Abstract—Circadian rhythms in mammals are regulated by a system of circadian oscillators that includes a light-entrainable pacemaker in the suprachiasmatic nucleus (SCN) and food-entrainable oscillators (FEOs) elsewhere in the brain and body. In nocturnal rodents, the SCN promotes sleep in the day and wake at night, while FEOs promote an active state in anticipation of a predictable daily meal. For nocturnal animals to anticipate a daytime meal, wake-promoting signals from FEOs must compete with sleep-promoting signals from the SCN pacemaker. One hypothesis is that FEOs impose a daily rhythm of inhibition on SCN output that is timed to permit the expression of activity prior to a daytime meal. This hypothesis predicts that SCN activity should decrease prior to the onset of anticipatory activity and remain suppressed through the scheduled mealtime. To assess the hypothesis, neural activity in the SCN of mice anticipating a 4–5-h daily meal in the light period was measured using FOS immunohistochemistry in vivo and in vitro multiple unit electrophysiology. SCN FOS, quantified by optical density, was significantly reduced at the expected mealtime, spanning pauses in locomotion, compared to ad libitum-fed and acutely fasted controls. Group differences were not significant when FOS was quantified by other methods, or in mice without running disks. SCN electrical activity was markedly decreased during locomotion in some mice but increased in others. Changes in either direction were concurrent with locomotion, were not specific to food anticipation, and were not sustained during longer pauses. Reduced FOS indicates a net suppression of SCN activity that may depend on the intensity or duration of locomotion. The timing of changes in SCN activity relative to locomotion suggests that any effect of FEOs on SCN output is mediated indirectly, by feedback from neural or systemic correlates of locomotion.

Key words: suprachiasmatic nucleus, circadian rhythms, food entrainment, food anticipation, multiple unit activity, FOS.

INTRODUCTION

Circadian activity rhythms in rodents are regulated by a light-entrainable circadian pacemaker in the suprachiasmatic nucleus (SCN), and by food-entrainable circadian oscillators (FEOs) located elsewhere in the brain and/or body. When food availability is unrestricted, nocturnal rodents are active and eat primarily at night, under control of the SCN pacemaker. If food is restricted to the middle of the day, when nocturnal rodents normally sleep, a daily rhythm of food anticipatory activity (FAA) emerges. The rhythm has canonical properties of circadian clock control, but does not require the SCN pacemaker (Boulos and Terman, 1980; Mistlberger, 1994; Stephan, 2002). The location of FEOs hypothesized to mediate FAA remains uncertain, although candidate sites include the hypothalamus, cerebellum and striatum (Sutton et al., 2008; Mendoza et al., 2010; Mistlberger, 2011; Gallardo et al., 2014).

In nocturnal rodents, the SCN pacemaker actively promotes sleep and suppresses activity in the light period and does the opposite in the dark period (Mistlberger, 2005; Flesher et al., 2011). The SCN is comprised of a population of coupled circadian clock cells which, in aggregate, generate an approximately sinusoidal daily rhythm of multiple unit electrical activity (MUA) with a peak at mid-day and a trough at mid-night (Houben et al., 2014). FEOs responsible for FAA are presumed to generate a similar daily rhythm, but synchronized to mealtime rather than light-dark (LD). For meals scheduled in the light period, this creates a conflict between SCN and FEO output. FEOs have been hypothesized to resolve this conflict in two ways, by directly activating arousal circuits and by suppressing SCN output prior to mealtime (Mistlberger, 2006; Moriya et al., 2009; Acosta-Galvan et al., 2011; Landry et al., 2011).

If FEOs directly impose a daily rhythm of inhibition on SCN output, then SCN neural activity should decrease prior to the onset of FAA, and remain suppressed through the end of the expected mealtime, spanning pauses in anticipatory or consummatory behavior. This hypothesis...
is supported indirectly by evidence that in nocturnal rodents fed *ad libitum*, SCN MUA (Meijer et al., 1997; Yamazaki et al., 1998; Schaap and Meijer, 2001; Van Oosterhout et al., 2012) and FOS expression (a molecular marker of neural activity) (Janik and Mrosovsky, 1992; Antle and Mistlberger, 2000) are reduced by locomotor activity in the light period. However, suppression of SCN MUA, when evident, appears concurrent with or after the onset of activity. It has therefore been interpreted as activity-dependent, rather than predictive of, or causal to activity (van Oosterhout et al., 2012). The suppression of SCN FOS following stimulated activity or arousal has also been interpreted as a response to behavior, mediated by inhibitory inputs to the SCN from the intergeniculate leaflet (NPY, GABA) and midbrain raphe (5HT, GABA), both of which are activated when animals are aroused and moving (Janik and Mrosovsky, 1992; Grossman et al., 2000; Saderi et al., 2013; Webb et al., 2014).

Few studies have recorded SCN electrical activity during restricted feeding schedules, and no quantitative data are available on changes in SCN activity prior to mealtime (Inouye, 1982). At least 10 studies have quantified SCN FOS expression in rats, mice or Syrian hamsters anticipating a mid-day meal, with four reporting a increase (Nakahara et al., 2004; Girotti et al., 2009; Mitra et al., 2011) and three no change (Angeles-Castellanos et al., 2004; Begriche et al., 2012; Dantas-Ferreira et al., 2015). Regardless of the direction of any change, FOS is an indirect measure of neural activity integrated over time. Without sampling at multiple time points during the hours immediately preceding mealtime, analysis of FOS cannot reveal whether changes in SCN neural activity precede or follow the onset of FAA, or whether SCN activity recovers during pauses in FAA.

To obtain more direct evidence by which to evaluate the hypothesis that anticipation of a daytime meal involves inhibition of SCN activity, we made electrical recordings from the SCN of freely moving mice with food available *ad libitum* and then restricted to a 4-h daily meal in the light period. To gain insights into the variability of results from previous studies of SCN FOS, we also assessed FOS expression immunohistochemically, using three different quantification methods and two recording conditions (i.e., with and without a running disk).

**EXPERIMENTAL PROCEDURES**

**Subjects and housing**

Experiments to quantify the immediate early gene product FOS in the SCN were conducted at Simon Fraser University (Burnaby, Canada) and were approved by the University Animal Care Committee. Male C57Bl/6J mice (N = 37, Charles River PQ) received at 45 days of age were single-housed in standard plastic mouse cages equipped with wire mesh food hoppers, corn cob bedding, and a plastic Igloo house (11 cm diameter, 5.7 cm tall; Igloo Fast-Trac, BioServ, Flemington, NJ, USA) with or without a running disk (depending on group assignment) mounted horizontally at a shallow angle on the top of the Igloo. Running disk rotations were recorded continuously using a commercial interface and data acquisition system (Clocklab, Actimetrics, USA). Passive infrared motion sensors were mounted above each cage to continuously record movement. Water was available *ad libitum*. Cages were housed in cabinets in a climate controlled vivarium (22 ± 2°C, ~50% humidity), with a 12:12 LD cycle (12 h of ~70 lux light provided by white LEDs).

Experiments to record electrical activity from multiple SCN neurons were conducted at the Leiden University Medical Center (Leiden, The Netherlands) in accordance with the regulations of Dutch law on animal welfare and were approved by the institutional ethics committee for animal procedures. Male C57Bl/6J mice were housed in a controlled environment (22°C, 40–50% humidity) in LD 12:12. Following surgical procedures (described below) the mice were housed in standard type III cages (425 × 266 mm) with stainless steel wire lids and a hinged divider. The cage was enriched with a 1-cm layer of sawdust bedding (Woody Clean S 8/15, Rettenmaier, Germany) and paper tissues (no running disk).

**Feeding schedules**

Mice used for quantification of FOS immunoreactivity were acclimated to the recording cages for 12 days with food available *ad libitum* (rodent chow 5001, BioServ, USA). The mice were then assigned randomly to one of three groups. Mice in the restricted feeding group (N = 13) were food deprived for 14 h and then provided food 7 h after lights-on (Zeitgeber Time (ZT) seven, where ZT0 is lights-on by convention) for 28 days. The duration of food access was gradually decreased from 10 h to 5 h over the first 2 weeks, and maintained at 5 h for the remainder of the schedule. Mice in the acute food deprivation group (N = 13) were provided food *ad libitum* for 28 days and were then food deprived for 20 h, beginning at ZT12 (lights-off). Mice in the food *ad libitum* group (N = 11) were not food deprived. Five mice in each group were maintained without running disks throughout the experiment. After day 28 of restricted feeding, mice in all three groups were euthanized and perfused for brain extraction at ZT8 (mice in the food restriction and acute food deprivation groups were not fed on that day).

Mice used for recording SCN MUA were provided food (rodent chow RM3, Special Diet Services, Sussex, UK) and water *ad libitum* prior to restricted feeding. Food was then removed for 28 h, and provided at ZT7 for 4 h daily for 4–24 days. In two cases, the mice were maintained in constant dark (DD) during the last 2 days of restricted feeding.

**In vivo electrophysiological SCN recordings**

For SCN electrode implantation, mice were anesthetized using a mixture of Ketamine (100 mg/kg, Aescocket, Boxtel, The Netherlands), Xylazine (10 mg/kg, Bayer AG, Leverkusen, Germany) and atropine (0.1 mg/kg, Pharmachemie, Haarlem, The Netherlands) and mounted in a stereotactactic device (Digital Just for Mouse
Stereotaxic Instrument, Stoelting Co, Wood Dale, IL, USA). Tripolar stainless steel electrodes were used, with two insulated electrodes twisted together and a third, uninsulated electrode for use as reference (125 µm, Plastics One, Roanoke, Virginia, USA). The reference electrode was placed in the cortex and the twisted electrode pair was implanted at a 5° angle in the coronal plane and aimed at the SCN, 0.61 mm lateral to the midline, and 5.38 mm ventral to the surface of the cortex. Following a 7-day recovery period, mice were placed in a recording cage (floor size 35 × 36 cm) and the electrodes were connected to a custom-made recording system. The connection consisted of lightweight cables suspended on a counter-balanced rotating contact, allowing animals to move around freely. Neuronal signals from the electrodes were differentially amplified and band-pass filtered (0.5–5 kHz) before being fed into amplitude-based spike triggers that converted action potentials into pulses that were counted every 10 s and stored on a computer for off-line analysis (CircV1.9 custom-made software). Behavioral activity was recorded by passive infrared sensors positioned 30 cm above the floor of the cage. Raw data files from individual recordings were analyzed offline using Igor Pro (version 6.3, Wavemetrics, Inc., USA).

All mice were recorded for several days to confirm the presence of a circadian rhythm in the recorded action potential frequency. Mice that did not show a 24-h rhythm in action potential frequency with a daytime peak were excluded from the experiment, as that normally indicates an electrode placement outside of the SCN (e.g., Nakamura et al., 2008, 2011; Van Oosterhout et al., 2012). A total of seven mice that did show a 24-h rhythm with a daytime peak were retained and recorded continuously until the end of the experiment. The mice were then euthanized and the electrode placement was determined by brain sectioning and Nissl stain.

**FOS immunohistochemistry**

On the final day of behavioral recording, mice in the FOS study were euthanized (100 mg/kg sodium pentobarbital via intraperitoneal injection) at ZT8, without feeding at ZT7. The mice were then perfused through the heart with cold 0.01 M phosphate-buffered saline (PBS), followed by cold 4% paraformaldehyde PBS solution. Brains were extracted and post-fixed for a minimum of 48 h in 4% paraformaldehyde in PBS. Brains were transferred to a 30% sucrose solution in PBS for cryoprotection, then sectioned at 30 µm in the coronal plane using a cryostat.

Indirect immunohistochemistry was used for cell visualization and tissue was treated as free-floating sections. Unless otherwise indicated, each washing step consisted of three 5-min washes to clear the previous solution, and washes and incubations were performed under agitation and at room temperature.

The tissue was first washed in PBS solution followed by a 30-min incubation in 0.3% hydrogen peroxide solution. Tissue was then washed once for 5 min in PBS with 0.3% Triton X-100 detergent (SigmaUltra; PBSx) and incubated for 1 h in a blocking solution of 20% Avidin (Avidin/Biotin Blocking Kit; Vector Laboratories Inc, Burlingame, CA 94010, USA) and 10% Normal Goat Serum (Vector Laboratories Inc; NGS) in PBSx. All subsequent washes used PBSx solution. The tissue was then incubated for 48 h at 4°C in a 1:30,000 dilution of anti-FOS antibody raised in rabbit (Calbiochem, EMD Millipore, Billerica, MA 01821, USA) with 1% NGS and 20% Biotin (Vector Laboratories Inc., Burlingame, CA 94010, USA) in PBSx.

After removal from the primary antibody solution, tissue was washed and transferred to the secondary antibody solution consisting of a 1:250 dilution of biotinylated affinity-purified anti-Rabbit antibody made in goat (Vector Laboratories Inc.) with 1% NGS in PBSx. After a 1-h incubation, tissue was washed and incubated for 45 min in a blocking solution consisting of 0.5% Avidin and 0.5% Biotin in PBSx (ABC Elite Kit, Vector Labs, Inc.). The stain was developed in a saturated solution of 3,3'-diaminobenzidine tetrahydrochloride (SigmaUltra, DAB) in trizma-buffered saline with 0.1% Hydrogen Peroxide and 0.08% Nickel Chloride to create a dark blackish color. After 8 min of development, the reaction was quenched by transferring the tissue into PBSx solution.

Tissue was wet-mounted onto Superfrost plus slides (VWR), dehydrated through a graded series of ethanol, clarified in Xylene solution, and coveredslipped with Permount (Fisherchemicals).

**FOS quantification**

Four slices per brain were selected for analysis to represent the SCN from its anterior to posterior extent, at approximately −0.34 mm, −0.46 mm, −0.70 mm, and −0.82 mm relative to Bregma, using a stereotaxic mouse brain atlas (Franklin and Paxinos, 2008). SCN slices were inspected with a light microscope (Nikon Eclipse 80i) under 200× magnification and hand tracings of all stained cells were made using an attached drawing tube. A low selection threshold was utilized so that all cells with clear borders demonstrating staining were included regardless of stain intensity. Photomicrographs were taken at 100× and 200× magnification using an attached digital camera (Retiga 2000R, QImaging). FOS staining was quantified using three methods: (1) hand counting, (2) automatic object recognition, and (3) optical density (OD) (Rieux et al., 2002).

**Hand counting.** After tracings and photomicrographs were obtained, the same tissue was counterstained for Nissl bodies with cresyl-violet to determine accurate SCN boundaries. Nissl-stained slices were projected over the previously made tracings to draw in the SCN boundaries. The number of stained cells within the SCN boundaries was counted bilaterally by a condition-blind research assistant. Total SCN FOS per brain was calculated by adding the stained cells from all four slices.

**Object recognition.** Photomicrographs were assessed using ImageJ software (U.S. National Institutes of Health) using a previously described method for automatic object recognition (Acosta-Galvan et al., 2011). Briefly, a background measurement of OD was taken from an area devoid of staining, and two times this background measurement...
was set as the threshold. Objects exceeding this threshold within a defined region of interest were identified by the software and counted as stained cells. Standard overlays of each SCN level were prepared from a subset of five representative sections to define the region of interest for the cell counting program. A subset of 32 measurements taken with standard overlays was correlated very strongly \((r = 0.99)\) with measurements taken using individually traced SCN boundaries. Additional overlays were prepared from photomicrographs of mouse SCN sections stained for arginine vasopressin (AVP). These overlays were used to determine dorsal/shell (AVP+ cell bodies) and ventral/core subdivisions within the SCN. The number of stained cells within the total and subdivided SCN was determined bilaterally by the program.

OD. Measurements of OD in the total and subdivided SCN were obtained with ImageJ using the same set of standard overlays. OD measurements were corrected for background staining by subtracting the OD reading from a nearby unstained area. OD is a measure of overall darkness in a region of interest and unlike the previous two techniques does not yield a count of stained cells. Total SCN OD was defined as the mean OD value of the four assessed slices per mouse.

Data analysis

FOS data are reported as group means and standard error. Group differences in SCN FOS were evaluated by separate ANOVAs run for each quantification method. The Pearson Product Moment Correlation Coefficient was also calculated for each possible pair of quantification strategies, by using the total SCN FOS values they produced for the set of animals.

SCN MUA data were analyzed using custom MATLAB (Mathworks Inc., Natick, MA, USA) software, as described previously (Vanderleest et al., 2007). SCN rhythm range was defined as the difference between the daily peak and trough. To determine whether FAA and bouts of activity at other times of the day were associated with a change in SCN electrical activity, mean MUA counts during locomotor activity bouts were expressed as a percent change from MUA during the immediately preceding rest bout. To determine whether changes in SCN electrical activity predicted daytime activity bouts, linear regression was used to calculate the slope of electrical activity counts in 10-s time bins over the last 2 min and 10 min of rest prior to activity.

RESULTS

Behavior

Visual inspection of actograms and average waveforms confirmed the presence of food anticipatory locomotor activity in mice fed once daily at ZT7, in both the FOS (Fig. 1) and neurophysiology experiments (Fig. 5). In the FOS study, the duration of food anticipatory running in mice housed with running disks \((158 \pm 21 \text{ min})\) was significantly greater than the duration of anticipatory activity detected by motion sensors in mice housed without running disks \((103 \pm 14 \text{ min})\) (Mann–Whitney \(U\) test, \(p = .0455\)). Daytime food anticipatory disk running was compensated by reduced nocturnal running, an effect not evident in motion sensor data from mice housed without running disks (Fig. 1).

Mice in the acute food deprivation group in the FOS study showed a trend for increased running activity during the 18-h food deprivation test \((t_{(7)} = 2.272, p = .057)\) but activity was low during the last 3 h of deprivation, and similar to the same 3 h (ZT5–8) during ad libitum food access \((3.0 \pm 1.4 \text{ counts vs. } 0.5 \pm 0.2 \text{ counts}; t_{(7)} = 2.02, p = .08)\).

SCN FOS

FOS staining was evident in all SCN sections from anterior to posterior, but was concentrated in the ‘shell’ region (Fig. 2), where daily rhythms of FOS expression are reported to be strongest (Butler et al., 2012). FOS staining in the SCN ‘core’ was minimal, with average counts below 10 stained nuclei in each group. Differences across feeding groups, with or without running disks, could not be reliably detected by cursory visual inspection (i.e., were not obvious to the eye).

Quantification of the total number of FOS+ nuclei (bilaterally, in four sections rostral to caudal) by visual counting revealed no significant main effect of feeding condition \((F_{(2,28)} = .28, p = .75)\), disk availability \((F_{(1,28)} = .05, p = .82)\) or interaction \((F_{(2,28)} = 1.07, p = .35)\) (Fig. 3A). Differences in cell counts for each of the four levels, rostral to caudal, were also not significant.

Quantification of FOS+ nuclei by object identification revealed a similar lack of main effect for feeding condition \((F_{(2,31)} = 0.57, p = .55)\), disk availability \((F_{(1,31)} = .78, p = .43)\) and interaction \((F_{(2,31)} = 1.56, p = .22)\). Given the prediction that SCN FOS would be decreased prior to mealtime in food-anticipating mice, specific contrasts were explored using t-tests, and this revealed a significant reduction in stained nuclei in the food-anticipating mice with running disks, compared to the acute food-deprived mice with running disks \((p = 0.03; \text{Fig. 3B})\). No other contrasts were significant.

Quantification of FOS by OD revealed a near significant main effect of feeding condition in the groups housed with running disks \((F_{(2,19)} = 3.44, p = .053)\) but not in the groups housed without running disks \((F_{(2,12)} = 0.125, p = .883)\; \text{Fig. 3C})\). Among the groups with running disks, the food anticipating group exhibited significantly lower OD compared to both the ad libitum-fed group \((p = .011)\) and the acute food deprivation group \((p = .039)\). The acute food deprivation group did not differ from the ad libitum-fed group \((p = .225)\).

To explore possible regional differences in the effects of restricted feeding on SCN FOS, OD was compared across groups in the SCN shell and core separately, in mice housed with running disks. There was a significant main effect of feeding condition on OD in the shell \((F_{(2,18)} = 4.91, p = .019; \text{Fig. 4A})\) but only a trend for an effect in the core \((F_{(2,18)} = 2.51, p = .10; \text{Fig. 4A})\). In the shell, OD was significantly lower in the restricted feeding group compared to both the ad libitum-fed \((p = .009)\) and acute food deprivation groups \((p = .006)\). In the SCN core, OD was lower in the
restricted feeding group compared to the food deprivation group \((p = .027)\) and the \textit{ad libitum} fed group \((p = .045)\), but these differences can be considered significant only without adjusting the significance threshold for multiple comparisons.

Potential regional differences in the effects of restricted feeding on SCN FOS were further examined by comparing OD separately at four levels of the SCN including anterior, medial-anterior, medial-posterior and posterior (Fig. 2A–D). In the restricted feeding group, OD was significantly reduced relative to both the \textit{ad libitum}-fed and acute food deprived groups in the anterior and medial-posterior SCN sections \((p < .05, \text{Bonferroni corrected})\), but was reduced only as a trend in the medial-anterior and posterior sections (Fig. 4B). The \textit{ad libitum}-fed and acute food deprived groups did not differ significantly at any level.

**Correlations between FOS quantification methods**

Pearson Product Moment Correlation Coefficients were calculated for each pair of quantification techniques, using total SCN FOS data, and pooling all groups (with and without running disks). Object identification and OD were strongly correlated \((r = 0.741)\). Hand counts were weakly correlated with both object identification \((r = 0.263)\) and OD \((r = 0.102)\).

**In vivo electrophysiology**

To explore the relationship between SCN electrical activity and locomotion, the data were first plotted in the format conventionally used for displaying food
anticipatory locomotor activity. Accordingly, the 10-s time bins were summed into 10-min bins, and plotted in raster style with consecutive days aligned vertically (e.g., Fig. 5A, B, E, F). To facilitate comparison between MUA and locomotion, the data for each mouse were then normalized, averaged across days of ad libitum feeding (2–7 days) and restricted feeding (the last 2–7 days in LD) and plotted as average waveforms on the same axis (e.g., Fig. 5C, D, G, H). Visual inspection of waveforms for ad libitum food access days revealed the expected inverse association between SCN MUA, which peaks in the middle of the light period (a criterion for inclusion), and locomotor activity, which is concentrated at night. Correlations between locomotion and SCN MUA in 10-min bins across the 24-h day were strongly negative in all the mice \( (r = -0.66 \pm 0.04) \).

Despite this inverse association between locomotion and SCN MUA in 10-min bins on a circadian timescale, the waveforms also reveal that SCN MUA can change rapidly with variations in locomotor activity within the daily rest and active phases. The mice could be grouped according to the direction of change of MUA during bouts of activity. Of the seven mice, SCN MUA was suppressed in three mice, activated in two mice, and activated during the day but suppressed during the night in two mice. These response patterns were consistent across ad libitum and restricted food access conditions.

Results for the mice showing SCN suppression are presented first. During restricted feeding, the mice showed the expected rise of FAA beginning 1–2 h prior to mealtime. SCN MUA changed in the opposite direction at this time, as evident in data plotted at 10 min (Fig. 5D, H) and 10 s (Fig. 6) resolution. In two of the three mice (Figs. 5D and 6C–F), the daily waveform of MUA evident during ad libitum food access was preserved during restricted feeding, with the daily peak occurring in the middle of the light period and a negative correlation between MUA and locomotion in 10-min bins across the 24-h day \( (r = -0.59 \pm 0.07) \). In the remaining case (Figs. 5E–H and 6A, B), the peak of the daily waveform of MUA was shifted later by 6.3 h (acrophase of a cosine fit), and the correlation with locomotor activity across the 24-h day was lost \( (r = 0.07, p > .2) \). Despite the relatively lower level of SCN electrical activity during the day in this mouse, FAA was not enhanced either in duration or peak level compared to the other two mice (Fig. 5H).

To quantify the changes in SCN MUA during transitions from behavioral rest to activity, MUA during the first 10 s of active bouts of any duration was expressed as a percent change from the last 10 s of the preceding bout of inactivity, i.e., the least stringent definition of rest and activity bouts possible. Three days of ad libitum food access and three days of restricted feeding were analyzed from each mouse. Surprisingly, the distribution of changes was Gaussian and centered close to zero, indicating that for all activity bouts, the direction of change in SCN electrical activity during the first 10 s of movement is essentially random. The distributions did not differ between food access conditions (ad libitum vs scheduled) or between day and night. Similar Gaussian distributions were obtained employing 30-s or 2-min criteria for defining rest and active bouts. Although Gaussian distributions centered at zero were unexpected, the finding is not inconsistent with prior observations that SCN electrical activity does
not always change during active states, and that the magnitude and direction of change may vary with different behaviors (Van Oosterhout et al., 2012).

Despite variability in direction and magnitude of MUA change, suppressions were clearly evident in the MUA waveforms during many bouts of daytime activity. These bouts were used to determine whether suppressions were enhanced during restricted feeding, and whether changes in SCN MUA predict activity onset. Bouts of daytime activity associated with MUA suppression were taken from 2 days of ad libitum food access and 2 days of restricted feeding. For this analysis, changes in MUA counts were expressed as a percent of the MUA daily range for each mouse. During ad libitum food access, MUA counts during the first 2 min of these bouts decreased by 42.2 ± 5.2% of the daily range, relative to the 2 min of preceding rest. During restricted feeding, the decrease averaged 41.7 ± 3.1% ($p > .2$ relative to ad libitum; Fig. 7A).

Linear regression was then used to calculate the slope of MUA during the 2 min of rest immediately preceding these activity bouts. This analysis revealed no
consistent direction of change in SCN MUA prior to activity. The group mean slopes of \(4.49 \pm 5.08\) (food ad libitum) and \(0.51 \pm 1.78\) (food scheduled; \(p = .4\) vs ad libitum), reflect a near equal mix of positive and negative values, few of which were significantly different from 0 (Fig. 7B). Similar results were obtained using the last 10 min of rest prior to activity bouts of at least 30-s duration. However, in this sample of 200 contiguous rest bout – active bout pairs (combining ad libitum and scheduled feeding), slopes were on average slightly positive, and this was marginally significantly different from a theoretical mean of 0 (mean = 1.14 ± .56; 1 sample t-test, \(t(199) = 2.02, p = .045\)).

Four mice exhibited increases rather than decreases in SCN MUA during daytime activity (Fig. 8A–H). By comparison with the last 2 min of rest, average MUA during the first 2 min of activity in these mice increased by 36.6 ± 4.4% of the daily range during ad libitum food access days, and 49.5 ± 5.2% of the range during restricted feeding days \((p > .05; \text{Fig. 7C})\). In two mice the activations were evident during both the light and the dark period, and thus were classified as light-independent (Fig. 8E–H). In the two other cases the increases were evident only during the light period, while activity at night was associated with MUA suppression (Fig. 8A–D). The daytime increases in these two cases were therefore classified as light-dependent. One of these mice (Fig. 8A, B) exhibited a sustained increase of SCN MUA during mealtime, with small suppressions when locomotor activity counts were registered (Fig. 9A, B). This pattern suggests a combination of light-dependent activation (animal is awake with eyes open at mealtime) modulated by movement-related suppression.

Analyses of the slope of SCN MUA prior to activity bouts in this group of four mice revealed a preponderance of positive values (Fig. 7D). Again no differences were evident between ad libitum and restricted food access, and data from those conditions were therefore pooled. Using the last 2 min of rest prior to active bouts, MUA slope averaged 20.13 ± 4.5 (1-sample \(t(81) = 4.46, p < .0001\)). In 10-min rest bouts prior to active bouts, slopes averaged 4.33 ± .56 (1 sample \(t(212) = 7.66, p < .0001\)).

**Histology**

Histological examination of electrode placements in six of seven mice confirmed that electrodes were in the SCN, as predicted based on the timing of the electrical activity rhythm during ad libitum food access. Due to the size of the recording electrodes relative to the SCN, localization of the recording site within the SCN can only be approximated (Fig. 10). In the mice that exhibited suppression of SCN electrical activity during locomotor activity, the electrode tips appeared to be located more dorsally in the SCN. In three of four mice that exhibited activation of MUA in association with locomotor activity, the electrode tips extended to the ventral SCN (e.g., Fig. 10B). The electrode location in one case could not be determined.
DISCUSSION

Multiple lines of evidence indicate that output from the LD-entrained SCN pacemaker opposes activity and promotes sleep during the rest phase of the circadian rest–activity cycle (Mistlberger, 2005; Fleshner et al., 2011). Despite this, nocturnal rodents readily anticipate a daily meal scheduled in the light period, when they normally would be sleeping. The SCN pacemaker in food-restricted rats and mice typically remains entrained to the LD cycle (Damiola et al., 2000; Kalsbeek et al., 2000; Stokkan et al., 2001). The food-entrained circadian oscillators believed to drive daytime FAA must therefore compete with the SCN pacemaker. One possibility is that FEOs impose a daily rhythm of inhibition on sleep-promoting SCN output, thereby permitting expression of food-seeking behavior (Mistlberger, 2006; Moriya et al., 2009; Acosta-Galvan et al., 2011; Landry et al., 2011). Expression of the immediate early gene product FOS, a molecular signature of neural activity integrated over time, has been found to decrease in the SCN of nocturnal rodents in association with behavioral arousal in the usual sleep period, but evidence for this relationship in food-anticipating mice has been inconsistent (Challet et al., 1997; Angeles-Castellanos et al., 2004; Acosta-Galvan et al., 2011; Landry et al., 2011). The food-entrained circadian oscillators believed to drive daytime FAA must therefore compete with the SCN pacemaker. One possibility is that FEOs impose a daily rhythm of inhibition on sleep-promoting SCN output, thereby permitting expression of food-seeking behavior (Mistlberger, 2006; Moriya et al., 2009; Acosta-Galvan et al., 2011; Landry et al., 2011). Expres-
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period, but evidence for this relationship in food anticipat-
A. Direct recordings of SCN electrical activity in vivo enabled us to explore this issue, and to also determine whether decreases in neural activity inferred from FOS data precede or follow the onset of FAA and persist or reverse during pauses in activity. We observed considerable heterogeneity in the relationship between SCN MUA and locomotion. Daily waveforms revealed unambiguous suppression of MUA during FAA and other bouts of movement in three of seven mice, a light-
dependent increase of MUA during activity in two mice, and a light-dependent increase of MUA with suppressions during nocturnal activity in two mice. Automated quantification of changes in SCN MUA across the transition from rest to active states was challenged by variability in responses. As noted in previous studies (e.g., Van Oosterhout et al., 2012), not every activity bout is associated with an acute change in MUA, and our results suggest a stochastic process in the direction of change from the last 10 s of rest to the first 10 s of activity. Nonetheless, in each mouse bouts of loco-
motion could be readily identified during which SCN MUA
showed a large change in a consistent direction. Analyses of these bouts revealed that the magnitude of change did not differ between ad libitum and scheduled feeding conditions and was similar at different times of the day. Furthermore, during pauses in activity prior to mealtime, MUA changes reversed, and in some cases reversal began before the end of the activity bout. In the group of mice that predominantly exhibited suppression of MUA during locomotion, the slope of MUA prior to activity onset varied stochastically, with a slight bias toward more positive slopes, whereas in mice that exhibited increased MUA during activity, MUA tended to increase in advance of activity onset.

The electrophysiological results permit several conclusions. First, the firing rate of SCN neurons sampled by multi-unit micro-electrodes can increase or decrease in association with locomotion. Second, these changes in SCN neural activity do not distinguish FAA from bouts of activity occurring at other times of the day or during ad libitum food access. Third, in mice that predominantly exhibited decreases in SCN MUA during activity, the slope of MUA during rest does not predict activity onset. Taken together, the results do not lend support to a conceptual model in which FEOs directly impose a daily rhythm of inhibition on the SCN, to suppress sleep-promoting daytime output and permit expression of food anticipatory behavior. Rather, the data are more parsimoniously interpreted as further evidence that neural circuits encoding behavioral output or sensory feedback from movement project to the SCN and alter its activity (Webb et al., 2014). Changes in SCN activity during FAA therefore appear to represent another example of SCN sensitivity to neural and/or endocrine correlates of behavior. This conclusion leads to the prediction that suppression of SCN neural activity during bouts of FAA should be blocked by inactivation of SCN inputs, such as those from the thalamic intergeniculate leaflet and median raphe, that have previously been iden-
tified as critical for effects of behavior on SCN pacemaker cycling (Morin, 2013; Webb et al., 2014).

We did not expect to see activations of SCN neural activity during movement in a majority of mice (four of seven), but such responses have been noted previously (De Boer et al., 2003; Van Oosterhout et al., 2012; Sakai, 2014). In two of the mice, the activations were evident in the day, but not at night, so these responses can be classified as light-dependent. In the other two cases, the neurons sampled may respond differently to the same inputs that suppress other SCN neurons, or they may respond to different inputs encoding different behaviors. Previous studies of SCN electrophysiology have primarily utilized running wheels or non-specific motion sensors to quantify locomotion, and have emphasized suppression of SCN MUA as the primary correlate of movement. However, video and EEG analyses have shown that SCN neurons may become more active during some behaviors, or on transitions between sleep stages, or between sleep and wake (De Boer et al., 2003; Van Oosterhout et al., 2012; Sakai, 2014). More work will be needed to determine how varying responses of different SCN neurons are integrated to produce a coherent functional output response. At present, the FOS data may represent the best indicator of cumulative SCN output during activity.

It is possible that neurons activated during locomotion are inhibitory to SCN output cells, which would contribute to a net reduction of FOS.

Perhaps more surprising than the prevalence of MUA activations during locomotion in four mice was the significant tendency for SCN MUA to rise in advance of activity onset. This was evident in both 2-min and 10-min segments of continuous rest immediately preceding...
locomotion. At present, it is not clear how these changes should be interpreted. Visual inspection of the MUA waveforms in these cases indicates that during rest bouts following active bouts, MUA first decreases and then rises. This U-shaped response may reflect ultradian variations in sleep–wake states. For example, SCN units activated during movement (whether in response to locomotion or to light exposure) may be quiescent during slow wave sleep (De Boer et al., 2003), which normally is concentrated early in sleep bouts. Some SCN units that are quiescent during slow wave sleep become active during REM sleep (De Boer et al., 2003), which normally follows slow wave sleep. Wake (and thus activity) onset is more likely after REM sleep. SCN MUA may be predictive of activity onset because sequences of slow wave sleep and REM sleep are predictive of waking.

One of the seven mice showed an ~6-h delay in the peak of SCN MUA when food was restricted to the midday. In this mouse, SCN MUA was low throughout the daytime prior to scheduled feeding, a result that would seem to fit with the hypothesis that daytime FAA requires suppression of SCN output. However, the result is limited to the one case, and FAA in this mouse was unremarkable. The phase of the SCN circadian clock, as defined by rhythms of clock gene expression, is assumed to be impervious to daytime feeding schedules, but modalities of phase have been observed when feeding schedules are maintained for several weeks or longer (Challet et al., 1997; Pavlovski et al., 2004). Whether or not the change in MUA waveform in this mouse reflects a shift in the SCN molecular clock, it is possible that the effect is limited to a particular subgroup of SCN neurons sampled by the electrode in this mouse.

The results of this study indicate that on average, SCN neural activity is suppressed prior to mealtime in food-restricted mice. Suppression is most likely to be evident in an aggregate measure of neural activity such as FOS expression, and in mice with access to a wheel or disk to facilitate running. Direct recordings of SCN electrical activity in freely moving mice confirm that SCN MUA can also be activated during locomotion. Whether activations are limited to specific cell groups within the SCN, and how these may contribute to the net suppression of neural activity reflected in FOS expression, remain to be determined. Changes in SCN MUA, in either direction, were not unique to food anticipation, were coincident with activity and were not sustained during pauses in activity. The evidence therefore suggests that changes in SCN activity prior to mealtime represent a response to ongoing behavior, rather than an imposed, FEO-dependent rhythm of inhibition that is permissive to FAA onset. Nonetheless, a reduction of SCN output following the onset of locomotor activity may serve to reinforce the alerting function of FEOs and promote a sustained state of behavioral arousal that is necessary for animals to exploit feeding opportunities in the day.

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