Persistence of a behavioral food-anticipatory circadian rhythm following dorsomedial hypothalamic ablation in rats

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Landry, G. J., M. M. Simon, I. C. Webb, R. E. Mistlberger. Persistence of a behavioral food-anticipatory circadian rhythm following dorsomedial hypothalamic ablation in rats. Am J Physiol Regul Integr Comp Physiol 290: R1527–R1534, 2006. First published January 19, 2006; doi:10.1152/ajpregu.00874.2005.—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 radiofrequency 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 3-h 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 48-h food deprivation test and reappeared during a 72-h 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.

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 2–4 h 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 24-h 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 30 yr 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, knockout 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 hypothypophysis (25) eliminates the food-anticipatory rhythm of body temperature, but not of behavior. FAA also persists after ablation of the hypothalamic subparaventricular zone (30, 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 adenectomy 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.

**Materials and Methods**

**Subjects and apparatus.** Adult male Sprague-Dawley rats (n = 14, 310–320 g; Charles River) were housed individually in polypropylene cages (45 × 24 × 20 cm) equipped with wire floors and tops, a water bottle, and a black opaque tube (15 cm long × 8 cm diameter) for sleeping or light avoidance (12:12-h light-dark cycle, ~1,000 lux). Food was available in a metal cup mounted on a manually controlled carousel, accessed via a 4 × 4 cm 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 10-min intervals and stored for analysis off-line using Circadia (Dr. T. A. Houpt, Florida State University, Tallahassee, FL) operated on a Macintosh computer.

**Surgery and histology.** Seven rats received bilateral radiofrequency lesions of the DMH. The rats were anesthetized for stereotaxic surgery using ketamine (90 mg/kg Ketalar; Bimeda-MTCAnimal 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 0.5 mm of the flattened tip. Current was supplied by a Grass LM3 Lesion Maker. Stereotaxic coordinates were ±0.5 mm lateral and 3.5 mm posterior to bregma and 8.5 mm 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 50-μm 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.

**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 18 h, and for the next 30 days was provided for 3 h each day beginning 6 h before lights off. Food consisted of powdered rat chow mixed with corn oil to the consistency of a wet sand. Food bins were manually rotated into position each day and removed and weighed 3 h later. Water bottles were also weighed daily to track fluid consumption. After 30 days, food was removed for 51 h. 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. 1 for activity charts illustrating this sequence. To minimize stress and activity artifacts, the rats were not weighed during the behavioral recording.

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 3 h 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 3 h 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 ±SE.

RESULTS

Histology. Figure 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 100–200 μm 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 pre-mammillary nuclei. The rostrocaudal extent of the DMH can be estimated at ~1.6 mm based on Paxinos and Watson (52) and ~1.12 mm 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. 2, A–C.

The lesion parameters were intended to produce ablations centered on the DMH and extending 2 mm 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. 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. 3A). A third case was also classified as partial; although the cavity extended ~1.6 mm 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. 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. 2, I–J and 3, C–E). In all of these cases, the lesion cavities subsumed the fornix and mammilothalamic 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 (reunions, 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.

Fig. 1. Activity records of representative rats with no lesion (A), partial dorsomedial hypothalamic nucleus (DMH) damage (B), and total DMH ablation (C). Each line represents a day, with time plotted from the left in 10-min bins. Bins in which 3 or more activity counts were registered are represented by a vertical bar. The 12-h 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.
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. 1A, 2D, and 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. 4A). During restricted feeding and food deprivation, nocturnal activity levels increased in the DMH lesion rats, but not in the intact rats (Fig. 4, B and 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.

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. 1, A–C; 2, D, H, and I; 3, F–J; and 4B) 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 3 h 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. 5A). Each of the six blocks of restricted feeding days was significantly different from the baseline block in both the intact group \( F_{(11,6)} = 10.24, P < 0.0001 \); pairwise comparisons significant at \( P = 0.02 \) or better and DMH lesion group \( F_{(11,6)} = 7.92, P < 0.0001 \); pairwise comparisons significant at \( P = 0.003 \) or better. FAA counts remained high during 2 days of food deprivation immediately following the last scheduled meal (e.g., Fig. 1, A–C), declined when food was provided ad libitum, and increased again when food was removed for 3 days (Figs. 1, A–C and 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. 5B); ratios during restricted feeding were significantly different from baseline by the first 5-day block in the DMH lesion rats \( F_{(11,6)} = 11.58, P < 0.0001 \); by the second 5-day block in the intact rats \( F_{(11,6)} = 4.92, P < 0.0001 \); and by the second 5-day block in the DMH lesion group \( F_{(11,6)} = 4.92, P < 0.0001 \) vs. intact rats \( P < 0.05 \) 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.

Food and water. Daily food intake increased over the first 7–19 days of restricted feeding (Fig. 6). This was more appar-
ent 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. 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.

**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 3 h 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. 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.”

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