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Metabolic Consequences in Humans of Prolonged Sleep Restriction Combined with Circadian Disruption

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Abstract

Epidemiological studies link short sleep and circadian disruption with risk of metabolic syndrome and diabetes. We tested the hypotheses that prolonged sleep restriction with concurrent circadian disruption, as can occur with shift work, impairs glucose regulation and metabolism. Healthy adults spent >5 weeks in controlled laboratory conditions including: sleep extension (baseline), 3-week sleep restriction (5.6 h sleep/24 h) combined with circadian disruption (recurring 28-h 'days'), and 9-day recovery sleep with circadian re-entrainment. Prolonged sleep restriction with concurrent circadian disruption significantly decreased resting metabolic rate, and increased postprandial plasma via inadequate pancreatic beta cell responsivity; these normalized with 9 days of recovery sleep and stable circadian reentrainment. Thus, in humans, prolonged sleep restriction with concurrent circadian disruption alters metabolism and could increase risk of obesity and diabetes.

Introduction

Short sleep duration and disordered sleep have been linked to numerous adverse metabolic changes (1-4), increased risk of chronic disease including obesity and type 2 diabetes (5, 6), and early mortality (7, 8).

Less is known about the adverse consequences of misalignment between endogenous circadian physiological rhythms and the daily environmental and behavioral rhythms, as occurs chronically in nightworkers and rotating shift workers (9-12). Endogenous circadian rhythms emanate from the central circadian pacemaker in the suprachiasmatic nucleus of the hypothalamus, and these rhythms help synchronize the molecular circadian clocks in the peripheral cells and tissues that optimize physiological functions to match the daily patterns of behavior, such as feeding, activity and sleep. However, suboptimal alignments between endogenous circadian rhythms and daily behaviors occur in the many millions of people who perform shift work, and this circadian disruption may contribute to the adverse health consequences of shift work, including fatigue and poor sleep, gastrointestinal complaints, detrimental metabolic changes, and increased risks of developing obesity and diabetes (13-19). For example, over a 3-year follow-up, the risk of progressing from impaired fasting glucose or impaired glucose tolerance to clinically classified diabetes was five-fold higher among night shift workers than among those who did not work at night (20).

Author Contributions: OMB, SAS, CAC, and JFD designed the study; SWC, OMB, and JFD supervised the data collection teams; SPO, OMB, and SWC collected data; SPO and JHP assisted OMB with data management and analysis; WW performed statistical analyses; OMB and SAS drafted the manuscript and all authors contributed to and approved the final version.

In humans, a laboratory study of acute (a few days) misalignment of circadian rhythms with respect to sleep/wake and meal schedules revealed higher post-prandial blood glucose, despite higher insulin release. This magnitude of hyperglycemia was comparable to a pre-diabetic state in a third of individuals (21). In mice, a laboratory study of prolonged (10 weeks) circadian disruption revealed broad adverse changes in the brain (dendritic reorganization and loss), behavior (learning and response to novel environments), and glucose metabolism (increased insulin at the same glucose level and weight gain) (22). It is not known whether these adverse effects of sustained circadian disruption would also occur in humans, but if true, this could mechanistically explain the epidemiological findings of the increased incidence of diabetes in shift workers.

It is also notable that both sleep patterns and circadian rhythms change profoundly with age. Older people experience less sleep, more frequent awakenings, a reduction of slow wave sleep (23) and blunting of the amplitudes of circadian rhythms such as core body temperature (24) and activity (25). Also, the phase relationships between those rhythms and the timing of sleep change with age (26). Given the possible adverse metabolic changes caused by short sleep and circadian disruption, it is also plausible that the sleep and circadian changes with age could contribute to the increased incidence of obesity and diabetes in the elderly (1). Thus, we tested the hypotheses that prolonged sleep restriction with concurrent circadian disruption impairs glucose regulation and metabolism, particularly in older individuals.

We have previously used a forced desynchrony protocol in humans to assess the separate effects of sleep and of circadian rhythms upon cognitive performance (12, 22, 27-29). This protocol was also recently translated for use in mice (22), In this protocol, behaviors, including the wake-sleep cycle and the feeding-fasting schedule, are scheduled to occur on ‘days’ that are much shorter or much longer than 24 h (e.g., 28 h), and light levels during wakefulness are kept dim so that the endogenous circadian pacemaker oscillates at its intrinsic period rather than being reset by daily exposure to the light/dark cycle (12, 28, 29). An imposed 28-h duration of environmental and behavioral cycles was selected in the current study to be well outside the range of entrainment of the circadian pacemaker, forcing desynchronization between endogenous circadian rhythms and the scheduled dark-light, fasting-feeding, and sleep-wake cycles. We have combined this model of circadian disruption with sleep restriction of 5.6 h per 24 h for ~3 weeks to test our hypotheses that prolonged sleep restriction combined with prolonged circadian disruption would impair glucose regulation and metabolism, and that such effects would be more pronounced in older people.

Results

Participant Characteristics

Twenty-four participants completed the full protocol (12 young plus 12 older participants). Three participants were excluded from group analyses (see Materials and Methods), leaving 11 young (mean 23 ± 2 years old, 5 female) and 10 older (60 ± 5 years old, 5 female) participants in the analyses.

Measurement Periods

To ensure participants were not suffering from sleep loss prior to starting the study and had stable circadian phase alignment, they were instructed to spend 10 h in bed each night, with a self-selected but constant bedtime and normal exposure to daytime light, for at least 3 consecutive weeks immediately before entry into the laboratory. Thereafter, each participant lived in an individual laboratory suite for 39 days in dim light and without time cues. Figure

1 schematically portrays the protocol, and highlights the three experimental phases during which assessment of body weight, resting metabolic rate (RMR), and metabolic responses to a standardized meal were performed: (1) baseline "sleep replete" condition with stable circadian phase alignment (after at least 27 days of 10-16 h sleep opportunity per 24 h, including at least 21 days when at home and 6 days in the laboratory; (2) after up to 3 weeks of exposure to sleep restriction with concurrent circadian disruption induced by imposition of 28-h fasting-feeding, and sleep-wake cycles, performed in dim light; and (3) after 9 days of circadian re-entrainment and recovery sleep opportunity of 10 h per 24 h. These three intensive assessment periods were timed to occur when the sleep episode was at the normal optimal circadian phase for each subject and standardized meals were at similar circadian phases (see Materials and Methods).

Prolonged Sleep Restriction with Concurrent Circadian Disruption Reduced Resting Metabolic Rate, and Caused Relative Hyperglycemia in Response to a Standardized Meal

The combined challenge of 3 weeks of sleep restriction with circadian disruption significantly reduced RMR relative to baseline (-8% on average for all subjects) and RMR returned towards baseline levels in the recovery phase, but without complete recovery (Figure 3). There were no statistical differences between age groups in the relative magnitude of the reduction in RMR.

Following a standardized breakfast eaten at a consistent circadian phase, exposure to prolonged (3 weeks) sleep restriction with concurrent circadian disruption significantly increased both fasting and postprandial plasma glucose levels relative to the responses to that same meal at baseline [Figs 2, A, B, E and F, and 3, A, C, and E; overall changes: fasted, +8%, $p=0.0019$ (signed rank test); postprandial peak,+14%, $p=0.0004$ (t-test); integrated postprandial response over 90 min, +15%, $p<0.0001$ (t-test)] There were no statistical differences between age groups in the effects of prolonged sleep restriction with concurrent circadian disruption on fasting or postprandial glucose levels. The relative hyperglycemia following this breakfast meal was apparently caused by inadequate pancreatic beta-cell compensation, as fasting plasma insulin and postprandial peak and integrated plasma insulin levels were significantly reduced [Figs. 2, C, D, G, and H, and 3, B, D, and F; overall changes: fasted, -12%, $p=0.0064$ (signed rank test); postprandial peak, -27%, $p<0.0001$ (t-test); integrated postprandial response over 90 min, -27%, $p<0.0001$ (t-test)] Glucose and insulin meal responses reverted back to baseline levels by the end of the 9-day sleep recovery with circadian re-entrainment period in both age groups (Fig. 3, D to F), with the exception that the glucose peak in the older subjects was still slightly elevated by the end of the recovery phase and in young subjects peak glucose was lower at recovery (this emerges as a significant interaction between age and condition for the peak postprandial glucose; $p=0.012$; Figure 3). Glucose responses expressed as 2-hour postprandial levels (for comparison to 140 mg/dL clinical threshold indicating impaired glucose tolerance and pre-diabetes) revealed that 3 of 21 participants had prediabetic postprandial glucose levels during sleep restriction with circadian disruption, whereas no participants had abnormal responses during baseline or recovery/realignment (see profiles, Fig 2).

Baseline percent body fat was not a significant independent contributor in the models derived to quantify the fasting and postprandial glucose and insulin responses (baseline, peak, area under the curve [trapezoidal method], and 90-minute profiles). The effects of sleep restriction and circadian disruption on glucose (peak, fasted, AUC) were independent of sex.

Circadian Rhythms of Glucose, Insulin, and Cortisol during Prolonged Exposure to Sleep Restriction with concurrent Circadian Disruption

To determine if there were changes over the course of the exposure to sleep restriction with concurrent circadian disruption, plasma samples were taken upon awakening while fasted on each experimental 'day' during the first and third weeks of the recurring 28 h cycles (first and third "beat cycles" of the forced desynchrony - Figure 1). Sampling times when grouped across each week encompassed the full range of circadian phases. There were significant endogenous circadian rhythms in glucose, insulin and cortisol (all $p<0.01$; Figure 4), with similar amplitudes and timing between the first and third weeks of exposure. For each analyte, peaks occurred around 60 circadian degrees relative to the nadir of the core body temperature rhythm (which was assigned a value of 0°), which corresponds to the usual morning when under normally entrained conditions. For comparison, fasted (pre-breakfast) values were collected in the baseline condition at an average circadian phase of 73° ± 27°, which equates to 4.9 h after the core body temperature minimum. Mean fasted cortisol and mean fasted glucose were similar from week 1 to week 3 (i.e., no progressive changes). However, fasting insulin levels remained close to baseline levels during the first week of exposure, then declined below baseline levels by the third week of exposure ($p<0.0001$; Figure 4). There were no significant differences in any of these circadian rhythms by age group or sex ($p>0.05$).

24-hour profiles of Leptin and free Ghrelin

For 24-hour profiles of leptin, there was no statistical significant difference between age groups across conditions as a main effect and no significant interactions between age group and condition, but there was a significant interaction between age group and time ($p=0.04$). For 24-hour profiles of free ghrelin,(Fig. 5, A to D), though there was no statistical significant differences between age groups across conditions as a main effect, there was significant interactions between age group and condition ($p=0.02$), and between age group and time ($p<0.0001$). Compared to baseline, the 24-hour profiles of leptin were slightly lower during sleep restriction ($p=0.05$) and recovery ($p<0.05$), whereas 24-hour profiles of ghrelin were slightly higher during sleep restriction and recovery (both $p<0.05$). Interestingly, ghrelin levels in young subjects were higher during time in bed than in older subjects.

Body Weight during Exposure to Sleep Restriction with Circadian Disruption

Young participants were significantly heavier than older participants at baseline (72.3 ± 12.0 kg vs. 67.9 ± 11.8 kg; $p=0.04$), whereas there was no significant difference in BMI (24.2 ± 2.6 kg/m^2 vs. 23.3 ± 1.9 kg/m^2). Dual energy x-ray absorptiometry scans at baseline revealed that the average whole body fat was not significantly different between age groups (17.2 ± 7.3 kg fat [24.5 ± 8.5% of body weight] in young; 20.6 ± 2.8 kg [31.3 ± 5.6% of body weight] in older subjects; $p=0.11$). Average trunk fat was slightly lower in the young subjects (7.9 ± 3.7 kg [23.0 ± 8.7% of trunk mass] versus 10.4 ± 2.0 kg [31.2 ± 5.2% of trunk mass]; $p=0.05$). Both groups lost some mass during the study as assessed by fasted, post-void weights measured at scheduled wake time on the day prior to each of the three standardized metabolic assessments (condition: $p<0.0001$; age*condition: p=n.s.). On average they lost 1.2 ± 1.3% body mass through sleep restriction with circadian disruption ($P=0.0003$), and had lost 1.7 ± 1.7% by the end of the recovery condition compared to baseline ($P=0.0003$). In a multiple linear regression model, changes in bodyweight were unrelated to changes in RMR, consumed calories (total kcal increased by an average of only 6 kcal/24 h from baseline to sleep restriction), or actigraphically-assessed physical activity (which increased by 58 ± 37% from baseline to sleep restriction, presumably due to the increased duration of wakefulness). Thus, the increase in activity without an increase in food intake would be the most likely cause of the small reduction in body weight throughout the

protocol. In multiple linear regression models comparing baseline to either sleep restriction with circadian disruption or recovery, weight changes were not associated with changes in 24 hr mean leptin levels (percent fat-adjusted), body temperature, activity levels, food consumed or metabolic rate. Average core body temperature actually decreased slightly (0.09 F° ± 0.37 stdev) and was positively correlated with weight change from baseline to sleep restriction with circadian misalignment (weight difference [in lbs] = -0.91+2.03*[core temperature difference (in F°)]; P=0.043, r^2=0.30), suggesting that on average one degree change of core temperature corresponds to 2.03 lbs of change in body weight. Effects of the combined exposure on changes in glucose AUC and insulin AUC were unrelated to changes in bodyweight (Pearson correlation r=0.28, p=0.22 and r=0.35, p=0.12, respectively). In multiple regression analyses, changes in post-prandial insulin and glucose were unrelated to energetics (i.e., changes in RMR, diet consumed, body temperature, and wrist physical activity levels).

Discussion

Our findings provide a potential mechanistic link between animal laboratory work and human epidemiological findings in terms of how prolonged sleep restriction and prolonged circadian disruption impair glucose regulation and reduce metabolism. The robust changes we observed with exposure to chronic and concurrent circadian disruption and sleep restriction have potential relevance to the millions of people who experience these challenges on a daily basis and who are more likely to develop the metabolic syndrome and diabetes. Findings of particular clinical relevance for exposure to chronic sleep restriction with circadian disruption include a 32% decrease in insulin secretion in response to a standardized meal, a very large effect that led to inadequate glucose regulation: glucose levels were higher for a longer time and rose to pre-diabetic levels in some participants. Finally, the 8% drop in RMR with sleep restriction and circadian disruption, assuming no changes in activity or food intake, would translate into ~12.5 pounds increase in weight over a single year (120 kcal/day × 365 days / 3500 kcal of fat mass), which has clear clinical relevance as chronic sleep restriction with circadian disruption is endemic in our society.

The seminal work of Spiegel et al in 1999 described adverse metabolic effects in humans when sleep was restricted to 4 h/night for 1 week, leading the authors to hypothesize "…*that chronic sleep loss could increase the severity of age-related pathologies, such as diabetes*…" (1). In general, the primary mechanisms in the development of impaired glucose metabolism are changes in insulin secretion, the ability of the pancreatic beta-cells to respond to a glucose stimulus, and insulin sensitivity, the ability of peripheral tissues to respond to an insulin signal, such as occurs after a meal, by increasing glucose uptake. A host of secondary mechanisms can further modulate insulin sensitivity and insulin secretion. In a recent study of one week of sleep restriction to 5 h/night, young adult men had reductions in insulin sensitivity (measured using both euglycemic hyperinsulinemic clamps and intravenous glucose tolerance tests), with no change in the acute insulin response, and without a change in RMR (3). These findings are consistent with those observed in middle-aged adults exposed to sleep restriction of 5.5 h/night for two weeks, who exhibited significant decreases in insulin sensitivity (30). Thus, we are beginning to understand the extent to which sleep deficiency impairs glucose metabolism, but need more information about the extent, mechanisms, and dynamics of these changes. The magnitude (hours of sleep per night) and duration of sleep restriction (days to weeks) are likely to be important factors in determining the speed and extent of any diabetogenic changes, i.e., elevations of circulating glucose levels caused by reduced insulin sensitivity of peripheral tissues and/or insufficient insulin secretion by the pancreas. Our current findings are consistent with recent epidemiological work demonstrating that lifestyle factors, including habitually short sleep duration, increase the risk of weight gain over the life course (31), and provide mechanistic

insights into how this may occur via alterations of glucose metabolism and energy expenditure.

Another recent study demonstrated that acute circadian misalignment (sleeping during the biological day and eating during the biological night), as occurs with jet lag and shift work, results in similar adverse effects on glucose metabolism: increased postprandial glucose despite increased circulating insulin levels, suggesting reduced insulin sensitivity coupled with an inability of the pancreas to sufficiently increase insulin secretion (21). Circadian misalignment usually also involves some degree of sleep restriction (sleep efficiency declined by 17% when misaligned in that study), representing a combined physiological challenge. Here we demonstrate the effects of a much more prolonged and more severe combined challenge in both young and older subjects, exposure to sleep restriction of 5.6 h sleep per 24 h with concurrent circadian disruption for an average of 19 days (range 15-22 days). Sleep restriction alone in younger men and middle-aged adults leads to no change in RMR (3, 32). However, we observed a notable decrease in RMR with prolonged sleep restriction and circadian disruption, along with significantly elevated glucose in response to a meal, but quite unexpectedly, a decreased insulin response suggesting pancreatic dysfunction that was unrelated to the small loss of body weight. Previous sleep restriction studies have shown reduced insulin sensitivity with no change in the insulin response to a tolerance test (3, 30) or meal response (1). Acute circadian misalignment results in an inadequate post-prandial insulin response (21). Our new findings demonstrate that with chronic exposure to sleep restriction and circadian disruption the pancreas exhibits more severe dysfunction as evidenced by the fact that insulin levels actually decreased despite elevated plasma glucose levels. Insulin clearance might also change. This physiological mechanism could explain the association of habitual sleep deficiency and elevated risk for obesity and diabetes (5, 6), and on weight gain and diabetes in a longitudinal study of male nightworkers (33, 34). Notably, in our study these effects in these healthy individuals were reversible with 9 days of stable circadian re-entrainment and recovery sleep.

The metabolic assessments during baseline and after exposure to chronic sleep restriction and circadian disruption were made at the same transiently realigned phase of the central circadian pacemaker. Controlling for the central circadian pacemaker phase of the metabolic assessment ensured that the effects observed were due to the combination of the prior histories of prolonged sleep restriction and of circadian disruption, rather than acute misalignment. Such effects have been proposed as important factors in the development of metabolic dysregulation via desynchronization of the central circadian pacemaker with respect to sleep-wake, fasting-feeding, and dark-light cycles (35).

Peripheral clocks are entrained by timing of food intake in rodents (36). Although no data are available from human studies, it is possible that the effects we observed reflect a reduced temporal coordination between central circadian pacemaker and peripheral tissues (37) such as the pancreas that may be responding to changes in meal timing independently of central circadian clocks (38). Misalignment of peripheral oscillators (e.g., in the pancreas and liver) with respect to the phase alignment of the central circadian pacemaker may thus also play a role in metabolic dysregulation. If the central circadian pacemaker and peripheral pacemakers were out of phase then the normally coordinated response to a meal may be dysfunctional, and lead to abnormal physiological responses to food intake. Peripheral oscillators relevant to metabolism coordinate both metabolic and circadian pathways (39) required for normal hepatic lipid metabolism and homeostasis (40). These findings and others (35), taken together with our current results, suggest that synchronized central and peripheral circadian processes are necessary for the optimal regulation of energy homeostasis in mammals.

Previous studies of sleep restriction in healthy young men have shown a decrease in plasma leptin and an increase in total ghrelin coupled with increased hunger and appetite (41, 42), possibly reflecting relative underfeeding in those studies. A study of young women exposed to a single night of partial sleep restriction revealed an elevation of fasted morning leptin levels (43). Epidemiological studies of short sleep duration have observed an association of shorter self-reported habitual sleep duration with lower leptin levels and higher total ghrelin levels (44, 45). In contrast, another laboratory study in middle-aged men and women exposed to either 8.5 or 5.5 h/night of time in bed for 2 weeks but with *ad libitum* food observed no differences in leptin or ghrelin levels between the sleep duration conditions. Instead, caloric consumption in the form of snacking increased, such that the subjects consumed over 200 kcal more food per day in the sleep-restricted condition (32). This increased food intake may have thereby normalized the leptin- and ghrelin-related hunger signal associated with sleep restriction. Although, we observed no change in fasted morning leptin levels in either young or older subjects exposed to prolonged sleep restriction with concurrent circadian disruption, and with controlled caloric intake, we did observe that, at the end of this period and through recovery, leptin was lower and ghrelin was higher, albeit a very small effect size (Figure 5). The combination of sleep restriction plus circadian disruption may be a qualitatively different challenge than either sleep restriction or circadian disruption alone, or the prolonged duration of our stimulus (averaging 19 days) may have contributed to our findings. Further studies are needed to better quantify the relationships between adipocyte physiology and the extent of sleep restriction (hours in bed per night) and duration of sleep restriction or circadian disruption (days to weeks to months).

Glucose and cortisol profiles were higher during the first week of the combined disruptions (relative to baseline) and these raised levels persisted at all circadian phases throughout the third week of exposure. In contrast, fasting insulin levels were the same for the first week of the exposure as occurred at baseline, yet lower throughout the third week of exposure and not accompanied by further alterations in glucose. These different responses between variables suggest that multiple adaptive or maladaptive processes are at play, and that the extended duration of the combined challenge in our study revealed this dynamic system. The changes in the levels of fasted insulin from the first to the third weeks of exposure in the current study are presumably a result of repetitive and maladaptive insulin responses to meals during the prolonged exposure period.

We observed some age-related differences (e.g. adiposity, RMR, fasted leptin, body weight) but generally the effects of the exposure were age-independent: both young and older participants responded to prolonged sleep restriction combined with circadian disruption with higher glucose levels and lower insulin responses to a standard meal eaten at a consistent circadian phase. This lack of age effect was contrary to our expectations. We note that we studied healthy non-obese participants to ensure that comorbidities did not influence results. Thus, our older participants were very likely to be more healthy that the general older population at large, in whom responses may be different.

Limitations

We investigated the effects of up to three weeks of exposure to the combined challenge of a relatively moderate degree of sleep restriction (5.6 h in bed per 24 h) and circadian disruption. This degree of sleep restriction is similar to that observed in permanent night workers who meet criteria for shift work disorder (15). We assumed that the results of this 3-week challenge would reveal any adaptive or maladaptive physiological effects that would emerge beyond the immediate acute metabolic effects observed in previous sleep restriction studies or circadian misalignment studies in humans (1, 3, 21, 30, 32, 41). Metabolic assessments were made from standardized meal responses at the end of each condition (baseline, sleep restriction with concurrent circadian disruption, and recovery). Although we

did see differences between the first and third week of the challenge, to determine the actual mechanisms that yield the time course of the associated metabolic deficits, future studies will need to be performed with more frequent assessments for weeks to months of exposure, and with perturbation analyses using such challenges as meal responses, intravenous glucose tolerance tests, and or glucose clamp studies. We did not assess changes by, or control for, the phase of the menstrual cycle in female participants, but there were no notable differences by sex or between age groups, possibly suggesting no difference between responses in these younger premenopausal and older postmenopausal women. The weight loss observed during the sleep restriction and circadian disruption period may have reflected a relatively underfed state that could have induced physiological changes. However, the average observed weight loss was quite minor (1.2% of body weight throughout the exposure). Moreover, in correlation analyses, the degree of weight loss in individual participants was not significantly related to the metabolic changes observed, and the weight loss persisted during the recovery phase when metabolic responses had returned to baseline. Thus, the reduction in insulin levels with sleep restriction and circadian disruption in the current study likely occurred via mechanisms unrelated to body mass or any underfeeding. Future studies might avert weight change by measuring metabolic rate rather than estimating metabolic rate prior to calculating dietary requirements, responding to small weight changes with caloric intake changes, and assessing body composition at multiple timepoints across a challenge and recovery (we only assessed body composition at baseline).

Conclusions

Sleep restriction combined with circadian disruption for up to three weeks reduced RMR, caused a relative reduction in insulin secretion to a standardized meal and resulted in relative hyperglycemia, presumably due to an inadequate pancreatic beta cell response. This last effect may underlie the elevated risk of diabetes in conditions of chronic exposure to sleep deficiency and recurrent circadian disruption. Despite some age-related differences between participants (trunk fat mass, basal levels of insulin), a surprising observation in this study was the age-independent effects of the exposure on metabolism: young and older participants both exhibited increases in postprandial glucose and decreases in postprandial insulin. Efforts to reduce the health impact and risk of diabetes in shift workers could thus focus on improving sleep duration and circadian re-alignment strategies to minimize circadian disruption and desynchrony of central and peripheral circadian oscillators via enhancing the strength of synchronizers of circadian rhythms such as the daily cycles of light and meals.

Materials and Methods

Study Design

All participants were studied in the Intensive Physiological Monitoring Unit of the Center for Clinical Investigation at Brigham and Women’s Hospital, Boston, Massachusetts and provided written informed consent. All procedures were approved by the Partners Human Research Committee and were conducted in accordance with the Declaration of Helsinki.

Participant Recruitment and Screening

Healthy adult participants were recruited using newspaper advertisements, flyers, and website postings. To ensure stable circadian rhythms, participants had no history of regular night shift work for the three years prior to the study and no history of travel >2 time zones in the three months prior to the study. Licensed physicians and clinical psychologists performed physical examinations and psychological screenings. Participants underwent an all-night clinical polysomnogram to rule out sleep-disordered breathing and other sleep

disorders. Participants were free of any disorders of sleep, circadian rhythms, and metabolism and passed a urine toxicology screen during screening and upon admission to the inpatient study. Participants received payment for volunteering in this study, equivalent to about ~$10 per hour when in the laboratory.

Pre-Study Conditions

Participants were instructed to maintain a consistent sleep-wake schedule for at least 21 days (mean 29.3 days ± 12.3, range 21-69 days) prior to admission with a 10 h per night scheduled time in bed, at a self-selected, regular time, and compliance with these instructions was confirmed by wrist actigraphy for at least three weeks prior to admission (Minimitter, Bend OR). A sleep diary and calls to a time-stamped phone answering system assured compliance.

Inpatient Study Conditions

Participants were admitted to the Intensive Physiological Monitoring Unit of the Center for Clinical Investigation at Brigham and Women's Hospital for the 39-day inpatient stay (Figure 1) in a controlled laboratory environment free of time cues, maintained at a temperature of 75 ± 3°F. The first three days each included a 12-hour nighttime sleep opportunity; days 2-4 had a nap near the middle of the normal waking period in order to achieve 'sleep satiation'. Days 4-6 included a 10-h nighttime sleep opportunity (referred to as 'baseline'). This was followed by the Forced Desynchrony portion of the protocol, consisting of eighteen, 28-h sleep-wake cycles with a 21.47 h wake episode and a 6.53 hour sleep opportunity (equivalent to 5.6 h of sleep opportunity per 24 h) over 3 weeks. For recovery, participants were realigned to their baseline circadian phase for light-dark, meal, and sleep-wake cycles, then spent 10 inpatient days each at an entrained circadian phase with a 10-h nighttime sleep opportunity before being discharged. During wakefulness participants were allowed to perform activities such as writing, reading, board or card games, movie viewing, arts and crafts, listening to or playing music, and mild stretching (exercise was prohibited). Research technicians observed participants throughout the study, either by closed circuit television or direct observation during waking episodes throughout the laboratory protocol. Light levels were maintained at <0.02 lux during sleep opportunities and at <15 lux during wake episodes to avoid circadian phase-resetting effects of light.

Timing of Metabolic Assessments and Recovery Sleep Episodes

During baseline, exposure to circadian disruption plus sleep restriction, and recovery segments, metabolic assessments of at least 24 h were made at similar circadian phases (± 4 h), determined by core body temperature (CBT) collected at 1-minute intervals throughout the exposure using a rectal thermistor (Measurement Specialties, Hampton, VA). Circadian period and phase of CBT throughout the exposure was analyzed using non-orthogonal spectral analysis (NOSA), as previously described (28). The NOSA analysis was also used to determine the initial endogenous CBT minimum. Although all participants were exposed to the same duration of 21 days challenge, to ensure that standardized metabolic measurements occurred between conditions at closely matched internal circadian phases within each participant, and because there were slight differences in the intrinsic circadian period between participants, this necessitated that the measurements during the challenge occurred on slightly different protocol days across participants. The average period of the internal circadian pacemaker was 24.13 ± 0.22h (range 23.46 – 24.50; p=n.s. for age), the average duration of challenge at the time of the metabolic assessments was 19.2 ± 2.8 24-h days (range of 15-22 days), and the average difference in circadian phase relative to baseline at the times of these measurements equated to +0.7 ± 1.8 h.

Forced-Desynchrony (Circadian Disruption) Metabolic Assessments

Upon waking each day during weeks 1 and 3 of the exposure to sleep restriction combined with circadian disruption, fasted blood samples were drawn through an indwelling IV catheter for assessment of glucose, insulin, cortisol, leptin and free ghrelin. By design, these samples were collected across a range of circadian phases to quantify the acute and chronic effects of both circadian misalignment and circadian disruption with the scheduled light/dark, food/fasting, and sleep/wake schedule.

Controlled Diet

During the inpatient portion of the study, participants received an isocaloric, controlled nutrient diet, consisting of 55-60% CHO, 15-20% protein, 15-30% fat, 150 mEq Na+ (+/-20%), 100 mEq K+ (+/-20%), and a minimum of 2.5 liters fluid per 24 h. The initial diet was calculated based upon the Harris Benedict equation with an activity factor of 1.4 (46). Each participant was given identical breakfast meals during the three intensive 24 h sampling intervals at baseline, the end of the Forced Desynchrony, and after 9 days of stable circadian re-entrainment and recovery sleep. Participants were required to finish all food. A eucaloric diet was maintained by increasing or decreasing kcals when changes in wake-time, fasted, post-void weights exceeded 1 kg. Weighed foods confirmed that actual consumed diet changed from baseline by a mean of only 6 kcal/24 h during the Forced Desynchrony period.

Resting Metabolic Rate

A validated and FDA-approved indirect calorimeter (Medgem 100, HealtheTech Inc) was used to estimate RMR in kcal/day from expired gases (47). Measurements were made after waketime for 12-15 minutes, prior to standardized breakfasts.

Blood Sampling

Fasting blood samples were taken on multiple days throughout the inpatient stay. During the three intensive 24-h sampling intervals, blood samples were scheduled to be taken every ten minutes for an hour following breakfast, every 30 minutes during the following two hours, and hourly at all other times.

Assays

Glucose was assayed using gluco-quant Glucose/HK kits (Roche Diagnostics GmbH, Mannheim, Germany) with a sensitivity of 2 mg/dL, an inter-assay precision CV of 1.7%, and an intra-assay precision CV of 1.0%. Insulin and cortisol were assayed using kits from Beckman Coulter, Inc. (Fullerton, CA). The insulin assay had a sensitivity of 0.03 μIU/mL, an inter-assay precision of 3.1-5.6%, and an intra-assay precision of 2.0-4.2%. The cortisol assay had a sensitivity of 0.4 μg/dL, an inter-assay precision of 6.4-7.9%, and an intra-assay precision of 4.4-6.7%. Leptin and active ghrelin were measured using ELISA kits (Millipore Corporation, Billerica, MA). The leptin assay had a sensitivity of 0.5 ng/mL for the standard assay (25 μL samples size) and 0.125 ng/mL for the sensitive assay and an inter-assay precision of 2.6–6.2% for the standard assay and 1.3–8.6% for the sensitive assay and an intra-assay precision of 2.6–4.6% for the standard assay and 1.4–4.9% for the sensitive assay. The sensitive assay was used to repeat samples with results below the threshold of the standard assay. The ghrelin assay had a sensitivity of 8 pg/mL, an inter-assay precision of 3.5–6.6%, and an intra-assay precision of 1.6–3.6%.

Study Participants

Of the 24 participants to complete the study protocol, 21 were included in these analyses, 11 young (5 female, mean 23 ± 2 years old) and 10 older (5 female, 60 ± 5 years old). One

participant was excluded from these analyses as blood samples were not obtained during intensive sampling intervals due to intravenous sampling difficulties. One young and one older participant were excluded from these analyses because their circadian temperature phase on the evaluation day following exposure to circadian disruption and sleep restriction was >4 h different from that at baseline.

Statistical Methods

Data are presented as mean ± SD unless otherwise indicated. Linear or generalized mixed-effects models were applied to study the effects of the history of sleep restriction combined with circadian disruption on metabolic measures. Participants were treated as random effects. For studying the postprandial responses (baseline, peak, AUC by the trapezoidal method, and 90-minute profiles), condition (baseline, exposure, recovery), age group, sex, and percent body fat were treated as fixed effects and were entered into the initial model, but only significant variables are reported in the final models. Because age was a main interest, age and the interaction of age and condition were retained in all models. For studying the effect of 'exposure' to the combination of circadian disruption and sleep restriction on fasted sample measures during that exposure, we used identical models adding a term for number of weeks into the exposure ("beat cycle"). Linear regression models were used to study the relationship between changes in insulin and bodyweight and changes in RMR, diet consumed, body temperature, and wrist physical activity levels. Significant effects were defined as p-values < 0.05. All tests are two-sided.

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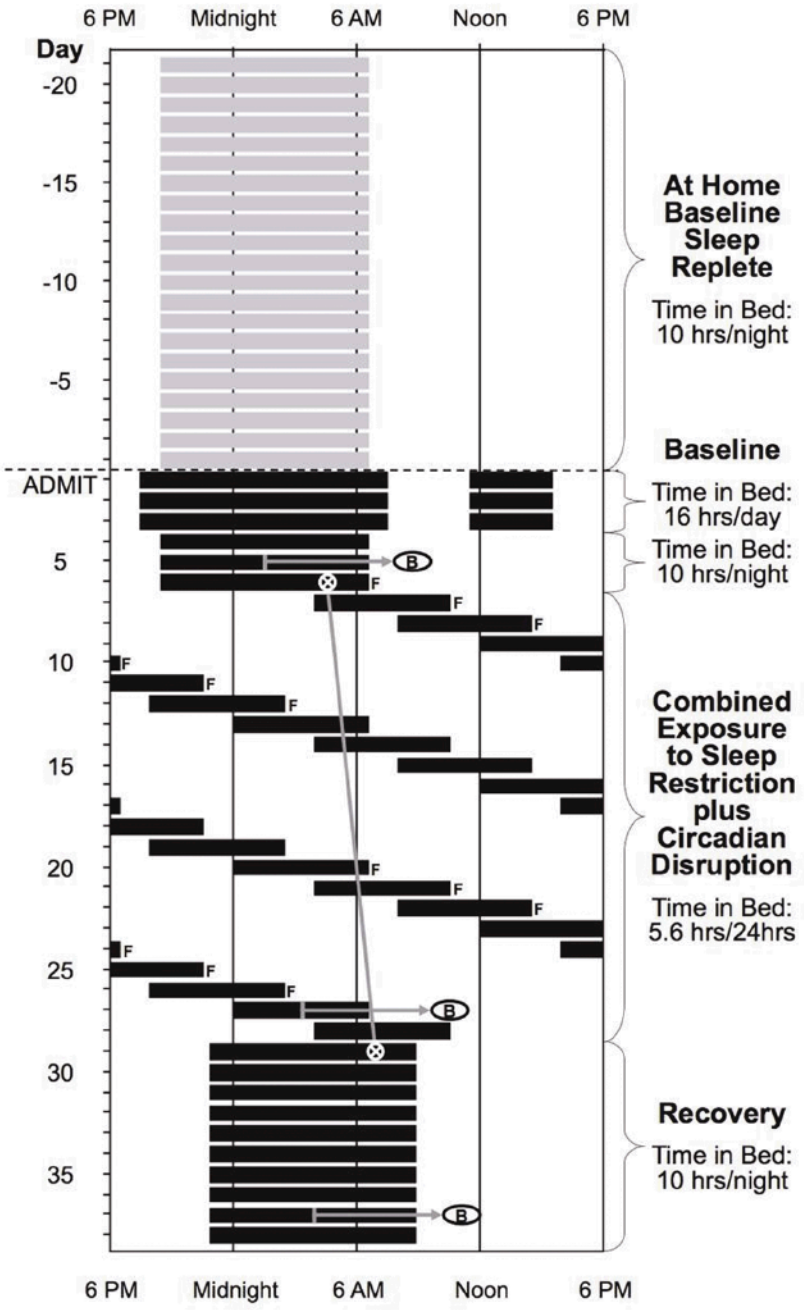

Figure 1. Study schedule

Dark bars represent the scheduled sleep episodes. Subjects completed a 39-day protocol with a baseline 'sleep replete' condition with 3 weeks of 10 h/day of time in bed (TIB) at home, then 6 days with ≥10 h TIB per day. Sleep opportunities were then spread across the circadian cycle on a 28-h "forced desynchrony" (FD) protocol, with 6.5 h TIB (equivalent to 5.6 h per 24h) and 21.5 h of monitored wakefulness for 3 weeks. A subsequent period of 10 days of circadian re-entrainment with sleep recovery (10 h TIB/24 h) with the sleep period adjusted to the same circadian phase as the baseline sleep condition by modification of the duration of the wake period after the last FD day. Standardized breakfast meal responses (B); daily fasted blood samples for assessment of glucose, insulin, cortisol (F); core body

temperature minimum (white X). Time from midpoint of sleep to start of breakfast (grey horizontal arrow) was maintained by choosing a day in the last week of sleep restriction plus circadian disruption such that the standardized meal occurred during this exposure at the same circadian phase as baseline within 4 h (0.7 ± 1.8 h), resulting in an average exposure duration of 19.2 ± 2.8 24-h days (range 15-22 days).

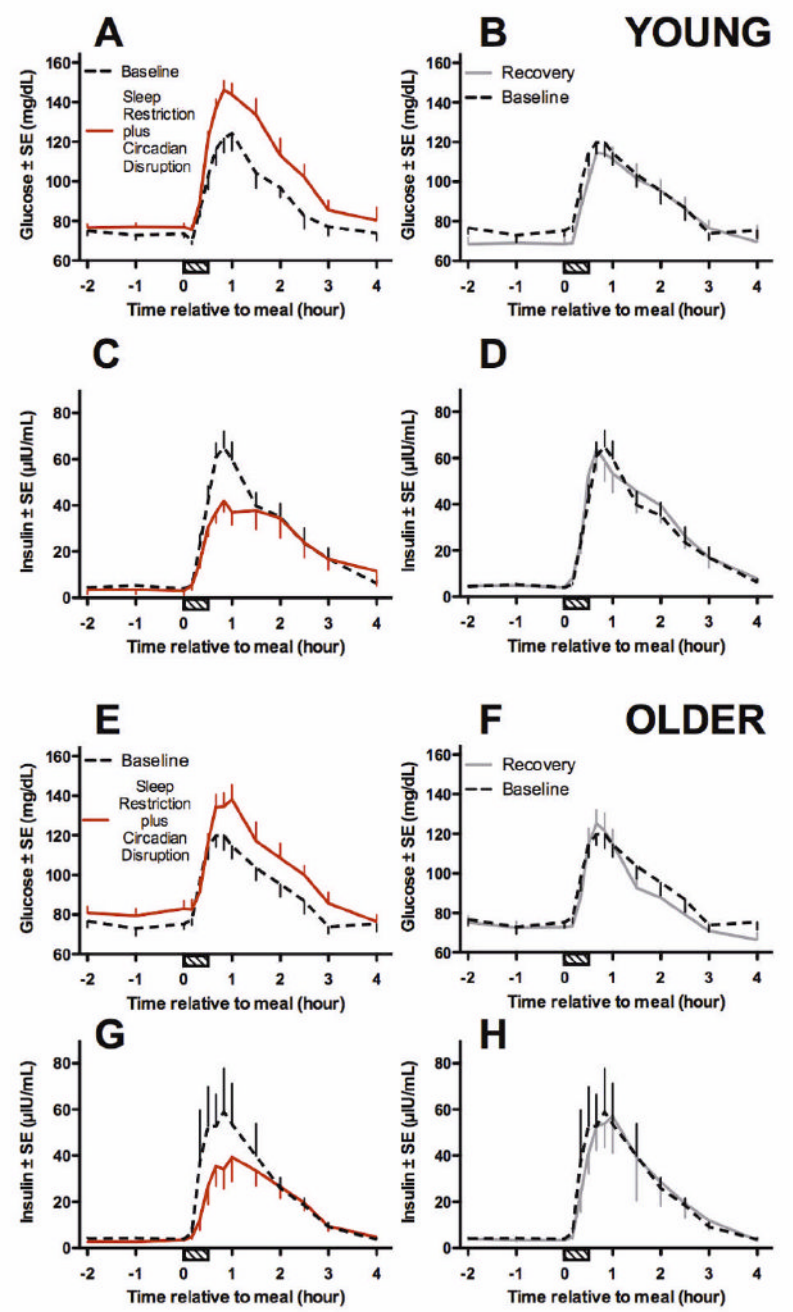

Figure 2. Glucose and insulin response to a meal in young and older participants at baseline, following an average of 19 days of prolonged sleep restriction combined with circadian disruption, and following 9 days of stable re-entrainment and recovery sleep

In young (*panels A-D*) and older subjects (*panels E-H*), mean profiles (± 95% C.I) are depicted for glucose (*panels A, B, E, F*) and insulin (*panels C, D, G, H*) responses to an identical, standardized breakfast (striped horizontal bar at time=0) under conditions of baseline sleep replete (≥10 h TIB/24 h [dashed black line]), history of prolonged sleep restriction combined with circadian disruption (5.6 h TIB/24 h [solid red line; left panels]), and following 9 days of stable circadian re-entrainment and recovery sleep (10 h TIB/24 h [solid grey line; right panels]). In each condition, breakfast was served at the same circadian temperature phase ± 4 h (0.7 h ± 1.8 h).

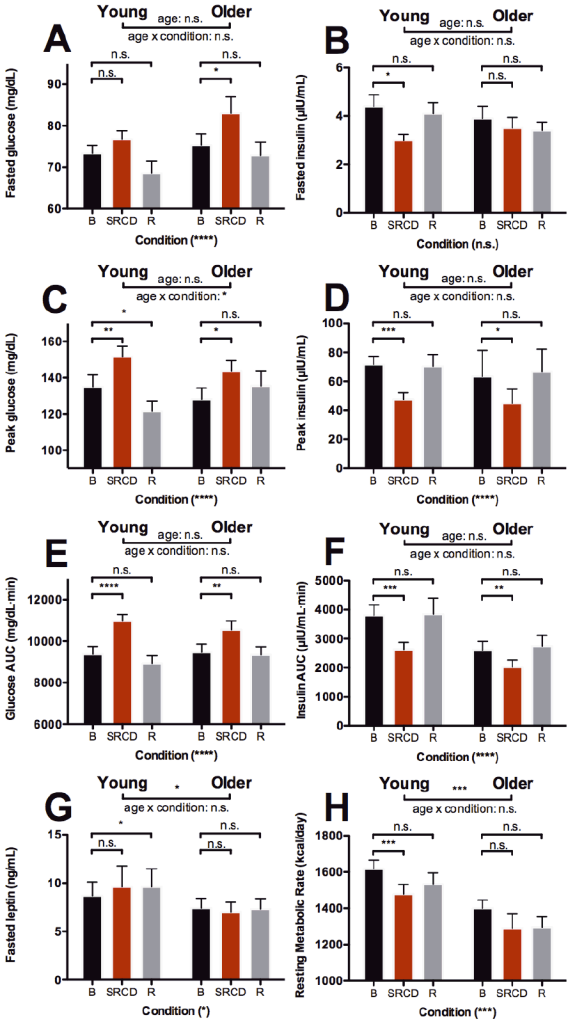

Figure 3. Metabolic effects of prolonged exposure to sleep restriction combined with circadian disruption in young and older participants

Young and older participants were assessed during the conditions of baseline sleep replete (10 hTIB/24 h [B, black bars]), following an average of 19 days of sleep restriction combined with circadian disruption (5.6 h TIB/24 h [SRCD, red bars]), and after 9 days of stable circadian re-entrainment and recovery sleep (10 h TIB/24 h [R, grey bars]). In each condition, a fasted sample was collected prior to an identical breakfast and assayed for glucose (*panel A*), insulin (*panel B*), and leptin (*panel G*). For an hour after the identical breakfast, samples were taken every 10 minutes, and another at 90 minutes post meal and assayed for glucose and insulin. Peak (*panels C-D*) and area under the curve (AUC) values (*panels E-F*), were calculated over the first 90 postprandial minutes. Resting metabolic rate (panel H) was determined prior to the meal. Insulin and leptin were log-transformed prior to statistical testing. Values are means ± SE. Bonferroni-adjusted P-values were based on mixed-effects models with age, condition, and age*condition (and gender for RMR) as the fixed effects and participants as the random effects, and are depicted as follows; $p \leq 0.05$ *; $p \leq 0.01$ **; $p \leq 0.001$ ***; $p \leq 0.0001$ ****. Bonferroni adjustments were applied to each age group separately.

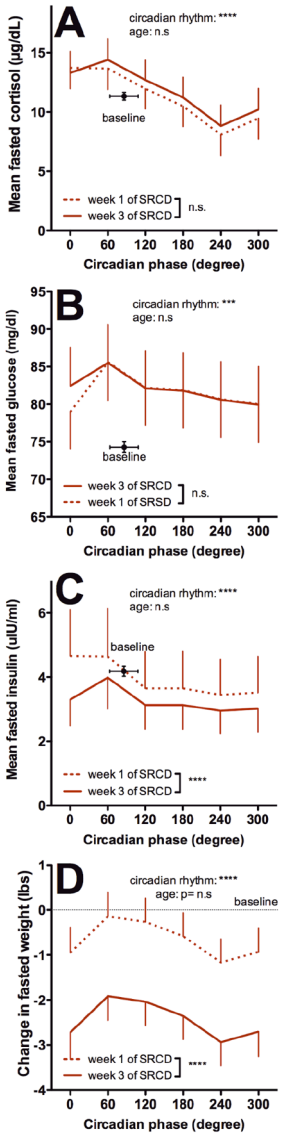

Figure 4. Circadian rhythms of fasted glucose, insulin, and cortisol during the first week (dotted red lines) and third week (solid red lines) of exposure to prolonged sleep restriction combined with circadian disruption

Mean fasted levels (±95% CI) of glucose (*A*), log insulin (*B*), cortisol (*C*) from samples collected within an hour of awakening at all circadian phases and post-void body weight (*D*). For reference, the mean level (±95% CI) of the fasted value at baseline for each measure is depicted at the approximate circadian phase of the baseline assessment (black circle). Circadian phase of the sample collection was determined using core body temperature recordings (see Methods). There was a significant circadian variation, but no significant effects of age for all measures. Week 1 differed from week 3 for insulin levels and weight. Significant p values are depicted as follows; p≤0.05 *; p≤0.01 **; p≤0.001 ***; p≤0.0001 ****.

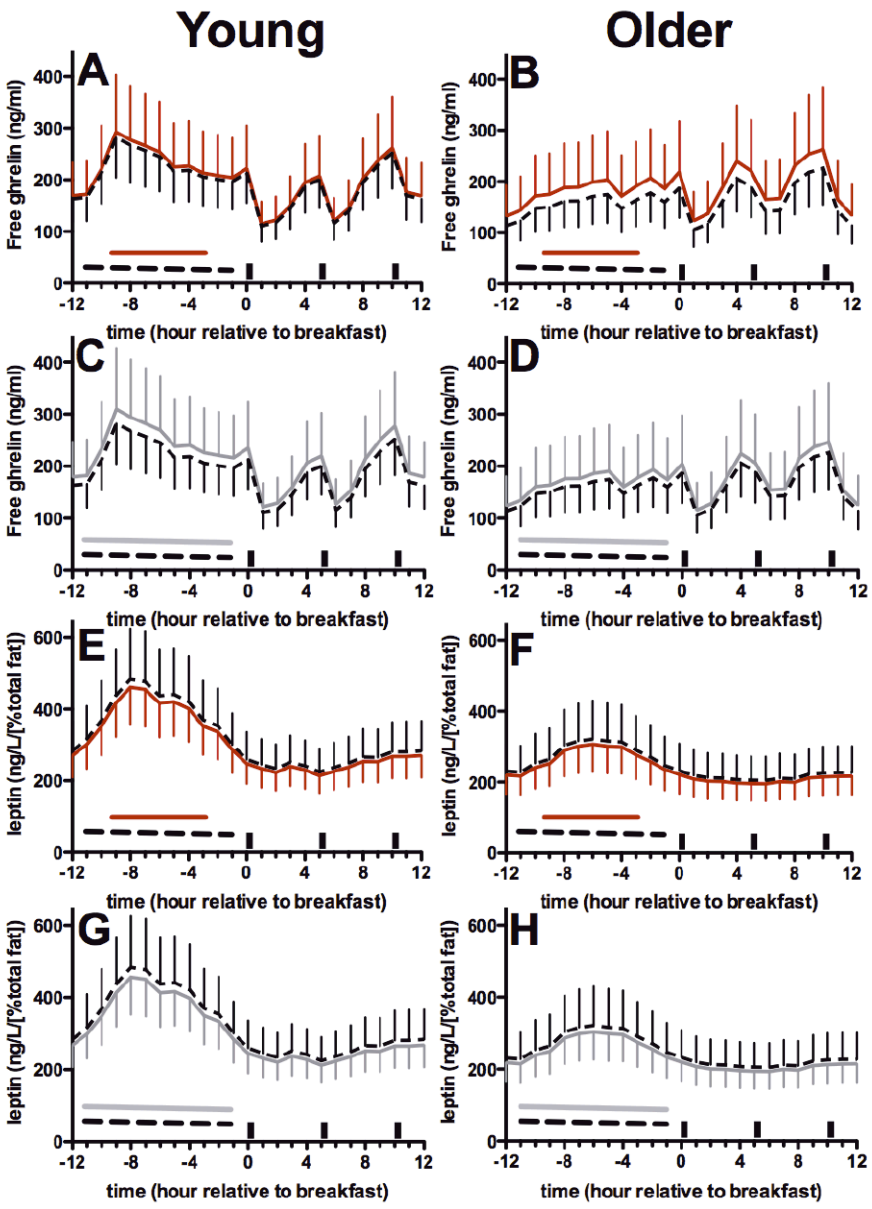

Figure 5.

Free ghrelin and leptin response to a meal in young and older participants at baseline, following an average of 19 days of prolonged sleep restriction combined with circadian disruption, and following 9 days of stable re-entrainment and recovery sleep. In young (panels A, C, E, G) and older subjects (panels B, D, F, H), mean profiles (± 95% C.I) are depicted for free ghrelin (panels A-D) and leptin per percent body fat (panels E-H) and aligned relative to an identical, standardized breakfast (time=0) under conditions of baseline sleep replete (≥10 h TIB/24 h [dashed black line]), history of prolonged sleep restriction combined with circadian disruption (5.6 h TIB/24 h [solid red line]), and following 9 days of stable circadian re-entrainment and recovery sleep (10 h TIB/24 h [solid grey line]). In each condition, breakfast was served at the same circadian temperature phase ± 4 h (0.7 h ± 1.8 h). Sleep periods are depicted by horizontal bars, meals by vertical bars.