Endocrinology, Vol. 156, No. 5, (May, 2015): 1685-1691. DOI.

Contents

The Melanocortin-4 Receptor Integrates Circadian Light Cues and Metabolism

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Abstract

The melanocortin system directs diverse physiological functions from coat color to body weight homoeostasis. A commonality among melanocortin-mediated processes is that many animals modulate similar processes on a circannual basis in response to longer, summer days, suggesting an underlying link between circadian biology and the melanocortin system. Despite key neuroanatomical substrates shared by both circadian and melanocortin-signaling pathways, little is known about the relationship between the two. Here we identify a link between circadian disruption and the control of glucose homeostasis mediated through the melanocortin-4 receptor (Mc4r). Mc4r-deficient mice exhibit exaggerated circadian fluctuations in baseline blood glucose and glucose tolerance. Interestingly, exposure to lighting conditions that disrupt circadian rhythms improve their glucose tolerance. This improvement occurs through an increase in glucose clearance by skeletal muscle and is food intake and body weight independent. Restoring Mc4r expression to the paraventricular nucleus prevents the improvement in glucose tolerance, supporting a role for the paraventricular nucleus in the integration of circadian light cues and metabolism. Altogether these data suggest that Mc4r signaling plays a protective role in minimizing glucose fluctuations due to circadian rhythms and environmental light cues and demonstrate a previously undiscovered connection between circadian biology and glucose metabolism mediated through the melanocortin system.

The melanocortin system directs diverse physiological functions from coat color to body weight homoeostasis. A commonality among melanocortin-mediated processes is that many animals modulate such processes on a circannual basis in response to longer, summer days, suggesting an underlying link between circadian biology and the melanocortin system. A growing body of literature links biological rhythms to metabolism. Circannual (seasonal) rhythms of food intake, energy expenditure, and body weight are associated with day length (1). Circadian (daily) rhythms, which are controlled by photic-sensitive circuits in the hypothalamic suprachiasmatic nuclei (SCN), orchestrate metabolic processes that, when disrupted, can lead to the development of the metabolic syndrome (2–4). However, little is known of the mechanism(s) that link circadian light cues to metabolism.

Multiple nutrient and hormonal signals convey information about energy status to the brain through the melanocortin system, and in particular through the melanocortin-4 receptor (Mc4r), to regulate energy balance (5). Thus, Mc4r-deficient humans and rodents are hyperphagic and obese and have impaired glucose tolerance (6, 7). Mc4r may also be important in mediating information about circadian light cues. When light stimulates the retina, photic information is relayed via the retinohypothalamic tract to the SCN and onto key metabolic areas that express Mc4r such as the arcuate nucleus via the melanocortin transmitter, α-MSH (8), and the paraventricular nucleus (PVN) (9). The overlap between anatomical pathways and physiological effects raises the possibility that Mc4r may function as an integrator of circadian light cues with the control of energy balance. Here we provide data that support the conclusion that Mc4r integrates circadian light cues with glucose metabolism.

Materials and Methods

Animals

LoxTb*Mc4r* (stock number 006414; Jackson laboratory) knockout (KO) mice and wild-type littermate controls (WT) were ordered from Jackson laboratory and bred in-house. WT mice (C57BL/6J; stock number 000664) were ordered from the Jackson laboratory. *Ob/ob* mice (stock number 000632) were ordered from The Jackson Laboratory. *Sim1*-Cre mouse models were kindly provided by Dr Joel Elmquist (University of Texas Southwestern, Dallas, Texas) and bred in-house to obtain homozygous loxTbMc4r (KO−), which lack Mc4r expression globally, and homozygous loxTbMc4r mice carrying at least one *Sim1*-Cre allele (KO+) as well as *Sim1*-Cre WT littermates (WT+), as previously described (10). All mice were males unless otherwise mentioned in text. Mice were singly housed and fed an ad libitum standard chow diet (Teklad number 7002, 18% calories from fat, 3.1 kcal/g) unless otherwise mentioned. To create obese WT animals, singly housed mice were fed a 45% high-fat diet (Research Diets number D12451, 45% fat, 4.54 kcal/g). For all studies, groups were age matched but otherwise divided to ensure equal body weights among similar genotyped animals. The total number per experiment is noted in each experimental figure legend. The investigators were not blinded to the animals' genotype or light condition. All studies were approved by and performed according to the guidelines of the Institutional Animal Care and Use Committee of the University of Cincinnati.

Light protocols

Mice were housed in a standard vivarium room with a minimum of 80 lux during the light period. Standard 12-hour light, 12-hour dark (LD) consisted of 12 hours of light followed by 12 hours of dark. Constant light (LL) occurred in a neighboring room with otherwise similar environmental conditions. Similarly, a 2L2D cycle was achieved by using repeated cycles of 2 hours of light followed by 2 hours of dark (2L2D).

Automated phenotype analysis

Animals were singly housed, maintained on the appropriate light-dark cycle, and placed into an automated system to measure data in increments of 5–45 minutes (Phenotyping Systems International Group). Activity counts (via beam break) and feeding patterns were measured simultaneously over the course of the 1–2 weeks. Data analysis began after animals had acclimated to the metabolic chambers for at least 48 hours. For consistency, the first 24 hours of data in the chamber or after a light switch were excluded from analysis to remove differences in habituation.

Twenty-four-hour fasting glucose levels

Animals were habituated to handling by 2+ weeks of at least twice a week gentle handling prior to testing. Animals were fasted for 12 hours (overnight) prior to the first blood glucose measurement at Zeitgeber time (ZT) 2. Blood glucose was measured by glucometer every 4 hours on each animal by tail clip and gentle handling. Animals remained fasting until all measures were complete (eg, ZT 22).

Glucose tolerance test

Animals were fasted for 6 hours prior to an ip injection of 25% dextrose at a dose of 8 µl/g at ZT 6. Blood glucose was measured by tail clip and gentle handling by glucometer prior to glucose injection (time 0), and at 15, 30, 60, and 120 minutes after injection. Animals were excluded from analysis based on

preestablished criteria, eg, if blood glucose did not rise significantly over the 2-hour period, indicating a failed glucose injection.

Catheterization for hyperinsulinemic-euglycemic clamps

After 2 weeks of LD or LL exposure, mice underwent surgery for the hyperinsulinemic-euglycemic clamp. Mice were catheterized in the left common carotid artery and right jugular vein as previously described (11). Immediately after the surgery, animals were given sc injections of buprenorphin (0.28 mg/kg Buprenex; Reckitt Benckiser Healthcare), meloxicam (0.25 mg/100 g body weight; Metacam), and 1 mL warm saline.

Hyperinsulinemic-euglycemic clamp

After 5–10 days of recovery from surgery, hyperinsulinemic-euglycemic clamps were performed as previously described (11). On the day of the clamp procedure, mice were fasted for 4 hours. At time 0 (∼10:00 am; ZT 4), a primed (2.5 μCi) continuous infusion (0.05 μCi) of [3-^{3}H]glucose (PerkinElmer Life Sciences) was started and continued throughout the clamp. One hundred twenty minutes after the start of the [3-^{3}H]glucose infusion, insulin was infused at a rate of 3 mU/kg · min, and exogenous glucose was infused to maintain blood glucose levels between 130 and 150 mg/dL. A bolus of 50 μL of 2-[^{14}C]2-deoxyglucose ([^{14}C]2-DG; 0.013 mCi) was infused into the jugular vein 40 minutes prior to the end of the clamp procedure to assess tissue-specific glucose uptake. Mice were euthanized with an injection of sodium pentobarbital and tissues collected. Plasma and tissues were stored at −80°C for analysis. Blood sampling, glucose turnover, and tissue-specific glucose uptake calculations were performed as previously described (12).

Statistics

Statistical analysis was performed using GraphPad Prism version 5.0 (GraphPad Software). Statistical significance was determined by unpaired Student's *t* test, one-way ANOVA followed by Tukey's multiple comparison post hoc test, two-way ANOVA followed by Bonferroni's multiple comparison post hoc test, or a repeated-measures ANOVA followed by Bonferroni's multiple comparison post hoc test as referenced in the text. Results were considered statistically significant when $P < .05$.

Results

Mc4r-deficient mice have amplified daily variations in baseline blood glucose and glucose tolerance

Circadian variations in circulating glucose and glucose tolerance occur in both rodents and humans (13–16). In fasting conditions, WT mice maintained under a normal LD cycle demonstrate a rise in the fasting blood glucose levels at the end of the light phase followed by a fall at the end of the dark phase (Figure 1A) as previously shown (14–16). However, mice lacking Mc4r (hereafter KO) display an exaggerated fluctuation in daily fasting glucose compared with WT littermate controls (Figure 1A, $P < .001$).

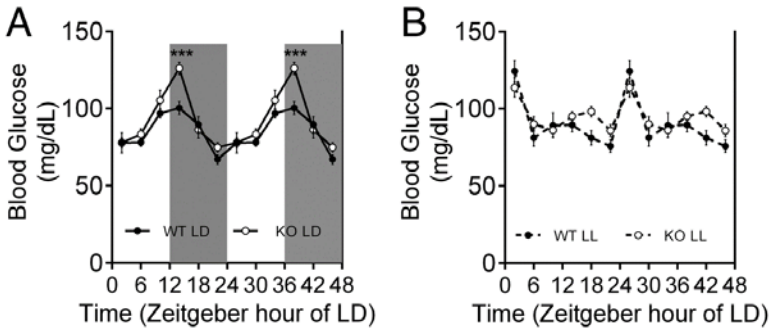

Figure 1 Twenty-four-hour baseline fasting glucose in C57BL/6J and Mc4R-deficient mice housed in a standard light-dark (LD) cycle or continuous light exposure (LL). A, Baseline fasting glucose levels in WT C57BL/6J mice (WT; black symbols, n = 11) and Mc4R-deficient mice (KO; white symbols, n = 10) housed in a standard LD cycle (solid lines). B, Baseline fasting glucose levels in WT (black symbols, n = 10) and KO (white symbols, n = 10) mice housed in LL (dashed lines). Data are expressed as mean ± SEM and double plotted (repeated measures two way ANOVA). Asterisks indicate significant posttest differences: *, *P* < .05; **, *P* < .01; ***, *P* < .001.

In addition to fasting baseline glucose, daily variation in glucose tolerance was also significantly altered in the KO compared with the WT, with the KO having a significantly amplified fluctuation in glucose tolerance (Figure 2A, *P* < .01). Glucose tolerance and baseline fasting glucose was similar between the genotypes during the early morning hours (eg, ZT 1) but became significantly different toward the end of the light phase (eg, ZT 11) (Figure 2A, *P* < .01; Figure 2B, *P* < .001).

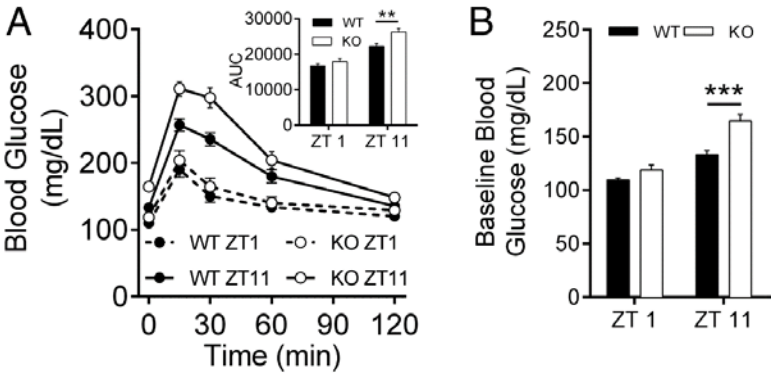

Figure 2 Daily variation in glucose tolerance of WT and KO mice. A, Glucose tolerance of both WT (black symbols) and KO (white symbols) are markedly improved at ZT 1 (dashed lines). Glucose tolerance is impaired at ZT 11 (solid lines) in both genotypes; however, KO mice have more of an impairment in glucose tolerance (area under the curve inset). Repeated-measures ANOVA was used for glucose tolerance test and an unpaired, two-sided *t* test for area under the curve (AUC). B, Baseline fasting glucose levels in WT (black) and KO (white) mice at ZT 1 and ZT 11 (cross-over design, two-way ANOVA, n = 21/group).

Acute exposure to constant light reduces daily variations in blood glucose and improves glucose tolerance in KO mice without altering body weight or food intake

Lesioning the SCN or disrupting its function by exposing mice to constant light (17) will lead to a blunting of glucose rhythms and insulin sensitivity (14, 18). To determine how the KO mouse would respond to desynchronized conditions, we housed KO mice in constant light (LL). In contrast to the KO mice housed

under standard LD, KO mice housed in LL for a week displayed similar fasting glucose levels to WT mice (Figure 1B).

To investigate the role of Mc4r in the control of glucose homeostasis, we performed a glucose tolerance test. Although the glucose tolerance of WT mice was unaffected by 1 week of LL exposure (Figure 3A), KO mice had significantly improved glucose tolerance after LL (Figure 3B, $P < .05$). This effect occurred independently of body weight because the 1 week of LL exposure was insufficient to lead to body weight changes in KO mice (data not shown). A similar improvement in glucose tolerance was also observed in female KO mice (Supplemental Figure 1, $P < .001$).

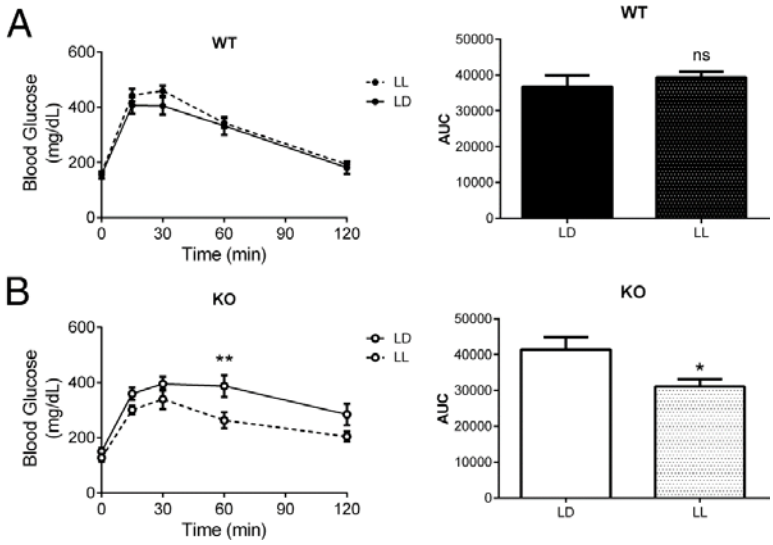

Figure 3 Glucose tolerance of WT and KO mice after 1 week of LL (dashed line) within the automated phenotyping system or 1 week of LD exposure (solid line) in standard housing. A, WT mice have no significant change of glucose tolerance after 1 week of LL exposure [repeated measures, two way ANOVA for glucose tolerance test, nonsignificant F (1, 13) = 0.9842, $P = .34$, unpaired, two sided *t* test for area under the curve (AUC), nonsignificant (ns) $P = .44$; WT LD (n = 8), WT LL (n = 7)]. B, KO mice have significantly improved glucose tolerance after 1 week of LL exposure [repeated measures, two way ANOVA for glucose tolerance test, significant effect of the LL exposure, F (1, 14) = 6.201, $P < .05$, Bonferroni posttest, unpaired, two sided *t* test for area under the curve (AUC), $P < .05$; KO LD (n = 8); KO LL (n = 8)]. Data are expressed as mean ± SEM.

To assess the impact of constant light on food intake and activity, KO and WT mice were maintained for 1 week in LD and then changed to LL for an additional week. When maintained on LD, KO mice had significantly lower physical activity (Supplemental Figure 2A, $P < .05$) than WT, although both exhibited similar dark-light fluctuations. During LL, WT mice had significantly reduced daily locomotor activity ($P < .001$), whereas there was no change in KO mice. Food intake (Supplemental Figure 2B) did not differ between the 1-week LL and the 1-week LD conditions for either genotype.

Improvement in glucose tolerance of Mc4r-deficient mice in LL is not due to obesity

To determine whether the LL-induced improvement in glucose tolerance was due to the obese state of the KO mice as opposed to a specific loss of Mc4r signaling, we assessed the glucose tolerance of other obese models exposed to LL. LL did not affect glucose tolerance of diet-induced obese WT mice (Supplemental Figure 3) or genetically obese *ob/ob* mice (Supplemental Figure 4).

Long-term exposure to constant light improves glucose tolerance and suppresses weight gain in Mc4r-deficient mice

Although light therapy can lead to weight loss in obese humans (19), long-term LL combined with a high-fat diet disrupts circadian rhythms and leads to weight gain in rodents (20). To test the effect of long term expose to LL on body weight and food intake in chow-fed animals, KO mice (8–12 wk old) and their WT littermates were maintained on LD or LL for 24 days. As expected, KO mice gained more weight than WT mice in both LD and LL. However, weight gain of KO mice was significantly attenuated in the LL relative to the LD condition (Table 1, $P < .05$). KO mice ate significantly more calories than WT mice in LD ($P < .05$) but not LL conditions. Within KO mice, there was no significant difference in caloric intake between LD and LL (Table 1). Similar to the acute LL exposure, long-term exposure to LL (eg, up to 3 wk) improves glucose tolerance, at least in part by increasing the insulin-dependent glucose uptake in the KO mice (Figure 4).

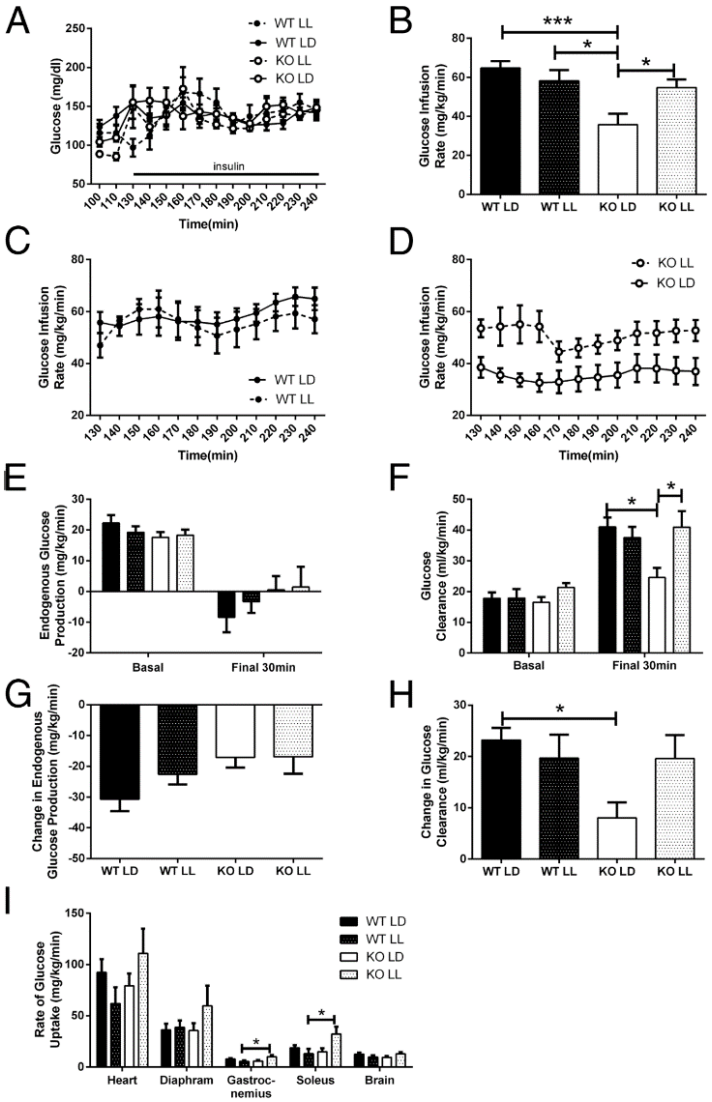

Figure 4 Hyperinsulinemic-euglycemic clamp in KO and WT littermates during LD or LL. A, Blood glucose levels were maintained at comparable levels for all groups. B, During steady-state conditions (final 30 min of the clamp),

the KO LD mice required significantly less glucose compared with all other groups to maintain euglycemia. Notably, KO LL had a similar glucose infusion rate as WT mice and required more glucose than KO LD. C, Glucose infusion rate in WT mice after 3 weeks of LD or LL. D, Glucose infusion rate in KO mice after 3 weeks of LD or LL. KO mice had a significantly higher rate of glucose infusion on LL than on LD ($P < .05$). E, During baseline conditions, there were no differences in endogenous glucose production (EGP). During the final 30 minutes, EGP was suppressed in all groups. F, During baseline conditions, there were no differences in glucose clearance. During the final 30 minutes, glucose clearance of WT LD and KO LL was significantly higher than in KO LD. G, There were no significant changes in EGP among groups. H, The insulin infusion significantly increased glucose clearance over baseline conditions in all groups except the KO LD. Moreover, KO LL mice increased their glucose clearance to a level similar to that of WT mice. I, [^{14}C]2-DG uptake was significantly enhanced in KO LL compared with WT LL in the gastrocnemius and soleus muscles, with a trend toward increased uptake in heart and brain. ($P < .05$). *, $P < .05$; ***, $P < .001$ (repeated measures, two way ANOVA; KO LD, n = 7, except gastrocnemius, n = 6; WT LL, n = 6; WT LD, n = 8; KO LL, n = 8). Data are expressed as mean ± SEM.

Table 1 Effects of Constant Light in WT and Mc4r-Deficient Mice

	Light Cycle	**WT**	**Mc4r Deficient**	***P* Value**
Change in body weight, g ± SEM	LD	2.83 ± 0.43 (n = 7)	9.84 ± 0.51 (n = 6)	<.001
	LL	2.27 ± 0.50 (n = 5)	6.97 ± 1.10 (n = 7)	<.001
Food intake, kcal ± SEM	LD	298.68 ± 21.58 (n = 7)	459.93 ± 23.21 (n = 6)	<.01
	LL	334.37 ± 17.15 (n = 5)	405.19 ± 40.03 (n = 8)	ns

Abbreviation: ns, not significant.

Constant light increases insulin-dependent glucose uptake in muscle of Mc4r-deficient mice

We performed hyperinsulinemic-euglycemic clamps in KO and WT littermate mice after 3 weeks of LD or LL exposure. Glucose was infused to achieve a stable blood glucose level of 150 mg/dL (Figure 4A). Consistent with the glucose tolerance tests, there was no difference in the glucose infusion rate in WT mice after LD or LL exposure (Figure 4, B and C). KO mice in LL required a significantly higher glucose infusion rate to achieve stable plasma glucose levels compared with KO mice in LD, indicating an increase in whole-body insulin sensitivity (Figure 4, B and D, $P < .05$). Constant light had no effect on endogenous glucose production (Supplemental Figure 5, A, C) but increased glucose clearance in KO but not WT mice (Supplemental Figure 5B and D, $P < .05$). This increase in clearance was associated with an increased ^{14}C-2-DG uptake in muscle (Supplemental Figure 5E, $P < .05$). These data suggest that the improved glucose tolerance by the KO in LL is due to an increase in insulin sensitivity at the level of the skeletal muscle.

Exposure to other, nonentraining light cycles improves glucose tolerance in Mc4r-deficient but not WT mice

To determine whether the changes in glucose homeostasis were specific to constant light exposure or whether they could be elicited using other circadian disruption protocols, we exposed mice to a nonentraining 4-hour day, or 2L2D cycle, for 10 days. Similar to what we observed during the LL

conditions, KO mice in 2L2D exhibited an improvement in glucose tolerance (Supplemental Figure 6, $P < .05$).

Reactivation of Mc4r in the PVN prevents LL-induced improvements in glucose tolerance

Lastly, we hypothesized that Mc4r expression in the PVN represented a key integration site for light and metabolic signaling. Using a *Sim1*-Cre reactivation model (10), we exposed 18- to 28-week-old loxTB Mc4r-deficient (eg, KO−), *Sim1*-Cre loxTB (eg, KO+), and age-matched wild-type littermate controls (*Sim1*-Cre WT; eg, WT+) to 2L2D for 2 weeks and then assessed glucose tolerance. As described (10), this model reactivates Mc4r expression within the PVN, supraoptic nucleus, and amygdala. Whereas 2L2D resulted in an improvement in glucose tolerance in the whole-body KO−, reactivation of Mc4r in the PVN (eg, KO+) severely attenuated the improvement (Figure 5).

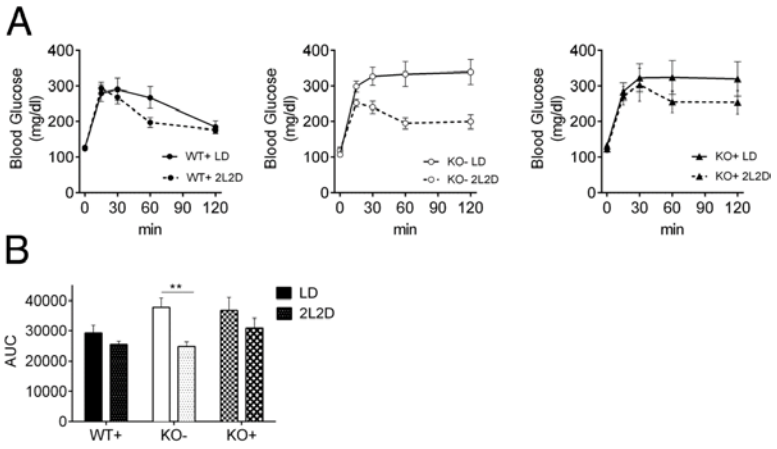

Figure 5 Glucose tolerance of *Sim1*-Cre reactivation mice after 2 weeks of 2L2D exposure (dashed lines) or 2 weeks of the LD exposure (solid lines). A, Neither WT+ mice nor KO+ mice have significant change of glucose tolerance after 2 weeks of 2L2D exposure. However, KO− mice have a robust improvement in glucose tolerance. B, Area under the curve (AUC) calculations for glucose tolerance indicates that only KO− had a significant improvement in glucose metabolism after 2 weeks of 2L2D exposure (n = 7–10/group). Data are expressed as mean ± SEM.

Discussion

Central nervous system sensing of available energy and the time of day is key for the normal control of energy balance. Aberrant amounts of either available nutrients or light exposure disrupt metabolic control and exacerbate susceptibility to develop the metabolic syndrome. Our data provide evidence that the melanocortin system, classically associated to the control of nutrient availability, is also involved in regulating the circadian control of glucose homeostasis. Specifically, these data suggest that Mc4r signaling, including that within the PVN, plays a protective role in minimizing glucose fluctuations due to circadian rhythms and environmental light cues. Likewise, we propose that circadian fluctuations contribute to the glucose intolerance of Mc4r-deficient mice (see schematic in Figure 6).

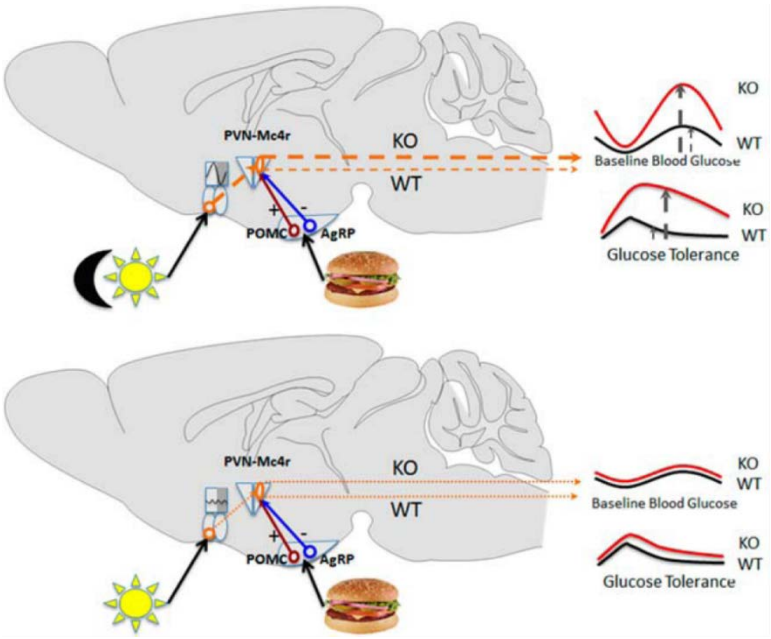

Figure 6 Mechanistic summary. Top panel, During a normal LD cycle, Mc4r expression in the PVN attenuates the impact of the normal output from the SCN on glucose homeostasis. Loss of Mc4r expression prevents this attenuation, leading to an exaggerated impact of the light cues on baseline glucose and glucose tolerance. Bottom panel, During constant light, the normal output from the SCN that regulates baseline glucose and glucose tolerance is disrupted, leading to a marked improvement in glucose tolerance in Mc4r KO mice. AgRP, Agouti related protein; POMC, proopiomelanocortin.

Most organisms have evolved to use light cues, and yet relatively little is known about how circadian rhythms go on to affect the multitude of physiological processes that are necessary for daily survival. From an evolutionary perspective, there is a benefit to closely associating photic cues with the melanocortin system. By using environmental light as a cue, animals can anticipate and predict the onset of day or night as well as the changing of the seasons. Circadian and circannual cues such as these are important indicators for the avoidance of predators, reproductive success, and the efficient use of metabolic resources. Interestingly, the melanocortin system is ideally posed to regulate many of these circadian and light-associated characteristics: coat color (21) (associated with the Mc1r), meal entrainment (22) (Mc3r), fat storage and glucose tolerance (21) (Mc3r, Mc4r), and sexual function (21) (Mc4r). Indeed, light directly regulates some aspects of the melanocortin system, including receptors responsible for pigmentation (21) and is linked to others by indirect mechanisms (23, 24).

Circadian fluctuations in the control of glucose homeostasis have been well documented in rodents and humans (14, 15, 25–27). This control requires the molecular (15) and neuroanatomical integrity (28) of the central clock, and their disruption leads to impaired glucose control. The exaggerated circadian fluctuations in baseline blood glucose and glucose tolerance exhibited by mice lacking Mc4r signaling demonstrate that rhythms, and the central clock, may be subjected to restraint by other neural systems.

Our results demonstrate that circadian disruption, as caused by disruptive environmental light exposure, in combination with lack of Mc4r signaling, results in an improvement in glucose tolerance independent of changes in body weight and food intake. This selective regulation of glucose homeostasis in response to circadian disruption is surprising, given the well-known critical role of Mc4r in the control of feeding

and fat mass. These data point to the unique role of Mc4r in regulating glucose homeostasis in response to circadian disruption and suggest that other central and/or peripheral factors are important for the changes in body weight and feeding associated with circadian disruption (20, 29, 30).

Taken together, these results, and previous reports on the neuroanatomical distribution of Mc4r expression, suggest that an integration site for light and the melanocortin system is downstream of the SCN, in sites that include the PVN. Indeed, we found no evidence to support the possibility that behavioral rhythms are directly altered by Mc4r mutation, suggesting that the SCN itself is not the critical region involved in the glucose response to light cues mediated by Mc4r signaling. Moreover, the SCN of mice, rats, and humans do not express Mc4r (31–33). However, the SCN, through projections to the PVN, plays a significant role in the control of glucose homeostasis (34). Reactivating Mc4r in the PVN and restoration of a wild-type glucose regulation response supports the conclusion that the PVN is relatively more important than the SCN for our observed changes in glucose. However, *Sim1*-Cre-dependent Mc4r reactivation is not specific to PVN (10), and therefore, a potential contribution of other brain regions coexpressing Sim1 and Mc4r to the circadian control of glucose cannot be excluded. Notably, *Sim1*-Cre Mc4r reactivation does not lead to improved glucose tolerance but instead nullifies the effect of the light exposure. These data suggest that although Mc4r in *Sim1*-expressing neurons, including those in the PVN, are critical for the modulation of glucose during circadian disruption, it is not principally involved in regulating other aspects of metabolism during circadian disruption (eg, feeding). The precise mechanism(s) by which Mc4r signaling controls glucose metabolism in response to circadian signals remains to be determined, although potential mechanisms could involve preautonomic neural circuits and/or neuroendocrine axes.

These results have important implications for humans. More and more, societies are running around the clock, leading to more people being active (and working) during the evening as well as experiencing increased light exposure at night. Mounting evidence suggests that these events can lead to circadian disruption, which is associated with obesity and cardiometabolic diseases (35, 36). However, it remains unclear exactly how circadian disruption translates into metabolic impairment. As others have noted (37), the combination of obesity and poor metabolic control leads to detrimental health, thus making it crucial to use a multiapproach strategy to fight disease. We expect that continued research focusing on how neural circuits, including the melanocortin system, and circadian light cues are connected can lead to novel approaches to treatment metabolic diseases such as obesity and diabetes.

Acknowledgments

We thank F. W. Turek (Northwestern University, Chicago, Illinois) and D. D'Alessio (University of Cincinnati, Cincinnati, Ohio) for their comments on the draft of the manuscript and C. Raver and S. Amburgy for technical assistance.

This work was supported by the National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases Grants F32 DK097867–01 (to D.M.A.), R01 DK093848–01 (to R.J.S.), R01 DK082480–01 (to D.A.S.), and R01 DK077975 (to D.P.-T.). R.J.S. receives support from Ethicon Endo-Surgery, Ablaris Therapeutics, Inc, Novo Nordisk, Novartis, Angiochem, Eisai, Forest Pharmaceuticals, Givaudan, Zealand Pharmaceuticals, and Boehringer Ingelheim International. D.A.S. receives support from Ethicon Endo-Surgery, Novo Nordisk, and Boehringer Ingelheim International. D.P.-T. receives support from Calibrium Ltd.

Disclosure Summary: D.M.A., R.J.S., D.A.S., and D.P.-T. receive support from various sources. J.H., N.P., J.S., J.P., R.M., S.W., and J.H. have nothing to disclose.

Abbreviations

[^{14}C]2-DG 2-[^{14}C]2-deoxyglucose

KO knockout

LD standard 12-hour light, 12-hour dark

2L2D repeated cycles of 2 hours of light followed by 2 hours of dark

LL constant light exposure

Mc4r melanocortin-4 receptor

PVN paraventricular nucleus

SCN suprachiasmatic nuclei

WT wild type

ZT Zeitgeber time.

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