Neural Mechanisms of Food-Anticipatory Circadian Rhythms in Rats

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Abstract

Circadian rhythms of behavior and physiology in rodents are regulated by a system of endogenous circadian oscillators synchronized to the external environment by two distinct pacemakers that are mutually coupled under normal conditions. The suprachiasmatic nucleus (SCN) is the master light-entrainable pacemaker (LEP) that mediates synchrony to the day-night cycle. The second pacemaker is a food-entrainable pacemaker (FEP) and has yet to be identified, but is capable of synchronizing circadian rhythms to restricted feeding (RF) schedules independent of the SCN. Rat, hamster and mouse behavior and physiology entrains to RF despite complete SCN ablations. The studies that comprise this dissertation examine two candidate FEP structures: the thalamic paraventricular nucleus (PVT); and the dorsomedial hypothalamus (DMH). Both the PVT and DMH are promising targets given evidence suggesting they: 1) are active in anticipation of the feeding window (as described in glucose metabolism and c-fos imaging studies); 2) are involved in arousal, ingestive behavior, and visceral function; 3) have reciprocal connections with the SCN which could mediate proposed coupling between the LEP and FEP under normal conditions; and 4) have been implicated in prior lesion studies, whereby damage to these structures has been shown to attenuate food-anticipatory physiology or behavior. To determine whether the PVT or DMH are critical for the expression of food-entrainable circadian rhythms, in separate studies rats received unambiguously complete radiofrequency lesions of the target structures. PVT-lesioned rats as well as DMH-lesioned rats are essentially indistinguishable from intact controls in terms of their capacity to entrain to and anticipate RF schedules. These results indicate that neither the PVT, nor the DMH are necessary for circadian food entrainment and thus they can be ruled out as candidate FEP structures. A series of follow-up studies were conducted to identify and explain the reasons why the DMH lesion results described above were contrary to earlier reports that the DMH was critical for expression of food-entrainable circadian rhythms.

Keywords: circadian, entrainment, food-anticipatory activity, thalamic paraventricular nucleus, dorsomedial hypothalamus, food-entrainable oscillator
For Cathy, Taylor, & Lauren
Acknowledgements

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<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>ARC</td>
<td>Arcuate Nucleus; a population of NPY and Ghrelin expressing hypothalamic neurons involved in regulation of ingestive behavior, energy expenditure, and body weight</td>
</tr>
<tr>
<td>Bmal1</td>
<td>Brain &amp; Muscle ARNT-like Protein-1 (AKA: MOP3); a core clock gene whose protein products form heterodimers with CLOCK or NPAS2, activating Per &amp; Cry</td>
</tr>
<tr>
<td>c-fos</td>
<td>An immediate early gene often used as a marker or correlate of neuronal activation</td>
</tr>
<tr>
<td>Circadian Rhythm</td>
<td>An endogenous rhythm (i.e., originating from within the organism) of behavior or physiology with a period ~24h</td>
</tr>
<tr>
<td>Clock</td>
<td>Circadian Locomotor Output Cycles Kaput; core clock gene whose protein products form heterodimers with BMAL1, activating Per &amp; Cry</td>
</tr>
<tr>
<td>Cry</td>
<td>Cryptochrome (Cry1 &amp; Cry2); a core clock gene whose protein product dimerizes with PER to form a critical negative feedback loop in the mechanism of circadian timing</td>
</tr>
<tr>
<td>CT</td>
<td>Circadian Time; a time scale dividing the period of a free-running circadian rhythm into 24 equal parts</td>
</tr>
<tr>
<td>DD</td>
<td>Dark:Dark; constant darkness, eliminating light as a zeitgeber</td>
</tr>
<tr>
<td>DMH</td>
<td>Dorsomedial Hypothalamus; a population of NPY and Orexin expressing neurons involved in the integration and regulation of circadian rhythms of arousal, ingestive behavior, and corticosterone</td>
</tr>
<tr>
<td>Entrainment</td>
<td>The process of synchronizing endogenous rhythms to a zeitgeber in the external environment</td>
</tr>
<tr>
<td>FAA</td>
<td>Food-Anticipatory Activity; a circadian rhythm of behavioral arousal and activity in anticipation of a restricted feeding window</td>
</tr>
<tr>
<td>FD</td>
<td>Food Deprivation; a period of food deprivation eliminating feeding time as a zeitgeber</td>
</tr>
<tr>
<td>FEO</td>
<td>Food-Entrainable Oscillator; a circadian oscillator capable of entraining to ~24h restricted feeding schedules</td>
</tr>
<tr>
<td>FEP</td>
<td>Food-Entrainable Pacemaker; a circadian pacemaker capable of synchronizing other oscillators, using food availability as a zeitgeber</td>
</tr>
<tr>
<td>Free-run</td>
<td>A state in which a circadian rhythm is allowed to express its endogenous period in the absence of zeitgebers</td>
</tr>
<tr>
<td>IBO</td>
<td>Ibotenic Acid; an excitatory neurotoxin that selectively destroys neurons while sparing fibres of passage</td>
</tr>
<tr>
<td>LD</td>
<td>Light:Dark; refers to the daily cycle of light and dark, providing time cues</td>
</tr>
<tr>
<td>LEO</td>
<td>Light-Entrainable Oscillator; a circadian oscillator capable of entraining to ~24h Light:Dark cycles</td>
</tr>
</tbody>
</table>
LEP  Light-Entrainable Pacemaker; a master circadian pacemaker located in the suprachiasmatic nucleus that synchronizes all behavior and physiology to the solar day-night cycle

LH  Lateral Hypothalamus; a population of neurons with widespread neuroendocrine interactions involved in food acquisition and ingestion; plays a regulatory role in ingestive behavior signalling hunger

MAP  Methamphetamine; a psychostimulant that enhances arousal levels

MASCO  Methamphetamine-Sensitive Circadian Oscillator; an SCN-independent oscillator entrained by continuous and chronic MAP administration in drinking water or by osmotic mini-pump

NPAS2  Neuronal PAS domain protein-2; a paralog of CLOCK that can dimerize with BMAL1

NPY  Neuropeptide Y; a neuromodulator component of the melanocortin system with strongly orexigenic properties promoting robust increase in food intake

PBN  Parabrachial Nucleus; a population of neurons that relay visceral inputs as well as integrate conditioned responses to aversive ingestive behavior whereby the limbic and reward systems receive ingestion-related information

Per  *Period* (*Per1, Per2, & Per3*); a core clock gene whose protein product dimerizes with CRY to form a critical negative feedback loop in the mechanism of circadian timing

Phase  A reference point or series of points in a cycle (e.g., activity onset or active phase)

PVN  Paraventricular Nucleus of the Hypothalamus; a population of neurons with widespread neuroendocrine interactions involved in food acquisition and ingestion

PVT  Thalamic Paraventricular Nucleus; a population of neurons serving an integrative role in arousal, ingestive behavior, and visceral function; with inputs from the brainstem and hypothalamus and outputs to limbic, hypothalamic, and cortical regions regulating reward systems and arousal

REV-ERB  Nuclear receptor with α and β sub-types that form an accessory feedback loop contributing to molecular clock function

RF  Restricted Feeding; an experimental condition whereby food availability is temporally restricted to examine capacity for food entrainment

SCN  Suprachiasmatic Nucleus; a population of hypothalamic neurons that serve as the master light-entrainable pacemaker, synchronizing all circadian oscillators and thus entraining behavior and physiology to the solar day-night cycle

SEM  Standard Error of the Mean

T  Zeitgeber Period; the length of time required to complete one cycle of an environmental rhythm
$\tau$  Endogenous Period; the length of time required to complete one cycle of a
free-running circadian rhythm

$\tau$ Mutant  An organism with a genetic mutation resulting in abnormally short-period
free-running circadian rhythms when compared with wild-type rhythms

$T_b$  Core Body Temperature; often used in circadian rhythms research to
determine phase of a circadian rhythm

VMH  Ventromedial Hypothalamus; a population of neurons involved in
regulating ingestive behavior by signalling satiety

Zeitgeber  An environmental time cue capable of entraining circadian
rhythms (e.g., light or restricted food availability)

ZT  Zeitgeber Time; a time scale dividing the period of a zeitgeber into 24
equal parts (e.g., ZT0-24 for an environmental light:dark cycle)
1. Introduction

Circadian rhythms in mammals are synchronized to cycles in the external environment, to coordinate behavior and physiology with local time in an adaptive manner. This process of synchronizing internal rhythms with the external environment is called entrainment. The solar day-night cycle is the primary entrainment signal, or zeitgeber (i.e., environmental stimulus capable of entraining circadian rhythms). Entrainment to the external environment enables mammals to predict and adapt to cyclical changes, maximizing critical factors such as predator or competitor avoidance and food and mate availability. In mammals these rhythms are regulated by a system of endogenous self-sustaining light-entrainable oscillators (LEO), widely distributed throughout the brain and most peripheral organs and tissues. Given this vast number of LEO’s, it makes sense they would require a pacemaker to synchronize them, much like an orchestra needs a conductor. The suprachiasmatic nucleus (SCN), a set of ~10⁴ neurons situated above the optic chiasm, is the master circadian pacemaker synchronizing LEO’s to environmental rhythms. The SCN’s role in synchronizing rhythms is evidenced by complete SCN lesions, which result in loss of circadian rhythmicity and disassociation of behavior and physiology from the external environment. Deletion of function following SCN lesions does not in itself qualify the SCN as a master pacemaker. It could simply be a critical component in the entrainment pathway. However, a decisive demonstration of the SCN’s role as master circadian pacemaker was provided by a tau-mutant SCN transplant study using hamsters whose circadian rhythm was much shorter than that of wild-type hamsters (Ralph et al., 1990). Behaviorally arrhythmic SCN-lesioned wild-type hamsters were implanted with SCN from tau mutants. These implants rescued the recipient’s circadian rhythms, but not with the wild-type period of ~24h. Remarkably, when the recipient’s behavioral rhythm was allowed to free-run in the absence of external zeitgebers, the restored rhythms had the short-period characteristics of the tau mutant donor’s SCN. The donor SCN’s ability to determine the period of the recipient’s rescued rhythms is precisely what defines a circadian pacemaker. To date the SCN is unique among circadian oscillators in that it is the only structure known to be necessary for entrainment to environmental LD (Light:Dark) cycles. It is therefore considered the master light-entrainable pacemaker (LEP).
For rodents, when access to food and water is unrestricted, LD cycles are the primary zeitgeber, as discussed above. However when access to food is restricted to a particular time of day (RF), the middle of the light phase for example, rodents will become active 1-3h in advance of the feeding window, even though it is a time they would normally be sleeping. This activity, known as food-anticipatory activity (FAA), starts to emerge within a few days of RF. FAA is observed in a number of behaviors including general cage activity, running wheel activity, drinking, and unreinforced lever pressing. FAA is most robust in measures of feeding-specific behaviors including activity directed at food sources such as a food bin. FAA exhibits properties of circadian entrainment in that the rhythm takes time to emerge, shifts gradually if mealtime is shifted, persists under total food deprivation, and has limits of entrainment meaning FAA is absent when mealtimes are scheduled with a period (T) outside of the circadian range of entrainment: 19h < T < 29h (Mistlberger, 1994; Stephan, 2002). When feeding is restricted in the presence of an LD cycle, the SCN of food-entrained rodents remain entrained to the LD cycle, whereas peripheral oscillators are entrained by the feeding window. In fact, most circadian oscillators with the exception of the SCN are preferentially entrained by feeding time (Damiola et al., 2000; Davidson et al., 2002; Stokkan et al., 2001). When rodents are fed once daily under constant dark (DD), SCN rhythms can free-run with a period different from 24h, establishing that SCN rhythms can decouple from FAA, whereby both rhythms persist with distinct periods. SCN-lesioned rodents that are behaviorally and physiologically arrhythmic under ad-libitum feeding during recovery from surgery, can entrain to subsequent RF. The resultant FAA persists for several cycles when these rats are deprived of food (for reviews, see Mistlberger, 1994; Stephan, 2002). Later, if ad-libitum feeding is alternated with periods of food deprivation, FAA will return at or near the phase of prior RF. Taken together the evidence described above establishes the existence of a food-entrainable pacemaker (FEP) that is separate and anatomically distinct from the SCN.

Having known for decades the characteristics of an SCN-independent food-entrainable pacemaker (Krieger et al., 1977; Stephan et al., 1979a, b; Boulos et al., 1980), it is remarkable that despite countless efforts to localize the FEP, its structure and organization remain to be defined. Following the strategy used to successfully identify the SCN as the LEP, early efforts to localize the FEP focused on mechanisms of ingestive behavior and metabolism as the entrainment signal. Whereas the photic pathway provided the roadmap for identifying the SCN as LEP, it made sense that early efforts to define the FEP focused on structures and pathways activated by or involved in food intake. Given the large number and vast distribution of candidate structures associated with
feeding pathways, one needed a means by which the list could be narrowed down. Observations
detailed earlier suggest the LEP and FEP can be coupled under ad-libitum feeding, but this
coupling is weak enough such that these oscillators can drive rhythms and behavior independently
when feeding is restricted under DD. Thus the list of candidates can be limited to structures
involved in feeding that are also connected in some way with the SCN. A final assumption is that
the FEP is comprised within a single structure in the same way that the SCN is the LEP. The
validity of this final assumption is called into question more and more as many candidate structures
have been eliminated over decades of research, as described below.

Adrenal glands were first on the list of FEP candidates tested given adrenal corticosterone
release in advance of feeding time, but FAA persists in rodents when these glands are removed
(Boulos and Terman, 1980). Subsequent efforts concentrated on systematic lesions through
hypothalamic areas that integrate neural and endocrine stimuli to regulate ingestive behavior and
metabolism, including the ventromedial hypothalamus (VMH), paraventricular hypothalamus (PVN),
arcuate nucleus (ARC) and lateral hypothalamus (LH) as reviewed in (Mistlberger, 1994; Davidson,
2009). The VMH’s role in satiety made it a reasonable candidate and initial reports suggested
lesions to this area eliminated FAA immediately following surgery during a period of hyperphagia
(Krieger, 1980; Inouye, 1982). However, given longer recovery periods the capacity for food
entrainment was not permanently damaged. VMH-ablated rats can recover capacity to entrain to
and anticipate daily RF (Mistlberger and Rechtschaffen, 1984). In short summary, while lesions to
the areas listed above have predictable effects such as weight gain or loss as a function of changes
in food intake, and in some cases altered behavioral outputs of FAA depending on the variable
measured (e.g., general cage activity vs. food bin approaches), none of the lesions eliminated the
capacity for food entrainment. We can thus eliminate the adrenal glands, VMH, PVN, ARC, and LH
as candidate FEP structures.

Later efforts to identify the FEP focused on sensory signals of the feeding pathway. Alas these
signals are not necessary to elicit FAA, as shown by lesions of the olfactory, optic, trigeminal
(gustatory), or visceral autonomic nerves (Coleman and Hay, 1990; Davidson et al., 2001; Davidson
and Stephan, 1998; Mistlberger, 1994). As noted earlier, peripheral organs are preferentially
entrained by RF schedules suggesting a possible locus outside the brain for the FEP. As discussed
in (Mistlberger, 2011) food-entrainable clock-gene rhythm expressing peripheral organs may
provide behavioral timing signals through hormonal or autonomic neural outputs. Oxyntic gland cell
secretion of ghrelin is an interesting example given its role in regulating energy homeostasis via
action on hypothalamic circuits including stimulation of arcuate neuropeptide Y (NPY) neurons (Cowley et al., 2003) and the fact that peripheral administration of ghrelin stimulates ingestive behavior (Tolle et al., 2002). Ghrelin became a promising candidate FEO with the discovery of food-anticipatory secretion of ghrelin via oxyntic gland cells under RF schedules (Cummings et al., 2001; Drazen et al., 2006; LeSauter et al., 2009). However, this peripheral signal is not critical for entrainment to RF since FAA is enhanced following lesions of the arcuate nucleus (Mistlberger and Antle, 1999) and mice lacking ghrelin or ghrelin receptors can entrain to feeding schedules (Szentirmai et al., 2009). Gastric or other peripheral food-entrainable clocks could play a role in driving FAA, but as reviewed in (Mistlberger, 2011), dissociations between FAA and peripheral clock gene rhythms all but rule out this possibility. After entrainment to a single daytime meal, clock gene rhythms of peripheral organs are rapidly reset to a nocturnal phase after only a few days of ad-libitum feeding. Persistence of behavioral food-anticipatory rhythms at the phase of prior feedings as revealed by several days of food deprivation following ad-libitum feeding confirms the FEP is not rapidly reset along with peripheral organs (Davidson et al., 2003). Taken together these findings suggest the FEP is not located outside the brain, hiding in some peripheral organ, but instead it is likely in the brain.

A separate line of research has taken advantage of the cell autonomous nature of circadian clocks in mammals. Considerable progress has been made in characterizing the molecular mechanisms of circadian clock function. In brief, the circadian clock is composed of an auto-regulatory transcriptional network with interlocking feedback loops (reviewed in Reppert and Weaver, 2001). CLOCK, BMAL1, and NPAS2, are transcriptional activators of Period (Per1, Per2, & Per3) and Cryptochrome (Cry1 & Cry2) genes (Gekakis et. al., 1998; Bunger et. al., 2000; Kume et. al., 1999; Lee et. al., 2001). Note that NPAS2 is a paralog of CLOCK, each with similar amino acid sequencing and sharing many of the same characteristics such that they both dimerize with BMAL1 and are inhibited by Cryptochromes (Dudley et al., 2003). As PER and CRY protein levels accumulate during the light phase they dimerize and translocate into the nucleus early in the dark phase, where they inhibit their own transcription by suppression of CLOCK/BMAL1 and NPAS2/BMAL1 heterodimers. In addition to this primary feedback loop, auxiliary interlocking loops and post-translational modulators serve to stabilize and increase robustness of oscillations in addition to determining the ~24h period of free-running rhythms. REV-ERB-α and REV-ERB-β are components of one of the better understood auxiliary interlocking loops regulating circadian behavior and metabolism (Cho et. al., 2012). Advances in gene research and identification of the
genes and proteins involved in clock function have led to a better understanding of the mechanisms of entrainment. For example, entrainment to LD cycles is now understood to be the result of daily adjustments known as phase shifts via circadian phase-dependent changes in SCN Per levels following light-exposure (Shearman et al., 1997; for review, see Reppert and Weaver, 2001).

Recall how the tau mutant provided the definitive evidence that the SCN is the master LEP. With the core clock genes identified, a similar approach using gene mutations or knockouts has been used to examine the mechanisms of entrainment to RF. Mutation or knockout of each of the core clock genes comprising the positive and negative legs of the primary autoregulatory transcriptional loop have been studied under RF. However much like the lesion studies described earlier, the results are mixed and have yet to elucidate the molecular mechanism of food entrainment (for reviews, see Challet et al., 2009; Bechtold and Loudon, 2013). Clock-mutant mice were the first to be tested for capacity to entrain to RF schedules. Clock\textsuperscript{clk/clk} mice retain the ability to anticipate and entrain to RF, though the characteristics of behavioral anticipation are different from wild-types (Pitts et al., 2003; Horikawa et al., 2005). In particular, Clock\textsuperscript{clk/clk} mice are active for a longer period in advance of the feeding window and a larger percentage of their total daily activity appears to be contained in that anticipatory bout of activity, compared with wild-types. NPAS2\textsuperscript{−/−} mice have also been challenged with RF, initially with lethal effect (Dudley et al., 2003). Abrupt transition from ad-libitum feeding to 4h RF windows resulted in severe illness and in some cases death shortly into RF. However, NPAS2\textsuperscript{−/−} mice can be protected from the lethal effect of RF by increasing the duration of the feeding window to 6h and by placing food pellets directly on the cage floor instead of in overhead bins that require mice to reach up to feed. With these methodological modifications NPAS2\textsuperscript{−/−} mice retain the capacity to entrain to RF; but with delayed acquisition of FAA and decreased food intake and body weight, when compared with wild-types. Clearly Npas2 gene function alters ability to cope with RF, but it is not critical for the expression of circadian food entrainment. In contrast to Clock\textsuperscript{clk/clk} and NPAS2\textsuperscript{−/−} mice, the first report testing RF with Bmal1\textsuperscript{−/−} mice not only suggested that FAA was absent, but that capacity for RF entrainment in these mice could be rescued by injecting viral vector containing the Bmal1 gene into the DMH of Bmal1\textsuperscript{−/−} mice (Fuller et al., 2008). Taken at face value the story told was an elegant depiction of the mechanism of food entrainment first being deleted and then being rescued. Restoration of FAA by Bmal1 viral vector injections into the DMH fit beautifully with an earlier report from the same laboratory that the DMH was critical for the expression of food-entrainable circadian rhythms (Gooley et al., 2006). Alas upon close inspection of Fuller et al., 2008, the study’s serious methodological shortcomings
were initially reported in a technical comment (Mistlberger et al., 2008) and then later in a more detailed review (Mistlberger et al., 2009). Subsequent examinations of Bmal1−/− mice from three independent laboratories report intact FAA during RF (Mistlberger et al., 2008; Pendergast et al., 2009; Storch and Weitz, 2009). The Gooley et al., 2006 DMH findings will be discussed later in this section.

As described earlier, Period genes are key components in the mechanism of entrainment to light, so there was hope that these genes could help unlock the mechanisms underlying food entrainment. Once again though, reports are a mixed. Using Per1Brdm1 and Per2Brdm1 mutations, considered a null-mutant allele (as described in Zheng et al., 2001), Per1 was shown to be dispensable for food entrainment, whereas Per2 was reported to be critical for entrainment to RF (Feillet et al., 2006). However subsequent studies from two independent laboratories report intact FAA in Per1−/−;Per2−/− double knockout mice (Storch and Weitz, 2009) and Per1−/−;Per2−/−;Per3−/− triple knockout mice (Pendergast et al., 2012). Complicating matters further, a report using Cry1−/−;Cry2−/− double knockout mice that ruled out Cry genes as necessary for FAA (Iijima et al., 2005), was later contradicted by Mendoza et al., 2010, whose follow-up study to Feillet et al., 2006 showed a loss of stable FAA in Per1−/−;Per2Brdm1 double mutants as well as Per2Brdm1;Cry1−/− double mutants.

Taken together, the food entrainment literature examining lesions and mutations of core clock genes have yet to identify the FEP or clarify its underlying mechanisms. Some lesions or gene mutations have been identified that affect the expression of FAA, but with two exceptions (discussed below) food entrainment has been shown to persist in at least some measures of behavior. In brief summary, knockouts of a clock gene expressed in the neocortex and hippocampus (Npas2) (Dudley et al., 2003) or genetic ablation of the lateral hypothalamic peptide orexin/hypocretin (Akiyama et al., 2004; Mieda et al., 2004) have been reported to attenuate FAA in mice, but FAA has already been shown to persist in rats following complete ablation of these structures (Mistlberger, 1994; Mistlberger et al., 2003; Mistlberger and Mumby, 1992; Mistlberger and Rusak, 1988). Lesions of the nucleus accumbens core (Mendoza et al., 2005a) and the hypothalamic paraventricular nucleus attenuate or eliminate FAA in some measures of behavior (e.g., general locomotor activity) but not in others (e.g., wheel running or activity directed at a food bin) (Mistlberger and Mumby, 1992; Mistlberger and Rusak, 1988). Ablation of infralimbic neocortex (Recabarren et al., 2005) or the hypophysis (Davidson and Stephan, 1999) eliminates the food-anticipatory rhythm of body temperature, but not of behavior. FAA also persists after ablation of the hypothalamic subparaventricular zone (Gooley and Saper, 2003; Gooley et al., 2006), and following
mutations affecting the clock genes mCry1/Cry2 (Iijima et al., 2005), Clock (Pitts et al., 2003), and the leptin receptor gene (Mistlberger and Marchant, 1999).

Ultimately, to demonstrate that an oscillating, feeding-responsive structure is a critical driving pacemaker for a food anticipatory behavior, the oscillator must be disabled in some way, by site-specific gene manipulations, immunological or pharmacological inhibition, or neurotoxic or radiofrequency ablation. Here we report the results of a radiofrequency ablation study designed to establish whether the paraventricular nucleus of the thalamus (PVT) is critical for the generation of food anticipatory activity rhythms in rats. The PVT is notable in exhibiting mealtime synchronized circadian rhythms of glucose utilization (Pereira de Vasconcelos et al., 2006), immediate early gene expression (e.g., c-fos) and clock gene expression (e.g., Per1) (Nakahara et al., 2004; Mendoza et al., 2005b). The PVT receives input from brainstem and hypothalamic regions involved in arousal, ingestive behavior and visceral function, and sends outputs to limbic, hypothalamic and cortical regions that regulate reward and arousal states (Van der Werf et al., 2002; Parsons et al., 2006). The PVT also has reciprocal connections with the SCN (Moga et al., 1995; Moga and Moore, 1996; Moga and Moore, 1997; Watts et al., 1987), which could mediate proposed coupling between food and light-entrainable oscillators (Stephan, 1986). While one study reported that ablation of the anterior PVT, collateral to lesions of the nucleus accumbens (Mistlberger and Mumby, 1992), does not impair the generation of food anticipatory behavioral rhythms in rats, a more recent study reports that rats with complete PVT lesions fail to exhibit anticipatory locomotor activity to a midday meal (Nakahara et al., 2004). However, the latter study used a behavioral assay, non-specific cage activity, which sometimes fails to reflect anticipatory rhythms evident in other behaviors, such as a place preference for a feeding location (e.g., Mistlberger and Rusak, 1988).

Two other reports merit special attention. Rats with electrolytic or cell-specific lesions of the brain stem parabrachial nucleus (PBN) were shown to express either very little or no food-anticipatory circadian rhythms of core body temperature or activity directed at a food tray, a behavioral measure that in other studies has been resistant to disruption by lesions (Davidson et al., 2000). The PBN has also been shown to exhibit a food-anticipatory rhythm of c-fos expression that, unlike FAA, does not persist if the scheduled daily meal is omitted for one cycle (Angeles-Castellanos et al., 2005). Together, these results suggest that the PBN may be a critical component of the entrainment pathway to food-entrainable oscillators located elsewhere. The PBN is an integrative area for visceral and gustatory sensory information, and projects to a variety of forebrain areas, including the dorsomedial hypothalamic nucleus (DMH) (Saper and Loewy, 1980; Thompson
et al., 1996). The DMH has recently been conceptualized as an integrative area and final common output for circadian rhythms of sleep-wake, ingestive behavior, and corticosterone (Saper et al., 2005). The DMH receives direct and indirect input from the SCN, expresses neuropeptides (e.g., NPY, orexin) and receptors (e.g., leptin, cholecystokinin, ghrelin) implicated in the control of ingestive behavior and metabolism and is richly interconnected with hypothalamic, preoptic, and some brain stem nuclei involved in regulation of energy input/output or behavioral state (Aston-Jones et al., 2001; Chou et al., 2002; Chou et al., 2003; Deurveilher and Semba, 2005; Mistlberger, 2005; Thompson et al., 1996; Thompson and Swanson, 1998). Lesions of the DMH disrupt LD-entrained circadian rhythms in these functions (Bellinger et al., 1976; Chou et al., 2003). As mentioned earlier, DMH lesions have recently been shown to attenuate or eliminate anticipatory rhythms of general activity and core body temperature measured by telemetry in rats restricted to a 4h daily meal (Gooley and Saper, 2004; Gooley et al., 2006). DMH damage may explain our own occasional observations of rats or mice that failed to anticipate a daily feeding time following very large nonspecific radiofrequency lesions of the medial hypothalamus, resulting from presumably faulty electrodes aimed at the SCN or the paraventricular nucleus (Antle et al., 1996; Marchant and Mistlberger, 1997). These results suggest the DMH may be the site of food-entrainable circadian oscillators or a critical link between such oscillators and circadian outputs.

Given the importance of the behavioral measure in assessing circadian function following brain lesions, we sought to further examine the role of the PVT and DMH by using motion detectors with more spatial selectivity than is provided by telemetric movement sensors. To maximize the chances of evaluating rats with unambiguous, total PVT and DMH ablation, we used electrodes, stereotaxic placements, and radiofrequency current parameters designed to produce very large lesions that completely encompassed the target structures. We found that rats with large lesions completely ablating the PVT show essentially normal food anticipatory rhythms in activity near a food bin, indicating that the PVT is not a critical input, oscillator or output component of the circadian system by which rats predict daily mealtime. Similarly, behavioral anticipation of a daily meal was not attenuated in rats with unambiguous DMH destruction, though these lesions did produce ingestive deficits and attenuated LD-entrained circadian activity rhythms. Given our results contradicted findings that identified the DMH as critical for expression of food-entrainable circadian rhythms, the remainder of this thesis focused on exploring possible explanations for the discrepancy in results including methodological issues such as cage configuration and food delivery, behavioral measure using motion sensors versus telemetry, and lesion type (i.e.,
radiofrequency versus neurotoxin ibotenic acid). After exhaustive efforts to replicate Gooley’s 2006 findings, we confirm the DMH is NOT critical for entrainment of circadian food-anticipatory rhythms, but our results suggest that under RF the DMH may play a modulatory role in expression of FAA during the light period when nocturnal rodents normally sleep.
2. Robust Food Anticipatory Circadian Rhythms in Rats with Complete Ablation of the Thalamic Paraventricular Nucleus

Landry GJ, Yamakawa GRS, & Mistlberger RE (2007) Robust food anticipatory circadian rhythms in rats with complete ablation of the thalamic paraventricular nucleus. Brain Research 1141:108-118. doi:10.1016/j.brainres.2007.01.032 (GJL designed the experiment, performed surgeries, collected and analysed data, and wrote the manuscript.)

Abstract

Rats can anticipate a fixed daily mealtime by entrainment of a circadian timekeeping mechanism anatomically separate from the light-entrainable circadian pacemaker located in the suprachiasmatic nucleus. Neural substrates of this food-entrainable circadian system have not yet been fully elucidated. A role for the thalamic paraventricular nucleus (PVT) is suggested by observations that scheduled feeding synchronizes daily rhythms of glucose utilization and immediate early gene and circadian clock gene expression in this area. One study has reported absence of food anticipatory circadian activity rhythms in rats with PVT ablations. To determine whether this effect extends to other behavioral measures of food anticipation, rats received large radiofrequency lesions aimed at the PVT and were maintained on a 3h meal provided each day 6h after lights-on. Rats with unambiguously complete PVT ablation exhibited increased total daily activity, a change in the waveform of the nocturnal activity rhythm, but no change in the amplitude, duration, latency to appearance or persistence during total food deprivation of food anticipatory activity measured by activity at or near a food bin accessible via a small window in the recording cage. These results indicate that, while the PVT may modulate light-entrainable rhythms, it is not a critical input, oscillator or output component of the circadian system by which rats behaviorally anticipate a daily mealtime.
2.1. Introduction

For adult rodents with ad-libitum access to food, daily light-dark (LD) cycles are the primary environmental stimulus entraining circadian rhythms of behavior and physiology to local time. Under these conditions, nocturnal rats consume most of their food at night. However, if food is restricted to one or two meals provided at a fixed time in the middle of the light period, rats within a few days exhibit increased locomotor activity beginning 1-3 h before the daily meal (Aschoff, 1986; Mistlberger, 1994; Stephan, 2002). Physiological rhythms also reorganize, such that body temperature and plasma corticosterone rise before mealtime, and gastric, intestinal, pancreatic and hepatic rhythms shift to track ingestive behavior (Boulos and Terman, 1980). Food-synchronized behavioral rhythms persist if food is withheld for several days (Boulos et al., 1980; Coleman et al., 1982; Rosenwasser et al., 1984), and do not emerge if the schedule of food availability is outside of the circadian range (~22-31 h; Bolles and DeLorge, 1962; Boulos et al., 1980; Stephan, 1981). Ablation of the hypothalamic suprachiasmatic nucleus (SCN), an identified circadian clock (Klein et al., 1991), eliminates all circadian organization in rodents fed ad-libitum, but does not affect the emergence of food anticipatory rhythms of behavior and physiology in rats, mice or Syrian hamsters maintained on circadian schedules of food access (Krieger et al., 1977; Stephan et al., 1979a, b; Mistlberger, 1992; Marchant and Mistlberger, 1997). Consequently, the mammalian circadian system appears to include a light-entrainable circadian pacemaker located in the SCN and a food-entrainable pacemaker (or multiple oscillators) located elsewhere. In the absence of an LD cycle, SCN-driven activity rhythms can sometimes come under the control of feeding schedules (Stephan, 1986a, b; Abe et al., 1989; Mistlberger, 1993; Castillo et al., 2004; Lamont et al., 2005a), so by definition the SCN must also be considered food-entrainable, but it does not mediate food anticipatory rhythms. Some rhythmic processes, such as locomotor activity and body temperature, appear to be jointly controlled by both pacemakers. Other rhythmic processes, particularly those associated with digestive and metabolic functions, may be regulated directly by food-entrainable oscillators and synchronized to daily LD cycles only indirectly, by entrainment of these oscillators to the SCN-driven daily rhythm of food intake.

Circadian clock genes at the molecular core of SCN circadian clock neurons are also expressed in other brain areas and in many, possibly all peripheral organ systems and tissues (Abe et al., 2002; Amir et al., 2004; Balsalobre et al., 1998; Lamont et al., 2005b; Schibler et al., 2003; Sun et al., 1997; Yamazaki et al., 2000; Yoo et al., 2004; Segall et al., 2006). Clock gene rhythms in peripheral organs are food-entrainable and presumably serve to synchronize local organ functions
with predictable daily meals (Damiola et al., 2000; Hara et al., 2001; Stokkan et al., 2001; Wakamatsu et al., 2001). The available evidence suggests that these peripheral clocks do not drive behavioral food anticipatory rhythms (Davidson et al., 2003), although such a role has not been ruled out for all peripheral tissues and continues to be evaluated (Silver et al., 2005). Efforts to localize food-entrainable oscillators in the brain critical for food anticipatory behavioral rhythms have historically relied on the production of lesions in specific brain structures, guided by reasonable hypotheses about the likely input-output characteristics of a food-entrained pacemaker. While some lesions have been shown to attenuate one or more behavioral or physiological food anticipatory rhythms, no structure has yet been shown to be indispensable for all food anticipatory behavioral rhythms, although many have been evaluated (for brief reviews, see Landry et al., 2006; Davidson, 2006).

The SCN exhibits robust circadian rhythms of glucose utilization and expression of immediate early genes and circadian clock genes (Antle and Silver, 2005; Klein et al., 1991; Schibler et al., 2003). Light pulses that shift the SCN pacemaker alter these variables acutely and shift their rhythms. The problem of localizing food-entrainable oscillators within the brain seems amenable to functional mapping using similar indices of activation and rhythmicity. Ultimately, however, to demonstrate that an oscillating, feeding-responsive structure is a critical driving pacemaker for a food anticipatory behavior, the oscillator must be disabled in some way, by site-specific gene manipulations, immunological or pharmacological inhibition, or neurotoxic or radiofrequency ablation. Here we report the results of a radiofrequency ablation study designed to establish whether the paraventricular nucleus of the thalamus (PVT) is critical for the generation of food anticipatory activity rhythms in rats. The PVT is notable in exhibiting mealtime synchronized circadian rhythms of glucose utilization (Pereira de Vasconcelos et al., 2006), immediate early gene expression (e.g., c-fos) and clock gene expression (e.g., Per1) (Nakahara et al., 2004; Mendoza et al., 2005). The PVT receives input from brainstem and hypothalamic regions involved in arousal, ingestive behavior and visceral function, and sends outputs to limbic, hypothalamic and cortical regions that regulate reward and arousal states (Van der Werf et al., 2002; Parsons et al., 2006). The PVT also has reciprocal connections with the SCN (Moga et al., 1995; Moga and Moore, 1996, 1997; Watts et al., 1987), which could mediate proposed coupling between food and light-entrainable oscillators (Stephan, 1986a,b). While one study reported that ablation of the anterior PVT, collateral to lesions of the nucleus accumbens (Mistlberger and Mumby, 1992), does not impair the generation of food anticipatory behavioral rhythms in rats, a more recent study reports
that rats with complete PVT lesions fail to exhibit anticipatory locomotor activity to a midday meal (Nakahara et al., 2004). However, the latter study used a behavioral assay, non-specific cage activity that sometimes fails to reflect anticipatory rhythms evident in other behaviors, such as a place preference for a feeding location (e.g., Mistlberger and Rusak, 1988). We found that rats with large lesions completely ablating the PVT show essentially normal food anticipatory rhythms in activity near a food bin, indicating that the PVT is not a critical input, oscillator or output component of the circadian system by which rats predict daily mealtime.

2.2. Results

2.2.1. Histology

Lesion electrodes aimed bilaterally at rostral, middle and caudal levels of the PVT produced large lesion cavities that removed the dorsal and medial thalamus for at least 5mm rostro-caudally, 0.7mm laterally and up to 3mm ventrally (Figure 2.1). The PVT, including the anterior and posterior portions, was completely absent in all three cases. Numerous surrounding medial thalamic structures were either absent (e.g., the medial habenula) or substantially damaged (triangular septal n., subfornical organ, fornix, paratenial n., paracentral n., ventral hippocampal commissure, lateral habenula, anterodorsal n., centromedial n., interanterodorsal thalamic n., intermediodorsal thalamic n., mediodorsal n., posterior commissure and central gray). Despite the size of the lesions, the presence of clear anatomical landmarks dorsal, lateral and ventral to the cavities made assessment of PVT status unambiguous.
Figure 2.1. Coronal brain sections stained with cresyl violet from one intact rat (left column) and three rats (PVT x 1-3) sustaining complete ablation of the thalamic paraventricular nucleus (PVT, see outlined area in the sections for the intact rat)

Note. The numbers indicate the approximate level of each section in millimeters posterior to bregma. Abbreviations: 3V, third ventricle; AD, anterodorsal thalamic nucleus; AV, anteroventral thalamic nucleus; BSTMPM, bed nucleus of the stria medularis posteromedial; F, fornix; LHbM, lateral habenular nucleus medial; LHbL, lateral habenular nucleus lateral; MHB, medial habenular nucleus; OPC, oval paracentral thalamic nucleus; PT, paratenial nucleus; Rh, rhomboid nucleus; sm, stria medularis.
2.2.2. **Activity during ad-libitum food access**

The infrared photodetector and the overhead motion detectors produced very similar patterns of activity. However, outside of scheduled mealtimes, the number of counts registered by the infrared photodetectors was much lower than the counts registered by the motion detectors, therefore the latter were used for quantitative and qualitative analyses, except where indicated. By this measure, there was a significant effect of both lesion group \( F_{(1,32)} = 11.28, p = 0.028 \) and feeding condition \( F_{(8,32)} = 10.58, p < 0.0001 \) on average daily activity levels. PVT-ablated rats exhibited higher levels of activity by comparison with the intact rats at all phases of the study (Figs. 2.2 & 2.3A). During ad-libitum food access prior to food restriction, there was no group difference in the percent of total daily activity occurring in the dark (nocturnality ratio, Fig. 2.3B). However, actograms (Fig. 2.2) and average waveforms (Fig. 2.4A) revealed group differences in the distribution of nocturnal activity. All PVT lesion rats showed a high concentration of activity during the last 4h of the dark period, and the peak of the waveform for each rat occurred within 1h of lights-on. Intact rats showed a more even distribution, with a tendency toward smaller peaks near the beginning, middle and end of the dark period. The peak of the waveforms for these rats occurred within the first 4h of the dark period, significantly earlier by comparison with the PVT lesion rats \( t = 7.17, p = 0.001 \).
Figure 2.2. **Actograms illustrating locomotor activity rhythms from three intact control rats (C1-3) and three rats with complete ablation of the thalamic paraventricular nucleus (P×1-3)**

Note. For each chart, time within a day is plotted in 10min bins from left to right, and consecutive days are aligned vertically. The weight of the line represents activity level (0 counts in 10min = 1 pixel; 1–9 counts = 3 pixels; 11–20 counts = 5 pixels; > 20 counts = 7 pixels). Hours of lights-off (12–24) are indicated by shading. The rats were food deprived on the days indicated by the square brackets on the left margin of each chart. The onset of deprivation is indicated by an arrowhead, the end of deprivation by a triangle. The daily 3h mealtime during food restriction is indicated by the opaque rectangle.
Figure 2.3. Group mean activity metrics of intact rats (dashed red lines) and PVT-ablated rats (solid lines) during ad-libitum food access (5- and 3-day means for first and second Adlib, respectively), total food deprivation (FD1 = 66h, FD2 = 51h, FD3 = 72h) and 3h/day restricted feeding (RF1, 2, 3, 4 = 5-day means)

Note. Results of post-hoc Bonferroni comparisons of means are indicated by 'a' (within-group, significantly different from Adlib1 at p < 0.05) and 'b' (between group, significantly different at p < 0.05). (A) Mean daily activity, (B) nocturnality ratios (activity during the 12h dark period as a percentage of total daily activity), (C) food anticipatory activity (FAA, mean daily activity during hours 4–6 of lights-on in each feeding condition; this corresponds to the time of elevated activity prior to scheduled mealtimes during daytime restricted feeding), (D) Food anticipatory activity ratios (FAA counts divided by the sum of activity during lights-off and activity during the first 3h of lights-on). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
2.2.3. Activity during food restriction

During the first total food deprivation test, all rats showed substantially increased total daily activity (Fig. 2.3A). Nocturnality ratios declined in both groups, but short of statistical significance (Figs. 2.3B & 2.4B). During the restricted daily feeding schedule, all rats exhibited substantially increased daytime activity beginning 2-3h before mealtime and rising steeply to a peak at mealtime (Figs. 2.2 & 2.4C). There were significance increases in both the amount of premeal activity ($F_{(8,32)} = 10.11, p < 0.0001$) and the ratio of this activity relative to activity at other times of day (excluding mealtime and 3h postmeal) ($F_{(8,32)} = 12.12, p < 0.0001$) during the 19 days of restricted feeding in both groups. Regression lines fit to the mean food anticipation counts (Fig. 2.5A) and ratios (Fig. 2.5B) during ad-libitum food access and the 4 blocks of days of restricted daytime feeding clearly show that the rate at which anticipation emerged did not differ between groups. Although the PVT rats showed significantly more total premeal activity (Figs. 2.3C & 2.5A), there was no significant group difference in the magnitude of the food anticipation ratios ($F_{(1,32)} = 0.04, p = 0.85$; Figs. 2.3D & 2.5B). A direct comparison of activity waveforms obtained by averaging the last 4 days of restricted feeding reveals a marked similarity in the duration, slope and peak of premeal activity in the two groups (Fig. 2.4C).

The waveforms of nocturnal activity in the two groups were also very similar during food restriction. Rats with PVT lesions exhibited a modestly higher level of nocturnal activity, but both groups exhibited a prominent trimodal waveform, with activity peaks near the beginning, middle and end of the dark period (Fig. 2.4C). As a consequence of food anticipatory and mealtime activity, nocturnality ratios were substantially reduced in both groups during food restriction (Fig. 2.3B).

During the second bout of total food deprivation, immediately following the daytime feeding schedule, all of the rats showed elevated daytime activity at the expected mealtime (Figs. 2.3C, D; & 2.4D). Daytime activity dropped precipitously when food was provided ad-libitum for 72h, but increased again during a third bout of total food deprivation (Figs. 2.3C, D; & 2.4E).
**Figure 2.4.** Group mean average waveforms illustrating daily rhythms of activity

Note. Activity during: (A) 5 days of ad-libitum food access, (B) 66h of total food deprivation immediately preceding daytime restricted feeding, (C) last 4 days of restricted feeding (midday mealtime indicated by shading), (D) 54h of total food deprivation immediately following restricted feeding, and (E) 72h total food deprivation after 3 days of recovery ad-libitum food access. Solid line, paraventricular thalamic nucleus ablation group; dashed red line, intact control group. Hours of lights-off (12-24) indicated by the heavy bar along the x-axis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**2.2.4. Mealtime activity, food and water intake during food restriction**

During the 3h daily mealtime, there was a substantial group difference in the amount and distribution of activity, as detected by both the overhead motion detector and the food bin monitor. PVT lesion rats showed much more mealtime activity (729 ± 124 vs 270 ± 25 counts/3h, \( t = 4.4, p = \)
0.003; Fig. 2.6A) and were active throughout the 3h mealtime (Fig. 2.4C). Intact rats were active primarily during the first hour of food access and, like the PVT rats, showed a second peak when the food bins were removed at the end of the third hour.

**Figure 2.5.** Group mean (± SEM) FAA counts (A) and FAA ratios (B) for PVT-ablated rats (black triangles) and intact rats (red squares), for the first ad-libitum food access condition and the 4 blocks of days of restricted daytime feeding (RF1-4).

Note. Regression lines reveal a significant linear component to the change over time for both variables in both groups ($p < 0.02$ or better for each line). There were no significant group differences in the slopes of the regression lines. The intercepts differ between groups only for FAA counts ($F_{1,7} = 8.68, p = 0.02$), consistent with the higher levels of activity in the PVT-ablated rats. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Despite the different activity levels during mealtime, there was no significant group difference in the amount of food ingested over the 19 days of food restriction. Both groups showed a gradual increase in amount consumed during the first 5-7 days of food restriction before stabilizing at very similar levels (Fig. 2.6B). Mean daily water intake was more stable during the restricted feeding schedule (Fig. 2.6C). The PVT lesion group showed a trend for less water consumption over the last 10 days of the feeding schedule. To avoid disrupting the pattern of activity, which was the primary dependent variable in this study, body weights were not obtained during food restriction.
However, PVT lesion and intact rats showed no obvious differences in size and general appearance.

**Figure 2.6.** (A) Group mean activity counts during the 3h daily mealtimes in Intact (dashed red line) and PVT-ablated rats (solid line). (B) Food intake in grams during 3h daily meal. (C) Total daily water intake during the restricted feeding schedule.

Note. For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.
2.3. Discussion

The objective of this study was to determine whether the PVT is critical for the expression of food anticipatory circadian activity rhythms in rats maintained on a restricted daytime feeding schedule. The results permit a substantive conclusion that the PVT is dispensable for this circadian function. Rats with unambiguously complete PVT lesions showed robust food anticipatory rhythms in locomotor activity that were strikingly similar to intact rats in duration, magnitude and latency to emerge. In both groups these rhythms persisted during 2 days of food deprivation that followed the feeding schedule and reappeared during 3 days of food deprivation after 3 days of recovery feeding. While the PVT does appear to be sensitive to food restriction schedules, as demonstrated by measures of glucose metabolism (Pereira de Vasconcelos et al., 2006) and immediate early gene or clock gene expression (Angeles-Castellanos et al., 2007; Mendoza et al., 2005; Nakahara et al., 2004), the present results provide no evidence for a role of this structure as an input (entrainment pathway), oscillator or output component of the food anticipatory circadian system. Other work indicates that the PVT may play a modulatory role in the regulation of hypothalamo-adrenal activity and energy balance that is apparent only or primarily in the presence of chronic stressors (Bhatnagar and Dallman, 1999). Conceivably, the changes in glucose utilization and gene expression noted in the PVT of food restricted rats may reflect a response to chronic stress associated with reduced caloric input and increased locomotor activity rather than to feeding or anticipatory activity per se.

In Introduction, a distinction was made between two types of food entrainment. The behavioral properties of food anticipatory activity rhythms in intact and SCN-ablated rodents indicate that these rhythms are the product of a non-SCN circadian pacemaker that is entrained by some aspect of scheduled feeding. In intact rodents, the SCN itself can sometimes also be entrained by a scheduled daily meal. This is typically assessed by maintaining the rodents in constant dark with access to food once/24h. Under these conditions, the typical rat exhibits two independent circadian activity rhythms, a food anticipatory component coupled to mealtime (the subject of the present study) and a free-running component that corresponds to the activity rhythm previously entrained to LD. In some rats, if the period of the free-running rhythm is sufficiently close to that of the feeding schedule, it may become stably entrained (Stephan, 1986a; Mendoza et al., 2005). This is more common in hamsters (Abe and Rusak, 1992), particularly if free-running rhythms have become weak or disorganized by exposure to constant light (e.g., Mistlberger, 1993; see also Lamont et al., 2005a,b for a study of rats) and in some strains of mice (Abe et al., 1989; Castillo et al., 2004).
the present study, analysis was limited to the issue of whether the PVT is a critical component of the circadian mechanism driving food anticipatory rhythms. Although it is evidently not a critical part of this mechanism, the results do not rule out a role for the PVT as an input pathway by which the SCN can be entrained by scheduled meals in the absence of competing LD cues (Mendoza et al., 2005).

The clarity of the result with respect to our primary objective led us to terminate the experiment after testing three rats per group. Despite the small number of cases, some group differences were of sufficient magnitude to suggest an effect of the lesion on levels and patterns of activity and possibly feeding. When food was available ad-libitum, all rats with PVT lesions showed more total daily activity than any of the intact rats. This difference was due primarily to increased activity during the latter half of the dark period, when PVT-ablated rats showed a distinct concentration of activity culminating in a daily maxima just before lights-on. The intact rats, by contrast, all showed a more uniform distribution of activity throughout the dark period, with a weak trimodal waveform and peak activity levels during the first 4h of the dark period. The same effect has been reported previously in a study of blind rats with electrolytic lesions of the PVT (Moga and Moore, 2000). The meaning of this difference is uncertain, but changes in circadian waveforms have been observed following lesions in other brain systems (e.g., forebrain serotonin projections, Marchant et al., 1997; Smale et al., 1990). A concentration of activity late in the active phase has been associated with a longer circadian period in constant dark or dim light (e.g., Edgar et al., 1991; Mistlberger et al., 1998), and blind rats did show a lengthening of the circadian period following PVT ablation (Moga and Moore, 2000). As the PVT is the source of a significant excitatory input to the SCN circadian pacemaker (Moga and Moore, 1997; Moga et al., 1995), it is conceivable that eliminating this input may alter some property of the pacemaker (e.g., coupling among component oscillators) that affects its aggregate rate of oscillation and phase dependent output. Other work has shown that PVT lesions can alter the phase shifting effects of light pulses in rats (Salazar-Juarez et al., 2002). Thus, while the PVT may play little or no role in the generation of food anticipatory circadian rhythms, it does appear to have a modulatory influence on the functioning of the light-entrainable SCN circadian pacemaker.

PVT-ablated and intact rats also differed in the amount and distribution of activity during the scheduled daily mealtime. All three PVT-ablated rats exhibited activity throughout the mealtime, as measured both by the overhead motion detector and by the infrared photobeam positioned at the food access window, whereas the intact rats were active primarily during the first hour and when the
food bin was removed. The total amount of food consumed in the two groups was very similar, and nearly identical after the first 4 days of restricted feeding. If activity at the food bin reflects feeding, then the result may be indicative of a lesion-induced change in the microstructure of feeding behavior. Continuous activity at the food bin could reflect a pattern of nibbling extended over the 3h food access time, as opposed to gorging during the first hour. Alternatively, both groups may be eating more or less continuously, but intact rats may do this without leaving the food bin. Behavioral observations of intact rats in previous studies indicate that they tend to gorge during the first hour and pause before eating again during the third hour, thus the differences here likely represent a difference in the feeding pattern. Further study will be necessary to evaluate these hypotheses.

Reports that the PVT is responsive to a daily feeding opportunity and that food anticipatory activity was absent in rats with PVT lesions raised hope that the site of a food-entrainable pacemaker critical for anticipatory rhythms might have been identified (Nakahara et al., 2004). Similar excitement has been generated in recent years from studies of the dorsomedial hypothalamus and orexin-containing neurons in the perifornical and lateral hypothalamus (Gooley et al., 2006; Mieda et al., 2004; Akiyama et al., 2004). However, in each case, lesion studies designed to completely eliminate these structures, without worrying about the integrity of fibres of passage or surrounding structures, revealed no obvious deficit in food anticipatory rhythms by comparison with intact rats (Mistlberger et al., 2003; Landry et al., 2006). A crucial factor underlying the differences between these studies may be the configuration of the test apparatus. It appears that a capacity for food anticipation is much more likely to be revealed when rats can sleep and avoid light in a ‘pseudo’ den (an opaque PVC tube) from which they must periodically emerge to check for food accessible only via a small window on the far side of the cage. For other resources (e.g., salt; Rosenwasser et al., 1985, 1988) or in other species (e.g., mice; De Groot and Rusak, 2004), environmental signaling of mealtime may also be crucial (i.e., anticipation may be absent or attenuated if food or salt access time is signaled by environmental cues; see also Terman et al., 1984). Damage to a number of brain structures appears capable of attenuating food anticipatory activity in at least some measures of behavior, but this may depend on environmental characteristics and contingencies. There has been speculation over the years that food anticipatory rhythms may be mediated by a distributed system, and while direct evidence is still lacking, the present results are not inconsistent with this view.
2.4. Experimental procedures

2.4.1. Animals and apparatus

Male Sprague–Dawley rats (N = 6, ~ 500g, Charles River PQ) were housed individually in polypropylene cages (45 × 24 × 20cm) with wire floors over waste bins. The cages were housed in cabinets with a 12:12 light-dark (LD) cycle (~ 200lux during lights-on). Each cage was equipped with a 15cm long × 8cm diameter black opaque PVC tube for sleeping and light avoidance. Food was available in an external bin attached to the end of the cage and accessible through a 4 × 4cm window. Activity at the window was detected via infrared photobeam breaks. The sleeping tube was positioned such that the rat had to traverse the length of the cage to move from the tube to inspect the feeding window. A passive infrared motion detector (Quorum RR-150) was placed over the cage to register these movements. The motion detector registered a count for movements of 1cm or more, capped at 1 count/10s (i.e., 6 counts/min for continuous movement). Signals from the food bin monitor and the overhead motion detector were transferred to an interface of our own design, and counted and stored to disc at 10min intervals using in-house data acquisition software. Activity data were analyzed offline using Circadia (T.A. Houpt, Florida State University, Tallahassee, FL).

2.4.2. Surgery and histology

Rats were anesthetized for stereotaxic surgery using ketamine (90 mg/kg Ketalean; Bimeda-MTC Animal Health) and xylazine (9 mg/kg Rompun, Bayer), with isoflurane supplements as needed (0.5–1%, Aerrane, Baxter). Lesion electrodes (size 00 insect pins insulated with polyurothane to within 0.75mm of the flattened tips) and a Grass LM3 lesion maker were used to make a series of 5 radiofrequency lesions bilaterally (10 lesions/brain) at the following stereotaxic coordinates in millimeters relative to (1) bregma, posterior − 0.70, − 1.70, − 2.70, − 3.70, − 4.70, (2) the midline, lateral ± 1.65, 1.50, 1.50, 1.55, 1.55, and (3) the dura, ventral − 6.20, − 5.50, − 5.50, − 5.80, − 5.80. To avoid the sagittal sinus, the electrode was angled ± 15°. Metacam (1 mg/kg) was provided for postoperative analgesia. When behavioral testing was complete, the rats received an overdose of pentobarbital and were perfused transcardially with phosphate buffered saline followed by 10% formalin. The brains were removed, postfixed, cryoprotected overnight in sucrose–formalin and sectioned at 50μm intervals through the extent of the lesion and the PVT using a freezing cryostat. Sections were slide mounted and stained with cresyl violet, and the locus of damage determined via light microscope and the Paxinos and Watson rat brain atlas (1986). Sections were digitized and prepared for illustration using Adobe Photoshop 7.0 (Adobe Systems Inc.).
2.4.3. **Procedure**

After 13 days of recovery from surgery, three lesion rats and three intact rats were transferred to the recording cages and provided ad-libitum access to rodent chow (Purina Rodent Chow 5001) and water for 8 days. The rats were food deprived for 66h beginning at lights-off and then fed for 3h each day beginning 6h after lights-on. Food consisted of powdered 5001 chow mixed with corn oil to a consistency of wet sand (2.8 ml oil and 0.08 g sugar per gram chow). Food was mixed fresh and provided in food cups manually delivered and removed each day. Food and water consumption were measured at the end of the mealtime by weighing the cup and water bottle, respectively. After 19 days, the rats were food deprived for 51h, ending at lights-off. After 72h of ad-libitum food access, food was removed again for 72h, beginning and ending at lights-off.

2.4.4. **Data analysis**

Activity data were displayed visually in the form of actograms and average waveforms, using Circadia and GraphPad Prism 4.0b (GraphPad Software Inc, 2004). A nocturnality score was defined as the percentage of total daily activity occurring at night, as measured by overhead motion detectors. Food anticipatory activity was quantified in two ways, first, as the number of activity counts occurring during the 3h preceding the daily mealtime and, second, as the ratio of those counts against total daily activity (excluding activity 3h before, during and after mealtime). GraphPad Prism was used to test effects of lesion and of feeding schedules using repeated measures ANOVA with Bonferroni post-hoc tests. For these analyses, the activity variables (average daily activity, nocturnality ratios, food anticipatory activity counts and food anticipatory activity ratios) for each rat were averaged in blocks of days as illustrated in Fig. 2.3.
References


3. Persistence of a Behavioral Food-Anticipatory Circadian Rhythm Following Dorsomedial Hypothalamic Ablation in Rats

Landry GJ, Simon MM, Webb IC, & Mistlberger RE (2006) Persistence of a behavioral food-anticipatory circadian rhythm following dorsomedial hypothalamic ablation in rats. Am J Physiol Regul Integr Comp Physiol 290:R1527-R1534. doi:10.1152/ajpregu.00874.2005 (GJL designed the experiment, performed surgeries, collected and analysed data, and wrote the manuscript.)

Abstract

Circadian rhythms of behavior in rodents are regulated by a system of circadian oscillators, including a master light-entrainable pacemaker in the suprachiasmatic nucleus that mediates synchrony to the day-night cycle, and food-entrainable oscillators located elsewhere that generate rhythms of food-anticipatory activity (FAA) synchronized to daily feeding schedules. Despite progress in elucidating neural and molecular mechanisms of circadian oscillators, localization of food-entrainable oscillators driving FAA remains an enduring problem. Recent evidence suggests that the dorsomedial hypothalamic nucleus (DMH) may function as a final common output for behavioral rhythms and may be critical for the expression of FAA (Gooley JJ, Schomer A, and Saper CB. Nat Neurosci 9: 398 – 407, 2006). To determine whether the reported loss of FAA by DMH lesions is specific to one behavioral measure or generalizes to other measures, rats received large radio-frequency lesions aimed at the DMH and were recorded in cages with movement sensors. Total and partial DMH ablation was associated with a significant attenuation of light-dark-entrained activity rhythms during ad-libitum food access, because of a selective reduction in nocturnal activity. When food was restricted to a single 3h daily meal in the middle of the lights-on period, all DMH and intact rats exhibited significant FAA. The rhythm of FAA persisted during a 48h food deprivation test and reappeared during a 72h deprivation test after ad-libitum food access. The DMH is not the site of oscillators or entrainment pathways necessary for all manifestations of FAA, but may participate on the output side of this circadian function.
3.1. Introduction

Circadian rhythms in mammals are generated by a system of cell-autonomous circadian oscillators distributed within the brain and in peripheral organs (32, 57). A population of oscillators located in the retino-recipient hypothalamic suprachiasmatic nucleus (SCN) function as a master pacemaker critical for normal circadian organization of behavior and physiology and for entrainment of rhythms to daily light-dark (LD) cycles (34). Circadian rhythms can also be entrained by daily feeding schedules. If food access is restricted to a narrow daily temporal window (typically 2-4h in the middle of the lights-on period), nocturnal rodents, such as rats, mice, and hamsters, become behaviorally active in anticipation of the feeding time. This daily rhythm of food-anticipatory activity (FAA) takes a few circadian cycles to emerge, exhibits gradual shifting (transients) if mealtime is shifted, persists for at least 5 days during total food deprivation, and does not emerge if the interval between mealtimes is outside of the circadian range or its harmonics (reviewed in Refs. 40 and 58). These properties are consistent with a consensus view that FAA reflects the behavioral output of a food-entrainable circadian pacemaker. Notably, the pacemaker is not the SCN, because complete lesions of the SCN do not affect FAA in those species in which this has been examined (2, 15, 37, 60). Moreover, chronic ingestion of heavy water significantly slows the frequency of SCN-mediated rhythms, but has no effect on the timing of FAA (46). In the presence of an LD cycle, the SCNs of rodents exhibiting FAA to a daytime meal remain entrained to LD, as assessed by behavioral and physiological outputs and by the phase of circadian clock genes expressed in SCN neurons (20, 63); whereas in constant dark or dim light, the SCN may free run with a periodicity different from the 24h rhythm of FAA (9, 15, 28, 59). The SCN can entrain to daily feeding in some individuals in constant dark or dim light [more common in Syrian hamsters and certain strains of mice than in rats (1, 2, 16)] or under some conditions [e.g., in Syrian hamsters or rats in constant bright light, or when the period of the feeding cycle is close to the period of free-running rhythms (36, 42, 59)] and thus can also be described as a food-entrainable pacemaker, but the point to underscore is that the circadian rhythms of FAA are generated by mechanisms outside of the SCN. For peripheral organs, feeding time may be the dominant entraining stimulus, and the SCN may regulate the phase of these peripheral oscillators indirectly, by driving the daily rhythm of food intake when food is available ad-libitum (20, 31, 57, 63).

Convincing phenomenological evidence for the existence of circadian clocks in mammals was available for many decades before the SCNs were first identified by lesion studies as the likely site of a light-entrainable circadian pacemaker (50, 62). It has now been nearly 30yr since the first
lesion studies confirmed that the SCNs are not necessary for the generation or entrainment of food-anticipatory circadian rhythms (15, 35, 60, 61), yet the sites of oscillators and entrainment pathways necessary for this circadian function have so far eluded identification, despite considerable effort (40, 58). Some lesions or gene mutations have been identified that affect the expression of FAA, but with two exceptions (discussed below), food entrainment has been shown to persist in at least some measures of behavior. Briefly, knockouts of a clock gene expressed in the neocortex and hippocampus (nPAS2) (27) or genetic ablation of the lateral hypothalamic peptide orexin (3, 39) have been reported to attenuate FAA in mice, but FAA has already been shown to persist in rats following complete ablation of these structures (40, 44, 47, 49). Lesions of the nucleus accumbens core (38) and the hypothalamic paraventricular nucleus attenuate or eliminate FAA in some measures of behavior (e.g., general locomotor activity) but not in others [e.g., wheel running or activity directed at a food bin (47, 49)]. Ablation of infralimbic neocortex (54) or the hypophysis (25) eliminates the food-anticipatory rhythm of body temperature, but not of behavior. FAA also persists after ablation of the hypothalamic subparaventricular zone (29, 30a), arcuate nucleus (43), ventromedial nucleus (48), mutations affecting the clock genes mCry1/Cry2 (33) and Clock (53), and the leptin receptor gene (45). FAA is also not dependent on endocrine signals from the adrenal gland (15, 60), although adrenalectomy does eliminate a food-entrainable circadian rhythm of clock gene expression in the oval nucleus of the bed nuclei of the stria terminalis (4, 36). Finally, FAA is not dependent on sensory signals provided by olfactory, optic, trigeminal (gustatory), or visceral autonomic nerves (19, 21, 24, 40), although combined removal of these afferents has not been attempted. Although these studies have failed to identify oscillators and input pathways necessary for entrainment by scheduled feeding, they are instructive in demonstrating that lesions can dissociate behavioral and physiological food-anticipatory responses and may differentially affect behavioral outputs. Non-specific measures of locomotor activity, such as provided by telemetry or tilt cages, may be more susceptible to disruption by lesions, whereas behaviors directed at feeding locations seem to be more resistant (e.g., Ref. 49).

Two other reports merit special attention. Rats with electrolytic or cell-specific lesions of the brain stem parabrachial nucleus (PBN) were shown to express either very little or no food-anticipatory circadian rhythms of core body temperature or activity directed at a food tray, a behavioral measure that in other studies has been resistant to disruption by lesions (22). The PBN has also been shown to exhibit a food-anticipatory rhythm of c-fos expression that, unlike FAA, does not persist if the scheduled daily meal is omitted for one cycle (5). Together, these results
suggest that the PBN may be a critical component of the entrainment pathway to food-entrainable oscillators located elsewhere. The PBN is an integrative area for visceral and gustatory sensory information, and projects to a variety of forebrain areas, including the dorsomedial hypothalamic nucleus (DMH) (55, 64). The DMH, referred to as “enigmatic” by Thompson, Swanson and colleagues (64, 65), has recently been conceptualized as an integrative area and final common output for circadian rhythms of sleep-wake, ingestive behavior, and corticosterone (56). The DMH receives direct and indirect input from the SCN, expresses neuropeptides (e.g., neuropeptide Y, orexin) and receptors (e.g., leptin, cholecystokinin, ghrelin) implicated in the control of ingestive behavior and metabolism and is richly interconnected with hypothalamic, preoptic, and some brain stem nuclei involved in regulation of energy input/output or behavioral state (10, 17, 18, 26, 41, 64, 65). Lesions of the DMH disrupt LD-entrained circadian rhythms in these functions (13, 18) and have recently been shown to attenuate or eliminate anticipatory rhythms of general activity and core body temperature measured by telemetry in rats restricted to a 4-h daily meal (30, 30a). DMH damage may explain our own occasional observations of rats or mice that failed to anticipate a daily feeding time following very large, nonspecific radiofrequency lesions of the medial hypothalamus, resulting from presumably faulty electrodes aimed at the SCN or the paraventricular nucleus (6, 37). These results suggest that the DMH may be the site of food-entrainable circadian oscillators or a critical link between such oscillators and circadian outputs.

Given the importance of the behavioral measure in assessing circadian function following brain lesions, we sought to further examine the role of the DMH by using motion detectors with more spatial selectivity than is provided by telemetric movement sensors. To maximize the chances of evaluating rats with unambiguous, complete DMH ablation, we used electrodes, stereotaxic placements, and radiofrequency current parameters designed to produce very large medial hypothalamic lesions. We found that unambiguous DMH destruction produced ingestive deficits and attenuated LD-entrained circadian activity rhythms but did not attenuate behavioral anticipation of a daily meal.

3.2. Materials and methods

3.2.1. Subjects and apparatus

Adult male Sprague-Dawley rats \((n = 14, 310-320g; \text{Charles River})\) were housed individually in polypropylene cages \((45 \times 24 \times 20cm)\) equipped with wire floors and tops, a water bottle, and a
black opaque tube (15cm long × 8cm diameter) for sleeping or light avoidance (12:12h light-dark cycle, ~1,000:0lux). Food was available in a metal cup mounted on a manually controlled carousel, accessed via a 4 × 4cm window cut through one end of the cage. The window was covered by a hinged metal gate, which the rats were required to move with their snouts to reach the food cup. Movements of the gate were detected by a microswitch, monitored continuously by an interface and data collection system of our own design. The “sleeping” tube was fixed to the cage floor with the opening at the end of the cage opposite to the feeding window. The rats thus had to cross the length of the cage to move from the tube to the food cup. A motion detector (Quorum RR-150) was positioned above the cage to detect these movements. Activity counts were summed in 10min intervals and stored for analysis off-line using Circadia (Dr. T. A. Houpt, Florida State University, Tallahassee, FL) operated on a Macintosh computer.

3.2.2. Surgery and histology

Seven rats received bilateral radiofrequency lesions of the DMH. The rats were anesthetized for stereotaxic surgery using ketamine (90 mg/kg Ketalean; Bimeda-MTC Animal Health) and xylazine (9 mg/kg Rompun; Bayer) supplemented with isoflurane (0.5-1% Aerrane; Baxter) as needed. The lesion electrodes were stainless steel insect pins (size 0) insulated to within 0.5mm of the flattened tip. Current was supplied by a Grass LM3 Lesion Maker. Stereotaxic coordinates were ± 0.5mm lateral and 3.5mm posterior to bregma and 8.5mm ventral from the dura. Following surgery, body temperature and food and fluid intake were monitored over an 8-day recovery period. All rats survived the procedure.

At the completion of behavioral testing all lesioned rats and two intact control rats were euthanized via pentobarbital sodium overdose and perfused transcardially with saline followed by 10% formalin. The brains were removed, postfixed, cryoprotected in a formalin-sucrose mixture for at least overnight, and sectioned at 50µm intervals using a cryostat. All sections from the posterior optic chiasm to the medial mammillary nuclei were mounted on slides, stained using cresyl violet, dehydrated, cleared, and coverslipped.

Sections in which lesions or intact DMH were evident were examined under a microscope, photographed with a digital camera, and then carefully inspected on a computer. Lesioned brains were compared with the two intact brains and with the Paxinos and Watson (52) rat brain atlas, supplemented by published work on DMH cell bodies, afferents, and efferents (18, 64, 65). From these comparisons, a percentage of DMH intact was estimated.
3.2.3. **Test procedures**

After recovery from surgery, the rats were returned to their recording cages where they and seven intact rats had free access to pellet food (Purina Rodent Chow 5001) and water for 20 days. Food was then removed at dark onset for 18h, and for the next 30 days was provided for 3h each day beginning 6h before lights off. Food consisted of powdered rat chow mixed with corn oil to the consistency of wet sand. Food bins were manually rotated into position each day and removed and weighed 3h later. Water bottles were also weighed daily to track fluid consumption. After 30 days, food was removed for 51h. Pellet chow was then provided ad-libitum for 4 days, after which food was removed for 4 days, beginning at dark onset. Pellet chow was provided ad-libitum for a final 10 days. See Fig. 3.1 for activity charts illustrating this sequence. To minimize stress and activity artifacts, the rats were not weighed during the behavioral recording.

**Figure 3.1. Activity records of representative rats with no lesion (A), partial dorsomedial hypothalamic nucleus (DMH) damage (B), and total DMH ablation (C).**

*Note.* Each line represents a day, with time plotted from the left in 10min bins. Bins in which 3 or more activity counts were registered are represented by a vertical bar. The 12h dark period is indicated by shading. Mealtime (3 h/day) during food restriction is labeled and indicated by the open bar. Days during which no food was provided are indicated by the black bar to the left of each chart. V, beginning of food deprivation; inverted V, end of deprivation.
3.2.4. **Data analysis**

Activity data during ad-libitum food access were expressed as nocturnality scores (%total daily activity occurring during lights-off). Activity data during ad-libitum food access and restricted feeding were also expressed as FAA counts (total number of activity counts during the 3h preceding mealtime, i.e., hours 4-6 after lights on) and FAA ratios (ratio of FAA counts to activity occurring at night and during the first 3h of lights on). Group differences and effects of time were evaluated by ANOVA and planned Student’s *t*-tests. In the text, means are given as ± SEM.

3.3. **Results**

3.3.1. **Histology**

Figure 3.2 illustrates photomicrographs of the hypothalamus from an intact rat (A-C) and rats with partial (E-G) or total (I-K) DMH ablation. The DMH first appears caudal to the paraventricular and anterior hypothalamic nuclei, above the rostral ventromedial hypothalamic nucleus, below the diffuse dorsal hypothalamic area, and extending from the third ventricle laterally to within 100-200µm of the fornix. The caudal border is considered ambiguous (14); conservatively, it may merge with the arcuate nucleus at the level of the mammillary recess and premammillary nuclei. The rostrocaudal extent of the DMH can be estimated at ~1.6mm based on Paxinos and Watson (52) and ~1.12mm based on sagittal sections illustrated in Chou et al. (18). The latter estimate corresponds approximately to the range of sections illustrated for the intact rat in Fig. 3.2, A-C.

The lesion parameters were intended to produce ablations centered on the DMH and extending 2mm or more rostrocaudally. Six of seven cases sustained ablations of this size. In three of seven cases, some DMH tissue appeared to be present. In two of these three cases, the lesions were asymmetrical and clearly partial; the smallest lesion (Fig. 3.2, E-G) spared the lateral third of the DMH on one side and the caudal DMH bilaterally. A second partial lesion spared ~20% of one DMH laterally (Fig. 3.3A). A third case was also classified as partial; although the cavity extended ~1.6mm rostral to caudal, some intact DMH cells medial to the fornix on one side could not be ruled out, and the lesion was estimated at ≥90% complete (Fig. 3.3B).
In four of seven cases, the lesions were very large, producing cavities that extended laterally at least to the fornix on both sides, caudally from the paraventricular nucleus to the premammillary nuclei, and dorsally from the ventromedial nucleus well into the medial thalamus above the roof of the third ventricle (Figs. 3.2, I-J & 3.3, C-E). In all of these cases, the lesion cavities subsumed the fornix and mammillothalamic tract on at least one side. The dorsal hypothalamic area and the diffuse and compact regions of the DMH were completely absent. At least partial damage was sustained by the paraventricular (particularly the medial magnocellular portions), subparaventricular, anterior, periventricular, ventromedial (particularly dorsomedially), arcuate, and posterior hypothalamic nuclei, the midline thalamus (reuniions, rhomboid, centromedian nuclei), and the tuberal magnocellular nucleus. Abnormal cells, glia, and apparent debris were evident in parenchyma near the borders of the cavities, indicating that this analysis, based on the cavity size and position relative to key landmarks, is a conservative estimate of the extent of the damage.
Figure 3.3. Photomicrographs and average waveforms from rats with DMH lesions judged to be partial (A & F, B & G) or total (C & H, D & I, E & J).

Note. Arrows in A and B indicate location of possible intact DMH neurons. See Fig. 3.2 for abbreviations and waveform plotting formats.

3.3.2. Activity levels and nocturnality

During ad-libitum food access before restricted feeding, intact rats exhibited a high-amplitude daily rhythm of locomotor activity, averaging 1,828 ± 119 counts/day of which 83 ± 3% were registered at night (Figs. 3.1A, 3.2D, & 3.4A). DMH lesion rats averaged 929 ± 225 counts/day [$t_{(12)} = 2.60$, $p = 0.02$ vs. intact rats] of which 72 ± 6% occurred nocturnally [$t_{(12)} = 5.77$, $p < 0.0001$ vs. intact rats]. The reduction in activity in lesion rats was significant only at night [$t_{(12)} = 3.17$, $p =$
0.008 vs. intact rats; Fig. 3.4A]. During restricted feeding and food deprivation, nocturnal activity levels increased in the DMH lesion rats, but not in the intact rats (Fig. 3.4, B & C). Nonetheless, nocturnal activity remained significantly lower in the DMH lesion rats. Activity levels and nocturnality ratios were virtually identical in rats with total and partial DMH lesions.

**Figure 3.4.** Group mean average waveforms of activity in intact rats (dotted lines) and DMH-ablated rats (solid lines) during ad-libitum food access (A), the last week of food restriction (B), and 72h of total food deprivation (C).

Note. Feeding time in B is denoted by the vertical hatched bar. Lights-off (hours 12–24) is denoted by the heavy bar above the x-axis.
3.3.3. **Food-anticipatory activity**

During restricted feeding, all intact and DMH lesion rats exhibited activity in anticipation of the daily meal, as measured by an overhead motion sensor (Figs. 3.1 A-C; 3.2 D, H, & I; 3.3 F-J; & 3.4 B) and by a microswitch at the food bin. The number of counts detected by the microswitch was low throughout the day in intact and lesion rats; therefore only the motion detector activity data were used for quantitative analyses. Total motion detector activity counts during the 3h before mealtime (FAA) were averaged in 5-day blocks over the 30 days of restricted feeding and compared with activity at the same time of day during the preceding five baseline days when food was available ad-libitum (Fig. 3.5A). Each of the six blocks of restricted feeding days was significantly different from the baseline block in both the intact group \[F_{(11,6)} = 10.24, p < 0.0001; \text{pairwise comparisons significant at } p = 0.02 \text{ or better}\] and DMH lesion group \[F_{(11,6)} = 7.92, p < 0.0001; \text{pairwise comparisons significant at } p = 0.003 \text{ or better}\]. FAA counts remained high during 2 days of food deprivation immediately following the last scheduled meal (e.g., Fig. 3.1 A-C), declined when food was provided ad-libitum, and increased again when food was removed for 3 days (Figs. 3.1 A-C and 3.4C), demonstrating persistence of the FAA rhythm in the absence of a daily feeding stimulus in both groups. Between-group comparisons revealed that FAA counts were significantly higher in the DMH lesion group on the last 5-day block of restricted feeding during the 48-h food deprivation test and on subsequent blocks with ad-libitum food access.

FAA ratios showed a similar pattern of results (Fig. 3.5B); ratios during restricted feeding were significantly different from baseline by the first 5-day block in the DMH lesion rats \[F_{(11,6)} = 11.58, p < 0.0001; p < 0.05 \text{ for each comparison with baseline}\] and by the second 5-day block in the intact rats \[F_{(11,6)} = 4.92, p < 0.0001; p < 0.05 \text{ for each comparison with baseline}\]. FAA ratios were significantly higher in the DMH group at all time points, including the baseline ad-libitum food access days [two-way ANOVA, \(F_{(11,1)} = 3.27, p < 0.0001; p < 0.05 \text{ for each pairwise comparison}\]. This reflects, in part, the reduced amount of nocturnal activity in rats with DMH lesions. There was no apparent relation between the FAA statistics and the size or completeness of the lesions.
Figure 3.5.  A: group mean ± SEM activity counts registered during hours 4-6 after lights on, averaged in 5-day blocks with food available ad-libitum or restricted to 3h/day or in 1-day or 3-day blocks of total food deprivation (number of days/block is indicated above the x-axis); B: same plotting convention, with food-anticipatory activity expressed as a ratio relative to activity at night and during the first 3h of the day. For some data points, standard error bars are too small to see. FAA, food-anticipatory activity; DMHx, DMH lesions; ad lib, ad libitum; dep, deprivation.

3.3.4. Food and water

Daily food intake increased over the first 7-10 days of restricted feeding (Fig. 3.6). This was more apparent in the intact group than in the DMH lesion group. Mean intake during restricted feeding was 13.6 ± 0.4 g in intact rats and 13.9 ± 0.4 g in the lesion rats, excluding one hyperphagic outlier. The outlier sustained the smallest partial lesion (Fig. 3.2 E-G), averaged 22 ± 3 g/day, and exhibited apparent weight gain over the course of behavioral testing. Daily water intake during restricted feeding averaged 20.1 ± 1.2 ml/day in rats with DMH lesions, and 25.9 ± 1.2 ml/day in intact rats (p < 0.001). There was no difference between rats with partial and total lesions.
3.4. Discussion

The DMH has been conceptualized as a site where neural and endocrine signals conveying information about circadian phase, energy states, and other factors are integrated to determine the daily circadian program for sleep-wake, activity, body temperature, and at least some endocrine rhythms (56). Consistent with this hypothesis, daily rhythms of immediate-early gene expression in DMH neurons are regulated by scheduled mealtimes, and ablation of DMH neurons by ibotenic acid eliminates food-anticipatory temperature rhythms and attenuates a rhythm of FAA in proportion to the severity of cell depletion (5, 30, 30a). The DMH has thus been described as critical for entrainment of circadian rhythms by scheduled feeding (30, 30a, 56); conceivably, it could be the site of food-entrainable circadian oscillators or of a final common output pathway for such oscillators. If so, then complete lesions of the DMH should eliminate FAA in all measures of behavior. Alternatively, the DMH could be critical for the expression of FAA in some but not other measures of behavior.

The results of the present study support the latter of these hypotheses; very large radiofrequency lesions that unambiguously destroyed the DMH produced ingestive deficits and attenuated LD-entrained circadian rhythms but did not eliminate FAA detected by a motion detector or a microswitch at the food-bin window. The difference between this result and results reported previously (30, 30a) is presumably related to the measure of behavior and possibly the
configuration of the recording apparatus. In the previous study, activity was measured by a radiofrequency transmitter implanted in the abdominal cavity, which detects movement nonspecifically. Other work has shown that anticipatory activity in nonspecific cage activity can be eliminated by hypothalamic lesions that do not affect anticipatory activity directed specifically at a food-access window (49). In the present study, activity was measured by motion detectors situated overhead and at a food-access window. The cage was configured such that the rats could sleep in a dark tube that opened at the end of the cage opposite from the food-bin window. This may have minimized detection of nonspecific daytime activity and increased the amount of activity that was specifically food anticipatory.

Surprisingly, not only was FAA present in all of the DMH-ablated rats, but the magnitude of the rhythms was enhanced by comparison with intact rats. We have previously observed enhanced FAA in rats with other neural ablations or genetic defects (43, 45), but the interpretation of such effects is unclear. In the present case, the DMH lesions were associated with significant reductions in nocturnal activity. Reduced activity at night must have contributed substantially to the increased FAA ratios, given that nocturnal activity was the main part of the denominator in this ratio. The absolute amount of activity during the 3h before mealtime was also increased in the DMH-ablated rats, and this might be because of an effect of the lesions on one or both of two factors that normally constrain the level of daytime activity: 1) an inhibitory influence of the SCN pacemaker on locomotor activity during the rest phase of the circadian cycle (41) and 2) an inhibitory influence of environmental light on locomotor activity [so-called “negative masking” (8, 51)]. The same factors may explain why there was a tendency for the DMH-ablated rats to eat larger meals during the first week of restricted feeding. Cage lights were relatively bright in this study, and this may have served to amplify differences between intact and ablated rats.

DMH lesions have previously been shown to reduce both food and water intake when both resources are freely available (11, 12). In the present study, DMH-ablated rats drank significantly less (~22%) but did not eat less than intact rats during scheduled feeding. This may be because food was limited [DMH-ablated rats overeat relative to intact rats during the first hour following food deprivation (11)] and/or because the powdered chow was mixed with oil, which enhances palatability. The amount of food eaten per scheduled meal increased over the first 7-10 days of restricted feeding in the ablated and intact rats (Fig. 3.6), likely because of homeostatic factors [i.e., loss of body weight during the first few days of limited access to food (e.g., Ref. 7)] and circadian factors [i.e., gradual shifting of gastrointestinal circadian rhythms from a nocturnal phase to a
diurnal phase, thereby permitting larger meals (e.g., Ref. 23)]. Although meal size is only an indirect (and putative) measure of the phase of gastrointestinal rhythms, the gradual increase of meal size during the first week of restricted feeding in the DMH-ablated rats suggests that DMH lesions also do not affect entrainment of peripheral oscillators to the scheduled daytime meal.

The results of the present study rule out a role for the DMH as the exclusive site of oscillators mediating entrainment of behavioral rhythms by circadian feeding schedules. However, the results are not inconsistent with a role for the DMH as an integrative area that adjusts the daily timing of at least some physiological and behavioral variables under the influence of circadian, metabolic, and other factors. The DMH is most likely situated downstream, i.e., on the output side of the food-entrainable oscillators critical for anticipatory activity rhythms. Alternatively, a number of brain regions, including the DMH, may be capable of food-entrainable oscillations driven by local oscillating clock cells, and these may be more or less directly coupled to specific outputs. Such a “distributed” organization could explain why lesions in several areas (e.g., nucleus accumbens, infralimbic cortex, paraventricular nucleus, lateral hypothalamic orexin cells, DMH, PBN, hypophysis) have been shown to attenuate at least one food-anticipatory circadian rhythm (e.g., temperature, general locomotor activity), whereas no lesion has yet been shown to eliminate all manifestations of food entrainment in all animals tested. A quarter-century on, Stephan’s (60) warning remains pertinent: “If many oscillators exist which are entrainable by food restriction schedules, it may not be possible to abolish anticipatory activity by selective removal, or interference with, specific organ systems,” to which we might add “specific brain regions.”
References


4. The Dorsomedial Hypothalamic Nucleus Is Not Necessary for the Expression of Circadian Food-Anticipatory Activity in Rats


Abstract

Restricted daytime feeding generates food-anticipatory activity (FAA) by entrainment of a circadian pacemaker separate from the light-entrainable pacemaker located in the SCN. The dorsomedial hypothalamic nucleus (DMH) has been proposed as the site of food-entrainable oscillators critical for the expression of FAA, but another study found no effects of complete DMH ablation on FAA. To account for these different results, the authors examined methodological factors, including (1) cage configuration and feeding method and (2) use of social cues. Intact and DMH-ablated rats were maintained on one 4h daily meal in the middle of the light period, using caging and feeding methods matching those of Gooley et al. (2006). Rats with partial or complete DMH ablation were less nocturnal during ad-libitum food access but exhibited normal FAA during restricted feeding, as quantified by FAA magnitude, ratios, latency to appearance, duration, and precision. To evaluate the use of social cues, intact rats naive to restricted-feeding schedules were food deprived for 72h on 4 tests. Daytime activity increased during food deprivation, but the magnitude and waveform of this activity was not influenced by the presence of food-entrained rats exhibiting robust FAA in adjacent cages. Thus, hungry intact rats do not use social cues to anticipate a daily mealtime, suggesting that DMH-ablated rats do not anticipate meals by reacting to sounds from food-entrained intact rats in adjacent cabinets. These results confirm our previous finding that the DMH is not critical for normal expression of FAA in rats, and this observation is extended to food restriction methodologies used by other labs. The methodological differences that do underlie discrepant results remain unresolved, as does the location of food-entrainable oscillators, input pathways, and output pathways critical for FAA.
4.1. Introduction

Circadian rhythms in mammals are regulated by a system of cell-autonomous circadian oscillators, distributed across many brain regions and peripheral organ systems (Abe et al., 2002; Cermakian and Sassone-Corsi, 2002; Granados-Fuentes et al., 2004; Lamont et al 2005; Reick et al., 2001; Schibler et al., 2003). Oscillators at most of these sites are presumed to regulate cellular or organ functions particular to the tissues in which they reside. One population of neuronal oscillators in the hypothalamic SCN has a special status as a pacemaker that directly or indirectly regulates the phase and period of oscillators elsewhere and that mediates entrainment of the entire system to daily LD cycles (Klein et al., 1991). A second set of oscillators, located outside of the SCN, is believed to function as a food-entrainable timing system coordinating behavior and physiology with daily feeding schedules. This food-entrainable circadian system is a construct invoked to explain the circadian properties of food-anticipatory rhythms of activity (FAA), body temperature, and other variables that emerge in intact and SCN-ablated animals fed at a predictable time of day or at regular circadian intervals in otherwise constant conditions (Boulos and Terman, 1980; Mistliberger, 1994; Stephan, 2002). By contrast with the light-entrainable pacemaker, the food-entrainable circadian system lacks specification at both the anatomical and molecular levels. It may be a single “pacemaker” located at an anatomically discrete site, driving all food-entrainable rhythms, comparable to the SCN, or it may be a network of oscillators distributed across many brain regions, with different oscillator subgroups driving different food-entrained rhythms, with either some or no overlap in output. The distributed network concept is indirectly supported by (1) persistence of food-anticipatory rhythms following ablations in many brain regions (Mistliberger, 1994; Landry et al., 2006; Davidson, 2006) and (2) evidence that circadian clock genes defined by analysis of SCN neurons are also expressed in other brain regions and organs and exhibit rhythms that, unlike the SCN, are readily rephased by daily feeding schedules in the presence of competing LD cycles (Angeles-Castellanos et al., 2007; Schibler et al., 2003; Verwey et al., 2007; Lamont et al., 2007; Wakamatsu et al., 2001). FAA rhythms in mice have now been shown to be disrupted by knockout of the mPer2 clock gene (Feillet et al., 2006), but sitespecific knockouts or conventional lesions will be necessary to determine which, if any, sites of mPer2 expression outside of the SCN are specialized to act as a pacemaker.

Two studies have drawn attention to the hypothalamic dorsomedial nucleus (DMH) as a potential candidate site for a food-entrainable pacemaker. In mice, the DMH exhibits little or no mPer1 or mPer2 expression when food is freely available but strong circadian expression when
food is restricted to a limited time of day (Mieda et al., 2006). A critical role for the DMH in food-anticipatory circadian rhythms in rats has been proposed based on the observation that neurotoxic lesions destroying 75% to 90% of DMH neurons strongly attenuate food-anticipatory rhythms of locomotion and EEG-defined waking, as well as eliminate the premeal rise in core body temperature evident in intact rats (Gooley et al., 2006). While apparently all DMH-damaged rats exhibited at least some FAA in that study, a significant correlation between lesion size and FAA magnitude suggests that residual anticipatory behavioral rhythms may be explained by surviving DMH neurons.

These results could be interpreted as evidence that the DMH contains food-entrainable circadian oscillators that function collectively as a master food-entrainable pacemaker driving anticipatory rhythms of behavior and physiology. However, we found that rats sustaining unambiguously complete ablation of the DMH were capable of essentially normal FAA rhythms (Landry et al., 2006). Also, dissociations between food anticipation and DMH PER2 expression have now been reported; rats with ad-libitum food access can anticipate a daily palatable meal but exhibit no rhythm of PER2 expression in the DMH (Verwey et al., 2007). We therefore conclude that the DMH is neither a master pacemaker nor a critical input pathway for food-entrainable behavioral rhythms. The DMH may be critical for food-entrained temperature rhythms, but this effect of DMH ablation requires confirmation and is not unique, as lesions in other brain regions can also eliminate the food-anticipatory rise in body temperature in rats (Davidson and Stephan, 1999; Recabarren et al., 2005).

Why DMH lesions affected anticipatory activity so severely in Gooley et al. (2006) but not at all in Landry et al. (2006) remains to be clarified. The 2 studies differ in lesion method, food type, food location, activity measure, cage isolation, and cage configuration. We speculate that the configuration of the cage may be a crucial variable. Both studies used polypropylene cages of the same dimensions, but our cages had wire floors, an opaque tube for sleeping, and a small side window through which a food cup could be accessed, whereas the Gooley cages had solid floors with bedding, and food was accessed through the metal bars of the cage tops. We previously found that some rats with lesions of the paraventricular hypothalamic nucleus (PVN) failed to exhibit anticipatory activity measured by a tilt floor, yet showed robust anticipation in activity directed at a food cup accessed via a window in the cage (Mistlberger and Rusak, 1988). The lesions evidently increased the probability that rats would remain sedentary but awake at a feeding location, rather than move about the cage in anticipation of mealtime (floor tilts register gross body movements.
across a fulcrum under the middle of the cage floor). In Landry et al. (2006), the availability of a tube for sleeping and light avoidance in the day, coupled with a fixed feeding location at the opposite end of the cage from the tube exit, may promote food site “sampling” behavior, requiring excursions across the length of the cage. This configuration may increase the probability that rats with hypothalamic lesions will express anticipatory activity, as detected by an overhead motion sensor. Such activity could, in turn, increase and consolidate premeal waking (e.g., Welsh et al., 1988).

If cage configuration is crucial, then we should be able to replicate the strong attenuation of FAA reported by Gooley et al. (2006) by housing rats with DMH lesions in similar cages with bedding but no sleeping tube or food access window. Surprisingly, we report here another failure to detect any effect of complete DMH ablation on FAA in rats, despite using cages, feeding methods, and food as in Gooley et al. (2006). We also evaluated whether acutely food-deprived rats naive to feeding schedules might use auditory cues from food-entrained neighboring rats to anticipate a forthcoming midday meal, and we found that they do not. Thus, it is unlikely that our DMH-ablated rats anticipate feeding time by responding to the sounds of intact rats in adjacent cabinets.

4.2. Materials and methods

4.2.1. Animals and apparatus

Young adult male Sprague-Dawley rats ($N = 35$, Charles Rivers, PQ) were housed individually in polypropylene cages ($45 \times 24 \times 20$cm) in 2 adjacent cabinets, each with 3 shelves, incandescent lighting (LD 12:12, ~200lux), and open front doors. To examine effects of DMH lesions on FAA using the procedures of Gooley et al. (2006), the cages had solid floors, corncob bedding, and standard stainless steel tops with bars. The bars on one half of the top angled down toward the center of the cage, to serve as a hopper for Purina 5001 chow pellets and as a water bottle holder. To examine effects of social auditory cues on anticipatory activity in intact rats, the cages had wire floors, a PVC tube ($15 \times 8$cm) for sleeping, and a $4 \times 4$cm window in the wall at one end of the cage, providing access to a manually operated carousel holding a food cup. Activity within both types of cages was detected by a passive infrared motion sensor (Quorum RR-150) positioned 25cm above the center of the cage. Sensors were monitored continuously using an interface and data acquisition system of our own design. Counts were summed and stored to disk at 10min intervals.
4.2.2. **Surgery and histology**

Rats were anesthetized for stereotaxic surgery with isofluorane gas (Aerrane, Baxter, Deerfield, IL) and a cocktail of xylazine (ip, 9 mg/kg Rompun, Bayer, Toronto, ON, Canada) and ketamine (90 mg/kg Ketalean, Bimeda-MTC Animal Health, Inc., Cambridge, ON, Canada). Rats received bilateral radiofrequency lesions directed at the DMH (n = 6) using stainless steel insect pins, insulated to within 0.5mm of the flattened tips, and a Grass LM3 lesion maker. Stereotaxic coordinates were ±1.5mm lateral to the sagittal sinus, 3.45mm posterior to bregma, and 8.6mm ventral to the dura, with the electrode arm angled 10 degrees.

Upon completion of behavioral testing, the rats were administered an overdose of sodium pentobarbital (Euthanyl; Bimeda-MTC Animal Health, Inc.) and were perfused transcardially with saline followed by 10% formalin. Brains were removed, cryoprotected for 48h in a formalin-sucrose mixture, and sliced at 50µm intervals using a cryostat. The sections were mounted on glass slides, stained with cresyl violet, dehydrated, cleared, and coverslipped with Permount. Brain sections through the entire DMH were digitized using a standard light microscope (Nikon Eclipse 80i) connected to a digital camera (Retiga 2000R, QImaging Corporation, Burnaby, BC, Canada). To calculate the percentage of DMH damage, the Paxinos and Watson (1986) rat brain atlas, supplemented by published photomicrographs (Chou et al., 2003; Thompson et al., 1996; Thompson and Swanson, 1998), was used to create templates of the DMH on which the lesion boundaries were drawn. The DMH area was divided into quadrants, with each box representing 12.5% of total DMH area per section (Fig. 4.1), and the amount of DMH tissue in each box was estimated as a percentage of volume. This method may underestimate the size of partial lesions, as no attempt was made to discriminate between normal and abnormal cells.

4.2.3. **Procedures**

**DMH lesion experiment**

After 10 to 15 days of recovery from surgery, DMH-ablated rats were placed in the recording cages and maintained with ad-libitum access to water and rat chow pellets for 21 days. These rats, along with 6 intact rats, were then food deprived for 20h, fed 4h/day beginning 6h after lights-on for 31 days, food deprived for 50h, fed ad-libitum for 5 days, and food deprived for 72h.
Social cues experiment

Rats housed in isolation cabinets may hear food-anticipatory activity or vocalizations (e.g., ultrasonic) from rats in adjacent shelves or cabinets. Ad-libitum-fed rats ignore such cues, as they do not show increased activity prior to scheduled midday meals delivered to nearby food-entrained rats (unpublished observations). However, hungry rats may be more sensitive to such cues and, without prior experience of restricted daytime feeding, may nonetheless “anticipate” a midday meal by responding to sounds from food-entrained rats. To test this hypothesis, 12 rats were housed in cages, 2/shelf on each of 3 shelves in 2 cabinets. The rats were divided into 2 groups, 1 serving as the food-entrained group (n = 4) and 1 as the naïve group (n = 8). The middle shelf of each cabinet housed 2 food-entrained rats, with 2 naïve rats on each shelf above and below. All rats were fed ad-libitum for 1 week and were then food deprived beginning at dark onset. The food-entrained group was deprived for 66h and then received food (powdered 5001, mixed in corn oil) for 3h each day beginning 6h after lights-on for the next 19 days. The naïve group was deprived for 72h and then provided food ad-libitum for the next 11 days. On days 12 to 15 of the restricted-feeding schedule, by which time all food-entrained rats were exhibiting robust FAA, the naïve group was food deprived a second time for 72h, beginning at dark onset. After day 19 of food restriction, both groups were food deprived for 75h, beginning 3h before dark onset (the end of the last mealtime). Food was then provided ad-libitum to both groups for 4 days and removed for a final 72h deprivation.

4.2.4. Data Analysis

Actograms and average waveforms were generated using Circadia (Behavioral Cybernetics) and Prism (Graphpad Software, Inc.). Activity data were quantified by calculating nocturnality scores (percentage of total daily activity occurring during lights-off), FAA counts (total number of activity counts during the 3h prior to mealtime, i.e., hours 4-6 of lights-on), FAA ratios (FAA counts as a percentage of daily activity, excluding hours 4-12 of lights-on), FAA duration (in minutes from FAA onset to mealtime, with onset defined as the time bin when FAA exceeded 50% of the FAA peak value for that day), and FAA precision (standard deviation of FAA duration; all FAA bouts ended at mealtime, and thus this is equivalent to precision of FAA onset). Statistical comparisons within and between groups were made using mixed-design analysis of variance (ANOVA), with Greenhouse-Geisser-adjusted degrees of freedom and Bonferroni t tests as appropriate (SPSS). Means in the text are reported ± standard error.
4.3. Results

4.3.1. DMH histology

The DMH is estimated to extend up to 1.6mm caudally from the PVN, 1mm dorsally from the ventro-medial hypothalamus (VMH), and 1mm lateral from the third ventricle to within 100 to 200mm of the fornix (Paxinos and Watson, 1986; Chou et al., 2003) (Fig. 4.1A). All 6 rats with DMH lesions exhibited large lesion cavities over this region. In 3 cases, the cavities spanned 2.45 to 2.66mm rostrocaudally, 1.6 to 3.5mm dorsally from the VMH (damaging the VMH dorsally), and 0.8 to 1.4mm laterally from the midline to the fornix (e.g., Fig. 4.1C). These lesions completely encompassed the DMH and dorsomedial area and produced substantial damage to the PVN, nucleus reunions, zona incerta, periventricular area, anterior hypothalamic area, tubercinereum area, arcuate nucleus, and dorsal tubero-mammilary nucleus (TMN, E4 subgroup). In the other 3 cases, the compact subregion of the DMH was absent, but some portions of the diffuse subregion were present ventrally and laterally. These lesions were estimated to be 79%, 70%, and 68% (Fig. 4.1E) complete.
4.3.2. **DMH ablation attenuates nocturnality during ad-libitum food access**

Inspection of activity waveforms and actograms (Fig. 4.2) revealed no differences in nocturnality or FAA between rats with total and partial DMH lesions, and thus data from all 6 lesion rats were combined for statistical comparisons. Activity data were averaged in blocks of 5 days during ad-libitum food access (1 block immediately prior to restricted feeding, 1 after restricted feeding) and restricted feeding (6 blocks), as well as in 1 block of 2 days and 1 block of 3 days during the first and second food deprivation tests, respectively. Mean daily activity levels did not differ by group during ad-libitum feeding, food restriction, or food deprivation (Fig. 4.3A). However, during ad-libitum food access prior to and following food restriction, the DMH lesion rats exhibited more activity during the daily light period, resulting in significantly decreased nocturnality ratios by comparison with intact rats (group means prior to food restriction = .59 ± .02% vs. .76 ± .02% nocturnal, respectively; \( t_{(10)} = 5.38, p < 0.001 \); Figs. 4.3B & 4.4A). We previously observed both less total activity and attenuated nocturnality in DMH-ablated rats (Landry et al., 2006). The difference in
total daily activity between our 2 studies is presumably related to the different cage configuration (no light-avoidance sleeping tube in the present study).

Figure 4.2. Actograms representing locomotor activity of intact rats (A-F) and dorsomedial hypothalamic nucleus (DMH)-ablated rats (G-L) subjected to restricted daytime feeding and total food deprivation.

Note. On each panel, each line represents 1 day, plotted in 10min bins from left to right. Time bins during which activity exceeded 5 counts are denoted by a heavy black bar. Days of total food deprivation are indicated by the heavy black bars to the left of each panel. “V” indicates start of total food deprivation and inverted “V” the end of food deprivation. Meal onset and end during the restricted-feeding schedule (food available hours 6-10 of lights-on) are denoted by vertical opaque white bars. The data for these plots were subjected to a 3-point running average with the center point weighted 4 times, for the purpose of identifying the onset of daily food-anticipatory activity. Panels G, I, and K are from rats with complete DMH lesions. I and J correspond to the histology of panels E-G and I-K, respectively.
Figure 4.3. Group mean activity data in dorsomedial hypothalamic nucleus (DMH) lesion rats (solid lines) and intact rats (dashed lines) averaged in 5-day blocks for ad-libitum food access (AdLib) and daytime restricted food access (RF1-6), as well as in 2 or 3-day blocks of total food deprivation (FD1 and FD2, respectively).

Note. For each panel, statistically significant within-group differences relative to the first block of ad-libitum feeding days (effect of feeding condition) are denoted by “a” (p < 0.025 for FD comparisons and p < 0.008 for RF comparisons). Significant between-group differences for each block (effect of lesion condition) are denoted by “b” (p < 0.025 for FD and AdLib comparisons and p < 0.008 for RF comparisons). F test results for each activity metric are as follows. (A) Total daily activity, within-group $F_{(2,33)} = 45.35$, p < 0.001; between-group $F_{(1,10)} = 0.17$, p = 0.69. (B) Nocturnality ratios (percentage of total daily activity occurring during lights-off), within-group $F_{(3,33.38)} = 63.25$, p < 0.001; between-group $F_{(1,10)} = 5.28$, p = 0.04. (C) Food-anticipatory activity (FAA) counts (number of activity counts during the 3h prior to mealtime), within-group $F_{(2,23.29)} = 32.83$, p < 0.001; between-group $F_{(1,10)} = 3.62$, p = 0.09. (D) FAA ratios (number of FAA counts divided by activity occurring during lights-off and the first 3h of lights-on), within-group $F_{(2.22, 28.18)} = 31$, p < 0.001; between-group $F_{(1,10)} = 5.74$, p = 0.04. (E) FAA ratios expressed as percent changes from baseline, using the procedure of Gooley et al. (2006; see text), within-group $F_{(2.82, 23.07)} = 24.79$, p < 0.001; between-group $F_{(1,10)} = 3.814$, p = 0.08.
4.3.3. **DMH ablation has no effect on food-anticipatory activity**

All rats began to exhibit activity in anticipation of mealtime within the first week of food restriction (Fig. 4.2). By days 6 to 15 (blocks 2-3) of restricted feeding, FAA in both groups was significantly elevated relative to the same hours during ad-libitum food access (Fig. 4.3C). The DMH lesion group exhibited more FAA counts than did the intact group, but this was consistent with the higher levels of daytime activity in these rats during ad-libitum food access and was statistically significant only for the first block of restricted feeding, after adjusting for multiple comparisons. FAA counts and ratios increased monotonically over the first 20 days of restricted feeding, reaching an apparent asymptote by days 21 to 25 (block 5; Fig. 4.3C, D). FAA ratios were significantly elevated relative to ad-libitum food access by block 1 of restricted feeding in the DMH lesion group and by block 2 in the intact group. Mean FAA duration over the entire 31 days of restricted feeding tended to be greater in the DMH lesion group (109 ± 10min vs. 83 ± 9min, \( t_{(10)} = 2.06, p = 0.068 \)). However, by the last 5-day block of restricted feeding, the duration (118 ± 12min vs. 101 ± 7min, \( t_{(10)} = 1.25, p = 0.24 \)), slope, and peak level of FAA were virtually identical in the lesion and intact groups (Fig. 4.4B). The precision of FAA onset, as represented by the standard deviation of FAA duration, also did not differ between groups (mean standard deviations for the DMH lesion and intact groups, respectively = .75 ± .05h vs. .77 ± .04h, \( t_{(10)} = .63, p = 0.61 \)).

Similar results were obtained for the total food deprivation tests. Both groups exhibited elevated activity prior to and during the usual mealtimes. This was particularly robust during the 2 days of food deprivation immediately following the daily feeding schedule (Fig. 4.4C) but was also evident during 3 days of deprivation after a 72h refeeding interval (Fig. 4.4D). FAA counts during food deprivation did not differ between groups, although the ratios were significantly higher in the lesion rats during the 72-h food deprivation test (Fig. 4.3C, D).

During food restriction, the DMH lesion rats ate on average 22.6 ± 3.6g of chow pellets, compared to 17 ± 3g by intact rats (\( t_{(10)} = 4.18, p = 0.0019 \)). Group differences in spillage are possible, but the DMH lesion rats also lost less weight during food restriction (8.9 ± 12.2g vs. 41 ± 7.9g, \( t_{(10)} = 2.19, p = 0.053 \)), suggesting that apparent differences in meal size were real. Larger meals could result from collateral PVN or VMH damage.
Figure 4.4. Group-average waveforms of locomotor activity in intact (dashed lines) and dorsomedial hypothalamic nucleus (DMH) lesion rats (solid lines)

Note. (A) the last 5 days of ad-libitum food access prior to restricted feeding, (B) the last 5 days of restricted daytime feeding, (C) 2 days of total food deprivation immediately following daytime feeding, and (D) 3 days of total food deprivation after 5 days of recovery ad-libitum food access. Plotting conventions as in Figure 4.1, except that raw data were not smoothed by running averages.

4.3.4. Effects of data transformation procedures on FAA ratios

The individual and group average waveforms of locomotor activity in intact and DMH-ablated rats are strikingly similar, providing no evidence for an effect of DMH ablation on the generation and persistence of FAA during daytime feeding and subsequent food deprivation tests, respectively. By contrast, Gooley et al. (2006) reported a 73% reduction of FAA in rats sustaining DMH damage
estimated to be 75% to 90% complete. In that study, FAA ratios were calculated and represented differently. First, activity during the 3h before mealtime was divided by total daily activity (including FAA and mealtime activity evoked by delivery and removal of food each day, excluded in our ratios). The resulting ratio for days 8 to 21 of restricted feeding was then represented as a percent change from the same ratio calculated for 2 weeks of ad-libitum food access prior to restricted feeding. By this formula, our DMH-ablated rats also appear to have a markedly reduced anticipatory response to restricted feeding (Fig. 4.3E). Calculations based on days 8 to 21 of restricted feeding reveal a 131% ± 28% increase of the FAA ratio in the intact rats but only a 77% ± 21% change over baseline in the DMH-ablated rats (independent \( t_{(10)} = 2.98, \ p = 0.014 \)). However, this difference between groups is entirely accounted for by the increased daytime activity exhibited by DMH-ablated rats prior to restricted feeding (quantified by nocturnality ratios and by activity counts during hours 4-6 of lights-on). Assuming a ceiling in the expression of premeal daytime activity (defined as the peak level expressed by intact rats), DMH-ablated rats start food restriction closer to the ceiling, creating the appearance of attenuated FAA when ratios are reported as a percent change from baseline (as in Fig. 4.3E). This data transformation as applied to our data has the effect of obscuring the striking similarity of FAA in intact and DMH-ablated rats evident by inspection of average waveforms.

### 4.3.5. Social cues do not stimulate FAA in naive food-deprived rats

In the DMH ablation experiment, lesion and intact rats were housed in separate cabinets, 6 per cabinet, 2 per shelf. To examine whether rats might exhibit increased activity prior to mealtime by responding to social cues from neighboring food-entrained rats, 12 intact rats were housed in 2 cabinets. In each cabinet, 2 rats were food entrained, and 4 were naive to feeding schedules. The 2 groups exhibited typical nocturnal activity during ad-libitum food access (Fig. 4.5A) and comparable increases of daytime activity during the first 72h food deprivation, when all rats were naive to scheduled feeding (Fig. 4.5B). During the food restriction schedule, the food-entrained group exhibited robust FAA (Fig. 4.5C) that persisted during food deprivation (Fig. 4.5D) and reappeared during a final 72h deprivation after 4 days of recovery feeding (Fig. 4.5E). The naive rats during each of the 4 food deprivation tests exhibited increased daytime activity that was indistinguishable from the response to the first food deprivation test (Fig. 4.5F), when no rats were food entrained. FAA counts and ratios calculated for the naive rats were much lower than for food-entrained rats and showed no significant differences across food deprivation tests (FAA counts \( F_{(3,31)} = 0.53, \ p = 0.61 \); ratios \( F_{(3,31)} = 0.49, \ p = 0.4 \); Fig. 4.6).
Figure 4.5. Group mean activity data in rats naive to daytime restricted feeding (solid lines) and rats entrained to a daytime meal (dashed lines).

Note. Data were averaged into 5-day blocks for ad-libitum food access (AdLib) and daytime restricted food access (RF1-4), as well as in 3-day blocks of total food deprivation (FD1-4). For each panel, there are 2 rows of labels for the x-axis indicating group feeding condition. The upper row refers to the restricted-feeding naive group and the lower row to the food-entrained group. Statistically significant within-group differences relative to the first block of ad-libitum feeding days (effect of feeding condition) are denoted by “a” (p < 0.0125), and significant between-group differences for each block (effect of lesion condition) are denoted by “b” (p < 0.0125). F test results for each activity metric are as follows. (A) Nocturnality ratios (percentage of total daily activity occurring during lights-off), within-group $F_{(8, 80)} = 21.31, p < 0.001$; between-group $F_{(1, 10)} = 18.13, p = 0.002$. (B) Food anticipatory activity (FAA) counts (number of activity counts during the 3h prior to mealtime), within-group $F_{(2.44, 24.4)} = 12.93, p < 0.001$; between-group $F_{(1, 10)} = 5.84, p = 0.036$. (C) FAA ratios (number of FAA counts divided by activity occurring during lights-off and the first 3h of lights-on), within-group $F_{(3.31, 33.17)} = 20.97, p < 0.001$; between-group $F_{(1, 10)} = 68.64, p < 0.001$. 
Figure 4.6. Group-average waveforms of locomotor activity in food-entrained rats (dashed lines) and in “naive” rats that were food deprived but not subjected to daytime restricted feeding (solid lines)

Note. (A) Ad-libitum food access prior to the restricted-feeding schedule; (B) 72h total food deprivation prior to restricted feeding (FD1); (C) naive group 72h deprivation, entrained group days 12 to 15 of daytime restricted feeding; (D) concurrent 72h food deprivation in both groups, immediately after the restricted-feeding schedule; (E) final 72h food deprivation in both groups, after 4 days of recovery feeding; and (F) first 3 food deprivation tests in the naive group (FD test 1 = gray fill, FD test 2 = fine line, FD test 3 = heavy line). For this panel only, the waveforms were subject to a 3-point running average to facilitate visual comparisons. Other plotting conventions as in Figure 4.3.

4.4. Discussion

In this study, we tested a hypothesis that small differences in the configuration of the test apparatus might account for the lack of consistency across laboratories in the reported effects of DMH ablation on FAA rhythms (Landry et al., 2006; Gooley et al., 2006). Although we attempted to closely duplicate the caging and feeding methods used by Gooley et al. (2006), we again found that rats with complete ablation of the DMH exhibited essentially normal FAA. Thus, in our hands, the DMH-ablated rat retains normal competency in the generation and persistence of food-anticipatory behavioral rhythms. DMH neurons are responsive to restricted-feeding schedules, exhibiting c-fos expression prior to mealtime in rats (Gooley et al., 2006; Verwey et al., 2007) and daily rhythms of mPer1 and mPer2 gene expression, with peak expression at mealtime, in mice (Mieda et al., 2006). Whatever the functional significance of this responsivity, our results rule out an interpretation that the DMH serves as a critical input pathway, pacemaker, or output pathway for food-entrainable behavioral rhythms. The DMH may play such a role for other food-shifted rhythms.
that were not measured in this study, such as body temperature, but additional studies will be needed to determine if an apparent role is also conditional on methodological factors.

We cannot yet explain the different results obtained by Gooley et al. (2006) and our laboratory, but we do believe the answer is methodological. Differences in data analysis (e.g., emphasis on ratios vs. percent change of ratios) and data display (e.g., actogram formatting) likely accentuate but do not fully explain the differences. Differences in motion sensors (overhead motion sensors vs. intraperitoneal transponders) might also contribute, but it is difficult to see how a large increase in premeal locomotor activity detected by 1 method would not also be detected by the other.

In addition to cage configuration and feeding method, we also explored a possible role for social cues as a means by which rats might “cheat” to anticipate a daily meal. In our studies, lesion and intact rats were housed in separate cabinets, but rats may nonetheless be able to hear movements and vocalizations, including ultrasonic, from neighboring rats. If so, then hungry rats that have never been exposed to a restricted daytime feeding schedule (“naïve” rats) might express food-anticipatory behavior by responding to sounds from food-entrained rats. If daytime activity did increase in hungry naive rats exposed to food-entrained rats, with a positive slope culminating in a peak at mealtime, then “cheating” (via “positive masking” effects of social stimuli) would have support as a viable hypothesis to explain FAA in our DMH-ablated rats. However, we found no evidence to support this hypothesis. Naive hungry rats did exhibit increased daytime activity, but the form and the magnitude of this increase were unrelated to the presence of food-entrained rats exhibiting robust FAA. Indeed, previous studies have shown that external cues (lights or tones) predictive of mealtime suppress rather than stimulate anticipatory activities in rats (Terman et al., 1984). Differential exposure to social cues predicting mealtime does not appear to be a viable explanation for inconsistencies across studies.

Another methodological factor that has not received attention in studies of circadian FAA is the effect of ambient temperature and thermoregulation on activity levels. We have noted anecdotally that wheel running and FAA are attenuated when room temperature is increased. Rats and mice subjected to caloric restriction (e.g., 66% of baseline caloric intake) exhibit increased daytime activity, but this effect is absent if room temperature is raised to thermoneutral (Gutierrez et al., 2002; Williams et al., 2002). It is conceivable that DMH damage alters thermoregulation (Dimicco and Zaretsky, 2007), thereby affecting the expression of FAA during restricted daytime.
feeding, depending on cage temperature. According to this hypothesis, differences between intact and lesion rats in the expression of FAA would disappear at certain cage temperatures. Small differences between studies in the site of the lesion or the ambient cabinet temperature might thereby account for large differences in FAA expression. Increased temperature during lights-on has been suggested to explain housing effects on FAA in C57BL/6J mice, which exhibit robust food-anticipatory wheel running when housed on open racks but almost none when housed in light-tight isolation boxes (de Groot and Rusak, 2004). Conceivably, increased cage temperature during lights-on in light-tight boxes, combined with lesion-induced changes in thermoregulation, may have contributed to the attenuation of FAA reported by Gooley et al. (2006). While speculative, this hypothesis merits attention in interpreting effects of conventional lesions and gene manipulations on FAA.

A third factor to consider as an explanation for differences across studies is the method and extent of the hypothalamic ablations. In the study of Gooley et al. (2006), intracerebral injection of the cell-specific neurotoxin ibotenic acid was used to kill neurons in the DMH region. This method has the advantage of sparing fibres of passage and thus improving the anatomical specificity of the lesion. The disadvantage of this method is that lesions are often incomplete, in part due to differential sensitivity of different neuronal populations to excitatory amino acid toxicity. Also, there may be diffusion of toxin outside of the target area, as well as selective loss of neurons not readily detectable without cell phenotyping by immunolabeling. In our studies, radiofrequency current was used to produce large nonspecific lesions. As we did not detect an effect of these lesions on FAA, the considerable damage sustained by other structures does not represent an interpretive issue. The advantage of the technique is that the lesion produces a large cavity, with very clear boundaries relative to easily identifiable landmarks (e.g., the fornix and mammillothalamic tracts laterally and dorsally, the VMH ventrally, the PVN rostrally, and mammillary nuclei caudally). These landmarks provided considerable confidence in our assessments of complete ablations. We did note that our lesions spared on at least 1 side some or all of the perifornical area lateral and dorsal to the fornix. This area contains many hypocretin neurons, ablation of which is associated with fragmented waking and, in mice, loss of hyperactivity in response to total food deprivation (Yamanaka et al., 2003). Ablation of hypocretin neurons does not block the generation of FAA but may attenuate its expression depending on the behavioral measure of anticipation (Akiyama et al., 2004; Mieda et al., 2004; Mistlberger et al., 2003). It is conceivable that ibotenic acid infusions directed at the DMH might kill a sufficient number of hypocretin neurons in the DMH and perifornical
region to reproduce this known attenuating effect of hypocretin deficiency on behavioral activation during food restriction. Differences in the expression of FAA following lesions directed at the DMH may thus be due to differential impact of the lesion methods on the hypocretin cell population.

Damage to histaminergic neurons in the TMN is also possible following neurotoxic lesions directed at the DMH. There is intriguing correlational evidence linking food anticipation with activation of histaminergic neurons in the dorsal E4 and ventrolateral E2 subregions (Inzunza et al., 2000; Valdes et al., 2005; Meynard et al., 2005). However, FAA was not affected by substantial destruction of E4 collateral to DMH ablation (Landry et al., 2006, and present study) or by selective lesions of E2 (unpublished observations). Thus, it is unlikely that differences between studies are due to the differential impact of DMH ablations on the TMN.

The results of this study confirm for the DMH a place on the list of hypothalamic structures that appear to be dispensable for the generation and persistence of food-anticipatory circadian behavioral rhythms in rats. Knockout of the mPer2 gene is associated with loss of FAA in mice (Feillet et al., 2006), and mismatches between FAA and clock gene rhythms in peripheral organs suggest that circadian oscillators driving FAA are in the brain (Davidson et al., 2003). Additional clock gene mapping and lesion studies are needed to elucidate the neural basis of the food-entrainable circadian system.
References


5. Food Entrainment: Methodological Issues


5.1. Discussion

The earliest studies of circadian activity rhythms in rodents identified food availability as an important synchronizing cue, but analysis of the role and neurobiology of food as a zeitgeber remained decidedly out of the spotlight for many years, to the extent that food garnered no mention in the Cold Spring Harbour Biological Clocks symposium proceedings of 1960 and but a single line in a recent, otherwise highly commendable, textbook of chronobiology (Dunlap et al., 2004). With the recent discovery that virtually all organs and many brain regions exhibit circadian rhythms of clock gene expression and that feeding time is the dominant zeitgeber for these rhythms, the molecular and neural biology of food entrainment is now attracting widespread attention. To elucidate the biology of any process (e.g., entrainment), it is critical to remain clear on the phenomenology that one seeks to explain. We therefore welcome the opportunity to exchange commentaries with Gooley and Saper (2007 [this issue]) on the measurement and interpretation of brain lesion effects on food entrainment. Contrary to Gooley et al. (2006), we find that ablation of the dorsomedial hypothalamus (DMH) in rats does not affect entrainment of behavioral rhythms by daily feeding schedules. In their commentary, Gooley and Saper argue that “feeding-related” behaviors (including wheel running and activity directed at feeding locations) are not appropriate measures of food entrainment because such behaviors are affected by hourglass processes unrelated to oscillator entrainment. Gooley and Saper also comment on our discussion of quantitative procedures, lesion techniques, and lesion specificity. We respond to these comments in turn.

5.1.1. What is the appropriate measure of food entrainment?

Nocturnal rats restricted to a single daily meal provided in the middle of the light period exhibit increased activity prior to mealtime. This can be readily detected in wheel-running behavior, activity
directed at a food bin, operant lever pressing, or even general cage activity measured by tilt floors, infrared motion sensors, or changes in strength (position) of a radiofrequency signal from an intraperitoneal transmitter. This bout of food-anticipatory activity (FAA) typically emerges within a few days and stabilizes within 1 to 3 weeks, at which time it begins ~1 to 3h before mealtime (depending on the behavior) and rises steeply to a peak at mealtime. If food is withheld, the peak level of FAA plateaus for the usual duration of mealtime and then declines to low levels until the night. The next day, FAA reappears at its usual time and with its usual waveform, and this will be repeated for 3 to 5 days without food. This repetition defines a true rhythm, not an hourglass process (i.e., a timer that has to be reset each day, e.g., by food delivery). Indeed, careful studies of FAA in rats under different feeding schedules have revealed canonical properties of circadian clock regulation, including circadian limits to entrainment (no anticipation to meals at intervals < 22h or > 31h), phase angle dependence on the period (T) of the feeding schedule, aftereffects of T on FAA period during food deprivation, and transients (gradual shifting) in response to a shift of mealtime (reviewed in Boulos and Terman, 1980; Aschoff, 1986; Mistlberger, 1994; Mistlberger and Marchant, 1995; Stephan, 2002). To explain these rhythmic properties, the concept of a food-entrainable pacemaker (oscillator or clock) was invoked (Boulos and Terman, 1980). The great majority of these experiments used wheel running as the measure of FAA, although food bin activity produces parallel results.

Gooley and Saper (2007) now argue that such behaviors are inappropriate measures of food entrainment because they reflect hourglass processes (i.e., these behaviors are claimed to increase nonspecifically during caloric deprivation, creating the appearance of clock-controlled anticipation where in fact there may be none). To accept this argument, we have to ignore several things. First, we have to ignore the entire behavioral literature on which the concept of food-entrainable oscillators was built, which long ago invalidated hourglass clocks as a plausible mechanism for FAA. Indeed, when rats are food deprived for 54h prior to their first midday meal, they show no increase in wheel running (see Mistlberger, 1994, Fig. 1). Daytime activity does not begin to appear until prolonged deprivation, and the pattern of increase is different (e.g., Challet et al., 1996). Second, we have to ignore the properties of FAA displayed by DMH-ablated rats in Landry et al. (2006, 2007 [this issue]). In these rats, FAA rises until scheduled mealtime and then declines if food is not provided, and this is repeated on subsequent deprivation days. This fails the first criterion for an hourglass clock. Third, we have to ignore the results of the second experiment in the present study, in which we show clearly that rats that have never been exposed to restricted daytime feeding
schedules do not show a pattern of activity reminiscent of FAA during 72h of total food deprivation, even when exposed to sounds of other rats anticipating a midday meal. If the hourglass clock argument had any merit, these rats should show a marked peak of activity in the middle of the day during total food deprivation. They do not.

Gooley and Saper (2007) state that our DMH-ablated rats exhibit food anticipation on the first day of restricted feeding, before their first meal, and that this is consistent with an hourglass interpretation of anticipation. This is misleading because our DMH-ablated rats (consistent with Chou et al., 2003) exhibit reduced nocturnality prior to food restriction, and thus some daytime activity should also be present on the first day of food restriction. An entrainment process driving FAA is manifest by the gradual intensification of this activity over days, the marked positive slope of the activity waveform toward mealtime, and persistence of the FAA rhythm during total food deprivation.

Gooley and Saper (2007) further state that our motion sensor was placed near the food hopper and they imply that we therefore measured “feeding-related” behavior, whereas their motion sensors (an implanted transmitter) somehow do not measure feeding-related movements. This is misleading on both counts. As stated in our article, our motion sensors were positioned directly over the middle of the cage and detected activity anywhere in the cage. The telemetry motion sensors would do the same thing. The 2 studies used the same cage types, with food in the same place on top of the cage (we appreciate Gooley’s personal communication of this information prior to our study). FAA should be detected by either measure. The primary difference between the 2 sensors is that implanted transmitters will register movements such as grooming or postural adjustments during rest. These constitute background noise, which can obscure the “signal” of interest, because movements unrelated to food anticipation are lumped in with movements that may be food anticipatory, and the 2 types of movements are mutually exclusive. Our motion sensors are not sensitive to such small movements and hence serve to filter out this noise. Thus, as a general principle, implanted sensors are the least appropriate measures for detecting food-entrained behavioral rhythms. We believe that this does contribute to the low levels of FAA in Gooley et al.’s (2006) DMH lesion rats, but we assume that other factors are also involved (see below).

5.1.2. What are valid conceptual models of food-anticipatory activity?

For the reasons outlined above, the hourglass clock is not a valid concept to explain the properties of FAA observed in wheel running or the locomotor activity measures used in our 2 DMH
ablation experiments. Gooley and Saper (2007) argue that FAA evident in our DMH-ablated rats could also be due to associative learning, whereby rats would learn that food is available 6h after lights turn on. This idea is unsubstantiated by evidence from either the chronobiology or the animal learning literature. In fact, rats provided with external cues that precede feeding time by 2h or 4h exhibit less, not more, FAA (Terman et al., 1984). FAA does not require an LD cycle and, once established, does not shift in response to a 4h phase advance of LD, as demonstrated in a study of FAA in rats with paraventricular nucleus lesions (Mistlberger and Rusak, 1988). In the present study, FAA in intact and DMH-ablated rats did not even begin until ~3h after lights-on. Thus, there is no evidence that rats use light onset and interval timing to predict mealtime 6h later. More generally, given that FAA in our intact and DMH-ablated rats was virtually identical, as revealed by the average waveforms, it is implausible to ascribe anticipation to very different mechanisms (e.g., food entrainment vs. associated learning/interval timing) in the 2 groups.

5.1.3. **What is a valid quantitative metric of food anticipation?**

We directly compared our metric of FAA with Gooley et al.’s (2006) metric. Contrary to what they state in their commentary, we did not conclude that their data transformation fully explained the low levels of FAA in their rats. Rather, we stated that it likely contributed but did not fully explain their results. Gooley and Saper’s (2007) reanalysis of their data with our metric confirms that conclusion. These comparative analyses do serve to illustrate the importance of the metric. Using our method, the quantitative result matches the average waveforms. Using their method, the quantitative and graphical descriptions are disconnected, and we explained why. Is there one best metric? Probably not, but a bad metric would be one that relies on comparisons with baseline, where baselines differ between groups in critical ways. Although Gooley and Saper state that their DMH-ablated rats did not show significantly attenuated rhythms (nocturnality) in LD during ad-libitum food access (statistically, only a trend for reduced nocturnality in their rats), our rats did, and thus the metric that we used was appropriate for our data.

5.1.4. **What is the appropriate lesion technique?**

Gooley et al. (2006) used ibotenic acid to kill cells and spare fibres of passage, whereas we used radiofrequency current to ablate all cells and fibres. The advantage of our technique is that we were able to make complete ablations, whereas with ibotenic acid, some cells were spared. If we had observed a significant effect with radiofrequency lesions, then a logical next step would have been a neurotoxin that spares fibres of passage. Making partial lesions with neurotoxins that are
selectively effective is a potentially wasteful strategy, if results are negative. This is of particular concern in studies of circadian timekeeping, given observations that lesions of light-entrainable pacemakers do not eliminate and may marginally affect circadian parameters unless most of the tissue is removed (e.g., Block et al., 1995; Harrington et al., 1993). This is directly contrary to reports by Gooley et al. (2006) and Chou et al. (2003) of highly significant linear correlations between numbers of DMH cells spared and the amplitude of food-anticipatory and free-running rhythms, respectively. Such strong linear correlations between cell numbers and function are unusual in neural systems, which more often display marked nonlinearity (e.g., dopamine neuron loss and movement disorders; cholinergic cell loss and cognitive dysfunction).

5.1.5. The role of the hypothalamus in food entrainment

We believe that the very weak behavioral anticipation evident in Gooley et al.’s (2006) DMH lesion rats is due to a combination of factors. First, the measurement tool is too blunt and conflates incompatible behaviors, some that increase prior to mealtime (locomotion) and some that decrease (grooming, postural adjustments during rest). Second, the cage configuration does not give the rat opportunity to display intact function (see Landry et al., 2006, for discussion of how this, as well as cage temperature, may affect expression of FAA). Third, the neurotoxic lesions may have damaged a greater proportion of hypocretin/orexin cells than were killed by our radiofrequency lesions. Hypocretin/orexin ablation fragments sleep-wake and reduces FAA in blunt measures of activity (Akiyama et al., 2004; Mieda et al., 2004) but not in spatially specific measures of activity, such as the ones we used to examine the role of this cell type in FAA (Mistlberger et al., 2003). Gooley et al. (2006) did not count hypocretin/orexin cells, and such counts may have revealed even better correlations with their measure of FAA. We thus agree with their call for “rigorous quantitative assessment of differential cell loss in specific cell groups,” in those experiments where a lesion effect is reported.

Strong evidence is accumulating that the DMH is not the site of oscillators critical for food anticipation. Another recent study has shown that FAA is not disrupted in mice with DMH ablations (Moriya et al., in press). Although DMH neurons exhibit a circadian rhythm of Per2 expression during restricted feeding schedules in mice (Mieda et al., 2006; Moriya et al., in press) and rats (Verwey et al., 2007), the rat study further revealed that this was dependent on food deprivation and was independent of food anticipation. Thus, neither the DMH nor DMH Per2 expression is necessary for FAA. It will be important to thoroughly characterize, at multiple time points, the
circadian rhythm of DMH Per2 expression during total food deprivation in rats naive to restricted daytime feeding schedules.

A synthesis of the available evidence is that the hypothalamus modulates the expression of food-anticipatory behavioral rhythms; it must, given its critical role regulating energy input and output. Leptin, acting on NPY neurons in the arcuate nucleus, may be an inhibitory factor responsible for attenuated FAA in diet-induced obese rats (Mistlberger and Antle, 1999; Mistlberger and Marchant, 1999; Persons et al., 1993). Hypocretin/orexin promotes arousal and consolidated wakefulness in response to energy depletion (Beuckmann et al., 2004). The DMH is activated in association with food deprivation, and although not required for FAA, this may be important for regulation of autonomic effectors for body temperature and endocrine rhythms, such as corticosterone. This needs confirmation. Entrainment of behavioral and autonomic rhythms by feeding likely involves a distributed population of circadian clock cells, with different sites contributing to different rhythms and multiple sites contributing to behavioral rhythms. We urge increased sophistication in behavioral analysis of food-anticipatory rhythms.
References


6. Phenotyping Food Entrainment: Motion Sensors and Telemetry Are Equivalent


Abstract

Rats can anticipate a daily meal by entrainment of a circadian timekeeping mechanism that is anatomically separate from the light-entrainable circadian pacemaker located in the suprachiasmatic nucleus. The dorsomedial nucleus of the hypothalamus (DMH) has been claimed to be critical for the expression of circadian rhythms of food anticipatory activity, but efforts to confirm this finding have so far failed. Failure to confirm that DMH ablation disrupts or eliminates food anticipatory rhythms has been attributed to the use of overhead motion sensors rather than telemetry to measure locomotor activity. To examine the relationship between motion sensor and telemetric measures of locomotor activity, transponders were implanted into the peritoneal cavity of adult male rats, and activity was recorded continuously by both telemetry and infrared motion sensors. Activity counts were ~4 fold higher as detected by telemetry, but normalized activity patterns were virtually identical for the two measures during ad-lib food access, 4 h/day food restriction and total food deprivation after food restriction. Overhead motion sensors and telemetry are equivalent measures of food anticipatory activity in rats. Telemetry is an effective tool for continuous recording of body temperature but has no advantages over infrared motion sensors for measuring food anticipatory activity rhythms.
6.1. Discussion

Rats, mice, and a variety of other species can anticipate a daily meal scheduled at a fixed time each day. In rats, anticipation is based on a circadian timing mechanism. Evidence for this conclusion includes persistence under free-running conditions (absence of food for several days), the presence of circadian limits to food entrainment, and the presence of transients during phase shifts (Boulos and Terman, 1980; Aschoff, 1986; Mistlberger, 1994; Stephan, 2002). These circadian properties have been demonstrated in rats using wheel running, lever pressing, and infrared photosensors at the window to a food bin. Anticipation can also be detected in sleep-wake states measured polygraphically, and in general cage activity detected by light beams, tilt floors, infrared motion sensors, and implanted radiofrequency transmitters, in rats and mice housed in a barren cage. However, the magnitude and precision of food anticipatory rhythms in measures of activity obtained under such housing conditions are often poor by contrast with measures obtained in more enriched environments. Therefore, enriched environments (wheels, levers, feeding windows) that provide the opportunity to express foraging-like behaviors have been recommended for this type of work.

Food anticipatory rhythms persist in rats, mice, and hamsters with complete ablation of the suprachiasmatic nucleus, the site of the circadian pacemaker that generates light-entrainable circadian rhythms (Boulos et al., 1980; Stephan et al., 1979). It has recently been reported that partial neurotoxic lesions of the dorsomedial hypothalamic nucleus (DMH) severely attenuate or eliminate food anticipatory rhythms of general activity and body temperature measured by telemetry (intraperitoneal transmitters) in rats housed in barren cages (Gooley et al., 2006).

We attempted to confirm these results by making complete radiofrequency lesions of the DMH and testing rats in a cage “enriched” with an opaque sleeping tube and an external food bin accessible via a window (Landry et al., 2006). Despite completely destroying the DMH, food anticipatory rhythms were normal. In addition to the differences in the nature of the lesions, there were 2 other methodological differences between the studies. One was in the feeding and housing conditions. To examine the impact of feeding and housing differences, we repeated the experiment using the same feeding procedures and barren housing as in Gooley et al. (2006), and again could detect no differences among rats with complete DMH lesions, partial lesions, or no lesions (Landry et al., 2007).
The other experimental difference lay in the method for assessing rhythmic activity. We measured activity using a Quorum RR-150 infrared motion sensor (Quorum International Ltd) placed 25cm above the cage. The earlier study measured activity using implanted transponders. Saper and colleagues have suggested that our motion sensors are detecting foraging behavior, and that foraging behavior reflects homeostatic factors that could create the impression of a food anticipation rhythm in rats lacking a food-entrainable circadian clock (Gooley and Saper, 2007, but see Landry and Mistlberger, 2007). They have argued that telemetry, by contrast, yields an activity measure that provides a more pure assessment of food-entrainable circadian time keeping. More recently, this argument has also been used to parry criticism of the results underlying their recent claim that mice homozygous for a null mutation of the clock gene Bma11 fail to anticipate a daily meal, but can do so after retrovirally mediated restoration of Bma11 expression specific to the DMH (Fuller et al., 2008a, 2008b; Mistlberger et al., 2008). To test directly whether motion sensors obscure results revealed by telemetry, we compared food anticipatory activity measured by motion sensors and telemetry in the same rats.

Adult male Sprague-Dawley rats (n = 16, 480-520g; Charles River, Montreal, QC, Canada) received intraperitoneal radiofrequency transponder (ER-4000, Mini Mitter, Inc., Sunriver, OR) implants via laparotomy, using ketamine (90 mg/kg), xylazine (9 mg/kg), and isoflurane (0.5% to 2.0%) for anesthesia. Eleven of the rats also received 0.5µL injections of PBS into the DMH bilaterally, using stereotaxic procedures. After 7 to 10 days of recovery, all rats were housed in standard clear plastic cages (45 × 24 × 20cm) with metal tops (empty of wheels, levers, or feeding windows and thus expected to provide less activity than do environmentally enriched cages), in cabinets with controlled lighting (LD 12:12). The cages were placed on top of ER-4000 receivers interfaced with a microcomputer running VitalView data acquisition software (Mini Mitter Inc.). A Quorum RR-150 infrared motion sensor was hung 25cm over the center of the cage, which detects locomotion anywhere in the cage.

The sensors were interfaced with another computer running ClockLab data acquisition software (Actimetrics, Inc., Evanston, IL). After 5 days of ad-libitum food access, the rats were food deprived for 30h, starting at light onset (zeitgeber time, ZT0), and then provided with food (PMI Lab Diet 5001) for 4h per day at ZT6, for 21 days. Food pellets were placed on the cage top, as in Gooley et al. (2006). The rats were then food deprived for 50h. Activity and temperature data were examined using ClockLab and exported to Circadia (Dr. T.A. Houpt, Florida State University,
Tallahassee, FL) and Prism (Graphpad Software, Inc., San Diego, CA) for plotting in “actogram” (Fig. 6.1) and average wave (Fig. 6.2) formats.

Figure 6.1.  Group mean (n = 16) activity patterns from rats recorded by (A) overhead infrared motion sensors and (B) intraperitoneal transponders.

Note. Each line represents 24h, plotted from left to right in 10min time bins. Activity counts were normalized using the daily mean. Time bins with activity above the mean are denoted by heavy bars. * denotes removal of food prior to daily feeding from ZT6 to ZT10. Feeding time is outlined by a box. AL = ad-libitum food access; FD = food deprivation; RF = food restriction, 4h per day.
Figure 6.2. Group mean (n = 16) average waveforms of activity measured by overhead infrared motion sensors (thin lines, 5 day average) and intraperitoneal transponders (heavy lines) during ad-libitum food access (top panel), 4h per day food access (middle panel; averaged across restricted feeding days 15-21), and total food deprivation (bottom panel; day 2 of food deprivation after 21 days of restricted feeding).

Note. Shading indicates lights-off. Dotted line indicates mealtime during restricted feeding (middle panel) or prior mealtime during total food deprivation (bottom panel).

The implanted transponders registered on average ~1000 activity counts per day, whereas the motion sensors registered on average ~225 counts per day (the motion sensors have a 10-sec delay after each trigger). When the data were normalized (using the daily mean), the 2 measures of activity produced virtually identical patterns during ad-libitum food access, restricted
feeding, and food deprivation. Thus, telemetry and overhead motion sensors positioned above an open cage are equivalent measures of food anticipation in rats. This outcome is not at all surprising, given that locomotion sufficient to trigger an over-head motion sensor will also be registered by an intraperitoneal transmitter. We conclude with 2 practical points. First, telemetry is not required to study food anticipatory activity. Furthermore, it is more expensive and more invasive than motion sensors. Second, while motion sensors may be adequate to detect food anticipation in barren cages, we believe that control of behavior by a food-entrainable clock evolved to facilitate optimal foraging. Therefore, enriched environments that provide rats and mice with opportunities to express food-seeking behavior may provide more sensitive screens to phenotype this circadian function.
References


7. Evidence for Time-of-Day Dependent Effect of Neurotoxic Dorsomedial Hypothalamic Lesions on Food Anticipatory Circadian Rhythms in Rats

Landry GJ, Kent BA, Patton DF, Jaholkowski M, Marchant EG, & Mistlberger RE (2011) Evidence for time-of-day dependent effect of neurotoxic dorsomedial hypothalamic lesions of food anticipatory circadian rhythms in rats. PLoS ONE 6(9):e24187. (GJL designed the experiment, performed surgeries, collected and analysed data, and wrote the manuscript.)

Abstract

The dorsomedial hypothalamus (DMH) is a site of circadian clock gene and immediate early gene expression inducible by daytime restricted feeding schedules that entrain food anticipatory circadian rhythms in rats and mice. The role of the DMH in the expression of anticipatory rhythms has been evaluated using different lesion methods. Partial lesions created with the neurotoxin ibotenic acid (IBO) have been reported to attenuate food anticipatory rhythms, while complete lesions made with radiofrequency current leave anticipatory rhythms largely intact. We tested a hypothesis that the DMH and fibres of passage spared by IBO lesions play a time-of-day dependent role in the expression of food anticipatory rhythms. Rats received intra-DMH microinjections of IBO and activity and body temperature ($T_b$) rhythms were recorded by telemetry during ad-lib food access, total food deprivation and scheduled feeding, with food provided for 4h/day for 20 days in the middle of the light period and then for 20 days late in the dark period. During ad-lib food access, rats with DMH lesions exhibited a lower amplitude and mean level of light-dark entrained activity and $T_b$ rhythms. During the daytime feeding schedule, all rats exhibited food anticipatory activity and $T_b$ rhythms that persisted during 2 days without food in constant dark. In some rats with partial or total DMH ablation, the magnitude of the anticipatory rhythm was weak relative to most intact rats. When mealtime was shifted to the late night, the magnitude of the food anticipatory activity rhythms in these cases was restored to levels characteristic of intact rats. These results confirm that rats can anticipate scheduled daytime or nighttime meals without the DMH. Improved anticipation at night suggests a modulatory role for the DMH in the expression of food anticipatory activity rhythms during the daily light period, when nocturnal rodents normally sleep.
7.1. Introduction

When food is freely available, daily rhythms of foraging, food intake and physiology are synchronized to the solar day by entrainment of a retinorecipient master circadian pacemaker, the hypothalamic suprachiasmatic nucleus (SCN) [1]. The SCN of nocturnal rodents confers circadian organization by actively promoting arousal at night and rest during the day [2, 3], and by coordinating the phase of circadian clocks in peripheral organs via neural, hormonal and behavioral signals [4]. Under standard laboratory conditions, the timing of these rhythms relative to the daily light-dark (LD) cycle is typically stable, but can be markedly altered if food is restricted to the ‘wrong’ time of day. Nocturnal rats and mice fed only in the middle of the light period, when sleep normally predominates, exhibit an inversion of the phase of circadian clocks in most peripheral organs, and the emergence of a food-anticipatory activity rhythm, evident in general activity, wheel running or operant behaviors such as lever pressing and food-bin approaches [5-7]. During such daytime restricted feeding schedules, the SCN pacemaker does not invert its phase [8-10]. Moreover, while ablation of the SCN eliminates daily rhythms in rats with free access to food, it does not affect food anticipatory behavioral and physiological rhythms, which emerge if food is temporally restricted, and then persist during total food deprivation tests lasting several days [11-13]. These and other properties indicate that food anticipatory behavioral rhythms are controlled by a circadian mechanism outside of the SCN that can override signals from the SCN that normally suppress daytime activity and arousal. The mechanism has been conceptualized as a food-entrainable oscillator or pacemaker, analogous to the light-entrainable SCN pacemaker [5-7].

Two questions that arise in neurobiological analysis of food anticipatory rhythms are where in the brain (or body) are the driving food-entrainable circadian oscillators located, and how do these overcome sleep promoting signals from the SCN to induce arousal in the usual sleep phase? Numerous brain regions exhibit daily rhythms of immediate early gene or clock gene expression that are shifted or induced by daily feeding schedules [14]. One structure in which this occurs is the dorsomedial hypothalamus (DMH) [15-22]. Partial lesions of the DMH created by local infusion of the excitatory neurotoxin ibotenic acid were reported to markedly attenuate food anticipatory rhythms of activity, sleep wake and body temperature ($T_b$) rhythms in rats, supporting a conclusion that the DMH is critical for the expression of these rhythms [16]. However, efforts to support this conclusion using electrolytic and radiofrequency lesion techniques have met with failure, as rats and mice sustaining substantial or complete ablation of DMH cells and fibres of passage through
this area were found to exhibit near normal food anticipatory rhythms of activity, body temperature and clock gene rhythms in other brain regions [22-24]. Subsequent studies ruled out several procedural variables as possible explanations for the differing results, including cage configuration, method of feeding and measure of activity [24, 25].

Circumstantial evidence suggests that the lesion method might be important. SCN outputs to sleep-wake regulatory circuits project in part either directly or indirectly to and through the DMH area [2, 26-28]. The SCN receives or is surrounded by fibres from hypothalamic structures that process feeding and metabolism related signals, including the DMH [29-30] and arcuate nucleus [31]. Expression of the immediate early gene c-Fos (a marker of neural activity) or the clock gene Per1 in the SCN can be inhibited by behavioral arousal and some metabolic signals [31-33]. These observations have been connected in the following way [22, 30, 34]. During restricted daytime feeding schedules, SCN sleep promoting outputs, conveyed in part by direct or indirect projections through the DMH area, might be directly inhibited by inputs from nutrient sensitive structures such as the DMH that are induced to oscillate in phase with a scheduled mealtime. DMH lesions made by neurotoxins such as ibotenic acid [16] would eliminate neurons responsible for inhibiting SCN output, and would spare SCN fibres of passage through the DMH area. These SCN outputs would then suppress food anticipatory activity without opposition. By contrast, DMH lesions made by radiofrequency current [22-24] would destroy not only DMH neurons inhibitory to the SCN, but also SCN outputs that project through and around the DMH area. This would allow food anticipatory activity to be expressed without opposition from the SCN, assuming that food-entrainable oscillators that drive these rhythms are located in whole or in part outside of the DMH.

This model leads to two predictions. If SCN outputs are responsible for attenuation of food-anticipatory rhythms in rats with ibotenic acid-induced DMH lesions, then food-anticipatory rhythms in these rats should be restored by 1. SCN ablation, or 2. scheduling mealtime at night, when the SCN does not inhibit activity. A recent study has confirmed the first prediction; rats with attenuated daytime food anticipatory rhythms following DMH ablation exhibited robust food anticipatory rhythms when the SCN were subsequently ablated [30]. In the present study, we followed closely the recording and ablation methods of Gooley et al [16] and provide evidence consistent with the second prediction; anticipation of a daytime meal was weak in some rats with ibotenic acid-induced DMH lesions, and was restored to levels characteristic of DMH-intact rats when the scheduled mealtime was shifted to late in the night.
7.2. Materials and methods

7.2.1. Animals and recording apparatus

All animal work was conducted according to guidelines established by the Canadian Council on Animal Care and was approved by the University Animal Care Committee at Simon Fraser University (permit number 732P95). Young adult male Sprague Dawley rats (N = 29, Charles River, Montreal, Canada) were housed in group cages under a 12:12h light-dark (LD) cycle in a climate controlled vivarium (22 ± 1°C). The rats then underwent surgical procedures to create a neurotoxic or sham DMH lesion and implant a calibrated radiotelemetry transponder (ER-4000, Minimiter Inc., OR, USA). After recovery, the rats were housed individually in standard plastic cages (45 x 24 x 20cm) equipped with a food hopper and water bottle holder, as in Gooley et al [16] and Landry et al [24]. Each cage was placed on top of an ER-4000 radiotelemetry receiver, housed inside individual sound-attenuating recording chambers with controlled lighting and an exhaust fan (Lafayette Instruments, IN, USA). Radiotelemetry signals were converted by the VitalView data acquisition system (Minimiter, Inc.) into pulses at a rate proportional to $T_b$. Changes in signal strength caused by movement of the rat were converted to pulses proportional to locomotor activity. Pulses were summed and stored in 1 minute time bins.

7.2.2. Surgical procedures

Rats were anesthetized for stereotaxic surgery using ketamine (90 mg/kg), xylazine (9 mg/kg), and isoflurane (0.5% to 2.0%). Following Gooley et al [16], ibotenic acid (Sigma) was dissolved in phosphate buffered saline (PBS) at a concentration of 10%, using 6N NaOH, with pH adjusted to ~7.2 using 6N HCl. In pilot tests, concentrations of 1-2% produced very little DMH damage, while infusions into the hippocampus caused extensive cell loss, demonstrating bioactivity of the neurotoxin at these concentrations. At the 10% concentration, bilateral infusions into the DMH were associated with immediate and severe respiratory depression. A two-stage lesion procedure was therefore adopted, with ~4 weeks between unilateral 300nL (N = 8 rats) or 100nL (N = 6 rats) infusions delivered by Hamilton syringe, using the following stereotaxic coordinates relative to bregma (10° angle): 3.4mm posterior, ± 2.2mm lateral from midline, -7.7mm ventral from dura. Seven additional rats received vehicle injections, and 8 rats served as unoperated controls. Radiotelemetry transponders were implanted ~7 days after the second stereotaxic surgery.
7.2.3. Feeding schedules

Continuous recording of activity and $T_b$ in the temporal isolation chambers began 2 weeks after the transponder implants. The rats received food (rodent chow pellets, 5001) ad-libitum in LD 12:12h for 15 days, with one day in constant dark (DD; day 10). Food was removed for 42h, and then provided for 4h daily, beginning 6h after lights-on (zeitgeber time 6, where ZT0 is defined as lights-on, by convention). After 20 days, the lights were turned off for 3 days of DD. Food was provided at the usual time on the first DD day, and was removed for the final 50h. Food was then provided ad-libitum for 19 days, removed for 38h and then provided for 4h each night, beginning 9h after lights-off (ZT21). The lights were turned off for 3 days, a last meal was provided at ZT21, and the rats were then food deprived for the last 60h of constant dark. Food was then available ad-libitum for 5 days, removed for 54h, and then provided for 4h each day at ZT6, for 9 days. The rats then received an overdose of pentobarbitol (Euthanol) and were processed for histological analysis of the lesions.

7.2.4. Histological analysis

The rats were perfused transcardially with 50ml of 0.1 M phosphate buffered saline (PBS, pH ~7.3) followed by 50ml of 4% paraformaldehyde in PBS (PFA, pH ~7.3). Brains were removed, postfixed in PFA, cryoprotected in 20% sucrose overnight, and then frozen sectioned at 40µm in a cryostat for histological confirmation of the lesion site. Sections were mounted on slides and stained for Nissl with cresyl violet. Brain sections were evaluated using a Nikon Eclipse 80i light microscope. The extent of the lesions was assessed based on loss of neurons and presence of gliosis. Templates of the DMH were created based on the Paxinos and Watson rat brain atlas and published photomicrographs [29, 35]. The DMH area was divided into quadrants, with each box representing 12.5% of total DMH area per section. The amount of undamaged DMH tissue in each box was estimated as a percentage of volume.

7.2.5. Activity and $T_b$ analyses

Activity and $T_b$ data were evaluated in the form of raster plots displaying all days and waveforms averaged over blocks of days using Clocklab (Actimetrics, Inc., IL), Circadia (Dr. T. A. Houpt, Florida State U, USA), and Prism (Graphpad Software, Inc., San Diego) software. Food anticipation was quantified by expressing activity during the 3h prior to mealtime (ZT3-6) as a ratio relative to total daily activity [16] or to activity from ZT12-ZT3 [24]. The two methods produced
equivalent results, therefore only the latter is presented here. The amplitude of the day-night rhythm of activity was expressed as a nocturnality ratio by dividing 12h nighttime activity by the 24h daily total. The amplitude of the Tₙ rhythm was expressed as a difference between the mean at night and the mean in the day. Group means of activity and Tₙ data were calculated separately for the high IBO dose rats, the low IBO dose rats, and the DMH intact control groups (unlesioned and sham operated rats combined). Group differences were evaluated statistically using 2-way repeated measures ANOVA (Prism 5.0, Graphpad Software) and planned t-tests.

7.3. Results

7.3.1. DMH lesions

The hypothalamus of rats that received IBO infusions presented with an expanded 3rd ventricle and loss of identifiable DMH tissue ranging from minor (estimated to be = < 15%, e.g., Fig. 7.1B) to severe (estimated to be 100%; e.g., Fig. 7.1C). In the most severe case (Fig. 7.1C), the 3rd ventricle extended laterally to within ~200µm of the fornix, dorsally to the mammilothalamic tract, and ventrally to the arcuate nucleus. The ventromedial hypothalamus was absent on one side and reduced by 50% contralaterally. The medial 50% of the arcuate was also destroyed. Tissue surrounding the border of the lesion was heavily gliosed. The large size of the lesion cavity likely reflects the extended post-lesion survival time, sufficient to permit removal of cellular debris. Fibres of passage spared by the neurotoxin presumably run through intact and gliosed tissue surrounding the expanded 3rd ventricle. Most of the other rats receiving the 300nl volume injections also exhibited extensive damage to the DMH, but in each case some of the DMH pars compacta could be identified at least unilaterally in at least 2 sections, and total surviving DMH volume was estimated to range from 20% to 80% across rats. Rats receiving the 100nl volume injections showed much smaller lesions, characterized by some expansion of the 3rd ventricle, and damage estimated to be 20% or less.
Figure 7.1. **Digital images of 40µm cresyl violet stained coronal brain sections through the hypothalamus at two levels of the dorsomedial hypothalamic nucleus (DMH)**

![Figure 7.1](image)

Note. A-C: ~2.9mm posterior to bregma (Paxinos and Watson, 2009). D-F: ~3.24mm posterior to bregma. (A, D). An intact rat. (B, E). A rat sustaining minor (<20%) DMH damage. (C, F). The rat sustaining the largest lesion, with complete ablation of the DMH, including the DMH pars compacta (PC), and partial damage to the ventromedial hypothalamic nucleus (VMH) and the arcuate nucleus (ARC).

### 7.3.2. Activity during ad-lib food access and food deprivation

Based on group differences in lesion size, group means were calculated separately for rats receiving high-dose and low-dose IBO lesions. Raster plots and average waveforms of activity data from 4 rats are illustrated in Figure 7.2, to represent an intact rat with a strong food anticipatory activity rhythm (Fig. 7.2A), and the rat from each of the three groups that had the lowest magnitude daytime food anticipatory rhythms, based on food anticipation ratios (control rat, Fig. 7.2B; low-dose IBO rat with ~85% DMH intact, Fig. 7.2C; high-dose IBO rat with no DMH detectable, Fig. 7.2D.). Group average waveforms for each feeding condition are illustrated in Figure 7.3.
Figure 7.2. Activity and body temperature rhythms in representative intact and DMH-lesion rats.
Note. Raster plots of locomotor activity (A-D), and average waveforms of locomotor activity (E-H) and body temperature (I-L) from an intact control rat with strong daytime food anticipatory rhythms (A,E,I), an intact rat with the weakest daytime food anticipation (B,F,J; defined by the lowest food anticipation ratio during daytime feeding), the rat with the lowest daytime food anticipation ratio (C,G,K) from the group that received a low-dose ibotenic acid microinjection causing a small partial lesion, and the rat with the lowest daytime food anticipation ratio (D,H,L) from among rats receiving a high-dose lesion that induced a complete DMH lesion (Fig. 7.1C,F). The raster plots illustrate activity summed in 10min bins. Each line represents 24h of recording, with time of day plotted from left to right (144 time points/day), and consecutive days aligned vertically. Time bins in which activity counts were registered are represented by a heavy bar, the height of which signifies the amount of activity (1-10 counts, 11-20 counts and > 20 counts/10 min). Grey shading denotes lights-off (hours 12-24 of LD, or all day during constant dark tests). Scheduled mealtime during food restriction are denoted by opaque columns labeled '4-h meal', with red signifying a daytime meal (hours 6-10 of the light period) and blue signifying a late nighttime meal (hours 21-01). Small red arrowheads pointing down denote beginning of total food deprivation test, and small green arrowheads pointing up denote the end of food deprivation. Waveforms of activity (E-H) and temperature (I-L) data were creating by averaging across 5 days of ad-lib food access prior to food restriction (grey shaded waveforms), the last 5 days of the daytime feeding schedule (light weighted red lines) and the last 5 days of the nighttime feeding schedule (heavy weighted blue lines). 4-h mealtimes are denoted by the translucent columns. The daily lights-on period is denoted by the yellow horizontal bar above the x-axis. To improve the clarity of the waveforms, the data were subjected to a second order smoothing polynomial averaging across 4 neighbouring data points (Prism 5.0 for Mac OS X). Raw (unsmoothed) data were used for the raster plots and statistical analyses.

Mean daily activity levels were compared during ad-lib food access (in LD and DD), restricted feeding (the last 5-day block of daytime and nighttime feeding) and total food deprivation (2-day blocks prior to restricted feeding and after both daytime and nighttime feeding schedules), for a total of 7 feeding conditions. There was a significant main effect of lesion group ($F_{(2,27)} = 8.96, p = .001$) and feeding condition ($F_{(6,162)} = 34.21, p < .0001$; Fig. 7.4A). Relative to control rats, high-dose IBO rats exhibited significantly lower mean daily activity levels during all of the feeding conditions ($p < .05$ for each between-group contrast), with the exception of the first food deprivation test prior to restricted feeding. Activity levels in low-dose IBO rats were more variable and did not differ from the intact group, but trended to be higher than the high-dose IBO group. During the food deprivation tests and the last 5-day blocks of daytime and nighttime restricted feeding, mean daily activity levels were increased in all groups relative to ad-lib food access ($p < .05$ for each within-group contrast), with the exception of the daytime restricted feeding in the high-dose IBO rats.
Figure 7.3. Group mean waveforms of activity data in intact controls rats (black dotted curves with standard error bars above and below the means), and in rats receiving small partial DMH lesions created by low-dose ibotenic acid (IBO) microinjections (blue curves with error bars above the means) or larger lesions created by high-dose IBO injections (red curves with error bars below the means).

Note. A. Ad-lib food access in a LD cycle, with the light period (hours 0-12) denoted by the heavy yellow bar above the x-axis. B. Ad-lib food access during a day in constant dark. C. Average of the last 5-days of daytime restricted feeding, with the daily 4h mealtime (hours 6-10 of lights-on) denoted by the translucent column. D. Average of the last 5-day of nighttime restricted feeding (hours 21-01). E. Average of days 2-3 of total food deprivation following the daytime feeding schedule. F. Average of the days 2-3 of total food deprivation following the nighttime feeding schedule.
Figure 7.4. Total daily activity and nocturnal activity in intact and DMH lesion rats

Note. A. Group mean daily activity counts in intact rats (solid black bars), low-dose ibotenic acid lesion rats (stripped bars) and high-dose lesion rats (white bars), during adlib food access in LD and constant dark (DD), during 2 days of total food deprivation (FD) prior to daytime restricted feeding, during the last 5-day block of the 20-day daytime restricted feeding (RFL-B4), during total food deprivation after daytime restricted feeding (RFL-FD), during the last 5-day block of nighttime restricted feeding (RFD-B4) and during the food deprivation that immediately following nighttime restricted feeding (RFD-FD). B. Nocturnality ratios of activity data from each group under the same 7 food access conditions as in Panel A. Significant differences (p < .05) relative to the intact group within each condition are denoted by a star.

Nocturnality ratios for locomotor activity also differed significantly by group ($F_{(2,27)} = 6.62$, $p < .005$; Fig. 7.4B) and by feeding condition ($F_{(6,162)} = 86.91$, $p < .0001$), with a significant interaction ($F_{(12,162)} = 1.88$, $p = .04$). The high-dose and low-dose IBO groups did not differ, but compared to the control rats, both groups exhibited a significantly lower nocturnality ratio (percent of total daily activity occurring during the usual dark period) during ad-lib food access in LD and DD and during the 42h food deprivation prior to restricted feeding ($p < .05$). The group differences in the other conditions were not significant. Within groups, nocturnality ratios were not altered during the 42h food deprivation. Nocturnality ratios greatly decreased during daytime restricted feeding, and during the total food deprivation test that immediately followed, consistent with the presence of a persisting rhythm of daytime food anticipatory activity in all 3 groups.
7.3.3. Food anticipatory activity

All rats exhibited food anticipatory activity during both the daytime and the nighttime restricted feeding schedules, but daytime anticipation was weak in some DMH ablated rats (e.g., Fig. 7.2G, H), and more robust at night in all rats. Waveforms of activity averaged in 5-day blocks showed that during restricted feeding, all rats exhibited a progressive increase of activity during the 3h prior to mealtime, with maximal values reached during the 30min immediately preceding mealtime (Figs. 7.2 & 7.3). Turning the lights off during the last day of scheduled feeding had no effect on the timing or amount of anticipatory activity.

Food anticipation was quantified by calculating ratios of activity during the 3h prior to mealtime relative to activity during the rest of the day (excluding mealtime to lights-off, ZT6-12, when activity is partly evoked by delivery and removal of food, rather than spontaneous). Ratios were averaged in 5-day blocks during ad-libitum food access and the two 20-day feeding schedules in LD. Low- and high-dose IBO groups exhibited similar average waveforms and ratios and were therefore combined to increase power for statistical contrasts with the intact rats. Repeated measures ANOVA revealed a significant effect of time block ($F_{(11,286)} = 127.8$, $p < .0001$) and a significant interaction between time block and group ($F_{(22,286)} = 2.45$, $p = .0004$) but no effect of group alone ($F_{(2,26)} = 2.69$, $p = .09$). A series of planned one-tailed t-tests (statistically liberal) was also conducted to contrast anticipation ratios between the high-dose IBO and control group alone, for each of the 4 blocks of daytime and nighttime feeding schedules, but no group differences were significant at $p < .05$, even without bonferroni correction for multiple tests.

Although the group mean anticipation ratios did not differ during restricted feeding, the 6 rats with the lowest magnitude ratios during the daytime feeding schedule (expressed as differences from the ratio calculated for the ad-libitum food access baseline block) were all among the DMH lesion groups (Fig. 7.5A). In each case, when mealtime was shifted to the night, the magnitude of the anticipation ratio increased to within the range exhibited by intact rats (Fig. 7.5B). The improvement from daytime to nighttime food anticipation is best illustrated by the rat that sustained the largest DMH lesion (100% complete) following high-dose IBO (Fig. 7.2D, H). This rat exhibited the weakest anticipation of the daytime meal (defined as the lowest anticipation ratio during the last 5-day block of restricted feeding), but much stronger anticipation of the nighttime meal, at a level comparable to an intact rat.
Figure 7.5. Food anticipatory activity (FAA) ratios for each DMH lesioned (A) and intact (B) rat, averaged over the last 5 days of daytime restricted feeding (ZT6-10 meal) and nighttime restricted feeding (ZT21-1 mealtime).

Note. Ratios are calculated by dividing total activity during the 3h immediately preceding mealtime by total activity during the 12h night plus the first 3h of the light period, and are expressed as difference scores from the same ratios calculated for the ad-lib food access condition. Data points from individual rats are connected by lines. The upper and lower red dotted lines denotes the lowest anticipation ratios in the intact rats and the lesion rats, respectively.

The remaining 8 rats with DMH lesions showed daytime food anticipation ratios within the range exhibited by intact rats, and on average did not show enhanced anticipation ratios during nocturnal restricted feeding. All of the DMH lesions in these rats were partial, and tended to be smaller and less symmetrical than the lesions in the 6 rats with the lowest anticipation ratios. However, due to a wide range of lesion size within groups, the difference was not statistically significant (25 ± 8% vs 41 ± 26% complete, t_{12} = 1.08, p = 0.29).

Anticipation rhythms evident during the two feeding schedules persisted during 2 days of total food deprivation tests in DD (Fig. 7.3E, F). This was confirmed by inspection of average waveforms and by anticipation ratios for all individual rats. The magnitude of persistence appeared weaker following the nighttime restricted feeding in the high-dose IBO group, but this apparent difference was not statistically significant compared to the other groups.

Following the nighttime restricted feeding schedule and a week of ad-lib food access the rats were subjected to a 2-day food deprivation and a second and final daytime feeding schedule for 9 days. Food anticipation ratios during the last 5 days of this schedule did not differ from ratios during the second 5 day block of the first daytime feeding schedule, indicating that higher anticipation ratios during the nighttime restricted feeding were not due to a gradual recovery over time.
7.3.4. **Body temperature**

During ad-libitum food access in LD, $T_b$ was higher at night than during the day in all rats (Fig. 7.2I–L; Fig. 7.6A). The day-night $T_b$ difference was significantly greater in the intact rats by contrast with the low-dose and high-dose IBO rats combined (0.67° ± .04°C vs 0.51° ± .04°C, respectively; $p < .05$; Fig. 7.7A), although not by contrast with the two lesion groups separately. Similar daily rhythms and group difference were also evident in DD (0.68° ± .06° vs. 0.53° ± .04°C, respectively; $p < .05$; Fig. 7.6B & 7.7A).

The $T_b$ daily waveforms were markedly altered by the feeding schedules. During daytime restricted feeding, all three groups exhibited a relative hypothermia during the first 3-4h of the light period, with mean $T_b$ declining into the 36.0-36.4° range, or about 0.4-0.8°C below normal values for that time of day (Fig. 7.6C, E). During the 3h prior to the daily meal at ZT6, group mean $T_b$ increased monotonically in all 3 groups. Within the first 30min of meal onset, $T_b$ increased rapidly by another ~0.7°C, before briefly settling by ~0.2°C. This was followed by a gradual increase of ~0.3-0.5°C over the remaining 2.5h of food access. All rats in all groups exhibited this pattern, including the rat with the largest DMH lesion (Fig. 7.2L).

During the 2 days of total food deprivation, the $T_b$ daily waveforms were virtually identical to the waveforms during scheduled feeding, except for the absence of the initial rapid rise of $T_b$ that occurred during the first 30min of food access. $T_b$ declined abruptly at the end of the mealtime, during restricted feeding and during the total food deprivation days. During the nighttime restricted feeding schedule, $T_b$ again exhibited a monotonic increase prior to mealtime in all intact and DMH ablated rats, and this pattern persisted during 2 days of total food deprivation. Daytime hypothermia was absent during the nocturnal feeding schedule.
Figure 7.6. Group mean waveforms of body temperature ($T_b$) data

Note. $T_b$ data in intact controls rats (black dotted curves with standard error bars above and below the means), and in rats receiving small partial DMH lesions created by low-dose ibotenic acid (IBO) microinjections (blue curves with error bars above the means) or larger lesions created by high-dose IBO injections (red curves with error bars below the means) A. Ad-lib food access in a LD cycle, with the light period (hours 0–12) denoted by the heavy yellow bar above the x-axis. B. Ad-lib food access during a day in constant dark. C. Average of the last 5-days of daytime restricted feeding, with the daily 4h mealtime (hours 6–10 of lights-on) denoted by the translucent column. D. Average of the last 5-day of nighttime restricted feeding (hours 21–01). E. Average of days 2-3 of total food deprivation following the daytime feeding schedule. F. Average of the days 2-3 of total food deprivation following the nighttime feeding schedule.

Although there were no group differences in the effects of scheduled feeding on the waveforms of the $T_b$ rhythms, ANOVA revealed a significant effect of both group ($F_{(2,156)} = 9.20, p = .0002$) and feeding condition ($F_{(5,156)} = 14.43, p < .0001$) on average daily $T_b$ (Fig. 7.7B). During ad-
lib food access in LD and DD, mean daily $T_b$ was significantly lower in the two IBO lesion groups relative to the control group ($p < .05$). Group differences were not significant during restricted feeding (Fig. 7.6 & 7.7B). Across feeding conditions, mean daily $T_b$ in each group were significantly lower during the food deprivation tests relative to ad-lib food access. There was also a trend for lower mean daily $T_b$ during daytime scheduled feeding relative to nighttime scheduled feeding (Fig. 7.7B).

**Figure 7.7. Group mean body temperatures ($T_b$)**

Note. $T_b$ in intact rats (black bars), low-dose DMH lesion rats (stripped bars) and high-dose lesion rats (white bars), expressed as A. dark-light differences during ad-lib food access in LD and DD, and as B. 24h daily means during each of 7 feeding conditions. Abbreviations: RFL, last 5 days of restricted feeding in the light period; RFD, last 5 days of restricted feeding in the dark period; FD, 3-days of total food deprivation immediately following RFL or RFD.
7.4. Discussion

The landmark discovery of a retinorecipient, light-entrainable master circadian pacemaker in the SCN suggested by analogy that SCN-independent food-entrainable oscillators hypothesized to drive food anticipatory circadian rhythms might be similarly located in the hypothalamus, possibly in a single structure containing neurons responsive to nutrients or other feeding-related signals. Lesion studies of medial and lateral hypothalamic structures failed to identify a critical locus [6, 36] until a report that neurotoxic (axon sparing) lesions in the DMH strongly attenuated food-anticipatory behavioral and \( T_n \) rhythms in rats [16]. All of the lesions in that study were partial, and all of the ablated rats showed at least some anticipatory activity, but a significant correlation between the amount of DMH damage and the food anticipation ratios supported an inference that complete lesions would have eliminated anticipatory rhythms altogether. This was the basis for the conclusion that the DMH is ‘critical’ for food anticipatory rhythms. A prediction from this conclusion is that complete removal of the DMH using electrolytic or radiofrequency lesion methods, which kill all cells and fibres of passage, would eliminate anticipatory rhythms. The results of two studies failed to confirm that prediction, and showed instead robust food anticipatory rhythms in rats with very large lesions that unambiguously removed the entire DMH, based on the absence of medial hypothalamic tissue bilaterally between the fornix and the 3rd ventricle, from the top of the 3rd ventricle extending ventrally to the middle of the ventromedial hypothalamus [23, 24]. The presence of clear neuroanatomical landmarks, including the fornix, mammillothalamic tract and ventromedial hypothalamus, eliminated any doubt that the DMH was entirely removed. Differences in cage design, recording method, and feeding procedures were ruled out as relevant [24, 25]. That left the seeming paradox that partial DMH lesions created with a neurotoxin can markedly attenuate food anticipatory rhythms, while total DMH removal by radiofrequency current does not.

The neurotoxin ibotenic acid kills only a subpopulation of neurons and spares fibres of passage, while radiofrequency lesions destroy all neurons, glia and fibres of passage indiscriminately. Taken together, the results indicate that the DMH cannot be the exclusive site of food-entrainable oscillators sufficient to produce robust food anticipatory rhythms, and suggest that fibres of passage through this area may play some role in the expression of these rhythms. This led to a working hypothesis that fibres of passage damaged by very large radiofrequency DMH lesions might include direct or polysynaptic projections from the SCN pacemaker that are responsible for inhibiting activity and arousal during the daily sleep period (lights-on) in nocturnal
rats. If DMH neurons played a role inhibiting SCN output, then selective removal of DMH neurons by neurotoxin would attenuate food anticipatory activity, while removal of DMH neurons and SCN outputs concurrently by large radiofrequency lesions would leave food anticipatory rhythms largely spared. According to this model, food anticipatory rhythms in rats with neurotoxic DMH lesions should be restored by removing the SCN or by scheduling food at night, when the SCN does not oppose the expression of activity.

The present study therefore had two objectives. The first objective was to duplicate the recording (radiotelemetry), feeding and lesion methods used by Gooley et al [16] to determine if the results of that study could be replicated. The results do confirm that DMH lesions can attenuate daytime food anticipatory activity, but do not confirm a loss of food anticipatory increases in $T_b$. The second objective was to test the prediction that attenuation of food anticipatory activity to daytime meals in DMH lesioned rats could be corrected by shifting mealtime to the night. The results are consistent with that prediction, although group mean differences between lesion and intact rats in the magnitude of food anticipatory rhythms in the day and the night were not great.

Using two different volumes of 10% ibotenic acid, we were able to create DMH lesions varying in size from 10-100% complete. These lesions had modest but significant effects on activity and $T_b$ relative to intact rats. When food was available ad-libitum, total daily locomotor activity was lower in high-dose IBO rats, and nocturnality was lower in both IBO lesion groups. Average daily $T_b$ was also significantly lower in the lesion groups, as was the day-night $T_b$ difference. These effects on activity and $T_b$ are consistent with those reported in previous DMH lesion studies using radiofrequency [24] or ibotenic acid lesion methods [35]. Group differences in average daily activity were also evident during the restricted feeding schedules, while $T_b$ differences between groups were minimal.

Despite the group differences evident in the mean levels of activity and $T_b$ during ad-libitum food access, group differences in food anticipatory activity (anticipation ratios) or in the premeal rise of $T_b$ during daytime or nighttime restricted feeding schedules were not statistically significant. Nonetheless, a subset of 6 rats with DMH lesions exhibited low anticipation ratios relative to baseline during the daytime feeding schedule, and in all cases the magnitude of these ratios improved during nighttime feeding schedules to within the range exhibited by intact rats. These results are as predicted if the DMH participates in the expression of food anticipatory activity by...
exerting a time-of-day (circadian phase)-dependent inhibitory effect on sleep-promoting outputs from the light-entrained SCN pacemaker. The other 8 rats with DMH lesions exhibited anticipation ratios during restricted daytime feeding that were within the range exhibited by intact rats. This presumably reflects sparing of a sufficient population of DMH neurons.

An instructive individual case is that of the rat with the largest lesion, in which the DMH was completely destroyed (Fig. 7.1C & 7.2D). This rat exhibited the lowest food anticipation ratio during daytime feeding, and marked improvement of the ratio relative to baseline during the nighttime feeding schedule. While this result supports the hypothesis that the DMH may play a modulatory role in the expression of food anticipation, it does not support a conclusion that the integrity of the DMH is critical either for food anticipatory behavior or a premeal rise of $T_b$. Food anticipation ratios for this rat, although lower than for other rats, were significantly different from ad-libitum baseline days by the second 5-day block of daytime restricted feeding. Furthermore, the anticipation rhythm persisted in DD when meals were omitted for 2 consecutive days. Although the raster style plots for this rat (and even for some intact rats, e.g., Fig. 7.2B) suggest low amplitude food anticipatory rhythmicity at best, average waveforms and anticipation ratios reveal a persisting ability to coordinate behavior and physiology ($T_b$) with predictable daily mealtimes. Whatever role the DMH might play in this function, it is not essential.

Another notable detail in the data from this rat is that during the last 5-day block of daytime food restriction, $T_b$ rose by $\sim1^\circ C$ from ZT2 (2h after lights-on) to ZT6 (meal delivery time), as it did in the intact rats. However, within this 4h time block, $T_b$ decreased slightly from ZT4-5 (Fig. 7.2L). If only the 2-3h immediately before mealtime are used to describe $T_b$, without presenting full 24h waveforms from both the food restriction and the ad-libitum food access conditions, the results may be misleading. Inspection of full 24h waveforms for this rat, and for the group data, clearly shows that across hours 2-6 of the light period, $T_b$ does rise steeply when DMH lesion rats are fed at ZT6, by contrast with the $T_b$ waveform during ad-lib food access.

The lack of effect of even complete DMH ablation on the early daytime hypothermia and the subsequent premeal rise of $T_b$ prior to a daily feeding at ZT6 is consistent with the results of other studies showing that the DMH does not participate critically in metabolic and peripheral clock adaptations to restricted feeding schedules. A lower average $T_b$ during caloric restriction is adaptive by minimizing energy expenditure, and evidently this response is not dependent on the DMH. Another study has shown that resetting of circadian oscillators in peripheral organs is not affected
by very large DMH lesions that included significant damage to the ventromedial hypothalamus and arcuate nucleus [37].

The results of this study confirm that the DMH does not contain circadian oscillators critical for driving food anticipatory rhythms of activity or $T_n$, but might facilitate expression of food-entrainable oscillators located elsewhere in the brain by actively inhibiting outputs from the SCN pacemaker that normally promote sleep and inhibit activity in the day. These conclusions are consonant with the results and conclusions of another study that demonstrated a neural basis for inhibition of the SCN by the DMH, and that provided evidence for recovery of attenuated food anticipatory rhythms in DMH lesioned rats by subsequent removal of the SCN [30]. Although some DMH neurons express daily rhythms of circadian clock gene expression in nocturnal rats and mice on daytime restricted feeding schedules, clock genes in this area can be induced by food deprivation alone, and by randomized feeding schedules that do not generate food anticipatory rhythms [17, 38, 39]. These observations are consistent with a role for DMH neurons as sensors of caloric restriction that modulate SCN output during the day, to enable hungry animals to exploit the daytime temporal niche when nutritional needs are not being met by foraging at night. These results underscore the potential importance of employing both daytime and nighttime restricted feeding schedules to thoroughly phenotype food anticipatory rhythms in rats with neural or genetic deficiencies.
References


8. Discussion

The aim of this thesis was to assess the role of two candidate structures under RF to determine whether these structures could qualify as circadian pacemakers. As described in Chapter 2, the objective of our first study was to determine whether the PVT was critical for the expression of food-anticipatory circadian rhythms. Our results indicate that rats with unambiguously complete PVT lesions continue to show robust food-anticipatory rhythms in locomotor activity remarkably similar to intact control rats in terms of duration, magnitude and latency to emerge. Whatever role the PVT may play in modulating the function of the SCN under RF schedules, our results rule out the PVT as the site of the FEP. Our second study, Chapter 3, assessed the DMH as a candidate pacemaker for food-entrainable rhythms using the same methods we employed in the first study. Similar to results from the PVT lesion study we showed that rats with unambiguously complete DMH lesions are capable of anticipating RF schedules. We therefore concluded the DMH was not critical for the expression of food-entrainable circadian rhythms, contrary to findings from an earlier study (Gooley et al., 2006). The remainder of this thesis focused on investigating possible explanations for the discrepancy in findings with respect to the DMH’s role in generating food-anticipatory rhythms under RF schedules. Methodological differences in our DMH lesion study provided a reasonable starting point for explaining contradictory results in the Gooley et al., 2006 study. The two studies differ in lesion method, food type, food location, activity measure, cage isolation, and cage configuration.

In our first attempt to replicate the Gooley et al., 2006 findings, Chapter 4, we speculated that cage configuration may have been the crucial variable. Both studies used polypropelene cages of the same dimensions, but our cages had wire floors, an opaque tube for sleeping, and a small side window through which a food cup could be accessed, whereas the Gooley et al., 2006 cages had solid floors with bedding, and food was accessed through the metal bars of the cage tops. Presumably, our cage design would be more sensitive to food-seeking specific behavior as our rats would have to leave their sleeping tubes and traverse the length of the cage in order to check the food bins. Despite closely duplicating caging and feeding methods used in the Gooley et al., 2006 study, we found once again that rats with unambiguously complete DMH ablations show near
normal FAA and clearly retain the capacity for entrainment to RF schedules. As discussed in Chapter 5, having failed for the second time to replicate the Gooley et al., 2006 results, we engaged in a scholarly debate with Gooley and Saper discussing methodological issues and questions regarding food entrainment including: what are valid conceptual models of FAA?; what is a valid quantitative metric of food anticipation?; and what is the appropriate lesion technique? As part of this debate, Saper and colleagues suggested that the motion sensors we used detected foraging behavior, and that foraging behavior reflects homeostatic factors that could create the impression of intact FAA in rats that actually lack a functioning food-entrainable circadian clock. It was unclear to us how activity detected by our motion sensors would not also be detected by an implant transponder, but none the less we proceeded to rule out the possibility that our failure to replicate the Gooley et al., 2006 findings was due in part to use of motion sensors instead of implants and telemetry. As we expected, our study described in Chapter 6 confirmed that when activity counts are normalized using the daily mean, motion sensors and telemetry provide equivalent measurement of food anticipation and thus the use of motion sensors does not explain our failure to replicate the Gooley et al., 2006 findings.

Having ruled out several procedural differences as possible explanations for contradictory findings including cage configuration, method of feeding, and the measure used to assess FAA, we turned to the lesion method. Clearly the technique used to lesion the DMH was an obvious difference between the studies, but how could that explain the results? The radiofrequency lesions we used result in complete, unambiguous ablations of the target area including significant collateral damage of structures surrounding the target. In contrast Gooley et al., 2006 used the excitatory neurotoxin ibotenic acid (IBO), which typically results in partial lesions of DMH cells while sparing tissue including fibres of passage through and around the DMH. How could lesion technique explain contradictory findings, whereby partial lesions of the DMH attenuate FAA while complete DMH lesions resulted in essentially normal FAA? If the DMH is critical for FAA and a potential site of the FEP, one would expect the opposite findings (i.e., near normal FAA following partial lesions, but failure to entrain following complete lesions). Our studies repeatedly showed near normal FAA following very large unambiguously complete destruction of the DMH including all tissue and fibres of passage in the immediate area surrounding the DMH. In our final study we hypothesized that this surrounding tissue and the fibres of passage may be critical in explaining our failure to replicate the Gooley et al., 2006 findings. If the DMH plays a modulatory role whereby FAA is expressed or
amplified under RF via disinhibition of daytime activity, then partial DMH lesions that spare fibres of passage could attenuate FAA during the day as described in Gooley et al., 2006.

In Chapter 7 we hypothesized that under daytime RF the DMH may play a role in suppressing an SCN output signal which actively attenuates daytime activity. As discussed earlier, we know that the LEP and the FEP can be coupled under ad-libitum feeding, suggesting some degree of connectivity and interaction between these pacemakers. Perhaps during daytime RF the DMH modulates FAA by disconnecting or freeing the FEO from the LEO’s daytime inhibitory signal. If so, then the Gooley et al., 2006 lesions that substantially damaged the DMH while sparing fibres of passage, effectively disinhibited the SCN signal, which then attenuated the FEO and thus resulted in decreased FAA. Lesions that destroyed both the DMH and fibres of passage, such as the radiofrequency lesions we used would result in near normal FAA because the SCN’s signal was disconnected along with complete ablation of the DMH. This model leads to two predictions. If SCN outputs are responsible for attenuation of food-anticipatory rhythms in rats with IBO-induced DMH-lesions, then food-anticipatory rhythms in these rats should be restored by 1. SCN ablation, or 2. Scheduling mealtime at night, when the SCN does not inhibit activity. A recent study has confirmed the first prediction; rats with attenuated daytime FAA following IBO DMH-ablation exhibited robust FAA when the SCN were subsequently ablated (Acosta-Galvan et al., 2011). In the final study of this thesis we followed closely the recording and lesion methods of Gooley et al., 2006 to test the second prediction. The final study had two objectives. The first objective was to replicate the Gooley et al., 2006 findings by using the same lesion technique, behavioral recording, and feeding protocol. Our results do confirm that IBO DMH-lesioned rats show attenuated anticipation to daytime RF. The second objective was to test the prediction that anticipation could be restored when feeding was phase shifted to the night. Our results are consistent with this prediction, although group mean differences between lesion and intact rats in magnitude of FAA in the day and night were not great. Based on these findings, it would appear that differences in lesion technique explain our repeated failures to replicate attenuation of FAA following DMH-lesions.

Taken together the studies that comprise this thesis support several important conclusions: first, the DMH is not critical for entrainment to RF and can be ruled out as a candidate structure for FEP; second, the DMH likely plays a modulatory role under daytime RF schedules whereby the FEP is disinhibited or disconnected from the LEP (i.e., the DMH likely inhibits the SCN’s inhibition of daytime activity, allowing the FEP to drive FAA); third, telemetry, which is more expensive and more invasive than motion sensors, is no better than motion sensors as a behavioral measure of FAA;
and fourth, expensive single housed isolation chambers are not necessary for assessing food entrainment, since social cues are not an effective means by which rats might “cheat” to anticipate daily meals (i.e., lesioned rats cannot anticipate a daily meal simply by picking up on social cues of intact rats that are entrained to a RF schedule). Alas, we find ourselves at the end of this thesis in the same place that we started, looking for the FEP. We know now that the DMH may play a role in modulating FAA, but can rule it out as the FEP. So where is the FEP? Given that the ability to anticipate the timing and availability of food is essential for survival, and given how robust FAA has been to lesions of a litany of brain structures and manipulations of a multitude core clock genes, it would appear likely that the FEP is comprised of a system of multiple redundant structures, whereby the capacity to entrain to RF survives destruction of any single structure within the system. If so, different strategies are obviously needed to identify the structures that comprise the FEP system. Presumably, future lesion studies need to target multiple candidate structures simultaneously, a daunting task no doubt. Where would one begin?

As discussed in detail earlier, the list of candidate structures is long and it would be impossible to lesion them all at the same time. Even if surgeries could be staggered to produce combinations of lesioned structures (e.g., DMHx-ARCx or PVTx-DMHx-ARCx), there are too many candidates to consider without some way to group and prioritize them. Assuming rats can survive combined lesions, we need a way to narrow the list to two or three structures that could be lesioned in the same animal. Perhaps it would be helpful to change the way we think of the FEP. If we think of the FEP as a system of structures, the task would be to identify which structures are activated in response to RF and then distinguish between structures that are activated as a function of feeding versus those that are critical to the mechanism of foodentrainment. If we could entrain the FEP system using some means other than by feeding, we might be able to compare and contrast maps of brain activation for each mode of entrainment. Areas that were activated in common across modes would be FEP system candidates. This idea may not be as far-fetched as it seems. We do not eat food simply for the sake of sustenance, there is also a reward component to palatable meals. In fact ad-libitum fed rats can entrain to restricted treats or palatable meals, with corresponding activation of reward centers in the brain (Mistlberger and Rusak, 1987; Mendoza et al., 2005b, c; Angeles-Castellanos et al., 2008). The idea of using reward for entrainment is not novel. In addition to studies examining the reward component of feeding, the psychostimulant methamphetamine has been assessed as an entrainment signal. The methamphetamine-sensitive circadian oscillator (MASCO) is an SCN-independent oscillator (Honma et al., 1986; Honma et al.,
that has been the subject of many studies and has been linked to the FEP (for review, see Honma and Honma, 2009). Neither palatable meals nor MAP administration would qualify as distinct modes of activation of the FEP, however, given they both involve ingestive behavior. What is required is a non-ingestive reward stimulus capable of entraining circadian anticipatory activity.

In a series of experiments, not included in this dissertation (Landry et al., 2012), we examined whether male rats could anticipate daily windows of opportunity to mate (i.e., restricted sex). The results were mixed, in ways very reminiscent of the IBO DMH-lesion results that comprise this dissertation. In brief summary, male rats can anticipate restricted sex (RS) but anticipation is gated, likely by a circadian mechanism, whereby daytime mating windows result in little to no anticipation, but nighttime mating windows result in robust ejaculation-dependent anticipation. These results fit nicely with a report of diurnal variation in sexual reward (Webb IC et al., 2009b); and are suggestive of a reward-entrainable oscillator (REO) that is gated by the SCN. For a detailed review of bidirectional interactions between circadian and reward systems, see Webb et al., 2009a. Perhaps the SCN inhibits anticipation to RS during daytime, in much the same way that we saw attenuation of FAA to daytime RF, in the absence of a functional DMH. SCN inhibition of daytime reward signals would also explain why rats and mice often fail to anticipate palatable foods (Pecoraro et al., 2002; Verwey M et al., 2007; Hsu CT et al., 2010).

According to the model proposed here, the SCN gates entrainment capacity of reward stimuli (e.g., palatable food or sex) restricting anticipation to the nighttime in nocturnal rodents. Under extreme circumstances when survival requires it (i.e., when caloric intake during RF schedules reach a threshold level ~30% of total daily intake), the DMH is engaged freeing the FEP from the SCN’s daytime inhibitory signal. This model makes adaptive sense as rodents need not risk predation by seeking out palatable foods or sex during the day. When food is only available during a limited window, however, rodents require the capacity to overcome light avoidance or else they would starve. It may be that the FEO, REO, and MASCO are manifestations of the same pacemaker system. If so, we could illuminate critical structures in the entrainment pathway by comparison of differential brain activation during an anticipation window across 3 distinct modalities: RF in IBO DMH-lesioned and intact rats; ad-libitum feeding with restricted palatable treats (RPT); and RS during the day and night. Using different modes of activation (i.e., food for survival, food as reward, and sex) and by toggling the entrainment signal on and off for each mode (i.e., IBO DMH-lesion versus intact for RF; daytime versus nighttime for RPT and ad-libitum feeding; and daytime versus nighttime for RS), we might be able to narrow the list of candidate structures that comprise.
entainment pathway. If successful we could go a long way in characterizing what is currently known as the FEP, but may more accurately be termed the food- and reward-entrainable pacemaker.

In short summary, the findings herein show telemetry and over-head motion sensors are equivalent measures of behavioral FAA, and establish that the PVT and DMH can be ruled out as single structure FEP candidates. In contrast with earlier reports that the DMH is critical for entrainment to RF schedules, our results suggest instead that the DMH plays an important role in signalling caloric restriction, freeing the FEP from the SCN’s daytime inhibition of arousal and activity. By adding the PVT and DMH to an already long list of structures that have been eliminated as candidates for FEP, our results will only serve to increase the growing speculation that unlike the SCN as LEP, the FEP may be comprised of a system of multiple redundant structures. As such, we suggest a change in strategy for future efforts to identify the FEP.
References


