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## Neural control of lipid metabolism and inflammation : implications for atherosclerosis

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### Citation

Kooijman, S. (2015, November 18). *Neural control of lipid metabolism and inflammation : implications for atherosclerosis*. Retrieved from <https://hdl.handle.net/1887/36380>

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**Title:** Neural control of lipid metabolism and inflammation : implications for atherosclerosis

**Issue Date:** 2015-11-18

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## PROLONGED DAILY LIGHT EXPOSURE INCREASES BODY FAT MASS THROUGH ATTENUATION OF BROWN ADIPOSE TISSUE ACTIVITY

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## Abstract

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Disruption of circadian rhythmicity is associated with obesity and related disorders including type 2 diabetes and cardiovascular disease. Specifically, prolonged artificial light exposure associates with obesity in humans, although the underlying mechanism is unclear. Here, we report that increasing the daily hours of light exposure increases body adiposity through attenuation of brown adipose tissue (BAT) activity, a major contributor of energy expenditure. Mice exposed to a prolonged day length of 16 h and 24 h light, compared to regular 12 h light, showed increased adiposity without affecting food intake or locomotor activity. Mechanistically, we demonstrated that prolonged day length decreases sympathetic input into BAT and reduces  $\beta$ 3-adrenergic intracellular signalling. Concomitantly, prolonging day length decreased the uptake of fatty acids from triglyceride-rich lipoproteins as well as of glucose from plasma selectively by BAT. We conclude that impaired BAT activity is an important mediator in the association between disturbed circadian rhythm and adiposity and anticipate that activation of BAT may overcome the adverse metabolic consequences of disturbed circadian rhythmicity.

## Introduction

Modern world society is subjected to disturbances of circadian rhythms by shift work, sleep deprivation and environmental light pollution. Importantly, increasing prevalence of obesity is associated with disrupted sleep-wake pattern in humans (1) and coincides with the availability of artificial light (2,3). Additionally, a recent study revealed a relationship between exposure to light at night and obesity in a cross-sectional analysis of over 100,000 women (4). Light input is the most important cue for generation of circadian (~24 h) rhythms by the master clock. Both in rodents and humans the master clock is situated in the suprachiasmatic nucleus (SCN) of the hypothalamus. The SCN is responsible for synchronization of peripheral clocks throughout the body, which is mediated by endocrine and neuronal signals (5). A causal role for a disturbed circadian rhythm in the development of obesity has been demonstrated by animal studies. Mice with genetically dysfunctional clock genes develop obesity and insulin resistance (6-9). Moreover, specific ablation of the SCN induces acute weight gain (10). These results indicate a crucial role for the SCN in the regulation of adiposity.

Interestingly, we previously showed that prolonged light exposure only is sufficient to enhance weight gain in mice. Constant light disrupts the central circadian clock, evidenced by an immediate reduction in the circadian amplitude of SCN electrical activity. Moreover, constant light induces body weight gain and insulin resistance, even faster than high-fat diet, which was not due to increased food intake or reduced locomotor activity (11). Therefore, disruption of the central biological clock likely induces weight gain by decreasing energy expenditure.

Recently, it has been recognized that brown adipose tissue (BAT) importantly contributes to energy expenditure. BAT combusts high amounts of triglycerides (TG) into heat, a process called thermogenesis that is mediated by uncoupling protein 1 (UCP1). Interestingly, SCN neurons project onto BAT and injection of glutamate into the SCN increases BAT thermogenesis in rats (12,13). This indicates that BAT may mediate the association between circadian rhythmicity and energy expenditure. Therefore, the aim of this study was to shed light on the association between prolonged light exposure and obesity in humans by investigating the effect of day length on BAT activity in mice in relation to body fat gain, independent of ambient temperature. We demonstrate that daily light exposure negatively associates with the uptake of TG-derived fatty acids and glucose from plasma by BAT, pointing to decreased activity of the tissue. Furthermore, we show that increasing daily light exposure decreases BAT activity through reduced sympathetic stimulation.

## Materials & Methods

### Animal study

All animal experiments were approved by the institutional ethics committee on animal care and experimentation at Leiden University Medical Center (LUMC), Leiden, The Netherlands. 9-12 week old male C57Bl/6J mice (Charles River) were single housed in clear plastic cages within light-tight cabinets at constant room temperature of 22°C. Stable temperature inside the light-tight cabinets was verified in 12 h vs. 24 h light conditions. The cages were illuminated with white fluorescent light with an intensity of approximately 85  $\mu\text{W}/\text{cm}^2$ . Before start of the experiment, mice were kept on a regular 12:12 light-dark cycle. Mice had *ad libitum* access to standard laboratory chow (Special Diets Services, UK) and water throughout experiments. Mice were matched on body weight and light intervention consisted of subjecting mice to either 12, 16 or 24 h light exposure per day (i.e. 24 h) for the duration of five weeks (n= 9).

In a second study, mice were randomized to either bilateral selective sympathetic denervation (n=17) of iBAT or sham surgery (n=6). Mice were anesthetized (isoflurane inhalation) and a midline incision of the skin was made, exposing both iBAT pads. Sympathetic branches were visualized and cut on both sides. Wounds were closed and mice received post-operative analgesia (0.03 mg/kg buprenorphine, Temgesic, Merck). Successful denervation was confirmed retrospectively by absence of TH in iBAT sections (see below). After four days of recovery, mice that underwent denervation were randomized based on body weight and exposed to 12, 16, or 24 h light per day for five weeks while sham operated mice were exposed to 12 h light per day and served as a reference group.

### Body composition, food intake and locomotor activity

At the end of the experiment, body weight was measured and body composition (i.e., lean mass and fat mass) was determined in conscious mice using an EchoMRI-100 (EchoMRI, Houston, Texas). Food intake was monitored by weighing food on lids either during last two weeks of light intervention or throughout the five weeks of light exposure (denervation experiment). Behavioural activity of mice was assessed with passive infrared detectors and recorded using Actimetrics software (Wilmette, IL, USA).

### TG and glucose clearance

At the end of the experiments, the clearance of TG and glucose was assessed. Glycerol tri[ $^3\text{H}$ ]oleate ([ $^3\text{H}$ ]TO) labeled VLDL-like emulsion particles (80 nm) were prepared as previously described [14] and [ $^{14}\text{C}$ ]deoxyglucose ([ $^{14}\text{C}$ ]DG) was added (ratio  $^3\text{H}:^{14}\text{C} = 6:1$ ). After 5 weeks of light intervention, mice were fasted for 4 h (9AM

to 1PM clock time, corresponding to Zeitgeber time (ZT) 2-6 for 12 h group and ZT 4-8 for 16 h group) and intravenously injected with the radiolabeled emulsion particles (1.0 mg TG in 200  $\mu$ L PBS) and glucose via the tail vein. At time points  $t=2, 5, 10$  and  $15$  min after injection, blood was taken from the tail vein to determine the serum decay of both radiolabels. Immediately after the last blood withdrawal, mice were euthanized by cervical dislocation and perfused with ice-cold PBS for 5 min. Organs were harvested, weighed, and the uptake of  $^3\text{H}$  and  $^{14}\text{C}$  radioactivity was determined.

## Histology

Formalin-fixed paraffin-embedded iBAT and gWAT sections were cut ( $5 \mu\text{m}$ ). To determine gWAT cell size, sections were stained with Mayer's haematoxylin and eosin. White adipocyte size was quantified using ImageJ software. To determine sympathetic activation of iBAT a TH staining was performed. Sections were rehydrated and incubated 15 min with 10 mM citrate buffer (pH 6.0) at  $120^\circ\text{C}$  for antigen retrieval. Sections were blocked with 5% BSA/PBS followed by overnight incubation with anti-TH antibody (1:2000, AB-112, Abcam) at  $4^\circ\text{C}$ . Next, sections were incubated with a secondary antibody (anti-rabbit antibody, DAKO enVision), stained with Nova Red and counterstained with Mayer's haematoxylin. Percentage of area positive for TH staining was quantified using Image J software.

## Gene expression analysis and mitochondrial assays

A part of iBAT and sBAT was snap frozen and stored at  $-80^\circ\text{C}$  for gene expression analysis and protein analysis (see below). Total RNA was isolated using TriPure (Roche) according to the manufacturer's instructions.  $1 \mu\text{g}$  of total RNA was reverse-transcribed using M-MLV reverse transcriptase (Promega, Madison, WI, USA). Real-time PCR was carried out on a CFX96 PCR machine (Bio-Rad) using IQ SYBR-Green Supermix (Bio-Rad). Expression levels were normalized to *36B4* as housekeeping gene. Expression of mitochondrial genes was measured with quantitative PCR on a Roche Lightcycler 480 using Roche SYBR-green mastermix, using mitochondrial specific primers (Table S1). Mitochondrial DNA (mtDNA) abundance was quantified as described before (15). In short, total DNA was extracted from sBAT tissue, using the QIAamp DSP DNA Mini Kit (Qiagen). Citrate synthase activity was measured in sBAT tissue as described before (16).

## Western blot analysis

The iBAT samples stored at  $-80^\circ\text{C}$  were homogenized in lysis buffer. Samples were diluted and denatured for 5 min at  $95^\circ\text{C}$  after adding Laemmli Sample Buffer (1:1, vol/vol; Serva, Heidelberg, Germany). Proteins within homogenates ( $15 \mu\text{g}$ ) were separated on a 10% SDS-page gel and subsequently transferred onto blotting membranes. The blotting membranes were blocked with 5% milk powder and

incubated overnight at 4°C with the primary antibody  $\beta$ -actin, pCREB, pAMPK or pHSL S565 (Cell Signaling). Secondary antibody (anti-rabbit IgG HRP conjugate; 1:5000; Promega, Madison, WI, USA) was added and SuperSignal Western Blot Enhancer (Thermo Scientific, Rockford, IL, USA) was used to visualize protein bands. Blots were analysed with Bio-Rad Quantity One and normalized to  $\beta$ -actin.

## Statistical analysis

Data are presented as means  $\pm$  SEM. Correlations between two dependent variables were made using Pearson's correlation. Associations of variables with day length were assessed by linear regression analysis. Differences between groups were determined using T-tests for normally distributed data. Contribution of light exposure as a covariate to body weight gain was analysed by mixed model analysis using IBM SPSS Statistics version 20. To assess behavioural activity, actograms were analysed using Clock lab and rhythmicity F periodogram analysis was performed on activity bins of 10 minutes of the last 10 consecutively recorded days, based on the algorithm of Dörrscheidt and Beck (17). Differences at P values < 0.05 were considered statistically significant.

## Results

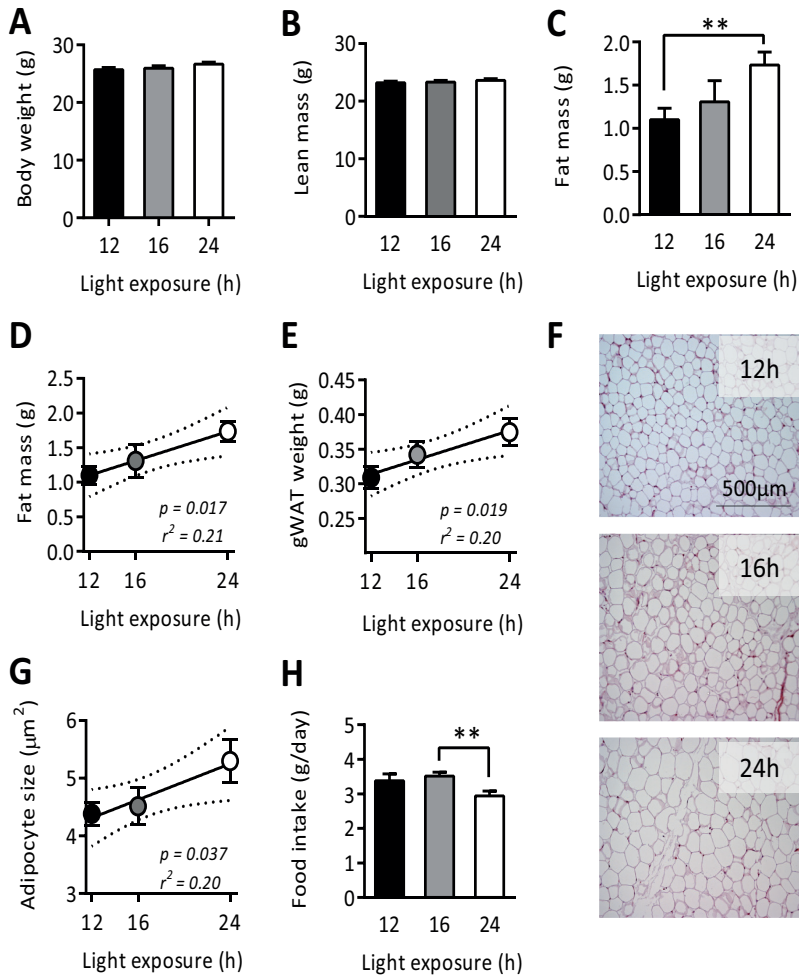
### Entrainment to light schedules

Male 12 week old C57Bl/6J mice, fed *ad libitum* a regular chow diet, were exposed to daily light exposure of either 12 h, 16 h or to 24 h during 5 weeks at a constant ambient temperature of 22°C. During the last 2 weeks of light intervention, circadian rhythm in behavioural activity was assessed in their home cages. As compared to a day length of 12 h (**Supplemental Figure 1A**), mice exposed to a day length of 16 h showed a retained circadian (24 h) rhythm in behavioural activity, with high activity during night-time (**Supplemental Figure 1B**). In contrast, circadian rhythmicity of behavioural activity was largely reduced in mice exposed to constant light (**Supplemental Figure 1C**).

### Prolonged daily light exposure increases adiposity without increasing food intake

After 5 weeks of light intervention, body weight was determined and body composition was assessed by EchoMRI. Prolonged light exposure did not significantly increase total body weight (**Figure 1A**) or lean mass (**Figure 1B**). Interestingly, we observed a daily light exposure-dependent increase in fat mass which reached significance for 24 h *versus* 12 h exposure (+57%;  $p=0.01$ ; **Figure 1C**). In fact, duration of light exposure positively correlated with the body fat mass ( $\beta=0.053$ ;  $r^2=0.21$ ;  $p=0.02$ ) (**Figure 1D**). Mixed model analysis of weekly body weight development showed that light exposure





**Figure 1** – Mice were exposed to either 12, 16 or 24 h light ( $n = 9$ ) for 5 weeks, and body weight (A), lean mass (B) and fat mass (C) were determined. Correlations are depicted between the light exposure period and total fat mass (D), gWAT weight (E) and adipocyte size in gWAT (G). Representative images of gWAT stained with haematoxylin and eosin are shown (F). Food intake of the last two weeks of light intervention was measured (H). Data are presented as means  $\pm$  SEM. Dotted lines represent 95% confidence interval of the regression line. \*\* $p < 0.01$ .

significantly contributed to weight gain ( $p = 0.028$ ). After 5 weeks of light intervention, mice were sacrificed after a kinetic experiment with radioactive tracers (see below), and gonadal white adipose tissue (gWAT) was quantitatively weighed and examined histologically. A positive correlation was found between light exposure duration and gWAT weight ( $\beta = 0.005$ ;  $r^2 = 0.20$ ;  $p = 0.02$ ) (Figure 1E) as well as average adipocyte size ( $\beta = 0.08$ ;  $r^2 = 0.20$ ;  $p = 0.04$ ) (Figure 1F,G). The gWAT weight and adipocyte size

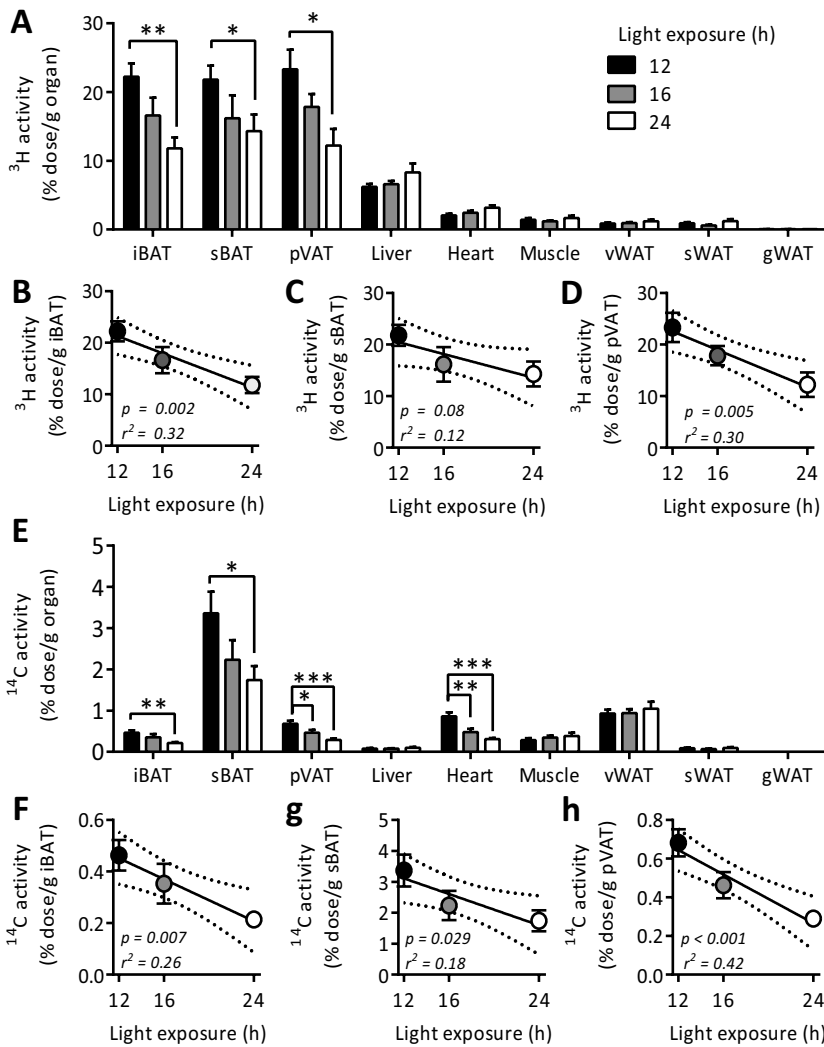
were significantly increased in mice exposed to 24 h light compared to 12 h (+21%;  $p=0.02$  and +21%;  $p=0.04$ , respectively). Notably, food intake was not different in mice exposed to 16 h light, and even tended to be reduced in mice continuously exposed to light (-13%;  $p=0.08$ ) compared to mice exposed to 12 h light per day, (**Figure 1H**). Therefore, the positive correlation between day length and adiposity is not explained by hyperphagia, consistent with our previous observations that exposure of mice to constant light decreases energy expenditure rather than increasing food intake (11).

## Prolonged daily light exposure decreases the nutrient uptake by brown adipose tissue

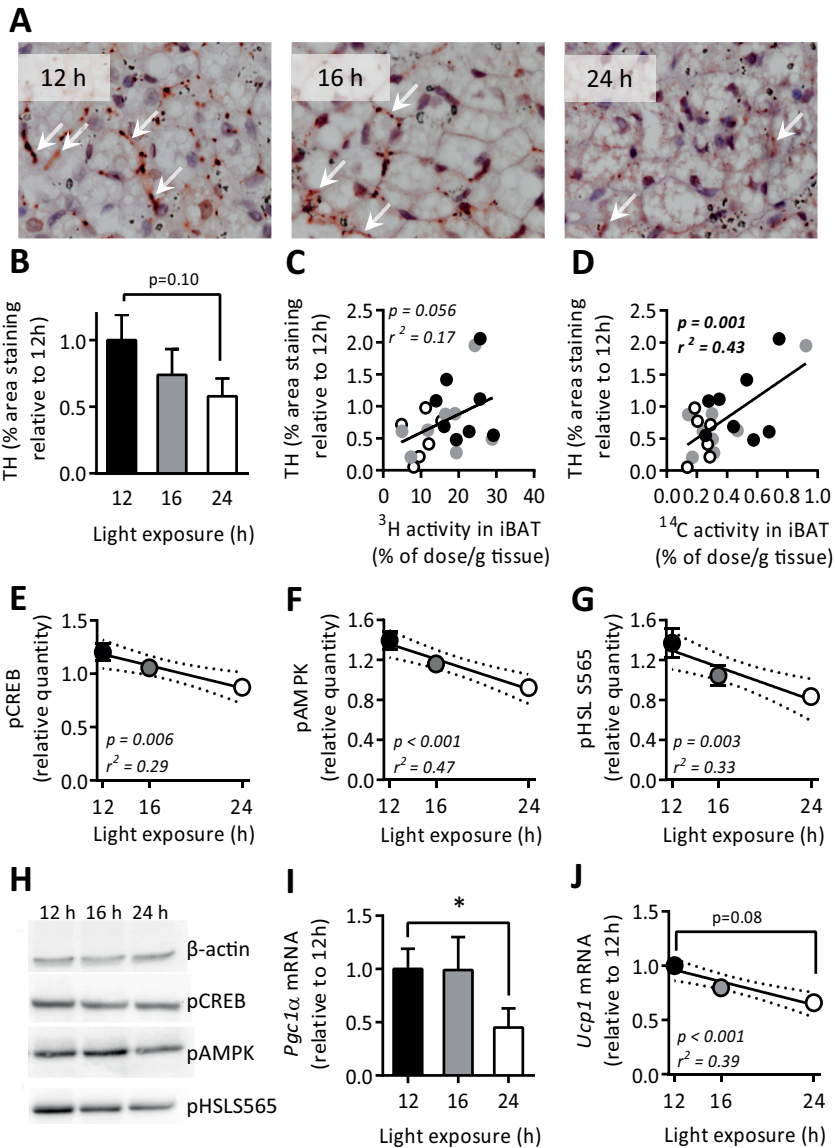
To investigate whether prolonged daily light exposure reduces BAT activity, consistent with decreased energy expenditure, we determined the effect of light exposure duration on the ability of BAT to take up TG-derived free fatty acids and glucose from plasma. Hereto, we assessed the kinetics of intravenously injected glycerol tri<sup>[3H]</sup>oleate ([<sup>3H</sup>]TO)-labeled VLDL-like emulsion particles and [<sup>14</sup>C]deoxyglucose ([<sup>14</sup>C]DG) and determined the distribution of radiolabels at 15 min after injection.

Prolonged daily light exposure did not substantially alter the kinetics of plasma clearance of [<sup>3H</sup>]TO and [<sup>14</sup>C]DG (**Supplemental Figure 2A,B**). In mice exposed to a 12 h light per day, the uptake of [<sup>3H</sup>]TO-derived radioactivity was much higher in the various BAT depots (interscapular BAT (iBAT), subscapular BAT (sBAT), and perivascular adipose tissue (pVAT)) as compared to liver (~3.5-fold), heart (~10-fold), muscle (~15-fold) and white adipose tissue (WAT) (~25-650-fold) (**Figure 2A**). Interestingly, the uptake of [<sup>3H</sup>]TO-derived activity by iBAT, sBAT and pVAT decreased with prolonged light exposure reaching -47% ( $p=0.001$ ), -34% ( $p=0.03$ ) and -48% ( $p=0.01$ ) for 24 h *versus* 12 h light exposure (**Figure 2A**). Accordingly, the day length negatively associated with the uptake of [<sup>3H</sup>]TO-derived activity by iBAT ( $\beta=-0.83$ ;  $r^2=0.32$ ;  $p=0.002$ ) (**Figure 2B**), sBAT ( $\beta=-0.58$ ;  $r^2=0.12$ ;  $p=0.08$ ) (**Figure 2C**) and pVAT ( $\beta=-0.90$ ;  $r^2=0.30$ ;  $p=0.005$ ) (**Figure 2D**). Prolonged light exposure did not alter the uptake of [<sup>3H</sup>]TO-derived radioactivity by organs other than BAT. Consistent with reduced TG-derived free fatty acid uptake by BAT, we found that prolonged light exposure associated to increased plasma free fatty acid levels ( $\beta=0.03$ ;  $r^2=0.45$ ;  $p<0.001$ ) (**Supplemental Figure 2C**).

Prolonged daily light exposure also decreased the uptake of [<sup>14</sup>C]DG by BAT. As compared to 12 h light exposure, 24 h light exposure decreased the uptake of [<sup>14</sup>C]DG by iBAT (-54%;  $p=0.002$ ), sBAT (-48%;  $p=0.02$ ) and pVAT (-57%;  $p=0.001$ ) (**Figure 2F**). Additionally, 16 h of light a day significantly decreased glucose uptake in pVAT (-32%;  $p=0.04$ ) compared to 12 h (**Figure 2E**). Similar to the uptake of [<sup>3H</sup>]TO-derived radioactivity, day length negatively associated with the uptake of glucose by iBAT ( $\beta=-0.02$ ;  $r^2=0.26$ ;  $p=0.007$ ) (**Figure 2F**), sBAT ( $\beta=-0.13$ ;  $r^2=0.18$ ;  $p=0.03$ ) (**Figure 2G**) and pVAT ( $\beta=-0.03$ ;  $r^2=0.42$ ;  $p=0.0005$ ) (**Figure 2H**).



**Figure 2** – Mice were exposed to either 12, 16 or 24 h light ( $n = 8-9$ ) for 5 weeks, and the VLDL-TG and glucose kinetics were assessed by injection of glycerol tri $^3\text{H}$ oleate ( $^3\text{H}$ TO)-labeled emulsion particles and  $^{14}\text{C}$ deoxyglucose ( $^{14}\text{C}$ DG). Uptake of  $^3\text{H}$ TO-derived activity by the various organs was determined **[A]**, and correlations were determined between light exposure and  $^3\text{H}$ TO-derived activity in iBAT **[B]**, sBAT **[C]** and pVAT **[D]**. Concomitantly, the uptake of  $^{14}\text{C}$ DG by the various organs was determined **[E]**, and correlations were determined between light exposure and the uptake of  $^{14}\text{C}$ DG by iBAT **[F]**, sBAT **[G]** and pVAT **[H]**. Data are presented as means  $\pm$ SEM. Dotted lines represent 95% confidence interval of the regression line. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . Abbreviations of organs: iBAT, interscapular BAT; sBAT, subscapular BAT; pVAT, perivascular adipose tissue; vWAT, visceral WAT; sWAT, subcutaneous WAT; gWAT, gonadal WAT.



**Figure 3** – Mice were exposed to either 12, 16, or 24 h light ( $n = 9$ ) for 5 weeks, and interscapular BAT was isolated. Histological sections were stained for tyrosine hydroxylase (TH), representative images are shown (**A**; arrows indicate TH staining), and quantified (**B**). Correlation was determined between TH staining and uptake of [ $^3$ H]TO-derived activity (**C**) and [ $^{14}$ C]DG (**D**). Also, correlations were determined between light exposure and protein levels of pCREB (**E**), pAMPK (**F**), and pHSL S<sup>565</sup> (**G**). Protein levels were normalized to  $\beta$ -actin levels. Representative blots for  $\beta$ -actin, pCREB, pAMPK and pHSL S<sup>565</sup> are shown (**H**). Gene expression of *Pgc1 $\alpha$*  (**I**) and *Ucp1* (**J**) were determined and normalized to *36B4* expression. Data are presented as means  $\pm$  SEM. Dotted lines represent 95% confidence interval of the regression line. \* $p < 0.05$ .

These data imply that prolonged daily light exposure decreases the uptake of nutrients from plasma quite specifically by the various BAT depots, indicating that prolonged daily light exposure decreases the activity of brown adipocytes.

### Prolonged daily light exposure decreases intracellular adrenergic signalling in BAT

As the SCN is directly connected to BAT via the sympathetic nervous system, we reasoned that prolonged daily light exposure decreases sympathetic activation of BAT. Indeed, immunohistochemical analysis of iBAT showed that the amount of tyrosine hydroxylase (TH), the rate-limiting enzyme in the synthesis of noradrenalin tended to decrease with increasing day length, up to -42% in 24 h compared to 12 h ( $p=0.10$ ) (**Figure 3A,B**). Additionally, the amount of TH detected in BAT correlated with the uptake of [ $^3\text{H}$ ]TO-derived activity by BAT ( $r^2=0.17$ ;  $p=0.056$ ) (**Figure 3C**) as well as with the uptake of [ $^{14}\text{C}$ ]DG by BAT ( $r^2=0.43$ ;  $p=0.001$ ) (**Figure 3D**).

Since activation of the  $\beta_3$ -adrenergic receptor by noradrenalin increases intracellular levels of cyclic AMP (cAMP) which activates protein kinase A (PKA), resulting in phosphorylation of cAMP response-binding element (CREB) and activates AMP-activated protein kinase (AMPK), we next determined the phosphorylation status of these proteins involved in thermogenesis in iBAT. Phosphorylated CREB (pCREB) was decreased in 24 h light exposure compared to 12 h (-27%;  $p=0.009$ ) (**Figure 3E**). Phosphorylated AMPK (pAMPK) was decreased in mice on a day length of 16 h (-14%;  $p=0.05$ ) and 24 h (-32%;  $p=0.002$ ) (**Figure 3F,H**) compared to 12 h of light exposure per day, independent of total AMPK levels (**Supplemental Figure 3A,B**). Daily light duration negatively associated with levels of both pCREB ( $\beta = -0.03$ ,  $r^2=0.29$ ,  $p=0.006$ ) (**Figure 3E, H**) and pAMPK ( $\beta = -0.04$ ,  $r^2=0.47$ ,  $p=0.0003$ ) (**Figure 3F,H**). Both pAMPK and pCREB induce phosphorylation of the lipolytic enzyme hormone-sensitive lipase (HSL). While day length did not affect PKA-mediated phosphorylation of HSL on serine 563 position (pHSL S<sup>563</sup>) (**Supplemental Figure 3A,C**), it reduced AMPK-mediated phosphorylation of HSL on serine 565 (pHSL S<sup>565</sup>). A day length of 24 h decreased pHSL S<sup>565</sup> compared to a day length of 12 h (-39%;  $p=0.009$ ) and day length negatively correlated with pHSL S<sup>565</sup> ( $\beta = -0.04$ ,  $r^2=0.33$ ,  $p=0.0031$ ) (**Figure 3G,H**).

Gene targets of pCREB include peroxisome proliferator-activated receptor 1 $\alpha$  (PPARGC1 $\alpha$  or PGC1 $\alpha$ ) that drives transcription of genes involved in mitochondrial biogenesis, and UCP1, which is essential for BAT thermogenesis. Prolonged daily light exposure (24 h vs. 12 h) decreased gene expression of *Pgc1 $\alpha$*  (-55%;  $p<0.05$ ) (**Figure 3I**) and tended to decrease gene expression of *Ucp1* (-37%;  $p=0.08$ ) (**Figure 3J**). Increasing day length negatively associated with expression of *Ucp1* ( $\beta = -0.03$ ,  $r^2=0.39$ ,  $p=0.0005$ ) (**Figure 3J**).

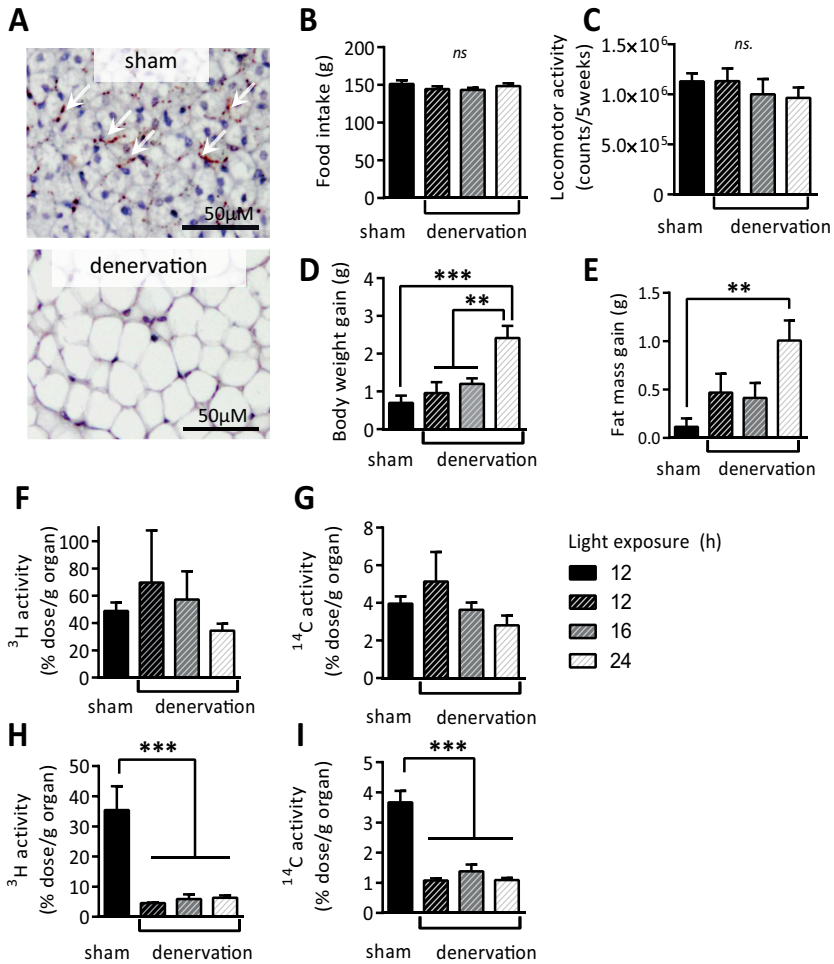
Next, we examined the possibility that prolonged light exposure reduces BAT thermogenic capacity by decreasing mitochondrial function. Prolonged light exposure did not affect gene expression of genes involved in mitochondrial biogenesis (*Tfam*, *Cox7a1*, *Cyc1*, *Atp5g1*), fatty acid oxidation enzymes (*Acadvl*, *Acadl*, *Acadm*) or mitochondrial fusion (*Mfn2*) (**Supplemental Figure 4A**). Additionally, the amount of mitochondrial DNA (**Supplemental Figure 4B**) as well as citrate synthase activity (**Supplemental Figure 4C**) was similar between the different light exposure groups. In line with this finding, total BAT amount was not different upon prolonged light exposure (**Supplemental Figure 4D**).

Together, these data indicate that prolonged daily light exposure reduces sympathetic signalling in BAT that is not accompanied by a decrease in mitochondrial capacity, but does result in reduced uptake of TG-derived fatty acids and glucose.

### Sympathetic denervation of iBAT largely reduces nutrient uptake and abolishes effects of prolonged light exposure

To confirm that sympathetic outflow is crucial for the observed effects of prolonged daily light exposure, we performed selective, bilateral denervation of the iBAT prior to exposing mice to 12, 16 or 24 h light per day. A reference group of mice underwent sham surgery and were exposed to 12 h light per day. Denervation completely abolished sympathetic input into iBAT, as evidenced by absence of TH (**Figure 4A**). In line with the previous experiment, light exposure did not affect food intake (**Figure 4B**). Additionally, spontaneous locomotor activity was similar between all groups (**Figure 4C**). Interestingly, body weight gain only increased in mice that were subjected to 24 h light exposure (**Figure 4D**). This increase was likely due to increased fat mass gain in these animals (**Figure 4E**).

Next, iBAT and sBAT activity was investigated by determining the ability to take up TG-derived fatty acids and glucose from plasma by injection of radiolabeled particles as described above. While the uptake of [<sup>3</sup>H]TO and [<sup>14</sup>C]DG by non-denervated sBAT remained high (**Figure 4F** and **Figure 4G** resp.), specific iBAT denervation lowered the uptake of [<sup>3</sup>H]TO-derived activity (**Figure 4H**) and [<sup>14</sup>C]DG (**Figure 4I**) by iBAT with approximately 70-80% compared to sham operated animals ( $p < 0.001$ ), indicating the importance of noradrenergic input in BAT activity. Importantly, prolonged daily light exposure did not further decrease the uptake of [<sup>3</sup>H]TO and [<sup>14</sup>C]DG. These data indicate the reduction in adrenergic signalling may be causal in the negative correlations of hours of light exposure and BAT activity.



**Figure 4** – Mice underwent bilateral sympathetic denervation of iBAT or sham surgery. Denervated mice were exposed to either 12, 16 or 24 h light (n = 5-6) while sham mice were exposed to 12 h light exposure (n=6). After 5 weeks, iBAT was isolated and histologically stained for tyrosine hydroxylase (TH). Representative images are shown (A). Total food intake (B), locomotor activity (C), body weight gain (D) and fat mass gain (E) were determined. VLDL-TG and glucose kinetics were assessed by injection of glycerol tri[<sup>3</sup>H]oleate ([<sup>3</sup>H]TO)-labeled emulsion particles and [<sup>14</sup>C]deoxyglucose ([<sup>14</sup>C]DG). Uptake of [<sup>3</sup>H]TO-derived and [<sup>14</sup>C]DG activity by sBAT (F,G) and by iBAT was determined (H,I). Data are presented as means ± SEM. ns. = not significant, \*p<0.05, \*\*p<0.01, \*\*\* p<0.001.

## Discussion

This study addressed the effect of daily light exposure (12, 16 and 24 h) on energy metabolism in chow-fed C57Bl/6J mice. We show that prolonging the daily light exposure increases adiposity and reduces the uptake of TG-derived fatty acids and glucose specifically by BAT, accompanied by decreased  $\beta$ -adrenergic signalling in BAT and decreased phosphorylation of intracellular proteins involved in thermogenesis.

Daily light exposure duration positively associated with body fat mass and white adipocyte hypertrophy. These data are in line with observations in field voles, switching the day length from 8 h to 16 h increased body weight by 24% in 4 weeks compared to animals that remained on a day length of 8 h (18). Accordingly, we previously showed that prolonging day length from 12 h to 24 h decreases energy expenditure in mice without increasing food intake or locomotor activity (11). Although acute light exposure at night can reduce locomotor activity (19) and prolonged light exposure affects wheel running activity (20,21), our present study confirms previous reports from us (11) and others (22) that prolonged light exposure does not decrease spontaneous locomotor activity. Together, these studies support the idea that prolonged daily light exposure increases body fat mass through a decrease in energy expenditure rather than to an increase in food intake or decrease of locomotor activity.

The present study strongly suggests that prolonged daily light exposure increases adiposity due to attenuation of BAT activity as reflected by the negative association between daily light exposure and the uptake of fatty acids and glucose by several BAT depots. Of note, prolonged daily light exposure did not affect uptake of nutrients by other metabolic organs, such as WAT. These data are consistent with our recent findings that attenuating BAT activity by inhibiting the central melanocortin system also reduces the influx of nutrients into BAT (23). In fact, activation of BAT, e.g. by cold exposure, increases expression of genes involved in fatty acid oxidation, glucose uptake and lipogenesis (24) and strongly increases the uptake of TG-derived fatty acids (25) and glucose (24).

Our data are consistent with the hypothesis that prolonged daily light exposure decreases BAT activity through reduction of the sympathetic outflow towards BAT. Tyrosine hydroxylase (TH), the rate limiting enzyme in noradrenalin production, correlates with nutrient uptake by BAT and we observed a decrease in sympathetic signalling pathways in BAT. Prolonged light exposure decreased phosphorylation of CREB and AMPK, two main targets of  $\beta$ 3-adrenergic signalling in the brown adipocyte. AMPK not only modulates intracellular lipolysis by phosphorylation of HSL, but also regulates uptake of lipids and glucose by inducing translocation of CD36, LPL and GLUT4 to the plasma membrane (26,27), which may explain the reduced nutrient



uptake by BAT. Moreover, we showed that in the absence of sympathetic input, BAT activity is equal among the various light exposure groups.

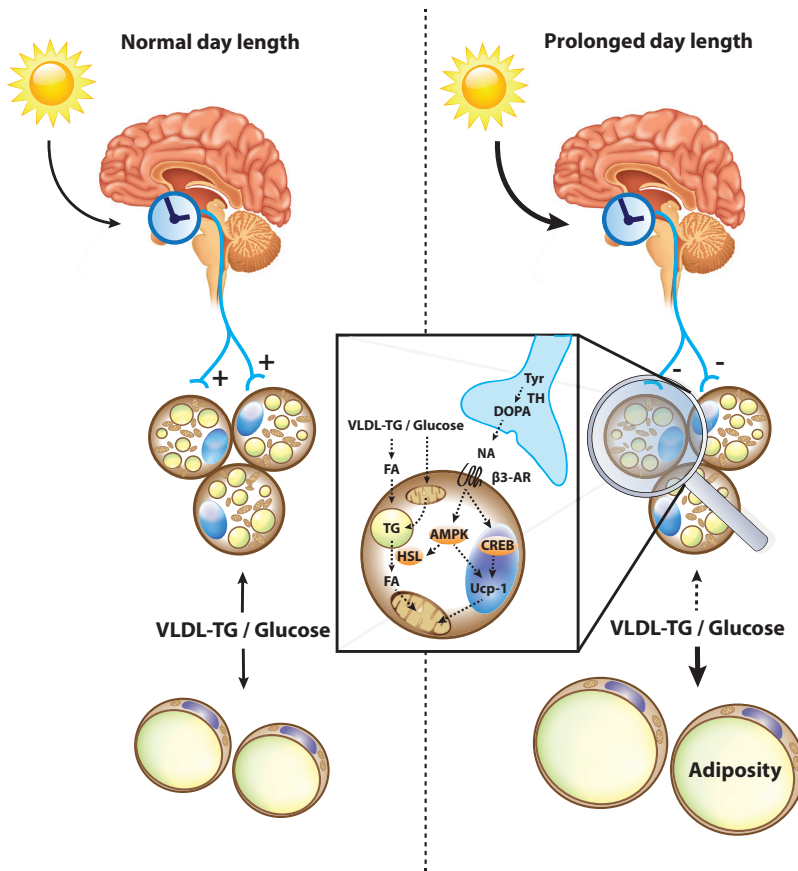
We propose that the SCN directly mediates the decrease in sympathetic outflow upon prolonging daily light exposure. In previous studies we demonstrated that prolonged light exposure dampens the amplitude of electrical activity in the SCN *in vitro* [28] and *in vivo* [10]. Interestingly, the amplitude of electrical activity in the SCN is linked to sympathetic outflow towards multiple organs [29]. This mechanism also explains previous findings that exposure of mice to dim light (5 lux) during 10 h nights for 4 weeks already results in an increase in body weight, which was accompanied by an attenuated amplitude of circadian gene expression in the hypothalamus [30]. Also, sympathetic outflow towards BAT depots other than interscapular BAT would explain our observations that denervation of iBAT does not increase body weight. Only 24 h light exposure decreases sympathetic outflow to such an extent, that mice increase significantly in body weight. Of note, the effects of light exposure on BAT are independent of melatonin secretion, since C57Bl/6J mice are genetically melatonin deficient [31]. However, we cannot exclude the possibility that in humans melatonin does play a role in the association between light pollution and adiposity as administration of melatonin increases BAT growth [32] and activity [33,34] in hamsters and rats.

Based on our collective data, we thus propose the following mechanism by which prolonging daily light exposure increases adiposity: prolonged day length dampens the SCN amplitude thereby lowering sympathetic outflow towards BAT resulting in decreased  $\beta$ 3-adrenergic signalling and thermogenesis in brown adipocytes. As a consequence, the uptake of VLDL-TG derived fatty acids and glucose by BAT is reduced. The decreased combustion of fatty acids by BAT at equal energy intake thus results in a positive energy balance and therefore storage of lipids in WAT (**Figure 5**).

Recent evidence suggests that BAT activity in humans is physiologically regulated by the biological clock. The detectability of BAT by [ $^{18}$ F]fluorodeoxyglucose (FDG)-PET-CT imaging at room temperature follows a circannual cycle, both in the northern and southern hemisphere [35-37], with low detectability of BAT in summer (i.e., short day) as compared to winter (i.e., long day). Although differences in outside temperature over the year would be a likely explanation for this phenomenon, the detectability of BAT showed a stronger correlation with day length than with outside temperature [35]. Based on our present data, the daily light exposure may thus well explain the circannual cycle of BAT detectability. Likewise, impaired BAT activity may also explain, at least partly, the relationship between obesity and disturbances in circadian rhythmicity in humans by light pollution [2,3,38], and possibly also by shift work [39-41] and sleep curtailment [1,42,43]. Additionally, our data may provide the link in the relationship between exposure to light in the bedroom and obesity [4]. The suggested causal relationship has clear implications for the prevention of

obesity in humans. Although the association between light in the bedroom and BAT activity in humans remains to be investigated, future lifestyle advice could include instructing people to darken their bedroom.

In conclusion, our study provides evidence that prolonged daily light exposure increases body fat mass through reduction of BAT activity. The present findings support the hypothesis that the relationship between disturbed circadian rhythmicity and adiposity in humans is mediated by impaired BAT activity.



**Figure 5 – Proposed model on how light exposure modulates body fat mass through brown adipose tissue activity.** Daily light exposure duration is perceived by the suprachiasmatic nucleus, that signals towards BAT via the sympathetic nervous system. At normal day length uptake of nutrients by BAT and WAT is in balance, while increasing daily light exposure result in reduced BAT activation and subsequent storage of excess energy in WAT. The decrease in noradrenaline (NA) availability for stimulation of the  $\beta$ 3-adrenergic receptor ( $\beta$ 3-AR) leads 1) reduced phosphorylation of CREB, which decreases transcription of UCP1; 2) reduced phosphorylation of AMPK resulting in decreased phosphorylation of HSL and thus decreased lipolysis.

## Acknowledgements

The authors thank Simone Denis, Julia Melia Aloma and Lianne van der Wee-Pals for excellent technical support. This research was supported by the Netherlands Organisation for Scientific Research (NWO-VENI grants 016.136.125 to NR Biermasz and 91613050 to RH Houtkooper), the European Foundation for the Study of Diabetes and the Programme Partner Novo Nordisk (grant 94802 to CP Coomans, JH Meijer and PCN Rensen), the Dutch Diabetes Research Foundation (grant 2013.81.1663 to CP Coomans), the Rembrandt Institute for Cardiovascular Science (RICS) (Rembrandt Research Award to MR Boon and RH Houtkooper) and an AMC PhD fellowship (to IA Chatzisyrou). PCN Rensen is an Established Investigator of the Netherlands Heart Foundation (grant 2009T038).

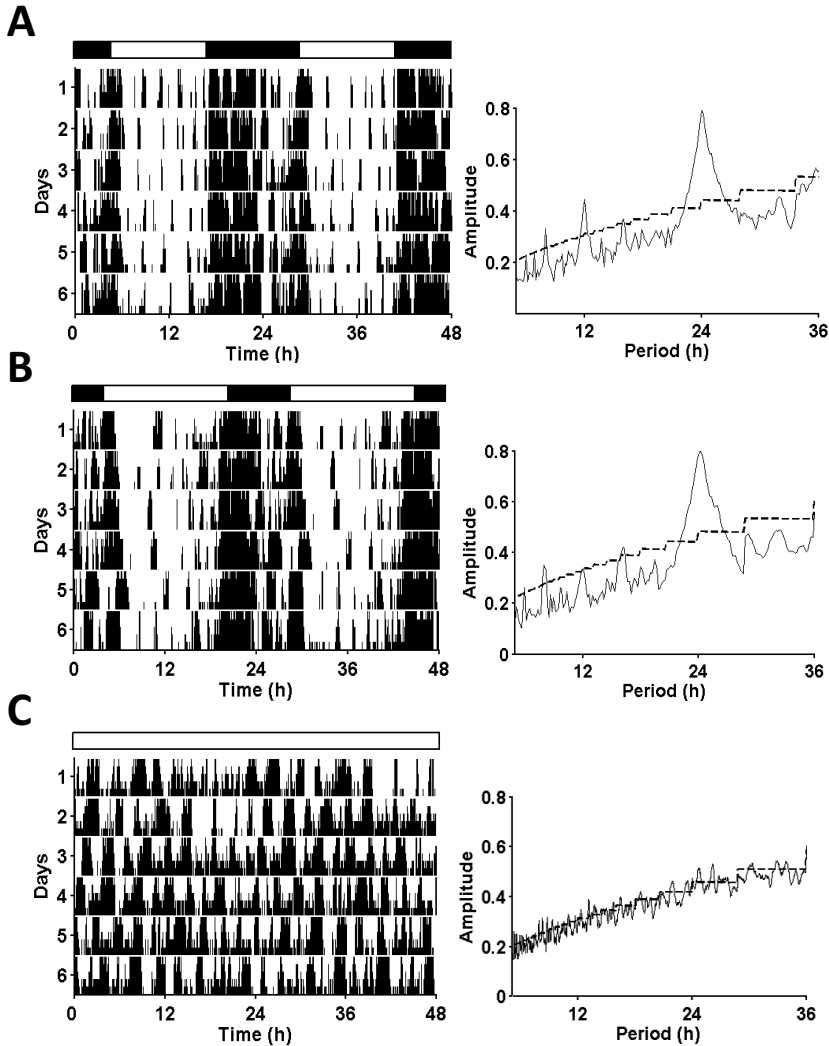
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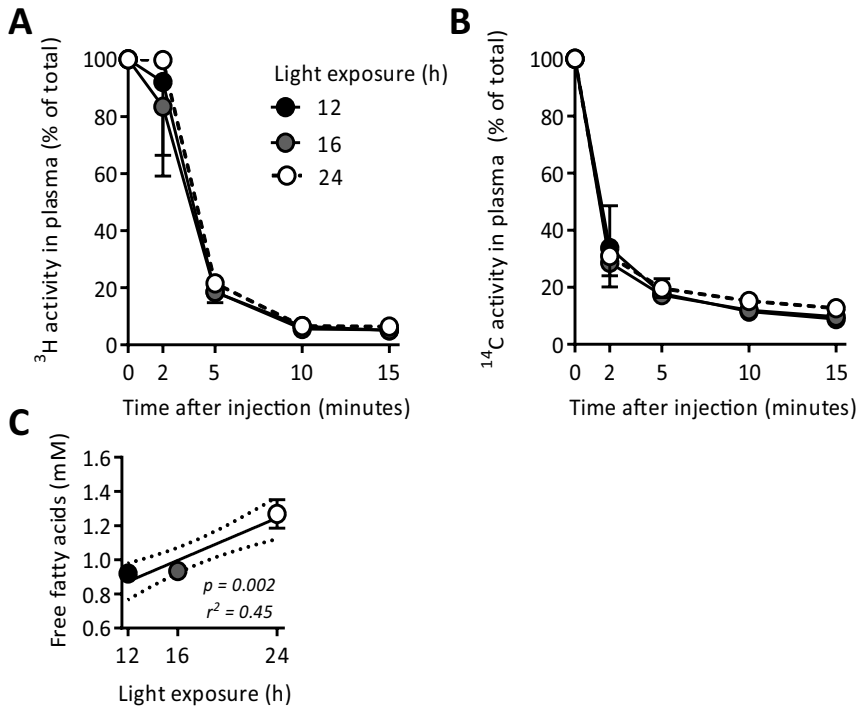
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## Supplementary appendix

4

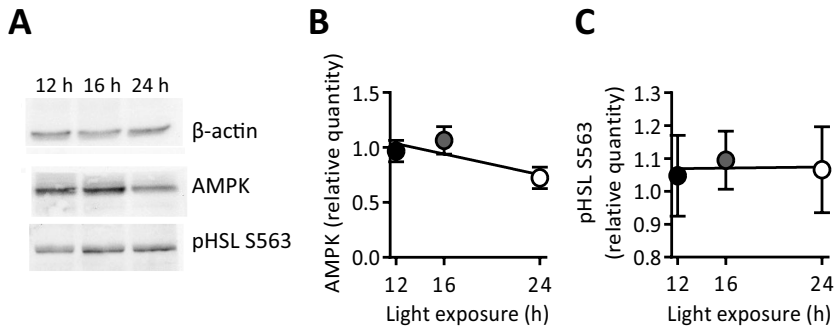


**Supplemental Figure 1 – Prolonged daily light exposure affects behavioural rhythms in mice.** Mice were exposed to either 12, 16 or 24 h light ( $n = 9$ ) for 5 weeks, and behavioural activity was monitored by passive infrared detectors. Representative actograms (left panels) and the pertaining F periodogram (right panels) are shown of a mouse exposed to 12 (A), 16 (B) and 24 h (C) of light. The double-plotted actograms show consecutive days on successive lines and the vertical black upticks indicate behavioural activity measured by passive infrared detectors. The light regimes are plotted on top of the actograms; white areas represent light and black areas represent darkness. Periodogram analysis visualizes the strength of behavioural rhythmicity. The dotted lines in the periodograms indicate the 0.05 level of significance.



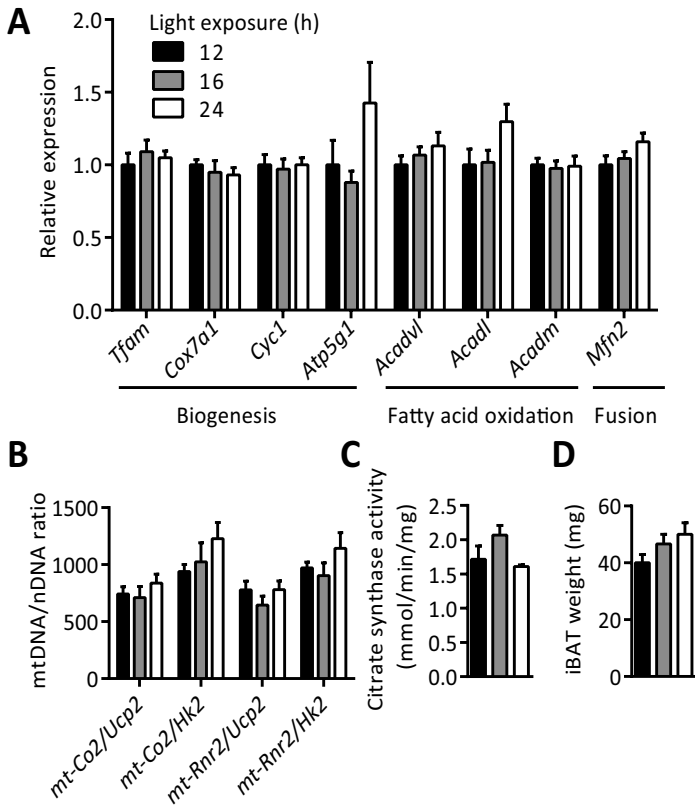
**Supplemental Figure 2 – Effect of light exposure on TG and glucose plasma clearance.**

Prolonged daily light exposure does not affect TG and glucose plasma clearance. Mice were exposed to either 12, 16 or 24 h light for 5 weeks, and the VLDL-TG and glucose kinetics were assessed by injection of glycerol tri $^3\text{H}$ oleate [ $^3\text{H}$ ]TO)-labeled emulsion particles and [ $^{14}\text{C}$ ] deoxyglucose [ $^{14}\text{C}$ ]DG. Blood was drawn 2, 5, 10 and 15 min after injection. [ $^3\text{H}$ ]TO (n=8-9) (**A**) and [ $^{14}\text{C}$ ]DG (n=6-7) (**B**) derived activity was assessed in plasma samples by liquid oscillation counting. Data are represented as means  $\pm$ SEM. Statistical significance was determined by unpaired two-tailed Student's t-test.



**Supplemental Figure 3 – Prolonged daily light exposure does not affect AMPK and pHSLS<sup>563</sup> in BAT.** Prolonged daily light exposure does not affect AMPK and pHSLS<sup>563</sup> in BAT. Mice exposed to either 12, 16, or 24 h light (n= 9) for 5 weeks, and interscapular BAT was isolated for protein quantification. Representative western blots are shown for  $\beta$ -actin, total AMPK and pHSLS<sup>563</sup> (A). Correlation was determined between hours of light exposure and protein levels of AMPK (B) and pHSLS<sup>563</sup> (C). Protein levels were normalized to  $\beta$ -actin levels. Data are represented as means  $\pm$  SEM. Statistical significance between groups was determined by unpaired two-tailed Student's t-test, linear regression analysis was performed to analyse the association of gene expression with light exposure.





#### Supplemental Figure 4 – Effects of prolonged light exposure on mitochondrial function.

Prolonged daily light exposure does not decrease structural mitochondrial function in BAT and quantity of BAT. Mice were exposed to either 12, 16, or 24 h light ( $n=9$ ) for 5 weeks, and interscapular and subscapular BAT was isolated for gene expression and mitochondrial function analysis. Expression of genes involved in mitochondrial biogenesis, fatty acid oxidation and fusion genes did not change upon prolonged light exposure (A). The same is true for relative mitochondrial DNA quantity (B) and mitochondrial citrate synthase activity (C). iBAT was removed quantitatively and weighed (D). Gene expression levels were normalized to *36B4* levels. Mitochondrial DNA quantity is expressed as ratio of mitochondrial gene expression relative to nuclear gene expression (genes indicated below) Data are represented as means  $\pm$  SEM. Abbreviations: *Tfam*, transcription factor A, mitochondrial; *Cox7a1*, cytochrome c oxidase subunit VIIa 1; *Cyc1*, cytochrome C-1; *Atp5g1*, ATP synthase, H<sup>+</sup> transporting, mitochondrial F0 complex, subunit C1; *Acadvl*, acyl-Coenzyme A dehydrogenase, very long chain; *Acadl*, acyl-Coenzyme A dehydrogenase, long-chain; *Acadm*, acyl-Coenzyme A dehydrogenase, medium chain; *Mfn2*, mitofusin 2; *mtCo2*, cytochrome c oxidase II, mitochondrial; *mt-Rnr2*, 16S rRNA, mitochondrial; *Ucp2*, uncoupling protein 2; *Hk2*, hexokinase 2.

