Metabolic rhythm abnormalities in mice lacking VIP-VPAC2 signaling

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The circadian pacemaker in the suprachiasmatic nuclei (SCN) controls endogenous near 24-h physiological and behavioral rhythms in metabolism, neuroendocrine function, and locomotor activity. Recently, we showed that vasoactive intestinal polypeptide (VIP) and its receptor, VPAC2, are critical to the intercellular communication between individual SCN neurons, and appropriate synchronization and phasing of these oscillatory cells. Mice defective in VIP signaling manifest grossly impaired circadian rhythms of SCN neuronal firing activity and are typically unable to maintain rhythmic wheel-running behavior in the absence of external time cues. Here we report that daily rhythms of metabolism and feeding behavior are also overtly altered in these animals. Under diurnal conditions (12:12-h light-dark; LD), metabolic and feeding rhythms are advanced in mice lacking either VIP or VPAC2 receptor expression, peaking in late day, rather than early night, as observed in wild-type mice. When placed in constant light (LL), both VIP-deficient and VPAC2 receptor-knockout mice exhibit dampening of metabolic and feeding rhythms, which deteriorate after a few days. In addition, overall metabolic rate is greatly reduced in VPAC2-knockout mice, when compared with wild-type mice, regardless of lighting condition. The advancement of metabolic and feeding rhythms in these mice under LD suggests that these rhythms are less sensitive to masking by light. These results demonstrate that altering SCN function not only affects neuronal and wheel-running activity rhythms but also dramatically impairs temporal regulation of metabolism and feeding.

MATERIALS AND METHODS

Animals. Vipr2−/− mice were obtained from Tony Harmar (University of Edinburgh), while VIP/PHI−/− mice were obtained from Chris Colwell and James Waschek (University of California at Los Angeles). The methods followed for generating these mice have been described previously (6, 10). Mice were introgressed onto a C57BL6 wild-type (WT) background for seven generations and maintained as a colony at the University of Manchester. All studies detailed herein were licensed under the Animals Act of 1986 (Scientific Procedures) and received ethical approval from the University of Manchester animal welfare committee. Male mice 12–16 wk of age were used for all experiments and were housed at an ambient temperature of 20–22°C and maintained in a 12:12-h light-dark cycle (LD), unless stated otherwise.

Indirect calorimetry. WT, Vipr2−/−, and VIP/PHI−/−, mice (n = 7, 11, and 6 mice, respectively) were housed individually in calorimetric cages (Columbus Instruments, Columbus, OH). Mice were monitored for a minimum of 5 days in LD (intensity with lights on: ~500 lux) followed by a minimum of 5 days in LL, during which time oxygen consumption (V̅O₂), carbon dioxide production (V̅CO₂), and respiratory quotient (RQ) were measured every 10 min. Cages were not equipped with running wheels, and environmental enrichment was limited to bedding material. Standard rodent chow (3.7 kcal/g, 20% diurnal; circadian; vasoactive intestinal polypeptide; VPAC2 receptor; suprachiasmatic

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protein, 10% fat by energy; BeeKay International, Hull, UK) and water were supplied ad libitum throughout these experiments. Measures of metabolic rate were carried out in four independent experiments. Illustrations of $\text{VO}_2$ and $\text{VCO}_2$ traces in Fig. 1 depict group (genotype) averages taken from a representative experiment. The location and setup of our calorimetric cages did not allow for adequate monitoring of the health and well-being of the mice by facility staff under conditions of constant darkness; therefore, this free-running condition was not examined.

Feeding studies. Activity at the food hopper was monitored within the calorimetric cages using a laser beam set across the opening of the hopper ($n = 7$ WT, $11 \text{Vipr}^2$ 
$\text{/}^2$, and $6 \text{VIP/PHI}^2$ mice). The number of beam breaks (minimum 1-s duration) was recorded for every 10-min time bin. Leading up to and during feeding studies, mice ($n = 6$ WT, $8 \text{Vipr}^2$, and $7 \text{VIP/PHI}^2$ mice) were housed individually, and food intake was monitored at four intervals in the diurnal cycle [zeitgeber time (ZT) 0, 6, 12, 18].

Analysis of metabolic rhythms. Rhythms in metabolic activity were analyzed using curve-fitting software developed in-house. Recordings from individual animals during LD and LL portions of the experiment were analyzed separately. Initially, data were normalized such that they spanned a range of values between 100 and 100. The normalized data were then fit with the equation $Y = A \sin(B(x + C))$ using the Newton-Raphson iterative method, where $A$ equaled the amplitude of the rhythm, $B$ equaled the period in radians per hour, and $C$ determined the phase. Initial values of $A, B,$ and $C$ were estimated from the best-fitting curve of a series of >3,000 standard curves, which exhibited periodicities between 3 and 34 h and a range of different amplitudes and phases.

Statistics. Data are presented as means ± SE, and generally, statistical significance was determined using one-way or two-way ANOVA with Bonferroni’s post hoc test using the software suite Prism (GraphPad, San Diego, CA). Statistical differences in mean $\text{VO}_2$ and $\text{VCO}_2$ were determined using general linear model analysis of covariance (ANCOVA) with mouse body weight as a covariant to metabolic gas exchange rate, using the SPSS software suite.

RESULTS

Reduced metabolic rate in mice lacking VIP or VPAC2 expression. Metabolic rate was assessed in WT and transgenic mice by indirect calorimetry, with gas sampling every 10 min for a period of $\approx$10 days. When subjected to LD photoperiod, clear daily rhythms in oxygen consumption ($\text{VO}_2$) and carbon dioxide production ($\text{VCO}_2$) were observed in WT mice, peaking in the first half of the night (Fig. 1). Rhythmic $\text{VO}_2$ and $\text{VCO}_2$ cycles were also observed in VIP/PHI$^2$ and Vipr$^2$ mice, although $\text{VO}_2$ and $\text{VCO}_2$ levels were greatly reduced in both strains during the dark (active) phase of the cycle ($\text{VO}_2$ WT: 3,110 ± 105 ml·kg$^{-1}$·h$^{-1}$, Vipr$^2$: 2,595 ± 85, $P < 0.01$, VIP/PHI$^2$: 2,789 ± 89; $\text{VCO}_2$ WT: 3,351 ± 137, Vipr$^2$: 2,660 ± 108, $P < 0.05$, VIP/PHI$^2$: 2,808 ± 90, $P < 0.05$; Fig. 1, B and D). The statistical significance of this observed
reduction was maintained when the V˙O2 readings for the VPAC2 knockout mice were averaged over the full 24-h period (V˙O2 WT: 2,884 ± 91, Vipr2−/−: 2,594 ± 109, P < 0.05). In contrast, VIP/PHI−/− mice were found to have a similar 24-h mean V˙O2 to those measured in WT mice (2,787 ± 111 ml·kg−1·h−1). Both strains of transgenic mice showed a reduction in RQ during ZT 12–24 and a slight increase during ZT 0–12, compared with WT mice.

The reduced metabolic activity of VIP/PHI−/− and Vipr2−/− mice over ZT 12–24, typically the active phase of the day for mice, appeared to be due, in part, to an alteration in the timing of activity in these mice. To address this issue, V˙O2 and V˙CO2 recordings for each animal were individually analyzed using curve-fitting software to generate a waveform representing the dominant rhythm in its daily metabolic gas exchange. Fig. 2 illustrates V˙O2 recordings overlaid with the resulting rhythm waveform from representative individuals of each genotype (genotype averages are illustrated in Fig. 5). An advancement in the peaks of V˙O2 and V˙CO2 are clearly visible in the VIP/PHI−/− and Vipr2−/− mice under LD. WT mice exhibited strong rhythms that peaked in the early night (V˙O2: ZT 15.7 ± 0.2 h; V˙CO2: ZT 16.3 ± 0.3 h). In contrast, Vipr2−/− and VIP/PHI−/− mice exhibited peaks in V˙O2 and V˙CO2 3–4 h earlier than those of WT individuals (V˙O2: ZT 12.5 ± 0.4 h and 11.9 ± 0.7 h, respectively; V˙CO2: 12.7 ± 0.2 and 11.9 ± 0.7, respectively; all P < 0.01 vs. WT).

Disrupted feeding behavior in VIP-VPAC2 signaling-impaired mice. A daily rhythm in feeding behavior was observed in WT animals, with both hopper visits (Fig. 3, A and B) and food intake (Fig. 3C) being maximal during the early night (ZT 12–18), similar to the pattern observed in metabolic rate. Unlike WT mice, which made over 70% of the visits to the food hopper at night (71.8 ± 2.6%), Vipr2−/− and VIP/PHI−/− mice exhibited a spread of food hopper activity throughout the day and night (Vipr2−/−: 51.6 ± 1.6% at night, VIP/PHI−/−: 50.3 ± 3.1% at night), with the highest level of activity observed in the late day (ZT 6–12). Measures of food intake paralleled hopper visits, with both transgenic strains of mice showing the highest level of consumption between ZT 6–12 (Fig. 3C).

Although not statistically significant compared with WT mice, average daily food intake was reduced in Vipr2−/− mice.

Fig. 2. Waveform analysis of V˙O2 and V˙CO2 rhythms under LD. V˙O2 and V˙CO2 rhythms for individual animals were analyzed with curve-fitting software (representative individuals shown in A and B). Peaks in the daily V˙O2 and V˙CO2 rhythms of both Vipr2−/− and VIP/PHI−/− mice were advanced in LD relative to wild-type (WT) mice, peaking close to the light-dark transition point (ZT 12, C and D). Vertical bars in C and D represent the median peak time for each genotype. **P < 0.01 vs. WT, Kruskal-Wallis test.
Effect of constant light on metabolic rhythms. Following a minimum of 5 days of monitoring metabolic gases under LD conditions, mice were switched to constant light (LL) for a further 5–6 days. Mean VO$_2$ and hopper activity records collected from representative individuals across the period of LL are illustrated in Fig. 4. Robust circadian rhythms of VO$_2$ were maintained in WT mice when switched to LL. VO$_2$ rhythms in LL were also observed in mice lacking either VIP or VPAC$_2$ receptor expression, although with much less consistency than WT mice. As with LD, patterns of hopper activity roughly corresponded with corresponding metabolic rhythms in all three genotypes under LL (Fig. 4).

Curve-fitting analysis of VO$_2$ recordings collected in LD and LL were performed and averaged across genotypes (Fig. 5, A and B). Similar results were obtained from analysis of VO$_2$ recordings (data not shown). Under LD, WT mice exhibited VO$_2$ rhythms with a period of $24\text{ h}$ (Fig. 5C). In contrast, many Vipr$_2^{-/-}$ and VIP/PHI$^{+/+}$ mice exhibited periods well below $24\text{ h}$, although a statistically significant reduction in period was only detected in the Vipr$_2^{-/-}$ mice ($P < 0.05$). This daily advancement of VO$_2$ rhythm (for example, the Vipr$_2^{-/-}$ mouse illustrated in Fig. 2A) suggests a disruption of the daily resetting of the endogenous clock in these mice. VO$_2$ rhythms under LD were weaker in the Vipr$_2^{-/-}$ mice, VIP/PHI$^{+/+}$ mice exhibited a similar 24-h mean VO$_2$ to WT mice.

DISCUSSION

Here, we provide the first examination of the loss of VIP or the VPAC$_2$ receptor on metabolic rhythms in mice. This signaling pathway plays a key role in the regulation of circadian rhythms by the SCN pacemaker. Specifically, VIP-
VPAC₂ signaling is crucial for appropriately timed cellular rhythms in SCN neurons (2–4, 18), and loss of either the receptor or its peptide ligand in mice leads to a profound disruption of circadian rhythms in wheel-running behavior when housed in constant darkness (6, 10). The current findings add to our understanding of circadian rhythmicity in these mice and demonstrate that they also exhibit altered rhythms in metabolism and feeding behavior that are apparent even under diurnal conditions, with daily peaks of metabolism that are considerably phase-advanced relative to WT mice. This is also the first study to examine the effects of constant light on circadian rhythms in mice with disrupted VIP-VPAC₂ signaling and demonstrates that such animals continue to express low-amplitude circadian metabolic rhythms in LL, albeit with a much shorter period than WT mice.

Altered clock function is often “masked” under LD due to the ability of light to suppress activity in nocturnal species such as mice. For example, wheel-running activity in mice with deficient VIP-VPAC₂ signaling is largely restricted to the dark phase of the day (6, 10, 13, 32), indicating that light is a strong repressor of this behavior and that such masking effects of light are maintained in animals lacking VIP-VPAC₂ signaling. In the present study, metabolic and feeding rhythms were advanced 3–4 h in both transgenic strains under LD, suggesting that light does not overly mask these behaviors. These findings are consistent with the abnormal phasing of wheel-running rhythms that have been observed in Vipr2⁻/⁻ and VIP/PHI⁻/⁻ mice upon release from LD to constant darkness (6, 13). Why light should strongly repress wheel running but not feeding and metabolic activity is unclear. Nonetheless, it raises some questions about how representative wheel-running activity may be of other behaviors, especially in animals with altered circadian phenotypes.

Increasing evidence suggests that VIP and VPAC₂ are important in the responsiveness of the SCN to light. Exogenous VIP application phase-shifts wheel-running rhythms in rodents (24) and SCN neuronal discharge rhythms in vitro (27) in a photic-like manner. Further, in contrast to WT mice, light pulses given during the subjective night do not induce expression of the clock genes per1 and per2 in the SCN of mice lacking the VPAC₂ receptor (10). However, components of the light-response cascade upstream of these clock genes are robustly activated in the SCN of these animals throughout the circadian cycle (13). Our observation of advanced feeding and metabolic rhythms in VIP/PHI⁻/⁻ and Vipr2⁻/⁻ mice held under LD are consistent with an alteration in the response of the SCN to light in these mutant animals. Our current findings and previous work on wheel-running behavior indicate that VIP-deficient and VPAC₂ knockout mice remain responsive to environmental lighting cues and can entrain their physiological rhythms to LD cycles, albeit with an abnormal phase angle. However, the timing of metabolic rhythms in Vipr2⁻/⁻ and VIP/PHI⁻/⁻ animals housed in LD was considerably more variable than in WT animals, with some individuals exhibiting periods substantially shorter than 24 h. This implies that such individuals free run though the LD cycle (for example, the...
VIPR2 mouse shown in Fig. 2). This possibility has never been investigated in detail and would require longer-term monitoring of metabolic rhythms under LD.

Our observation that VIP/PHI−/− and Vipr2−/− mice retain metabolic rhythms when housed in LL, without the extensive lengthening of circadian period seen in WT mice, suggests that the SCN circadian clock in these mice may be less responsive to photic conditions. The presence of VIP and VPAC2 receptor expression in the retina is consistent with such an interpretation (11, 17). However, as mentioned above, photic stimuli robustly activate c-Fos and other components of the light-responsive intracellular cascade in the SCN of Vipr2−/− animals at the same intensities that these stimuli elicit such changes in the WT SCN (13). It is also notable that under DD conditions, rhythmic Vipr2−/− and VIP/PHI−/− mice wheel-run with periods of ~22–22.5 h. Under LL conditions, we observe periods of ~23.2 h in the metabolic rhythms, suggesting that the endogenous period of these animals is lengthened in LL but not to the same magnitude as seen in WT mice. This raises the possibility of a role for VIP and the VPAC2 receptor in the parametric effects of light on the SCN clock.

Intriguingly, WT and Vipr2−/− mice exhibit a dampening of metabolic rhythms when housed in LL, whereas VIP/PHI−/− mice do not. Since Vipr2−/− mice still produce VIP, it is possible that VIP signaling through a non-VPAC2 receptor-dependent pathway may contribute to the effects of constant light on the amplitude of metabolic rhythms. An additional possibility is that these findings result also from the absence of PHI signaling in VIP/PHI−/− mice (for a review, see Ref. 34), although there are presently no known specific PHI receptors in the mammalian CNS.

The basis of the altered metabolism and feeding behavior in mice lacking VIP or VPAC2 is unclear. Neuronal output from the SCN of these mice is blunted (3, 4), and this likely has consequences for temporal regulation in SCN-recipient brain areas such as the paraventricular hypothalamus or dorsomedial hypothalamus, which are implicated in ingestive behavior and metabolism. Since VIP and VPAC2 receptors are also expressed in peripheral tissues, it is also possible that transgenic impairment in this pathway alters peripheral regulators of metabolism. The combination of alterations in central and peripheral mechanisms most likely accounts for the metabolic disturbances seen in this study. Daily food intake and metabolic rate were significantly reduced in mice lacking the VPAC2 receptor, when compared with WT mice. Interestingly, this pronounced reduction was not observed in the VIP/PHI−/− mice. Although two groups have previously reported that administration of VIP can reduce feeding in rats (15, 36), a direct role for VIP and VPAC2 in the regulation of feeding behavior remains speculative. Nevertheless, it is possible that the differences we observe in food intake between the two strains of transgenic mice may involve feeding-related action.

Fig. 5. Waveform analysis of VO2 rhythms. Waveforms representative of the VO2 rhythms observed under LD (A) and LL (B), averaged across genotypes. Daily VO2 rhythms of both Vipr2−/− and VIP/PHI−/− mice were advanced in LD relative to WT, peaking close to the light-dark transition point (A, see also Fig. 2). The period of daily VO2 rhythms lengthened in WT mice in response to LL (C). Most Vipr2−/− and VIP/PHI−/− mice exhibited a shortening of circadian period in LL, and both genotypes showed a significantly shorter-period mean when compared with WT mice (C). WT and Vipr2−/− mice experienced a dampening of daily metabolic rhythms in LL, reflected by significantly reduced peak-to-trough amplitudes (D). Vertical bars in C and D represent the median period and amplitude (respectively) for each genotype. *P < 0.05, **P < 0.01 vs. WT, #P < 0.05 vs. LD, two-way ANOVA with Bonferroni’s post hoc test.
of VPAC2 mediated through putitary adenyly cyclase-activating peptide (PACAP) signaling. The VPAC2 receptor binds PACAP with as equal affinity as VIP (9). PACAP knockout mice are reported to show reduced carbohydrate feeding and reduced expression of NPY mRNA in the arcuate nucleus (22), and central administration of PACAP decreases fast-induced feeding in rats and mice (5, 19, 20). Further, a recent study indicates that VPAC2 receptors still become activated in the VIP/PHI−/− mice, possibly due to endogenous PACAP signaling (3).

Daily metabolic rhythms are certain to reflect feeding-related arousal and activity, although feeding appears to be less responsive to light in the knockout mice. Sheward et al. (32) show that when Vipr2−/− mice are held on a restricted feeding schedule, rhythmic expression of clock genes in the liver corresponds with the time of feeding. Administration of a VIP antagonist can block food-induced increases in circulating ACTH and corticosterone in fasted rats (1). VPAC2 knockout mice lack a robust diurnal rhythm of corticosterone, although production of the steroid remains responsive to restricted feeding in these mice (32). This is in line with our own observations that WT and Vipr2−/− mice exhibit similar metabolic and feeding responses to a 48-h fast.

In conclusion, we demonstrate altered metabolic and feeding rhythms in mice with deficient VIP-VPAC2 signaling under both diurnal and constant lighting conditions, consistent with the proposed roles of this pathway in regulating the SCN clock. Additionally, we highlight differences in the circadian and metabolic profiles of VIP/PHI−/− and Vipr2−/− mice and provide evidence that the VPAC2 receptor may contribute to the regulation of feeding and metabolism independently from its role in the circadian clock.

Perspectives and Significance

The work presented here further links endogenous timekeeping pathways with key physiological outputs, namely, that of feeding behavior and metabolism. Examination of metabolic and feeding parameters has also provided a unique insight into the phenotype of VIP/PHI−/− and Vipr2−/− mice under diurnal conditions that is not apparent from monitoring their locomotor activity. These data suggest that the reinforcing properties of wheel running on behavioral rhythms can alter underlying circadian phenotypes; a finding that merits attention in future circadian studies.

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