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Response to Comment on “Differential Rescue of Light- and Food-Entrainable Circadian Rhythms”

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The points raised by Mistlberger *et al.* arise from a shortcoming in their approach, namely, that they measure the response to food restriction by using food-seeking behavior, which is confounded by homeostatic inputs. We used unrelated circadian-driven physiological responses, and we stand by our finding that the dorsomedial nucleus of the hypothalamus contains a food-entrainable oscillator that is sufficient for entrainment of circadian rhythms of body temperature and locomotor activity.

Mistlberger *et al.* (1) raise several questions about our recent study (2), most of which we previously addressed in a published debate on the methodology for recording food-entrainment of circadian rhythms (3, 4). We maintain that the methods they use measure food-seeking behaviors rather than circadian rhythms. Here, we summarize our position and answer specific questions about the *Bmal1*^{-/-} mice used in our study.

Our disagreement with Mistlberger *et al.* arises from a shortcoming in the design of their studies. We are interested in the brain mechanisms for regulating circadian rhythms and how they are influenced by feeding schedule. To measure this, it is critical to avoid using output measures that are related to feeding itself (which is the variable being manipulated and which has many homeostatic and cognitive drivers beyond circadian rhythms). We therefore measured body temperature as well as overall cage locomotor activity by telemetry (2) and have measured wake-sleep rhythms as well in our earlier work (5). For reasons discussed previously (3), we believe that Mistlberger *et al.* and others cited in (1) are using measures of “food-anticipatory activity” that are essentially food-seeking behaviors and reflect factors other than a circadian process. In their cited experiments involving DMH-ablated rats (6), the infrared movement sensor was placed over the food bin, where it is more sensitive to smaller movements near the food bin than those in distant parts of the cage. Hence, the study did not measure general cage activity but rather is biased toward movements in the vicinity of the food bin. In the mouse studies described in (1), Mistlberger *et al.* placed the food on the floor of the cage, where the hungry mice had to forage for it. Thus, the activity they measured as the hungry mice looked for food

after 16 hours of food deprivation contains a strong element of homeostatic food-seeking behavior, not only circadian rhythmicity. Other investigators use wheel-running as a measure, which is similarly related to foraging behavior in rodents, and which Mistlberger himself has shown is increased by hunger (7). We agree that it is not possible to eliminate food-seeking behavior in hungry animals by DMH lesions or clock gene deletions, because these behaviors have drivers other than circadian rhythms. It is for this reason that, in our studies, we use body temperature, general cage activity, and wake-sleep cycles as readouts of circadian rhythmicity, not food-seeking behavior.

The data presented in (1) strongly support the interpretation that the authors are measuring homeostatic, not circadian, responses in the *Bmal1*^{-/-} mice. First, the activity levels of *Bmal1*^{-/-} mice shown in figure 1 in (1) are nearly as high as those in the wild-type mice. As shown by McDearmon and colleagues (8) and our own work, *Bmal1*^{-/-} mice have overall activity levels about one-fifth those of wild-type mice. The high activity levels recorded by Mistlberger *et al.* likely represent increased food-seeking behaviors in hungry mice, rather than overall activity. These disparate findings clearly indicate that the Mistlberger laboratory and our laboratory are not measuring the same thing. Second, Mistlberger (4, 9) has advocated a period of food deprivation after restricted feeding, which is necessary to demonstrate a self-sustaining rhythm of activity that defines circadian entrainment. We did this by including one day of food deprivation at the end of restricted feeding, and not only did the control mice (but not the *Bmal1*^{-/-} mice) show a circadian increase in body temperature and locomotor activity before the time of the food deprivation but also they showed a reduction in these measures after the time of food presentation [see our figure 2 in (2)], as required for a circadian response. Hungry mice engaged in homeostatic food-seeking would continue to be active into the food presentation period, a critical control that is missing from figure 1 in (1). Instead, they released the

animals from restricted feeding into ad libitum food presentation. In their experiment, the wild-type animals persist in having increased activity before the previous onset of food presentation (i.e., the influence of the food-entrained oscillator persists for several days after the animals are placed back in ad libitum feeding). The *Bmal1*^{-/-} mice, when released from restricted feeding back into ad libitum feeding, show no remnant of the increase in activity before the habitual time of food presentation. In other words, their own experiment verifies our result: that a *Bmal1*-based clock is necessary for the circadian entrainment component of the food-seeking behavior they measure and that the anticipatory food-seeking behavior seen in the *Bmal1*^{-/-} mice is not circadian because it does not survive even a single cycle of ad libitum food presentation or reappear at the expected phase (6) during the subsequent fasting period.

Mistlberger questions whether our *Bmal1*^{-/-} mice under restricted feeding were healthy. We addressed this issue in our paper (2), noting that because our animals failed to show food anticipation, we woke the animals up during the feeding period so that they would have the opportunity to eat. This did not contribute to circadian entrainment, because the animals that required awakening to avoid starvation were the same animals that never showed circadian rhythms under food restriction. However, this method did allow the animals to remain healthy, as reflected by the fact that they did not lose weight during restricted feeding, and they ate about 85% of the number of calories per day ingested by animals on an ad libitum diet. Mistlberger *et al.* (1) also question whether the periods of torpor we record in our mice represent poor health. Torpor is a normal defense mechanism used by mice when faced with a 20-hour fast in a cool laboratory (22°C, below thermoneutrality for singly housed mice) (10). Even wild-type mice in our laboratory on 4 hours/day restricted feeding schedules show occasional periods of torpor. The only way to measure torpor is by doing temperature recordings in the mice, because torpor superficially resembles sleep, except that the animals' body temperature drops to about 30 to 31°C (in our laboratory under these conditions, the depth and duration of the periods of torpor depend on the room temperature and previous metabolic state of the animals). The methods described in (1) do not include measurement of body temperature. Thus, the assertion that their animals do not go into torpor is puzzling. We are confident that if other laboratories repeat our experiments using the measures that we used, they will find similar results.

Finally, the assertions made by Mistlberger *et al.* concerning the role of the DMH in food-entrainable circadian rhythms are not accurate. We and Mieda *et al.* (11) did not find that the DMH is one of “a number of” brain regions whose clock gene expression is “synchronized by scheduled feeding.” What was found is that the DMH is the only region of the brain that has self-sustained cycles of

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clock gene expression induced de novo by restricted feeding (11). We never stated that “the DMH is the site of the long-sought food-entrainable circadian pacemaker for behavior.” Rather, we showed that the DMH contains an inducible clock that is sufficient to restore food-entrained circadian rhythms (2). We do not claim that there are no other sites that can function as food-entrainable oscillators, because our experiments do not address that possibility, nor do we focus on “behavior” as the output (as Mistlberger does), precisely because food-seeking behavior is confounded by the food restriction itself. Instead, in both of our studies implicating the DMH in mediating entrainment to restricted

feeding (2, 5), we measured circadian physiology, using outputs (body temperature, overall locomotor activity, and wake-sleep states) that are independent of the food restriction manipulation.

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