Reduced preprandial dipping accounts for rapid elevation of blood pressure and renal sympathetic nerve activity in rabbits fed a high-fat diet

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Consumption of a high fat diet (HFD) by rabbits results in increased blood pressure (BP), heart rate (HR) and renal sympathetic nerve activity (RSNA) within one week. Here, we determined how early this activation occurred and whether it was related to changes in cardiovascular and neural 24h rhythms. Rabbits were meal-fed a HFD for three weeks then a normal fat diet (NFD) for one week. BP, HR and RSNA were measured daily in the home cage via implanted telemeters. Baseline BP, HR and RSNA over 24h were 71±1mmHg, 205±4b/min and 7±1 normalised units (nu). The 24h pattern was entrained to the feeding cycle and values increased from preprandial minimum to postprandial maximum by 4±1mmHg, 51±6b/min and 1.6±0.6nu each day. Feeding of a HFD markedly diminished the preprandial dip after two days (79-125 % of control, $P<0.05$) and this reduction lasted for three weeks of HFD. 24h BP, HR and RSNA concurrently increased by 2%, 18% and 22%, respectively. Loss of preprandial dipping accounted for all of the BP increase and 50% of the RSNA increase over three weeks and the 24h rhythm became entrained to the light-dark cycle. Resumption of a NFD did not alter the BP preprandial dip. Thus, elevated BP induced by a HFD and mediated by increased sympathetic nerve activity results from a reduction in preprandial dipping, from the first day. Increased calories, glucose, insulin and leptin may account for early changes whilst long-term loss of dipping may be related to increased sensitivity of sympathetic pathways. (Author correspondence: geoff.head@baker.edu.au)

Keywords: 24-Hour rhythm, blood pressure, heart rate, obesity, rabbits, sympathetic nervous system

INTRODUCTION

One of the consequences of the current obesity epidemic is the increased incidence of cardiovascular disease and its associated risks (Murphy *et al.*, 2006). It has been well documented in studies involving humans and in animal models that hypertension and sympathetic activation result from a hypercaloric diet (Verwaerde *et al.*, 1999; Barnes *et al.*, 2003; Lambert *et al.*, 2007; Prior *et al.*, 2010a; Adachi *et al.*, 2011; Armitage *et al.*, 2012). There is a strong correlation between body weight and blood pressure and many studies have suggested that the development of obesity related hypertension is a consequence of weight gain (Reisin *et* and there is increased muscle sympathetic nerve activity and renal noradrenaline spillover within 1 hour of subjects consuming a high-energy meal (Cox *et al.*, 1995).

Disruption of the normal 24h rhythm by changes to diet provides a mechanism for immediate and prolonged elevation of cardiovascular and sympathetic nerve activity. Studies in humans and other species show altered behavioural, cardiovascular, body temperature, metabolic and hormonal rhythms are associated with a diet high in calories or fat (Havel *et al.*, 1999; Cha *et al.*, 2000; Carroll *et al.*, 2005; Kohsaka *et al.*, 2007; Mendoza *et al.*, 2008). Importantly, lack of nocturnal dipping of blood pressure in humans is a serious risk factor for cardiovascular disease and its incidence is increased with obesity (Kario *et al.*, 2003; de la Sierra *et al.*, 2009). In animals with unlimited access to food, the circadian clock is synchronised to the light cycle and rabbits fed *ad libitum* have a nocturnal eating pattern in which cardiovascular parameters are highest in the dark (Eijzenbach *et al.*, 1986). However, rabbits on a food restricted normal diet display patterns of blood pressure, heart rate (HR) and renal sympathetic nerve activity (RSNA) that are strongly entrained to the feeding cycle (Van den Buuse & Malpas, 1997; Lim *et al.*, 2012). This pattern is characterised by a rapid increase in parameters in response to presentation of food followed by a decline to a minimum level in the hours prior to presentation of food on the next day. This minimum level before feeding is defined as "preprandial

al., 1978; Carroll *et al.*, 1996). However it now appears that the timecourse of the onset of hypertension and sympathetic activation is much more rapid and they occur before there are significant changes to body weight or adipose tissue. We recently reported that rabbits exposed to a high fat diet (HFD) develop increased blood pressure within one week (Armitage *et al.*, 2012). This was mediated by an elevation in sympathetic drive and occurred at a time when metabolic factors such as glucose, insulin and leptin were also increased. On an even shorter time scale, rabbits given access to increased quantities of food have raised blood pressure on the first day (Antic *et al.*, 2003)

dipping". Antic and colleagues reported that in rabbits fed a HFD, the preprandial lowering of blood pressure and HR was abolished after one week, effectively suppressing the 24h rhythm (Antic *et al.*, 2001). Furthermore, the loss of the preprandial dipping accounted for the increased blood pressure measured over a full day and whilst adrenergic blockade data led the authors to infer that this process was sympathetically mediated, sympathetic nerve activity was not measured directly (Antic *et al.*, 2001). We hypothesise that a HFD would rapidly suppress preprandial dipping of blood pressure, mediated by a loss of dipping of RSNA. The first aim of the present study was therefore to determine how soon cardiovascular activation follows the introduction of a HFD and whether it could be explained by changes to neural and cardiovascular rhythms. Using rabbits fitted with telemeters, we recorded blood pressure, HR and RSNA over 24h from the first day to the third week of exposure to a HFD and examined the 24h rhythm of these parameters to determine whether pattern changes would account for their overall elevation. The second aim of our study was to determine whether the HFD altered the meal entrainment of the cardiovascular and autonomic 24h patterns.

Although a reduction in food intake is an important target for obese individuals, the often transient nature of calorie restriction may have negative consequences. We have shown that blood pressure and RSNA remain elevated one week after resumption of a normal diet in rabbits previously fed a HFD (Armitage *et al.*, 2012). However, few studies have explored the short-term effects of a reduction in calories, without weight loss, on the cardiovascular system (Antic *et al.*, 2003). The final aim was therefore to determine whether the HFD-induced effects on the cardiovascular rhythm would be reversed by resumption of a normal diet.

MATERIALS AND METHODS

Ethical Approval

Experiments were approved in advance by the Alfred Medical Research Education Precinct Animal Ethics Committee and conducted in accordance with the Australian Code of Practice for Scientific Use of Animals. The study conforms to international ethical standards (Fortaleza *et al.*, 2012). Experiments were conducted in 38 male New Zealand White rabbits (initial body weight 2.6 – 3.3 kg, age 13 – 23 weeks). The rabbits were housed in individual cages with a telemetry blood pressure receiver (model RLA 1020, Data Sciences International, St. Paul, MN, U.S.A) fitted to the door of each cage. They received water *ad libitum* and were fed daily at 12:00 h. The room was kept under controlled light (lights on 06:00 h to 18:00 h) and temperature conditions. It should be noted that other data from some of the rabbits has been published elsewhere (Armitage *et al.*, 2012).

Experimental Procedures and Protocol

Rabbits underwent two preliminary surgical operations under isoflurane anaesthesia (3-4 % in 1L/min oxygen; Abbot, Botany, NSW, Australia) following induction with propofol (10 mg/kg, Fresenius Kabi, Pymble, NSW, Australia). A radiotelemetry transmitter (model TA11PA-D70, Data Sciences) and catheter (150 mm long with 0.7 mm diameter tip) was implanted in the aorta via a small branch of the left iliac artery. Two weeks later, the rabbit received a radiotelemetry implant for measurement of RSNA (model TR76S or TR46S, Telemetry Research, Auckland, New Zealand) with the battery and transmitter pack placed subcutaneously on the rabbit's flank. Carprofen (3 mg/kg, Pfizer, Noth Ryde, NSW, Australia) was given before and 24h after surgery for analgesia.

After one week recovery, baseline mean arterial pressure (MAP), HR and RSNA were measured in the laboratory and the maximal RSNA response measured (in duplicate) by exposure to a noxious stimulus (smoke) for calibration of the RSNA signal (Burke & Head, 2003). MAP was measured both by telemetry and by a catheter in the central ear artery. The telemetry signal was calibrated to the ear artery signal and this adjustment was applied to MAP measured in the home cage to minimise the possibility of drift of the signal with time. Baseline home cage MAP, HR and RSNA were recorded for 1-2 days. Rabbits were then randomised into two groups and meal fed 190g of HFD (13.3 % fat, 3.34 kcal/g, Specialty Feeds, Glen Forrest, Australia, n = 13) or 120g of a normal fat diet (NFD, 4.2 % fat, 2.63 kcal/g, Specialty Feeds, n = 10) for three weeks (Prior *et al.*, 2010a). The HFD fed rabbits were returned to a NFD for the last week of the 4 week treatment protocol. Home cage measurements were made continuously over this period. Body weight and calorie intake were measured daily in a subset of rabbits (n = 5-6 per group) and food intake was measured hourly for seven hours in another cohort of rabbits (n = 5) on days one to four. Ear artery pressure and the response to smoke were measured in the laboratory at weekly intervals. Arterial blood samples (4 ml) were taken weekly for glucose measurement by glucometer (Optium Xceed, Abbott, Doncaster, Victoria, Australia) and plasma stored at -80°C for analysis of insulin (high-sensitivity ELISA with rabbit insulin standard, CrystalChem Downers Grove, Illinois, USA) and leptin by radioimmunoassay using a multispecies kit (LINCO Research, St Charles, Minnesota, USA). Neuronal activation in the suprachiasmatic nucleus (SCN) in 6 rabbits was detected using Fos-related antigens (FRA) as described previously (Davern & Head, 2007).

Data Analysis

MAP, HR, derived from the pressure pulse, and RSNA were digitised online at 500 Hz using an analog-to-digital data acquisition card (National Instruments 6024E, Austin, Texas, USA) and averaged over 2s. An index of locomotor activity was obtained from changes in the received signal strength, which occurred during

movement of the animal. For detection of activity the transmitter had to move so that slight movements occurring during grooming or eating were not registered as activity. MAP, HR and activity were collected continuously over each 24 hour period and averaged over one hourly intervals. RSNA was collected for 15 minutes every two hours and was normalized to the maximum RSNA recorded during the nasopharyngeal response evoked by smoke which was taken to be 100 normalised units (nu) (Burke & Head, 2003). A linear regression was used to estimate the value of the nasopharyngeal response on the days between the weekly measurement. RSNA responses during the first week of treatment were measured in seven rabbits per group; during the second and third weeks and on the first day of return to NFD in 5-6 rabbits per group. However, an insufficient number of rabbits had viable RSNA for the remainder of week four for statistical analysis, due to loss of battery power and the decay of the signal after almost five weeks of recording.

The effect of feeding was measured by comparing data over the six hours between 03:30 h and 09:30 h (preprandial), when the animals were quiet and values were stable, and the 6 hours in the period after feeding (13:00 h – 19:00 h, postprandial). To test whether the 24h pattern of parameters was linked to the light cycle, we determined the difference between values measured over the 12 hours when the lights were on (06:00 h-18:00 h) with those measured over 12 hours in the dark (18:00 h-06:00 h). To minimise the effect of feeding on the responses to the light cycle, parameters collected during the five hours of darkness from 00:00 h to 05:00 h were also compared with those collected from 06:00 h to 11:00 h when the lights were on but prior to feeding. At the end of the study, white adipose tissue was dissected from the mesenteric viscera, perirenal area, epididymis, bladder and heart and weighed.

Values were expressed as mean ± SEM or mean difference ± SE of the difference (SED). Data were analysed by split plot repeated measures analysis of variance which is a mixed model allowing for within-animal and between-animal (group) contrasts. Type 1 error was controlled using Bonferroni and Greenhouse-Geisser corrections (Ludbrook, 1994). A probability of $P < 0.05$ was considered significant.

RESULTS

Effect of the HFD on 24h Averages of MAP, HR, RSNA, Calorie Intake and Body Weight

Baseline home cage values averaged over 24h were 71 ± 1 mmHg (n = 18), 205 ± 4 b/min (n = 18) and 7.3 ± 0.5 nu (n = 14) for MAP, HR and RSNA, respectively. Within two days of commencing the HFD, values had increased by 2 %, 18 % and 22 %, respectively (Fig. 1). MAP and RSNA averaged over 24h continued to increase, and after three weeks of the diet were 7 % and 34 % greater than baseline, respectively ($P < 0.01$, n=5-11, Fig. 1). The effect of HFD on 24h HR measurements was an initial increase of 14 % in the first week, beginning on day one, which diminished to a level 6 % greater than baseline in the 3rd week ($P < 0.001$, Fig. 1). Calorie intake was double the NFD intake as the rabbits consumed their first high fat meal but was maintained for only four days when its decline mirrored that of HR (Fig. 1). In the third week, calorie intake was 35 % greater than baseline intake on a NFD. Three weeks of HFD produced a 14 % gain in body weight (Fig. 1).

Following replacement of the HFD with a NFD, MAP over 24h remained elevated at 5 % of the baseline response ($P < 0.001$, $P_{groups} < 0.001$, Fig. 1). Similarly, body weight remained elevated but HR decreased to a level that was not significantly different from baseline (Fig. 1). On the first day following return to a NFD, RSNA remained elevated at 38 % of the baseline level ($P = 0.011$, $n = 5$) compared with RSNA of NFD rabbits which had not changed from baseline (Fig. 1). In three rabbits, RSNA continued to be viable for the entire week after return to NFD and remained elevated (data not shown). Calorie intake rapidly diminished after the change in diet, indeed the rabbits consumed fewer calories than when previously on a NFD ($P < 0.001$).

In NFD fed rabbits, there was no detectable change in 24h MAP or RSNA over the 4 week experiment period (Fig. 1). By contrast, HR showed a steady time-related decline until the 4th week of recording when it was 8 % lower than baseline ($P < 0.001$, Fig. 1). Body weight in rabbits on a NFD increased by 5 % over the course of the experiments.

Effect of the HFD on 24h patterns

MAP, HR and RSNA in all rabbits during the baseline period showed a pattern over 24h that was closely related to feeding. These parameters were at their lowest level in the preprandial period from 03:30 h – 09:30 h, when average MAP, HR and RSNA were 69 ± 1 mmHg, 184 ± 4 b/min and 6.6 ± 1.0 nu, respectively (Fig. 2). When food was presented and eaten, MAP and HR rapidly increased until a stable postprandial level was reached between 13:00 and 19:00. The average increases from preprandial to postprandial were 4 ± 1 mmHg, 51 ± 6 b/min and 1.6 ± 0.6 nu ($P < 0.05$, for MAP, HR and RSNA, respectively, Fig. 2, 3). Locomotor activity followed a similar pattern (Fig. 2, 3).

The introduction of a HFD markedly altered the 24h patterns of MAP, HR, RSNA and activity. Changes began on the first day but on the second day the differences between preprandial and postprandial levels in MAP and HR were reduced by 79 % and 99 %, respectively ($P < 0.01$ for baseline vs day 2), and remained attenuated for the 20 day HFD feeding period ($P < 0.001$, Fig. 2, 3). The preprandial to postprandial difference in RSNA was also reduced during the first week of the HFD ($P = 0.05$ days 2-6) and elevated postprandial locomotor activity was prolonged (Fig. 3). In HFD fed rabbits, the reductions in the range of the responses were due mainly to a loss of the "dip" normally associated with the preprandial period in the

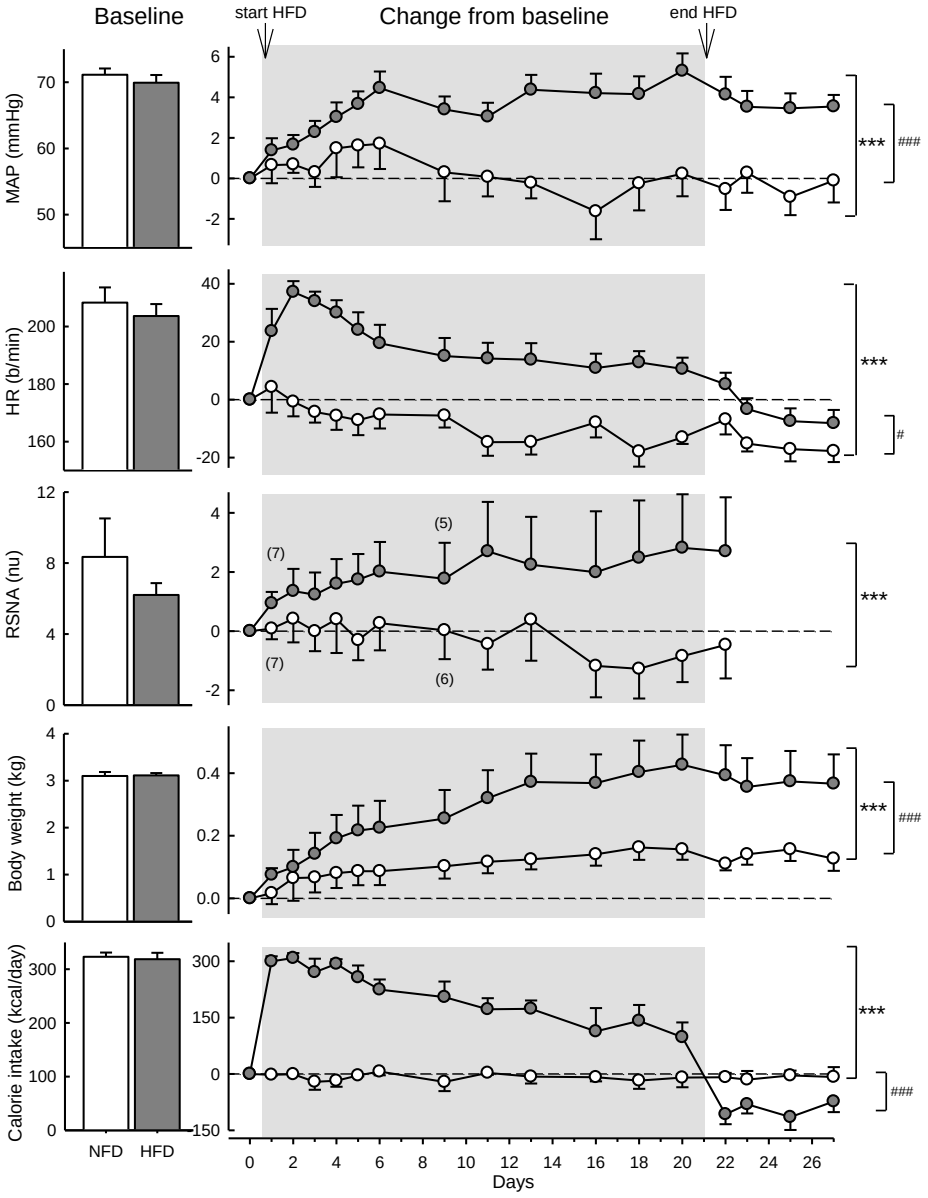

FIGURE 1. Baseline levels and effect of 3 weeks of a high fat diet and resumption of a normal diet. Left panels: Baseline values averaged over 24 hours in rabbits before beginning a normal (NFD, open bars) or high (HFD, gray bars) fat diet. Values are mean ± SEM. Right panels: Average changes from baseline from the first until the 20th day of a NFD (open circles) or HFD (gray circles). HFD is indicated by gray panel. Data from days 22 to 27 are average changes after rabbits on a HFD were returned to a NFD. RSNA data were collected in 7 rabbits in each group from days 0-6 then 5 HFD and 6 NFD rabbits from days 9-22. Values are mean difference ± SED indicating between animal variance. ***P<0.001 for HFD vs NFD (days 1-20); #P<0.05, ###P<0.001 for HFD vs NFD (days 22-27). Mean arterial pressure (MAP), heart rate (HR) and renal sympathetic nerve activity (RSNA, normalised units).

early morning. During weeks two and three, the average preprandial to postprandial differences in MAP and RSNA were 6 mmHg and 1.1nu less than at baseline and the average increases in MAP and RSNA over 24h were 4 mmHg and 2.3 nu, respectively. Thus loss of preprandial dipping accounted for 100 % of the increased 24h MAP but only 47 % of the RSNA increase. The postprandial level of MAP remained similar to the baseline level throughout the HFD but interestingly, postprandial HR progressively decreased so that in weeks 2 - 3 of the HFD it was 10 % lower than the baseline postprandial level (P < 0.001, Fig. 3). Thus the impact of the loss of dipping in preprandial HR (62 b/min) was far greater than the elevation in 24h HR over the same period (13 b/min). There was no change in the feeding related 24h pattern in rabbits fed a NFD over the 27 day period (Fig. 2, 4).

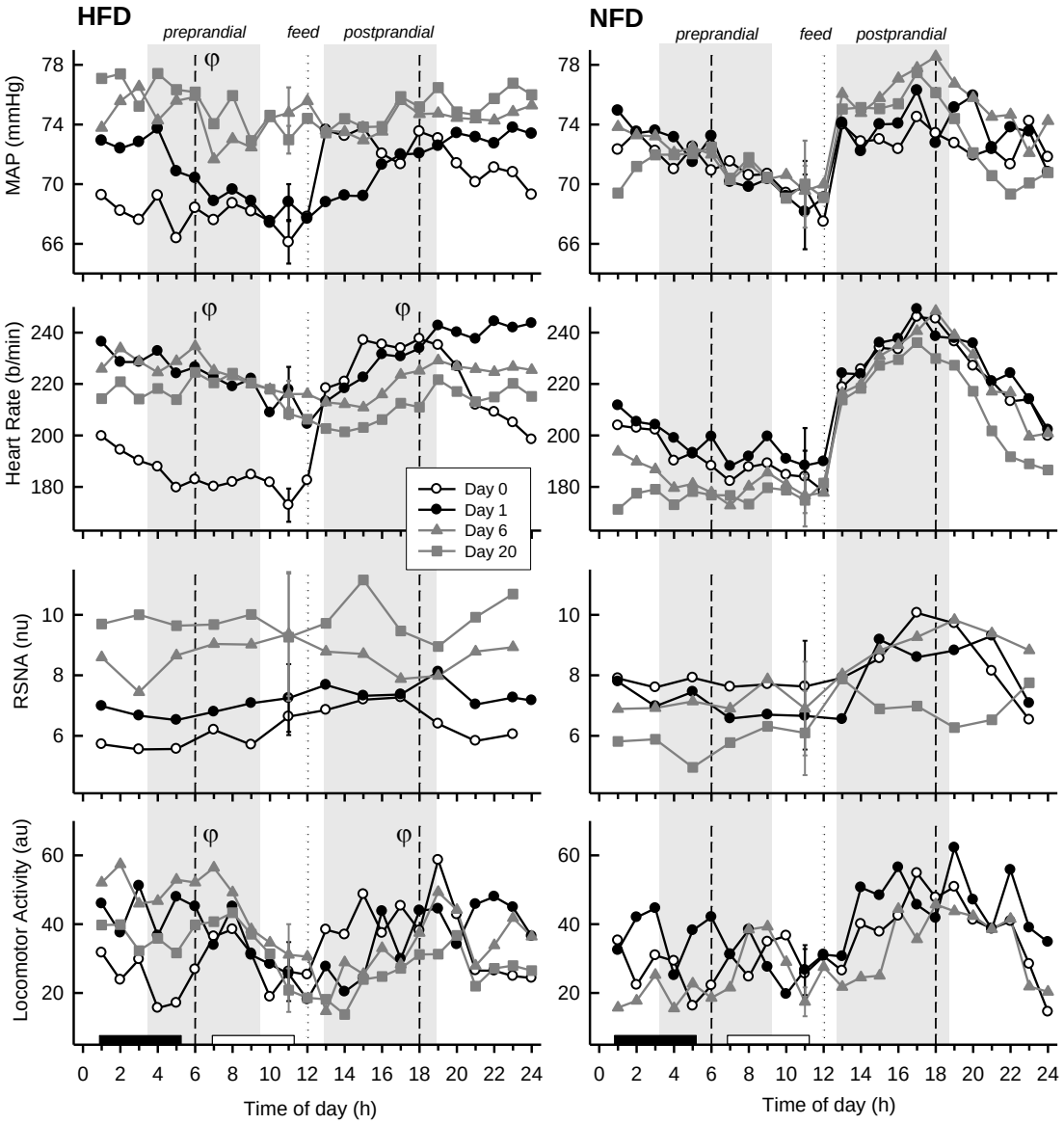

FIGURE 2. Data over 24 hours before and after 1, 6 and 20 days of a high fat or normal diet. Left: Hourly averaged data showing the variation over 24 hours of MAP, HR and activity (au, arbitrary units) in rabbits on a control diet on day 0 (open circles) and on days 1 (black circles), 6 (gray triangles) and 20 (gray squares) after the start of a HFD. Right: Hourly averaged data on the same days in rabbits fed a NFD on all days. Renal sympathetic nerve activity (RSNA) was averaged over 15 min every 2 hours. Rabbits were fed at 12:00 h (hatched bar) and the lights were on between 6:00 h and 18:00 h (dashed vertical lines). Values are mean ± SEM indicating between animal variance. The preprandial and postprandial periods (03:30 h - 09:30 h and 13:00 h-19:00 h) are shaded in gray; the preprandial dark and light periods (0:00 h-05:00 h and 06:00 h -11:00 h) are indicated by the black and white bars. φP<0.5 for Day 0 vs Day 1, 6, 20 during preprandial and postprandial periods. Abbreviations as for Fig. 1.

Effect of HFD on Light-Related 24h Patterns

To determine whether there was also a relationship between the light cycle and the 24h pattern, we measured the differences between data collected during the 12h of dark and during 12h of light. There were no dark-light differences in baseline values (Fig. 3). From the first day of the HFD there were marked increases in the dark-light difference for MAP, HR and activity ($P < 0.001$) but little change in RSNA (Fig. 3). Thus MAP, HR and activity were higher during the dark period on all days of the HFD (Fig. 3). By contrast, in rabbits fed a NFD for 27 days, there was little dark-light difference in MAP and RSNA but HR remained markedly lower in the dark (Fig. 4).

Because feeding occurred during the lights-on period, we also measured dark-light differences over a shorter period (five hours) before feeding, which minimised the influence of the feeding schedule. From the first day of the HFD, MAP and HR were greater in the dark than in the light, and continued so throughout

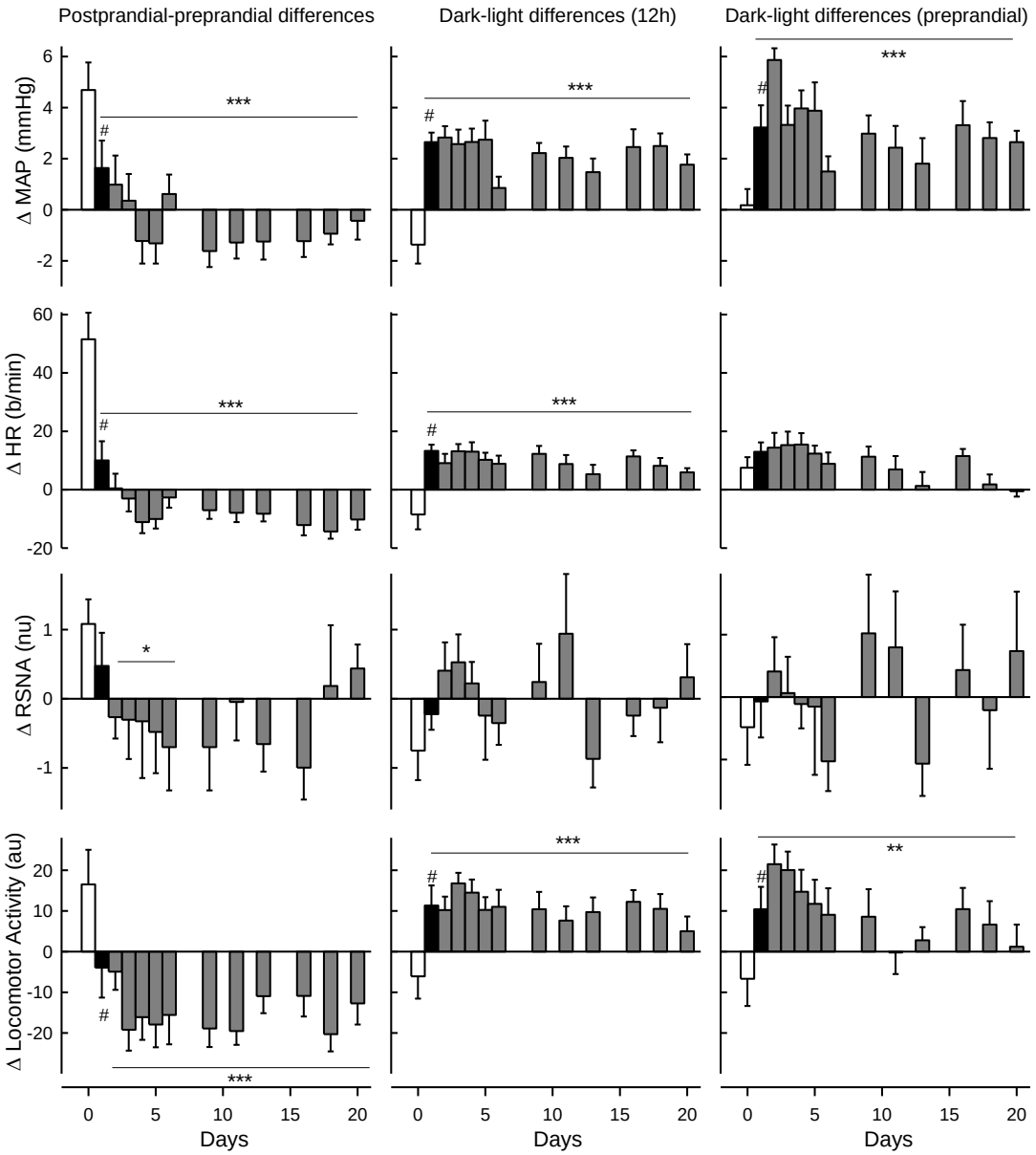

FIGURE 3. Postprandial-preprandial, dark-light (over 12h) and dark-light (over 5h preprandial) differences during 3 weeks of a HFD. Left: Average differences between values collected during 6 hours preprandial (03:30 h - 09:30 h) and those during 6 hours postprandial (13:00 h -19:00 h) before (open bar), on day 1 (black bar) then days 2 – 20 (dark gray bars) of HFD. Middle: Average differences between values collected during 12 hours of dark (18:00 h - 06:00 h) and 12 hours of light (06:00h - 18:00 h). Right: Average differences between values collected in the dark (0:00 h - 05:00 h) and the light (06:00 h - 11:00 h) preprandial period. All data from rabbits on a HFD. Values are mean difference ± SED indicating between animal variance. #P < 0.05 for day 1 HFD compared to baseline; ***P < 0.001 for days 1-20 compared to baseline; *P = 0.05 for RSNA on days 2-6 compared to baseline. Abbreviations as for Fig. 1 and 2.

the treatment period (P < 0.001, Fig. 3). There was a similar pattern in rabbits fed a NFD (Fig. 4).

Effect of the HFD on pattern of food intake

Food intake was measured over 7 hours from the time of feeding on days one to four. Rabbits fed a NFD consumed 51 ± 10g in the first hour (43% of the total, n = 3) and 106 ± 5g within 7 hours (88%, Fig. 5). On the second and fourth days of the HFD, the hourly rate of food consumption was not altered by the diet (diet x food intake interaction $F_{6,21}$ = 1.3, P = 0.3 and $F_{6,21}$ = 2.3, P = 0.07 for day two and day four, respectively, Fig. 5).

Effect on 24h pattern of return to normal diet after HFD

The feeding-related 24h pattern in MAP observed during the HFD was maintained and the difference between preprandial and postprandial MAP was not changed by the return to a NFD for one week (Fig. 6). The light-related 24h patterns in MAP and activity were also maintained (greater in the dark) although there was

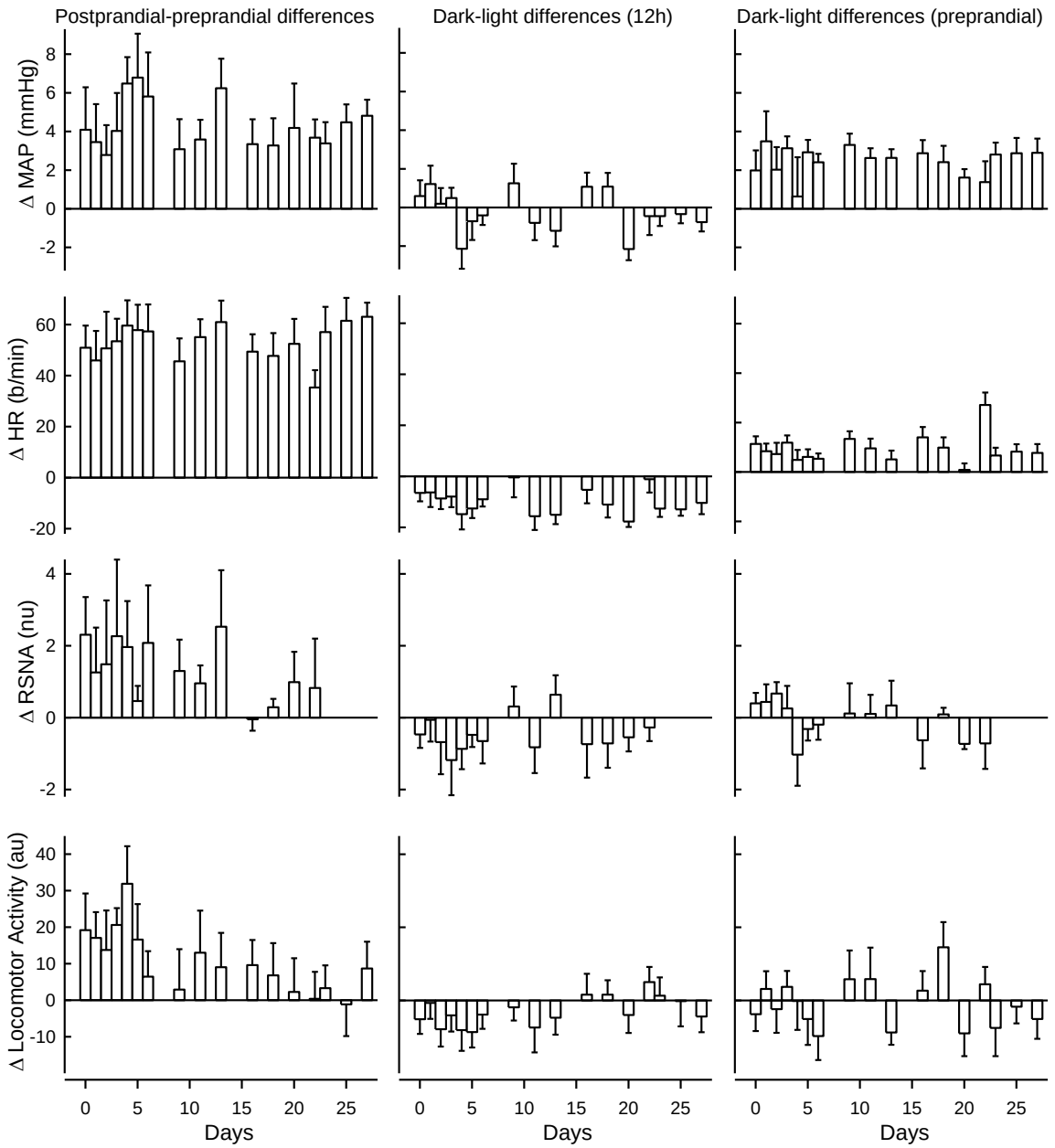

FIGURE 4. Postprandial-preprandial, dark-light (over 12h) and dark-light (over 5 h preprandial) differences over 3 weeks of a normal fat diet. Left: Average differences between values collected during 6 hours preprandial (03:30 h - 09:30 h) and those during 6 hours postprandial (13:00 h - 19:00 h) on days 0-27 of a NFD. Middle: Average differences between values collected during 12 hours of dark (18:00 h - 06:00 h) and 12 hours of light (06:00 h - 18:00 h). Right: Average differences between values collected in the dark (0:00 h - 05:00 h) and the light (06:00 h - 11:00 h) preprandial period. All data from rabbits on a NFD. Values are mean difference ± SED, indicating between animal variance. RSNA was recorded until Day 22. Abbreviations as for Fig. 1 and 2.

some attenuation of the dark-light differences ($P < 0.05$, Fig. 6). RSNA also remained elevated on the first day. Interestingly, the feeding-related 24h pattern of HR was altered by the return to a normal diet. The preprandial "dip" was progressively restored to levels similar to baseline, however the maximum HR reached after feeding, even after six days, remained similar to that observed during the HFD (Fig. 6). Thus the difference between preprandial and postprandial HR after resumption of the NFD (average +11±2 b/min) was markedly increased compared to the last week of the HFD (-12±2 b/min, $P < 0.001$) but still attenuated compared to baseline (Fig. 6). We were unable to detect changes to the 24h patterns of RSNA during the NFD period due to greater variance in the data.

Predictors of postprandial-preprandial differences

Regressions were made of the changes from baseline of preprandial - postprandial differences in MAP, HR and RSNA and changes from baseline in plasma hormones and body weight in weeks one, two and three of both a

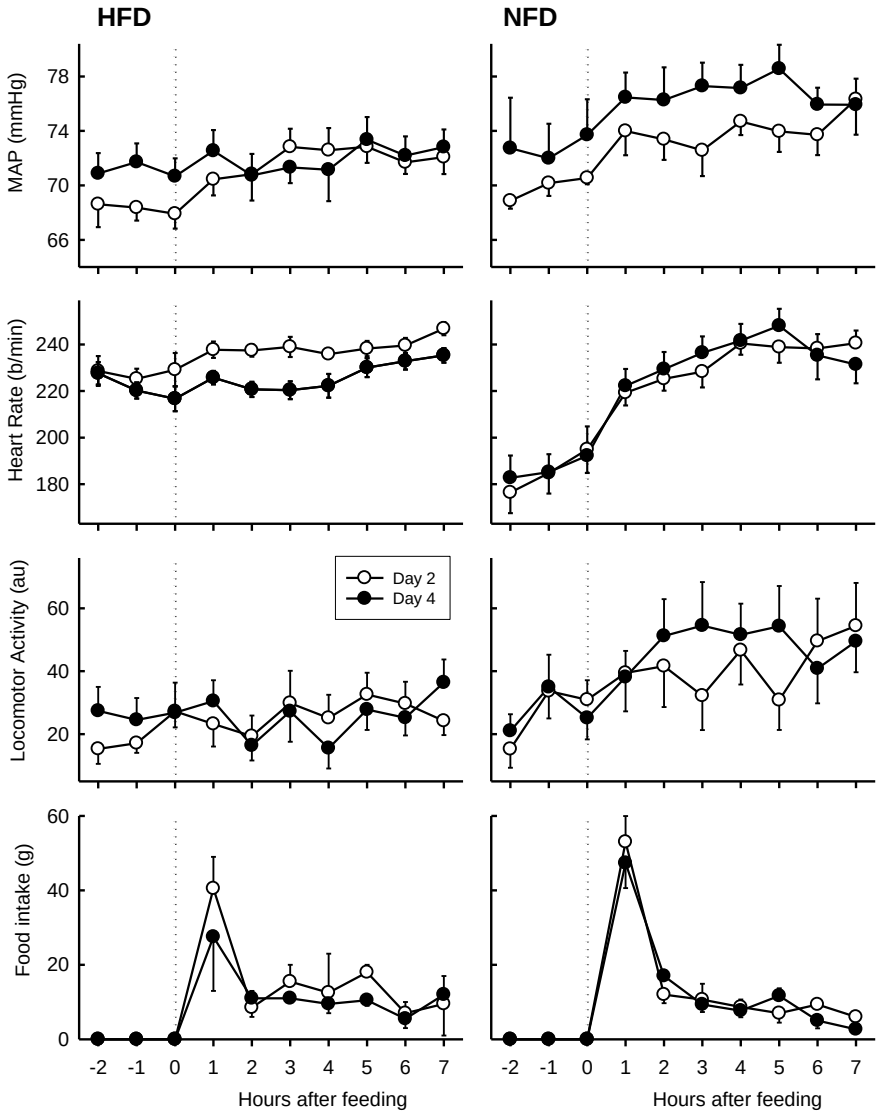

Figure 5. Hourly measurements of food intake. Average mean arterial pressure (MAP), heart rate, locomotor activity and food intake measured hourly over the 3 hours before feeding and 7 hours after feeding (dotted line). Data were collected on days 2 (open circles) and 4 (closed circles) of a high fat diet (HFD, left) or normal diet (NFD, right). Values are mean ± SEM indicating between animal variance.

NFD and HFD. There were strong relationships between changes in MAP, HR and RSNA and changes in leptin and insulin. By contrast, MAP and HR were also predicted by glucose and body weight but RSNA was not (Fig. 7).

Effect of HFD on neuronal activation in SCN

Neuronal activation in the SCN, as determined by FRA, after 2-3 weeks of HFD, was 4 fold greater than activation in rabbits fed a NFD (26±5 counts vs 5±1 counts, $P<0.05$, $n = 3$ in each group).

DISCUSSION

The present study shows that meal-fed rabbits on a normal diet have a 24h pattern dominated by preprandial low and postprandial high levels of MAP, HR and RSNA rather than a light dark pattern that has been observed for rodents (Head *et al.*, 2004) and humans (de la Sierra *et al.*, 2009). The main influence of changing the meal to a high fat daily meal was to reduce the normal preprandial dipping in MAP, HR and RSNA. The onset of the effect of the HFD was rapid, being observed within the first 24 hours for all variables and reaching a maximum within four days. These changes were maintained throughout the three week dietary period and returning to a normal diet partially reversed the effects on HR but not MAP or RSNA. Importantly, this disruption of the 24h rhythm accounted for the elevated arterial pressure which we have previously reported to occur from the first week of feeding a HFD (Armitage *et al.*, 2012).

Rapid alteration of 24 hour pattern with HFD

Antic and colleagues also observed a loss of "nocturnal" dipping (which coincided with the preprandial period as the rabbits in that study were fed at the end of the night cycle) one week after beginning a HFD (Antic *et al.*, 2001). The novelty of our study is that RSNA was recorded directly and concurrently with MAP over 24h periods which extended over 3 weeks. Alteration of the

24h rhythm as well as the changes to the average 24h levels followed a similar pattern for MAP and RSNA

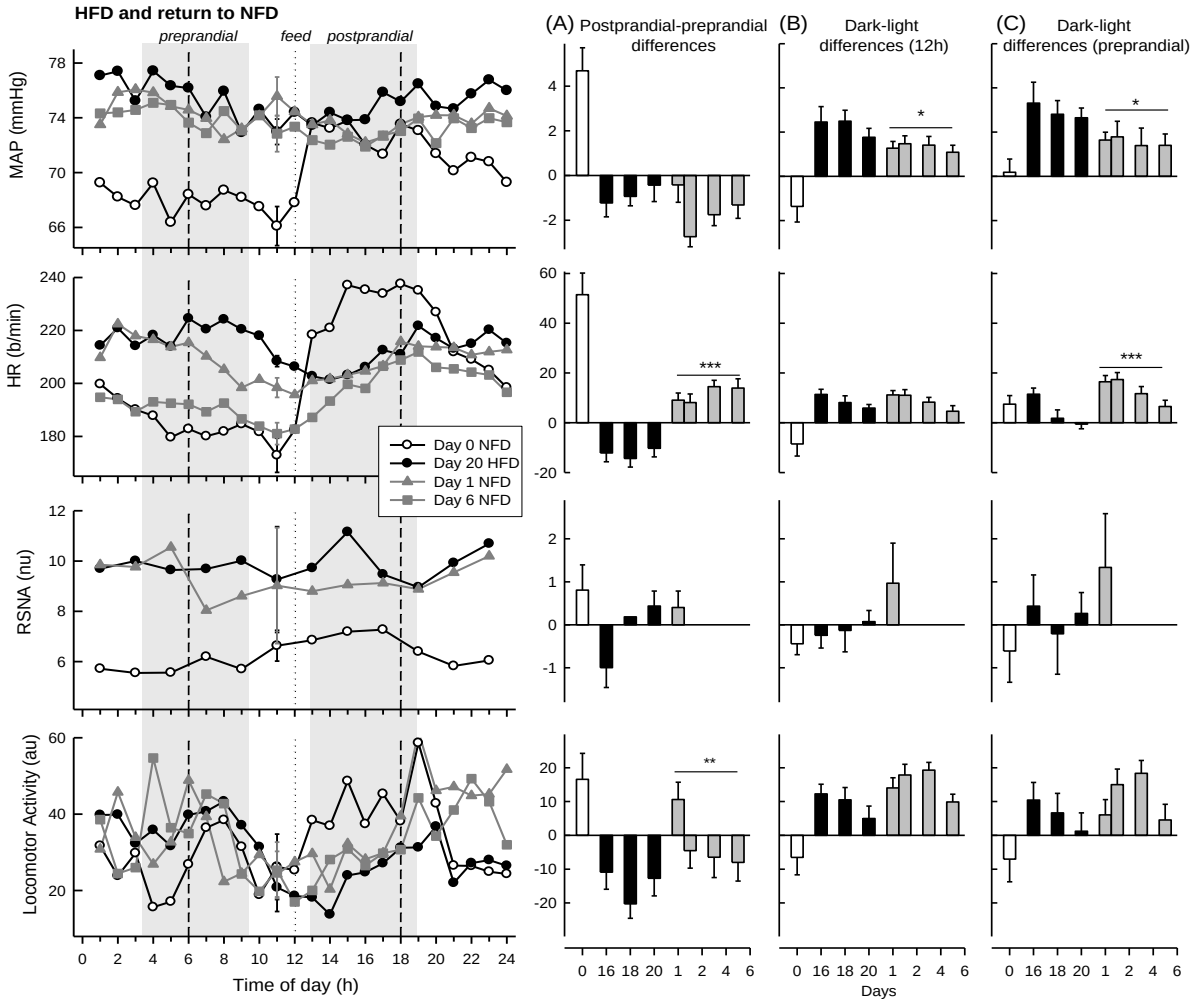

FIGURE 6. 24h data and postprandial-preprandial, dark-light (over 12h) and dark-light (over 5 h preprandial) differences over 3 weeks of a high fat diet and resumption of a normal diet. Left panel: Hourly averaged data showing the variation over 24 hours of MAP, HR, RSNA and activity in rabbits on a NFD on day 0 (open circles), on day 20 of HFD (black circles) and days 1 (grey triangles) and 6 (grey squares) after the return to a NFD. Description of graphs as for Fig. 2. Right panels: (A) Average differences between values collected during 6 hours preprandial (03:30 h - 09:30 h) and those during 6 hours postprandial (13:00 h - 19:00 h) before (open bar), on days 16-20 of HFD (black bar) then days 1,2,4 and 6 (light grey bars) after return to a NFD. (B): Average differences between values collected during 12 hours of dark (18:00 h - 06:00 h) and 12 hours of light (06:00 h - 18:00 h). (C): Average differences between values collected in the dark (0:00 h - 05:00 h) and the light (06:00 h - 11:00 h) preprandial period. RSNA was recorded to Day1 of return to NFD. Values are mean difference ± SED, indicating between animal variance. * $P < 0.05$, ***$P < 0.001$ for days 1-6 of recovery (NFD) compared to days 16-20 of HFD. Abbreviations as for Fig. 1 and 2.

and support our previous data that show that increased blood pressure induced by fat feeding is mediated by elevated sympathetic nerve activity (Armitage *et al.*, 2012). The loss of the preprandial dipping from the first day of the HFD accounted for all of the MAP increase over 24h and approximately half of the increase in RSNA. Adrenergic blockade in rabbits inhibits the postprandial increase in MAP suggesting that it is attributable to greater sympathetic outflow (Antic *et al.*, 2001). In humans, the nocturnal fall in urinary noradrenaline output was blunted in obese subjects and increased sympathetic tone underlies the morning surge in blood pressure and the higher level of blood pressure during daytime (Furlan *et al.*, 1990). The elevated MAP prior to feeding in the present study is therefore likely related to a failure of the preprandial inhibition of sympathetic nerve activity. By contrast, the magnitude of the reduction in the preprandial dipping of HR was far greater than the average 24h increase in HR, even in the early days of the HFD when 24h HR was at its highest. Although the 24h pattern of HR remained disrupted for the three weeks of exposure to a HFD, 24h HR measured at three weeks was markedly lower than HR measured in the first few days of the HFD, a trend that closely followed the reduction in caloric intake. A reduction in the postprandial increase in HR partly accounted for the overall reduction in HR over the three weeks of HFD. In humans, the morning surge in HR

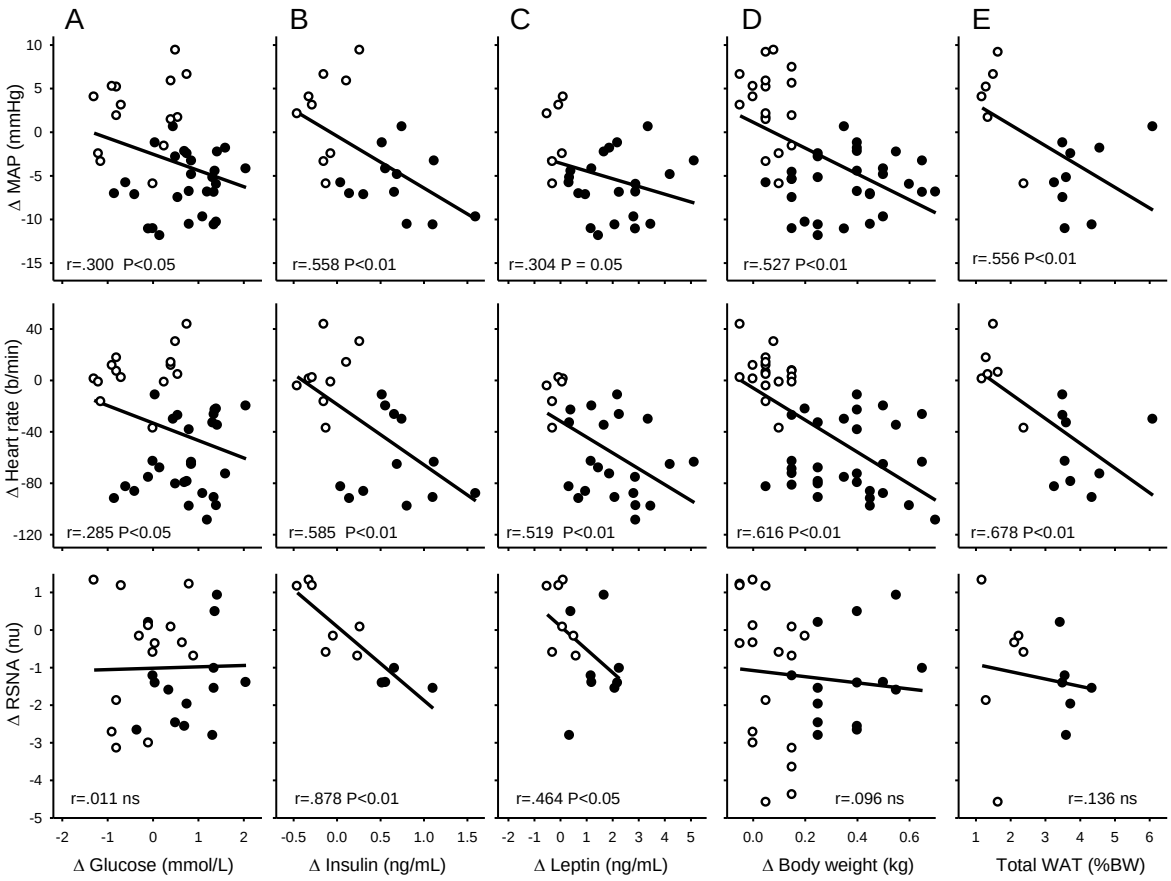

FIGURE 7. Predictors of postprandial – preprandial differences. Linear regressions between changes from baseline of postprandial - preprandial differences in mean arterial pressure (MAP), heart rate and renal sympathetic nerve activity (RSNA, normalised units) and changes from baseline in glucose (A), insulin (B), leptin (C) and body weight (D). Regressions were also made between postprandial - preprandial differences and total white adipose tissue (WAT) expressed as % of body weight (E). Data were collected over weeks 1, 2 and 3 in NFD (open circles) and HFD (filled circles) fed rabbits and at week 4 for WAT. r, regression coefficients.

results from an increase in sympathetic nerve activity and a reduction in parasympathetic activity (Furlan *et al.*, 1990). However, Antic and colleagues observed no effect of sympathetic blockade on the HR rhythm in rabbits (Antic *et al.*, 2001), suggesting that the 24h pattern of HR was chiefly being influenced by changes in cardiac vagal activity. It is likely that in the present study, the large effect of the HFD on the 24h pattern of HR is a result of a combination of a failure of sympathetic nerve activation to diminish and of vagal activity to increase in the preprandial phase.

Alteration of the 24h pattern began on the first day of the HFD when calorie intake increased; in this case there was a doubling of calorie intake with the first meal. This change clearly occurred before accumulation of adipose tissue, suggesting that the sympathetic nervous system is initially sensitive to signals arising from the consumption of calories rather than those originating in fat deposits. In support of this, Antic and colleagues reported rapid elevation of blood pressure and HR, also on the first day, in rabbits given access to normal chow but in increased quantities (Antic *et al.*, 2003). The eating pattern of rabbits during the first two days of the HFD was similar to that in meal fed rabbits on a NFD, with almost half the meal consumed in the first hour, suggesting that the changes to haemodynamics in the first few days of a HFD cannot be accounted for by changes in the feeding pattern. Locomotor activity can also be discounted as a source of the elevation in preprandial MAP, HR and RSNA. It closely follows the 24h pattern of these parameters and also switches to a light-dark related pattern. Thus in HFD fed rabbits, low periods of activity during lights-on correspond to high levels of cardiovascular parameters (see Figure 5).

There are a number of candidates for the stimulus that so rapidly alters the 24h pattern of cardiovascular and neural activity after exposure to a HFD. Blood glucose, insulin and leptin in plasma are elevated in the first week of feeding a HFD in rabbits (Armitage *et al.*, 2012) and it is known that glucose, leptin, insulin and adipokines are all expressed under the influence of a circadian clock which originates in the cells of the SCN of the hypothalamus and in

peripheral tissue (Ando *et al.*, 2005; Ramsey *et al.*, 2007). In humans, plasma glucose concentrations and glucose tolerance are reduced in the afternoon compared to the morning (Van Cauter *et al.*, 1997) and in rats, daily fluctuations in glucose tolerance are associated with circadian variation in insulin sensitivity (la Fleur *et al.*, 2001). Leptin secretion is at a maximum during the sleep phase in humans (Kalra *et al.*, 2003) and postprandially in food restricted rats (Bodosi *et al.*, 2004). Regression analysis of our data shows that the increases in insulin and leptin resulting from a HFD are predictors of the loss of preprandial dipping in RSNA and may be the initial stimulus to increase blood pressure. There are a number of studies that demonstrate that a HFD disrupts the 24h rhythms of circulating hormones. Mice exposed to a HFD have altered or blunted diurnal variations in leptin, glucose, insulin, adiponectin, free fatty acids, and corticosterone whilst a HFD in rats abolished the circadian fluctuations in plasma leptin (Cha *et al.*, 2000; Ando *et al.*, 2005; Kohsaka *et al.*, 2007). In humans, a single high fat meal reduces the normal postprandial levels of leptin and postprandial fluctuations in insulin are blunted in obese subjects (Havel *et al.*, 1999; Emdin *et al.*, 2001; Poppitt *et al.*, 2006). Importantly, muscle sympathetic nerve activity and noradrenaline spillover to kidneys increase in response to peripheral insulin infusion or within one hour of a high fat meal (Vollenweider *et al.*, 1993; Cox *et al.*, 1995) and RSNA increases with CNS administration of leptin (Prior *et al.*, 2010a). Two of the CNS regions that are innervated by fibres from the SCN are the dorsomedial (DMH) and ventromedial (VMH) nuclei of the hypothalamus and both have key roles in regulating circadian rhythms (Ramsey *et al.*, 2007). Moreover, the DMH has also been implicated in the entrainment of rhythms to meal feeding (Gooley *et al.*, 2006). Importantly, leptin receptors are highly expressed in both the DMH and VMH and microinjections of leptin into those regions increase blood pressure and sympathetic vasomotor activity (Marsh *et al.*, 2003). Thus the marked effect of the HFD on the cardiovascular 24h patterns may be explained by changes to the action of leptin in the DMH or VMH where there is a close nexus between the cells regulating circadian rhythms and those controlling sympathetic outflow. Alternatively ghrelin, a peptide released by the stomach, has been suggested to act as a circadian clock (LeSauter *et al.*, 2009) and is known to inhibit sympathetic nerve activity. Under normal circumstances, plasma ghrelin concentrations are low postprandially and rise gradually over several hours to peak just before the anticipated time of feeding. Postprandial ghrelin sensitivity is diminished in obese humans (Maffeis *et al.*, 2006) but it is not known whether the sympathoinhibitory functions of ghrelin are affected by obesity. We have shown that the normal sympathoinhibitory actions of ghrelin are diminished in offspring of fat fed mothers during periods when ghrelin may be released prior to eating (Prior *et al.*, 2010b).

Maintained Elevation of MAP and RSNA Throughout HFD and After Withdrawal

Despite caloric intake declining by the third week, the effect on the 24h patterns of MAP and RSNA persisted. At this stage, body weight was markedly increased and there was more than a doubling of adipose tissue (Armitage *et al.*, 2012). Whilst caloric intake appears to be the initial stimulus for these changes, after the return to a normal diet caloric intake is less than in NFD fed rabbits. Thus other factors take over to maintain the altered 24h patterns and the elevated MAP and RSNA which remain even when the HFD is withdrawn. The mechanism of the maintenance of this cardiovascular activation is unknown. Circulating leptin and insulin concentrations are elevated throughout the HFD period but fall rapidly when the diet is withdrawn (Armitage *et al.*, 2012), suggesting a change in the relationship between these peptides and sympathetic outflow. One scenario may be that CNS sympathetic pathways have become sensitised to the higher levels of leptin and the 24h pattern continues to be blunted (for at least one week) even after circulating leptin levels are reduced. Another explanation is that whilst peripheral leptin levels were found to be lower, CNS levels may still be elevated due to the persistence of fat. Other signals arising from adipose tissue, such as from adipokines or cytokines, may be responsible for the continuing disruption of the 24h rhythms of MAP and RSNA. However, experiments in rabbits ingesting a short-term increase in calories (rather than an increase in fat intake) also showed that MAP and HR were not completely reversed within five days of the caloric intake returning to normal (Antic *et al.*, 2003) suggesting that deposition of fat is not the only factor involved.

Switch From Entrainment to Feeding Cycle to Synchronisation with Light Cycle

The 24h rhythms of MAP, HR and RSNA in the rabbits on a normal diet were strongly entrained to the feeding cycle. It has been well documented that in food restricted animals the circadian rhythmicity of behavioural activity and physiology is synchronised to the time of feeding (Van den Buuse & Malpas, 1997; Barrett *et al.*, 2001; Kaur *et al.*, 2008). By contrast, animals on an ad libitum diet display 24h rhythmicity which is entrained to the light cycle. Under those conditions, rodents have a nocturnal pattern of eating and behavioural activity (Head *et al.*, 2004) and rabbits eat more in the hours of darkness than in the light (Sanderson & Vanderweele, 1975) which is reflected in higher levels of MAP and HR in the dark (Eijzenbach *et al.*, 1986). However, the novel finding of our study is that in rabbits, there is a switch from a food-entrained 24h pattern to one synchronized to the light cycle within two days of consuming a HFD, despite the animals continuing to be meal fed and indeed eating their food in a pattern similar to those on a normal diet. The heart, liver and adipose tissue are some of the tissues and organs under the control of their own intracellular peripheral oscillators and meal feeding uncouples these

oscillators from the central circadian clock in the SCN (Lin *et al.*, 2008). Consumption of a HFD, as well as reducing the amplitude of the 24h variation, appears to re-entrain these tissues to the central light-responsive pacemaker. Furthermore, we found that neuronal activation in the SCN was markedly greater in rabbits on a HFD than in control rabbits. Lesions in the DMH abolish the preprandial rise in locomotor activity, body temperature and wakefulness in food restricted rats, suggesting that control of food entrainment also resides in the DMH (Gooley *et al.*, 2006). Thus pathways from the SCN conveying information about light intersect with food entrainment pathways in the DMH and provide a mechanism for the switching from food to light entrainment during a HFD.

Conclusion

Consumption of a HFD in rabbits rapidly attenuates cardiovascular and sympathetic nerve 24h rhythms by preventing the normal preprandial dipping. The disruption to the rhythm begins on the first day of the HFD and continues for at least one week after cessation of the diet and accounts for the increase in blood pressure that is initiated on the first day. Blunting of the 24h patterns of release of insulin, leptin and other lipid metabolites may provide a mechanism and leptin in particular appears to play a key role. In the CNS, the hypothalamus is a region where leptin sensitive pathways, control of sympathetic outflow and regulation of circadian rhythms intersect. Disturbances to 24h rhythms in obesity have important consequences for human health. For example the loss of rhythmicity of glucose metabolism may contribute to development of type 2 diabetes (Van Cauter *et al.*, 1997) and a blunted nocturnal dip in blood pressure, commonly associated with obesity, is a predictor of high cardiovascular risk (de la Sierra *et al.*, 2009).

DECLARATION OF INTEREST

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