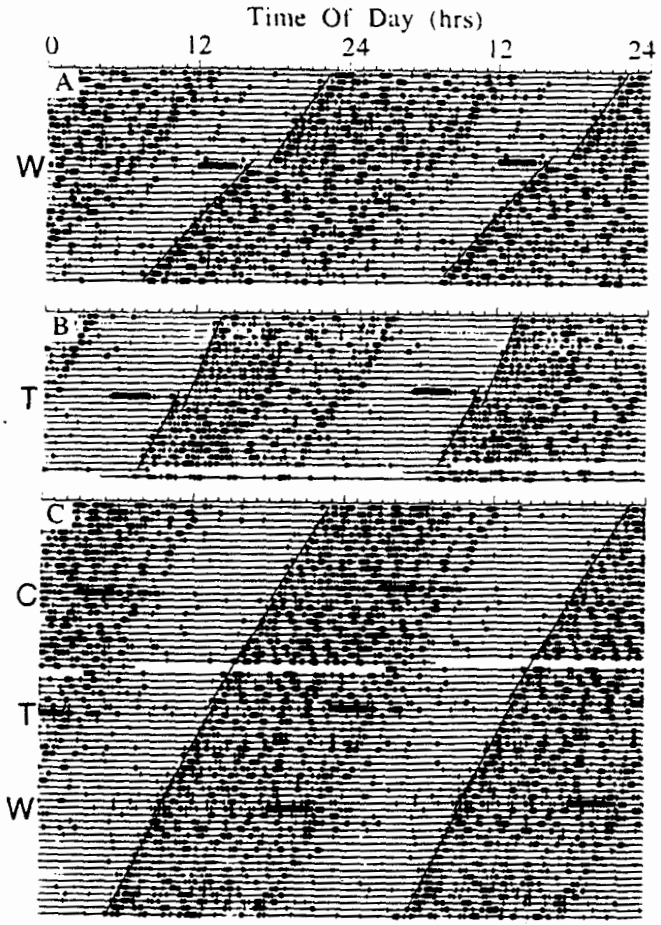
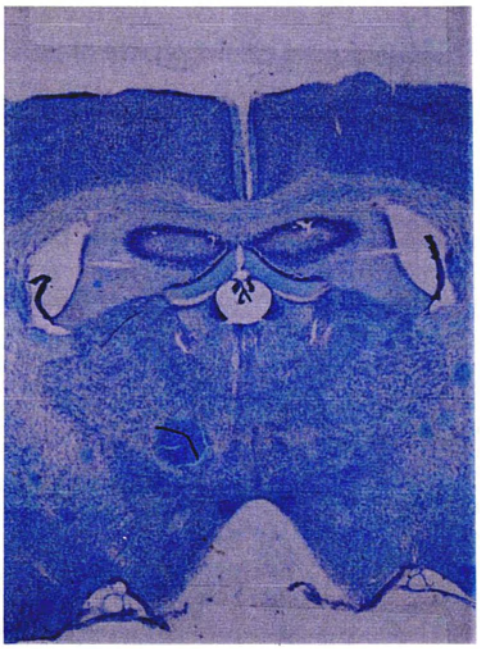


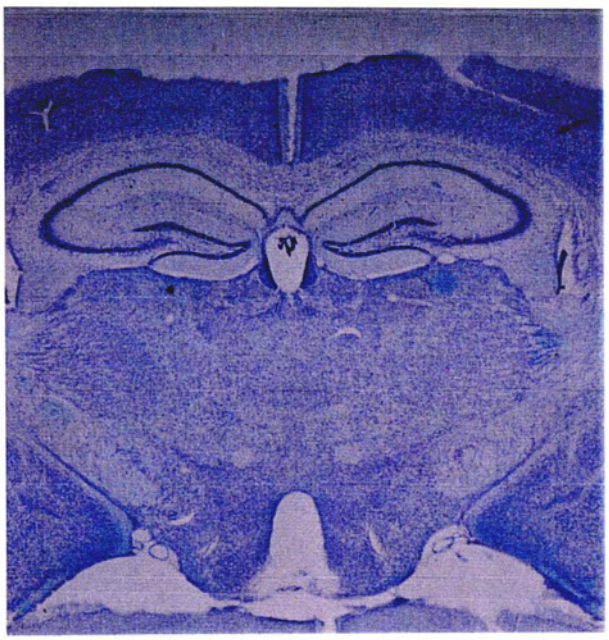
Figure 7. Photomicrographs of cresyl violet stained sections from mice with representative radiofrequency lesions. A. complete SCN ablation, judged to be unambiguous (activity chart Figure 8d). B. SCN ablation, judged to be ambiguous (activity chart Figure 9a). C. partial SCN ablation, judged to be 20 % complete, with considerable damage dorsal and caudal to the SCN (activity chart Figure 9c). D. no lesion of SCN (activity chart Figure 10d).

Figure 7.

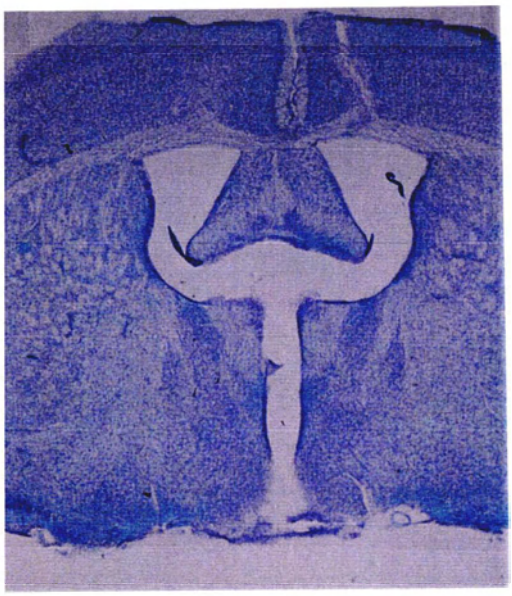
A.



B.



C.



D.

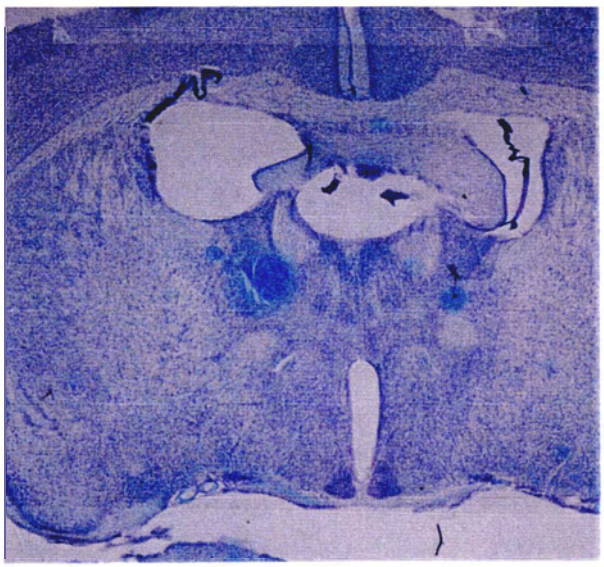


Figure 8. Activity charts from representative mice with complete SCN ablations judged to be unambiguous. A-D. Double-plotted actograms. Time is plotted left-to-right in 10 min. bins (144/day). Each bin is represented by a point (zero activity) or a vertical deflection (3 points = 1-9 wheel revs, 5 points = 10-19 refs, 7 points = >20 refs). Feeding time (4 h/day) during food restriction is indicated by the vertical hollow bar. Triangles indicate resumption of ad-lib food access. E-H. Average waveforms of activity during the last 2 weeks of food restriction from mice represented in panels A-D. Feeding time is indicated by the opaque column.

Figure 8.

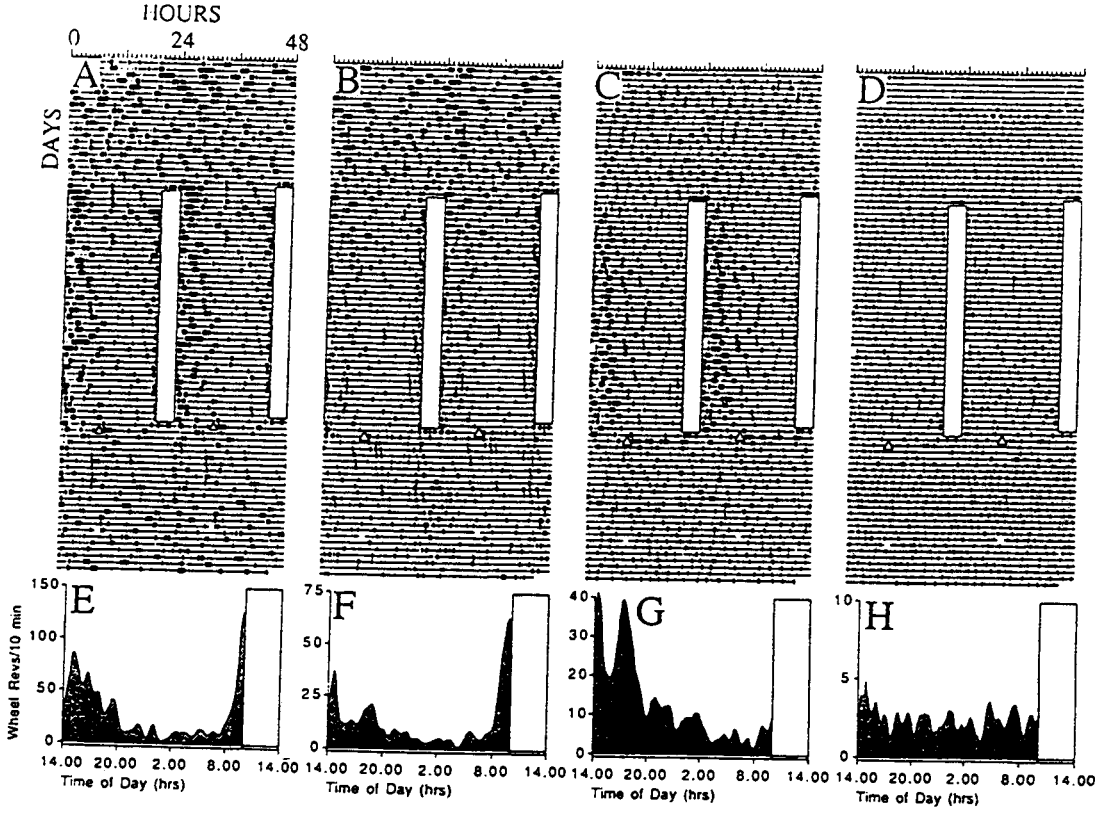

HOURS
0
24
48
A
B
C
D
DAYS
E
F
G
H
Wheel Revs/10 min
150
100
50
0
75
25
40
30
20
10
5
14.00 20.00 2.00 8.00 14.00
Time of Day (hrs)

Figure 9. Activity charts from representative mice with partial SCN ablations. A-D. Double-plotted actograms, in same format as Figure 8a-d. Chart A was reduced in size to match the length of the other charts. SCN damage estimates; A. 99% (ambiguous), B. 95%, C. 20%, D. 10%. E-H. Average waveforms of activity, in same format as Figure 8e-h.

Figure 9.

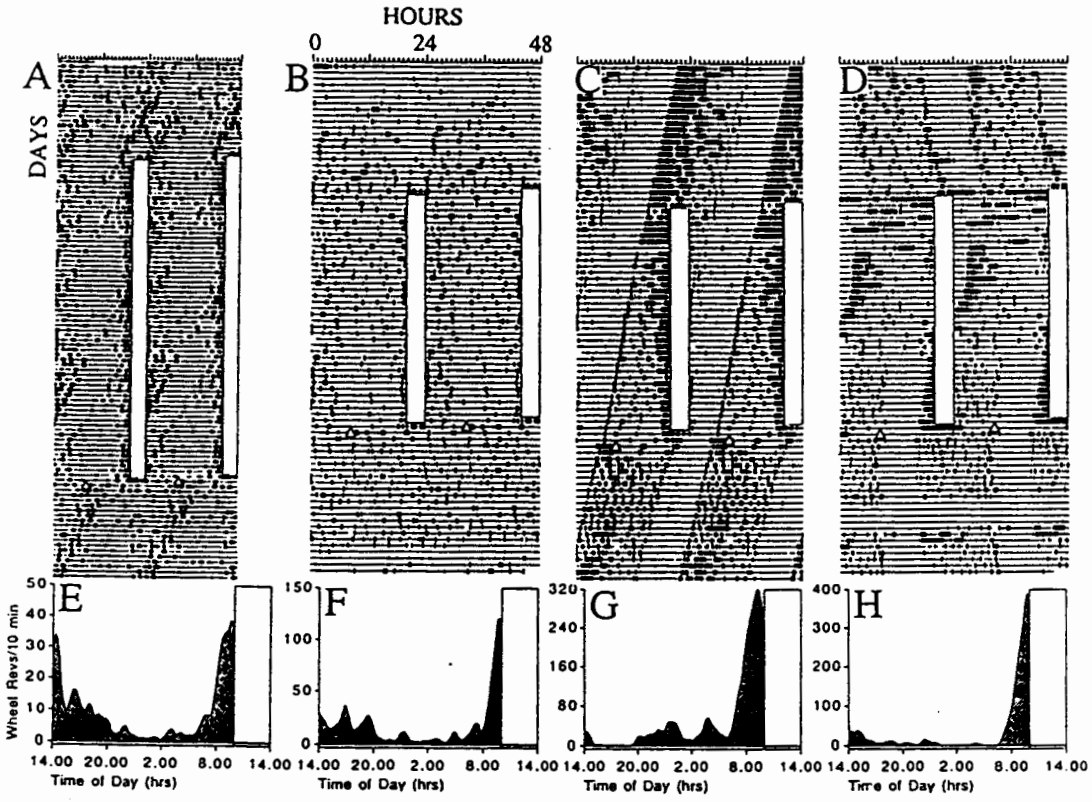

HOURS
0
24
48
A
B
C
D
DAYS
E
F
G
H
Wheel Revs/10 min
50
40
30
20
10
0
150
100
50
0
320
240
160
80
0
400
300
200
100
0
14.00 20.00 2.00 8.00 14.00
Time of Day (hrs)

Figure 10. Activity charts from representative mice with no SCN damage. A-D. Double-plotted actograms, in same format as Figure 8a-d. E-H. Average waveforms of activity, in same format as Figure 8e-h.

Figure 10.

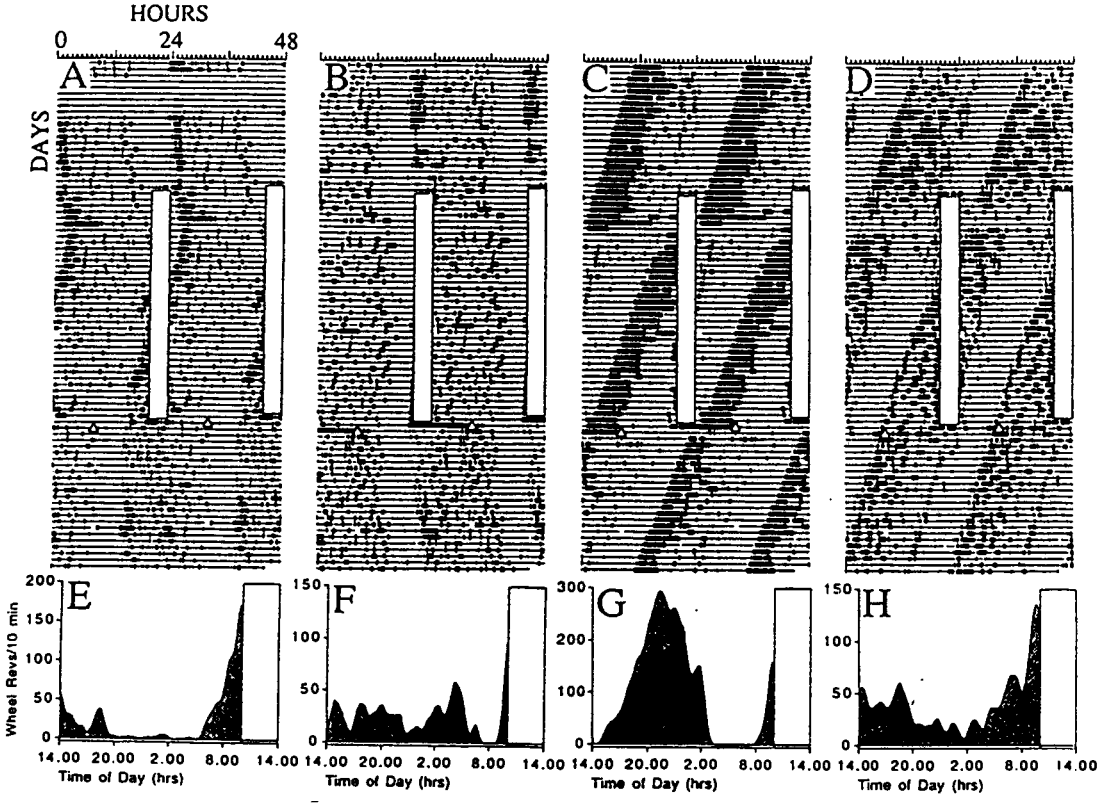

HOURS
0
24
48
A
B
C
D
DAYS
E
F
G
H
Wheel Revs/10 min
200
150
100
50
0
300
14.00 20.00 2.00 8.00 14.00
Time of Day (hrs)

Figure 11. Ratios of activity during 4 h blocks of time preceding and following the usual feeding time on the day of delayed feeding. Block 4 represents the usual feeding time. Food was provided after block 6. Ratios represent activity in each block divided by total activity during the 6 blocks. Solid circles: mice with complete SCN ablations that did not exhibit anticipatory activity (n=7). Diamonds: mice with complete or near complete SCN ablation that did anticipate mealtime (n=5). Squares: mice with partial SCN ablations, all of which anticipated (n=14). Triangles: mice with intact SCN, all of which anticipated (n=9).

Figure 11.

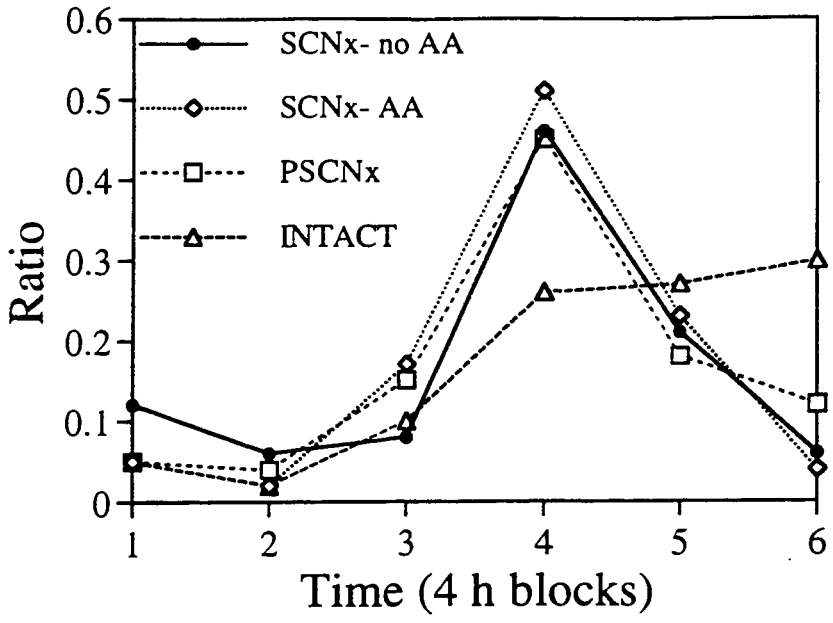

0.6
0.5
0.4
0.3
0.2
0.1
0
Ratio
SCNx- no AA
SCNx- AA
PSCNx
INTACT
1
2
3
4
5
6
Time (4 h blocks)

Figure 12. A. Average wheel revolutions per day during the last 2 weeks of food restriction in 1. mice with complete SCN ablations that did not anticipate (n=7), 2. mice with complete or near complete SCN ablations that did anticipate (n=5), 3. mice with partial SCN ablations (n=14), and 4. mice with intact SCN (n=9). B. Body weights measured at the end of food restriction in groups 1-4. C. Free-running τ measured by regression lines fit to wheel running data during the 10-14 days prior to food restriction in mice that exhibited free-running rhythms. Entrained: mice in which free-running rhythms entrained to feeding time. Free-ran: mice in which free-running rhythms did not entrain.

Figure 12.

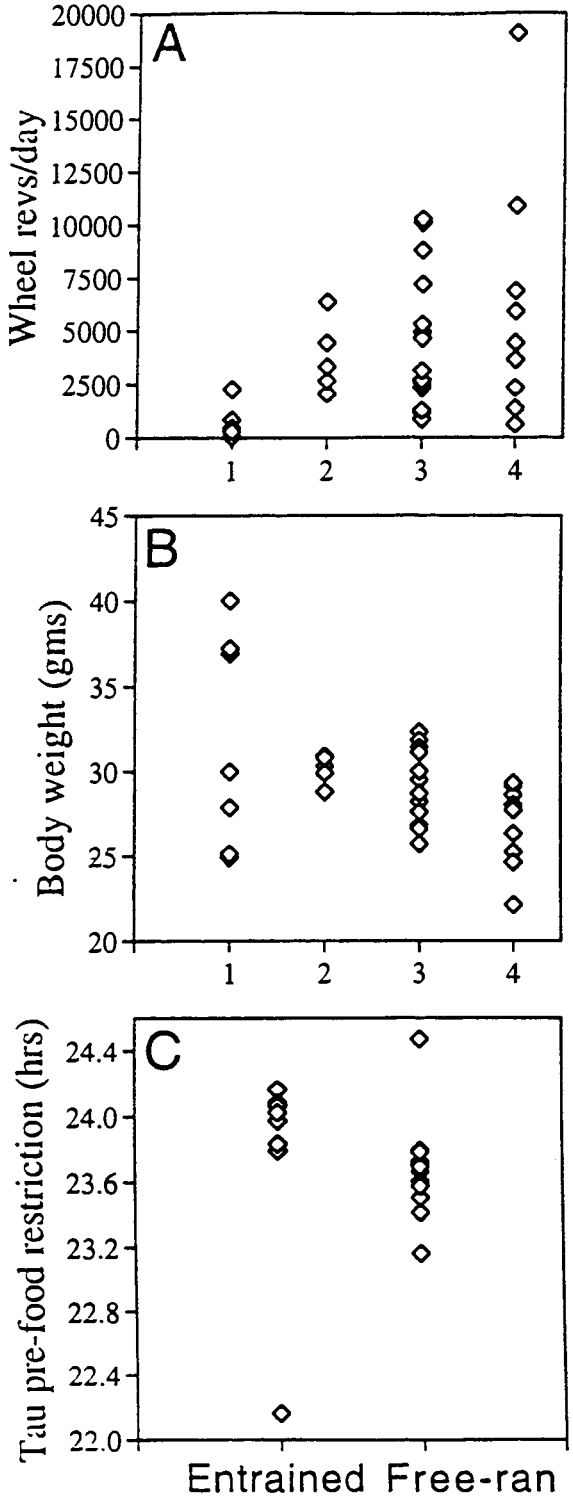
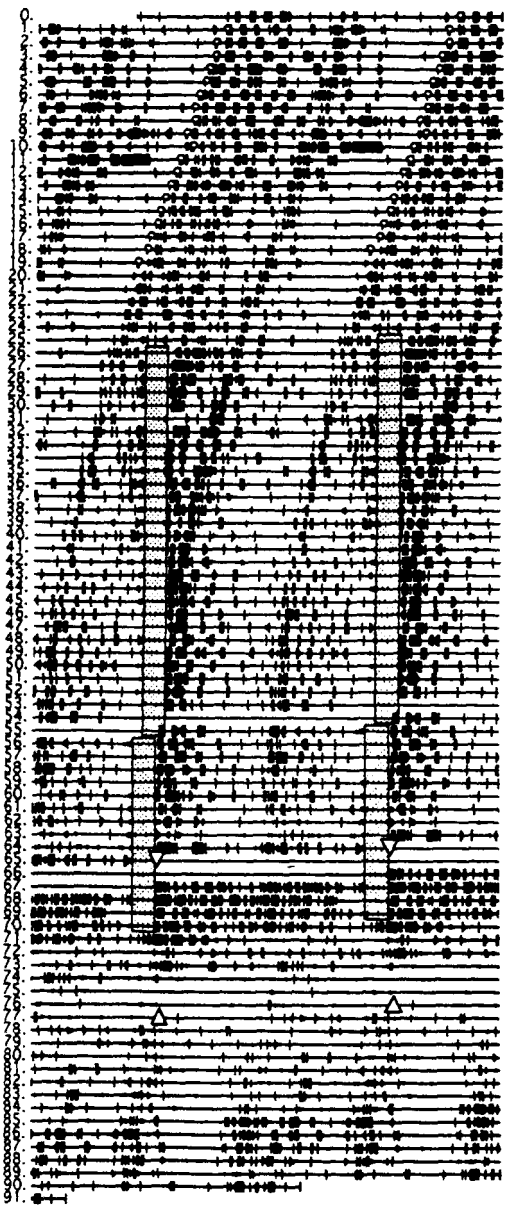

A
Wheel revs/day
20000
17500
15000
12500
10000
7500
5000
2500
0
1
2
3
4
B
Body weight (gms)
45
40
35
30
25
20
1
2
3
4
C
Tau pre-food restriction (hrs)
24.4
24.0
23.6
23.2
22.8
22.4
22.0
Entrained
Free-ran

Figure 13a, b. Double plotted actogram (a) and average waveform (b; days 67-70) showing the effects of TRY-free diet on the circadian rhythms of a C57 mouse that entrained to daily TM running (shaded vertical bar). After stable entrainment had occurred (CT 20-23), the TRY-free diet was initiated (inverted triangle). The special diet was discontinued (triangle) after 13 days.

Figure 13a, b.

A.



B.

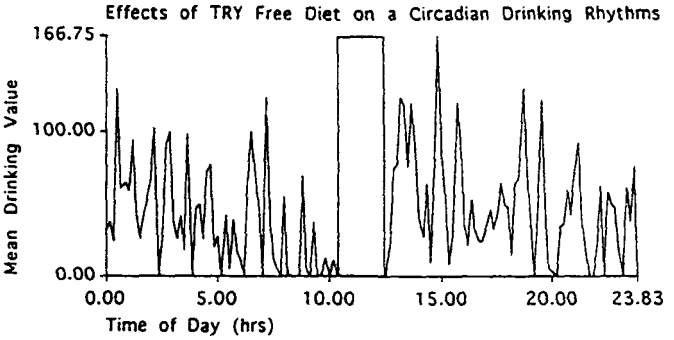
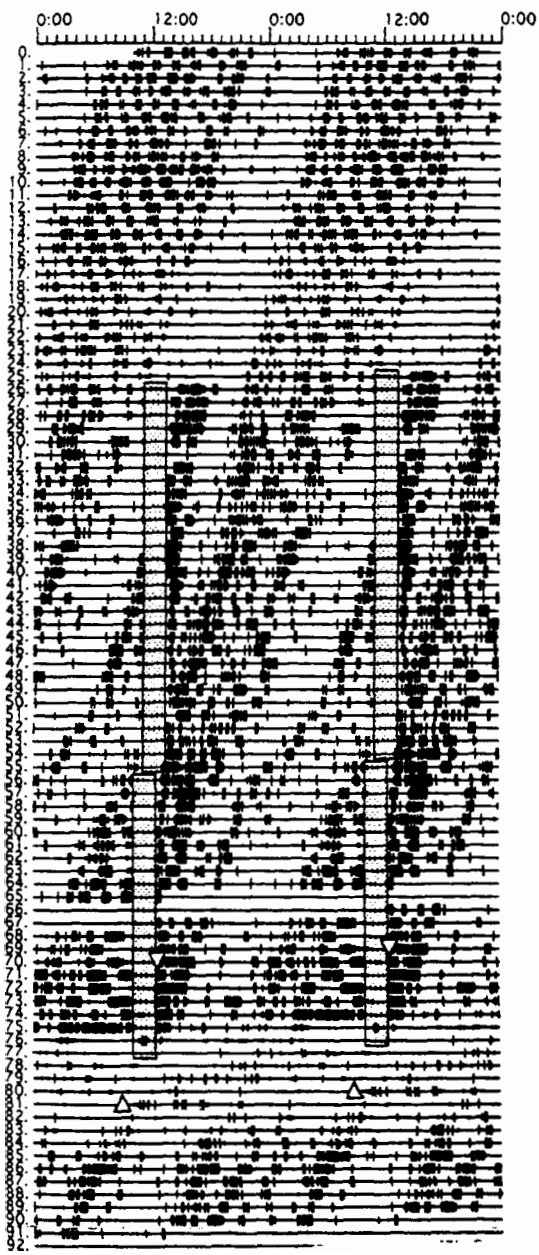


Figure 14a, b. Double-plotted actogram (A) and average waveform (B; days 74-76) showing the effects of 12 days of TRY-free diet on the circadian rhythms of a C57 mouse that failed to entrain to daily TM running (approximately 2 km/2 hours / day).

Figure 14a, b.

A.



B.

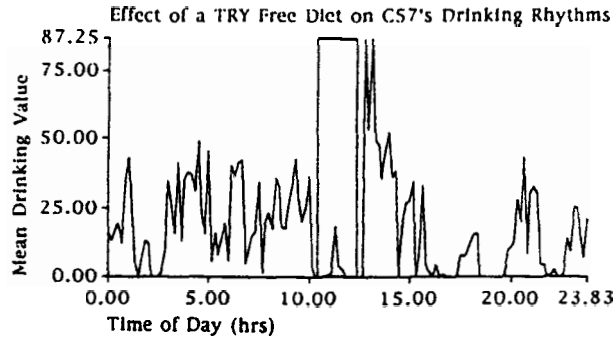


Figure 15. Average drinking behavior across the different experimental conditions. Drinking activity increased, but not significantly, when the TRY-free diet was initiated (TM-TRY), then decreased significantly when TM running was terminated (POST-TM-TRY; $t=2.305$, $p<.038$), and decreased again, although not significantly, when the TRY-free diet was terminated (POST-TRY).

Figure 15.

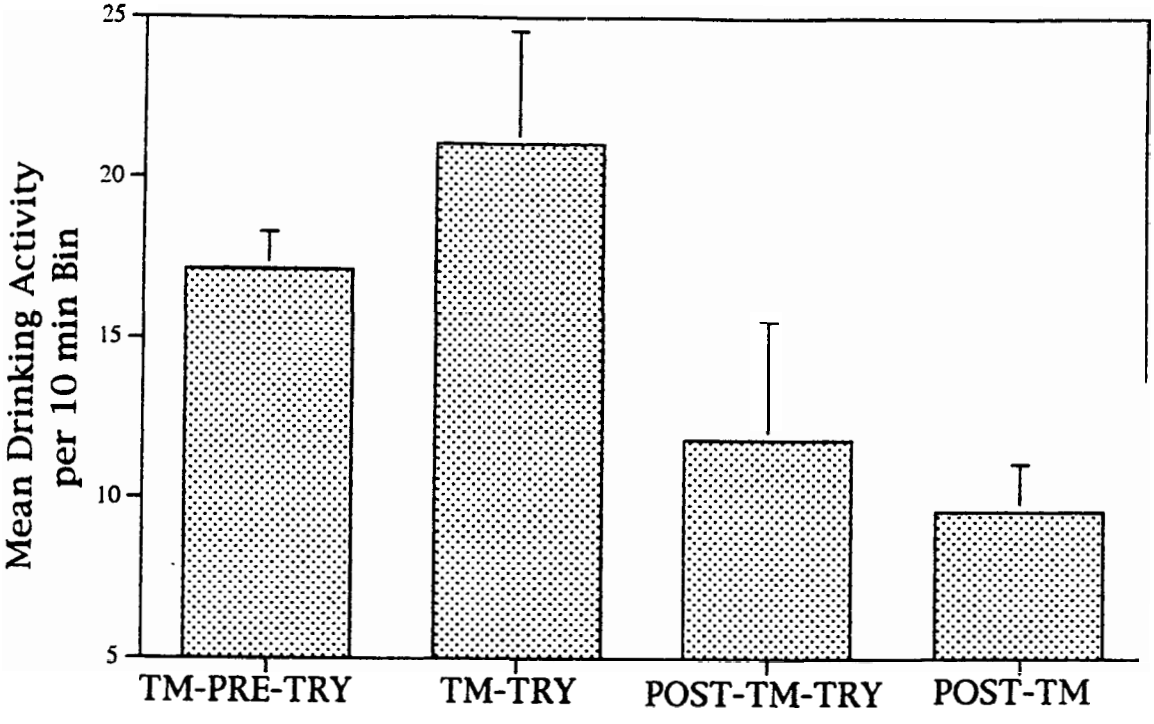
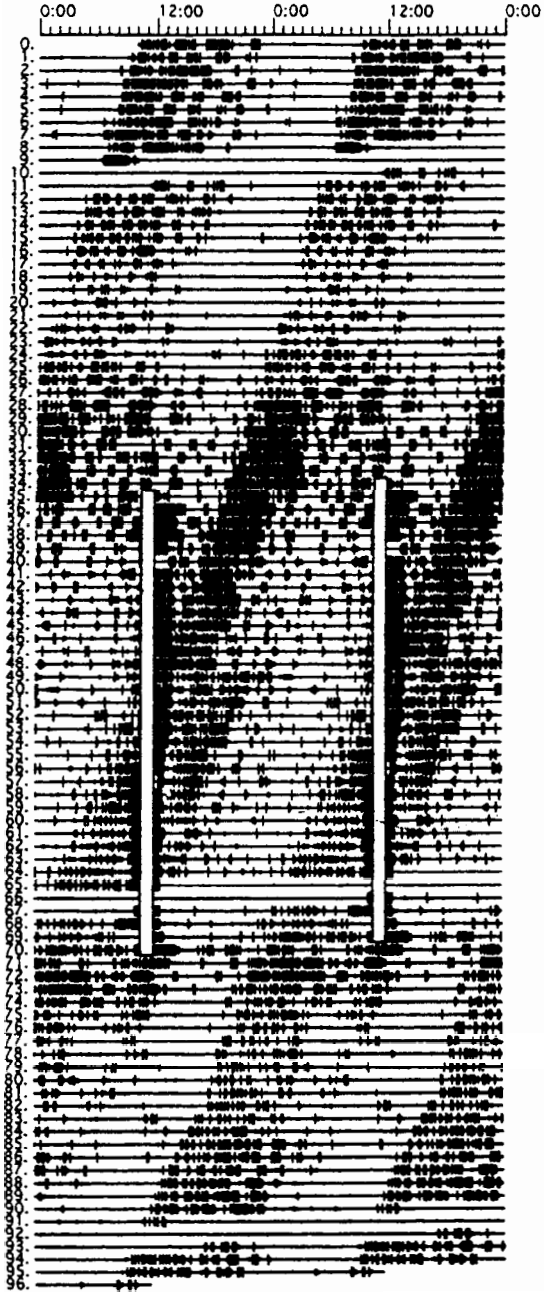

Mean Drinking Activity per 10 min Bin
25
20
15
10
5
TM-PRE-TRY
TM-TRY
POST-TM-TRY
POST-TM

Figure 16a, b. Double plotted actogram (A) and average waveform (B; days 63-65) from a C57 mouse maintained on a TRY-free diet with Isomil formula available for a fixed 90 minute period each day, represented by the opaque vertical bar.

Figure 16a &b.

A.



B.

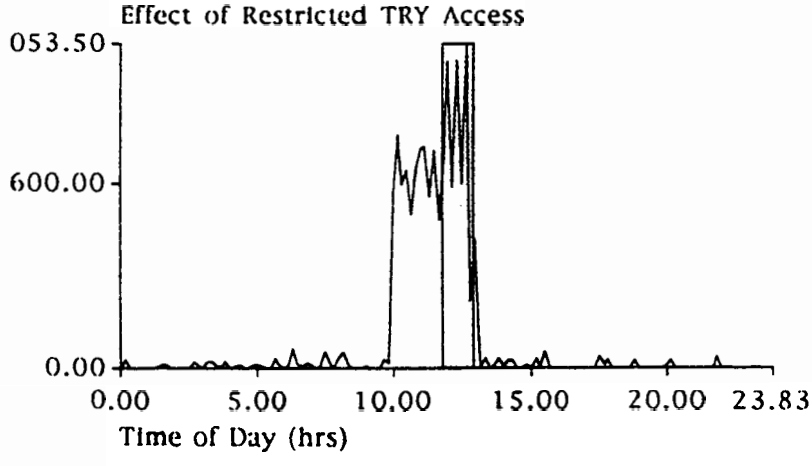
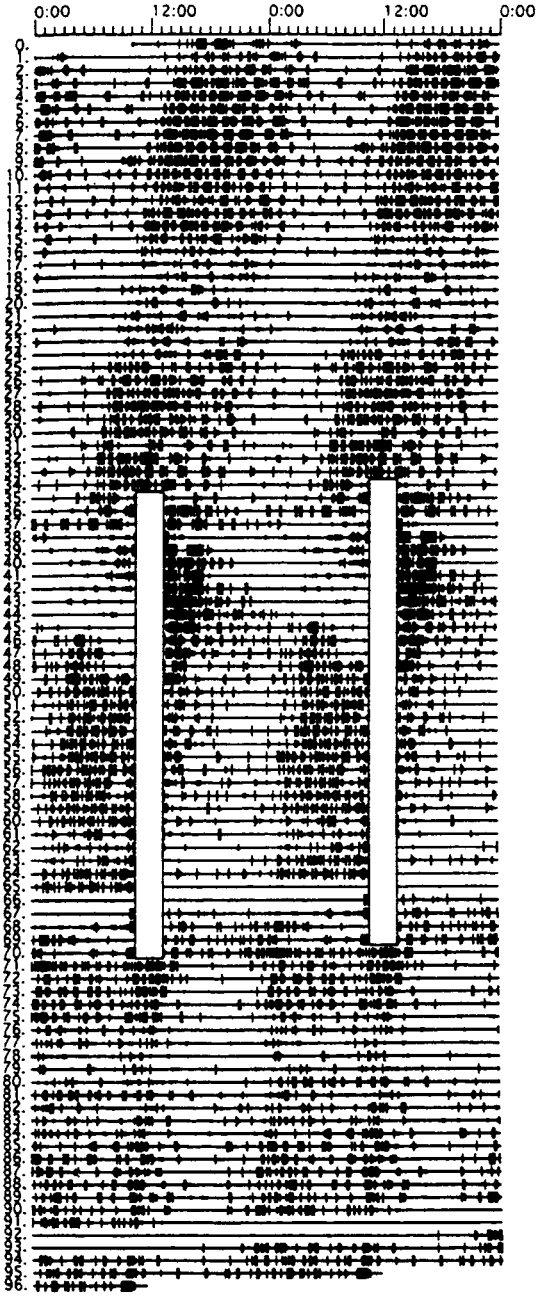


Figure 17a, b. Two double-plotted drinking charts of C57 mice showing the effects of restricted feeding (opaque vertical bar) on the free-running drinking rhythm. One mouse may have been close to entraining to the feeding schedule (A), while the other is representative of the rest of the mice, and clearly did not entrain (B).

Figure 17.

A.



B.

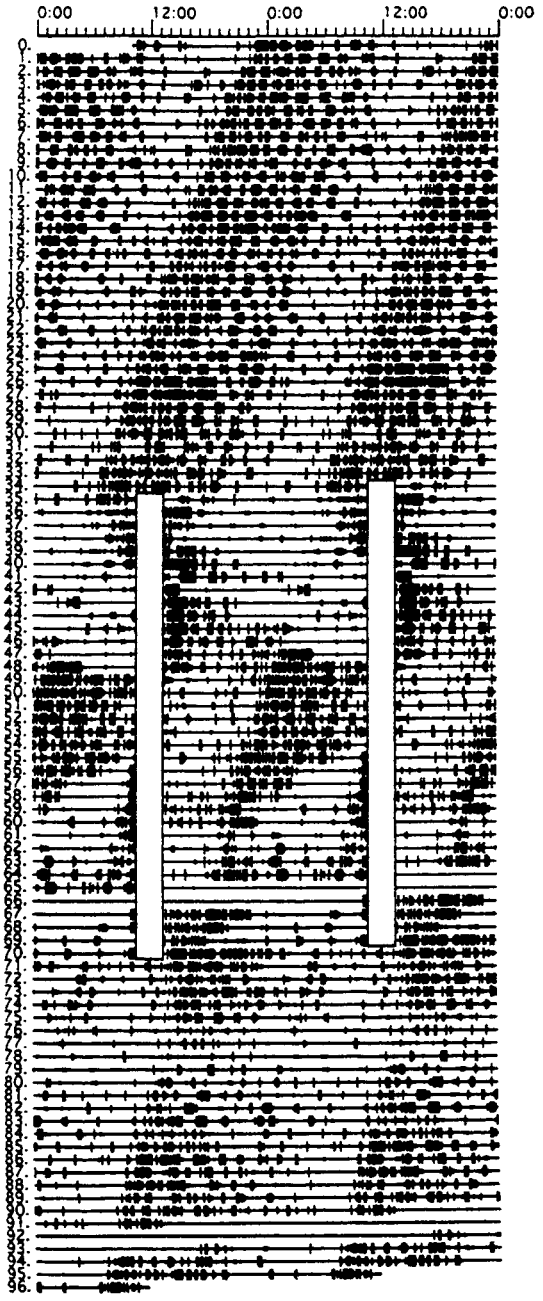
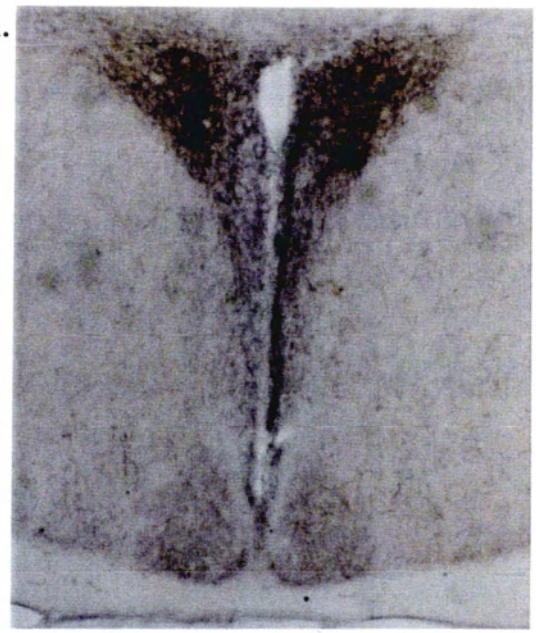


Figure 18. Photomicrographs of SCN in an intact mouse (A, B) and a mouse with a complete IGL ablation (C, D). The optic chiasm is at the bottom of each photo, and the third ventricle to the immediate right in C & D. Magnification: A,C = 32x; B, D = 200x.

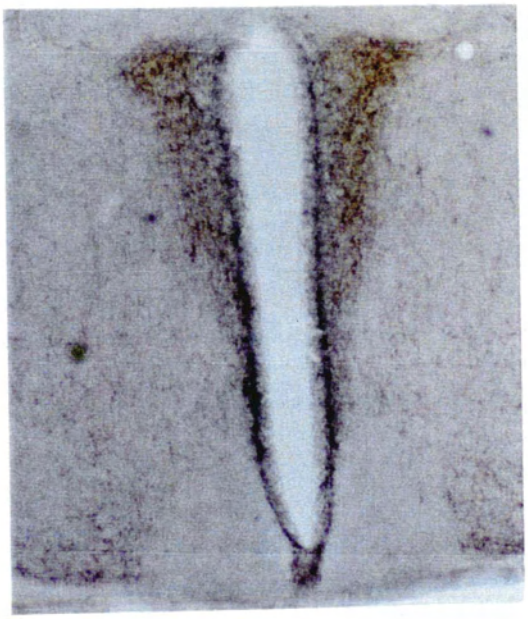
Figure 18.

A.



B.

C.



D.

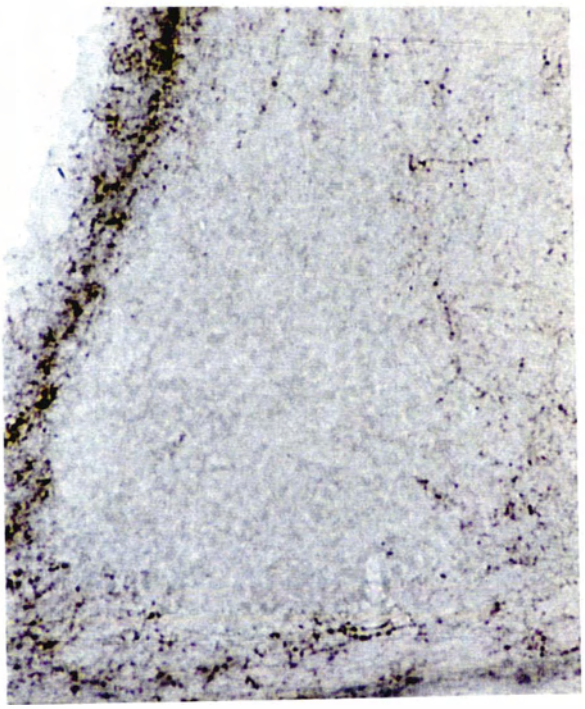
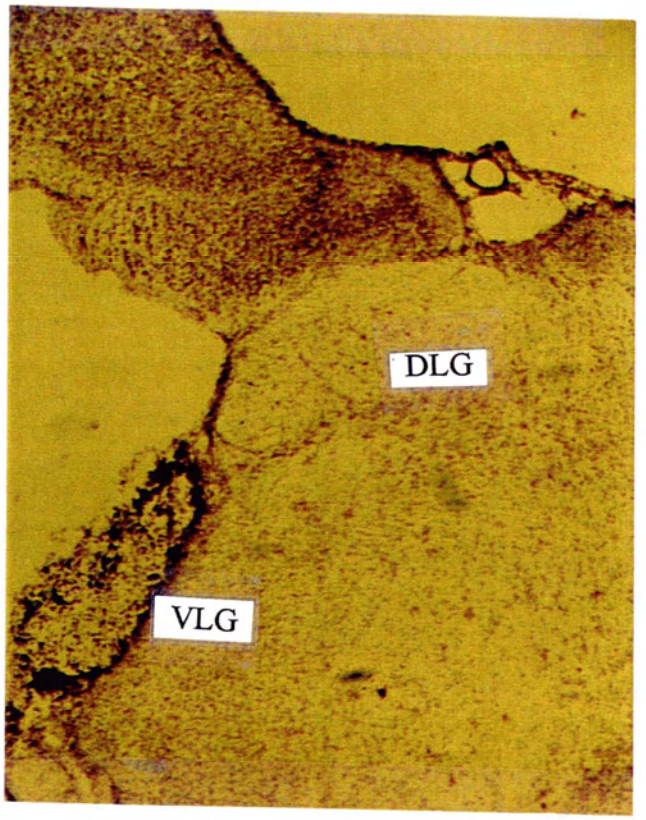


Figure 19. Representative partial (A) and complete (B) IGL lesions, visualized with nissl stain and viewed at 200x magnification. The lesion is represented by darker staining scar tissue, with an absence of nissl bodies. Both lesions are between the DLG and the VLG. However, in example A, some ventral-medial IGL was likely spared.

Figure 19a, b.

A.



B.

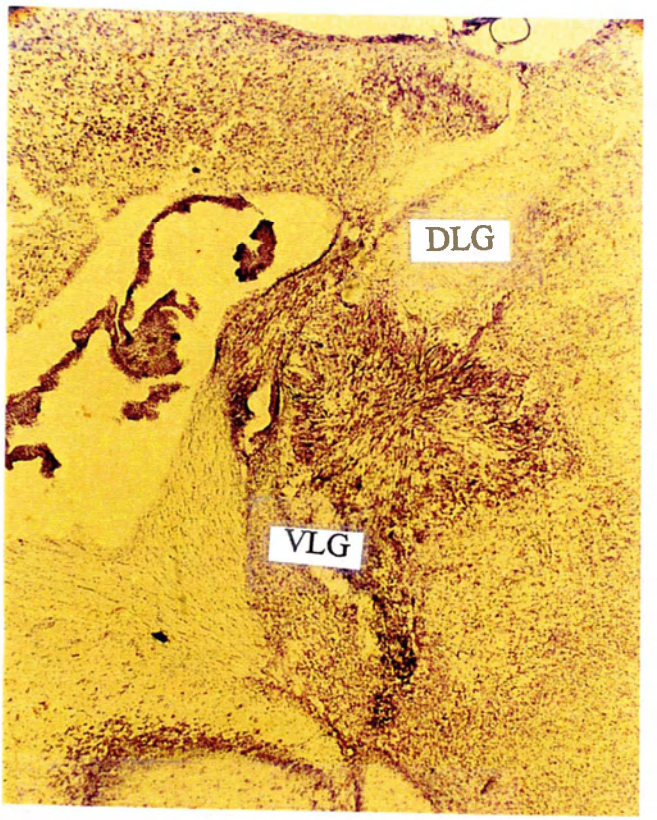
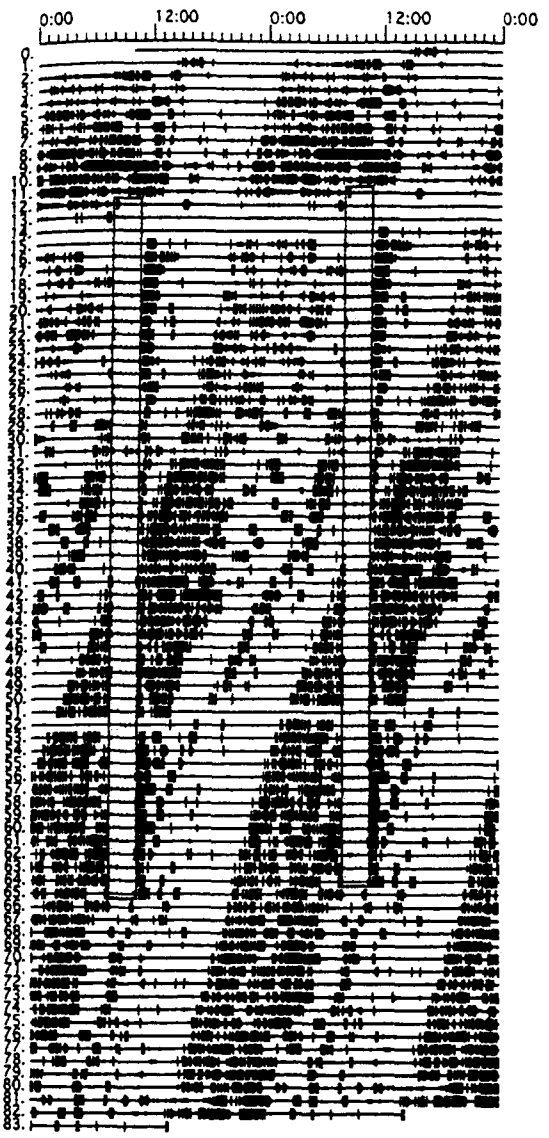


Figure 19c, d. Double-plotted drinking charts from 2 mice subjected to 3 hours per day of forced TM running. Actogram C represents a partial lesioned animal, while D represents a complete lesioned animal as rated by 2 blind investigators. Plotting conventions as in Figure 3.

Figure 19c & d.

C.



D.

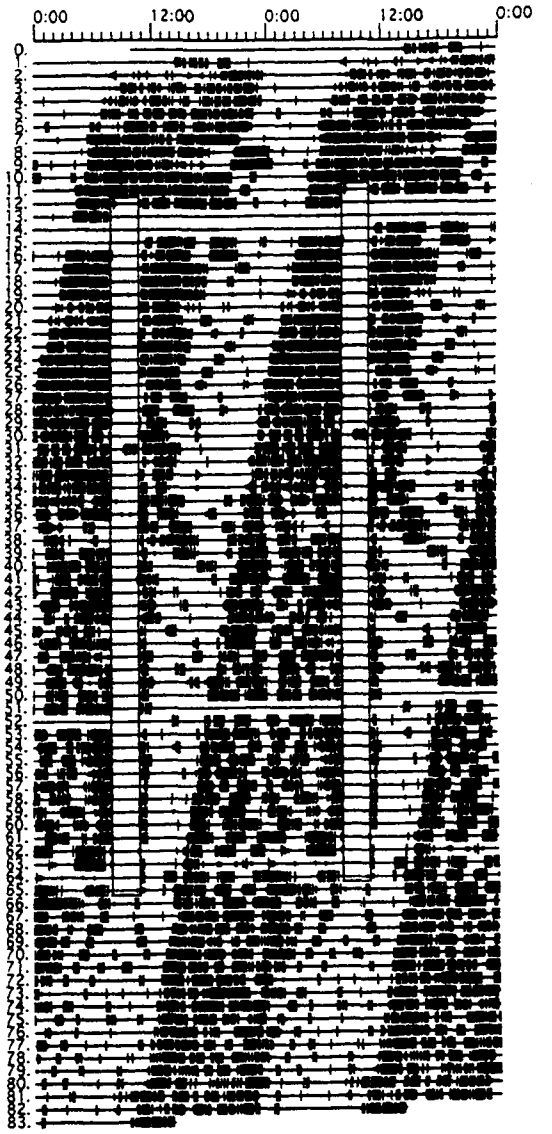
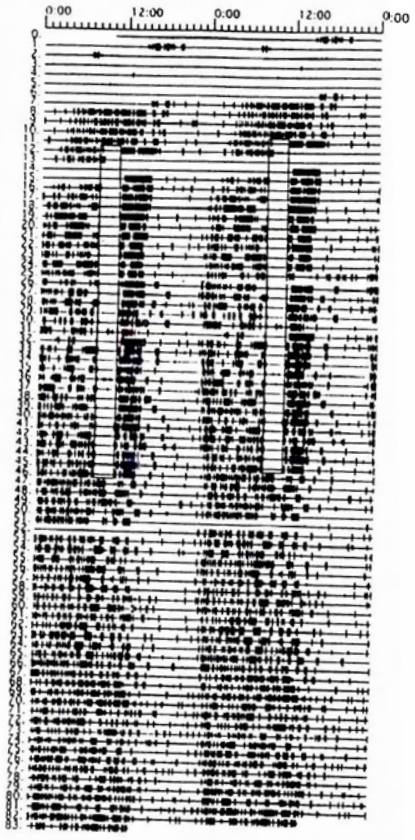


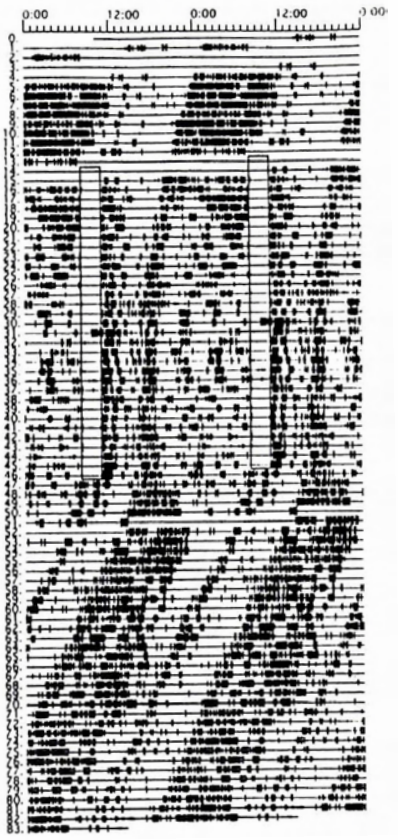
Figure 20. Double-plotted drinking charts (A,B) from 2 incomplete IGLx mice that entrained to 3 h/day of forced TM running and their corresponding histology at 32x magnification (c & d). Plotting conventions as in Figure 3

Figure 20.

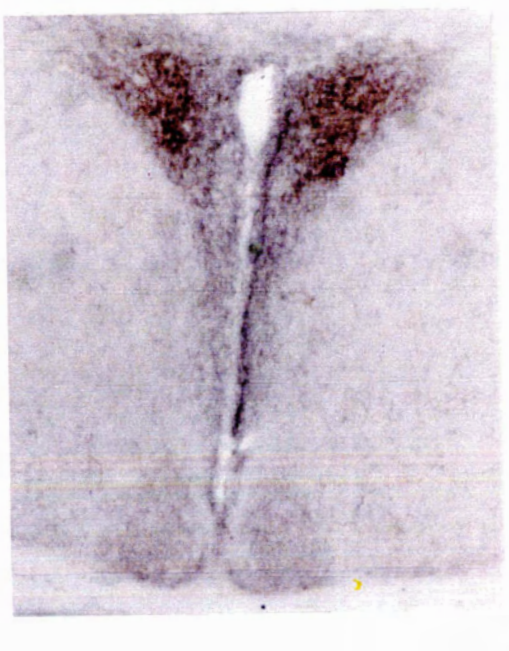
A.



B.



C.



D.

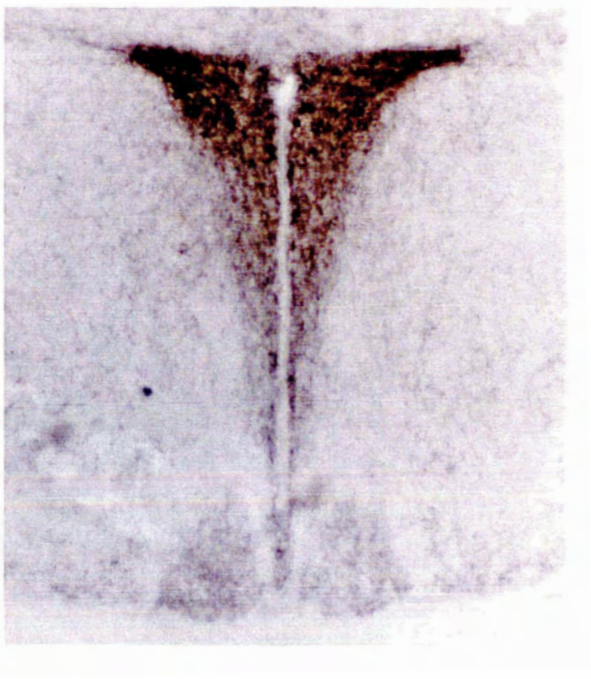
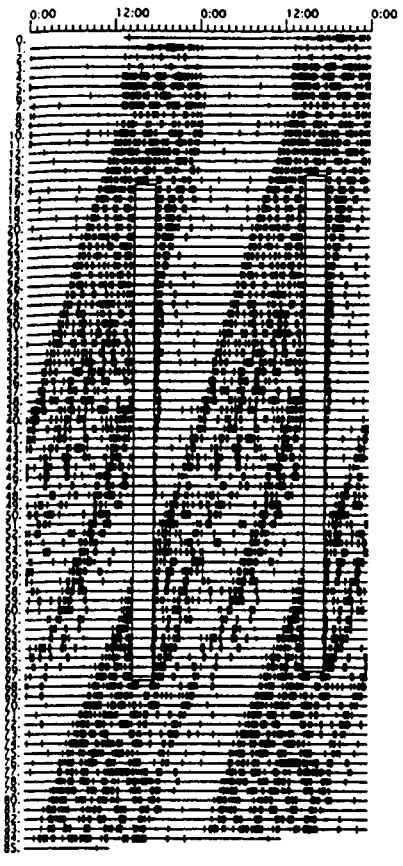


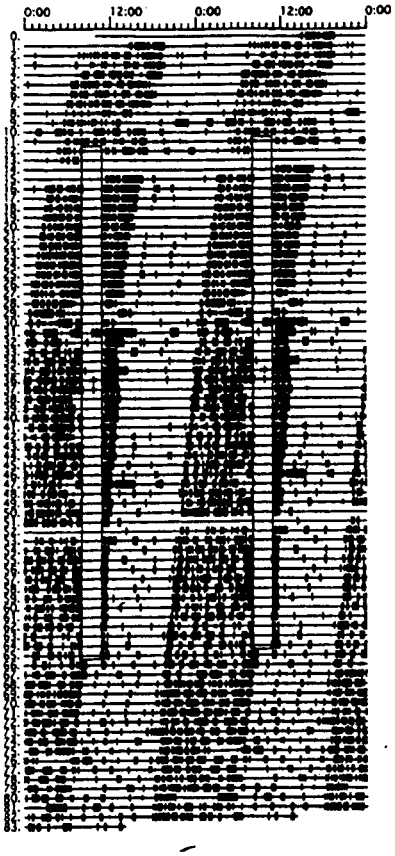
Figure 21. Double plotted drinking charts from 3 complete IGLx mice that did not entrain to 3 h/day of forced TM running. Plotting conventions as in Figure 3.

Figure 21.

A.



B.



C.

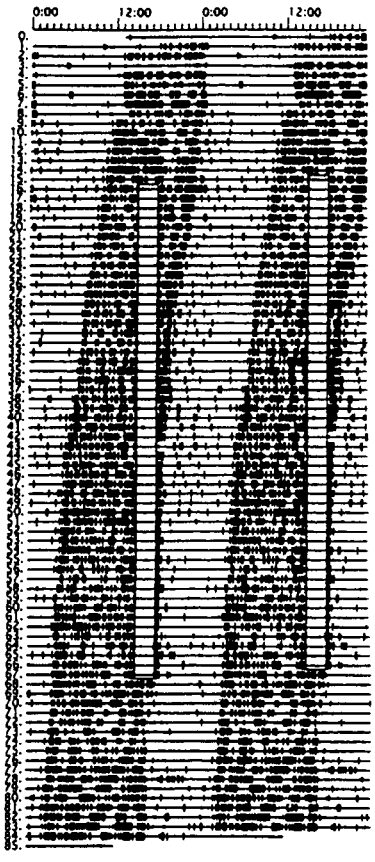
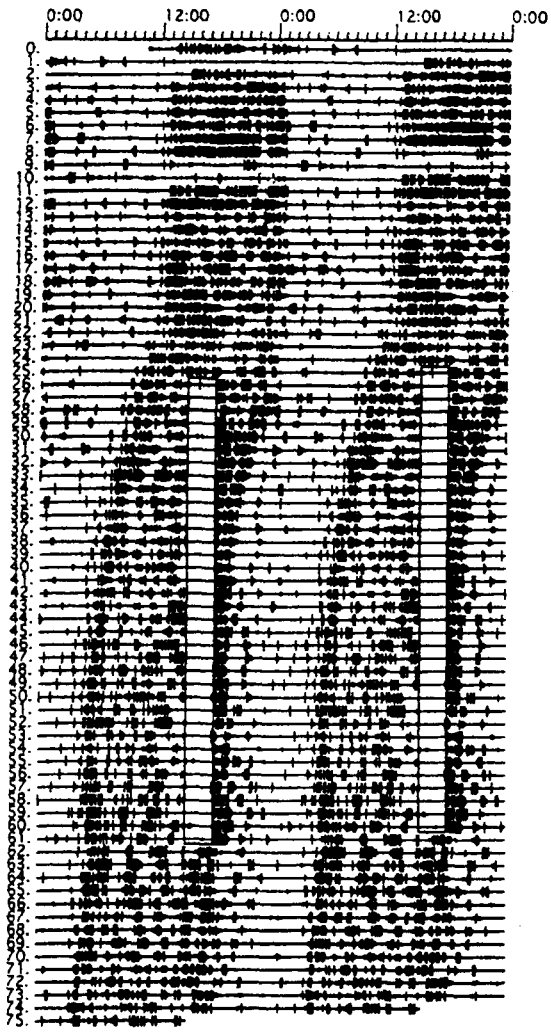


Figure 22. Double-plotted drinking charts from 2 DHT lesioned mice that entrained to forced running (3 hrs/day).

Figure 22.

A.



B.

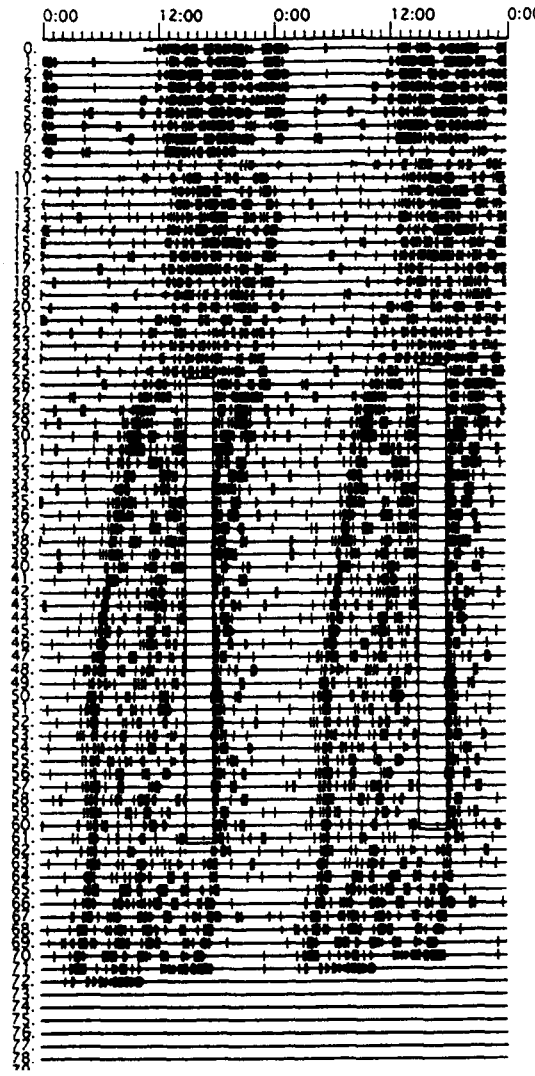
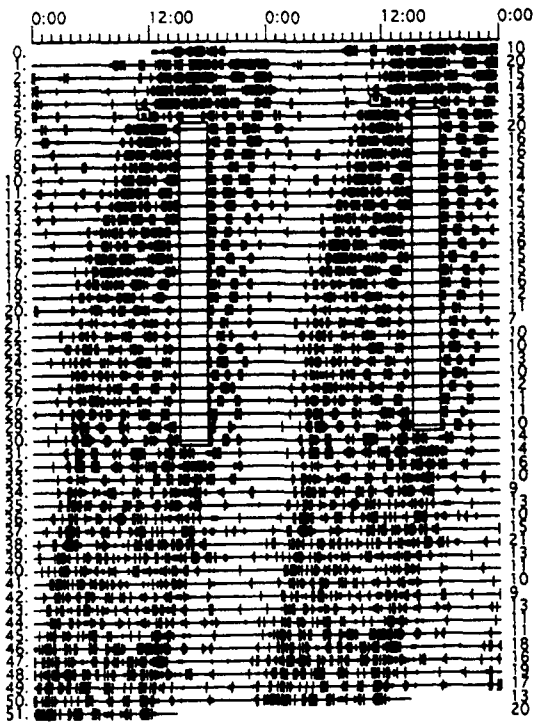


Figure 23. Double-plotted drinking charts from 2 intact control mice subjected to 3 forced TM running without home cage wheels using the modified procedure (control group 2c). Example of control groups 2a and 2b can be seen in experiment 2. Square represents enucleation time.

Figure 23.

A.



B.

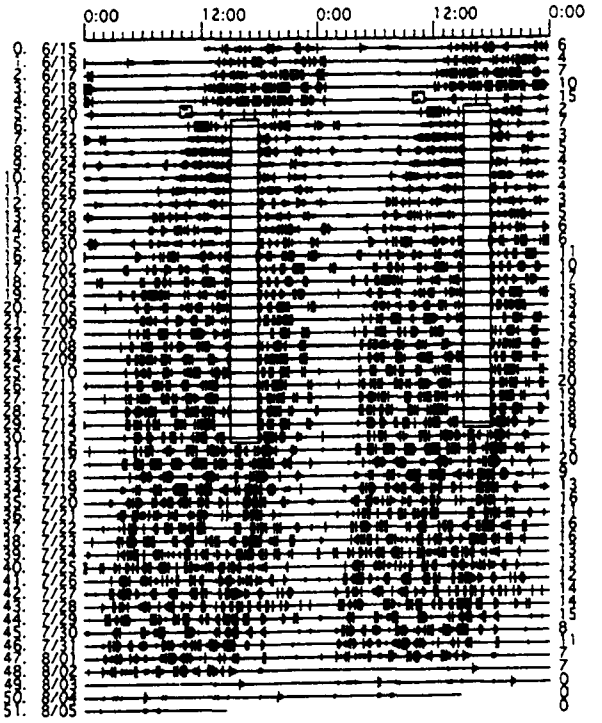
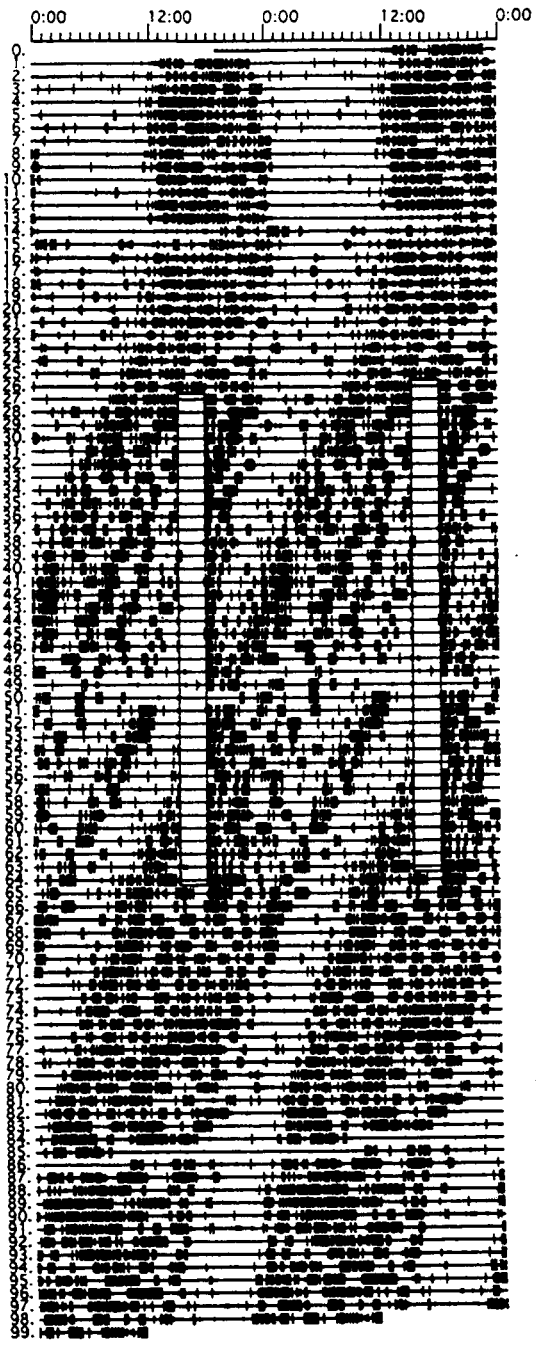


Figure 24. Double-plotted drinking charts from 2 DHT lesioned mice that did not entrain to forced TM running (3h/day) .

Figure 24.

A.



B.

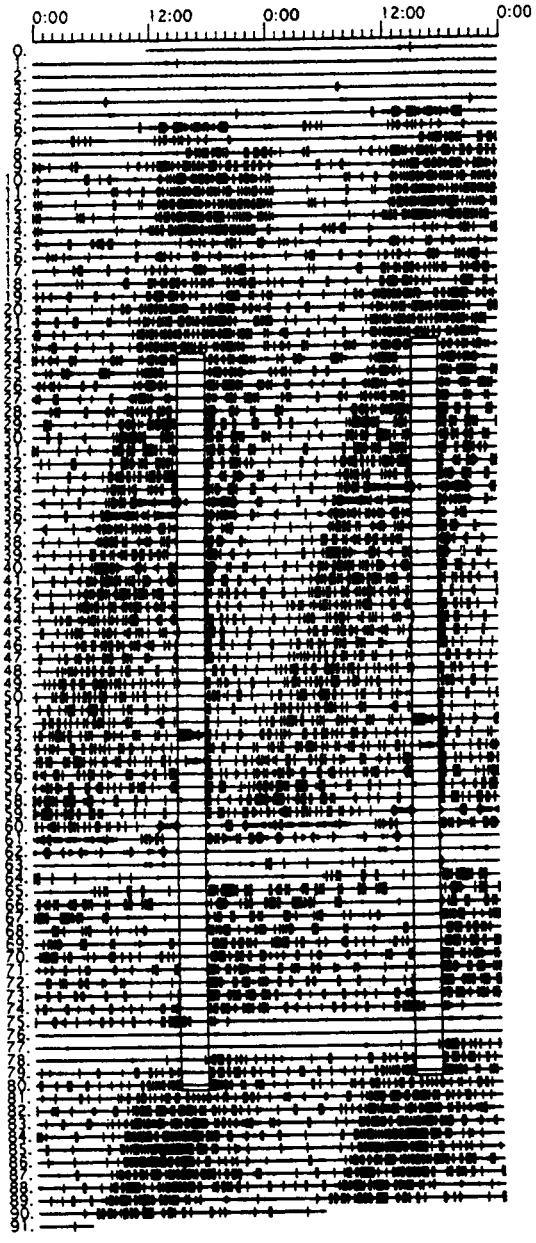
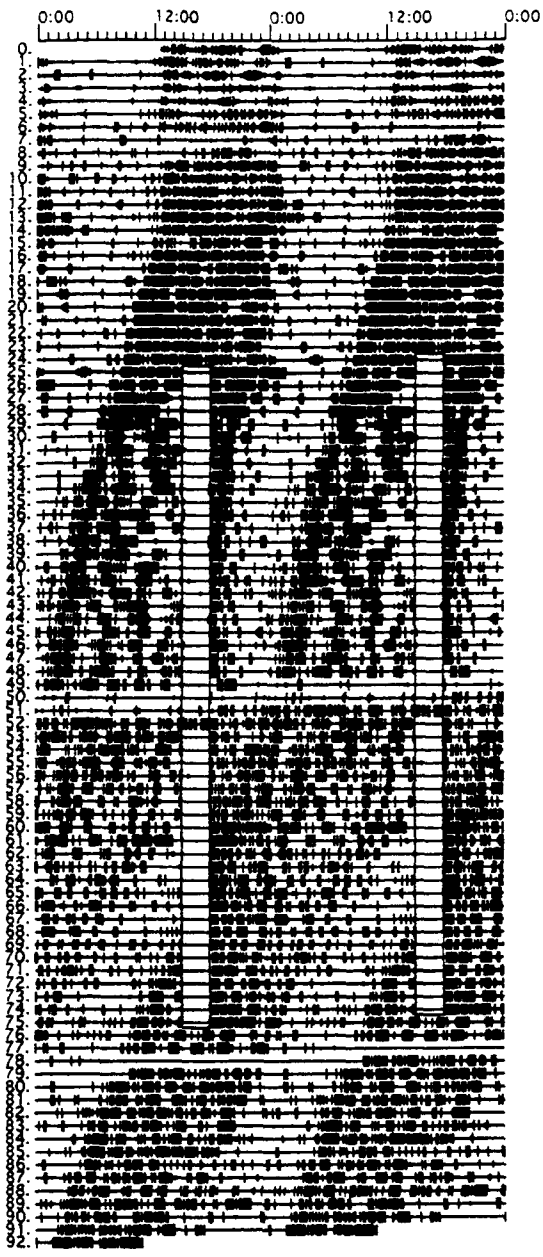


Figure 25. Double-plotted drinking charts from 2 DHT lesioned mice that failed to entrain but displayed a number of tau modulations. Plotting conventions as in Figure 3.

Figure 25.

A.



B.

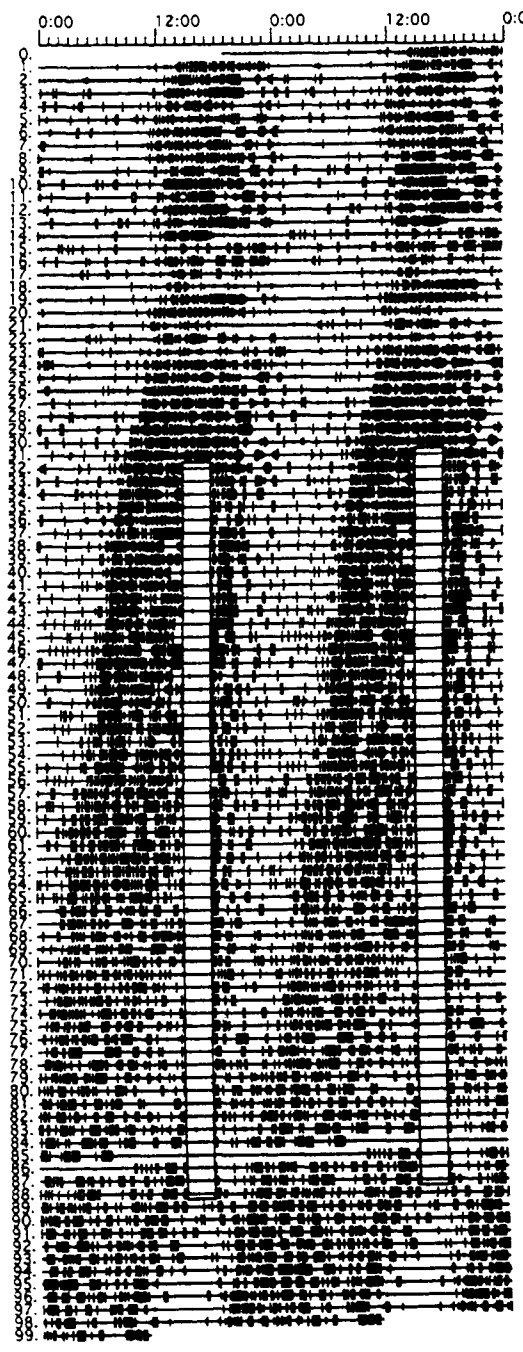


Figure 26. Raw tau scores prior to the TM period. Animals who entrained to forced TM running are indicated a dark circles around the square data point.

Figure 26.

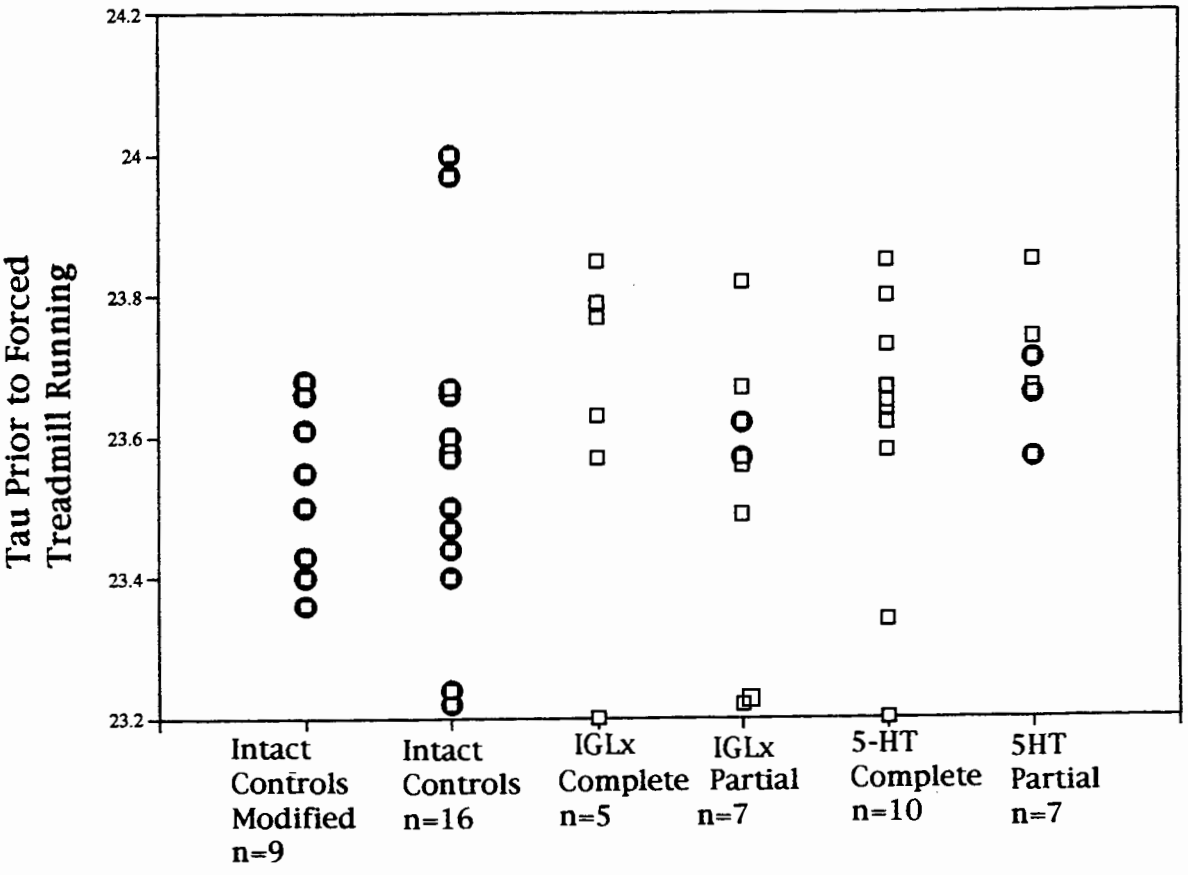
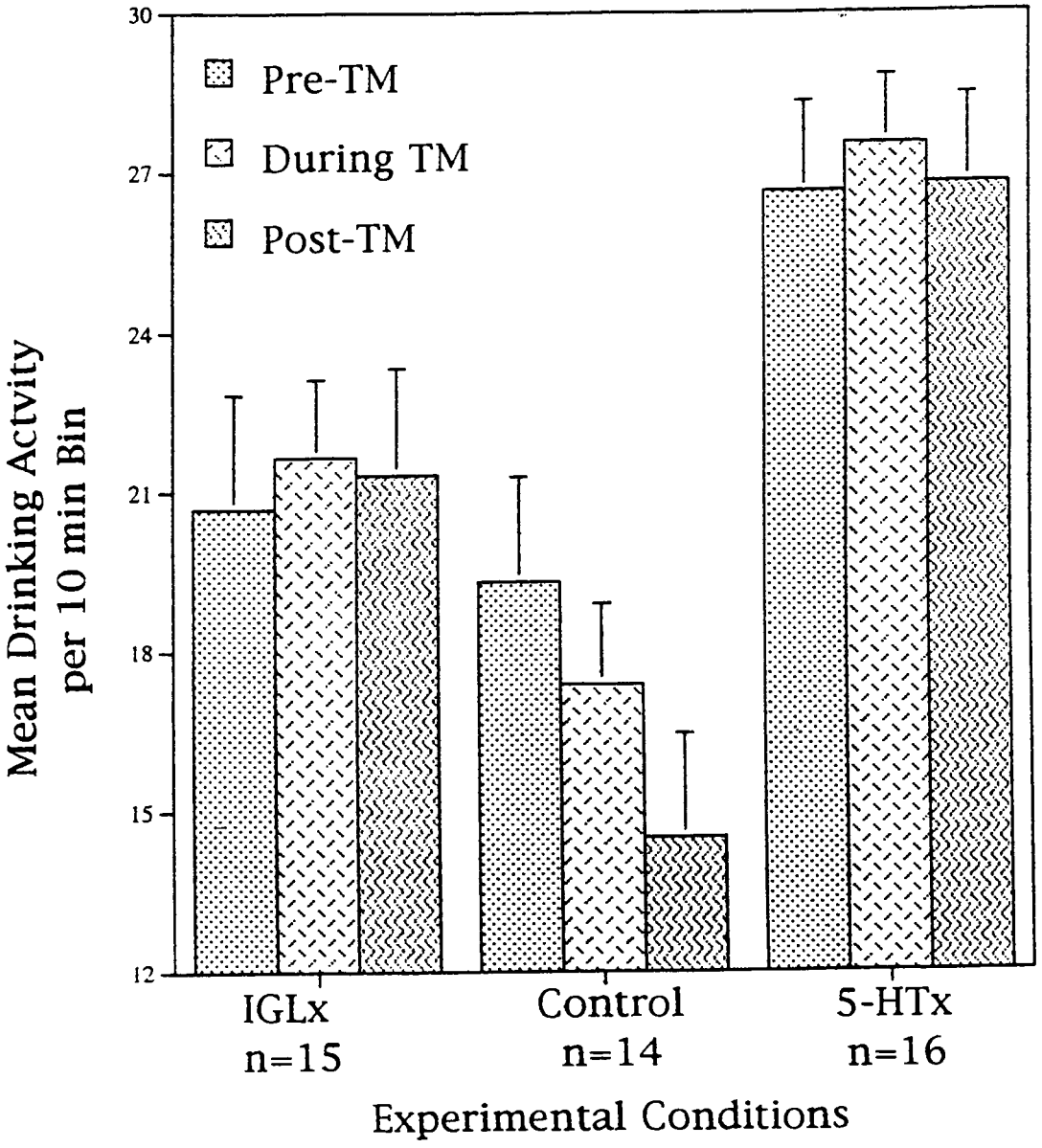

Tau Prior to Forced Treadmill Running
24.2
24
23.8
23.6
23.4
23.2
Intact Controls Modified n=9
Intact Controls n=16
IGLx Complete n=5
IGLx Partial n=7
5-HT Complete n=10
5HT Partial n=7

Figure 27. Bar graph displaying the mean drinking across experimental conditions of various groups of mice.

Figure 27.


Pre-TM
During TM
Post-TM
Mean Drinking Actvity per 10 min Bin
30
27
24
21
18
15
12
IGLx n=15
Control n=14
5-HTx n=16
Experimental Conditions

APPENDIX B

Areas Damaged by SCN Lesions in the TM mice

Area	Complete Lesions (n=8)	Partial Lesions (n=4)
SCN	99.750	23.750
MNPO	48.125	13.750
MPO	37.500	11.250
PVN	69.125	42.500
RCh	97.375	62.500
ARC	40.375	21.250
VMH	43.000	12.500

Table 1. Areas damaged by SCN lesions. Tissue was visualized using CV nissl stain.

IGL and SCN Lesion Coordinates

Location	Anterior/ Posterior	Dorsal/Ventral (DV)	Lateral
IGLx Lesions	-1.6, -2.0	-3.0, 3.4	±2.1
DHT 5HT Lesions	.1, -.3	-5.5	0.0

Table 2. All coordinates were taken after head leveling and the DV measurement was taken from dura.

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