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MECHANISMS AND POTENTIAL THERAPY ON DISRUPTED BLOOD PRESSURE CIRCADIAN RHYTHM IN DIABETES

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MECHANISMS AND POTENTIAL THERAPY ON DISRUPTED BLOOD PRESSURE CIRCADIAN RHYTHM IN DIABETES

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in
the College of Medicine at the University of Kentucky

By

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Lexington, Kentucky

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2018

ABSTRACT OF DISSERTATION

MECHANISMS AND POTENTIAL THERAPY ON DISRUPTED BLOOD PRESSURE CIRCADIAN RHYTHM IN DIABETES

Arterial blood pressure (BP) undergoes a 24-hour oscillation that peaks in the active day and reaches a nadir at night during sleep in humans. Reduced nocturnal BP fall (also known as non-dipper) is the most common disruption of BP circadian rhythm and is associated with increased risk of untoward cardiovascular events and target organ injury. Up to 75% of diabetic patients are non-dippers. However, the mechanisms underlying diabetes associated non-dipping BP are largely unknown. To address this important question, we generated a novel diabetic *db/db-mPer2*^{Luc} mouse model (*db/db-mPer2*^{Luc}) that allows quantitatively measuring of mPER2 protein oscillation by real-time mPer2^{Luc} bioluminescence monitoring *in vitro* and *in vivo*. Using this model, we demonstrated that the *db/db-mPer2*^{Luc} mice have a diminished BP daily rhythm. The phase of the mPER2 daily oscillation is advanced to different extents in explanted peripheral tissues from the *db/db-mPer2*^{Luc} mice relative to that in the control mice. However, no phase shift is found in the central oscillator, the suprachiasmatic nucleus (SCN). The results indicate that the desynchrony of mPER2 daily oscillation in the peripheral tissues contributes to the loss of BP daily oscillation in diabetes.

Extensive research over the past decades has been focused on how the components of food (what we eat) and the amount of food (how much we eat) affect metabolic diseases. Only recently has it become appreciated that the timing of food intake (when we eat), independent of total caloric and macronutrient quality, is also critical for metabolic health. To investigate the potential effect of the timing of food intake on the BP circadian rhythm, we simultaneously monitored the BP and food intake profiles in the diabetic *db/db* and control mice using radiotelemetry and BioDAQ systems. We found the loss of BP daily rhythm is associated with disrupted food intake rhythm in the *db/db* mice. In addition, the normal BP daily rhythm is altered in the healthy mice with abnormal feeding pattern, in which the food is available only during the inactive-phase. To explore whether imposing a normal food intake pattern is able to

prevent and restore the disruption of BP circadian rhythm, we conducted active-time restricted feeding (ATRF) in the *db/db* mice. Strikingly, ATRF completely prevents and restorers the disrupted BP daily rhythm in the *db/db* mice. While multiple mechanisms likely contribute to the protection of ATRF on the BP daily rhythm, we found that ATRF improves the rhythms of energy metabolism, sleep-wake cycle, BP-regulatory hormones and autonomic nervous system (ANS) in the *db/db* mice. To further investigate the molecular mechanism by which ATRF regulates BP circadian rhythm, we determined the effect of ATRF on the mRNA expressions of core clock genes and clock target genes in the *db/db* mice. Of particular interest is that we found among all the genes we examined, the mRNA oscillation of *Bmal1*, a key core clock gene, is disrupted by diabetes and selectively restored by the ATRF in multiple peripheral tissues in the *db/db* mice. More importantly, we demonstrated that *Bmal1* is partially required for ATRF to protect the BP circadian rhythm.

In summary, our findings indicate that the desynchrony of peripheral clocks contributes to the abnormal BP circadian pattern in diabetes. Moreover, our studies suggest ATRF as a novel and effective chronotherapy against the disruption of BP circadian rhythm in diabetes.

KEYWORDS: Diabetes, Blood pressure circadian rhythm, Clock genes, Time-restricted feeding, Sympathetic nervous system, *db/db* mice

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12-03-2018

MECHANISMS AND POTENTIAL THERAPY ON DISRUPTED BLOOD PRESSURE CIRCADIAN RHYTHM IN DIABETES

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CHAPTER I. INTRODUCTION

1.1 Diabetes

1.1.1 Background

The prevalence of diabetes is increasing steadily over the decades, from 4.3% in 1980 to 8.5% in 2014 worldwide. This accounts for a total of 422 million adults (9% of men and 7.9% of women) that year (2016). This number exceeds the previous projection that 439 million adults will have diabetes in 2030 (Shaw, Sicree et al. 2010). The newest International Diabetes Federation (IDF) diabetes atlas projects that the number of adults affected by diabetes is expected to rise to 521 to 829 million by 2040, which accounts for 10.4% of adults globally (Ogurtsova, da Rocha Fernandes et al. 2017).

Diabetes is classified into four general categories: 1) type 1 diabetes (T1D); 2) type 2 diabetes (T2D); 3) gestational diabetes and 4) specific types of diabetes due to other causes, such as monogenic diabetes syndromes, diseases of the exocrine pancreas, and drug- or chemical-induced diabetes (2018). Among the different forms of diabetes, T2D accounts for 90-95% of all diabetes in the US (Prevention 2017). T2D, previously known as “non-insulin dependent diabetes mellitus”, is a chronic metabolic disease and results from the progressive loss of β-cell insulin secretion; it is characterized by hyperglycemia, insulin resistance and relative insulin deficiency (Chatterjee, Khunti et al. 2017).

1.1.2 Complications of type 2 diabetes

People with T2D are more susceptible to various forms of short- and long-term complications, which can lead to their premature death. The Centers for Disease Control and Prevention (CDC) of US reported that diabetes is the seventh leading cause of death in the US (Prevention 2017). The world Health Organization (WHO) projects that diabetes will be the seventh leading cause of death in 2030 in the whole world (Mathers and Loncar 2006). The short-term, or acute complications associated with T2D include a hyperglycemic hyperosmolar state (HHS) due to acute hyperglycemia (Pasquel and Umpierrez 2014) as well as coma due to hypoglycemia, resulting from an adverse effect of diabetes treatment using insulin and sulfonylureas (Frier 2014). The long-term, or chronic complications of diabetes include microvascular diseases (due to the damage to small vessels, e.g. retinopathy, nephropathy, and neuropathy) and macrovascular diseases (due to the damage to the arteries, e.g. coronary artery disease leading to angina or myocardial infarction and cerebrovascular disease contributing to stroke) (Forbes and Cooper 2013). In addition, T2D has also been found to be associated with cancer (Tsilidis, Kasimis et al. 2015), depression (Semenkovich, Brown et al. 2015), dementia (Biessels, Strachan et al. 2014), tuberculosis (Jeon and Murray 2008) and sexual dysfunction (Isidro 2012). The incidence and mortality rate of the short-term complications is relatively low in T2D. The most devastating consequence of T2D is the long-term complications, among which cardiovascular disease (CVD) is the leading cause of mortality and morbidity associated with T2D (Organization 2014). In 1979, Kannel et al used the data from the Framingham Heart Study (FHS), which includes 13,861 men

and 18,928 women of 45 to 74 years of age at the time of the study, first identified that people with diabetes are two to three times more likely to have CVD than those without (Kannel and McGee 1979). Several decades later, due to effective prevention and treatment, the incidence of CVD has declined in people with diabetes (Fox, Coady et al. 2004, Dale, Vatten et al. 2008), which is in accordance with the overall reduction of CVD (Rosamond, Chambless et al. 1998, Cooper, Cutler et al. 2000). Despite this, diabetes is still associated with 2-fold higher incidence in the rate of CVD (Fox, Coady et al. 2004, Collaboration 2010) . A recent systematic literature review demonstrated that CVD affects more than 30% of persons with T2D and accounts for approximately half of all death among people with diabetes between the year 2007 to 2017 (Einarson, Acs et al. 2018).

1.1.3 Hypertension in type 2 diabetes

The mechanisms linking T2D with CVD are not well understood currently. Researchers have demonstrated there are several risk factors for developing CVD in people with T2D. Studies have shown that hyperglycemia (Klein 1995, Gerstein and Yusuf 1996, Lehto, Ronnemaa et al. 1996, Turner, Millns et al. 1998, Stratton, Adler et al. 2000), hypertension (1993, Stamler, Vaccaro et al. 1993, Lehto, Ronnemaa et al. 1996, Turner, Millns et al. 1998), increased low density lipoprotein (LDL) cholesterol and decreased high density lipoprotein (HDL) cholesterol (Stamler, Vaccaro et al. 1993, Turner, Millns et al. 1998), and smoking (Stamler, Vaccaro et al. 1993, Turner, Millns et al. 1998, Qin, Chen et al. 2013, Pan, Wang et al. 2015) significantly increase the incidence and mortality of CVD. However, the contribution of each risk factor to the

development of CVD is not equal. The population attributable fraction (PAF) is a widely used epidemiologic measure to assess the impact of exposures to the public health in populations (Mansournia and Altman 2018). It is equivalent to the reduction of mortality with reduced risk factor exposured. For ischaemic heart disease, the PAF for hyperglycemia, high cholesterol, hypertension and smoking is 21%, 45%, 47% and 12%, respectively (Danaei, Lawes et al. 2006). And for stroke, the PAF for hyperglycemia is 13%, which is equal to high cholesterol, for hypertension is 54% and for smoking is 8% (Lopez, Mathers et al. 2006). Therefore, hypertension has the highest PAF value in CVD. Indeed, a comparative risk assessment showed that hypertension is the leading risk factor for deaths for CVD, chronic kidney disease and diabetes and causes more than 40% deaths from these diseases worldwide (2014).

Hypertension and T2D often coexist. Hypertension occurs twice as often in people with diabetes than in those without diabetes (El-Atat, McFarlane et al. 2004). The prevalence of hypertension in T2D is 40 to 60 percent (1993, Kabakov, Norymberg et al. 2006, Colosia, Palencia et al. 2013). When people have both hypertension and T2D, their risk for CVD increases (Gomes 2013). Multiple studies report that antihypertensive treatment lowers the incidence of cardiovascular events among diabetic patients (Curb, Pressel et al. 1996, Group 1998, Hansson, Zanchetti et al. 1998). The UK Prospective Diabetes Study Group (UKPDS) found that each 10 mm Hg decrease in updated mean systolic blood pressure was associated with reductions in risk of 12% for any complication related to diabetes (95% confidence interval 10% to 14%, $P<0.0001$), 15% for deaths related to diabetes (12% to 18%, $P<0.0001$), 11% for

myocardial infarction (7% to 14%, P<0.0001), and 13% for microvascular complications (10% to 16%, P<0.0001) (Adler, Stratton et al. 2000). A recent meta-analysis showed that a 10-mmHg reduction in systolic blood pressure was significantly associated with lower risk of all-cause mortality (RR, 0.87 [95% CI, 0.78-0.96]) and cardiovascular events (RR, 0.89 [95% CI, 0.83-0.95]) (Emdin, Rahimi et al. 2015).

Accumulating studies have shown that diabetic patients have not only an increased prevalence of high blood pressure, but also the incidence of abnormal BP daily variation is elevated compared to nondiabetic people. In addition, the abnormal BP variation is independently associated with increased risk of cardiovascular events and target organ injury.

1.2 Blood pressure circadian rhythm

1.2.1 Background

Arterial blood pressure (BP) displayed distinct diurnal variation, characterized by lowest value during sleep (around 3 a.m.), a slow increase during late sleep, an abrupt rise upon morning awakening, and two daytime peaks (first one around 9 a.m. and second one around 7 p.m.); the latter are separated by a small mid-afternoon drop (around 3 p.m.), and a gradual decline thereafter till the nadir during sleep (Millar-Craig, Bishop et al. 1978, Hermida, Ayala et al. 2007, Smolensky, Hermida et al. 2017). The 24-hr BP in humans can be measured using a device called ambulatory blood pressure measurement (ABPM); this is a noninvasive and autonomic BP monitor that measures BP at regular intervals. The BP during the nighttime mean is normally 10-20% lower

compared to the daytime mean, which is also called a dipping pattern. Abnormal BP rhythm patterns include: non-dipping (nocturnal BP fall is less than 10% of daytime BP mean), reverse dipping (nocturnal BP mean is higher than daytime BP mean), and extreme dipping (nocturnal BP fall is greater than 20% of daytime BP mean).

In the general population, 50.3% of people are dippers, 23.5% are non-dippers, 20.3% are extreme-dippers and 5.9% are reverse-dippers as assessed by the International Database on Ambulatory blood pressure monitoring in relation to Cardiovascular Outcomes (IDACO). This study was based on the ABPM data of 7458 individuals (Boggia, Li et al. 2007). The prevalence of circadian BP patterns in hypertensive patients has been assessed by the Spanish Society of Hypertension ABMP Registry. Based on the results of 42947 hypertensive patients, dippers accounts for 50.2% of previously untreated patients (n=8384), extreme dippers, non-dippers and risers (reverse-dippers) account for 8.8%, 35% and 6% respectively of this population (de la Sierra, Redon et al. 2009). In patients receiving antihypertensive treatment (n=34563), 39.9%, 7.2%, 39.4% and 13.5% are dipper, extreme dippers, non-dippers and risers (de la Sierra, Redon et al. 2009). Therefore, non-dipping BP is the most prevalent abnormal BP circadian pattern in both the general population and in hypertensive patients.

1.2.2 Circadian variation of BP and CVD

Many cardiovascular events follow a well-defined frequency in their onset throughout the day; this pattern is closely related to the diurnal variation of BP. For example, the frequency of acute myocardial infarction onset is lowest during the

nighttime sleep and highest during the awake time, between 6 AM to noon (Muller, Stone et al. 1985, Hjalmarson, Gilpin et al. 1989, Willich, Linderer et al. 1989, Gilpin, Hjalmarson et al. 1990, Hansen, Johansson et al. 1992, Behar, Halabi et al. 1993, Boari, Salmi et al. 2007). Sudden cardiac death also exhibits 24-hour variation in the occurrence, with trough onset during the night and peak onset during the morning hours (Muller, Ludmer et al. 1987, Willich, Levy et al. 1987, Levine, Pepe et al. 1992, Aronow and Ahn 1993, Aronow, Ahn et al. 1994). Stoke is another cardiovascular events that is shown to be more frequent during the morning hours (Tsementzis, Gill et al. 1985, Marler, Price et al. 1989, Kelly-Hayes, Wolf et al. 1995, Manfredini, Gallerani et al. 1997, Casetta, Granieri et al. 2002). Meta-analyses of the three cardiovascular events demonstrated that there is a 40%, 29% and 49% excess risk of acute myocardial infarction, sudden cardiac death and stroke, respectively, that happen between 6 AM to noon (Cohen, Rohtla et al. 1997, Elliott 1998). In addition, both the aortic rupture and dissection happen more frequently during the early morning hours (Gallerani, Portaluppi et al. 1997, Manfredini, Portaluppi et al. 1999, Manfredini, Boari et al. 2004, Lasica, Perunicic et al. 2006, Killeen, Neary et al. 2007). The apparent time-of-day difference in CVD onsets suggests there may be an association between the circadian pattern of BP and CVD.

In 1988, O'Brien *et al* reported for the first time that hypertensive patients with a less marked decrease in nighttime BP had a greater prevalence of stroke incidents and named these patients non-dippers, in contrast to the normal dippers (O'Brien, Sheridan et al. 1988). Since then, numerous studies have investigated the importance of

nighttime BP and the circadian variation of BP. The majority of these studies found that blunted nighttime BP fall and abnormal night-to-day ratio of BP is associated with increased risk of CVD (Verdecchia, Schillaci et al. 1993, Verdecchia, Porcellati et al. 1994, Zweiker, Eber et al. 1994, Staessen, Thijs et al. 1999, Ohkubo, Hozawa et al. 2002, Kikuya, Ohkubo et al. 2005, Sega, Facchetti et al. 2005, Ingelsson, Bjorklund-Bodegard et al. 2006, Boggia, Li et al. 2007, Mancia, Bombelli et al. 2007, Fagard, Celis et al. 2008, Fagard, Thijs et al. 2009, Muxfeldt, Cardoso et al. 2009, Bastos, Bertoquini et al. 2010, Hansen, Li et al. 2011, de la Sierra, Banegas et al. 2012) and target organ damage in heart, kidney and brain, including greater ventricular mass index or left ventricular hypotrophy (Kuwajima, Suzuki et al. 1992, Palatini, Penzo et al. 1992, Suzuki, Kuwajima et al. 1992, Mayet, Shahi et al. 1995, Verdecchia, Schillaci et al. 1995, Cuspidi, Macca et al. 2001, Hoshide, Kario et al. 2003, Hoshide, Kario et al. 2003, Cuspidi, Meani et al. 2004), albumin excretion or albuminuria (White 1992, Lurbe, Redon et al. 1993, Lindsay, Stewart et al. 1995, Timio, Venanzi et al. 1995, Equiluz-Bruck, Schnack et al. 1996), and silent cerebrovascular damage (Kario, Matsuo et al. 1996, Kario, Pickering et al. 2001). Results from the Ohasama study showed that on average, each 5% decrease in the decline in nocturnal systolic/diastolic BP was associated with an approximately 20% greater risk of cardiovascular mortality in the Japanese general population (Ohkubo, Hozawa et al. 2002). In addition, the nighttime BP was demonstrated to be a better predictor of future cardiovascular events than daytime or office BP (Staessen, Thijs et al. 1999, Dolan, Stanton et al. 2005, Kikuya, Ohkubo et al. 2005, Boggia, Li et al. 2007, Fagard, Celis et al. 2008, Hansen, Li et al. 2011, O'brien, Parati et al. 2013). Fan et al in

2010 published results from a large prospective study in 8711 individuals from 10 populations for a mean follow-up of 10.7 years and found that the isolated nocturnal hypertension, defined as daytime BP <135/85 mmHg and night-time BP ≥120/70 mmHg, predicts cardiovascular outcomes in patients, even though their office or daytime ambulatory BP is at normal range (Fan, Li et al. 2010). A recent review of nine cohorts from Europe, Brazil and Japan of 13844 individuals also supports the claim that nighttime BP better predicts cardiovascular outcomes than daytime and clinic BP (Roush, Fagard et al. 2014).

Despite massive evidence supporting the prognostic role of nighttime BP in predicting cardiovascular events, the relationship between the dipping status of BP and cardiovascular outcomes is not consistent. For example, a few prospective studies with relatively small sample size found that the nocturnal fall of BP is not associated with increased left ventricular mass index or left ventricular hypotrophy (Roman, Pickering et al. 1997, Cuspidi, Lonati et al. 1999, Yi, Shin et al. 2014). The results from meta-analyses also showed inconsistent conclusions. For example, Fagard *et al* reported that night-to-day ratio of arterial BP only predicts all-cause mortality but is not associated with cardiovascular risk (Fagard, Celis et al. 2008). On the contrary, Hansen reported that night-to-day ratio predicts total cardiovascular events but not mortality after adjusting for the 24-hour BP (Hansen, Li et al. 2011). Such discrepancy may be possibly because of differences in sample size, study population, end point, the low-reproducibility of a single 24-hour ABPM data and the definition of day and night periods (fixed or diary time) that may significantly affect the classification of BP dipping pattern (Henskens,

Kroon et al. 2008). Therefore, the Ambulatory Blood Pressure Collaboration in Patients with Hypertension (ABC-H) recently published a largest known meta-analysis on ABPM database from 3 continents; the study consists of a total of 17312 hypertensive individuals for follow-up of 4 to 8 years and found that after adjusting for 24-hour systolic blood pressure (SBP), the systolic night-to-day ratio predicts all outcomes. In particular, reduced nocturnal BP fall is associated with a significant 27% higher incidence of all cardiovascular events compare to normal nocturnal BP fall (Salles, Reboldi et al. 2016). This largest meta-analysis confirmed the prognostic significance of BP dipping status on CVD.

Among the different abnormal circadian BP patterns, reverse-dipping, in which the nighttime BP is higher than the daytime BP, seems to have the worst cardiovascular prognosis. In particular, the Ohasama study showed that reverse-dippers have the highest mortality risk, followed by non-dippers (Ohkubo, Imai et al. 1997). Kario et al found that the incidence of stoke is 6.7% in dipper, 7.6% in non-dippers, 12% in extreme-dipper and 22% in reverse-dippers for an average duration of 41 months (Kario, Pickering et al. 2001). In addition, intracranial hemorrhage was also more common in reverse-dippers (29% of strokes) than in other subgroups (7.7% of strokes, P=0.04) (Kario, Pickering et al. 2001). In another study Eguchi *et al* found that reverse-dippers have the highest cardiovascular events rate, followed by non-dippers; these findings are associated with approximately 150% increased risk of CVD (Eguchi, Pickering et al. 2008).

1.2.3 Circadian variation of BP in diabetes

Previous studies have demonstrated that type 2 diabetes is significantly associated with abnormal BP circadian rhythm in both the general population (Boggia, Li et al. 2007) and in hypertensive patients (de la Sierra, Redon et al. 2009). Impaired BP circadian rhythm is found to be more common in people with diabetes than those without, although the exact prevalence various among different studies. Fogari *et al* found that an abnormal circadian BP pattern is detected in 30% of the normotensive and 31% of the hypertensive diabetic patients compared to 6% of the normotensive and 6.4% of the hypertensive nondiabetic subjects (Corradi, Zoppi et al. 1993). However, Pistrosch *et al* observed non-dipping BP in 73% of hypertensive diabetic patients (Pistrosch, Reissmann et al. 2007), while Oh *et al* found non-dipping BP occurs in 58.2% vs. 48.2% of hypertensive patients with and without diabetes (Oh, Han et al. 2015). The reasons for the discrepancy of the prevalence of an abnormal BP circadian pattern in diabetic patients are probably similar to the reasons that lead to inconsistent conclusions between the relationship of BP dipping status and cardiovascular outcomes, such as relatively small sample size, low-reproducibility of a single 24-hour ABPM data and the definition of daytime and nighttime periods. In addition, the complications of diabetes may also affect the BP dipping status. For example, Equiluz-Bruck *et al* observed non-dipping BP in 80% of the macroalbuminuric, 74% of the microalbuminuric, and 43% of the normoalbuminuric T2D patients compare to 37% of nondiabetic subjects (Equiluz-Bruck, Schnack et al. 1996). Therefore, Ayala *et al* evaluated the influence of diabetes on the circadian pattern of BP from the data of Hygia project, where 12765

hypertensive patients (2954 T2D patients) were maintained on a diary listing times of going to bed at night and awakening in the morning and their ABPM were monitored for 48-hours. They found that the prevalence of non-dipping BP was detected in 62.1% of hypertensive diabetic patients compared to 45.9% hypertensive nondiabetic patients (Ayala, Moya et al. 2013). In addition, the largest difference between diabetic and nondiabetic patients is the prevalence of the riser pattern (19.9% in diabetes vs. 8.1% in nondiabetes), which is also shown to be associated with the worst cardiovascular prognosis (Ohkubo, Imai et al. 1997, Kario, Pickering et al. 2001, Eguchi, Pickering et al. 2008).

In patients with diabetes, reduced nocturnal BP fall is also associated with increased risk of CVD and target organ damage. In a 4-year prospective study, the incidence of fatal and nonfatal vascular (cerebrovascular cardiovascular, peripheral vascular arteries, and retinal artery) events is higher in T2D patients with reverse-dipping BP than in those with dipping BP (Nakano, Fukuda et al. 1998). The intima-media thickness and left ventricular mass index, which are found to be increased in non-dippers, are also greater in diabetic non-dippers than dippers (Di Flaviani, Picconi et al. 2011). The nocturnal fall in systolic blood pressure (SBP), independent of 24-hour BP, is negatively associated with pulse wave velocity (PWV), lower albumin: creatinine ratio (ACR) and positively associated with glomerular filtration rate (GFR) in T2D patients (Jennersjo, Wijkman et al. 2011). The rate of decline of creatinine clearance is higher in diabetic non-dippers than in dippers (-7.9 vs. -2.9 ml/min/year) over a 6-year period (Farmer, Goldsmith et al. 1998). The prevalence of non-dipping BP is highest in diabetic

patients with macroalbuminuria compared to diabetic patients with microalbuminuria and normoalbuminuria (Equiluz-Bruck, Schnack et al. 1996). There are conflicting results on the relationship of BP dipping status and the progression of diabetic nephropathy. Knudsen *et al* found the night: day BP ratio is an independent predictor of nephropathy progression in T2D patients (Knudsen, Laugesen et al. 2009) while Palmas *et al* concluded nocturnal non-dipping, if not with nocturnal BP rise, is not an independent predictor of nephropathy (Palmas, Pickering et al. 2008). However, Pierdomenico *et al* analyzed the results of both studies and demonstrated that the overall risk ratio (RR) for nephropathy progression in non-dippers was 1.73, 95% CI: 0.97–3.06, P=0.061 (Pierdomenico and Cuccurullo 2010).

1.2.4 Mechanisms of BP circadian rhythm

1.2.4.1 Role of hemodynamics

BP is determined by total peripheral resistance (TPR) ×cardiac output (CO), in which the CO equals heart rate (HR)×stroke volume (SV). Several human studies have investigated the daily variations of TPR and CO and found the CO has an oscillation pattern parallel to that of BP, whereas TPR remains unchanged throughout the day or increases during the night (Khatri and Freis 1967, Miller and Horvath 1976, Mori 1990, Idema, van den Meiracker et al. 1994, Veerman, Imholz et al. 1995). Veerman *et al* concluded the nocturnal decrease of CO is mainly due to nocturnal fall of HR as the nighttime SV is comparable to daytime (Veerman, Imholz et al. 1995); while Miller *et al* found the decreased SV leads to nocturnal fall of CO as HR is not different between day

and night (Miller and Horvath 1976). The discrepancy may be due to differences in physical activity as the day and night changes in HR and CO are found to be correlated with physical activity (Cavelaars, Tulen et al. 2004). In non-dippers, an increment in nighttime TPR or a smaller decline in nocturnal CO and SV are found as compared to dippers. (Takakuwa, Ise et al. 2001, Cavelaars, Tulen et al. 2004).

1.2.4.2 Role of sleep

The sleep-wake cycle is the most evident circadian rhythm of life and is also a significant determinant of BP circadian rhythm. As reviewed by Smolensky *et al* (Smolensky, Hermida et al. 2007), the stages of sleep have a profound effect on the BP 24-hour oscillation. The sleep stage 3 and 4, which are the deepest sleep stage, correspond to the lowest BP levels during sleep, whereas the less deep sleep stage 1 and 2 and the rapid eye movement (REM) sleep, coincide with higher BP levels, although these values are still lower than the awake BP levels. Human study has shown that the levels of BP decline significantly from wakefulness to stage 4 of non-rapid eye movement (NREM) sleep in normotensive subjects (Somers, Dyken et al. 1993). Another influence of sleep on the BP 24-hour oscillation is exerted through the variation of respiration. During the NREM sleep, the breathing is regular and the BP declines. Whereas during REM sleep, the breathing and HR are irregular; central apneas or hypopneas occur sporadically, and the peaks of BP, sometimes as great as 30–40 mmHg from baseline, happen abruptly during the whole REM episode. The relationship between sleep and BP circadian rhythm has been investigated mainly in patients with obstructive sleep apnea (OSA). Studies have shown that 43% to 84% of OSA patients,

either hypertensive or normotensive, are non-dippers (Suzuki, Guilleminault et al. 1996, Loredo, Ancoli-Israel et al. 2001). Severe OSA, compared to mild OSA or habitual snorers, is associated with greater BP night-to-day ratio (Pankow, Nabe et al. 1997) and the severity of sleep apnea is demonstrated to be an independent predictor for BP circadian rhythm (Nabe, Lies et al. 1995).

1.2.4.3 Role of autonomic nervous system

It is well-known that the autonomic nervous system (ANS) is an important regulator of BP homeostasis. The ANS is also critical to the BP circadian rhythm. The activity of the sympathetic and parasympathetic nerve system (SNS and PNS), two efferent arms of ANS, exhibit day and night difference. The sympathetic nerve activity, in accordance with BP, is typically higher during the active or awake period and lower during sleep. In human studies, the concentrations of plasma norepinephrine (NE) and epinephrine (EPI) peak during the awake and reach a nadir at night during sleep in normal men (Prinz, Halter et al. 1979, Stene, Panagiotis et al. 1980, Lightman, James et al. 1981, Sowers and Vlachakis 1984, Linsell, Lightman et al. 1985). The low- to high-frequency ratio (LF: HF), a value that is calculated from the frequency domain method of heart rate variability (HRV) and considered as an indicator of sympatho-vagal balance in some studies, is significantly higher in the morning than in the evening (Huikuri, Niemelä et al. 1994, Kawano, Tochikubo et al. 1994, Nakagawa, Iwao et al. 1998, Massin, Maeyns et al. 2000). Using microneurography, which provides a direct measurement of efferent sympathetic nerve activity related to muscle blood vessels, Somers *et al* demonstrated that the mean amplitude of bursts of sympathetic nerve activity decreased significantly

from wakefulness to deep sleep in normal subjects (Somers, Dyken et al. 1993). In laboratory animals, the plasma (McCarty, Kvetnansky et al. 1981, De Boer and Van der Gugten 1987) and urinary (Fu, Patel et al. 2005) NE and EPI concentrations are higher during the active phase than rest phase. The LF: HF also displays day and night difference, with significantly greater LF: HF in the active phase than rest phase (Hashimoto, Kuwahara et al. 1999, Kuwahara, Suzuki et al. 1999, Matsunaga, Harada et al. 2001, Kuwahara, Tsujino et al. 2004). Direct measurement of stellate ganglion nerve activity in dogs revealed that there is a higher sympathetic outflow to stellate ganglia during the daytime (Jung, Dave et al. 2006). The direct measurement of sympathetic nerve activity in rodents can be obtained by measuring renal sympathetic nerve activity (RSNA) (Ling, Cao et al. 1998). However, whether the RSNA exhibits circadian variation in rodents has not been examined. On the other hand, the parasympathetic nerve activity, which is mostly demonstrated by the HF power of HRV, has an opposite diurnal pattern as the sympathetic nerve activity. An increased HF of HRV during the night has been found in several human studies (Furlan, Guzzetti et al. 1990, Lombardi, Sandrone et al. 1992, Huikuri, Niemelä et al. 1994, Yamasaki, Kodama et al. 1996, Nakagawa, Iwao et al. 1998, Massin, Maeyns et al. 2000). The HRV can also be calculated using a time domain method, in which the square root of the mean of the sum of squares of differences between adjacent RR intervals (rMSSD), and the percentage of differences between adjacent RR intervals that are greater than 50 msec (pNN50), correlate with the HF of HRV (Sztajzel 2004). In humans, the rMSSD and pNN50 increase at night and decrease during the day (Massin, Maeyns et al. 2000). In animals, a 24-hour variation of

HF was found in rats (Hashimoto, Kuwahara et al. 1999), dogs (Matsunaga, Harada et al. 2001) and miniature swine (Kuwahara, Suzuki et al. 1999, Kuwahara, Tsujino et al. 2004) and the pattern of HF is opposite to LF: HF in these animals.

The circadian variation of ANS activity is altered in non-dippers as compared to normal individuals. The difference (nocturnal dip) of the excretion rate of both NE and EPI between the awake and asleep period was reduced in non-dippers compare to dippers (Sherwood, Steffen et al. 2002). In addition, the normal difference of the urinary NE between work and nonwork periods in day shift workers is absent in evening+night shift workers, who also have a higher prevalence of non-dipping BP (Yamasaki, Schwartz et al. 1998). Direct measurement of muscle sympathetic nerve activity (MSNA) showed a close inverse association between the degree of sympathetic activation and the magnitude of nighttime BP falls. The most severe form of non-dipping BP pattern, the reverse-dipping, has the highest level of MSNA in hypertensive subjects (Grassi, Seravalle et al. 2008). These studies suggest that the nighttime sympathetic activity is increased in non-dippers vs. dippers. On the other hand, the normal increase of parasympathetic activity during nighttime, as assessed by power spectral analysis of HRV, is significantly blunted in non-dippers compare to dippers (Kohara, Nishida et al. 1995). The ANS contributions to BP circadian rhythm are further demonstrated in sinoaortic denervated (SAD), guanethidine sulfate-induced sympathectomized, and atropine-induced parasympathetic blockade rats (Makino, Hayashi et al. 1997). The findings from the SAD rats showed that the MAP daily rhythm was lost due to increased MAP during the light phase. The daily rhythm of MAP in sympathectomized rats was

also diminished because of decreased MAP during the dark phase, although the decrease was not significant. The parasympathetic blockade induces compromised MAP daily rhythm in rats by increasing MAP only during the light phase. In summary, both human and animal studies indicate that increased sympathetic activity and/or decreased parasympathetic activity at night participate in BP circadian rhythm disruption.

1.2.4.4 Role of baroreflex

The baroreflex is a rapid mechanism to buffer acute BP sudden change. The baroreflex reflects the reciprocal responses between SNS and PNS: when the BP increases, baroreceptors activate and trigger the inhibition of SNS and the activation of PNS, resulting in decreased HR and TPR, and thus lower BP. An inversed reflex will be triggered when BP decreases. The baroreflex sensitivity (BRS), defined as the change in inter beat interval (IBI) of HR in milliseconds per unit change in BP, is used to quantify baroreflex function. The BRS has a daily rhythm that is opposite to BP rhythm in both humans (HOSSMANN, FITZGERALD et al. 1980, Takakuwa, Ise et al. 2001) and mice (Xie, Su et al. 2015). The role of the baroreflex in BP circadian variation is not clear. Some studies investigated BRS between dippers and non-dippers but did not find a significant difference (Vaile, Stallard et al. 1996, Takakuwa, Ise et al. 2001, Myredal, Friberg et al. 2010). Myredal *et al* calculated the baroreflex effectiveness index (BEI), defined as the number of SBP ramps that are followed by the respective reflex RR interval ramps fulfilling the BRS criteria, divided by the total number of SBP ramps and found the BEI is decreased in non-dippers compared to normal dippers, indicating the baroreflex

function might be impaired in non-dippers (Myredal, Friberg et al. 2010). In mice with smooth muscle-specific deletion of the clock gene *Bmal1*, diminished BP daily rhythm is found to be associated with abolished BRS daily rhythm (Xie, Su et al. 2015).

1.2.4.5 Role of hormonal systems

BP is regulated by multiple hormones, among which the renin-angiotensin-aldosterone system (RAAS) is the most important hormonal system for BP homeostasis. The RAAS regulates BP through modulating vasoconstriction and extracellular fluid volume. Interestingly, the components of the RAAS all exhibit circadian rhythm. Renin is the rate-liming enzyme of the RAAS and is secreted by the kidney. It hydrolyzes angiotensinogen (AGT) to angiotensin I (Ang I). In humans, numerous studies have demonstrated an apparent circadian rhythm of plasma renin activity (PRA), characterized by peak in the early morning, and nadir around midnight (Gordon, Wolfe et al. 1966, KATZ, ROMFH et al. 1975, Modlinger, Sharif-Zadeh et al. 1976, Cugini, Manconi et al. 1980, Beilin, Deacon et al. 1983, Cugini, Salandi et al. 1983, Kawasaki, Uezono et al. 1983, Stern, Sowers et al. 1986, Kawasaki, Cugini et al. 1990, Portaluppi, Bagni et al. 1990, Brandenberger, Follenius et al. 1994). Despite the massive evidence of PRA circadian rhythm, the relationship of the PRA and BP circadian rhythm is vague. PRA is comparable in dippers, non-dippers and extreme dipper in both the supine and tilting positions (Kario, Mitsuhashi et al. 2002). No significant correlation of PRA and the night/day ratio of MAP has been found (Fukuda, Urushihara et al. 2012). However PRA increases less in non-dippers during tilting as compared to dippers and to extreme dippers (Kario, Mitsuhashi et al. 2002). The BP circadian rhythm is inverted in transgenic

TGR (mREN2)27 rats that harbor the mouse salivary gland renin gene (mREN2) (Mullins, Peters et al. 1990), (Lemmer, Mattes et al. 1993, Lemmer, Witte et al. 2003). AGT is mostly produced and secreted by the liver and is the only known substrate of renin. Recent studies suggest urinary AGT is a biomarker of the intrarenal RAAS activity (Kobori and Navar 2011). However, the urinary AGT levels are not significantly different between day and in healthy people (Nishijima, Kobori et al. 2014, Isobe, Ohashi et al. 2015); but it is significantly higher during the daytime in patients with chronic kidney disease, who also exhibit a riser pattern of BP (Isobe, Ohashi et al. 2015). The renal proximal tubular AGT is found to be significantly higher in non-dippers than dippers and correlate with the night/day ratio of BP (Fukuda, Urushihara et al. 2012). A recent animal study demonstrated that deletion of AGT in brown adipose tissue (BAT) leads to decreased BP only during the rest phase (Chang, Xiong et al. 2018). Angiotensin-converting enzyme (ACE) is found predominantly in the lung and converts Ang I to Ang II. An ACE inhibitor is a widely used pharmaceutical drug to treat hypertension. Only a few studies have reported a circadian rhythm of ACE activity, probably because the ACE rhythm is not apparent due to the low amplitude (Veglio, Pietrandrea et al. 1987, Cugini, Letizia et al. 1988). Ang II acts on multiple tissues to increase BP. Plasma Ang II peaks in the early morning and is lowest in the evening (Kala, Fyhrquist et al. 1973). The effects of Ang II on BP circadian rhythm are revealed in rats with chronic low dose Ang II infusion, which inverts the circadian pattern of BP (Baltatu, Janssen et al. 2001, da Silva Lemos, Braga et al. 2005). The effect of Ang II on BP circadian rhythm is likely mediated by brain RAS as TGR(ASrAOGEN) rats with low brain AGT levels are resistant to Ang II

induced BP circadian alternation (Baltatu, Janssen et al. 2001). In the TGR (mREN2)27 rats, which exhibit inverted BP circadian rhythm, the levels of Ang II are significantly elevated and the rhythm of Ang II is lost (Schiffer, Pummer et al. 2001). *In vitro* study demonstrated that Ang II can induce clock gene expression in vascular smooth muscle cells (VSMCs) via the Ang II type 1 (AT1) receptor (Nonaka, Emoto et al. 2001). Aldosterone plays a critical role in maintaining electrolyte balance by promoting sodium reabsorption and potassium secretion. In humans, aldosterone is highest in the morning and lowest around midnight (Liddle 1966, Kem, Weinberger et al. 1973, GRIM, WINNACKER et al. 1974, Armbruster, Vetter et al. 1975, KATZ, ROMFH et al. 1975, Ryoyu, Isamu et al. 1984, Portaluppi, Bagni et al. 1990). The effects of aldosterone on BP circadian rhythm are controversial. Several studies reported that the BP circadian rhythm is disrupted in patients with primary aldosteronism (Tanaka, Natsume et al. 1983, Middeke and Schrader 1994, Rabbia, Veglio et al. 1997), while others found no significant change (Imai, Abe et al. 1992, Penzo, Palatini et al. 1994). This discrepancy is probably due to pathogenesis of aldosteronism (adenoma or idiopathic hyperaldosteronism) and the dietary sodium intake. Uzu *et al* demonstrated that in patients with unilateral adenoma, BP circadian rhythm is diminished during normal sodium diet but not low sodium diet; and both sodium restriction and adrenalectomy are able to restore BP dipping (Uzu, Nishimura et al. 1998).

Besides RAAS, BP is also regulated by other hormones, such as cortisol, thyroid and atrial natriuretic peptide (ANP). Cortisol, the major form of human glucocorticoid, regulates BP homeostasis through multiple mechanisms (Saruta 1996). Cortisol is

produced in the adrenal gland and the release of cortisol is stimulated by adrenocorticotropic hormone (ACTH) from the pituitary gland. Excessive cortisol, commonly seen in people with Cushing's syndrome, is associated with hypertension (Whitworth, Williamson et al. 2005). Plasma cortisol has a 24-h oscillation with highest level around awakening and lowest level shortly after sleep (Bridges and Jones 1966, ORTH, ISLAND et al. 1967, Weitzman, Fukushima et al. 1971, Désir, Van Cauter et al. 1980, Ockenfels, Porter et al. 1995). The circadian rhythm of BP is blunted or reversed in excessive glucocorticoid conditions, either endogenous (Cushing's disease due to pituitary adenoma) or exogenous (patients with glomerulonephritis (CGN) and systemic lupus erythematosus (SLE) who received glucocorticoid treatment) (Imai, Abe et al. 1988, Imai, Abe et al. 1989, Imai, Abe et al. 1990). Glucocorticoid is considered as a zeitgeber of circadian rhythm as it can entrain the rhythm of the circadian clock (Dickmeis 2009). Therefore, the effects of glucocorticoid on BP circadian rhythm may be also mediated through regulation of clock genes. Thyroid has complex effects on BP (Danzi and Klein 2003). Hypertension is observed in both hyper- (Merillon, Passa et al. 1981) and hypo- (Streeten, Anderson Jr et al. 1988) thyroidism. A graded independent relation is found between lower levels of free thyroid 3 (FT3) and the risk of non-dipping BP (Kanbay, Turgut et al. 2007). ANP is a peptide hormone synthesized and secreted by cardiac muscle cells of the atria and works to decrease BP (Laragh 1985). ANP has an apparent circadian rhythm that is almost antiphase to BP oscillation: highest level during sleep and lowest level in the afternoon and evening (Winters, Sallman et al. 1988, Portaluppi, Montanari et al. 1989, Portaluppi, Bagni et al. 1990). A temporal inverse

relationship is found between the rhythm of ANP and BP (Sothern, Vesely et al. 1995). In addition, in patients with chronic renal failure (CRF), the loss of nocturnal BP fall is associated with loss of circadian variation of ANP (Portaluppi, Montanari et al. 1992).

1.2.4.6 Role of vasculature

Blood vessels are the major organ in maintaining BP. Studies have demonstrated that the vascular tone exhibits circadian variation. The vascular contractile responses to various agonists have been investigated extensively in isolated aortas and mesentery arteries in rodents. Results from these studies have demonstrated there is a time-of-day variation in the *in vitro* VSM contractile responses to phenylephrine (PE, α1-agonist), Ang II, high K^+ and 5-hydroxytryptamine (5-HT); this variation is characterized by generally higher responses during the rest phase and lower responses during the active phase (Keskil, Gorgun et al. 1996, Gorgun, Keskil et al. 1998, Witte, Hasenberg et al. 2001, Su, Xie et al. 2011). In addition, the *in vivo* MAP responses to intravenous PE and Ang II injection also exhibit similar variations in rodents (Masuki, Todo et al. 2005, Su, Xie et al. 2011). In humans, the BP response to L-noradrenaline infusion is lowest at 0300 (HOSSMANN, FITZGERALD et al. 1980). On the other hand, the vascular responses to vasodilators also exhibit daily rhythm. Keskil *et al* found both the endothelium-dependent and -independent relaxations are more pronounced in rats aorta at ZT19 than the other times of the day (Keskil, Gorgun et al. 1996). Witte *et al* found higher endothelium-dependent relaxation at ZT2 than at ZT14 (Witte, Hasenberg et al. 2001). However, Witte *et al* did not examine vascular relaxation at ZT19, so they may have missed the peak response. In humans, both endothelium-dependent vasodilation

induced by acetylcholine (ACh) and flow-mediated dilation is lower in the morning than in the afternoon (Etsuda, Takase et al. 1999, Elherik, Khan et al. 2002). The results of endothelium-independent vasodilation induced by sodium nitroprusside (SNP) are not consistent: Panza *et al* did not find significant difference of vasodilation in response to SNP at 0700, 1400 and 1700 (Panza, Epstein et al. 1991) while Elherik *et al* found the response peaks at 1600 and lowest at 0400 and 0800 (Elherik, Khan et al. 2002). Again, the difference may due to limited sampling time points.

Studies have demonstrated that altered circadian variation in vascular tone is associated with abnormal BP circadian rhythm. Blunted time-dependent variations in vascular contraction to Ang II and endothelium-dependent relaxation by ACh are found in TGR(mREN2)27 rats, who have an inverted BP circadian rhythm (Witte, Hasenberg et al. 2001). Deletion of smooth muscle specific *Bmal1* in mice resulted in attenuated BP circadian rhythm and abolished time-of-day variation in agonist-induced vasoconstriction of the mesenteric arteries (Xie, Su et al. 2015). Mice lacking Cryptochrome (*Cry*), one of the clock genes, also exhibit diminished MAP circadian rhythm, which is accompanied by blunted day and night difference in MAP responses to PE (Masuki, Todo et al. 2005). Mutation of clock gene Period 2 (*Per2*) in mice leads to attenuated BP circadian rhythm (Vukolic, Antic et al. 2010) and diminished diurnal variation in endothelium-dependent relaxations (Viswambharan, Carvas et al. 2007). In subjects with non-dipping BP, the dose needed to increase the MAP to 25 mmHg using PE is lower than subjects with dipping BP, suggesting heightened α1-adrenergic receptor (α1-AR) responsiveness in non-dippers (Sherwood, Steffen et al. 2002). In addition, a

long-acting α1-AR blocker, doxazosin, lowers the nighttime SBP only in non-dippers but not in dippers (Ebata, Hojo et al. 1995). In non-dippers, endothelium-dependent vasodilation is reduced compared to dippers (Higashi, Nakagawa et al. 2002). In people with coronary artery disease, blunted BP circadian rhythm is associated with diminished variation in ACh induced vasodilation (Shaw, Chin-Dusting et al. 2001).

1.2.4.7 Role of kidney

The kidney regulates BP homeostasis through modulating extracellular fluid volume and electrolyte concentrations. It has long been known that the urine flow and excretion of sodium, potassium and chloride electrolyte are lower during sleep than when awake (Manchester 1933, Sirota, Baldwin et al. 1950). Such rhythms persist with the same amounts of food and water consumption at certain intervals throughout the 24 hours (Simpson 1924, Nom 1929, Borst and De Vries 1950, Mills and Stanbury 1952). The water and electrolyte variations are in phase with the variation of glomerular filtration rate (GFR) and antiphase with tubular reabsorption rhythm (Koopman, Koomen et al. 1989). As reviewed by Burnier *et al* (Burnier, Coltamai et al. 2007), changes in GFR and/or tubular reabsorption lead to increased daytime sodium retention, which results in elevated nighttime BP via the pressure-natriuresis mechanism. The pressure-natriuresis mechanism is a long-term regulator of BP, in which increased renal perfusion pressure increases sodium excretion and decreases sodium reabsorption (Ivy and Bailey 2014). The nocturnal BP needs to increase to excrete excessive sodium in order to reach sodium balance. Indeed, daytime urinary sodium is found to be independently associated with nocturnal SBP fall (Nishijima and Tochikubo

2003, Bankir, Bochud et al. 2008). People with high-sodium sensitivity have a diminished nocturnal BP fall (Uzu, Kazembe et al. 1996, Kimura 2001). In people with chronic kidney disease (CKD), the GRF is reduced and the sodium reabsorption is enhanced. Increased prevalence of abnormal BP circadian rhythm is observed in people with CKD (Farmer, Goldsmith et al. 1997, Mojón, Ayala et al. 2013). In addition, such prevalence increases with the progression of CKD (Farmer, Goldsmith et al. 1997, Mojón, Ayala et al. 2013). Renal transplantation normalizes non-dippers to dippers (Gatzka, Schobel et al. 1995), suggesting a critical role of kidney in BP circadian rhythm.

In summary, BP circadian rhythm is regulated by multiple factors and pathways. And these factors and pathways are interconnected and cannot be separated. For instance, sleep deprivation leads to derangement of CNS and endothelial dysfunction (Tobaldini, Costantino et al. 2017). There is a close interaction between SNS and RAAS: SNS stimulate renin and aldosterone release (Gordon, Küchel et al. 1967) while Ang II activates SNS and modulates baroreflex control of HR (Reid 1992). Both the vascular tone and kidney function are influenced by SNS, RAAS, glucocorticoid and thyroid hormones (Dibona and Kopp 1997, Mangos, Whitworth et al. 2003, Brewster and Perazella 2004, Danzi and Klein 2004, Yang and Zhang 2004, van Hoek and Daminet 2009, Cat and Touyz 2011, Amiya, Watanabe et al. 2014).

1.2.5 Mechanisms of diabetes associated BP circadian rhythm disruption

As described above, BP circadian rhythm is influenced by multiple factors, including the sleep-wake cycle, the ANS, the hormonal systems (RAAS, glucocorticoid,

thyroid and atrial natriuretic peptide), the vasculature and the kidney. These factors interact with each other and work together to determine BP circadian rhythm. Alternations of these factors and pathways have been observed in diabetes. For example, people with diabetes have increased TPR compared to nondiabetic subjects (Sole, Lucas et al. 2014). Findings from the Sleep Heart Health Study (SHHS) showed that diabetes is associated with periodic breathing (Resnick, Redline et al. 2003). Over 86% of obese patients with T2D have OSA (Foster, Sanders et al. 2009). Diabetic patients are known to have sympatho-vagal imbalance, in which the vagal activity is impaired while the SNS is overactive (Perin, Maule et al. 2001). In people with T2D, increased nighttime sympathetic nerve activity (Spallone, Maiello et al. 2001, Perciaccante, Fiorentini et al. 2006) and decreased nighttime vagal activity (Bernardi, Ricordi et al. 1992) are observed. In addition, there is a positive relationship between the nocturnal BP change and the nocturnal plasma NE change in T2D patients with neuropathy (Nielsen, Hansen et al. 1999). The diurnal variations of plasma PRA, aldosterone and ANP are altered in diabetic patients, especially in those with reversed BP rhythm (Nakano, Uchida et al. 1994). Diabetic nephropathy is a major compilation of diabetes (Gross, De Azevedo et al. 2005). In patients with T2D, there is a positive correlation between urinary albumin excretion rate (UAER) and non-dipping BP (Equiluz-Bruck, Schnack et al. 1996, Nakano, Ishii et al. 1996). In diabetic animal models, such as *db/db* mice (Hummel, Dickie et al. 1966) and SHRcp rats (Takaya, Ogawa et al. 1996), non-dipping BP circadian rhythm is observed (Su, Guo et al. 2008, Sueta, Kataoka et al. 2013). In *db/db* mice, the LF component of the SBP variability, indicative of sympathetic input to the vasculature

(Stauss 2007), loss the circadian variation (Senador, Kanakamedala et al. 2009) and the HRV linked to sympathetic control is altered at different time of day (Stables, Auerbach et al. 2016). The diurnal rhythm of plasma corticosterone is also altered *db/db* mice (Saito and Bray 1983). In addition, the time-of-day variations in VSM contractile responses and MAP responses to PE and Ang II are attenuated in *db/db* mice (Su, Xie et al. 2011). In SHRcp rats, the day and night difference in LF of HRV is lost (Sueta, Kataoka et al. 2013).

In addition to the above factors, diabetes is a metabolic disorder that is associated with obesity, hyperglycemia and insulin resistance. The metabolic abnormalities are also implicated in BP regulation and may contribute to diabetes associated BP circadian rhythm disruption.

Obesity is known to be associated with increased BP (Mikhail, Golub et al. 1999). Whether obesity is associated with the abnormal BP circadian rhythm is controversial. The incidence of non-dipping BP is significantly higher in obese subjects (Kotsis, Stabouli et al. 2005). In obese children and adolescents, the nighttime BP are higher than in healthy controls (Hvidt, Olsen et al. 2014). The results from both the Oman family study and Spanish Society of Hypertension registry demonstrated increased BMI or obesity is associated with non-dipping BP (Hassan, Jaju et al. 2007, de la Sierra, Redon et al. 2009). In addition, the body weight loss induced by gastric bypass surgery has been shown to restore the normal BP rhythm in morbidly obese hypertensive subjects (Czupryniak, Strzelczyk et al. 2005). In contrast, Diamantopoulos et al. did not find any difference in

the BMI between dipper and non-dippers (Diamantopoulos, Andreadis et al. 2006). The result from the Korean Ambulatory Blood Pressure Monitoring Registry also demonstrated the central obesity has no influence on the BP dipping patterns (Kang, Pyun et al. 2013). In an animal study, the BP dipping pattern was also not significantly different between diet-induce obese and lean mice (Prasai, Mughal et al. 2013).

High blood glucose is the major diagnostic criteria of diabetes and plays an important role in the pathogenesis of the micro- and macro-complications of diabetes (Ohkubo, Kishikawa et al. 1995) (1998). The 24-h variation in fasting blood glucose is disrupted in people with prediabetes or T2D (Gubin, Nelaeva et al. 2017). In diabetic *db/db* mice, the daily rhythm of blood glucose is almost antiphase to that of control mice (Grosbellet, Dumont et al. 2015). Both the diabetic and non-diabetic non-dippers have greater postprandial glucose levels and are more glucose intolerant than dippers (Pistrosch, Reissmann et al. 2007) (Chen, Jen et al. 1998). In addition, the postprandial glucose is considered as an independent predictor for non-dipper BP in diabetes (Pistrosch, Reissmann et al. 2007).

Various studies have investigated the roles of insulin in the BP regulation. More than 40% of hypertensive patients have hyperinsulinemia (Zavaroni, Mazza et al. 1992, Vanhala, Pitkajarvi et al. 1998). Insulin resistance is associated with hypertension, even in the absence of obesity and diabetes and there is a protective association between greater insulin sensitivity and lower BP (Goff, Zaccaro et al. 2003). The effects of insulin on BP regulation are through multiple pathways, including induction of vasodilation by

stimulating the release of nitric oxide (NO) in the endothelial cells (Scherrer, Randin et al. 1994), regulation of sodium handling by enhancing sodium absorption in the diluting segment of the distal nephron (DeFronzo, Cooke et al. 1975) and increases in sympathetic nervous activity (Rowe, Young et al. 1981). Studies have shown that non-dippers have a greater degree of insulin resistance and a delayed insulin secretion phase during oral glucose tolerance test (OGTT) (Chen, Jen et al. 1998, Pistrosch, Reissmann et al. 2007). In addition, the insulin sensitivity, measured by the steady-state plasma glucose (SSPG) method, is negatively associated with the nocturnal BP fall (Suzuki, Kimura et al. 2000). In the diabetic *db/db* mice, non-fasting plasma glucose and insulin only correlate with the light-phase BP but not the dark-phase BP (Su, Guo et al. 2008).

1.2.6 Therapies for abnormal BP circadian rhythm

1.2.6.1 Chronotherapy

Since the abnormal BP circadian rhythm is usually characterized by inadequate decrease of sleep BP, it is not surprising to speculate that different treatment times of antihypertensive drugs yield different results on the pattern of BP variation. A great number of clinical trials have been done to test possible relationship between timing of antihypertensive medicine and nocturnal BP . The results demonstrated that single evening dose of calcium channel blockers (CCB), AngII receptor blocker (ARB) or angiotensin-converting enzyme inhibitors (ACEIs), compare to single morning dose, significantly reduce asleep BP in individuals with essential hypertension (Umeda, Naomi et al. 1994, Kohno, Iwasaki et al. 1997, Hermida, Calvo et al. 2003, Hermida, Ayala et al.

2007, Hermida and Ayala 2009, Hermida, Ayala et al. 2009, Hermida, Ayala et al. 2010). A similar effect is also observed in patients with T2D receiving olmesartan at bedtime (Tofé Povedano and García De La Villa 2009). In addition, the bedtime administration of antihypertensive drugs also reduces microalbuminuria (Hermida, Calvo et al. 2005, Kario, Hoshide et al. 2010), plasma fibrinogen (Hermida, Ayala et al. 2005, Hermida, Ayala et al. 2009), cholesterol (Hermida, Ayala et al. 2005) and CVD morbidity and mortality (Hermida, Ayala et al. 2010, Hermida, Ayala et al. 2011). In people with T2D, the CVD morbidity and mortality is also significantly decreased with treatment of ≥1 hypertension medications at bedtime (Hermida, Ayala et al. 2011).

1.2.6.2 Targeting sodium handling

Since the salt sensitivity is associated with BP dipping (Uzu, Kazembe et al. 1996, Kimura 2001), studies have investigated whether decreasing salt intake in salt-sensitive hypertension or reducing salt sensitivity can restore BP dipping. The results demonstrated that salt restriction can shift non-dippers to dippers in patients with salt-sensitive hypertension (Uzu, Ishikawa et al. 1997) and in patients with primary aldosteronism, a typical salt-sensitive secondary hypertension (Uzu, Nishimura et al. 1998, Takakuwa, Shimizu et al. 2002). In addition, diuretics, which lower BP by reducing sodium sensitivity (Saito and Kimura 1996), restore BP from the non-dipping to the dipping pattern (Uzu and Kimura 1999, Uzu, Harada et al. 2005). ARB also restores normal BP circadian rhythm (Fukuda, Yamanaka et al. 2008) by suppressing tubular sodium reabsorption (Fukuda, Wakamatsu-Yamanaka et al. 2011). In patients with T2D,

combination therapy of diuretic and ARB, but not monotherapy of ARB, restores nocturnal BP fall (Uzu, Sakaguchi et al. 2009).

1.3 Circadian rhythm

1.3.1 Background

Almost all the physiological processes in living organisms have a daily oscillation that is thought to adapt to the 24-hour environmental light-dark cycle. The circadian oscillation in mammals is generated by cell autonomous transcription-translational negative feedback networks. As shown in Figure 1.3.1 (Golombek, Bussi et al. 2014), the core of the clock networks is composed of the Bmal1-Clock/Per-Cry loop: the brain-muscle arnt-like protein 1 (BMAL1) and circadian locomotor output cycles kaput (CLOCK), which form a heterodimer that activates the transcription of their repressors Period (*Per1* and *Per2*) and Cryptochrome (*Cry1* and *Cry2*). The PER and CRY proteins accumulate and form a complex that translates into the nucleus and, in turn, suppresses *Bmal1* and *Clock*; this results in a rhythmic expression of the loop (Gekakis, Staknis et al. 1998, Jin, Shearman et al. 1999, Kume, Zylka et al. 1999, Bunger, Wilsbacher et al. 2000).The cycle of the loop takes about 24 hours. There are additional feedback loops interacting with *Bmal1*, among which the *Rev-erb*α and *Ror*α loop is the most prominent one. The REV-ERBα competes with RORα and directly represses the transcription of *Bmal1* while RORα promotes its transcription (Preitner, Damiola et al. 2002, Sato, Panda et al. 2004).

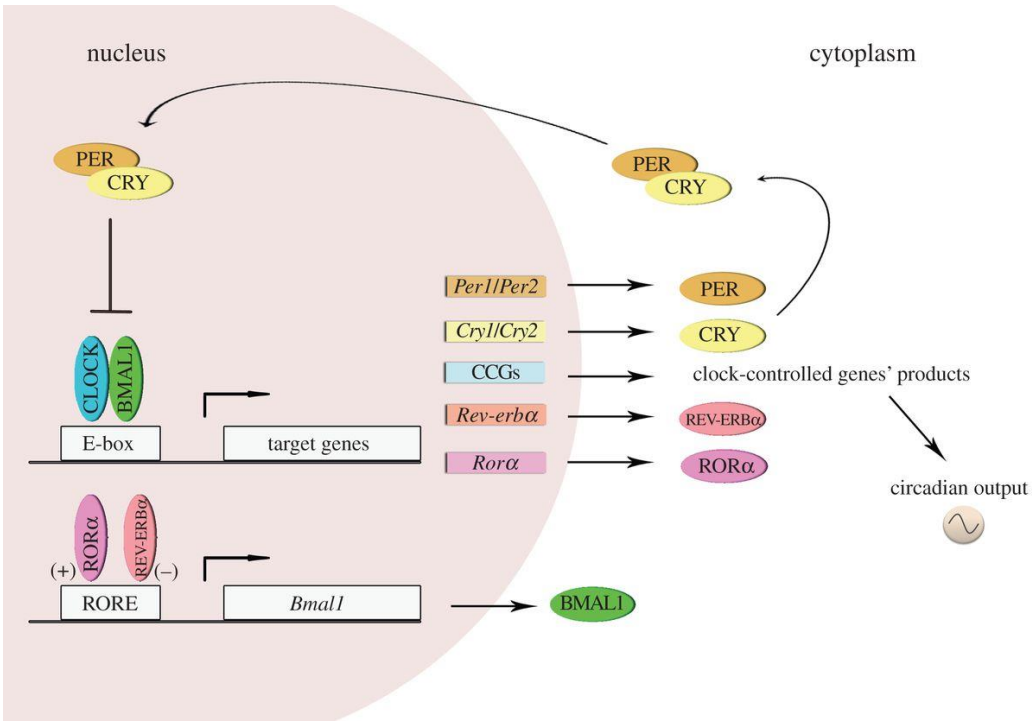


Figure 1.3.1 The molecular mechanisms of circadian clock.

The circadian system in mammals consists of the central and peripheral oscillators. The central oscillator is located in the hypothalamus suprachiasmatic nucleus (SCN), which acts as a master pacemaker and generates behavioral rhythms (locomotor activity and feeding). With the observation of rhythmic expression of clock genes and proteins in cells and tissues throughout the body, circadian oscillators are realized to ubiquitously exist in peripheral tissues (Balsalobre, Damiola et al. 1998, Yamazaki, Numano et al. 2000, Yoo, Yamazaki et al. 2004). The circadian system is regulated in a hierarchical manner with the SCN being the master pacemaker that controls the clocks of peripheral tissues. Animals with SCN lesions are missing the circadian rhythmicity in behavioral and endocrine oscillations (Moore and Eichler 1972, Stephan and Zucker 1972), including the rhythm of BP (Sano, Hayashi et al. 1995, Witte, Schnecko et al. 1998).

Tissue-specific gene expression is regulated by both the local clock and by the signals from the SCN. As demonstrated in mice with an intact SCN clock and conditionally inactive liver clock (Kornmann, Schaad et al. 2007), the rhythmic transcription of most hepatic genes becomes arrhythmic when the liver oscillator is arrested, but some genes, including the core clock gene, *Per2*, still oscillate robustly. In contrast, in liver explant culture, the rhythms in PER2 can only be observed in the liver with functional oscillators. The peripheral clocks are also regulated by SCN, which synchronizes peripheral clocks through several signals, including the autonomic innervation; hormonal signals (such as glucocorticoid), body temperature and behavioral processes (such as feeding) (reviewed in (Mohawk, Green et al. 2012)). Briefly, the sympathetic innervation from the SCN to the liver and adrenal gland regulates the daily rhythm of plasma glucose (Kalsbeek, La Fleur et al. 2004, Cailotto, La Fleur et al. 2005, Kalsbeek, Bruinstroop et al. 2010) and modulates adrenal sensitivity to adrenocorticotropic hormone (ACTH) and the release of glucocorticoid (Buijs, Wortel et al. 1999). Glucocorticoid is a hormone that can shift the phase of peripheral tissues as demonstrated by the glucocorticoid analog, dexamethasone (Balsalobre, Brown et al. 2000). This may be accomplished by the regulation of clock genes transcription as the glucocorticoid-response elements (GREs) are found in the regulatory regions of the core clock genes *Per1* and *Per2* (Yamamoto, Nakahata et al. 2005, So, Bernal et al. 2009). The daily rhythm of body temperature is under the control of the SCN. Peripheral oscillators are sensitive to temperature changes (Abraham, Granada et al. 2010) and can be reset by a low temperature pulse (Brown, Zumbrunn et al. 2002, Buhr, Yoo et al. 2010). The peripheral clocks can also be

entrained by the feeding activity generated by the SCN. Rodent studies have shown that the rhythms of liver clock genes and protein expression rapidly shift their phase following the time of feeding (Damiola, Le Minh et al. 2000, Stokkan, Yamazaki et al. 2001). The feeding cue is of particular interest, given that scheduled, time-restricted feeding is capable of resetting the circadian outputs and organizing the peripheral clocks in the absence of SCN (Stephan, Swann et al. 1979, Hara, Wan et al. 2001). More importantly, recent studies have demonstrated that time-restricted feeding is closely related to the metabolic health.

1.3.2 Time-restricted feeding

Extensive research over the past decades has focused on how the components of food (what we eat) and the amount of food (how much we eat) affect metabolic diseases. Only recently has it become appreciated that the timing of food intake (when we eat), independent of total caloric and macronutrient quality, is also critical for metabolic health. Results from animal and human studies demonstrated that it is beneficial to the organism when the feeding time is in alignment with the endogenous circadian clock and *vice versa*. In mice fed normal diet *ad libitum*, the food intake pattern has a daily rhythm, with most of the food (~80%) consumed during the dark-phase, which is the active period in nocturnal mice. The high-fat fed mouse is a commonly used animal model of obesity and diabetes. Close monitoring of food intake reveals that when mice are fed a high-fat diet *ad libitum*, their food intake pattern is altered as the percentage of daily food intake during the light-phase (rest period) is increased (Kohsaka, Laposky et al. 2007). Importantly, the change in feeding pattern

exceeds the onset of weight gain. Accompanied with the changes in food intake pattern, the 24-h profiles of circulation metabolic markers, including leptin, glucose, insulin, free fatty acids (FFA), and corticosterone are also altered (Kohsaka, Laposky et al. 2007). These results suggest a critical role in the timing of food intake on metabolic health. Arble *et al* (Arble, Bass et al. 2009) first reported that nocturnal mice fed a high-fat diet only during the 12-hour dark-phase gain significantly less weight than the mice fed only during the 12-hour light-phase without changing the calories intake. Later studies have confirmed the effects of time-restricted feeding on the body weight. They found active-time (dark-phase in nocturnal animals) restricted feeding (ATRF) prevents high-fat diet induced obesity in animals (Hatori, Vollmers et al. 2012, Sherman, Genzer et al. 2012, Tsai, Villegas-Montoya et al. 2013, Chaix, Zarrinpar et al. 2014, Yasumoto, Hashimoto et al. 2016). Other metabolic profiles, including total cholesterol, triglyceride, glucose intolerance, insulin and insulin resistance, are also improved under ATRF in high-fat fed animals (Hatori, Vollmers et al. 2012, Sherman, Genzer et al. 2012, Tsai, Villegas-Montoya et al. 2013, Adamovich, Rousso-Noori et al. 2014, Chaix, Zarrinpar et al. 2014). In addition to metabolism, the inflammatory biomarkers, such as Interleukin-6 (IL-6), tumor necrosis factor-alpha (TNFα), Interleukin 1 Beta (IL1β) and C-reactive protein (CRP) are also reduced with ATRF (Sherman, Frumin et al. 2011, Hatori, Vollmers et al. 2012, Sherman, Genzer et al. 2012, Chaix, Zarrinpar et al. 2014). The daily fluctuation of the gut microbiome composition is dampened with high-fat diet and ATRF partially restores the fluctuation (Chaix, Zarrinpar et al. 2014).

In humans, a late eating pattern correlates with increased incidence of metabolic syndromes. Epidemiological studies demonstrate that night eating syndrome, characterized by delayed time of eating, is positively associated with BMI (Colles, Dixon et al. 2007). More daily energy intake consumed in the evening is associated with higher risk of obesity compared to a schedule where more energy is consumed at midday (Wang, Patterson et al. 2014). People who ate lunch after 1500 hours lost less weight and displayed a slower weight-loss rate than those who consumed their lunch before 1500 hours in a weight-loss treatment (Garaulet, Gomez-Abellan et al. 2013). The late dinner eating in Japanese adults is significantly associated with hyperglycemia (Nakajima and Suwa 2015). In addition, in-laboratory late meal timing (lunch at 16:30) or large dinner (60% energy consumed at dinner) worsens insulin sensitivity and reduces glucose tolerance (Morgan, Shi et al. 2012, Bandin, Scheer et al. 2015). A recent published clinical trial demonstrated that time-restricted feeding (a 6-hour feeding period before 1500 hours) in males with prediabetes improves insulin sensitivity, β cell responsiveness, oxidative stress, appetite and lowers morning BP compared to the 12-hour feeding controls (Sutton, Beyl et al. 2018). All these data suggest eating at the "right" time is beneficial while eating at the "wrong" time is detrimental to metabolic health.

1.3.3 Circadian rhythm and diabetes

Accumulated evidence from human and animal studies demonstrates that circadian misalignment between environmental/behavioral cycles and the endogenous circadian clock is associated with increased incidence of obesity and type 2 diabetes. In

humans, results from epidemiology studies show that night or rotating shift workers have a higher prevalence of type 2 diabetes (Gan, Yang et al. 2015). The social jet lag--mismatching sleep timing between workdays and freedays, is found to be associated with increased body weight (Roenneberg, Allebrandt et al. 2012, Parsons, Moffitt et al. 2015, Wong, Hasler et al. 2015), elevated glycated hemoglobin in obese individuals (Parsons, Moffitt et al. 2015), enhanced fasting plasma insulin and insulin resistance (Wong, Hasler et al. 2015). Short duration and poor quality of sleep is associated with increased risk of future diabetes (Cappuccio, D'elia et al. 2009, Nedeltcheva and Scheer 2014) (Holliday, Magee et al. 2013). Prolonged nighttime light exposure in an uncontrolled home setting is associated with a significant increase in body weight, triglyceride levels and low-density lipoprotein cholesterol levels (LDL-C) and a decrease in high-density lipoprotein cholesterol (HDL-C) levels in elderly individuals (Obayashi, Saeki et al. 2013). Laboratory human studies that mimic circadian misalignment also reveal the association between circadian disruptions and diabetes. As described before, late food consumption or a large dinner worsens insulin sensitivity and reduces glucose tolerance (Morgan, Shi et al. 2012, Bandin, Scheer et al. 2015). In addition, sleep restriction in normal subjects enhances plasma cortisol levels and sympathetic nervous activity and reduces insulin sensitivity (Stamatakis and Punjabi 2010, Broussard, Ehrmann et al. 2012). Sleep disturbance, such as sleep fragmentation and selective suppression of slow-wave sleep, without changing the sleep duration, also decreases glucose tolerance (Tasali, Leproult et al. 2008, Stamatakis and Punjabi 2010). Moreover, laboratory circadian misalignment, achieved by forced desynchrony or 12-h reversed

eating and lighting protocols, uncouples behavioral cycles (fasting/feeding and sleep/wake cycles) and/or light/dark cycles from the central circadian clock. Such circadian misalignment causes increased postprandial glucose and BP, and reduced leptin, sleep efficiency, glucose tolerance and insulin sensitivity (Scheer, Hilton et al. 2009, Buxton, Cain et al. 2012, Morris, Yang et al. 2015, Morris, Purvis et al. 2016). In animals, as described before, time-restricted feeding only during the rest phase is associated with accelerated weight gain (Arble, Bass et al. 2009) (Bray, Ratcliffe et al. 2013) and flattened fluctuation of plasma glucose (Bray, Ratcliffe et al. 2013). In addition, light exposure significantly increases food intake and weight gain, reduces glucose tolerance and impairs insulin sensitivity (Fonken, Workman et al. 2010, Coomans, van den Berg et al. 2013). Imposing chronic jet-lag in mice is associated with enhanced body weight and glucose intolerance (Oike, Sakurai et al. 2015) and leptin resistance (Kettner, Mayo et al. 2015).

There seems be a reciprocal relationship between circadian disruptions and diabetes as diabetes also alters circadian rhythm. As descripted before, diabetes disrupts rhythms of BP and BP regulatory factors. In addition, people with prediabetes or type 2 diabetes have disrupted 24-hour variation in body temperature (Gubin, Nelaeva et al. 2017). In animal models of diabetes, altered circadian rhythms in body temperature (Murakami, Horwitz et al. 1995, Grosbellet, Dumont et al. 2016), locomotor activity (Kohsaka, Laposky et al. 2007, Su, Guo et al. 2008) (Kudo, Akiyama et al. 2004) and food intake pattern (Kohsaka, Laposky et al. 2007, Grosbellet, Dumont et al. 2016) are observed.

The mechanisms underlying the reciprocal relationship between circadian misalignment and diabetes are not fully understood. Results from animal studies reveal that alternations in clock genes participate in linking circadian rhythm disruptions and diabetes. Genetic modulations of clock genes in animals lead to metabolic disorders. In mice, either global (Rudic, McNamara et al. 2004, Kondratov, Kondratova et al. 2006, Shi, Ansari et al. 2013) or tissue-specific *Bmal1* deletion in liver (Lamia, Storch et al. 2008, Jacobi, Liu et al. 2015) or pancreas (Marcheva, Ramsey et al. 2010) causes impaired glucose homeostasis and/or insulin resistance. Mutation of *Clock*, either globally or in pancreas, leads to hyperglycemia and obesity (Turek, Joshu et al. 2005, Marcheva, Ramsey et al. 2010). Deletion of *Clock* in primary myotubes decreases insulin response to glucose and promotes lipid utilization (Perrin, Loizides-Mangold et al. 2018). Both *mPer1/2/3* triple-deficient and *mPer3* single-deficient mice gain more weight on high-fat diet than control mice (Dallmann and Weaver 2010). The *mPer1/2*-defecient mice have significantly higher fat composition and leptin levels than wild type mice (Kettner, Mayo et al. 2015). Mice with double knockout of *Cry1/2* develop hyperglycemia (Tanida, Yamatodani et al. 2007) while overexpression of *Cry1* reduces blood glucose concentration and improves insulin sensitivity in diabetic *db/db* mice (Zhang, Liu et al. 2010). Dual depletion of *Rev-erb-α/β* in mice disrupts lipid hemostasis (Cho, Zhao et al. 2012). On the other hand, circadian genes expressions are altered in liver (Kudo, Akiyama et al. 2004, Ando, Oshima et al. 2006, Kohsaka, Laposky et al. 2007), adipose tissues (Kohsaka, Laposky et al. 2007, Caton, Kieswich et al. 2011), aorta

(Su, Xie et al. 2011, Nernpermpisooth, Qiu et al. 2015), mesentery arteries (Su, Xie et al. 2011), heart (Su, Xie et al. 2011) and kidney (Su, Xie et al. 2011) in diabetic animals.

1.3.4 Clock genes in BP circadian rhythm

With the discovery of clock genes, the roles of clock genes in the regulation of BP were investigated. In humans, genetic variations in Bmal1 are associated with hypertension (Woon, Kaisaki et al. 2007) and non-dipping BP pattern in hypertensive patients (Leu, Chung et al. 2015). Deletion of *Bmal1* in mice, either embryonical (Bunger, Wilsbacher et al. 2000) or tamoxifen inducible (Yang, Chen et al. 2016), leads to decreased BP during the active-phase, resulting in flattened BP circadian rhythm (Curtis, Cheng et al. 2007, Yang, Chen et al. 2016). In smooth-muscle-specific *Bmal1* knockout (*SM-Bmal1–KO*) mice, the circadian rhythm of BP is comprised, characterized mainly by decreased SBP during the dark phase (Xie, Su et al. 2015). Deletion of mice *Bmal1* in the brown adipocytes (*BA-Bmal1-KO*) including perivascular adipose tissue (PVAT) reduces the BP during the rest period, resulting in an extreme-dipping BP pattern (Chang, Xiong et al. 2018).

The Clock is another component of the active arm of the clock molecular loop. The BP in the *Clock*-KO mice preserves the circadian rhythm, but the levels are significantly lower during both the light- and dark-phase compared with the wild type mice (Zuber, Centeno et al. 2009). The *Clock* mutant ($Clock^{mut}$) mice on different backgrounds, including C57BL/6J (Curtis, Cheng et al. 2007), Jcl/ICR (Sei, Oishi et al. 2008) and BALB/c (Nakashima, Kawamoto et al. 2018), all exhibit increased BP during

the light-phase, resulting in a non-dipping BP pattern. The cardiomyocyte-specific *Clock* mutant (*CCM*) mice have a decreased heart rate (HR) during both the light- and dark-phase, in which the decrease of HR is greater during the dark-phase than the light-phase, resulting in attenuated circadian variation of HR (Bray, Shaw et al. 2008). However, the BP circadian rhythm and average levels are not significantly different between the *CCM* and wild type mice.

Per is one of the components of the negative arm of the clock molecular loop. In humans, the mRNA expression of *Per1* in the renal medulla is significantly increased in hypertensive patients compared to normotensive controls (Marques, Campain et al. 2011) and a tag single-nucleotide polymorphisms (SNPs) in *Per2* are found to be significantly associated with a non-dipping BP pattern in hypertensive patients (Leu, Chung et al. 2015). The 129/sv mice lacking *Per1* exhibit decreased BP during both light- and dark-phase, while the rhythm of BP is preserved (Stow, Richards et al. 2012). The *Per1-KO* mice on the C57BL/6J background have normal BP level and circadian rhythm on control diet, while they exhibit a non-dipping BP pattern on high-salt diet plus desoxycorticosterone pivalate, a treatment that is used to generate salt-sensitivity hypertension (Solocinski, Holzworth et al. 2017). *Per2* mutant mice have increased 24-hour HR, decreased 24-hour diastolic BP and attenuated the day and night difference in HR and BP without changes in locomotor activity (Vukolic, Antic et al. 2010). The mice lacking *Per2* display normal BP and locomotor activity rhythm on normal diet in LD condition, but have moderate increase in light-phase BP accompanied with loss of locomotor activity rhythm in constant dark, and exogenous administration of Ang II on

normal diet induces non-dipping BP in *Per2-KO* mice in constant dark (Pati, Fulton et al. 2016). In addition, low salt diet causes no-dipping BP in *Per1/2/3* triple knockout (*Per-TKO*) mice (Pati, Fulton et al. 2016).

Another component of the circadian repressor is Cry. Deletion of both *Cry1 and Cry2* leads to increased BP during the light phase, resulting in flattened BP circadian rhythm (Masuki, Todo et al. 2005, Doi, Takahashi et al. 2010).

1.4 Diabetic mouse model-*db/db* mouse

The most widely used type 2 diabetic mouse model is the *db/db* mouse model. The syndromes in *db/db* mice are similar to those in maturity-onset diabetes in humans, characterized by obesity, infertility, hyperphagia and marked hyperglycemia (Ktorza, Bernard et al. 1997). Diabetes in *db/db* mice is caused by a spontaneous point mutation in the "leptin receptor" gene (lepr), resulting in abnormal splicing of the gene transcript, leading to defective in leptin signaling (Chen, Charlat et al. 1996, Lee, Proenca et al. 1996). The BP level and its circadian rhythm in the *db/db* mice are preserved when they are young (Senador, Kanakamedala et al. 2009). However, the *db/db* mice develop hypertension, along with non-dipping BP around 12 weeks old (Su, Guo et al. 2008, Senador, Kanakamedala et al. 2009). The diabetic and non-dipping BP phenotypes make the *db/db* mouse a good animal model to study BP circadian rhythm disruption in diabetes

CHAPTER IA. STATEMENT OF THE PROBLEM

Diabetes affected approximately 422 million adults worldwide in 2014 (2016). 90-95% of these people are type 2 diabetes (T2D) in the US (Prevention 2017). People with T2D suffer from short- and long-term complications, among which cardiovascular disease (CVD) is the leading cause of mortality and morbidity associated with T2D (Organization 2014). It is known that hypertension is a major risk factor for CVD. Hypertension and T2D often coexist and the risk of CVD increases when both hypertension and T2D are present (Gomes 2013).

With the use of ambulatory blood pressure monitoring (ABPM), people are aware that blood pressure (BP) exhibits 24-h oscillation that is lowest at night and peaks before awaking (Millar-Craig, Bishop et al. 1978). The BP during the nighttime mean is normally 10-20% lower compared to the daytime mean, which is also called a dipping pattern. In a large study that included 12765 hypertensive patients (2954 T2D patients), more than 80% of hypertensive, type 2 diabetic patients were found to have an abnormal BP circadian pattern (non-dipping or reserved-dipping) (Ayala, Moya et al. 2013). The abnormal BP circadian pattern is associated with increased risk of CVD and target organ damages (described in chapter 1.2.2). Importantly, the dipping status of systolic blood pressure (SBP) predicts all CVD outcomes independent of 24-hour SBP levels (Roush, Fagard et al. 2014). Therefore, understanding the mechanisms and restoring the disruption of BP circadian rhythm in T2D may help prevent or delay the onset of CVD.

The underlying mechanisms of diabetes associated BP circadian rhythm disruption are not well-understood. As described in Chapter 1.2.4, BP circadian rhythm is regulated by multiple factors, including the sleep-wake cycle, the ANS, the hormonal systems (RAAS, glucocorticoid, thyroid and atrial natriuretic peptide), the vasculature and the kidney. Alterations of any one of the above factors, along with diabetes induced metabolic abnormalities, such as obesity, hyperglycemia and insulin resistance, may be expected to contribute to diabetes associated BP circadian rhythm disruption.

With the identification of mammalian clock genes in the last two decades, the molecular mechanisms of mammalian circadian clock are revealed. Investigations of clock genes point out that the clock genes may also participate in diabetes associated BP circadian rhythm disruption. For example, mutation or knockout of core clock genes, such as *Bmal1* (Curtis, Cheng et al. 2007, Yang, Chen et al. 2016), *Clock* (Curtis, Cheng et al. 2007, Sei, Oishi et al. 2008, Nakashima, Kawamoto et al. 2018), *Per* (Vukolic, Antic et al. 2010) and *Cry* (Masuki, Todo et al. 2005, Doi, Takahashi et al. 2010) leads to abnormal BP circadian rhythm in rodents. On the other hand, the expressions of circadian genes are altered in liver (Kudo, Akiyama et al. 2004, Ando, Oshima et al. 2006, Kohsaka, Laposky et al. 2007), adipose tissues (Kohsaka, Laposky et al. 2007, Caton, Kieswich et al. 2011), aorta (Su, Xie et al. 2011, Nernpermpisooth, Qiu et al. 2015), mesentery arteries (Su, Xie et al. 2011), heart (Su, Xie et al. 2011) and kidney (Su, Xie et al. 2011) in diabetic animals. However, the results of clock genes expressions in diabetes are achieved using real-time PCR or Western blotting in tissues collected every 4 to 6 h

in only one day. Consequently, the time resolution of circadian rhythm analysis is limited by sampling intervals and duration.

To overcome this barrier, we crossed *db/db* mice, a widely used type 2 diabetic mice, with *mPer2*^{Luc} knock-in mice and generated a novel *db/db-mPer2*^{Luc} mouse model. The *mPer2*^{Luc} mice have an in-frame 3'-end fusion of the luciferase reporter gene to the endogenous mPer2 gene, which allows real-time monitoring of mPer2^{Luc} bioluminescence *ex vivo* and *in vivo* (Yoo, Yamazaki et al. 2004, Tahara, Kuroda et al. 2012). Therefore, the *db/db-mPer2*^{Luc} mice enable a novel, continuous monitoring of Per2 oscillation under a diabetic condition.

Extensive research over the past decades has focused on how the components of food (what we eat) and the amount of food (how much we eat) affect metabolic diseases. Only recently has it become appreciated that the timing of food intake (when we eat), independent of total caloric and *macronutrient* quality, is also critical for metabolic health. Results from animal and human studies demonstrated that it is beneficial to the metabolic health of an organism when the feeding time is in alignment with the endogenous circadian clock and *vice versa*. However, it is not known whether the timing of food intake affects BP circadian rhythm. It is also not known whether active-time restricted feeding (ATRF, the feeding time is restricted to the active-phase) is able to restore disrupted BP circadian rhythm in diabetes.

CHAPTER IB. HYPOTHESIS

Specific Aim 1: To real-time monitor clock gene oscillation in diabetes by generating *db/db-mPer2*^{Luc} mice.

Specific Ami 2: To examine the effects of active-time restricted feeding (ATRF) on BP circadian rhythm in diabetic *db/db* mice and to explore underlying mechanisms.

CHAPTER II. MATERIALS AND METHODS

2.1 Project 1: A Novel Diabetic Mouse Model for Real-time Monitoring of Clock Gene Oscillation and Blood Pressure Circadian Rhythm.

2.1.1 Generation of the *db/db-mPer2^{Luc}* mice

The heterozygous leptin receptor (*Lepr^{db}*) mutation *db/+* mice on the C57BL/KsJ background (Stock No.: 000642; also known as *C57BL/KsJ-db/+*) and the homozygous *mPer2^{Luc}* mice on the C57BL/6J background (Stock No.: 006852; also known as *C57BL/6J-mPer2^{Luc}*) were purchased from the Jackson Laboratory. Since the homozygous *C57BL/KsJ-db/db* mice are infertile, the heterozygous male *C57BL/KsJ-db/+* mice and homozygous female *C57BL/6J-mPer2^{Luc}* mice were used as breeders to generate the homozygous diabetic *db/db*-m*Per2^{Luc}* mice and heterozygous non-diabetic *db/+*-m*Per2^{Luc}* control mice (Figure 2.1.1A). Of note, both *db/db*-m*Per2^{Luc}* and *db/+*-m*Per2^{Luc}* control mice have a mixed C57BL/KsJ and C57BL/6J background. The genotyping protocol for the *db/db* mice is listed in the Jackson Laboratory website. The genotyping protocol for the *mPer2^{Luc}* mice was described previously (Yoo, Yamazaki et al. 2004). The representative agarose gels for PCR genotyping of the *mPer2^{Luc}* and *db/db* mice are shown in Figure 2.1.1B and 2.1.1C. The mice were fed normal chow diet and housed under 12:12 light: dark condition. Only the 4-6 month-old male *db/db*-m*Per2^{Luc}* and age- and gender-matched *db/+*-m*Per2^{Luc}* control mice were used in the current study. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

Figure 2.1.1

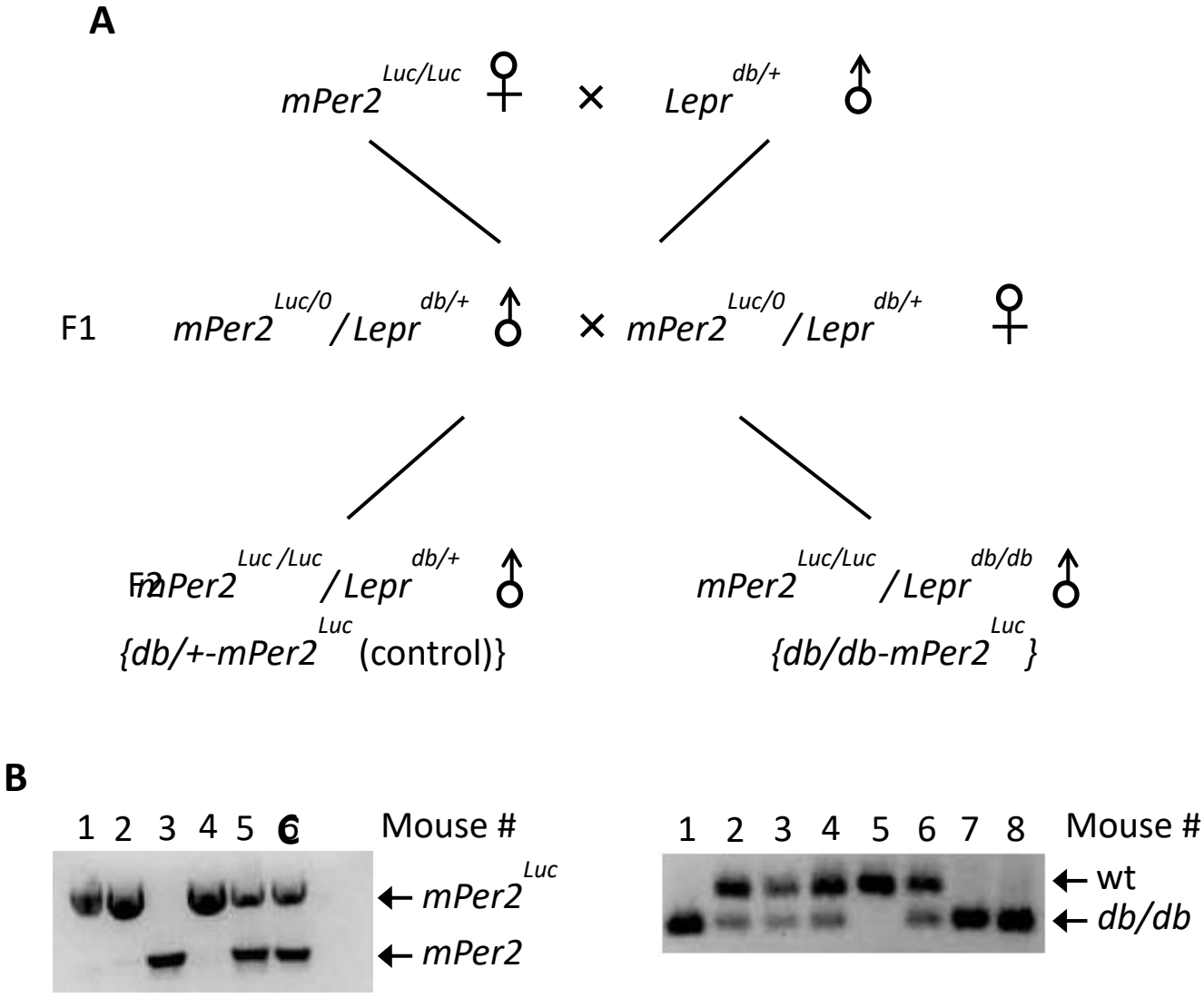


Figure 2.1.1 Generation of the *db/db-mPer2^{Luc}* mice. (**A**) Breeding strategy to generate *db/db-mPer2^{Luc}* and control male mice. (**B**) Representative image for genotyping *mPer2^{Luc}* mice. Of note, mouse # 1, 2, and 4 are homozygous *mPer2^{Luc}* mice. (**C**) Representative image for genotyping *db/db* (*lepr$^{-/-}$*) mice. Of note, mouse #1, 7, and 8 are *db/db* mice whereas mouse # 2, 3, 4, and 6 are *db/+* control mice.

2.1.2 Metabolic characterization of animals

2.1.2.1 Body composition

Body composition (lean mass and fat mass) was assessed in the light phase by NMR spectroscopy according to manufacturer's instructions (Echo MRITM-100H, Houston, TX, USA).

2.1.2.2 Blood glucose

Non-fasting blood glucose was measured between Zeitgeber Time (ZT; ZT0 is defined as light on and ZT12 is defined as light off) ZT9 to ZT10 from the tail vein using StatStrip® XepressTM glucometer (NOVA® biomedical, Waltham, MA, USA).

2.1.2.3 Plasma insulin

Blood was collected between ZT10 and ZT11 in EDTA-coated tubes. Then the blood was centrifuged at 5000rpm for 10 mins and the supernatant was collected as plasma. Plasma insulin was determined by ELISA according to manufacturer's instructions (Chrystal Chem, Elk Grove Village, IL, USA).

2.1.2.4 Intraperitoneal glucose tolerance test (IPGTT)

IPGTT was performed at ZT3 after 6-h fasting. The basal blood glucose was measured, followed by intraperitoneal (i.p.) injection of 1 mg/kg body weight (BW) glucose dissolved in 0.9% NaCl. The blood glucose was then measured at 15, 30, 60, 90 and 120 mins after glucose injection.

2.1.3 Implantation of radiotelemetry

The radiotelemetry probe (TA11PA-C10, Data Sciences International, St. Paul, MN, USA) was chronically inserted inside the left common carotid artery of the mice as described previously (Su, Guo et al. 2008, Su, Xie et al. 2013, Xie, Su et al. 2015). Briefly, the mice were anesthetized using isoflurane. Then the left carotid artery was isolated and the catheter of the radiotelemetry probe was inserted into the isolated carotid artery and tied. The body of the radiotelemetry was slipped subcutaneously to the flank close to the left hindlimb. Then the neck incision was sutured. After the surgery, the mice were watched closely until fully awake from anesthesia. After 7-10 days of recovery from the surgery, blood pressure (BP), heart rate (HR), and locomotor activity were recorded in conscious free-moving mice. The signals from the radiotelemetry were received to the receiver (model RPC-1) and then transferred as described in the Acquisition software. The data were sampled at 500Hz and analyzed using the Dataquest A.R.T. software. In order to demonstrate the daily rhythms of BP, HR and locomotor activity, at least 72-h continuous data were collected.

2.1.4 Baroreflex sensitivity analysis

Spontaneous baroreflex sensitivity (BRS) was analyzed by sequence techniques using Hemolab software downloaded from: http://www.haraldstauss.com/HemoLab/HemoLab.html. The data collected from radiotelemetry were generated into short (1-h) data sets. Then the short data sets were filtered at fourth order, and corner frequency 40 Hz. For an effective BRS, at least four consecutive sequences where the systolic arterial pressure and pulse interval were

positively correlated (r^2>0.80) were counted. Baroreflex sensitivity was calculated as the average slope of the systolic pressure-pulse interval relationships with auto threshold at 3 beats in delay. The BRS were averaged in each corresponding hour over 3 days and one 24-hour BRS was generated.

2.1.5 Metabolic chamber measurement of locomotor activity, food and water intake, respiratory exchange ratio (RER) and energy expenditure (EE)

The locomotor activity, food and water intake, RER, and EE were determined by indirect gas calorimetry LabMaster system (TSE System, Bad Homburg, Germany; also known as metabolic chambers). The mice were kept under 12:12 light: dark cycle and were individually housed in the acclimation cages for seven days before being transferred to the metabolic chambers. The mice were put in the metabolic chamber to collect data for at least three consecutive days. The concentrations of oxygen and carbon dioxide inside the metabolic chambers, the weights of food and water containers and the counts of locomotor activity were measured every 30 minutes. The data were calculated by the accompanied TSE PhenoMaster software.

2.1.6 Real-time monitoring of mPer2 oscillations in explant tissues by LumiCycle

The procedure for real-time monitoring of mPer2 oscillations in explant tissues by LumiCycle was adapted from previous report (Yamazaki and Takahashi 2005). Briefly, the aorta, mesenteric artery (MA), kidney, liver, white adipose tissue (WAT), thymus, lung, adrenal gland (AG), and brain were isolated from mice between ZT10 and ZT11. The aorta was cleaned, cut open longitudinally, and denuded of endothelium cells. The

MA was dissected to remove fat tissues. The kidney, liver, WAT, thymus, and lung were cut into small pieces, with a diameter varying between approximately 2 and 6 mm depending upon the tissue. The total AG was used. The brain containing the SCN was cut into 250 μm thick sections by using NVSL manual advance vibroslice (World Precision Instruments, Sarasota, FL, USA). Each tissue was cultured in a well-sealed 35-mm Petri dish containing Dulbecco's Modified Eagle Medium (DMEM) and 0.1 mM D-luciferin (Gold Biotechnology Inc., St. Louis, MO). Details of the medium constituent were described previously (Yamazaki and Takahashi 2005). The light emission from the cultured tissues was measured with photon-counting photomultiplier tubes that count photons for 1 min over a 10 min interval using a LumiCycle 32 system (Actimetrics, Wilmette, IL, USA) as described (Yamazaki and Takahashi 2005). The bioluminescence data obtained from the explanted tissues were analyzed using LumiCycle Analysis software (Actimetrics, Wilmette, IL, USA). To detrend the signal drift over time, the 24-hour moving average was subtracted from the raw data. To eliminate the influence of exposure to environmental lighting before recording, the first 12-hour data collected in the explant culture were excluded. The data collected from 12 hours to 36 hours in the culture were used to determine the oscillation amplitude and acrophase. The data collected from 12 hours to 120 hours in the culture were used to determine the oscillation period by the dampened sine-curve fitting method. The data with a goodness of fit >0.8 were used for analysis in all the tissues except in kidney where data with a goodness of fit > 0.7 were used due to the rapid dampening of the oscillation.

2.1.7 *In vivo* imaging of mPer2 time-of-day variation in the kidney, liver, and submandibular gland (SG)

The procedure for *in vivo* imaging of mPer2 time-of-day variation in the kidney, liver, and SG was adapted from previous report (Tahara, Kuroda et al. 2012). Briefly, at ZT5, 11, 17 and 23, mice were anesthetized with 2.5-4% isoflurane and subcutaneously injected with D-luciferin (15 mg/kg body weight in PBS). The mice were imaged 7 minutes later for dorsal side up and 10 minutes later for later ventral side up for 5 seconds by using the IVIS spectrum (IVIS spectrum *in vivo* imaging system, PerkinElmer, Waltham, MA, USA). Bioluminescence from the liver of each mouse was quantified (photon/s/cm^2/sr) by setting the region of interest to the same shape and size using Living Image software (IVIS Imaging System). The bioluminescence intensity was expressed as an absolute value or as the percentage of the average value throughout the day as described (Tahara, Kuroda et al. 2012).

2.1.8 Cosinor analysis of circadian rhythm

The daily rhythms of BP, HR, locomotor activity, food and water intake, RER and EE were analyzed using Cosinor analysis as previously reported (Refinetti, Lissen et al. 2007). Briefly, a cosine wave with a known period (24 hours) was fitted by the least squares to the data as an estimate of the pattern of the smooth rhythm. The model equation was written as $x_i=M+A\cos(\vartheta_i+\varphi)$, where M is mesor, A is amplitude, φ is acrophase, and ϑ_i is trigonometric angles corresponding to the sampling time.

2.1.9 Tissue collection and quantitative analysis of mRNA expression

2.1.9.1 Tissue collection

Mesenteric arteries (MA) were isolated at ZT5 and ZT17 and immediately placed in RNAlater solution. Then the fat and adventitious tissues were carefully cleaned off under microscope from the MA.

2.1.9.2 Quantitative analysis of mRNA expression

The cleaned tissues were homogenized using tissue homogenizer (Bullet Blender ® Next Advance, Troy, NY) in RNase- DNase-free tubes, followed by total RNA extraction using RNeasy® Mini Kit (Qiagen, Hilden, Germany). Then the total RNA was used to synthesize cDNA by M-MLV reverse transcriptase kit (Invitrogen, Carlsbad, CA) using random hexamers. The real-time PCR primers for each gene are described in Table 2.1.1.

Table 2.1.1. Real-time PCR primer information for project 1.

Gene	**Primer**	**Sequence**
Bmal1	Forward	5'-ATCAGCGACTTCATGTCTCC-3'
	Reverse	5'-CTCCCTTGCATTCTTGATCC-3'
ROCK1	Forward	5'-GACTGGGGACAGTTTTGAGAC-3
	Reverse	5'-ATCCAAATCATAAACCAGGGCAT-3'
ROCK2	Forward	5’-TTTCTAAACATGCGAAGAATCTCATATG-3’
	Reverse	5’-CTTCTACCCCATTTCTTCCAAGTC-3’
Calponin-1	Forward	5'-GCACATTTTAACCGAGGTCCT-3'
	Reverse	5'-CTGATGGTCGTATTTCTGGGC-3'

Table 2.1.1 (continued)

Calponin-2	Forward	5'-GCGGGAACATGACACAGGT-3'
	Reverse	5'-CATGGTGGCGTCGTCAAAGT-3'
Calponin-3	Forward	5’-AGGCAGAATACCCCGATGAA-3’
	Reverse	5’-GGTCGTCGCCATACTGGTACTC-3’
Tropomyosin 1 (α)	Forward	5'-CTGGTTGAGGAGGAGTTGGA-3'
	Reverse	5'-ATGTGCTTGGCCTCTTTCAG-3'
Tropomyosin 2 (β)	Forward	5’-AGGCCACCGACGCTGAA-3’
	Reverse	5’-CCTGTGCCCGATCCAACT-3’
SM22α	Forward	5’-ACCGTGGAGATCCCAACTGGTTTA-3’
	Reverse	5’-CATTTGAAGGCCAATGACGTGCT-3’

Bmal1: Brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1; ROCK1/2: Rho kinase 1/2; SM22α: Smooth muscle protein 22-α.

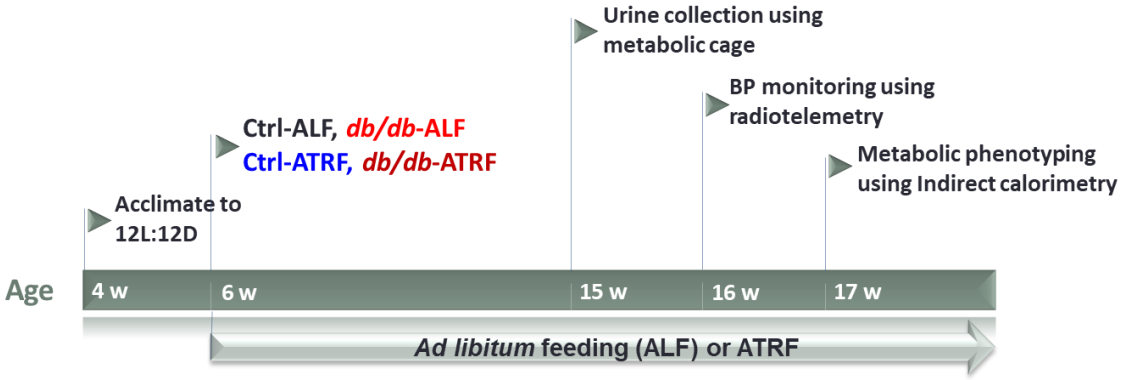
2.1.10 Statistical analysis

All data were expressed as mean ± SEM. For comparison of 1 parameter between 2 strains of mice, unpaired 2-tail Student's t-test was used. For comparison of one parameter across a time period between 2 strains of mice, 2-way ANOVA with repeated measures and Bonferroni's post-test were performed. For comparison of multiple parameters between 2 strains of mice, regular 2-way ANOVA with Bonferroni's post-test was performed. $P < 0.05$ was defined as statistically significant.

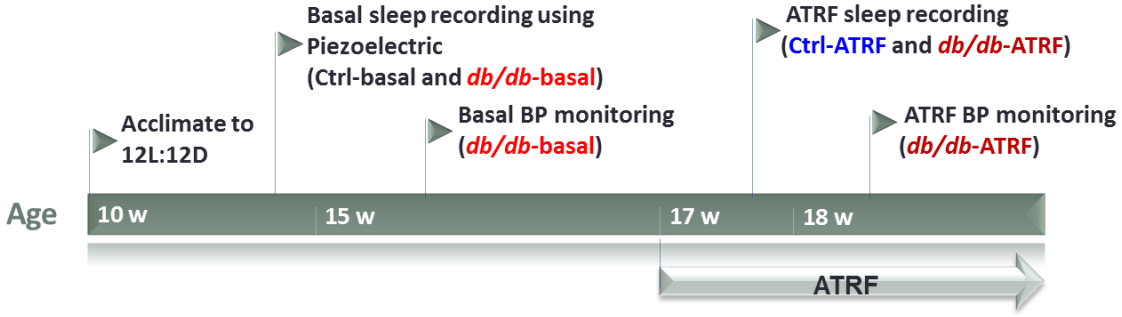
2.2 Project 2: Active-Time Restricted Feeding Restores Blood Pressure Circadian Rhythm via Autonomic Nervous System in Type 2 Diabetic *db/db* Mice.

2.2.1 Experimental design

Experimental 1: To test if active-time restricted feeding (ATRF) can prevent *db/db* mice from disruption of BP daily rhythm.



Experimental 2: To test if ATRF can restore the already disrupted BP daily rhythm in *db/db* mice.



2.2.2 Animals

2.2.1.1 C56BL/6J and *db/db* mice

C56BL/6J mice were purchased from the Jackson Lab (Stock No.: 000664). *Db/db* on the C57BL/KsJ background and age-, gender-matched nondiabetic *db/+* mice were purchased from the Jackson Lab (Stock No.: 000642; Bar Harbor, ME). Upon arrival, the

mice were housed under 12:12 light: dark condition in a light-tight box and fed with normal chow diet *ad libitum*.

2.2.1.2 Generation of the inducible global Bmal1 knockout (*iG-Bmal1-KO*) mice

The homozygous *Bmal1*$^{flox/flox}$ mice (Storch, Paz et al. 2007)(Stock No.: 007668) and heterozygous *UBC-Cre-ERT2*$^{+/-}$ mice (Stock No.: 007001) were purchased from the Jackson lab. The *Bmal1*$^{flox/flox}$*/UBC-Cre-ERT2*$^{+/-}$ mice were generated by crossing *Bmal1*$^{flox/flox}$ mice and *UBC-Cre-ERT2*$^{+/-}$ mice. The genotyping protocols for the *Bmal1*$^{flox/flox}$ and *UBC-Cre-ERT2*$^{+/-}$ mice are listed in the Jackson Laboratory website. To generate *iG-Bmal1-KO* mice, 100 ul 20mg/ml tamoxifen dissolved in sesame oil was i.p. injected daily for 5 days.

2.2.3 Feeding schedule

All the mice were housed under 12:12 light: dark (LD) condition upon arrival and fed *ad libitum* before being subjected to experiments. The mice were fed with normal chow diet and had free access to water throughout the study.

Inactive-time restricted feeding (ITRF): the mice were allowed access to food for 10 hours between ZT2 and ZT12 during the light-phase.

8-h active-time restricted feeding (ATRF): the mice were allowed access to food for 8 hours from ZT13 to ZT21 during the dark-phase.

12-h ATRF: the mice were allowed access to food for 12 hours from ZT12 to ZT24 during the dark-phase.

2.2.4 Metabolic characterization of animals

2.2.4.1 Body composition

Please see chapter 2.1.2.1.

2.2.4.2 Blood glucose

Blood glucose was measured from the tail vein using StatStrip® XepressTM glucometer (NOVA® biomedical, Waltham, MA, USA). For *db/db* and control mice that began 8-h ATRF at 6-week-old, non-fasting blood glucose was measured every other week at ZT13 and ZT21. For the *db/db* and control mice that began 8-h ATRF at 16-week-old, non-fasting and fasting blood glucose was measured at ZT21, in which the fasting blood glucose was measured after 4-h fasting started at ZT17.

2.2.4.3 Plasma insulin, non-esterified fatty acids (NEFA) and total cholesterol measurement

Endpoint blood was collected at ZT5, ZT11, ZT17 and ZT23 in tubes with 10ul 0.5mM EDTA and plasma was separated as described in chapter 2.1.2.3. Plasma insulin was determined by ELISA kit (Chrystal Chem, Elk Grove Village, IL, USA). Plasma NEFA was determined using NEFA kit (FUJIFILM Wako Diagnostics, Richmond, VA, USA). Total cholesterol was determined by a commercial cholesterol reagent set (Pointe Scientific, Canton, MI, USA).

2.2.4.4 Intraperitoneal insulin tolerance test (IPTTT)

IPITT was performed at ZT1 after 4-h fasting. The basal blood glucose was measured, followed by i.p. injection of 1 unit/kg body weight of insulin dissolved in 0.9% NaCl. The

blood glucose was then measured at 15, 30, 60, 90 and 120 mins after insulin injection. Area under the curve (AUC) was calculated for the area between the basal and decreased blood glucose after insulin injection over 120 mins in each mouse.

2.2.5 Implantation of radiotelemetry

Please see chapter 2.1.3.

2.2.6 Sleep-wake state monitoring

The sleep-wake state of the mice was monitored using PiezoSleep system (Signal Solutions LLC, Lexington, KY, USA) (Flores, Flores et al. 2007). The PiezoSleep system distinguishes between the sleep and wake state according to the body movements of the mice. During sleep, the primary gross body movements are associated with respiration and are rhythmic. During awake, the respiratory movements are masked by other activity and are erratic. The PiezoSleep system detects the body movements using a high-sensitive motion detector on the bottom of the animal cages. Control and *db/db* mice were habituated to the piezo device in LD schedule with normal chow diet for 4 weeks. The sleep-wake state was recorded at 15-week-old for 7 days as baseline. Then the mice were subjected to 8-h ATRF and sleep-wake state was continued to be recorded for 5 days after ATRF.

2.2.7 Urine collection and catecholamines, aldosterone and corticosterone measurement

Urine was collected using metabolic cages (Tecniplast, S.p.A.). The mice were acclimated in the cage for over 12 hours, then the urine was collected during the periods ZT0 to 6,

ZT6 to 12, ZT12 to 18 and ZT18 to 24. ELISA kits were used to determine the concentrations of urinary epinephrine, norepinephrine and normetanephrine (Abnova, Taiwan) and aldosterone (Enzo Life Sciences, Inc.). The urinary concentrations of corticosterone were determined using EIA kit (Arbor Assay, Ann Arbor, MI). Total contents of urinary epinephrine, norepinephrine, normetanephrine, aldosterone and corticosterone were calculated as concentrations × urine volumes.

2.2.8 Effects of prazosin on BP

The α1-adrenergic receptor antagonist, prazosin, was i.p. injected at 1mg/kg body weight in the mice. Radiotelemetry was recorded 1 hour before injection as baseline and 2 hours after the injection (total three hours). The data were averaged over an interval of 3 mins. Baseline BP was calculated as the average of 1h data before injection. The values of BP after injection of prazosin were selected as the lowest points after injection.

2.2.9 Baroreflex sensitivity analysis

Please chapter 2.1.4

2.2.10 Heart rate variability (HRV) analysis

HRV was analyzed from the radiotelemetry data by frequency domain and time domain methods using Ponemah Software (DSI, version 6.32).

For frequency domain analysis, 2-min segments at 20-min interval over 72 hours were selected and scanned to ensure they were free of artifacts. Each segment was interpolated to 20Hz using quadratic method. Subsequently, the data were subdivided

into 50 overlapping series and computed by Fast Fourier Transform (FFT) using Hanning window method. The cut-off frequency range for low-frequency (LF) was 0.15-0.6Hz, optimized by Baudrie's group (Baudrie, Laude et al. 2007). The high-frequency (HF) range was 1.5-4Hz.

For time domain analysis, 5-min segments over 72 hours were calculated and the root-mean-square successive beat-to-beat difference (rMSSD) was plotted as the marker of parasympathetic heart rate control.

For both the frequency and time domain data, The HRV were averaged in each correspondent hour over 3 days and one 24-hour HRV was generated.

2.2.11 Tissue collection and quantitative analysis of mRNA expression

2.2.11.1 Tissue collection

The mice were euthanized at ZT5, 11, 17 and 23. The liver, mesenteric arteries (MA), kidney, heart and adrenal gland were removed and immediately placed in RNAlater solution. Then the fat and adventitious tissues of the MA were carefully cleaned off under microscope. The real-time PCR primers for each gene are described in Table 2.2.1.

Table 2.2.1. Real-time PCR primer information for project 2.

Gene	**Primer**	**Sequence**
Bmal1	Forward	5'-ATCAGCGACTTCATGTCTCC-3'
	Reverse	5'-CTCCCTTGCATTCTTGATCC-3'
Clock	Forward	5'-GGCGTTGTTGATTGGACTAGG-3

Table 2.2.1 (continued)

	Reverse	5'-GAATGGAGTCTCCAACACCCA-3'
Per1	Forward	5’-TCGAAACCAGGACACCTTCTCT-3’
	Reverse	5’-GGGCACCCCGAAACACA-3’
Per2	Forward	5'-AAAGCTGACGCACACAAAGAA-3'
	Reverse	5'-ACTCCTCATTAGCCTTCACCT-3'
Cry1	Forward	5'-TCGCCGGCTCTTCCAA-3'
	Reverse	5'-TCAAGACACTGAAGCAAAAATCG-3'
Cry2	Forward	5’-CCTCGTCTGTGGGCATCAA-3’
	Reverse	5’-GCTTTCTTAAGCTTGTGTCCAGATC-3’
Rev-erbα	Forward	5'-CCCTGGACTCCAATAACAACACA-3'
	Reverse	5'-GCCATTGGAGCTGTCACTGTAG-3'
Rorc	Forward	5’-TCCACTACGGGGTTATCACCT-3’
	Reverse	5’-AGTAGGCCACATTACACTGCT-3’
Th	Forward	5’-TCTCCTTGAGGGGTACAAAACC-3’
	Reverse	5’-ACCTCGAAGCGCACAAAGT-3’
Dbh	Forward	5’-CTGGGTGCCAAGGCATTTTAC-3’
	Reverse	5’-GAACTTCCAGTCGGAGAAACG-3’
Pnmt	Forward	5’-AGACCTGAGCAACCCTGATG-3’
	Reverse	5’-TGGTGATGTCCTCAAAGTGG-3’
Comt	Forward	5’-AACACGCAAAGCCTGGAGA-3’
	Reverse	5’-CATGGTGAGAAGCCTGGCTC-3’
MaoA	Forward	5’-GGTCCTCCTTGGGGATAAAG-3’

Table 2.2.1 (continued)

	Reverse	5’-TCTCAGGTGGAAGCTCTGGT-3’
MaoB	Forward	5’-ATGAGCAACAAAAGCGATGTGA-3’
	Reverse	5’-TCCTAATTGTGTAAGTCCTGCCT-3’
Angiotensinogen	Forward	5’-TCTCTTTACCCCTGCCCTCT-3’
	Reverse	5’-CAGGCAGCTGAGAGAAACCT-3’
Renin	Forward	5’-TCAGGGAGAGTCAAAGGTTTCC-3’
	Reverse	5’-ACAGTGATTCCACCCACAGTCA-3’
Ace	Forward	5’-AGCCCAAGTGTTGTTGAACGA-3’
	Reverse	5’-TGGATACCTCCGTGCTTTTCT-3’
Ace2	Forward	5’-TCCAGACTCCGATCATCAAGC-3’
	Reverse	5’-TGCTCATGGTGTTCAGAATTGT-3’
At1a	Forward	5’-CCAAGAAAGCCATCACCAGATC-3’
	Reverse	5’-TTTCTGGGTTGAGTTGGTCTCA-3’

Bmal1: Brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1; Clock: Circadian locomotor output cycles kaput; Per: Period; Cry: Cryptochrome; Rorc: RAR-related orphan receptor c; Th: Tyrosine hydroxylase; Dbh: Dopamine beta (β)-hydroxylase; Pnmt: Phenylethanolamine N-methyltransferase; Comt: Catechol-O-methyltransferase; MaoA and MaoB: Monoamine oxidase A and B; Ace: angiotensin-converting enzyme; At1a: angiotensin II receptor type 1a.

2.2.11.2 Quantitative analysis of mRNA expression

Please see chapter 2.1.9.2.

2.2.12 Statistical analysis

All data were expressed as mean ± SEM. For comparison of 1 parameter between 2 groups of mice, paired 2-tail Student's t-test was used. For comparison of 1 parameter between more than 2 groups of mice, one-way ANOVA with Newman-Keuls's post-test was used. For comparison of two parameters between ≥2 groups of mice, 2-way ANOVA with Bonferroni's post-test were performed. $P < 0.05$ was defined as statistically significant.

CHAPTER III. RESULTS

3.1 Project 1: A Novel Diabetic Mouse Model for Real-time Monitoring of Clock Gene Oscillation and Blood Pressure Circadian Rhythm.

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3.1.1 *Db/db-mPer2^{Luc}* mice are obese and diabetic

The *db/db* mouse is an extensively used monogenic type 2 diabetic mouse model. The syndrome in *db/db* mice is similar to that in maturity-onset diabetes in humans, characterized by obesity, infertility, hyperphagia and marked hyperglycemia (Ktorza, Bernard et al. 1997). The diabetic phenotype of the *db/db* mice, however, varies depending on the genetic background. Currently, there are two *db/db* mouse models: one is on the C57BL/KsJ background with severe hyperglycemia and temporarily elevated plasma insulin; the other one is on the C57BL/6J background with transient hyperglycemia and marked hyperinsulinemia (Hummel, Coleman et al. 1972). To study the disruption of circadian rhythms in type 2 diabetes, we crossed the *C57BL/KsJ-db/db* mice that have severe diabetes with the *mPer2^{Luc}* mice that contain a knock-in luciferase gene fused to mouse Period2 (mPer2) as a clock gene reporter (Yoo, Yamazaki et al. 2004), and generated a novel *db/db-mPer2^{Luc}* mice. Since the *mPer2^{Luc}* mice are on the C57BL/6J background, the generated *db/db-mPer2^{Luc}* mice have a mixed background (C57BL/KsJ and C57BL/6J). It is unclear to what extent the *db/db-mPer2^{Luc}* mice retain

obesity and diabetes. Therefore we first characterized this novel mouse model with respect to obesity, hyperglycemia, hyperinsulinemia and insulin resistance.

The *db/db-mPer2*^{Luc} mice had significantly increased body weight when compared to their littermate *db/+-mPer2*^{Luc} control mice (Figure 3.1.1A). The body weight increase was mostly attributable to an increased fat mass as the lean mass was comparable between the *db/db-mPer2*^{Luc} and control mice (Figure 3.1.1B). The non-fasting blood glucose and plasma insulin levels in the *db/db-mPer2*^{Luc} mice were also markedly elevated relative to those in the control mice (Figure 3.1.1C and 3.1.1D). Moreover, the *db/db-mPer2*^{Luc} mice exhibited a severely impaired glucose tolerance (Figure 3.1.1E). These results indicate that the *db/db-mPer2*^{Luc} mice manifest the common characteristics of type 2 diabetes, e.g. obesity, hyperglycemia, hyperinsulinemia, and impaired glucose tolerance.

Figure 3.1.1

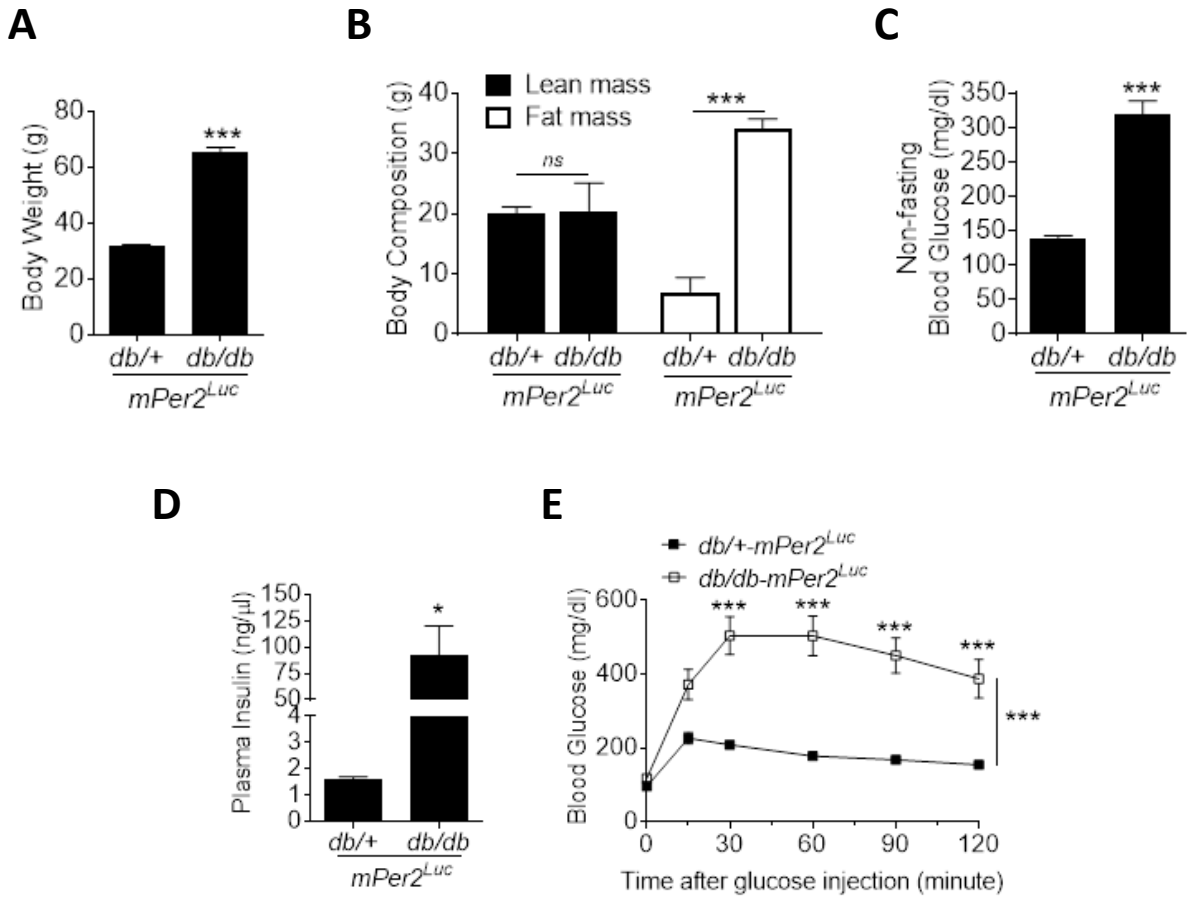


Figure 3.1.1 The *db/db-mPer2^{Luc}* mice are obese and diabetic. Body weight (**A**; n = 12), body composition (**B**; n = 4-6), non-fasting blood glucose (**C**; n = 12), and plasma insulin (**D**; n = 4-5) were measured between ZT9 and ZT11 in the *db/db-mPer2^{Luc}* and control *db/+-mPer2^{Luc}* mice. Glucose tolerance test (**E**; n = 11-12) was performed at ZT3 after 6-hour fasting. All data were expressed as mean ± SEM. *, P < 0.05; ***, P < 0.001; ns, not significant.

3.1.2 *Db/db-mPer2*^{Luc} mice have a compromised BP daily rhythm that is associated with the disruption of the daily rhythm in baroreflex sensitivity but not heart rate

To determine whether the BP daily rhythm is disrupted in the *db/db-mPer2*^{Luc} mice, we recorded BP by radiotelemetry under normal 12:12 light-dark cycle for 72 consecutive hours. We found that the daily oscillations of mean arterial pressure (MAP), systolic blood pressure (SBP), and diastolic blood pressure (DBP) were diminished in the *db/db-mPer2*^{Luc} mice compared to that in the control mice (Figure 3.1.2A, 3.1.3A and 3.1.3D). The compromised daily rhythms of the MAP, SBP, and DBP were primarily caused by the decreased dipping during the inactive light phase with no change during the active dark phase in the *db/db-mPer2*^{Luc} mice relative to the control mice (Figure 3.1.2A, 3.1.3A and 3.1.3D). Quantitative analysis of the daily (24-hour) average of MAP, SBP, and DBP showed no difference between the *db/db-mPer2*^{Luc} and control mice (Figure 3.1.2B 3.1.3B and 3.1.3E), indicating that the *db/db-mPer2*^{Luc} mice are normotensive, unlike the *C57BL/KsJ-db/db* mice (Park, Bivona et al. 2008, Su, Guo et al. 2008, Goncalves, Tank et al. 2009, Senador, Kanakamedala et al. 2009). Further quantitative analysis of the BP during either the light or dark phase BP (12-hour) revealed a 50% reduction in the difference between light phase and dark phase in the MAP, SBP, and DBP in the *db/db-mPer2*^{Luc} mice compared to that in the control mice (Figure 3.1.2C, 3.1.3C and 3.1.3F). Cosinor analysis of the oscillations showed that the amplitude (half of the range of oscillation) and robustness of daily rhythms in the MAP, SBP, and DBP were significantly attenuated in the *db/db-mPer2*^{Luc} mice compared to that in the control mice (Figure 3.1.2D and 3.1.2E; Table 3.1.1). Interestingly, no differences were found in the

acrophase (the time when the cycle peaks) between the *db/db-mPer2*^{Luc} and control mice (Figure 3.1.2F; Table 3.1.1).

Baroreflex is an important rapid negative feedback mechanism for maintaining normal BP. Therefore we investigated whether the compromised BP daily rhythm in the *db/db-mPer2*^{Luc} mice is associated with an alteration of the time-of-day variations in baroreflex sensitivity. We analyzed spontaneous baroreflex sensitivity by sequence techniques in the *db/db-mPer2*^{Luc} and control mice as previously described (Xie, Su et al. 2015). In the *db/+-mPer2*^{Luc} control mice, baroreflex sensitivity was significantly higher during the light-phase than during the dark-phase (Figure 3.1.4). In contrast, such time-of-day variations of baroreflex sensitivity were abolished in the *db/db-mPer2*^{Luc} mice. This result implicates the loss of daily variation in baroreflex sensitivity in the compromised BP daily rhythm.

Because heart rate is an important factor that determines the cardiac output and BP level (Reule and Drawz 2012), we investigated whether the daily heart rate oscillation is also altered in the *db/db-mPer2*^{Luc} mice. We found that the daily heart rate, the difference between light phase and dark phase heart rate, and its rhythmicity, including amplitude, robustness, and acrophase, were not significantly altered in the *db/db-mPer2*^{Luc} mice compared to that in the control mice (Figure 3.1.5A-3.1.5F).

Figure 3.1.2

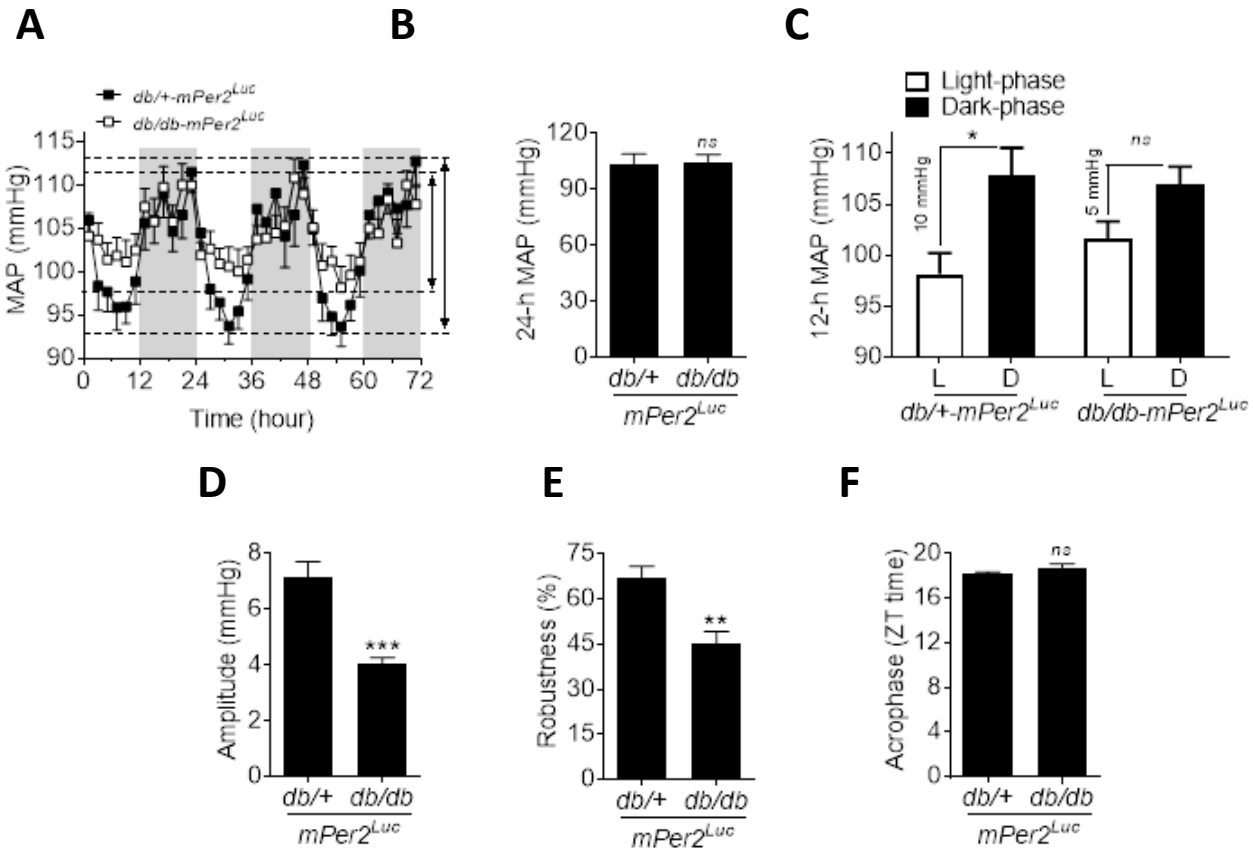


Figure 3.1.2 The daily rhythm of mean arterial pressure (MAP) is disrupted in the *db/db-mPer2^{Luc}* mice. MAP was recorded by radiotelemetry in the *db/db-mPer2^{Luc}* and control *db/+-mPer2^{Luc}* mice. (**A**) The 72-hour recording of MAP. The light grey box indicates the dark-phase and the length of the arrowhead lines indicates the BP difference between the light and dark phase in the two mouse strains. (**B)** The 24-hour MAP. (**C**) The 12-hour MAP during the light phase (L) and dark phase (D). (**D-F**) The amplitude, robustness, and acrophase of the MAP daily oscillation. All data were expressed as mean ± SEM (n = 6). *, P < 0.05; **, P < 0.01, ***, P < 0.001; ns, not significant.

Figure 3.1.3

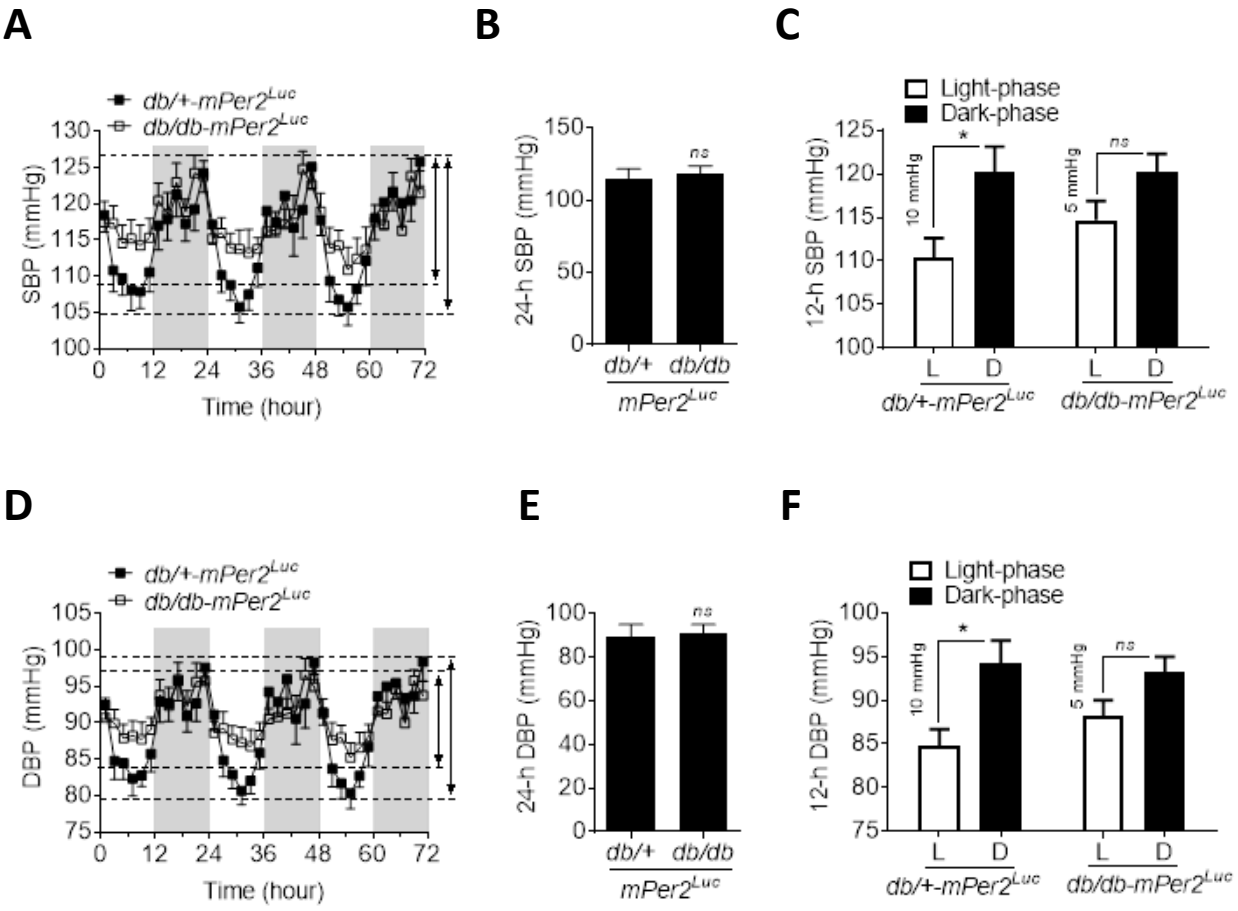


Figure 3.1.3 The daily oscillations of systolic blood pressure (SBP) and diastolic blood pressure (DBP) are diminished in *db/db-mPer2^{Luc}* mice. SBP (**A-C**) and DBP (**D-F**) were recorded by radiotelemetry. The 72-hour recording of SBP (**A**) and DBP (**D**) where the grey box indicates the dark phase and the length of the arrowhead lines indicate the difference of BP between two strains of mice. The 24-hour average of SBP (**B**) and DBP (**E**). The 12-hour SBP (**C**) and DBP (**F**) during the light phase (L) and dark phase (D). The difference in the day and night BP was indicated in the figures. All data were expressed as mean ± SEM (n = 6). Unpaired t test was used for (**B** and **E**). Two-way ANOVA was used for (**C** and **F**). The difference between two mouse strains was indicated in the figures. *, $p<0.05$; ns, $p>0.05$.

Figure 3.1.4

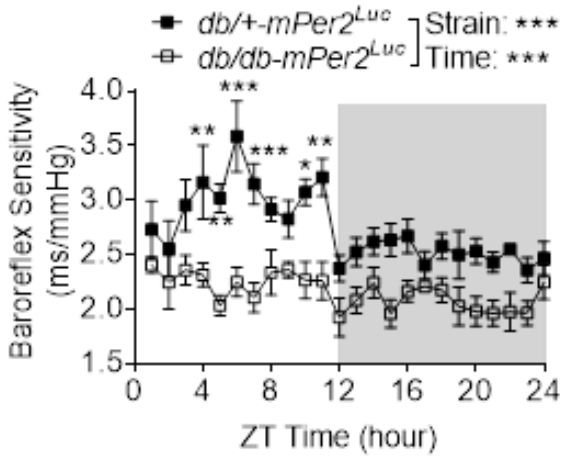


Figure 3.1.4 The day and night variation of baroreflex is abolished in the *db/db-mPer2^{Luc}* mice. Baroreflex was calculated every hour using the Hemolab software in the *db/db-mPer2^{Luc}* and control *db/+-mPer2^{Luc}* mice. The light grey box indicates the dark-phase. All data were expressed as mean ± SEM (n = 6). **, P < 0.01, ***, P < 0.001.

Figure 3.1.5

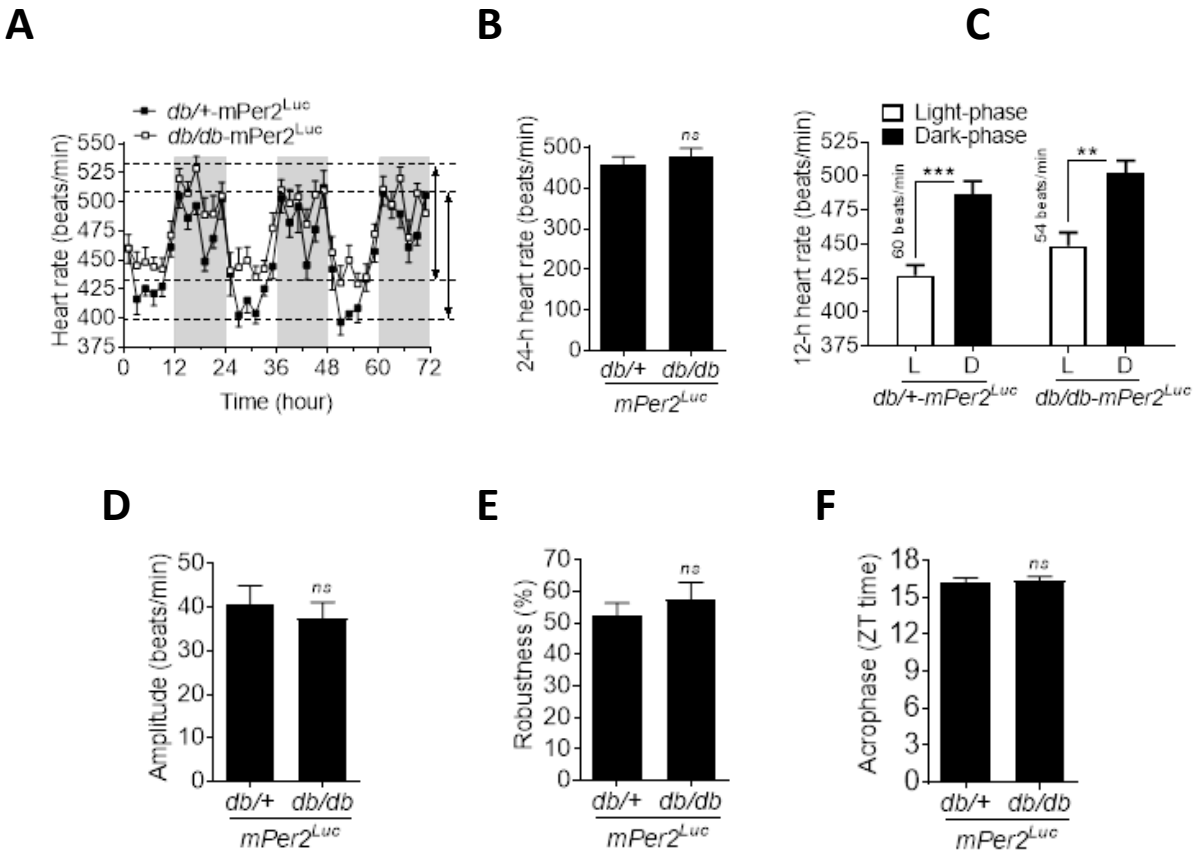


Figure 3.1.5 The daily oscillation of heart rate (HR) is not disrupted in *db/db-mPer2^{Luc}* mice. HR was recorded by radiotelemetry in the *db/db-mPer2^{Luc}* and control *db/+-mPer2^{Luc}* mice. (**A**) The 72-hour recording of HR. The light grey box indicates the dark-phase and the length of the arrowhead lines indicates the HR difference between the light and dark phase in the two mouse strains. (**B**) The 24-hour HR. (**C**) The 12-hour HR during the light phase (L) and dark phase (D). (**D-F**) The amplitude, robustness, and acrophase of the HR daily oscillation. All data were expressed as mean ± SEM (n = 6). **, $P < 0.01$; ns, not significant.

Table 3.1.1 The daily oscillations in systolic blood pressure (SBP) and diastolic blood pressure (DBP) were diminished in *db/db-mPer2^{Luc}* mice.

Blood Pressure	Circadian Rhythm	*db/+-mPer2^{Luc}*	*db/db-mPer2^{Luc}*	P value
SBP	Amplitude (mmHg)	7.421±1.546	4.353±0.5485	0.001 *
	Acrophase (ZT time)	18.34±0.5734	19.17±0.731	0.0555
	Robustness (%)	67.93±7.997	44.17±9.646	0.0009 ***
DBP	Amplitude (mmHg)	6.596±1.425	3.728±0.4713	0.0004 ***
	Acrophase (ZT time)	17.96±0.5435	18.54±0.8214	0.1741
	Robustness (%)	66.58±10.39	46.22±9.543	0.0054 **

The SBP and DBP were recorded by radiotelemetry. The amplitude, acrophase, and robustness were calculated by Cosinor analysis. *, P<0.05; **, p<0.01, ***, p<0.001.

3.1.3 The compromised BP daily rhythm is associated with the disruption of daily rhythms in locomotor activity and metabolism but not in food and water intake in the *db/db-mPer2^{Luc}* mice

Behavioral factors such as locomotor activity, food and water intake as well as metabolism may affect central and peripheral clock function through the release of neurotransmitters and hormones and thus impinge on BP circadian rhythm (Rudic and Fulton 2009). Therefore, the daily rhythms in locomotor activity, food and water intake, and metabolism were monitored by indirect calorimetry (also known as metabolic chamber) in the *db/db-mPer2^{Luc}* and control mice every 30 minutes over 72 consecutive hours under 12: 12 light: dark condition. We also used radiotelemetry to monitor locomotor activity independently to confirm the indirect calorimetry data. The results from both indirect calorimetry and radiotelemetry data consistently showed that the daily oscillation in locomotor activity was abolished in the *db/db-mPer2^{Luc}* mice compared with that in the control mice (Figure 3.1.6A, 3.1.6B, 3.1.6H and 3.1.6I). While the absolute counts regarding the daily locomotor activity from indirect calorimetry (Figure 3.1.6C) and radiotelemetry (Figure 3.1.6J) were not consistent, both methods showed a loss of the locomotor activity daily oscillation in the *db/db-mPer2^{Luc}* mice (Figure 3.1.6D and 3.1.6K). Cosinor analysis revealed that the amplitude and robustness of the locomotor activity daily oscillations were largely diminished in the *db/db-mPer2^{Luc}* mice (Figure 3.1.6E, 3.1.6F, 3.1.6L and 3.1.6M). Interestingly, in agreement with the compromised BP daily rhythm in the *db/db-mPer2^{Luc}* mice (Figure 3.1.2F), there were

also no differences in the acrophase of the locomotor activity daily oscillation between the *db/db-mPer2*^{Luc} and control mice (Figure 3.1.6G and 3.1.6N).

In contrast to the locomotor activity, the food and water intake daily oscillations appeared to be preserved in the *db/db-mPer2*^{Luc} mice (Figure 3.1.7A and 3.1.7B; Figure 3.1.8A and 3.1.8B), although the *db/db-mPer2*^{Luc} mice consumed more food and water than the control mice (Figure 3.1.7C and 3.1.8C). Since the *db/db-mPer2*^{Luc} mice consumed more food and water proportionally during both the light and dark phase than the control mice (Figure 3.1.7D and 3.1.8D), the percentages of daily food and water intake during the light and dark phase were similar between two strains of mice (Figure 3.1.7D and 3.1.8D). In accordance with these findings, there were also no differences in robustness and acrophase in food and water intake daily oscillations (Figure 3.1.7F, 3.1.7G, 3.1.8F and 3.1.8G). Interestingly, there was a trend towards an increased daily oscillation amplitude in food intake (Figure 3.1.7E) and a significant increase in water intake (Figure 3.1.8E) in the *db/db-mPer2*^{Luc} mice.

The respiratory exchange ratio (RER) and energy expenditure (EE) daily oscillations were acquired by the metabolic chamber. The RER is calculated as the ratio between the volume of carbon dioxide (VCO_2) produced and the volume of oxygen (VO_2) used in metabolism. It is an indicator of fuel sources (Even and Nadkarni 2012). The EE is calculated as the total daily energy expenditure (calories) in the metabolic chamber, including basal and physical activity expenditure, thermoregulation, and the thermic effects of food (Even and Nadkarni 2012). The RER daily oscillation was disrupted in the

db/db-mPer2^{Luc} mice compared with the control mice (Figure 3.1.9A vs. 3.1.9B). Although both strains of mice had a similar average RER (Figure 3.1.9C), the *db/db-mPer2*^{Luc} mice lost the RER daily oscillation compared to the control mice (Figure 3.1.9D). In agreement with these findings, the amplitude and robustness of the RER daily oscillation were suppressed (Figure 3.1.9E and 3.1.9F), and the acrophase was delayed in the *db/db-mPer2*^{Luc} mice (Figure 3.1.9G). In contrast, the EE daily oscillation was preserved in both strains of mice (Figure 3.1.10A and 3.1.10B), although the daily EE level was higher in the *db/db-mPer2*^{Luc} than the control mice (Figure 3.1.10C). Both strains of mice exhibited a similar EE daily oscillation pattern (Figure 3.1.10D). In agreement with these findings, there was no difference in amplitude and acrophase between the *db/db-mPer2*^{Luc} and control mice (Figure 3.1.10E and 3.1.10F). However, the robustness was suppressed in the *db/db-mPer2*^{Luc} mice (Figure 3.1.10G).

Figure 3.1.6

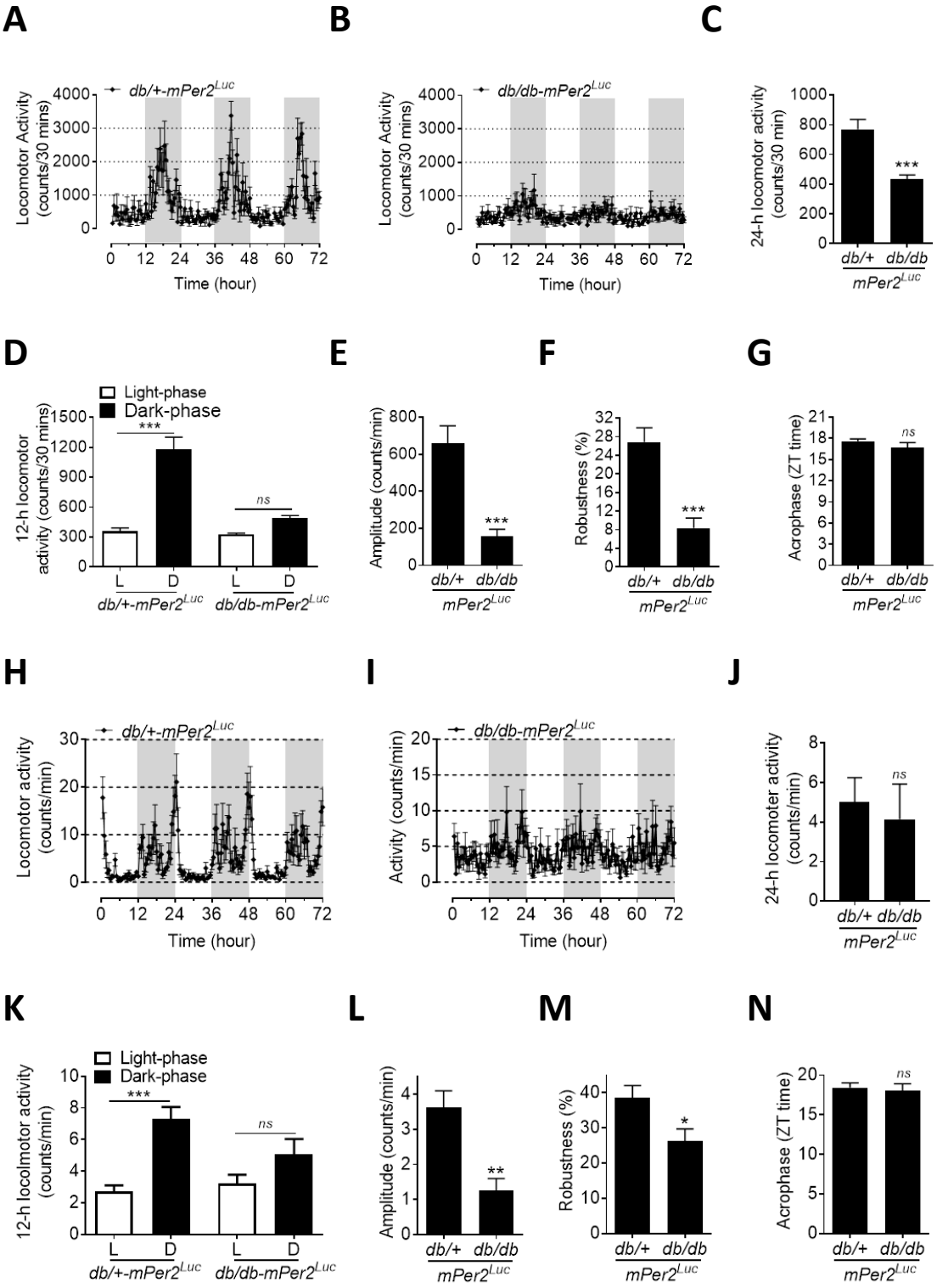

A
B
C
Locomotor Activity (counts/30 mins)
db/+-mPer2Luc
db/db-mPer2Luc
Time (hour)
24-h locomotor activity (counts/30 min)
***
db/+ db/db
mPer2Luc
D
E
F
G
Light-phase
Dark-phase
12-h locomotor activity (counts/30 mins)
***
ns
L D L D
db/+-mPer2Luc db/db-mPer2Luc
Amplitude (counts/min)
Robustness (%)
Acrophase (ZT time)
H
I
J
Locomotor activity (counts/min)
Activity (counts/min)
24-h locomoter activity (counts/min)
K
L
M
N
12-h locolmotor activity (counts/min)
Amplitude (counts/min)
**
Robustness (%)
*
Acrophase (ZT time)

Figure 3.1.6 The daily rhythm of locomotor activity is disrupted in the *db/db-mPer2^{Luc}* mice. Locomotor activity was recorded by indirect calorimetry (A-G) and radiotelemetry (H-N). (A and H) The 72-hour recording of locomotor activity in the control mice by indirect calorimetry (A) and radiotelemetry (H). The 72-hour recording of locomotor activity in the db/db-mPer2Luc mice by indirect calorimetry (B) and radiotelemetry (I) where the light grey box indicates the dark-phase. (C and J) The 24-hour locomotor activity calculated from indirect calorimetry (C) and radiotelometry (J) data. (D and K) The 12-hour locomotor activity during the light phase (L) and dark phase (D) calculated from indirect calorimetry (D) and radiotelometry (K) data. (E-F and L-N): The amplitude, robustness, and acrophase of locomotor activity daily oscillation calculated from indirect calorimetry (E-F) and radiotelometry (L-N) data. All data were expressed as mean ± SEM (n = 6). *, $p<0.05$, ***, $P < 0.001$; ns, not significant.

Figure 3.1.7

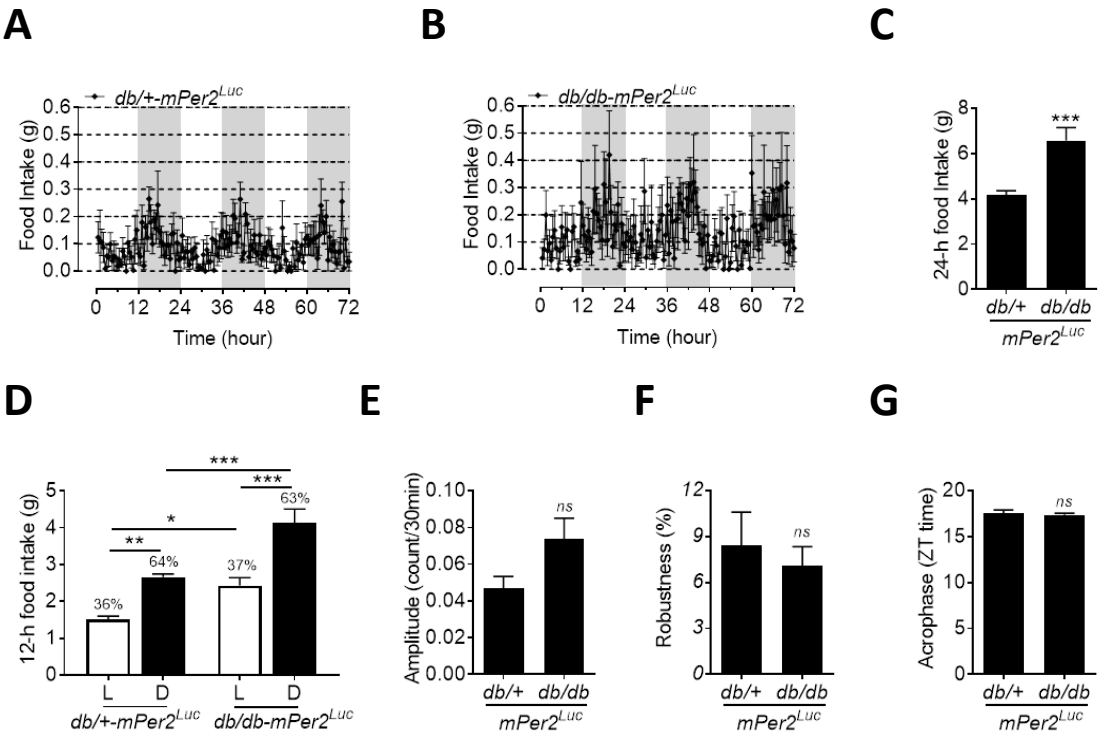


Figure 3.1.7 The daily rhythm of food intake is not diminished in the *db/db-mPer2^{Luc}* mice. Food intake was recorded by indirect calorimetry. (**A** and **B**) The 72-hour recording of food intake in the control (**A**) and *db/db-mPer2^{Luc}* (**B**) mice where the light grey box indicates the dark-phase. (**C**) The 24-hour food intake. (**D**) The 12-hour food intake during the light phase (L) and dark phase (D). (**E-F**): The amplitude, robustness, and acrophase of food intake daily oscillation. All data were expressed as mean ± SEM (n = 6). *, P < 0.05; **, P < 0.01, ***, P < 0.001; ns, not significant.

Figure 3.1.8

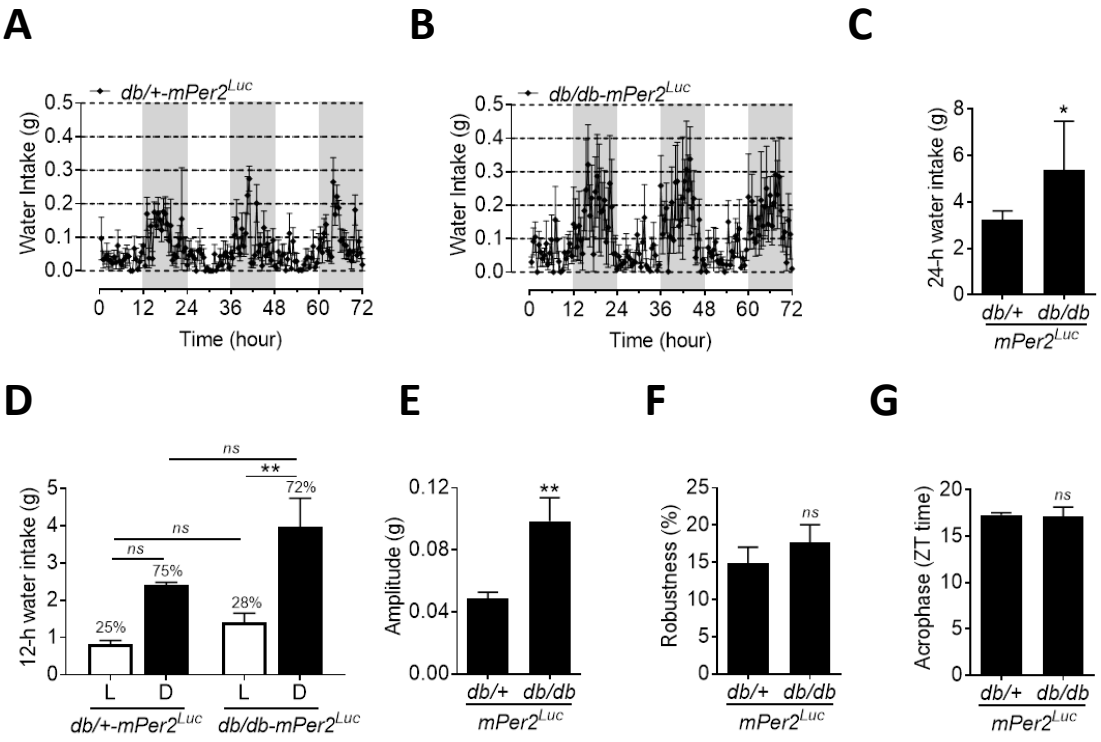


Figure 3.1.8 The daily rhythm of water intake is not diminished in the *db/db-mPer2^{Luc}* mice. Water intake was recorded by indirect calorimetry. (**A** and **B**) The 72-hour recording of water intake in the control (**A**) and *db/db-mPer2^{Luc}* (**B**) mice where the light grey box indicates the dark-phase. (**C**) The 24-hour water intake. (**D**) The 12-hour water intake during the light phase (L) and dark phase (D). (**E-F**): The amplitude, robustness, and acrophase of water intake daily oscillation. All data were expressed as mean ± SEM (n = 6). *, P < 0.05; **, P < 0.01; ns, not significant.

Figure 3.1.9

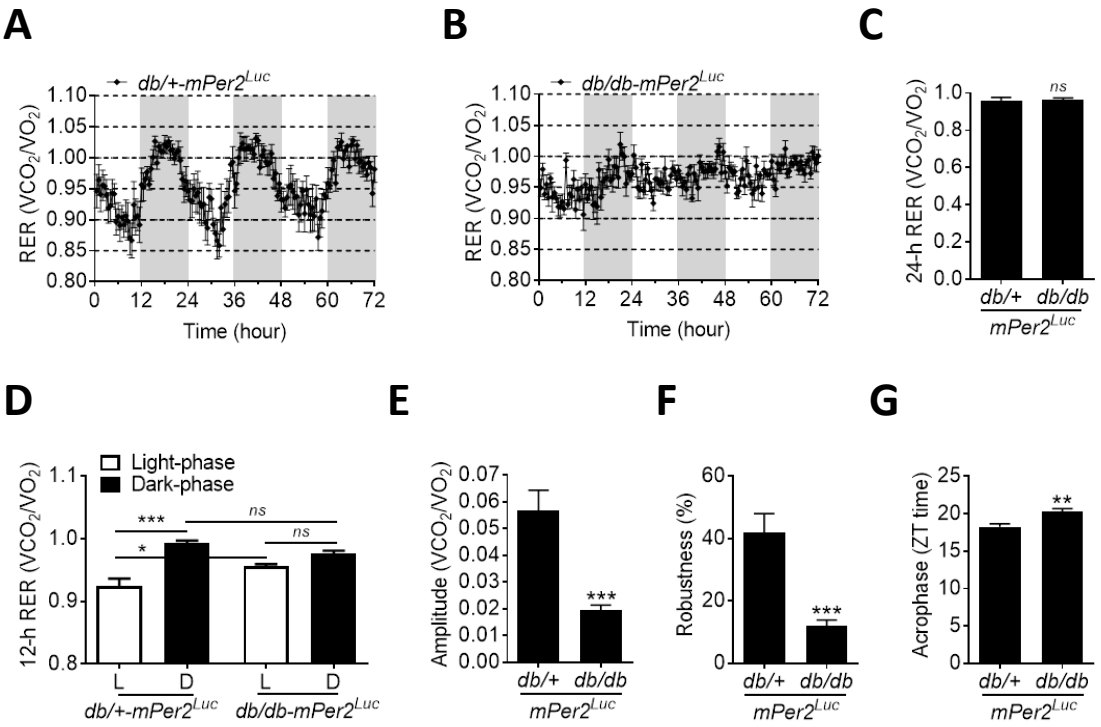


Figure 3.1.9 The daily rhythm of respiratory exchange ratio (RER) is disrupted in the *db/db-mPer2^{Luc}* mice. RER was recorded by indirect calorimetry. (**A** and **B**) The 72-hour recording of the RER in the control (**A**) and *db/+-mPer2^{Luc}* (**B**) mice. The light grey box indicates the dark-phase. (**C**) The 24-hour RER. (**D**) The 12-hour RER during the light phase (L) and dark phase (D). (**E** and **F**) The amplitude, robustness, and acrophase of the RER daily rhythm. All data were expressed as mean ± SEM (n = 6). *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$; ns, not significant.

Figure 3.1.10

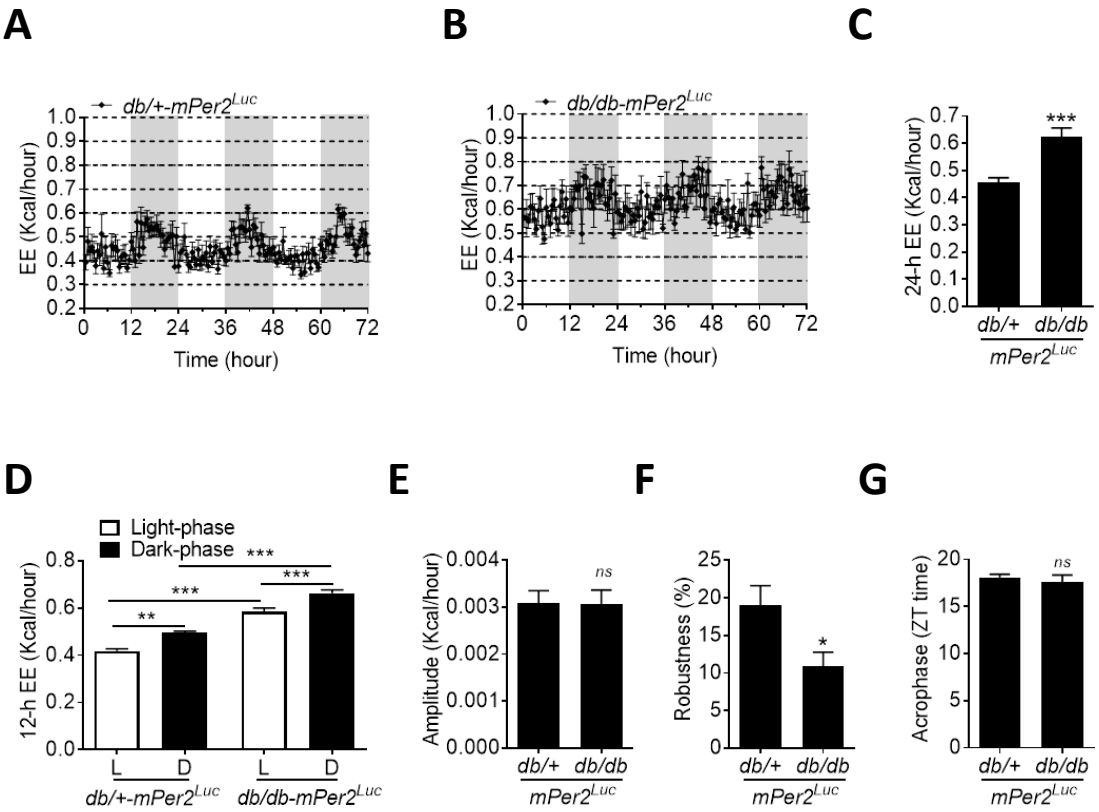


Figure 3.1.10 The daily rhythm of energy expenditure (EE) is not disrupted in the *db/db-mPer2^{Luc}* mice. EE was recorded by indirect calorimetry. (**A** and **B**) The 72-hour recording of the EE in the control (**A**) and *db/+-mPer2^{Luc}* (**B**) mice. The light grey box indicates the dark-phase. (**C**) The 24-hour EE. (**D**) The 12-hour EE during the light phase (L) and dark phase (D). (**E** and **F**) The amplitude, robustness, and acrophase of the EE daily rhythm. All data were expressed as mean ± SEM (n = 6). *, P < 0.05; **, P < 0.01, ***, P < 0.001; ns, not significant.

3.1.4 *Ex vivo* LumiCycle recording reveals that the phases of mPer2 daily oscillation are shifted to different extents in various peripheral tissues but not the SCN from the *db/db-mPer2^{Luc}* mice

Multiple systems coordinate to maintain the normal physiological BP circadian rhythm (Coffman 2011). To investigate in which tissue the clock genes are altered in the *db/db-mPer2^{Luc}* mice that may contribute to the compromised BP circadian rhythm, we monitored mPer2 bioluminescence in real-time in peripheral and central SCN tissues in explant organ culture in the *db/db-mPer2^{Luc}* and control mice. In the various tissues from the control mice, the acrophases of mPer2 oscillation varied but were orchestrated in a specific order (Figure 3.1.11A through 3.1.11J), with the earliest peak shown by the SCN (10.47 ± 0.82 hours) and later peaks shown by the lung (12.08 ± 0.24 hours), kidney (14.23 ± 0.11 hours), liver (14.39 ± 0.77 hours), adrenal gland (15.59 ± 0.20 hours), white adipose tissue (WAT; 15.59 ± 0.39 hours), aorta (16.17 ± 0.24 hours), thymus (19.61 ± 0.77 hours), and mesenteric arteries (MA; 19.69 ± 0.29 hours).

In the tissues from the *db/db-mPer2^{Luc}* mice, the acrophases of mPer2 oscillations were significantly advanced to different extents relative to the corresponding control in a tissue-specific manner (Figure 3.1.11J). The aorta, MA, and kidney, which are crucial for BP and cardiovascular homeostasis, had a 0.98 ± 0.40, 1.70 ± 0.42, and 2.21 ± 0.56 hour phase advance, respectively (Figure 3.1.11A to 3.1.11C). The liver and WAT, two tissues that are crucial for energy metabolism, had a 3.28 ± 0.77 and 4.65 ± 1.21 hour phase advance (Figure 3.1.11D and 3.1.11E). The thymus, a primary lymphoid organ, had a

4.24 ± 1.59 hour phase advance (Figure 3.1.11F). In contrast, the lung and adernal gland had no significant phase shift (Figure 3.1.11G and 3.1.11H). Interestingly, the SCN that has long been believed to be a major regulator of BP circadian rhythm, had also no significant phase shift (Figure 3.1.11I). In contrast to the shift in the acrophase in tissues from the *db/db-mPer2*^{Luc} mice, no significant change was detected in period and amplitude of mPer2 luciferase oscillations in most peripheral tissues from the *db/db-mPer2*^{Luc} (Table 3.1.2).

Figure 3.1.11(1)

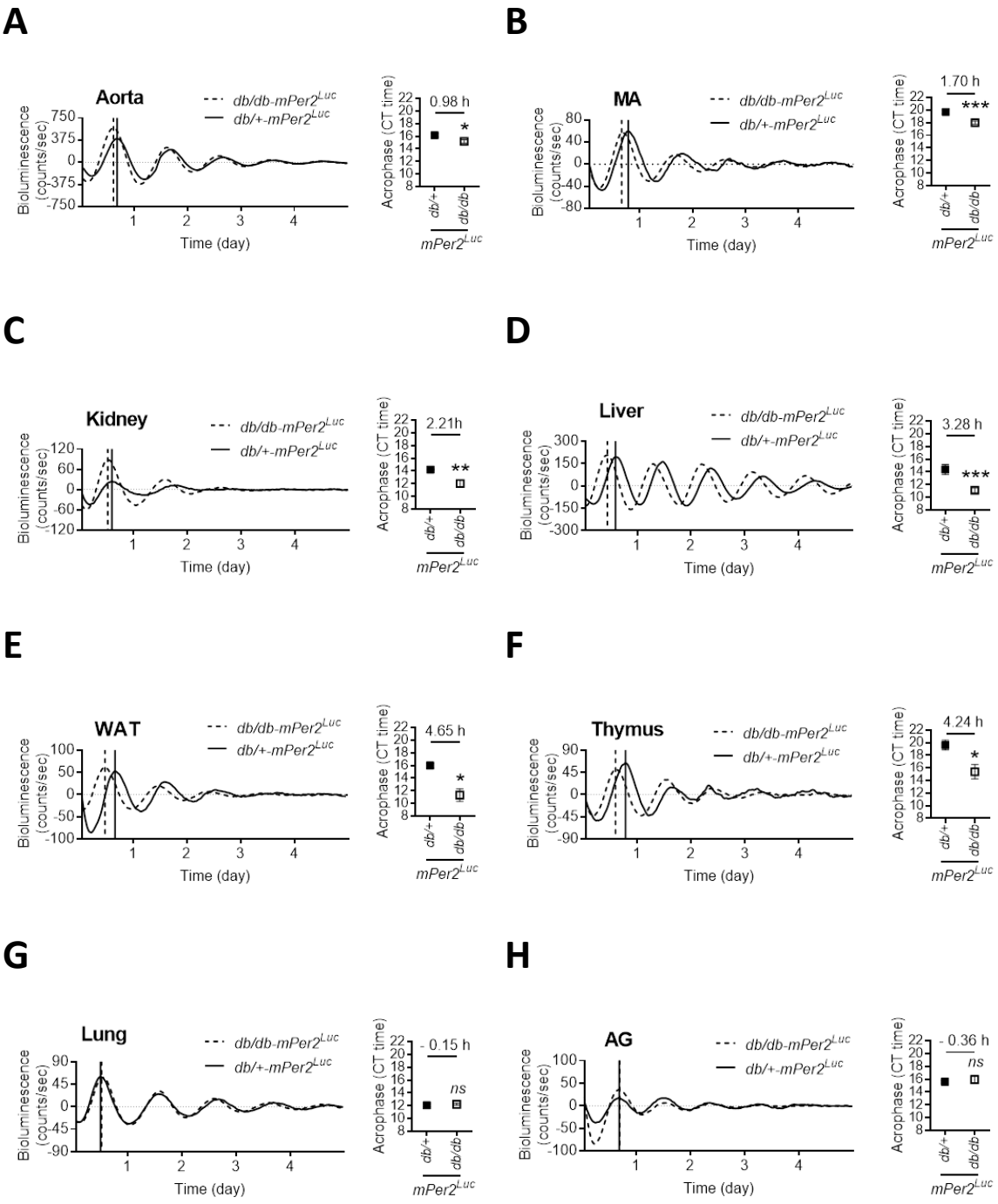


Figure 3.1.11(2)

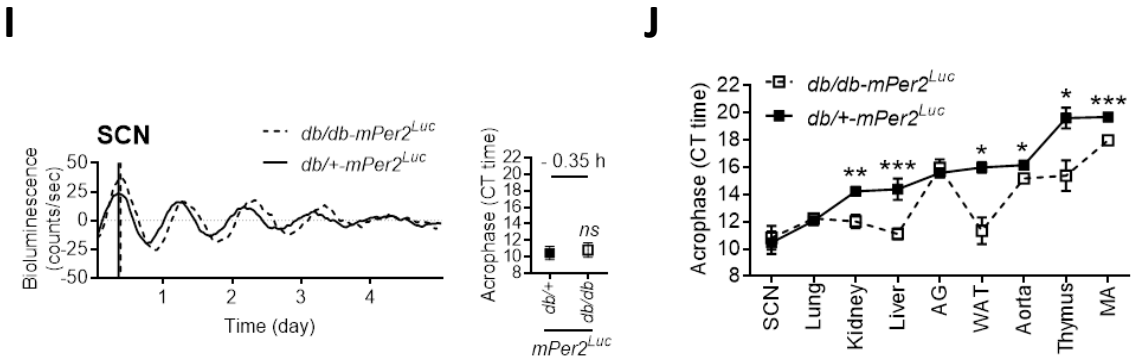


Figure 3.1.11 The phases of mPer2 protein daily oscillation are desynchronized in various explanted peripheral tissues from the *db/db-mPer2^{Luc}* mice. The bioluminescence of mPer2 protein daily oscillation was recorded by LumiCycle in explanted central SCN and peripheral tissues from the *db/db-mPer2^{Luc}* and control *db/+-mPer2^{Luc}* mice. The mPer2 oscillation acrophase of the tissues was calculated using the LumiCycle analysis software. In the representative mPer2 bioluminescence real-time recording (left panel), the solid vertical line indicates the acrophase of the non-diabetic *db/+-mPer2^{Luc}* control mice, whereas the dotted vertical line indicates the acrophase of the diabetic *db/db-mPer2^{Luc}* mice. In the acrophase (right panel), the number above the symbol indicates the difference of the acrophase between two strains of mice. All data were expressed as mean ± SEM from the aorta (**A**; n= 7-11), mesentery artery (MA; **B**; n = 8-12), kidney (**C**; n = 4-5), liver (**D**; n = 6-12), white fat tissue (WAT; **E**; n = 3-4), thymus (**F**; n = 3-5), lung (**G**; n = 4-6), adrenal gland (**H**; n = 3-6), and suprachiasmatic nucleus (**I**; SCN; n = 6-11). (**J**) The acrophase of various tissues in control and *db/db-mPer2^{Luc}* mice. *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$; ns, not significant.

Table 3.1.2 No significant changes were detected in the period and amplitude of mPer2 oscillations in most explanted peripheral tissues from the *db/db-mPer2^{Luc}* mice.

Tissues	Circadian Rhythm	*db/+-mPer2^{Luc}*	*db/db-mPer2^{Luc}*	P value
Aorta	Period (h)	23.76±0.2235	24.05±0.186	0.3409
	Amplitude (counts)	139.3±29.33	208.3±21.45	0.0706
MA	Period (h)	24.41±0.2349	24.4±0.1887	0.9672
	Amplitude (counts)	26.6±2.005	28.11±3.485	0.7458
Kidney	Period (h)	24.5±0.5553	23.78±0.2905	0.2608
	Amplitude (counts)	8.072±2.38	25.06±3.207	0.0049 **
Liver	Period (h)	21.08±0.3492	21.01±0.2061	0.8555
	Amplitude (counts)	44.42±10.53	55.54±11.6	0.5471
WAT	Period (h)	24.41±0.2349	24.4±0.1887	0.9672
	Amplitude (counts)	26.6±2.005	28.11±3.485	0.7458
Thymus	Period (h)	25.23±0.9905	23.96±0.6772	0.3133
	Amplitude (counts)	23.83±10.15	20.5±5.679	0.7641
Lung	Period (h)	23.9±0.7106	23.85±0.3052	0.9431
	Amplitude (counts)	22.37±2.039	33.32±2.855	0.0233 *
AG	Period (h)	22±0.4155	20.67±1.014	0.1821
	Amplitude (counts)	5.882±1.914	16.16±7.113	0.1004
SCN	Period (h)	23.47±0.1764	23.88±0.273	0.311
	Amplitude (counts)	11.4±2.262	11.82±2.781	0.9212

The aorta, mesentery artery (MA), kidney, liver, white adipose tissues (WAT), thymus, lung, adrenal gland (AG), and suprachiasmatic nucleus (SCN) were isolated from the

db/db-mPer2^{Luc} and *db/+-mPer2*^{Luc} control mice and cultured in organ culture. The mPer2 bioluminescence was monitored and recorded by the LumiCycle system. The period and amplitude of these tissues were analyzed by using the LumiCycle analysis software. *: $p<0.05$; **: $p<0.01$

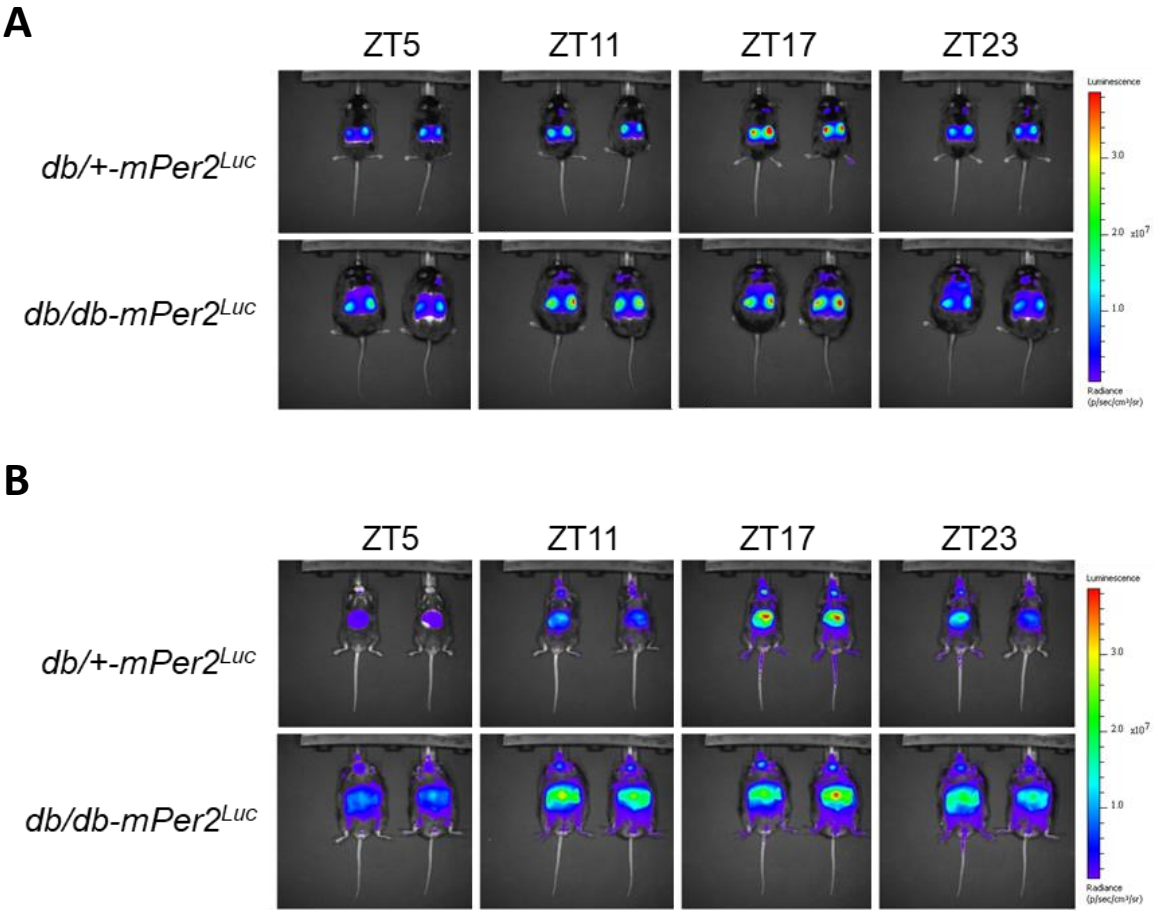
3.1.5 *In vivo* imaging verifies that the phase of mPer2 oscillation is also advanced in the kidney, liver, and submandibular gland (SG) in the *db/db-mPer2^{Luc}* mice

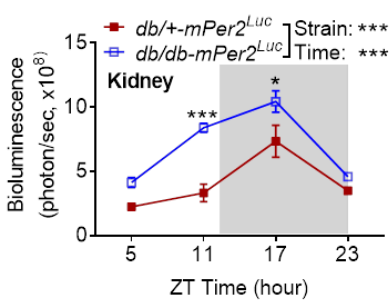
To investigate whether the phase advance of the mPer2 oscillation observed in explant tissue culture manifests the *in vivo* tissue oscillation, we used IVIS spectrum and monitored the mPer2 oscillations of the kidney, liver, and SG by IVIS spectrum in the intact *db/db-mPer2^{Luc}* and control mice. The in vivo mPer2 bioluminescence images were obtained with 6 hours interval at ZT5, ZT11, ZT17, and ZT23, respectively. In accordance with the result from the *ex vivo* LumiCycle recording (Figure 3.1.11C and 3.1.11D), the *in vivo* mPer2 bioluminescence of the kidney, liver, and SG exhibited apparent time-of-day variations. The lowest absolute bioluminescence intensity was detected at ZT5 and the highest absolute bioluminescence intensity was detected at ZT17 in all three tissues (Figure 3.1.12A and 3.1.12B). The absolute bioluminescence intensities were significantly higher in the *db/db-mPer2^{Luc}* mice as compared to the control mice at ZT11 and ZT17 in the kidney (Figure 3.1.12C), at ZT11, ZT17, and ZT23 in the liver (Figure 3.1.12F) and at ZT17 in the SG (Figure 3.1.12I).

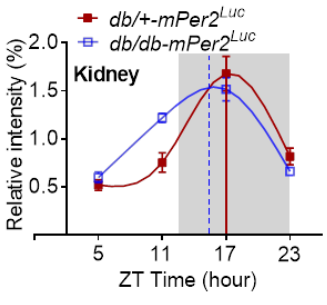
To better quantify the mPer2 oscillation in all three tissues between the two mouse strains, we normalized the absolute mPer2 bioluminescence intensities to the average of the four ZT time points absolute mPer2 bioluminescence intensities, in accordance with a previous report (Tahara, Kuroda et al. 2012). The resulting analysis revealed that the relative mPer2 bioluminescence signal from the *db/db-mPer2^{Luc}* mice peaked earlier in all three tissues than those of the control mice (Figure 3.1.12D, 3.1.12G, and 3.1.12J).

Moreover, cosinor analysis further illustrated that the phase of the mPer2 oscillation was significantly advanced in all three tissues in the *db/db-mPer2*^{Luc} mice compared with that in control mice, with 2.60 ± 0.82, 1.54 ± 0.59, and 1.571 ± 0.61 hour advance in the kidney, liver, and SG (Figure 3.1.12E, 3.1.12H, and 3.1.12K), respectively.

Figure 3.1.12(1)







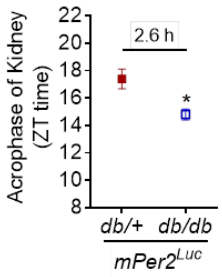


Figure 3.1.12(2)

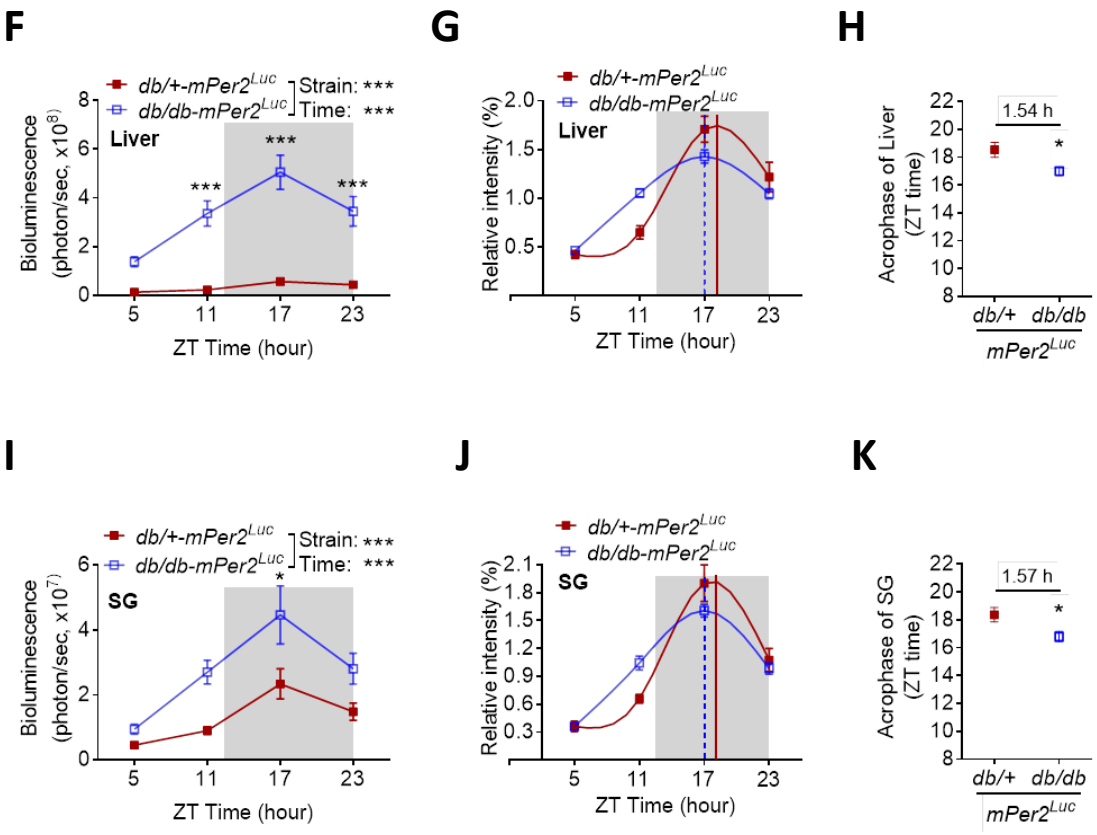


Figure 3.1.12 The in vivo imaging shows a phase shift in the kidney, liver, and submandibular gland (SG) in the *db/db-mPer2^{Luc}* mice. The in vivo imaging of mPer2 bioluminescence by the IVIS spectrum show a time-of-day variation in the kidney, liver, and SG. (**A**) Representative in vivo imaging of the mPer2 bioluminescence in the kidney in the *db/db-mPer2^{Luc}* (upper panel) and control mice (lower panel). (**B**) Representative in vivo imaging of the mPer2 bioluminescence in the SG and liver in the *db/db-mPer2^{Luc}* (upper panel) and control mice (lower panel). The absolute bioluminescence intensity detected in the kidney (**C**), liver (**F**), and SG (**I**). The relative bioluminescence intensity obtained by normalizing to the average of the four-time points' data in the kidney (**D**), liver (**G**), and SG (**J**). The brown color solid vertical line indicates the acrophase of the control *db/+-mPer2^{Luc}* mice, whereas the blue dotted vertical line indicates the acrophase of the *db/db-mPer2^{Luc}* mice. The acrophase of the two strains of mice in the kidney (**E**), liver (**H**), and SG (**K**) where the number above the symbol indicates the difference of the acrophase between the two strains of mice. All data were expressed as mean ± SEM (n = 4-5). *, P < 0.05; ***, P < 0.001.

3.1.6 Altered time-of-day variations of gene expressions in the mesenteric arteries from the *db/db-mPer2^{Luc}* mice

Db/db mice exhibit alterations in the daily mRNA expressions of clock and clock-controlled genes that are involved in the regulation of BP, as we have previously shown (Su, Xie et al. 2012). In addition, we have demonstrated that smooth muscle BMAL1 participates in the control of the BP daily rhythm by regulating one of the contraction regulatory proteins Rho-kinase 2 (ROCK2) in wild-type mice (Xie, Su et al. 2015). To test whether any putative clock-controlled BP-associated genes are dysregulated in *db/db-mPer2^{Luc}* mice, we determined mRNA expressions of Bmal1 and several contractile regulatory genes in the MA at ZT5 and ZT17. As shown in Figures 3.1.13A through 3.1.13I, Bmal1, ROCK1, calponin-1, tropomyosin-2, and smooth muscle protein-22α (SM22α) mRNA expression exhibited a significant time-of-day variation. Importantly, an attenuation or loss of the time-of-day variations was found in the *db/db-mPer2^{Luc}* mice compared with the control mice. In contrast, no time-of-day variations were detected in ROCK2, calponin-2, calponin-3, and tropomyosin-1 mRNA in either genotype (Figure 3.1.13C, 3.1.13E, 3.1.13F, and 3.1.13G).

Figure 3.1.13

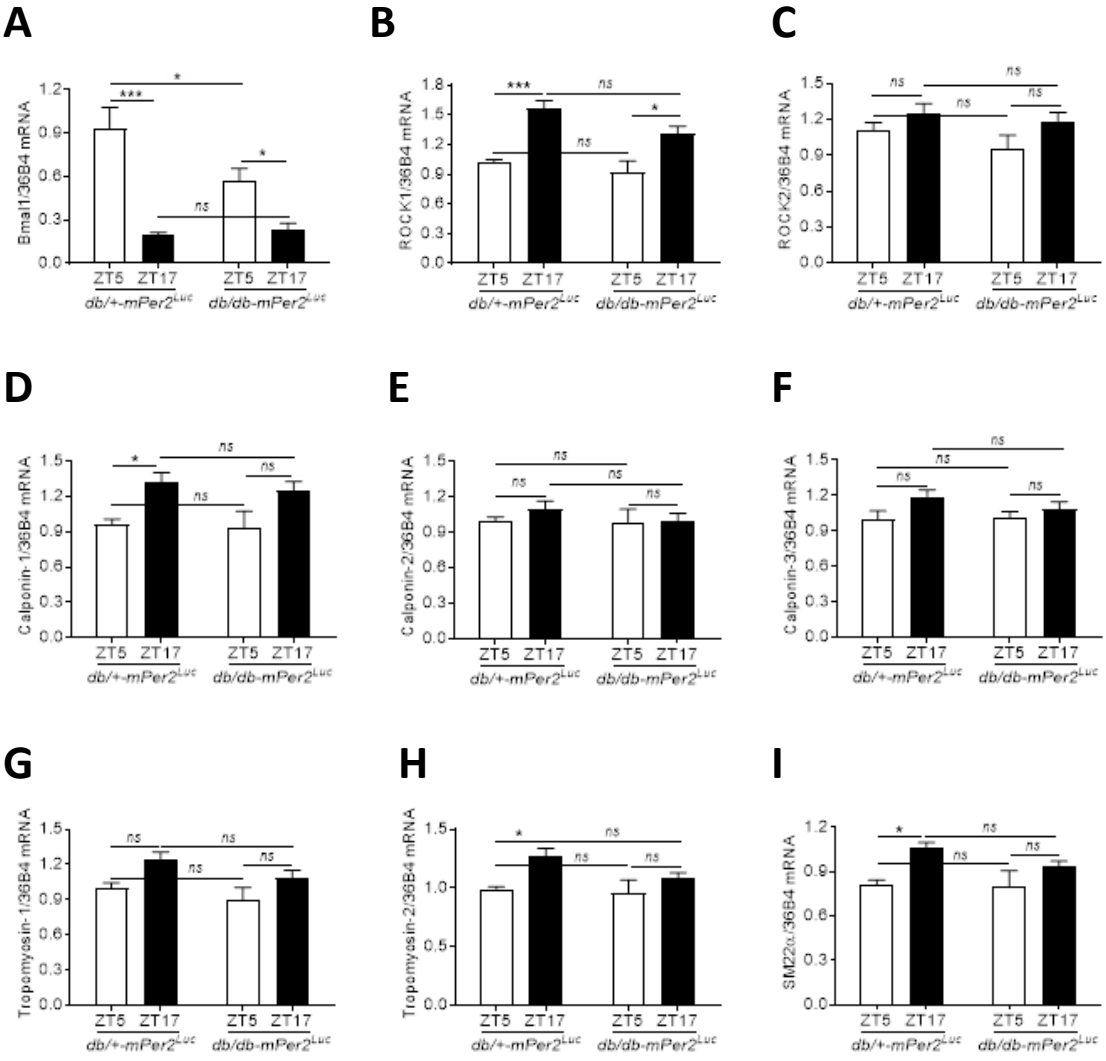


Figure 3.1.13 Altered time-of-day variations of gene expressions in the mesenteric arteries from the *db/db-mPer2^{Luc}* mice. Control and *db/db-mPer2^{Luc}* mice were euthanized at ZT5 and ZT17 and mesenteric arteries were harvested. Mesenteric arteries mRNAs were quantified using real-time PCR. (**A**) Bmal1. (**B**) ROCK1. (**C**) ROCK2. (**D**) calponin-1. (**E**) calponin-2. (**F**) calponin-3. (**G**) tropomyosin-1. (**H**) tropomyosin-2. (**I**) smooth muscle protein 22-α (SM22α). n = 4-5 for each mouse strain at each time point. Data were analyzed by 2-way ANOVA. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ns, not significant.

3.2 Project 2: Active-Time Restricted Feeding Restores Blood Pressure Circadian Rhythm via Autonomic Nervous System in Type 2 Diabetic *db/db* Mice

(Manuscript in preparation)

Authors: Tianfei Hou, Wen Su, Chanuang Wang, Shreyas Joshi, Bruce O'Hara, Zhenheng Guo and Ming C. Gong

3.2.1 Investigation of the effect of timing of food intake on BP circadian rhythm.

3.2.1.1 The disruption of BP daily rhythm is associated with altered food intake rhythm in the *db/db* mice.

To investigate whether the disruption of BP daily rhythm in the *db/db* mice is associated with an altered food intake rhythm, we simultaneously monitored the daily rhythms of BP and food intake using radiotelemetry and Bio-DAQ system in 12-week-old freely moving control and *db/db* mice. Consistent with previous studies (Su, Guo et al. 2008) : the control mice exhibited apparent 24-hour oscillation in the mean arterial pressure (MAP); whereas the *db/db* mice showed diminished daily rhythm in the MAP, mainly due to a reduced MAP fall during the light-phase (Fig. 3.2.1.1A). The 12-hour average of MAP during the light- and dark-phase showed that although the levels of MAP during both the light- and dark-phase were higher in the *db/db* mice than those of the control mice, the extent of increase from *db/db* mice compared to control mice in the light-phase MAP was greater than that of the dark-phase MAP, resulting in diminished difference between the light- and dark-phase in the MAP of the *db/db* mice compared to that of the control mice (Fig. 3.2.1.1B).

The food intake was monitored at 1-min interval using the Bio-DAQ system. As illustrated in figure 3.2.1.1C and 3.2.1.1D, the food consumption in the control mice was concentrated during the dark-phase, while only sporadic consumption occurred during the light-phase; however, the food consumption in the *db/db* mice was distributed throughout the whole day. When calculating the accumulative food intake during the light- and dark-phase, the *db/db* mice consumed more food during both the light- and dark- phase than that of the control mice (Fig 3.2.1.1E). In addition, a significant higher percent of food out of the daily food intake was consumed during the light-phase in the *db/db* mice compared to that of the control mice (11.3$\pm$4.2% in the control mice *vs.* 36.0$\pm$2.9% in the *db/db* mice, $p<0.0001$).

Figure 3.2.1.1

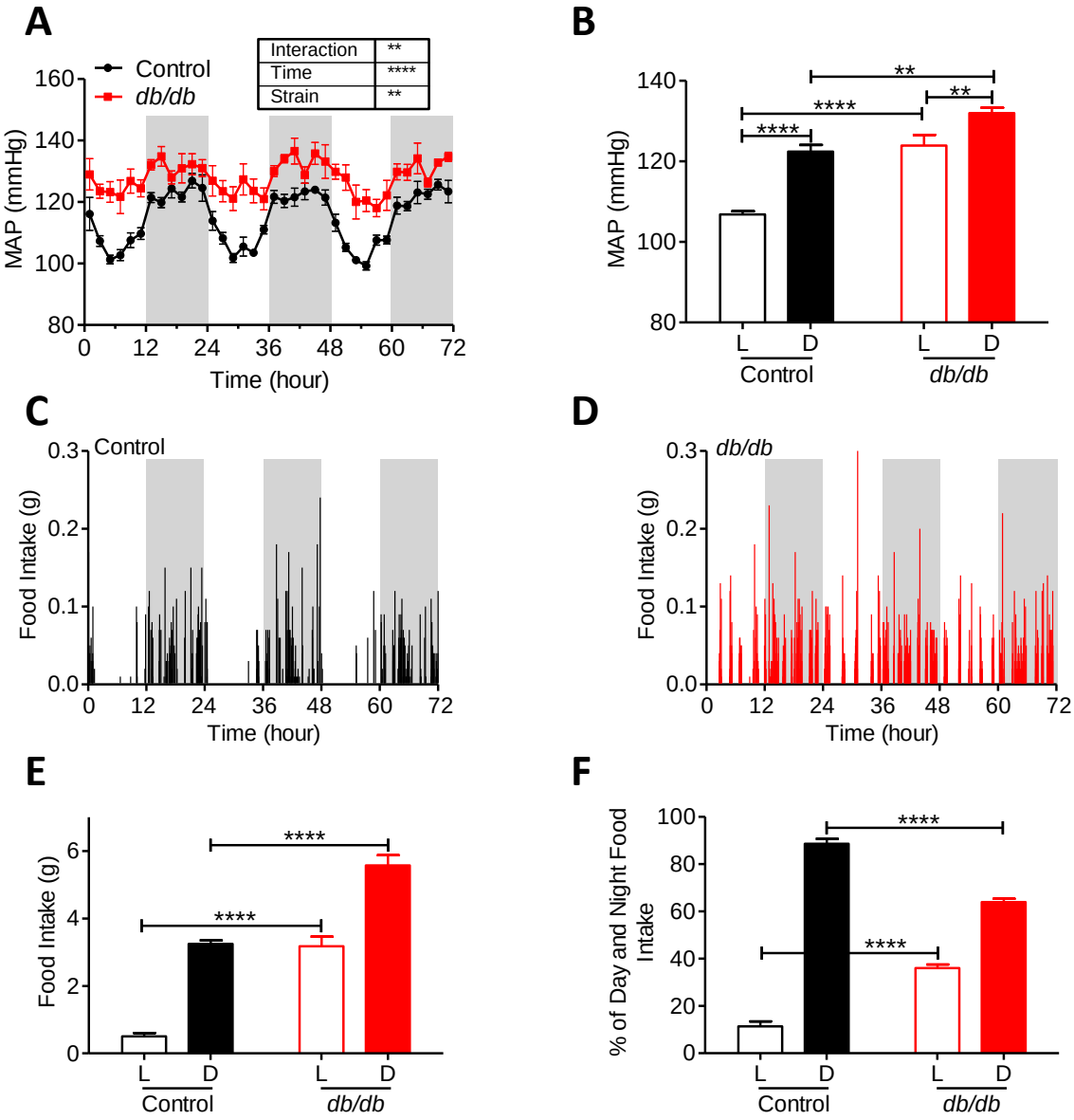


Figure 3.2.1.1 The disruption of blood pressure daily rhythm is associated with altered food intake rhythm in the *db/db* mice. **(A)** 2-hour average mean arterial pressure (MAP) in the control and *db/db* mice. The light grey box indicates the dark-phase. **(B)** 12-hour average MAP during the light-phase (L) and dark-phase (D). **(C and D)** Representative figures of food intake collected at 1-min interval using the Bio-DAQ system in the control **(C)** and *db/db* **(D)** mice. **(E)** Food intake during the light- (L) and dark- (D) phase. **(F)** Percents of food intake during the light- (L) and dark- phase (D). n=4. **, p<0.01; ****, P<0.0001.

3.2.1.2 Inactive-time restricted feeding (ITRF) altered the BP daily rhythm in the healthy mice.

To investigate whether the alternation of food intake daily rhythm contributes to the disruption of BP daily rhythm, we fed the healthy C57BL/6J mice, whose BP daily rhythm is normal, on an ITRF regimen, in which the food was only available between ZT2 and ZT12 (i.e., during the inactive-phase (light-phase)). As shown in figure 3.2.1.2A, the MAP daily rhythm was apparent in the healthy C57BL/6J mice at baseline. After 4 days of ITRF, although the MAP started to rise at the beginning of the dark-phase, it was not sustained at that level during the late dark-phase and dropped to the level that was similar to the light-phase MAP. In agreement with that, the dark-phase MAP after ITRF was significantly lower than that at baseline (Fig. 3.2.1.2B). Cosine analysis of the MAP oscillation revealed the amplitude and robustness were significantly decreased after ITRF (Fig. 3.2.1.2C and 3.2.1.2D). In addition, the acrophase of MAP oscillation was advanced (Fig. 3.2.1.2E).

The effect of ITRF on the level and daily rhythm of SBP was similar to that on the MAP (Fig. 3.2.1.3A-3.2.1.3D), except the acrophase of SBP was not significantly altered after ITRF (Fig. 3.2.1.3E). Interestingly, besides the similar effects of ITRF on the level of dark-phase and the oscillation of DBP as those of the SBP (Fig. 3.2.1.3F-3.2.1.3J), ITRF also significantly increased the DBP level during the light-phase (Fig. 3.2.1.3F and 3.2.1.3G).

Figure 3.2.1.2

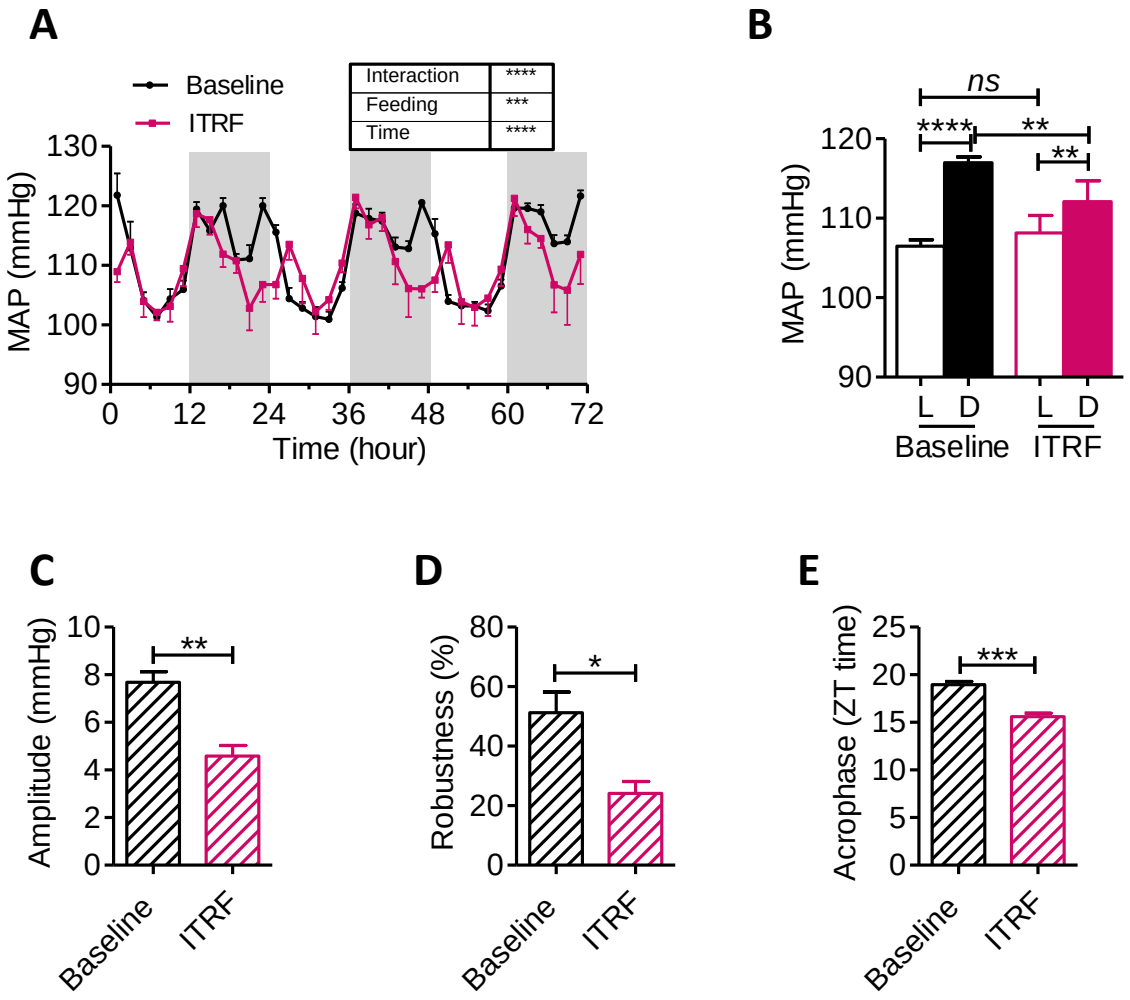


Figure 3.2.1.2 Inactive-time restricted feeding (ITRF) altered the MAP daily rhythm in the healthy mice. (A) 2-hour average MAP in the C57BL/6J mice at baseline and after 4 days of ITRF. The light grey box indicates the dark-phase. **(B)** 12-hour average MAP during the light-phase (L) and dark-phase (D). **(C-E)** The amplitude **(C)**, robustness **(D)** and acophase **(E)** of MAP oscillation. n=6. *, p<0.05; **, p<0.01; ***, p<0.001; ****, P<0.0001; ns, not significant.

Figure 3.2.1.3(1)

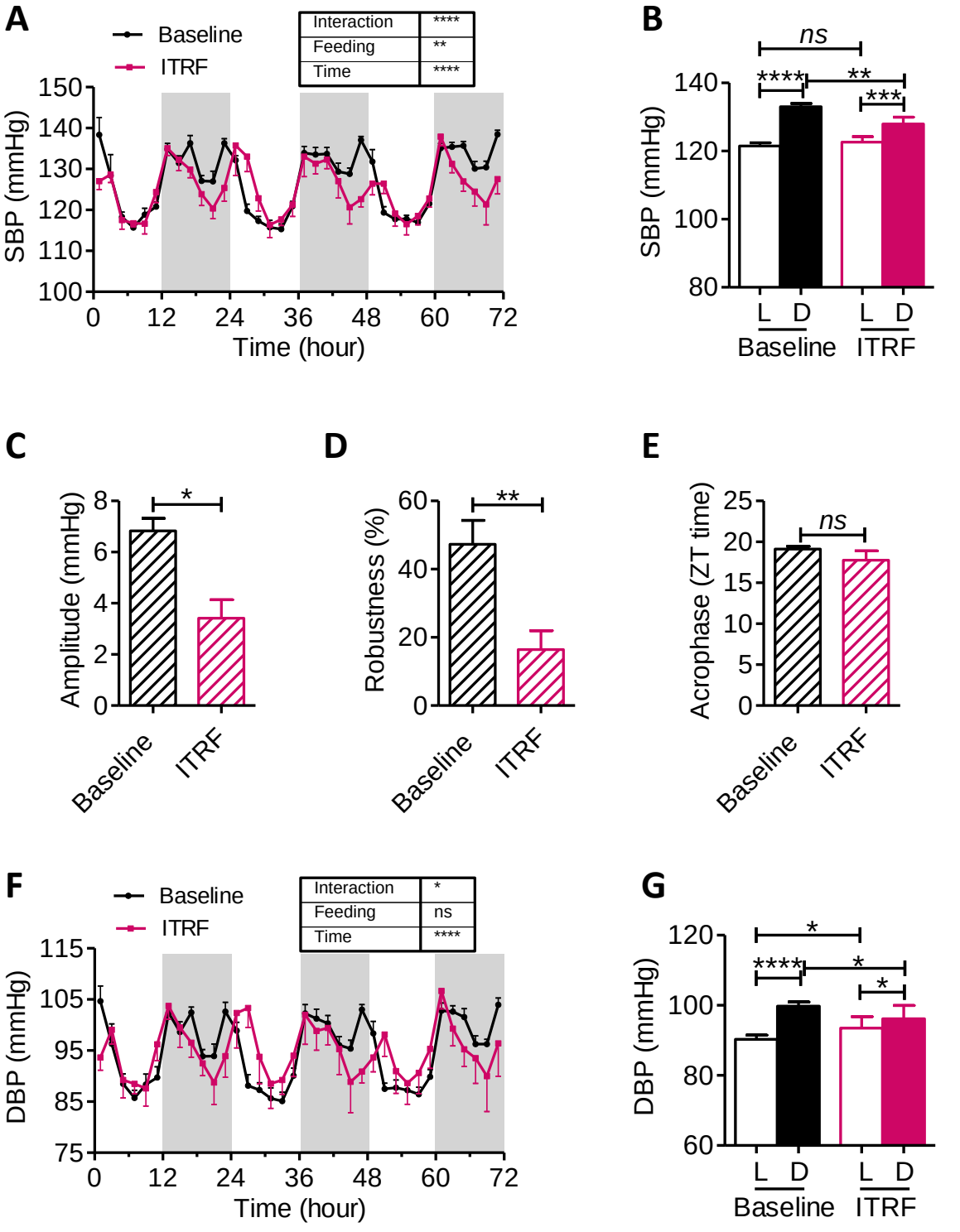


Figure 3.2.1.3(2)

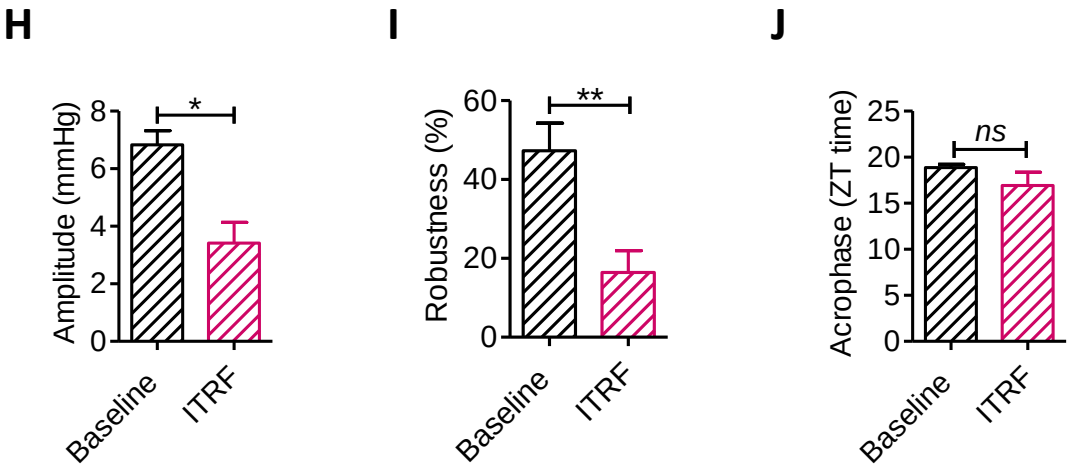


Figure 3.2.1.3 ITRF altered the systolic blood pressure (SBP) and diastolic blood pressure (DBP) daily rhythm in the healthy mice. (A and F) 2-hour average SBP **(A)** and DBP **(F)** in the C57BL/6J mice at baseline and after 4 days of ITRF. The light grey box indicates the dark-phase. **(B and G)** 12-hour average SBP **(B)** and DBP **(G)** during the light-phase (L) and dark-phase (D). **(C-E and H-J)** The amplitude, robustness and acrophase of SBP **(C-E)** and DBP **(H-J)** oscillation. n=6. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$; ns, not significant.

3.2.2 Determination of whether active-time restricted feeding (ATRF) restores BP circadian rhythm in diabetes.

3.2.2.1 ATRF prevented the disruption of BP daily rhythm in the *db/db* mice

To investigate whether ATRF prevents the disruption of BP daily rhythm, we fed the *db/db* and control *db/+* mice on an ATRF regimen, in which the food was only available between ZT13 and ZT21 during the active-phase (dark-phase). The ATRF was started at the age of 6-weeks-old when the normal BP daily rhythm was preserved in the *db/db* mice, as the disruption of BP daily rhythm in the male *db/db* mice begins around 12-weeks of age (Senador, Kanakamedala et al. 2009). As a control for the ATRF feeding paradigm, a group of age-matched *db/db* and control mice received *ad libitum* feeding (ALF). The BP was measured after 10-weeks of ATRF or ALF. As shown in Figure 3.2.2.1A and 3.2.2.1B, the *ad libitum* fed-control mice (Ctrl-ALF) exhibited 24-hour oscillation in mean arterial pressure (MAP): the MAP is lower during the light-phase than that during the dark-phase. In contrast, the *ad libitum* fed *db/db* mice (*db/db*-ALF) exhibited a non-dipping BP pattern: the decrease of MAP during the light-phase was severely compromised when compared with the control mice. ATRF had no apparent effect on the MAP in the control mice (Ctrl-ATRF vs. Ctrl-ALF), but effectively prevented the disruption of BP daily rhythm in the *db/db* mice (*db/db*-ATRF vs. *db/db*-ALF). As shown in Figure 3.2.2.1C, the MAP was increased in the *db/db*-ALF mice compared to the control mice during both the light- and dark-phase, but the extent of increase was larger during the light-phase than during the dark-phase, which resulted in a non-dipping

phenotype in the *db/db* mice. The ATRF selectively reduced the MAP during the light-phase thus preserved the normal MAP dipping in the *db/db*-ATRF mice (Fig. 3.2.2.1C).

Cosine analysis of the MAP daily oscillation revealed that the oscillation amplitude (Fig. 3.2.2.1D) and robustness (Fig. 3.2.2.1E) were significantly decreased in the *db/db*-ALF mice compared to the Ctrl-ALF mice. Importantly, the ATRF fed *db/db* mice maintained near normal oscillation amplitude and robustness as in the control mice (Fig. 3.2.2.1D and 3.2.2.1E). In contrast, the oscillation acrophase was not significantly altered in the *db/db*-ALF mice and ATRF did not modify it significantly (Fig. 3.2.2.1F).

ATRF had similar effects on the daily oscillations of systolic blood pressure (SBP) (Fig. 3.2.2.2A-3.2.2.2E) and diastolic blood pressure (DBP) (Fig. 3.2.2.2F-3.2.2.2J) as observed in the MAP (Fig. 3.2.2.1).

Figure 3.2.2.1

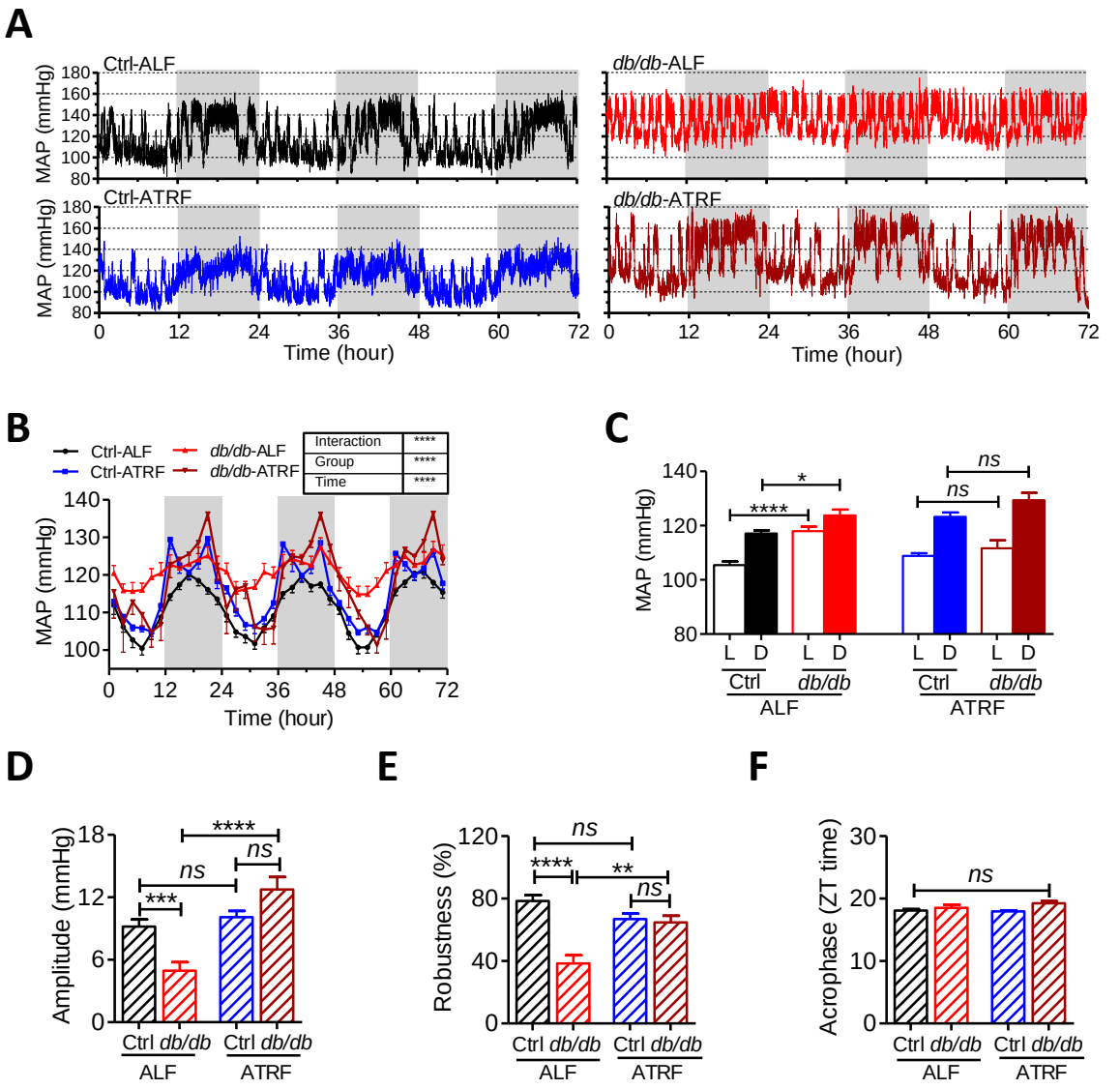


Figure 3.2.2.1 Active-time restricted feeding (ATRF) prevented *db/db* mice from the disruption of mean arterial pressure (MAP) daily rhythm. (A) Continuous 72-hour MAP in the control and *db/db* mice with *ad libitum* feeding (ALF) or ATRF. The grey box indicates the dark-phase. **(B)** 2-hour average MAP in the control and *db/db* mice with ALF or ATRF. **(C)** 12-hour average MAP during the light-phase (L) and dark-phase (D). **(D-F)** The amplitude **(D)**, robustness **(E)** and acrophase **(F)** of MAP oscillation. Ctrl-ALF: n=13; Ctrl-ATRF: n=12; *db/db*-ALF: n=12; *db/db*-ATRF: n=5. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001; ns, not significant.

Figure 3.2.2.2(1)

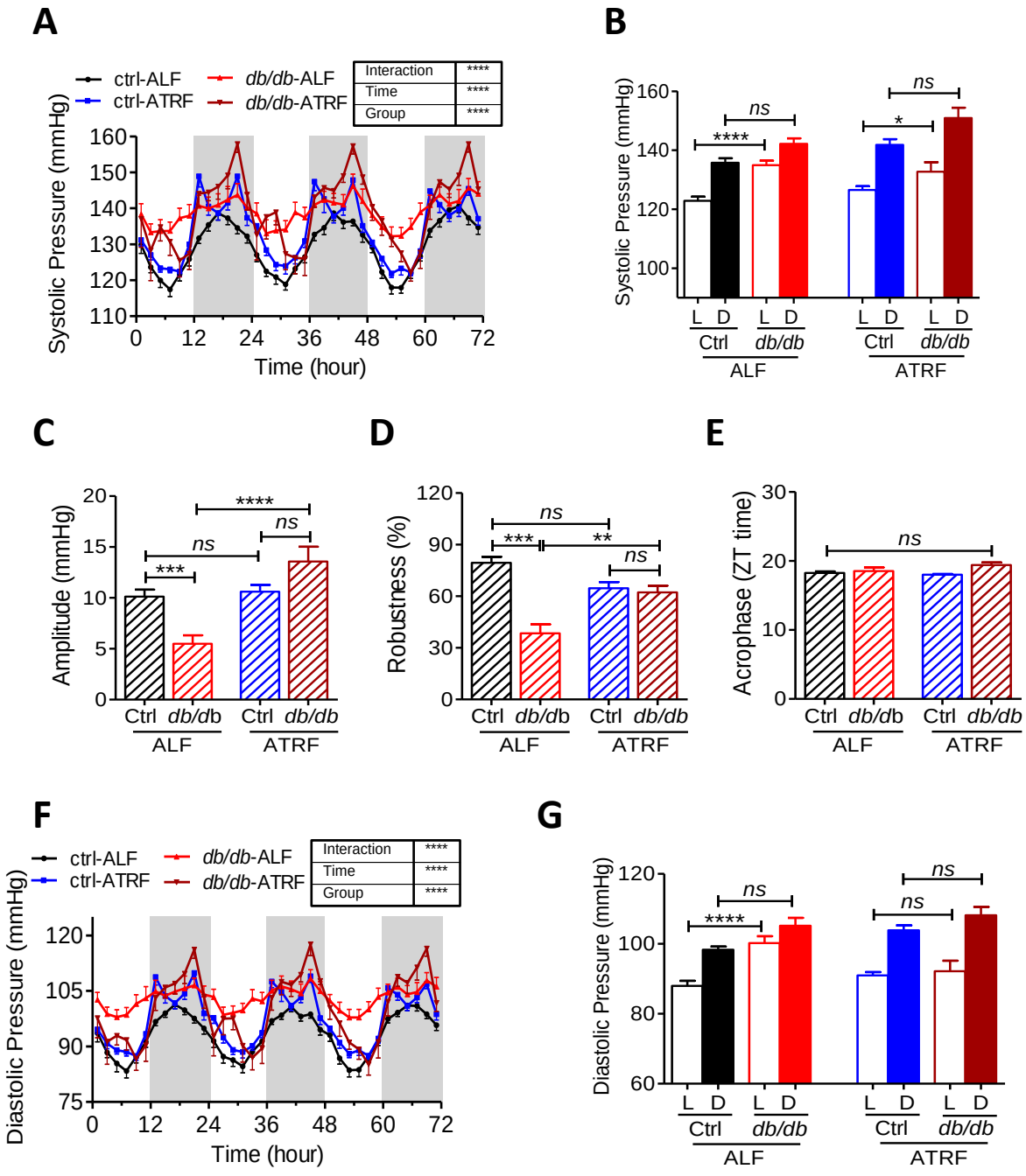


Figure 3.2.2.2(2)

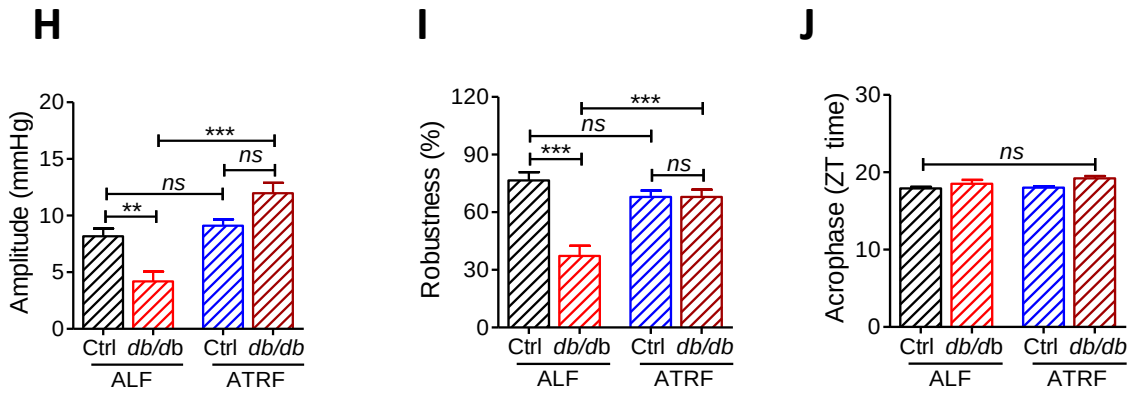


Figure 3.2.2.2 ATRF prevented the disruption of systolic blood pressure (SBP) and diastolic blood pressure (DBP) daily rhythm in the *db/db* mice. (A and F) 2-hour average SBP **(A)** and DBP **(F)** in the control and *db/db* mice with ALF or ATRF. The grey box indicates the dark-phase. **(B and G)** 12-hour average SBP **(B)** and DBP **(G)** during the light-phase (L) and dark-phase (D). **(C-E and H-J)** The amplitude, robustness and acrophase of SBP **(C-E)** and DBP **(H-J)** oscillation. Ctrl-ALF: n=13; Ctrl-ATRF: n=12; *db/db*-ALF: n=12; *db/db*-ATRF: n=5. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001; ns, not significant.

3.2.2.2 ATRF restored the already disrupted BP daily rhythm in the *db/db* mice

To test whether ATRF can restore the already disrupted BP daily rhythm, which is highly relevant to human situations, we administered ATRF to 16-week-old *db/db* mice when their BP daily rhythm is severely disrupted. BP was recorded at baseline with ALF and after 9 days of ATRF. As expected, *db/db* mice lost the normal MAP daily oscillation at baseline (Fig. 3.2.2.3A and 3.2.2.3B). Importantly, ATRF restored the MAP daily oscillation mainly by reducing the light-phase MAP (Fig. 3.2.2.3A and 3.2.2.3B). Cosine analysis demonstrated that the amplitude (Fig. 3.2.2.3C) and robustness (Fig. 3.2.2.3D) of the *db/db* mice MAP oscillation were increased after ATRF. Moreover, ATRF had similar effects on the SBP (Fig. 3.2.2.4A-3.2.2.4E) and DBP (Fig. 3.2.2.4F-3.2.2.4J) as that on the MAP (Fig. 3.2.2.3).

Figure 3.2.2.3

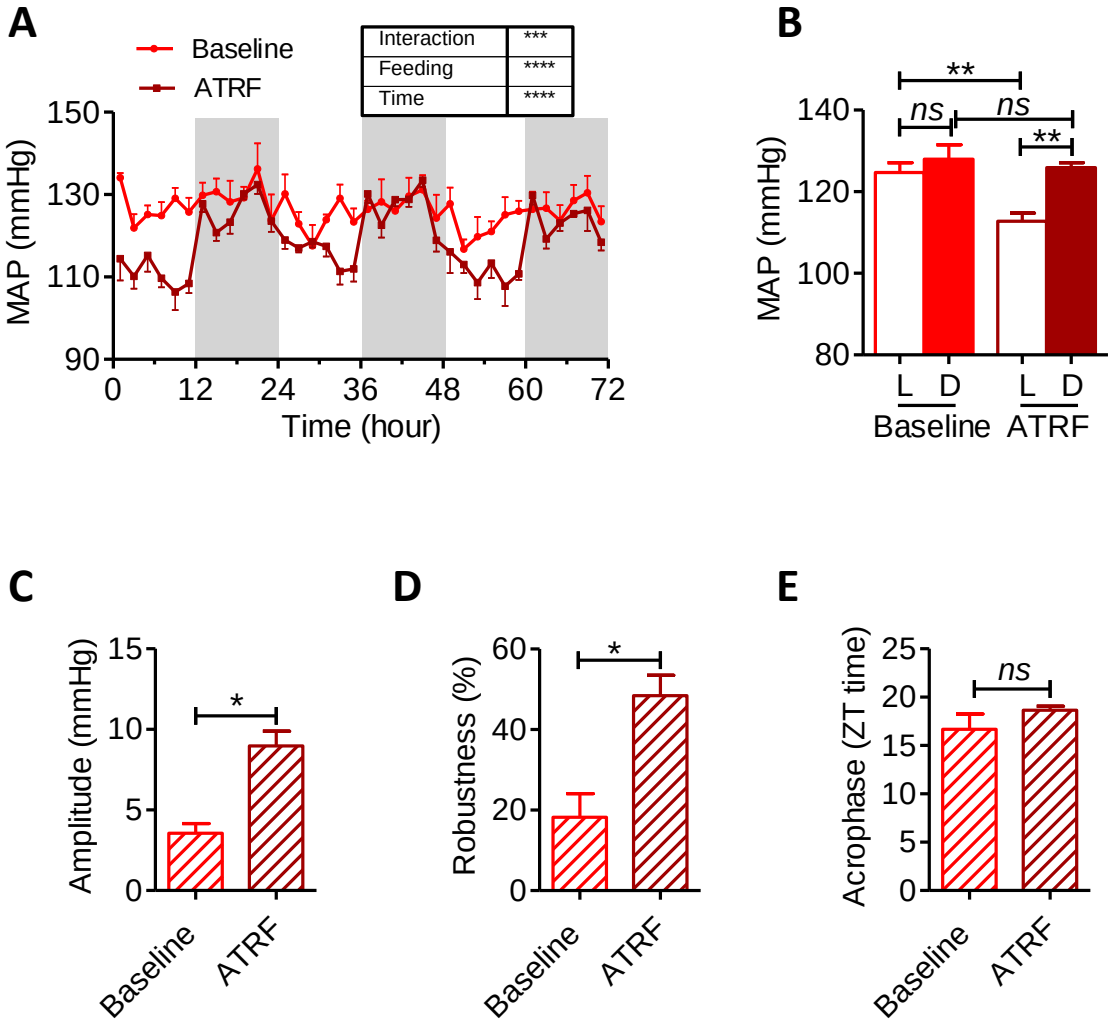


Figure 3.2.2.3 ATRF restored the disrupted MAP daily rhythm in the *db/db* mice. (A) 2-hour average MAP in the *db/db* mice at baseline and after 9 days of ATRF. **(B)** 12-hour average MAP during the light-phase (L) and dark-phase (D). **(C-E)** The amplitude **(C)**, robustness **(D)** and acrophase **(E)** of MAP oscillation. n=5. *, p<0.05; **, p<0.01; ***, p<0.001; ****, P<0.0001; ns, not significant.

Figure 3.2.2.4(1)

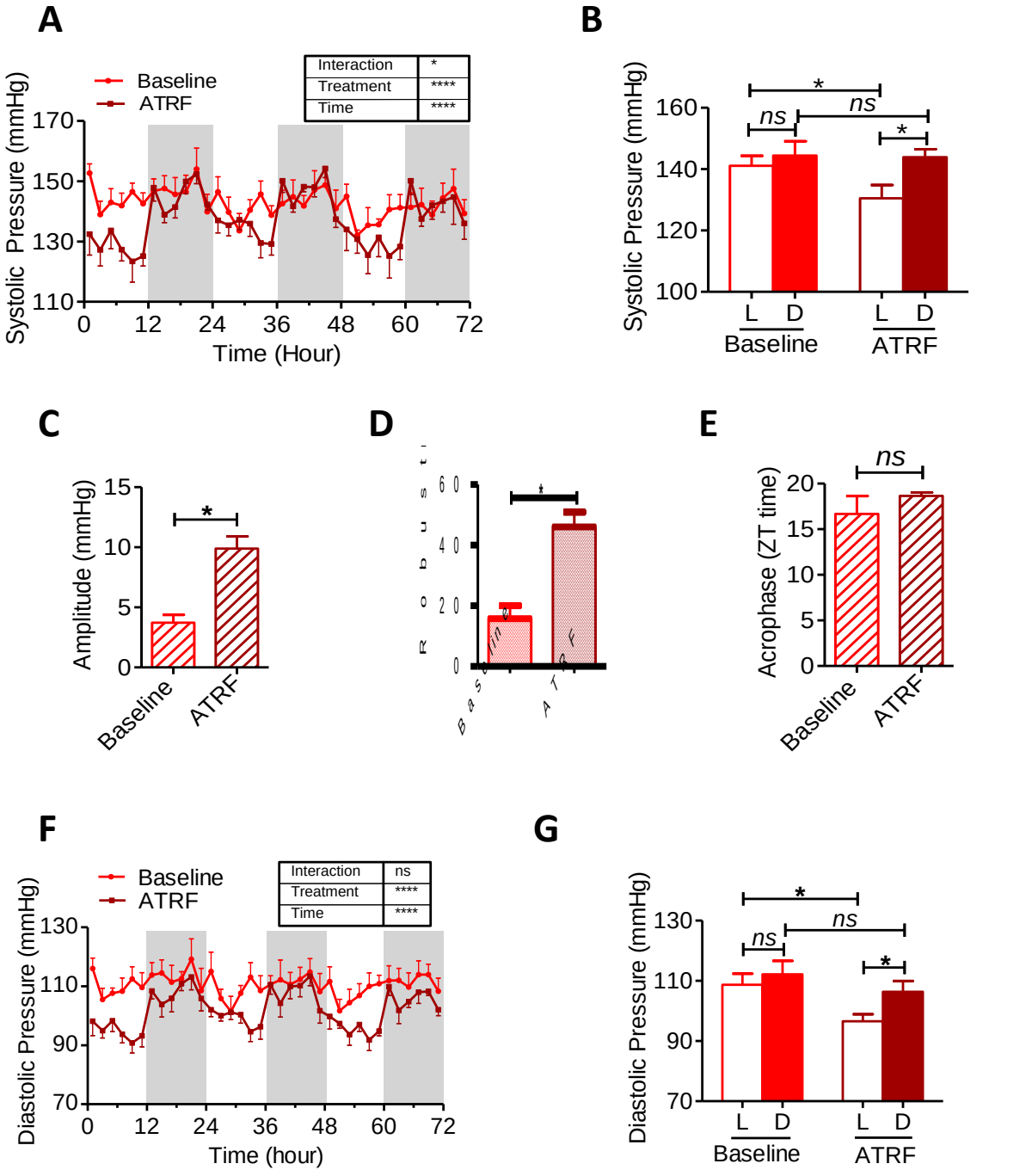


Figure 3.2.2.4(2)

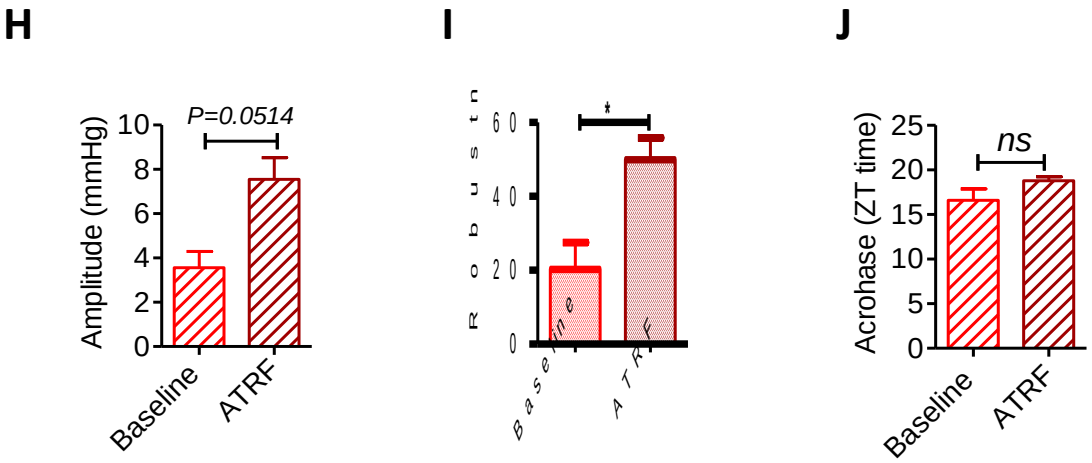


Figure 3.2.2.4 ATRF restored the disrupted daily rhythms of SBP and DBP in the *db/db* mice. **(A and F)** 2-hour average SBP **(A)** and DBP **(F)** in the *db/db* mice at baseline and after 9 days of ATRF. **(B and G)** 12-hour average SBP **(B)** and DBP **(G)** during the light-phase (L) and dark-phase (D). **(C-E and H-J)** The amplitude, robustness and acrophase of SBP **(C-E)** and DBP **(H-J)** oscillations. n=5. *, p<0.05; ****, p<0.0001; ns, not significant.

3.2.2.3 The effects of ATRF on the diabetic symptoms of the *db/db* mice

To investigate that in addition to the striking benefits in the BP daily rhythm, whether the ATRF improves the diabetic symptoms in the *db/db* mice, we measured the body weight, lean and fat body mass, blood glucose at ZT 13 and ZT 21 in the four groups of mice who started ATRF at 6-weeks of age. Neither the body weight (Fig. 3.2.2.5A) nor the lean- or fat-body mass (Fig. 3.2.2.5B) were altered by the 10-week-long ATRF when compared to the ALF fed groups. Of note, the body weight and fat mass remained significantly higher in the *db/db* than in the control mice throughout the entire feeding regimen (Fig. 3.2.2.5A and 3.2.2.5B). When measured at ZT13, a time point immediately after the fasting period for the ATRF fed groups; the blood glucose levels were significantly lower in the *db/db*-ATRF vs. *db/db*-ALF and in the Ctrl-ATRF vs. Ctrl-ALF groups respectively (Fig. 3.2.2.5C). In contrast, when measured at ZT21, a time point immediately after the active-phase feeding in the ATRF fed mice, the blood glucose was not different between the *db/db*-ATRF vs. *db/db*-ALF mice and between the control-ATRF vs. control-ALF mice respectively (Fig. 3.2.2.5D). Of note, the blood glucose remained at much higher levels in the *db/db* mice than in the control mice under both ATRF and ALF regimens (Fig. 3.2.2.5C and 3.2.2.5D).

We also investigated whether the recovery of BP daily rhythm in 16-week-old mice by ATRF is associated with improvements of body weight, body composition and blood glucose, insulin sensitivity, plasma insulin and non-esterified fatty acid (NEFA) and total cholesterol. The results demonstrated no significant improvements in the body weight,

body composition or fasting and non-fasting blood glucose (Fig. 3.2.2.5E-3.2.2.5H). For insulin sentivity, we carried out an intraperitoneal insulin tolerance test at ZT1 after 4-hour fasting. While insulin induced prompt blood glucose decrease in the control mice, there was a minimal decrease in the blood glucose of the *db/db* mice, indicating a severe insulin resistance in the *db/db* mice (Fig. 3.2.2.5I and 3.2.2.5J). Importantly, ATRF did not improve the insulin sensitivity in the *db/db* or the control mice (Fig. 3.2.2.5I and 3.2.2.5J). Plasma insulin, NEFA and total cholesterol were significantly higher in the *db/db*-ALF than in the Ctrl-ALF mice (Fig. 3.2.2.5K-3.2.2.5M). While ATRF significantly decreased plasma insulin and total cholesterol levels in the control mice (Ctrl-ALF vs. Ctrl-ATRF), it had no significant effects on the plasma NEFA levels in either the control or *db/db* mice, nor on insulin or total cholesterol levels in the *db/db* mice (*db/db*-ALF vs. *db/db*-ATRF) (Fig. 3.2.2.5K-3.2.2.5M).

Figure 3.2.2.5(1)

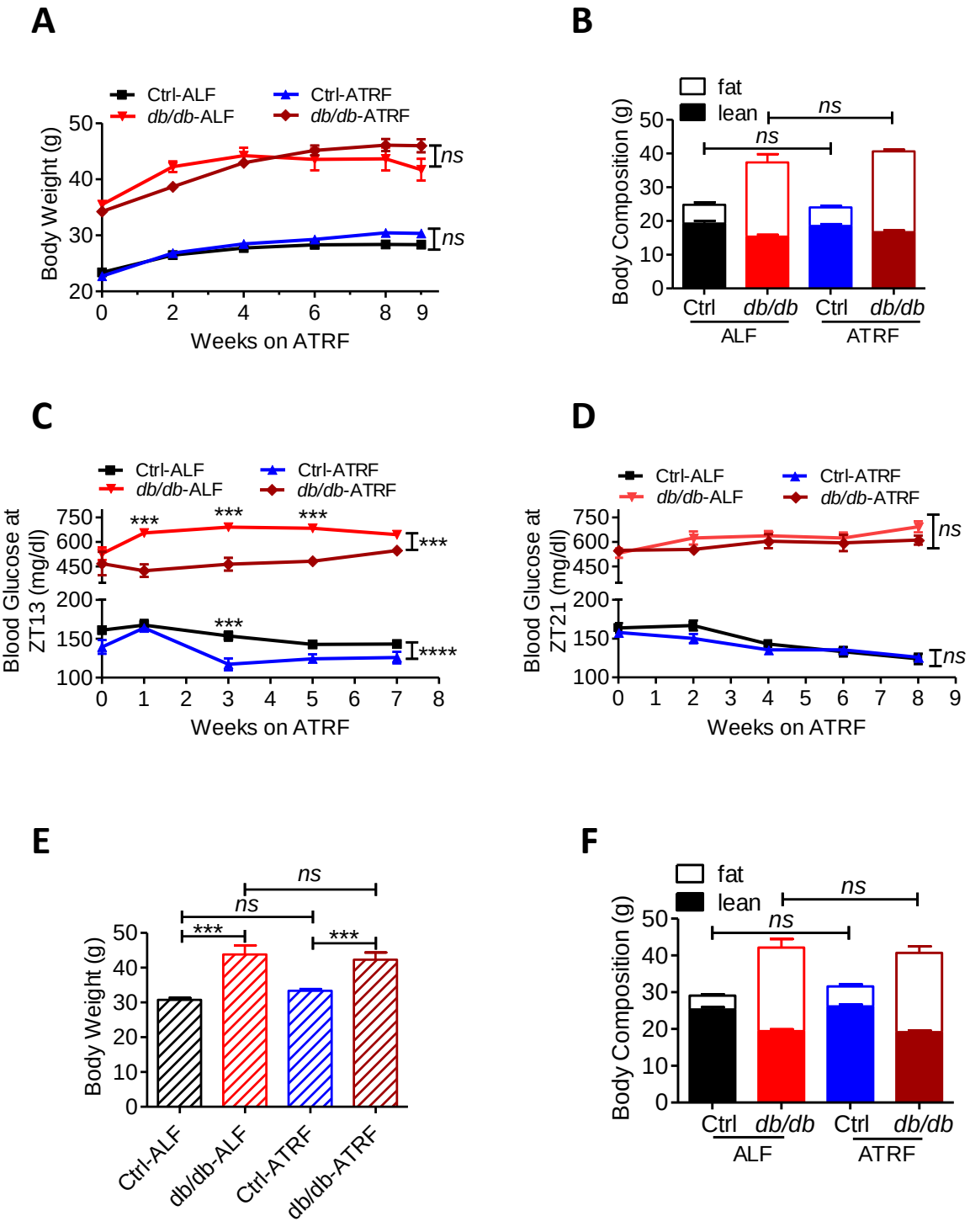


Figure 3.2.2.5(2)

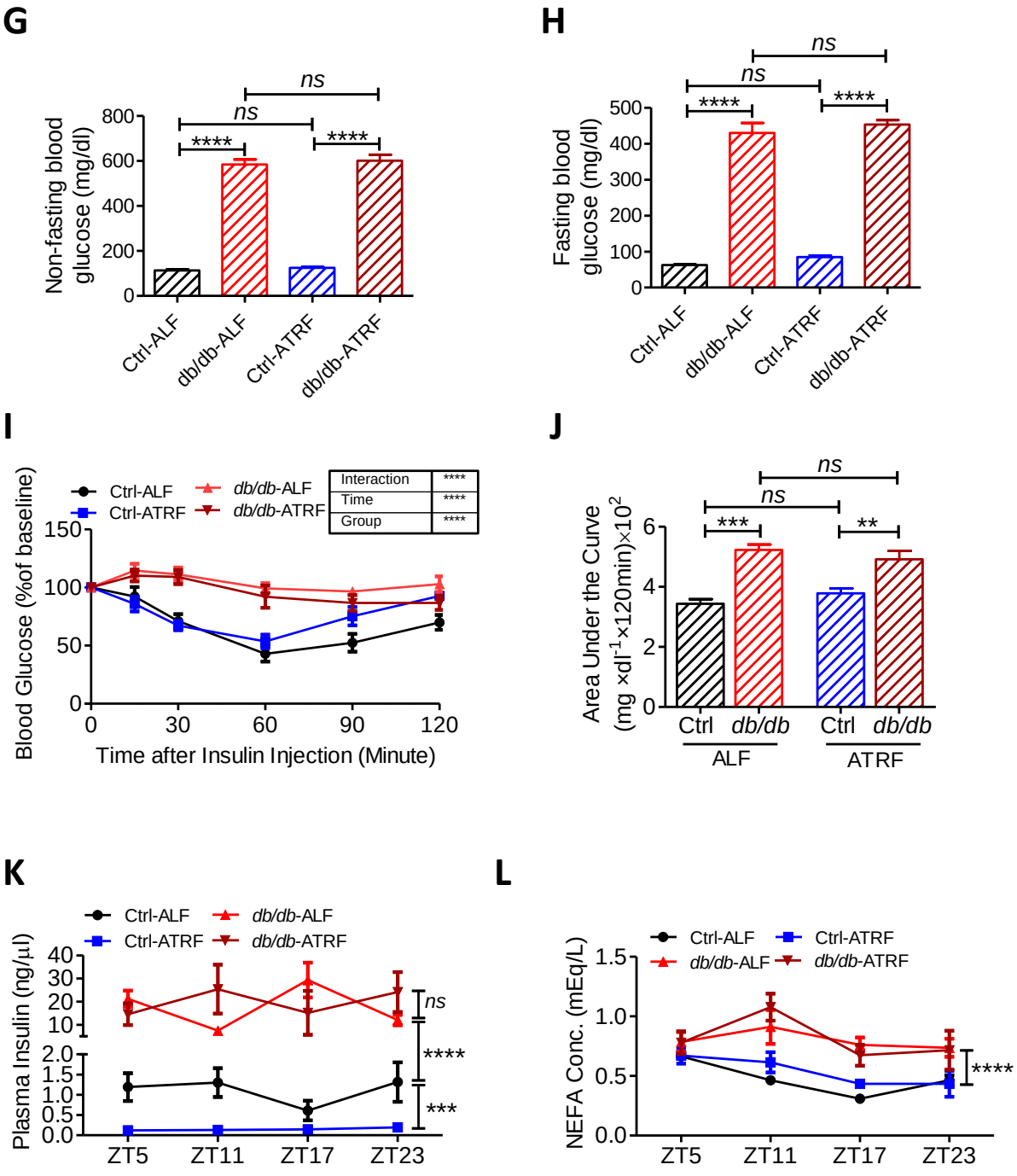


Figure 3.2.2.5(3)

M

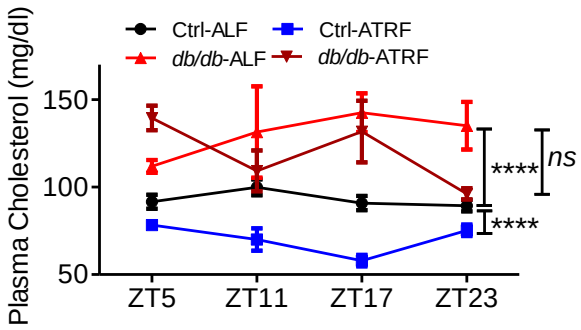


Figure 3.2.2.5 The effects of ATRF on the diabetic symptoms of the *db/db* mice. (A-D) data collection in the mice started with ATRF at 6-week-old. **(A)** Body weight; Ctrl-ALF: n=10; Ctrl-ATRF: n=14; *db/db*-ALF: n=8; *db/db*-ATRF; n=17. **(B)** Body composition; n=5 in each group; **(C and D)** Blood glucose at ZT13 **(C)** and at ZT21 **(D)**; n=7-10. **(E-M)** data collected in the mice started with ATRF at 16-week-old. **(E)** Body weight; n=8-10; **(F)** Body composition; n=8-10; **(G and H)** Non-fasting blood glucose **(G)** and fasting blood glucose **(H)** measured at ZT21; Ctrl-ALF: n=3-8; Ctrl-ATRF: n=12; *db/db* groups: n=5-8; **(I)** Insulin resistance test was performed at ZT1 by i.p. injection of 1 unit/kg insulin after 4-hour fasting and the blood glucose was measured at indicated times; n=7-9; **(J)** Areas under the curve of insulin resistance test; **(K-M)** Plasma insulin **(K)**, non-esterified fatty acid (NEFA, **L**) and cholesterol **(M)**; control groups: n=4-7; *db/db* groups: n=3-5. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001; ns, not significant.

3.2.2.4 ATRF improved the daily rhythm of energy metabolism in the *db/db* mice

We had previously shown that the diabetic mice had disrupted daily rhythm in the energy metabolism (Fig 3.1.9 and 3.1.10). It is not known, however, whether ATRF improves the oscillation in the energy metabolism of the *db/db* mice. The respiratory exchange ratio (RER) and energy expenditure (EE) were recorded using indirect calorimetry in the four groups of mice which started ATRF at 6-weeks of age. The results demonstrated that the daily rhythm of RER seen in the Ctrl-ALF mice was lost in the *db/db*-ALF mice (Fig. 3.2.2.6A). ATRF induced an even more robust RER oscillation in the control mice by decreasing the light-phase RER and increasing the dark-phase RER (Fig. 3.2.2.6A and 3.2.2.6B) and established a modest RER oscillation in the *db/db* mice by decreasing the light-phase RER (Fig. 3.2.2.6A and 3.2.2.6B). Of note, the average RER during the light- and dark-phase and 24-hour day was lower in the *db/db* mice compared with the control mice in both feeding regimens (Fig. 3.2.2.6C and 3.2.2.6D). In agreement with the observed oscillation, cosine analysis revealed that the RER oscillation amplitude (Fig. 3.2.2.6E) and robustness (Fig. 3.2.2.6F) were significantly decreased in the *db/db*-ALF mice compared with the Ctrl-ALF mice. ATRF increased the oscillation amplitude and robustness in both the control and *db/db* mice (Fig. 3.2.2.6E and 3.2.2.6F). In addition, the delayed acrophase of RER oscillation in the *db/db* mice was corrected with ATRF (Fig. 3.2.2.6G). Regarding the EE, ATRF significantly decreased the mean EE in the *db/db* mice during the light phase (Fig. 3.2.2.7B and 3.2.2.7C) and consequently the 24-hour energy expenditure (Fig. 3.2.2.7D). In contrast, ATRF did not significantly modify the EE in the control mice (Fig. 3.2.2.7A to 3.2.2.7D). Cosine analysis

revealed that ATRF significantly enhanced the daily oscillations of EE amplitude (Fig. 3.2.2.7E) and robustness (Fig. 3.2.2.7F) in both the control and *db/db* mice. In addition, ATRF corrected the delayed acrophase in the daily oscillation of EE in the *db/db* mice (Fig. 3.2.2.7G).

Figure 3.2.2.6

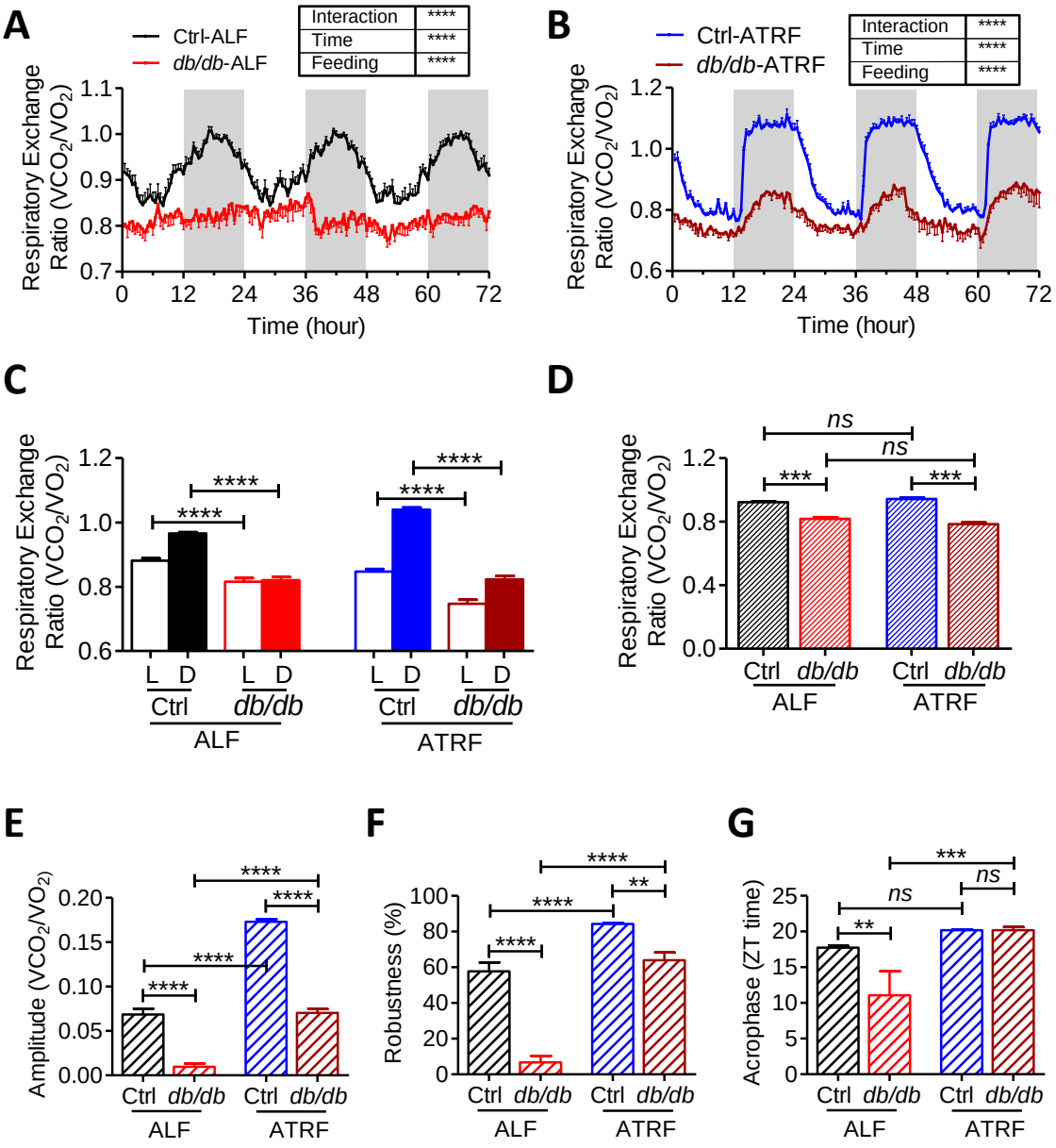


Figure 3.2.2.6 ATRF improved the daily rhythm of respiratory exchange ratio (RER) in the *db/db* mice. RER was recorded by indirect calorimetry. (**A** and **B**) The 72-hour recording of the RER in the control and *db/db* mice with ALF (**A**) or ATRF (**B**). The light grey box indicates the dark-phase. **(C)** 12-hour average RER during the light-phase (L) and dark-phase (D). **(D)** Daily RER. **(E-G)** The amplitude **(E)**, robustness **(F)** and acrophase **(G)** of RER oscillation; control groups: n=10; *db/db* groups: n=5-6. *, p<0.05; **, p<0.01; ***, p<0.001; ****, P<0.0001; ns, not significant.

Figure 3.2.2.7

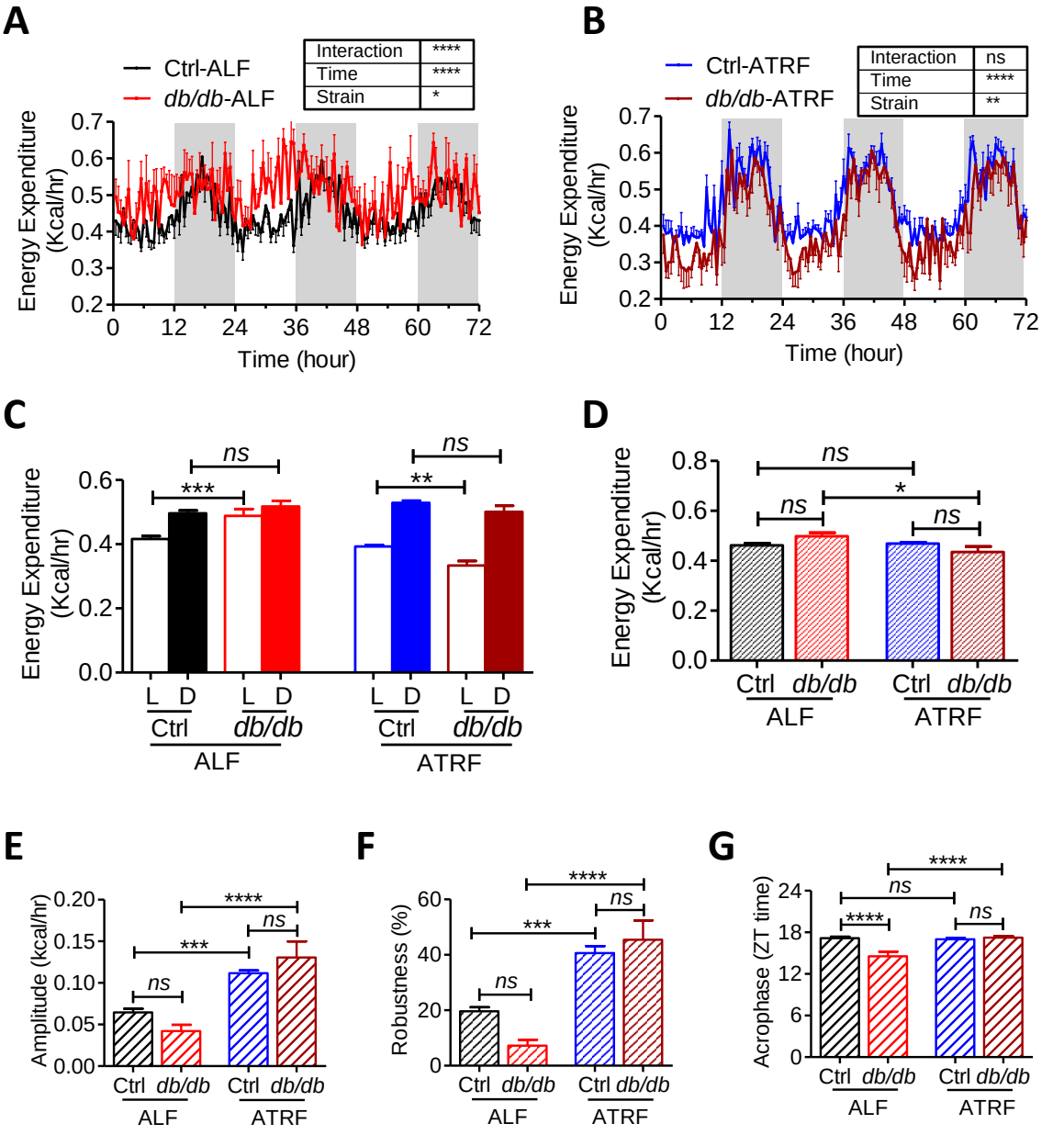


Figure 3.2.2.7 ATRF improved the daily rhythm of energy expenditure (EE) in the *db/db* mice. EE was recorded by indirect calorimetry. (**A** and **B**) The 72-hour recording of the EE in the control and *db/db* mice with ALF (**A**) or ATRF (**B**). The light grey box indicates the dark-phase. **(C)** 12-hour average EE during the light-phase (L) and dark-phase (D). **(D)** Daily EE. **(E-G)** The amplitude **(E)**, robustness **(F)** and acrophase **(G)** of EE oscillation; control groups: n=10; *db/db* groups: n=5-6. *, p<0.05; **, p<0.01; ***, p<0.001; ****, P<0.0001; ns, not significant.

3.2.3 Exploration of possible mechanisms underlying ATRF initiated protection of BP circadian rhythm in the *db/db* mice.

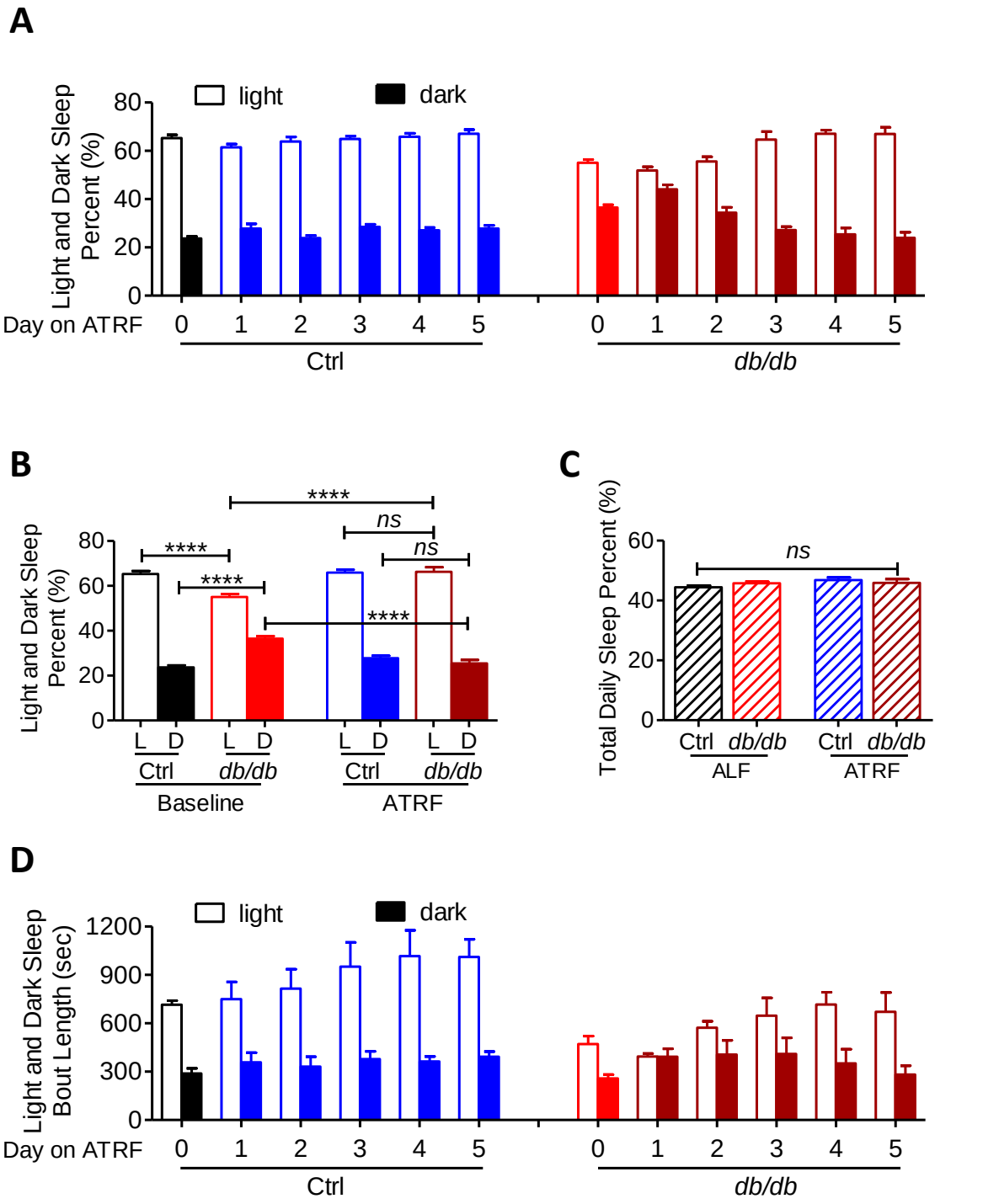
3.2.3.1 ATRF improved the daily rhythms in the sleep and locomotor activity

Since the daily cycles of sleep/wake and activity/rest significantly influence BP daily oscillation; we monitored the effects of ATRF on these behaviors. As shown in Fig. 3.2.3.1A and 3.2.3.1B, ALF-fed control mice slept about 65% of the time during the light-phase and 25% of the time during the dark-phase. ATRF did not significantly modify the sleep time distribution in the control mice. In contrast, ALF-fed mice slept significantly less (~55%) during the light-phase and more (~37%) during the dark-phase sleep compared with the ALF-fed control mice (Fig. 3.2.3.1A and 3.2.3.1B). Importantly, ATRF significantly increased the sleep time during the light-phase and significantly decreased the sleep during the dark-phase, resulting in a restoration of the sleep time distribution during the light- and dark-phase similar to the control mice (Fig. 3.2.3.1A and 3.2.3.1B). Interestingly, there is no difference in the total daily sleeping time between the control and *db/db* mice (Fig. 3.2.3.1C). To investigate the sleep quality, we determined the the sleep bout length during the light- and dark-phase. In both the *db/db* and control mice, the sleep bout length was longer during the light-phase than during the dark-phase (Fig. 3.2.3.1D and 3.2.3.1E). Interestingly, while ATRF did not alter the sleep bout length during the dark-phase in either the *db/db* and control mice, ATRF significantly increased the sleep bout length during the light phase starting on the second day of ATRF and reached a plateau around the third day of ATRF (Fig. 3.2.3.1D).

With ALF, the daily rhythms in locomotor activity in the control mice, as demonstrated in Fig. 3.2.3.2A, were lost in the *db/db* mice (Fig. 3.2.3.2B). ATRF significantly decreased the locomotor activity during the light-phase and significantly increased the locomotor activity during the dark-phase. ATRF thus restored a light-dark phase locomotor activity difference in the *db/db*-ATRF group (Fig. 3.2.3.2C) without altering the mean locomotor activity during the 24-hour day (Fig. 3.2.3.2D). Surprisingly, ATRF also significantly increased the locomotor activity in the control mice during the dark-phase (Fig. 3.2.3.2C), which resulted in an increase in the mean locomotor activity during the 24-hour day in the control mice (Fig. 3.2.3.2D). Analysis of the daily locomotor activity oscillation demonstrated that, in the *db/db* mice, ATRF significantly enhanced the daily activity oscillation robustness (Fig. 3.2.3.2F) and forward shifted the acrophase (Fig. 3.2.3.2G) without changing the amplitude (Fig. 3.2.3.2E). Interestingly, in control mice, ATRF significantly increased the daily oscillation amplitude (Fig. 3.2.3.2E) and robustness (Fig. 3.2.3.2F) without shifting the acrophase (Fig. 3.2.3.2G).

The ATRF induced an increase in the sleep percentage and decrease in the locomotor activity during the light-phase, and had the opposite effects on the sleep percentage and locomotor activity during the dark-phase in the *db/db* mice; this shift likely contributed to the prevention/restoration of the BP daily rhythm in the *db/db* mice.

Figure 3.2.3.1(1)



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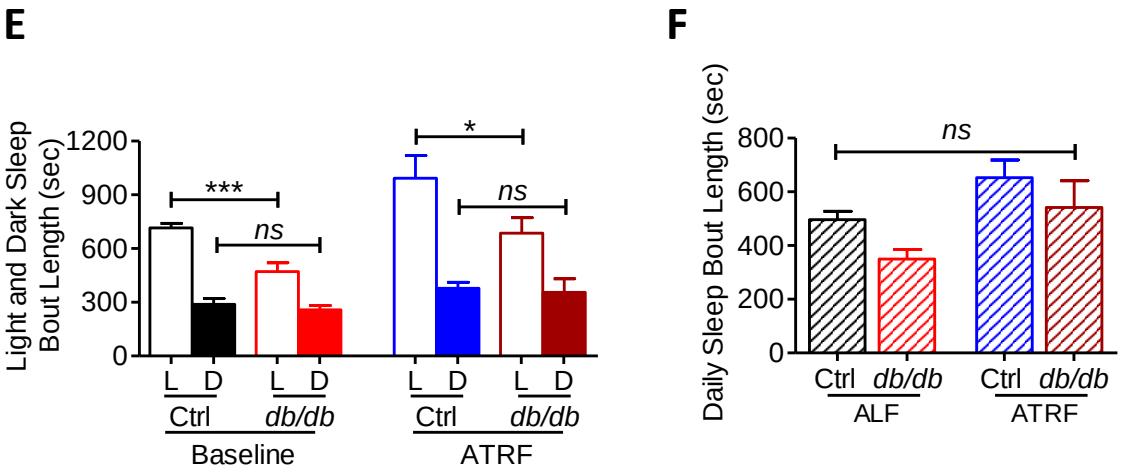


Figure 3.2.3.1 ATRF improved the daily rhythm of sleep time in the *db/db* mice. Sleep was monitored using the PiezoSleep system. **(A and D)** The time course of the percentage of sleep time **(A)** and sleep bout length **(D)** during the light- and dark-phase in the control (Ctrl) and *db/db* mice before and after ATRF. **(B and E)** The baseline and the average of day 3 to day 5 sleep time percentage **(B)** and sleep bout length **(E)** after ATRF during the light- and dark-phase. **(C and F)** The baseline and the average of day 3 to day 5 sleep time percentage **(C)** and sleep bout length **(F)** after ATRF during the 24-hour day. n=6-8. *, p<0.05; ***, p<0.001; ****, P<0.0001; ns, not significant.

Figure 3.2.3.2

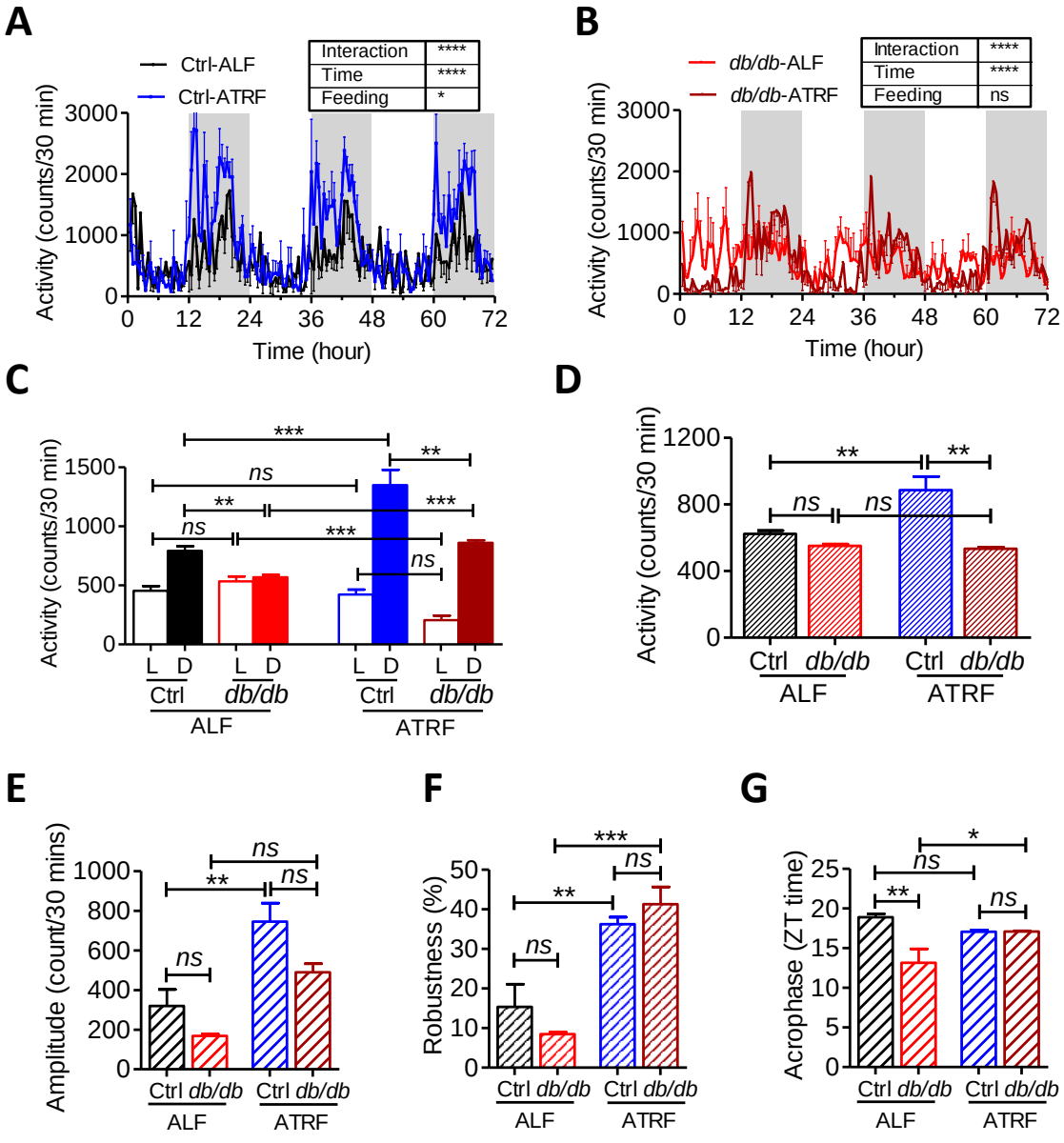


Figure 3.2.3.2 ATRF improved the daily rhythm of locomotor activity in the *db/db* mice. The locomotor activity was recorded by indirect calorimetry. (**A** and **B**) The 72-hour recording of the locomotor activity in the control (**A**) and *db/db* (**B**) mice with ALF or ATRF. The light grey box indicates the dark-phase. **(C)** 12-hour average locomotor activity during the light-phase (L) and dark-phase (D). **(D)** Daily locomotor activity. **(E-G)** The amplitude **(E)**, robustness **(F)** and acrophase **(G)** of locomotor activity oscillation; control groups: n=4-5; *db/db* groups: n=3. *, p<0.05; **, p<0.01; ***, p<0.001; ****, P<0.0001; ns, not significant.

3.2.3.2 The effects of ATRF on the BP-regulatory hormones

The BP-regulatory hormones play an important role in the BP homeostasis. We first explored the renin-angiotensin system (RAS) by determining the mRNA levels in the four groups of mice (Ctrl-ALF; *db/db*-ALF; Ctrl-ATRF and *db/db*-ATRF) at 6-hour intervals of the following genes: angiotensinegin [Fig. 3.2.3.4A (liver) and Fig. 3.2.3.4B (kidney)], renin (Fig. 3.2.3.4C, kidney), angiotensin converting enzyme [Fig. 3.2.3.4D (ACE, kidney), Fig. 3.2.3.4E (ACE2, kidney) and Fig. 3.2.3.4F (ACE/ACE2, kidney)], angiotensin II receptor 1a [Fig. 3.2.3.4G (kidney) and Fig. 3.2.3.4H (and renal gland)]. The results demonstrated that the kidney ACE was significantly diminished (Fig. 3.2.3.4D) while the kidney angiotensinogen (Fig. 3.2.3.4B) was significantly increased in the *db/db* mice when compared with that in the control mice in all four time points of the day. However, no dramatic effects were observed by ATRF in either the control or the *db/db* mice, suggesting the RAS does not play a critical role in the ATRF initiated protection of BP daily rhythm.

We then measured three hormones that are important for BP regulation: aldosterone, corticosterone, and epinephrine in urine samples collected every 6 hours from the *db/db*-ALF and *db/db*-ATRF groups. The results showed that in the *db/db*-ALF mice, all three hormones retained the time-of-day variations with a peak at the sample collected between ZT12-18 (Fig. 3.2.3.5A-3.2.3.5C). ATRF significantly improved the time-of-day variation by decreasing the aldosterone (Fig. 3.2.3.5A) and corticosterone (Fig. 3.2.3.5B) at the ZT6-12 sample and by enhancing the epinephrine (Fig. 3.2.3.5C) at the ZT12-18

sample. While these improvements of the time-of-day variations in humoral factors may participate in the protective effects of ATRF on the BP daily oscillation, additional factors are likely involved for the striking effective protection.

Figure 3.2.3.4

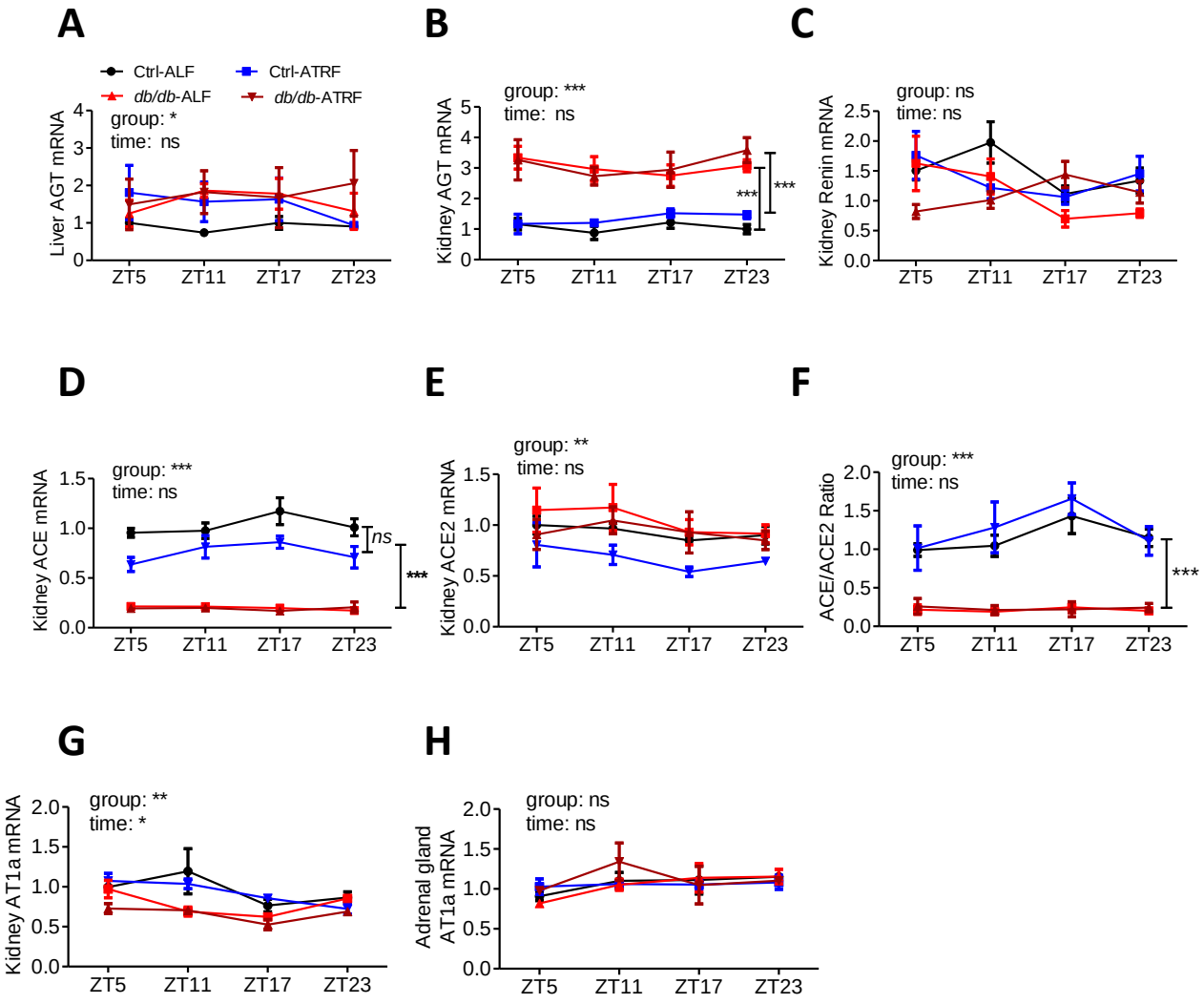


Figure 3.2.3.4 ATRF had no effect on the renin-angiotensin-system (RAS). Control and *db/db* mice with ALF or ATRF were euthanized at ZT5, ZT11, ZT17 and ZT23 and the time-of-day mRNA expression of RAS components were detected by real-time PCR. **(A and B)** *Agt* in the liver **(A)** and kidney **(B)**. **(C-F)** *Renin* **(C)**, *Ace* **(D)**, *Ace2* **(E)** and *Ace/Ace2* ratio **(F)** in the kidney. **(G and H)** *At1a* in the kidney **(G)** and adrenal gland **(H)**. *Agt: angiotensinogen; Ace: angiotensin-converting enzyme; At1a: angiotensin II receptor type 1a.* At each time point, Ctrl-ALF: n=6-7; Ctrl-ATRF: n=4-5; *db/db*-ALF: n=4-5, *db/db*-ATRF: n=3-5. *, p<0.05; **, p<0.01; ***, p<0.001; ns, not significant.

Figure 3.2.3.5

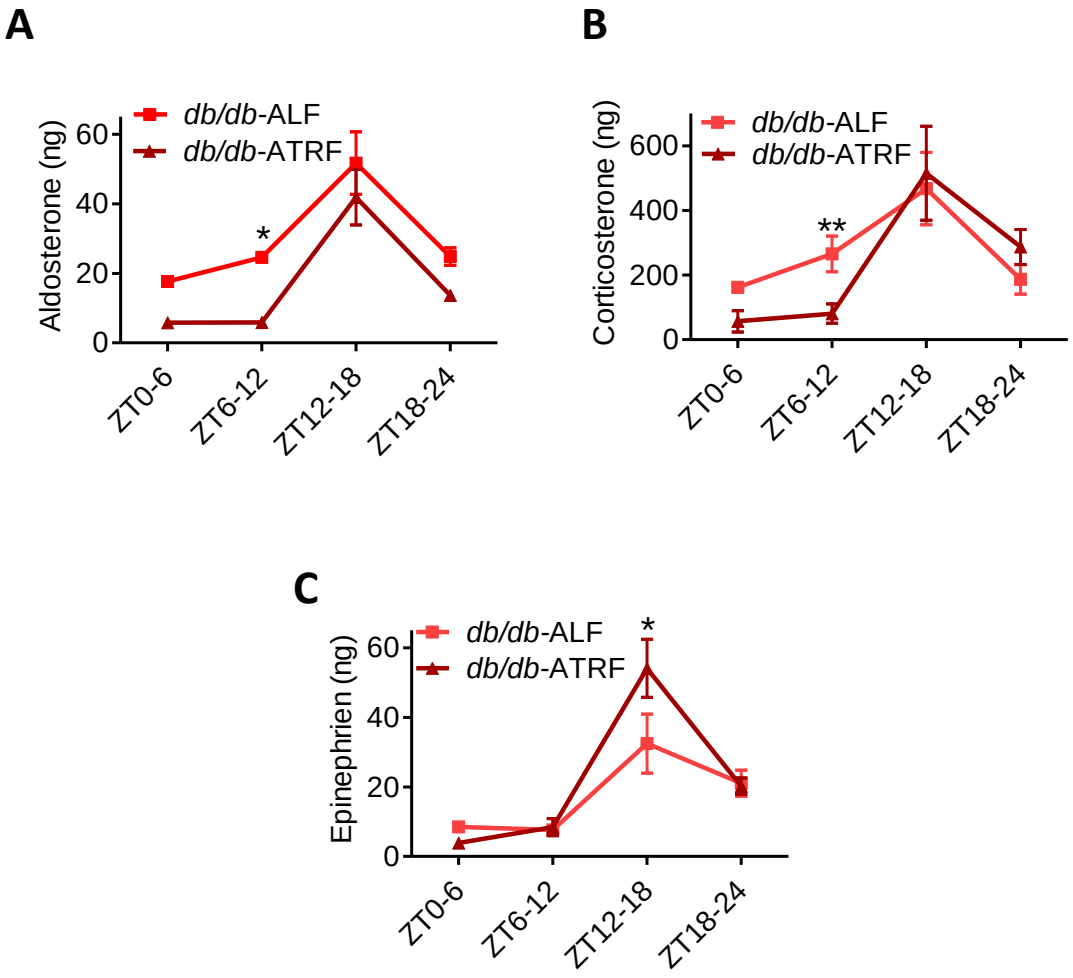


Figure 3.2.3.5 ATRF improved the time-of-day variations in the urinary excretion of aldosterone, corticosterone and epinephrine. Urine was collected every 6 hours at ZT6, ZT12, ZT18 and ZT0 from the *db/db*-ALF and *db/db*-ATRF mice. The urinary contents of aldosterone **(A),** corticosterone **(B)** and epinephrine **(C)** were calculated by the concentration times the urine volume. n=4-5. *, p<0.05; **, p<0.01.

3.2.3.3 The autonomic nervous system (ANS) mediates, at least in part, the protective effects of ATRF on BP daily rhythm

We next investigated the ANS as it is the prominent regulator of the BP circadian rhythm. Several approaches were employed to investigate the involvement of the ANS. Firstly, we determined the circadian rhythm of heart rate (HR) as the HR is primarly determined by the ANS. As shown in Fig. 3.2.3.6A and 3.2.3.6B, in mice that started the ATRF regimen at 6-weeks-old, the ALF-fed *db/db* mice had a similar HR oscillation as the ALF-fed control mice. Interestingly, ATRF had no effect on the light-phase HR in the control mice (Ctrl-ALF *vs.* Ctrl-ATRF), but dramatically decreased the light-phase HR in the *db/db* mice (*db/db*-ALF vs. *db/db*-ATRF)(Fig. 3.2.3.6A and 3.2.3.6B). Cosine analysis revealed the amplitude and robustness of the HR oscillation were significantly increased in the *db/db*-ATRF mice compared to the *db/db*-ALF mice (Fig. 3.2.3.6C and 3.2.3.6D). In addition, the HR oscillation amplitude of the *db/db*-ATRF mice was even greater than the Ctrl-ATRF mice (Fig. 3.2.3.6C). Similar effects of ATRF on the HR were observed in the *db/db* mice started on the ATRF regimen at 16-weeks-old (Fig. 3.2.3.7).

Secondly, we calculated the spontanous baroreflex sensitivity (BRS) across the 24-hour day in the consious and free-moving mice using the sequence method (Di Rienzo, Parati et al. 2001) . As shown in Fig. 3.2.3.8A, in the Ctrl-ALF group, there was a time-of-day variation in the BRS, as we and others have reported (Hossmann, Fitzgerald et al. 1980, Di Rienzo, Parati et al. 2001, Xie, Su et al. 2015). Such variation was abolished in the *db/db*-ALF group (Fig. 3.2.3.8A). ATRF induced the time-of-day variation of BRS in the

db/db mice that were on the ATRF regimen at both 6-weeks-old (Fig. 3.2.3.8B) and 16-weeks-old (Fig. 3.2.3.8C).

Thirdly, we determined the heart rate variability (HRV) across the 24-hour day as HRV is considered as an indication of autonomic function in some studies. The HRV was calculated by frequency domain and time domain. For frequency domian, there are inconsistent cut-offs of the low frequency (LF) and high frequency (HF) ranges for mice between different literatures. We chose the cut-off at 0.15-0.6Hz for the LF, as optimized by (Baudrie, Laude et al. 2007). The HF cut-off we used was 1.5-4Hz since Thireau *et al* found this cut-off allows for a better evaluation of parasympathetic modulation in mice (Thireau, Zhang et al. 2008). We first used atropine, a muscarinic receptor antagonist that inhibits parasympathetic nervous system (PNS) and metoprolol, a β1-adrenergic receptor antagonist that inhibits sympathetic nervous system (SNS) function to the heart, to test the cut-offs of the LF and HF. We found that when the cut-off was defined at 0.15-0.6Hz for the LF and 1.5-4Hz for the HF, atropine decreased both the LF and HF (Fig. 3.2.3.9A) and metoprolol only decreased the LF (Fig. 3.2.3.9B). This is consistent with the indications of LF and HF in humans, that the LF is determined by both the SNS and PNS, and the HF is dominated by the PNS (Draghici and Taylor 2016). Next we calculated the hourly LF and HF using the above cut-offs. As shown in Fig. 3.2.3.10A and 3.2.3.10C, there were time-of-day variations in both the LF and HF in the ALF-fed control mice: both the LF and HF tended to be higher during the light-phase and lower during the dark-phase; however, such variations were lost in the ALF-fed *db/db* mice. When the ATRF was started at 6-weeks-old, the ATRF increased the

light-phase LF and HF in the *db/db* mice, resulting in the time-of-day variations in the LF and HF in the *db/db* mice that were equivalent to those of the control mice (Fig. 3.2.3.10B and 3.2.3.10D). The LF/HF was lower in the *db/db*-ALF mice than in the Ctrl-ALF mice (Fig. 3.2.3.10E), and the difference persisted after ATRF (Fig. 3.2.3.10F). For the time domain, the root mean square of successive RR interval differences (rMSSD), which is demonstrated to be associated with HF, exhibited similar trend and response to ATRF as the HF of the *db/db* mice (Fig. 3.2.3.10G and 3.2.3.10H). Equivalent effects of ATRF on the HRV were observed in the *db/db* mice started on the ATRF regimen at 16-weeks-old (Fig. 3.2.3.11). These results suggest that at least, the day and night difference in the PNS activity was restored in the *db/db* mice when they were on the ATRF regimen.

Fourthly, we measured the sympathetic neurotransmitter norepinephrine (NE) and its metabolite normetanephrine in the 6-hour urine samples collected across a 24-hour day. As shown in Fig. 3.2.3.12A and 3.2.3.12B, no time-of-day variations in the NE and normetanephrine were detected in the ad libitum fed *db/db* mice ($p>0.05$ by JTK-CYCLE). The JTK-CYCLE is a algorithm that can distinguishes between rhythmic and non-rhytmic transcripts (Hughes, Hogenesch et al. 2010). The ATRF effectively recovered the time-of-day variations of the urinary NE and normetanephrine mostly by decreasing their excretion during the light-phase. The correlation between the decrease in urinary excretion of sympathetic transmitter and the decrease in BP during the light-phase suggest that modulation of sympathetic tone is involved in the ATRF's protection of the BP daily oscillation. The change in the urinary excretion of NE is unlikely due to the alternations of the biosynthesis and/or the disposition of NE, as the mRNA expression of

adrenal *tyrosine hydroxylase* (*Th*) and *dopamine beta (β)-hydroxylase (Dbh)*, enzymes resposible for the NE biosynthesis did not differ between the control and *db/db* mice with either ALF or ATRF (Fig. 3.2.3.13A and 3.2.3.13B). For the diposition of NE, the mRNA expression of the adrenal *phenylethanolamine N-Methyltransferase (Pnmt)*, an enzyme that methylates NE to form epinephrine, was higher at ZT11 in *db/db*-ATRF mice compared with the *db/db*-ALF mice (Fig. 3.2.3.13C). However, the *Pnmt* expression was still at a high level at ZT5 and increased PNMT is shown to be associated with increased BP (Nguyen, Peltsch et al. 2009), therefore, the increase in the *Pnmt* expression unlikely accounts for the ATRF initiated BP decrease during the light-phase.

Fifthly, to further explore a potential cause and effect relationship between the sympathetic activity and BP daily oscillation, we determined the extent of BP drop in response to the bloackade of sympathetic vascular function by α1 adrenergic receptor antagonist prazosin at ZT5 and ZT 17 respectively. As shown in Fig. 3.2.3.14A and 3.2.3.14B, the control mice had higher BP at ZT17 than at ZT5; prazosin decreased the BP to similar levels. These data indicated a major role of higher sympathetic vascular tone underlying the higher BP during the dark-phase than during the light-phase in the control ALF-fed mice. In contrast, in the *db/db*-ALF mice, no difference in the BP was detected between ZT5 and ZT 17, and prazosin induced comparable extents of BP drop (Fig. 3.2.3.14C and 3.2.3.14D). Importantly, the ATRF restored the BP day and night difference in the *db/db*-ATRF mice and the prazosin decreased the BP to similar levels (Fig. 3.2.3.14E and 3.2.3.14F), indicating a prominent role of sympathetic vascular tone in the restoration of the BP difference between the light- and dark-phase.

Figure 3.2.3.6

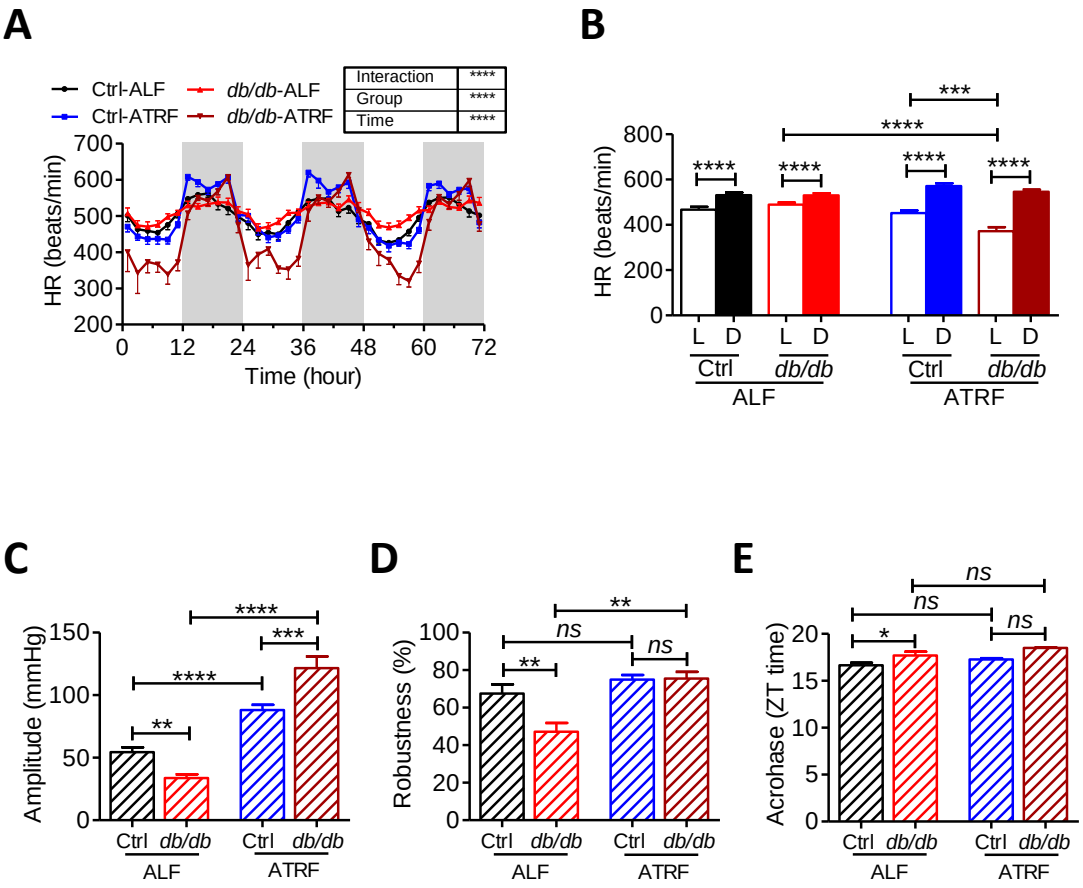


Figure 3.2.3.6 ATRF improved the heart rate (HR) oscillation in the *db/db* mice started on the ATRF regimen at 6-week-old. **(A)** 2-hour average HR in the control and *db/db* mice with ALF or ATRF. **(B)** 12-hour average HR during the light-phase (L) and dark-phase (D). **(C-E)** The amplitude **(C)**, robustness **(D)** and acrophase **(E)** of HR oscillation. Ctrl-ALF: n=13; Ctrl-ATRF: n=12; *db/db*-ALF: n=12; *db/db*-ATRF: n=5. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001; ns, not significant.

Figure 3.2.3.7

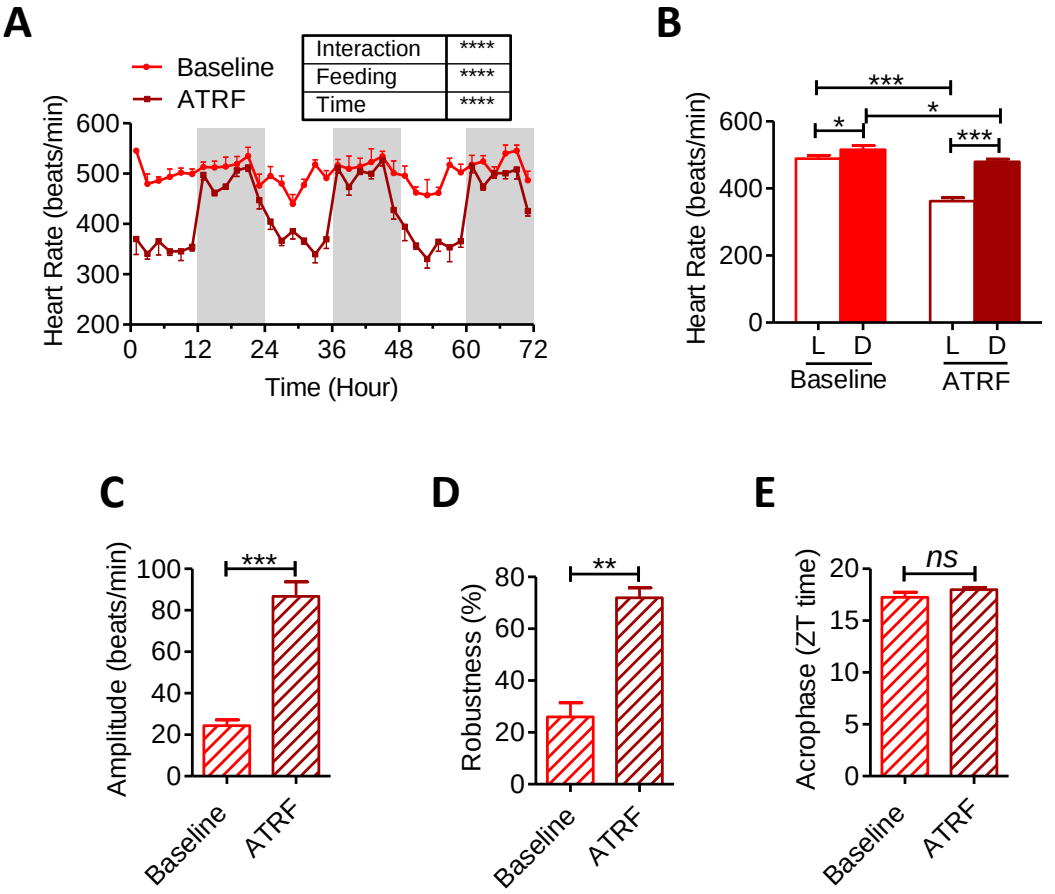


Figure 3.2.3.7 ATRF improved the HR oscillation in the *db/db* mice started on the ATRF regimen at 16-week-old. **(A)** 2-hour average HR in the *db/db* mice at baseline and after 9 days of ATRF. **(B)** 12-hour average HR during the light-phase (L) and dark-phase (D). **(C-E)** The amplitude **(C)**, robustness **(D)** and acrophase **(E)** of HR oscillation. n=5. *, p<0.05; **, p<0.01; ***, p<0.001; ****, P<0.0001; ns, not significant.

Figure 3.2.3.8

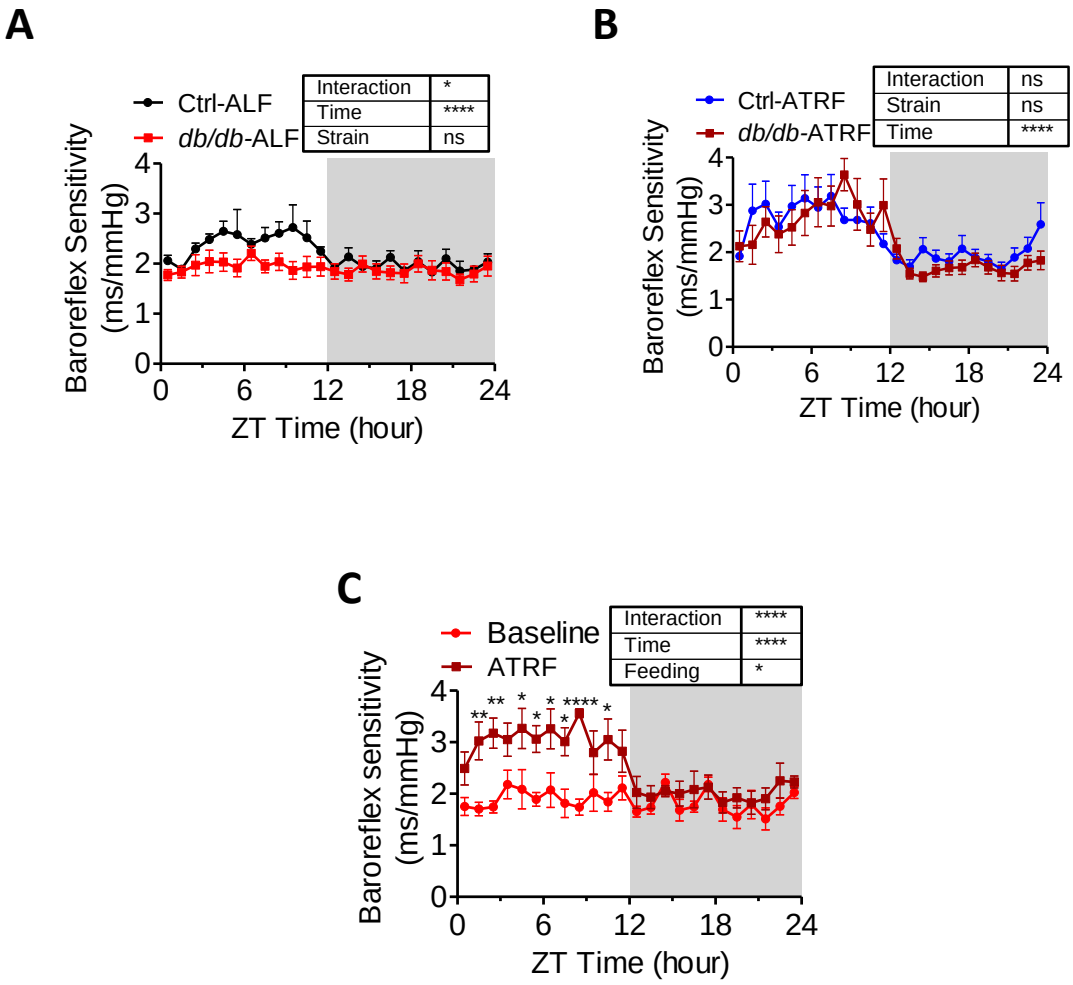


Figure 3.2.3.8 ATRF induced the time-of-day variation of baroreflex sensitivity (BRS) in the *db/db* mice. The hourly average BRS was calculated using the sequence method. **(A)** BRS in the Ctrl-ALF and the Ctrl-ATRF mice that started the ATRF regimen at 6-week-old. **(B and C)** BRS in the *db/db* mice that started the ATRF regimen at 6-week-old **(B)** and at 16-week-old **(C)**. Ctrl-ALF, n=4; *db/db*-ALF, n=10; Ctrl-ATRF, n=5; *db/db*-ATRF, n=6; baseline and ATRF, n=4. *, p<0.05; **, p<0.01; ****, P<0.0001; ns, not significant.

Figure 3.2.3.9

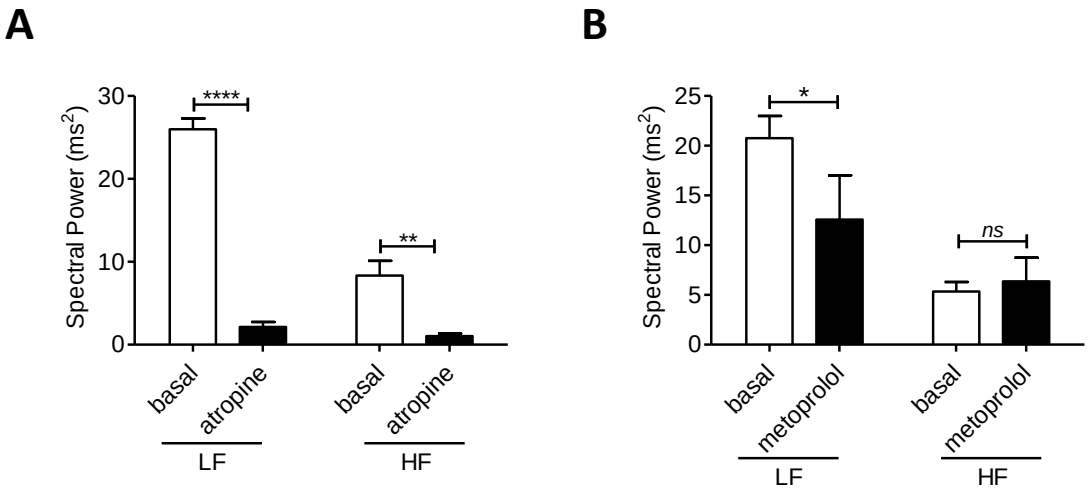
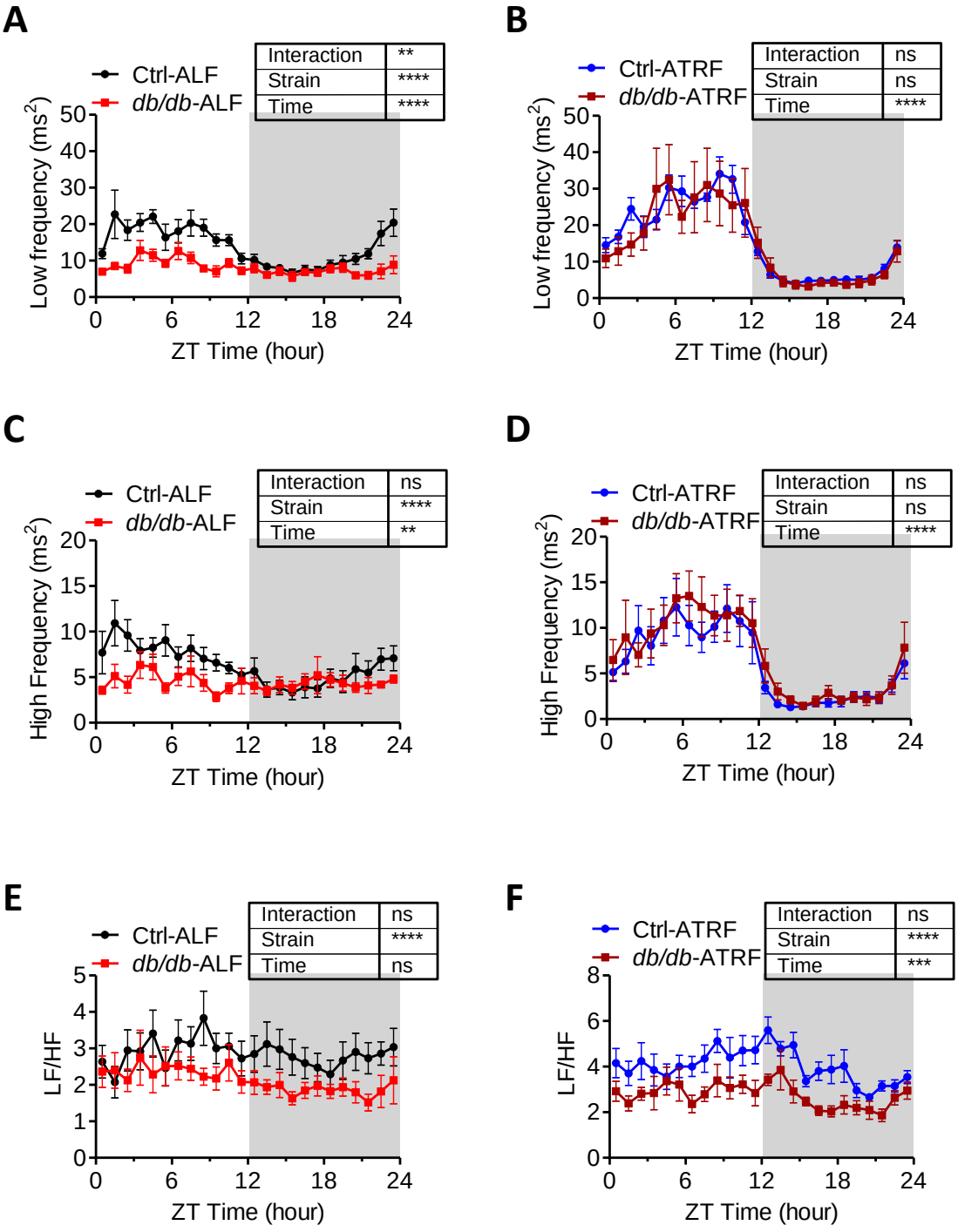


Figure 3.2.3.9 The effects of atropine and metoprolol on the low frequency (LF) and high frequency (HF) range of heart rate variability (HRV). **(A)** The LF and HF of HRV before and after i.p. injection of 1mg/kg atropine. **(B)** The LF and HF of HRV before and after i.p. injection of 4mg/kg metoprolol. The cut-off for the LF is 0.15-0.6Hz and for the HF is 1.5-4Hz**.** n=5-6. *, p<0.05; **, p<0.01; ****, P<0.0001; ns, not significant.

Figure 3.2.3.10(1)



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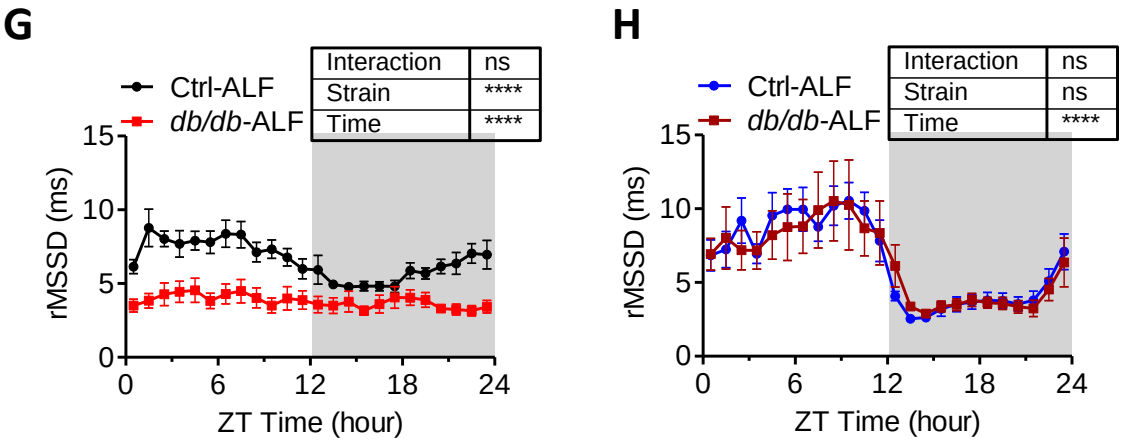

Figure 3.2.3.10 ATRF induced the time-of-day variations of the HRV in the *db/db* mice started on the ATRF regimen at 6-week-old. **(A-B)** LF of the control and *db/db* mice with ALF **(A)** or ATRF **(B)**. **(C-D)** HF of the control and *db/db* mice with ALF **(C)** or ATRF **(D)**. **(E-F)** LF/HF of the control and *db/db* mice with ALF **(E)** or ATRF **(F)**. **(G-H)** The rMSSD of the control and *db/db* mice with ALF **(G)** or ATRF **(H)**. rMSSD: root mean square of successive RR interval differences. n=6-8. **, p<0.01; ***, P<0.001; ****, P<0.0001; ns, not significant.

Figure 3.2.3.11

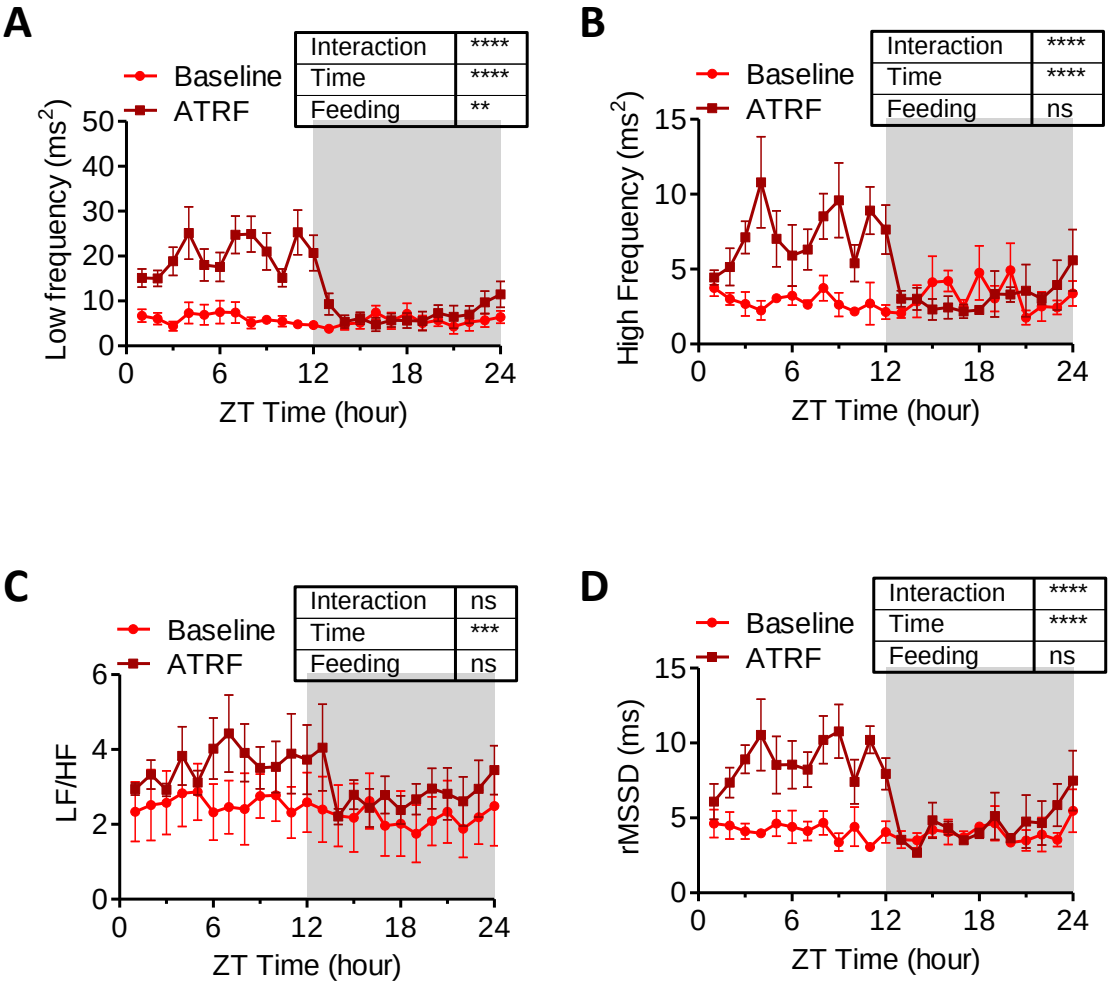

Figure 3.2.3.11 ATRF induced the time-of-day variations of the HRV in the *db/db* mice started on the ATRF regimen at 16-week-old. (A to D) LF **(A)**, HF **(B)**, LF/HF **(C)** and rMSSD **(D)** of the *db/db* mice before and after 9 days of ATRF. rMSSD: root mean square of successive RR interval differences. n=3-4. **, p<0.01; ***, P<0.001; ****, P<0.0001; ns, not significant.

Figure 3.2.3.12

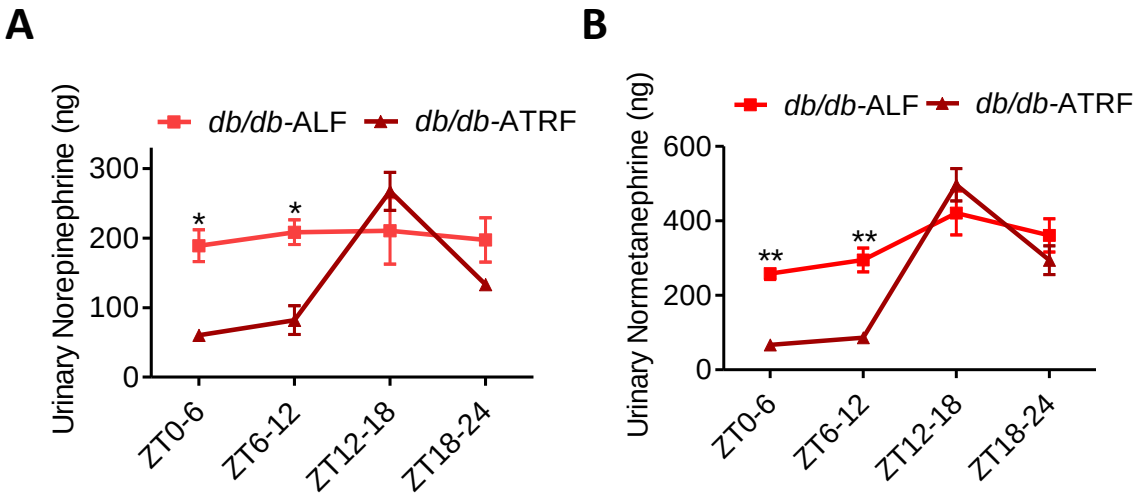

Figure 3.2.3.12 ATRF improved the time-of-day variations in the urinary excretion of norepinephrine (NE) and normetanephrine. Urine was collected every 6 hours at ZT6, ZT12, ZT18 and ZT0 from the *db/db*-ALF and *db/db*-ATRF mice. The urinary contents of NE **(A)** and normetanephrine **(B)** were calculated by the concentration times the urine volume. n=4-5. *, $p<0.05$; **, $p<0.01$.

Figure 3.2.3.13(1)

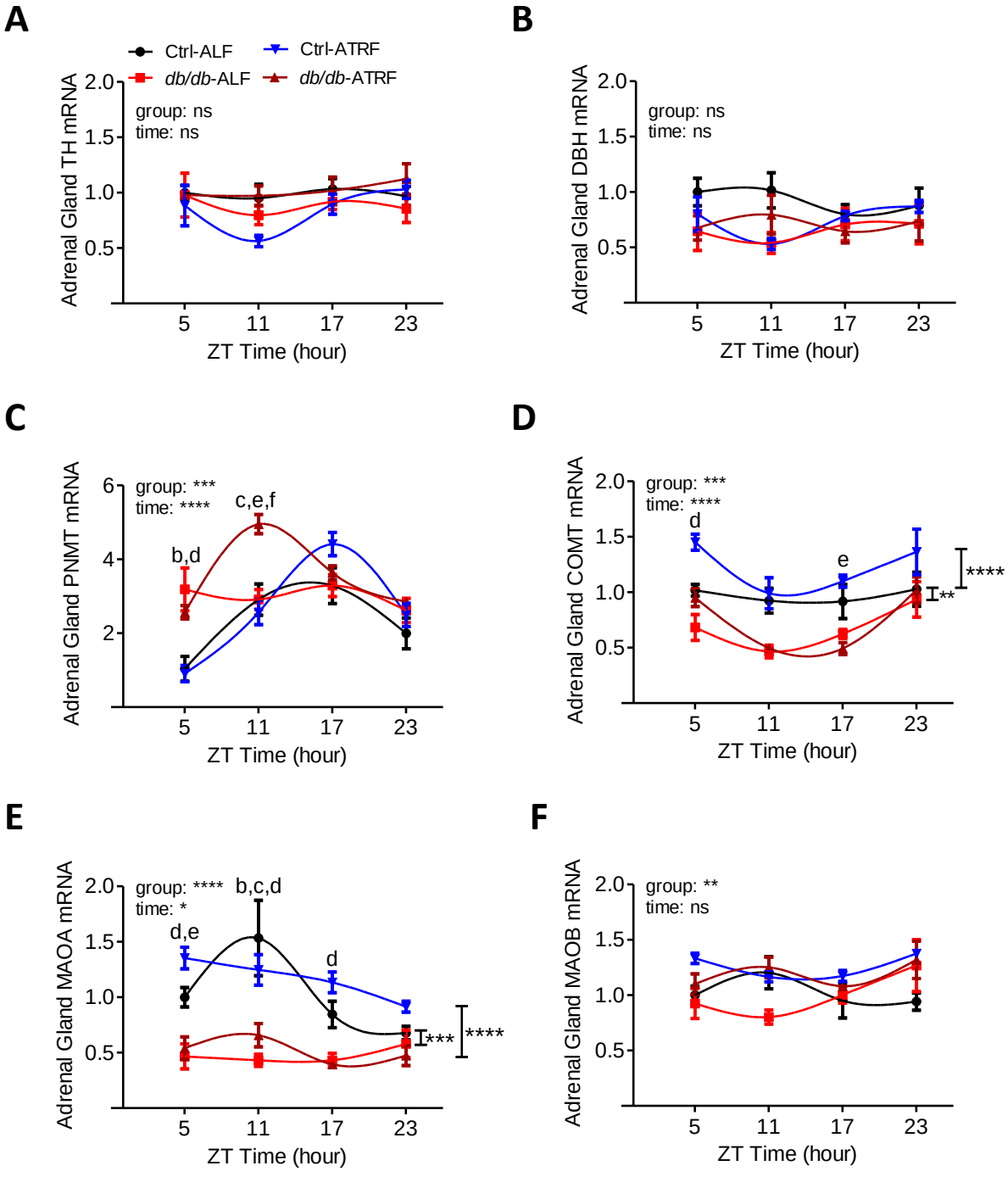

Figure 3.2.3.13 The effects of ATRF on the mRNA expression of the enzymes responsible for NE biosynthesis and disposition. Control and *db/db* mice with ALF or ATRF were euthanized at ZT5, ZT11, ZT17 and ZT23 and the time-of-day mRNA expressions of the enzymes responsible for NE biosynthesis and disposition were detected by real-time PCR. **(A-F)** The mRNA expressions of *Th* **(A)**, *Dbh* **(B)**, *Pnmt* **(C)**, *Comt* **(D),** *MaoA* **(E)** and *MaoB* **(F)** in the adrenal gland. *Th: tyrosine hydroxylase; Dbh: dopamine beta (β)-hydroxylase; Pnmt: phenylethanolamine N-methyltransferase; Comt: catechol-O-methyltransferase; MaoA and MaoB: monoamine oxidase A and B.* At each time point, Ctrl-ALF: n=6-7; Ctrl-ATRF: n=4-5; *db/db*-ALF: n=4-5, *db/db*-ATRF: n=3-5. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ns, not significant.

Figure 3.2.3.14

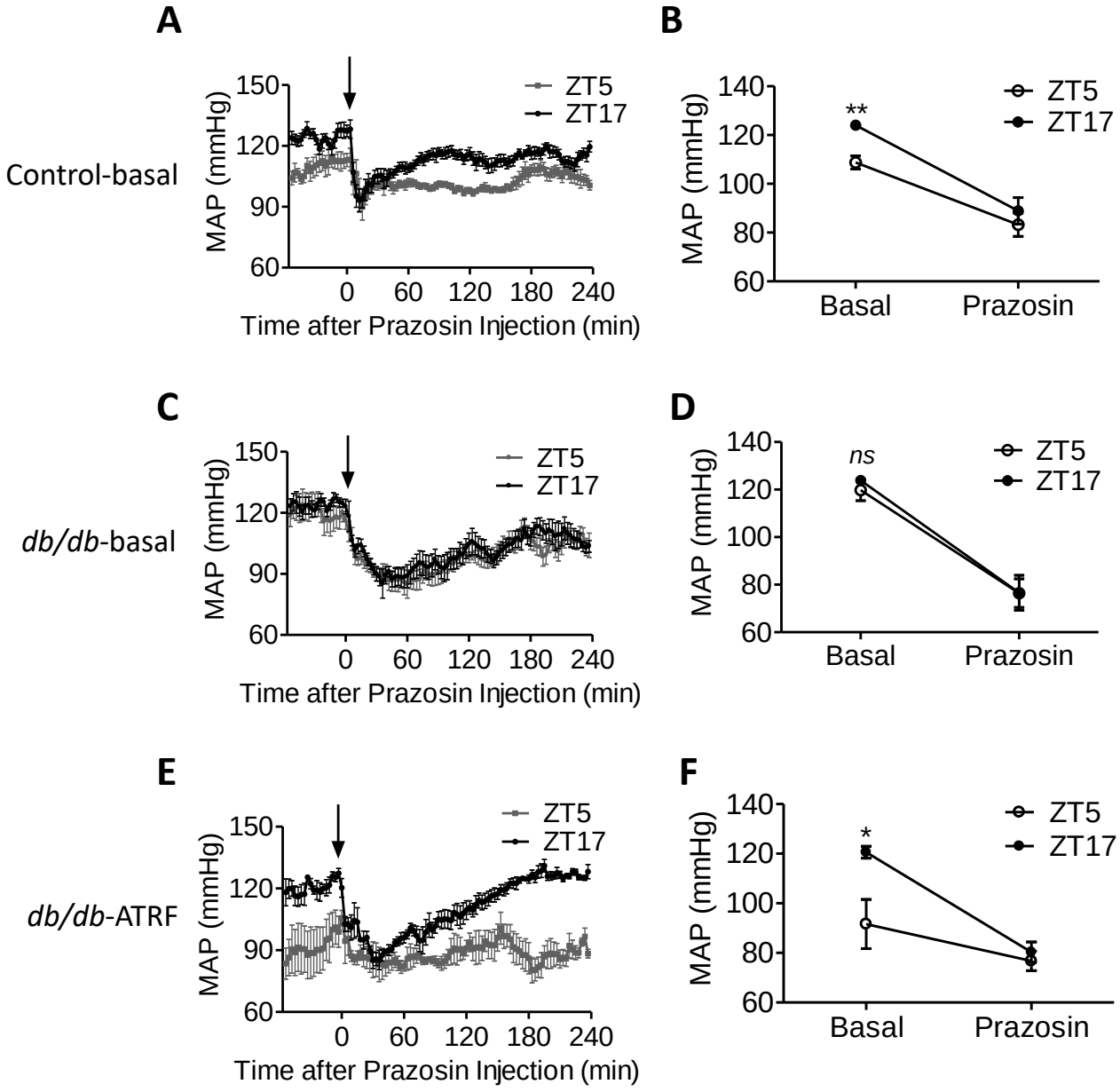

Figure 3.2.3.14. ATRF induced the day and night difference in prazosin induced BP reduction in the *db/db* mice. **(A, C and E)** The MAP response to prazosin (1mg/kg) i.p. injection at ZT5 and ZT17 in the control mice at baseline **(A)** and the *db/db* mice at baseline **(C)** and after ATRF **(E)**. **(B, D and F)** Average peak response of MAP to prazosin injection at ZT5 and ZT17 in the control mice at baseline **(B)** and the *db/db* mice at baseline **(D)** and after ATRF **(F).** control-basal, n=8; *db/db*-basal, n=9; *db/db*-ATRF, n=4. *, $p<0.05$, **, $p<0.01$, ns, not significant.

3.2.4 Investigation of whether the clock genes participate in the ATRF initiated protection of BP circadian rhythm in the *db/db* mice.

3.2.4.1 ATRF restored the clock gene Bmal1 mRNA daily oscillations in multiple tissues in the *db/db* mice.

Clock genes are the molecular mechanisms underlying the intrinsic biological rhythms. We and others have demonstrated that the clock gene daily oscillations were altered in the *db/db* mice (Su, Xie et al. 2012). To investigate whether ATRF restores the daily oscillation of clock genes in the *db/db* mice, we determined the mRNA expressions of clock genes *Bmal1, Clock, Per1, Per2, Cry1, Cry2, Rev-erbα and Rorc* at ZT5, ZT11, ZT17 and ZT23 in the liver, mesentery arteries (MA), kidney, heart and adrenal gland from the control and *db/db* mice fed ALF or ATRF. We found that the time-of-day expression of several clock genes showed tissue-dependent alternations in the *db/db*-ALF mice compared with the Ctrl-ALF mice (Fig 3.2.4.1-3.2.4.5). Moreover, ATRF recovered some of the altered clock genes in most of the tissues in the *db/db*-ATRF mice (Fig. 3.2.4.1-3.2.4.5). Among all the clock genes investigated, *Bmal1* mRNA showed the most consistent alterations in the *db/db*-ALF group and recovery in the *db/db*-ATRF group (Fig. 3.2.4.1A, 3.2.4.2A, 3.2.4.3A, 3.2.4.4A and 3.2.4.5A). The JTK cycle analysis showed the oscillations of *Bmal1* mRNA expressions were retained in the *db/db*-ALF and –ATRF mice ($P<0.05$); however, the oscillation acrophase was shifted forward by 6.32 hours in liver, 3.78 hours in the MA, 5.13 hours in the kidney, 5.51 hours in the heart and 4.73 hours in the adrenal gland in the *db/db*-ALF mice when compared with that in the Ctrl-

ALF mice (Table 3.2.4.1). ATRF nearly completely shifted the acrophase phase back to control mice in all the tissues investigated (Table 3.2.4.1). Another consistent change in *Bmal1* oscillation is the suppressed amplitudes of the oscillations, which are also nearly restored by ATRF (Table 3.2.4.1).

Figure 3.2.4.1

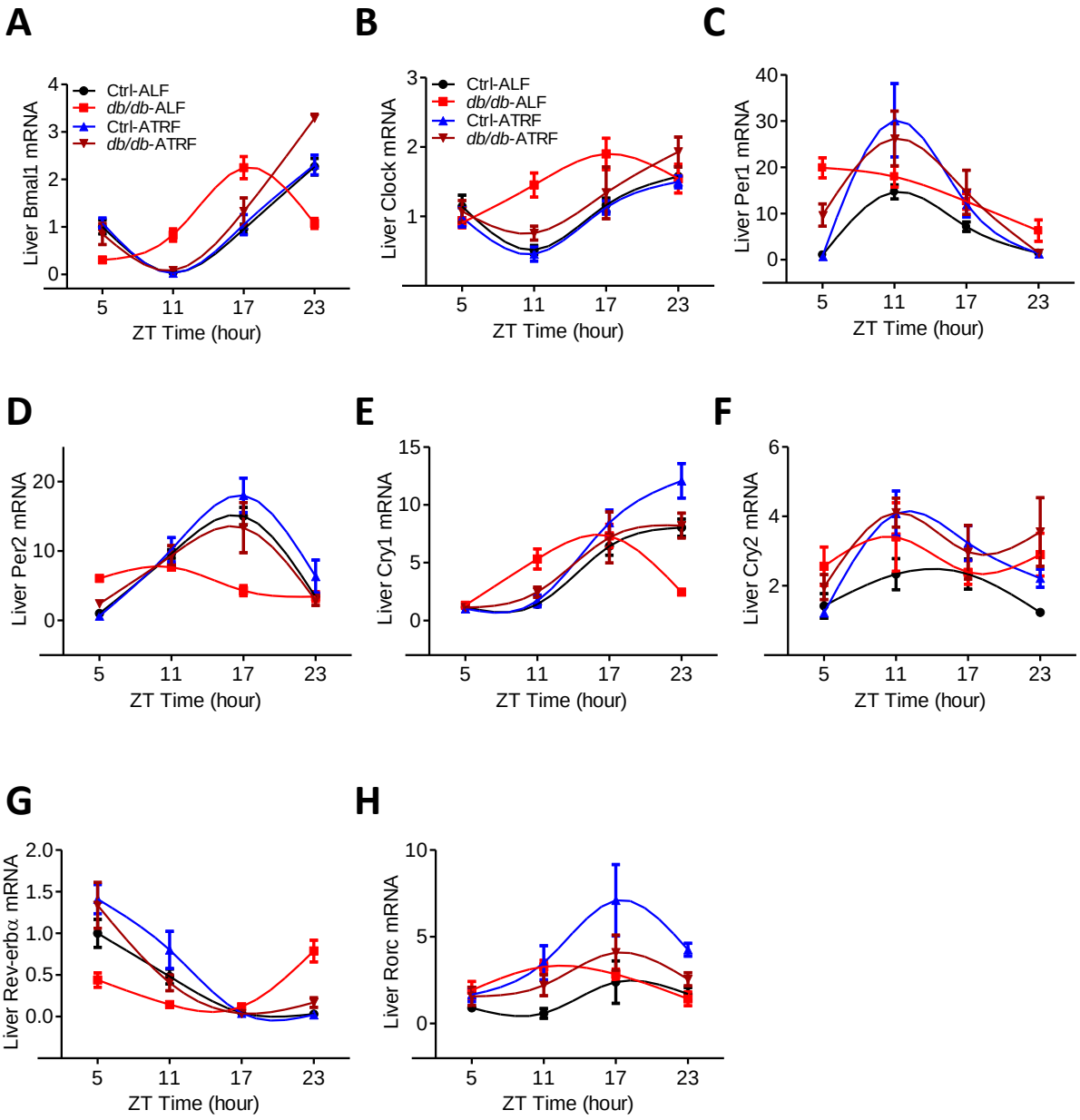

Figure 3.2.4.1 The mRNA expression of clock genes in the liver. Control and *db/db* mice with ALF or ATRF were euthanized at ZT5, ZT11, ZT17 and ZT23 and the time-of-day mRNA expressions of the clock genes were detected by real-time PCR. **(A)** *Bmal1*, **(B)** *Clock*, **(C)** *Per1*, **(D)** *Per2*, **(E)** *Cry1*, **(F)** *Cry2*, **(G)** *Rev-erbα* and **(H)** *Rorc*. At each time point, Ctrl-ALF: n=6-7; Ctrl-ATRF: n=4-5; *db/db*-ALF: n=4-5, *db/db*-ATRF: n=3-5. *, p<0.05; **, p<0.01; ***, p<0.001; ns, not significant.

Figure 3.2.4.2

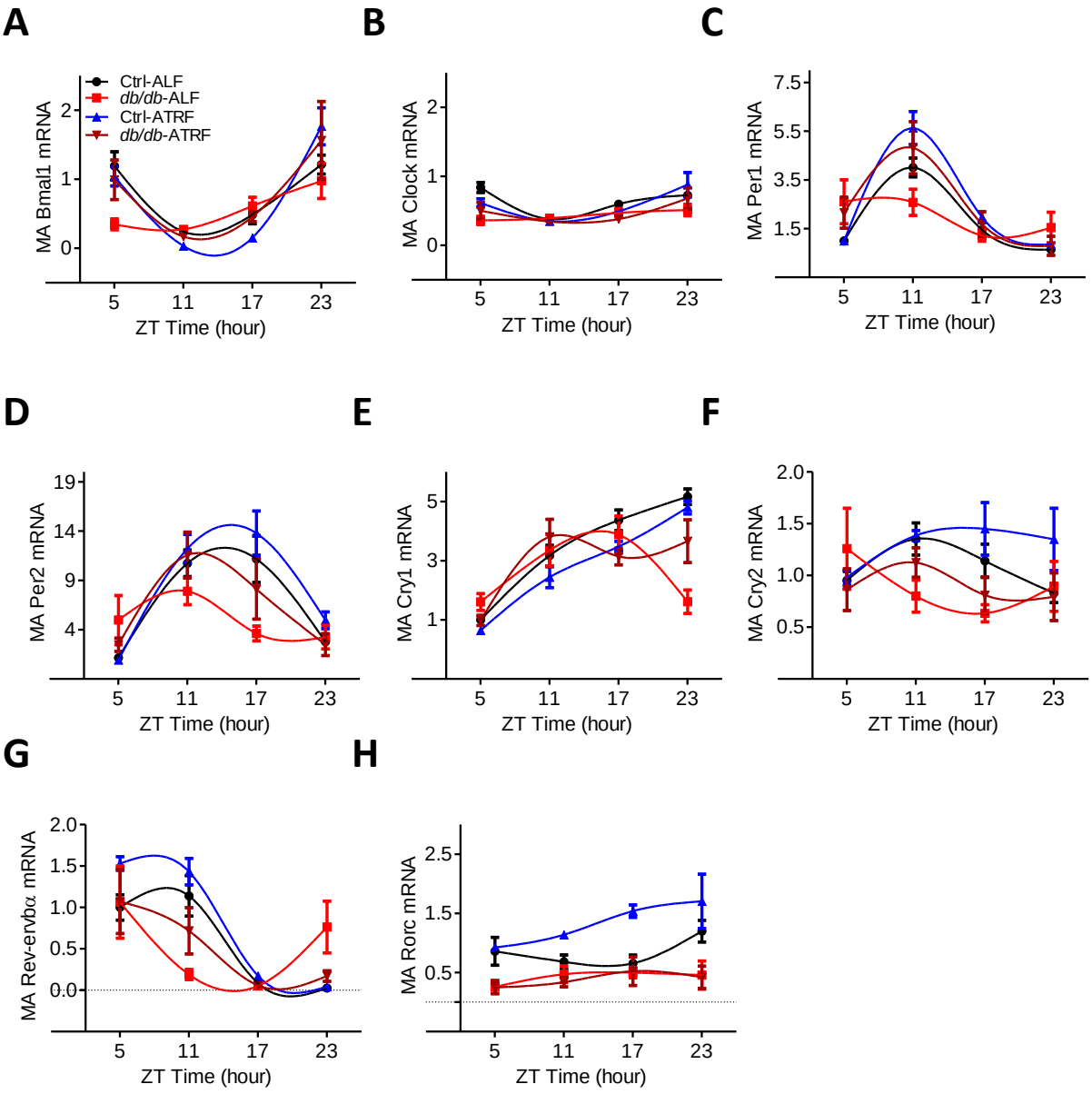

Figure 3.2.4.2 The mRNA expression of clock genes in the mesentery arteries (MA). Control and *db/db* mice with ALF or ATRF were euthanized at ZT5, ZT11, ZT17 and ZT23 and the time-of-day mRNA expressions of the clock genes were detected by real-time PCR. **(A)** *Bmal1*, **(B)** *Clock*, **(C)** *Per1*, **(D)** *Per2*, **(E)** *Cry1*, **(F)** *Cry2*, **(G)** *Rev-erbα* and **(H)** *Rorc*. At each time point, Ctrl-ALF: n=6-7; Ctrl-ATRF: n=4-5; *db/db*-ALF: n=4-5, *db/db*-ATRF: n=3-5. *, p<0.05; **, p<0.01; ***, p<0.001; ns, not significant.

Figure 3.2.4.3

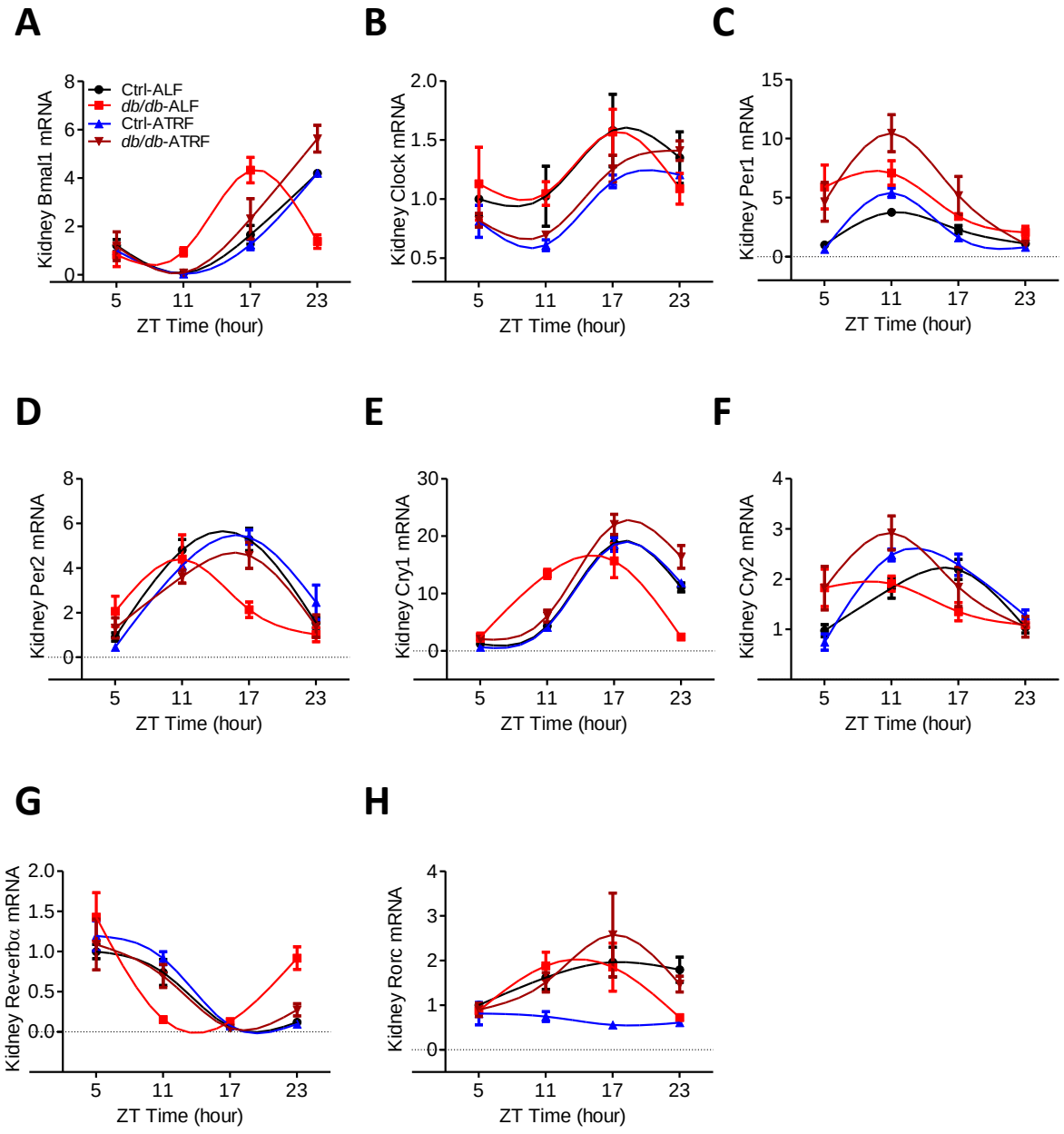

Figure 3.2.4.3 The mRNA expression of clock genes in the kidney. Control and *db/db* mice with ALF or ATRF were euthanized at ZT5, ZT11, ZT17 and ZT23 and the time-of-day mRNA expressions of the clock genes were detected by real-time PCR. **(A)** *Bmal1*, **(B)** *Clock*, **(C)** *Per1,* **(D)** *Per2*, **(E)** *Cry1*, **(F)** *Cry2*, **(G)** *Rev-erbα* and **(H)** *Rorc*. At each time point, Ctrl-ALF: n=6-7; Ctrl-ATRF: n=4-5; *db/db*-ALF: n=4-5, *db/db*-ATRF: n=3-5. *, p<0.05; **, p<0.01; ***, p<0.001; ns, not significant.

Figure 3.2.4.4

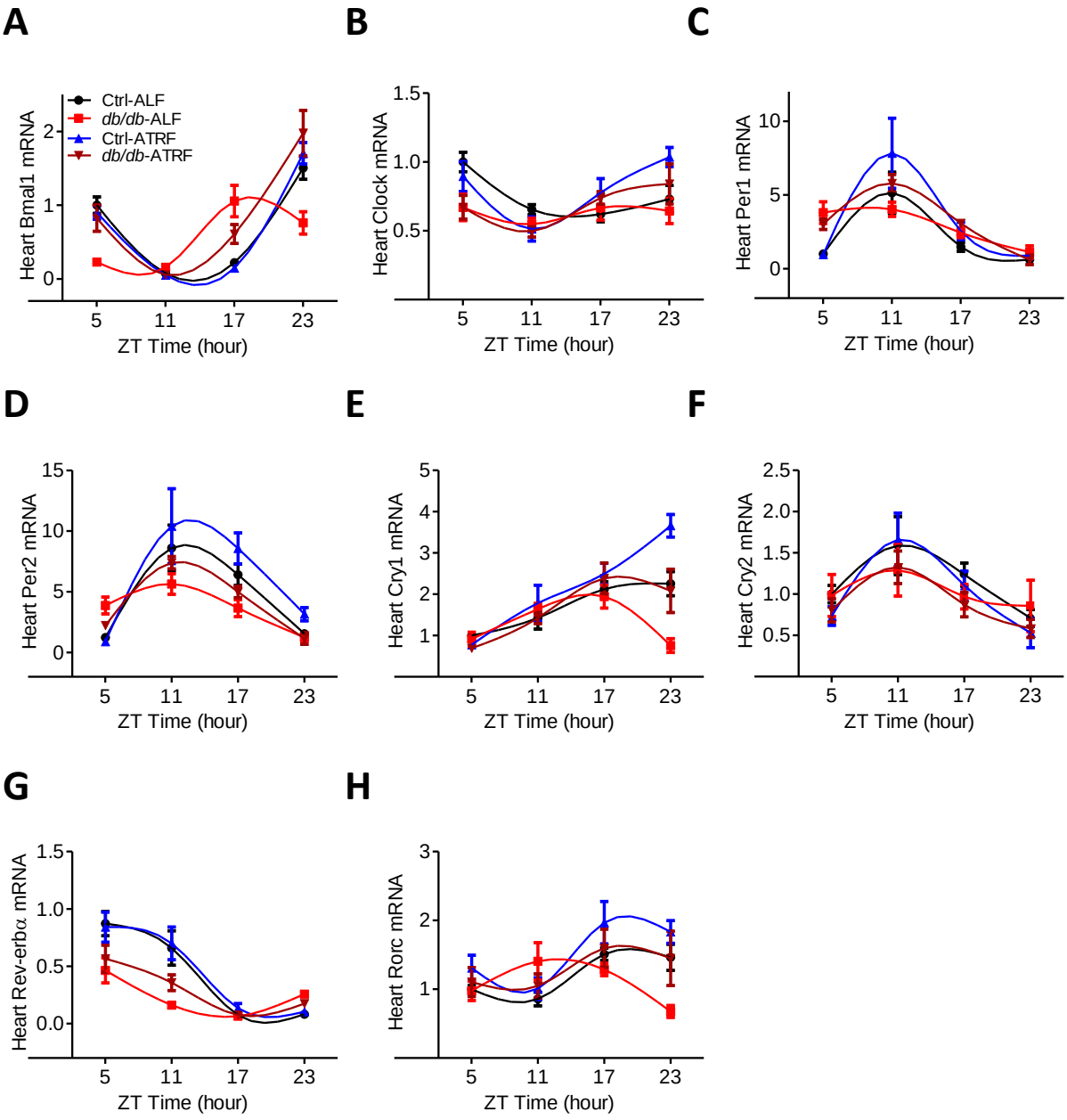

Figure 3.2.4.4 The mRNA expression of clock genes in the heart. Control and *db/db* mice with ALF or ATRF were euthanized at ZT5, ZT11, ZT17 and ZT23 and the time-of-day mRNA expressions of the clock genes were detected by real-time PCR. **(A)** *Bmal1*, **(B)** *Clock*, **(C)** *Per1*, **(D)** *Per2*, **(E)** *Cry1*, **(F)** *Cry2*, **(G)** *Rev-erbα* and **(H)** *Rorc*. At each time point, Ctrl-ALF: n=6-7; Ctrl-ATRF: n=4-5; *db/db*-ALF: n=4-5, *db/db*-ATRF: n=3-5. *, p<0.05; **, p<0.01; ***, p<0.001; ns, not significant.

Figure 3.2.4.5

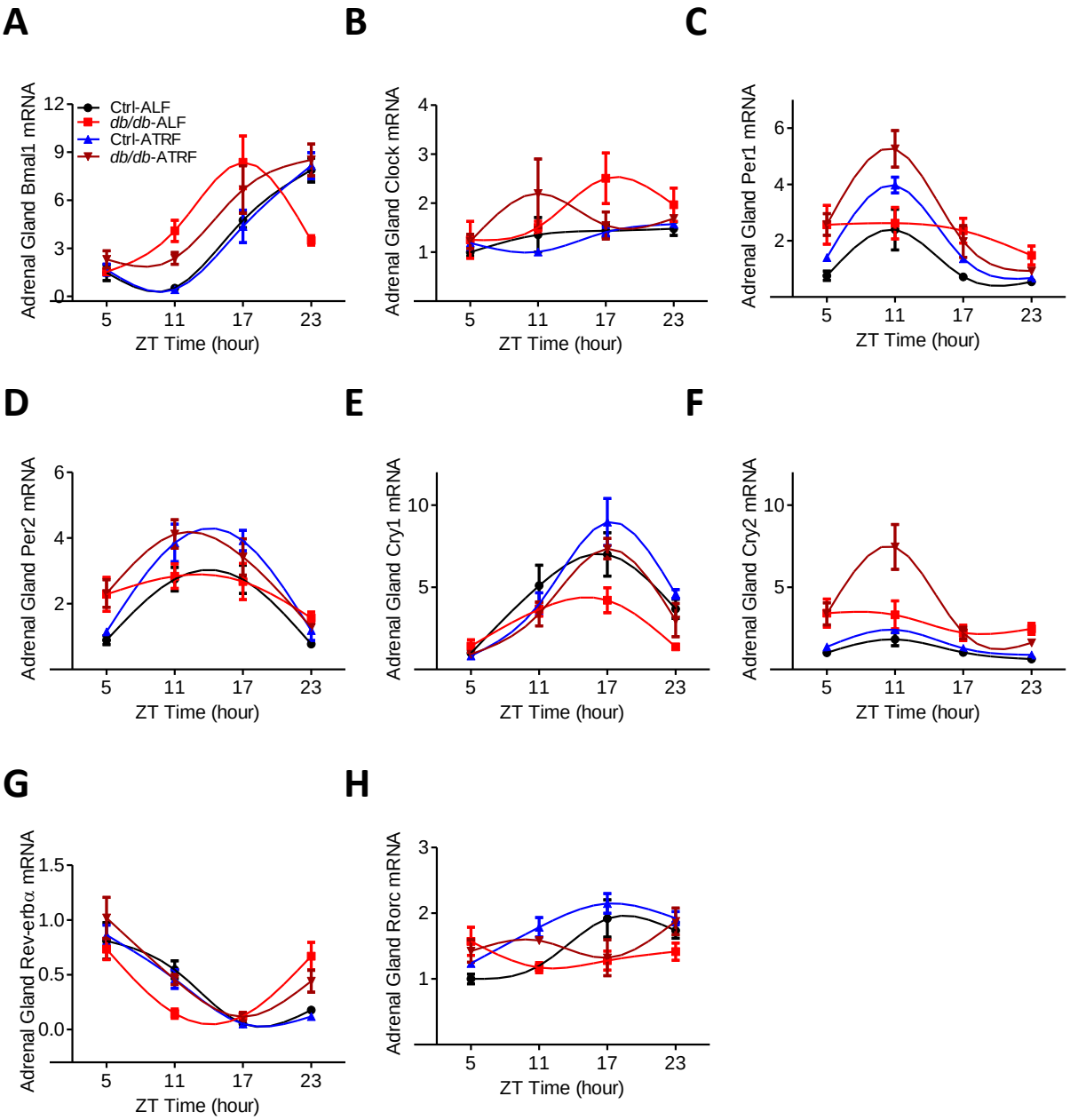

Figure 3.2.4.5 The mRNA expression of clock genes in the adrenal gland. Control and *db/db* mice with ALF or ATRF were euthanized at ZT5, ZT11, ZT17 and ZT23 and the time-of-day mRNA expressions of the clock genes were detected by real-time PCR. **(A)** *Bmal1*, **(B)** *Clock*, **(C)** *Per1*, **(D)** *Per2*, **(E)** *Cry1*, **(F)** *Cry2*, **(G)** *Rev-erbα* and **(H)** *Rorc*. At each time point, Ctrl-ALF: n=6-7; Ctrl-ATRF: n=4-5; *db/db*-ALF: n=4-5, *db/db*-ATRF: n=3-5. *, p<0.05; **, p<0.01; ***, p<0.001; ns, not significant.

Table 3.2.4.1 ATRF recovered the amplitude and acrophase of *Bmal1* mRNA time-of-day expressions in multiple tissues.

Tissue	Groups	Amplitude	Acrophase	ADJ.P
Liver	Ctrl-ALF	1.1153	23.08	1.32E-08
	Ctrl-ATRF	1.1393	23.06	9.94E-05
	db/db-ALF	**0.9791**	**17.48**	1.99E-06
	db/db-ATRF	1.6204	22.44	7.13E-06
Mesentery Artery	Ctrl-ALF	0.6011	1.4	5.80E-06
	Ctrl-ATRF	0.9758	0.81	4.85E-07
	db/db-ALF	**0.375**	**21.62**	0.013453
	db/db-ATRF	0.7463	0.4	9.94E-05
Kidney	Ctrl-ALF	2.0724	22.59	5.61E-09
	Ctrl-ATRF	2.0812	22.89	1.49E-05
	db/db-ALF	**1.7639**	**17.46**	0.000556
	db/db-ATRF	2.8298	22.23	5.55E-06
Heart	Ctrl-ALF	0.8144	0.91	1.43E-11
	Ctrl-ATRF	0.9091	0.64	3.73E-08
	db/db-ALF	**0.5121**	**19.4**	0.00265
	db/db-ATRF	0.9676	23.42	1.49E-05
Adrenal gland	Ctrl-ALF	4.0315	21.4	5.27E-11
	Ctrl-ATRF	4.0976	21.72	4.85E-07
	db/db-ALF	**3.4306**	**16.67**	0.000228
	db/db-ATRF	3.7691	20.64	0.015956

The amplitude, acrophase and ADJ.P of *Bmal1* mRNA expressions in the liver, mesentery arteries, kidney, heart and adrenal gland.

3.2.4.2 Bmal1 is partially required for ATRF initiated protection of BP daily rhythm

To further test whether the clock gene *Bmal1* is required for the protection of BP daily rhythm by ATRF, we investigated the BP daily rhythm in an inducible global Bmal1 knockout mouse model (*iG-Bmal1-KO*). As shown in Fig. 3.2.4.6A and 3.2.4.6B, the normal BP daily rhythm was abolished by global deletion of *Bmal1*, which is associated with a complete loss of food intake rhythm (Fig. 3.2.4.7). Imposing ATRF resulted in partial recovery of BP daily rhythm (Fig. 3.2.4.6A and 3.2.4.6B). Cosine analysis revealed the BP daily oscillation amplitude (Fig. 3.2.4.6C), robustness (Fig. 3.2.4.6D) and acrophase (Fig. 3.2.4.6E) in the *iG-Bmal1-KO* mice under ATRF condition remained significantly suppressed when compared with the control mice under ALF, indicating that Bmal1 is essential for the normal BP daily rhythm.

Since the daily rhythm of locomotor acitivity was associtated with ATRF induced protection of BP oscillation, we tested the locomotor activity in the *iG-Bmal1-KO* mice before and after ATRF. As expected, the locomotor activity daily rhythm was abolished in the *iG-Bmal1-KO*-ALF mice; however, ATRF restored the oscillation almost completely (Fig. 3.2.4.8A and 3.2.4.8B). The locomotor activity oscillation amplitude (Fig. 3.2.4.8C) and robustness (Fig. 3.2.4.8D) were restored to the levels that are similar to the Flox mice, although there was still a shift in the acrophase (Fig. 3.2.4.8E). As ANS plays a critical role in ATRF induced protection of BP daily rhythm, we then tested whether the time-of-day variations in the HR, BRS and urinary NE excretion are recoved by ATRF in the *iG-Bmal1-KO* mice. As shown in Fig. 3.2.4.9A-3.2.4.9F, the deletion of *Bmal1*

abolished oscillations of HR (Fig. 3.2.4.9A-3.2.4.9E), BRS (Fig. 3.2.4.9F) and attenuated the day and night difference in the urinary NE excretion (Fig. 3.2.4.9G) in the *iG-Bmal1-KO*-ALF mice. When the *iG-Bmal1-KO* mice were fed on the ATRF regimen, the oscillations of HR (Fig. 3.2.4.9A-3.2.4.9E) and BRS (Fig. 3.2.4.9F) were completely restored, except the overall level of HR was lower in the *iG-Bmal1-KO*-ATRF mice than the Flox-ALF mice (Fig. 3.2.4.9A and 3.2.4.9B). On the contrary, no significant difference of NE was found in the *iG-Bmal1-KO* mice between ALF and ATRF in either the light- or dark-phase (Fig. 3.2.4.9G).

Figure 3.2.4.6

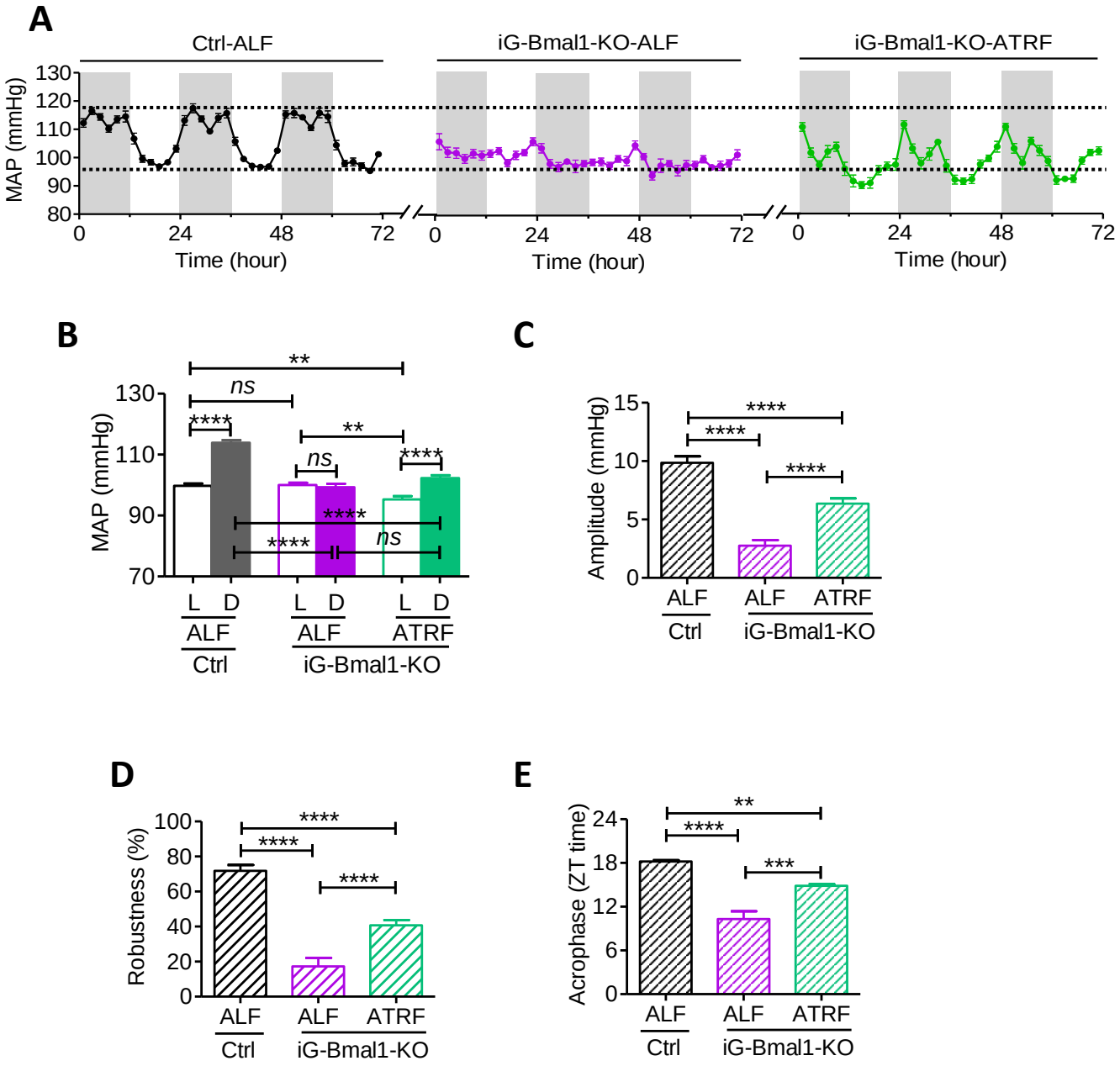

Figure 3.2.4.6 ATRF partially restored the abolished MAP oscillation in the *iG-Bmal1-KO* mice. (A) 2-hour average MAP in the Flox-ALF, *iG-Bmal1-KO*-ALF and *iG-Bmal1-KO*-ATRF mice. **(B)** 12-hour average MAP during the light-phase (L) and dark-phase (D). **(C-E)** The amplitude **(C)**, robustness **(D)** and acrophase **(E)** of MAP oscillation. n=12. **, p<0.01; ***, p<0.001; ****, P<0.0001; ns, not significant.

Figure 3.2.4.7

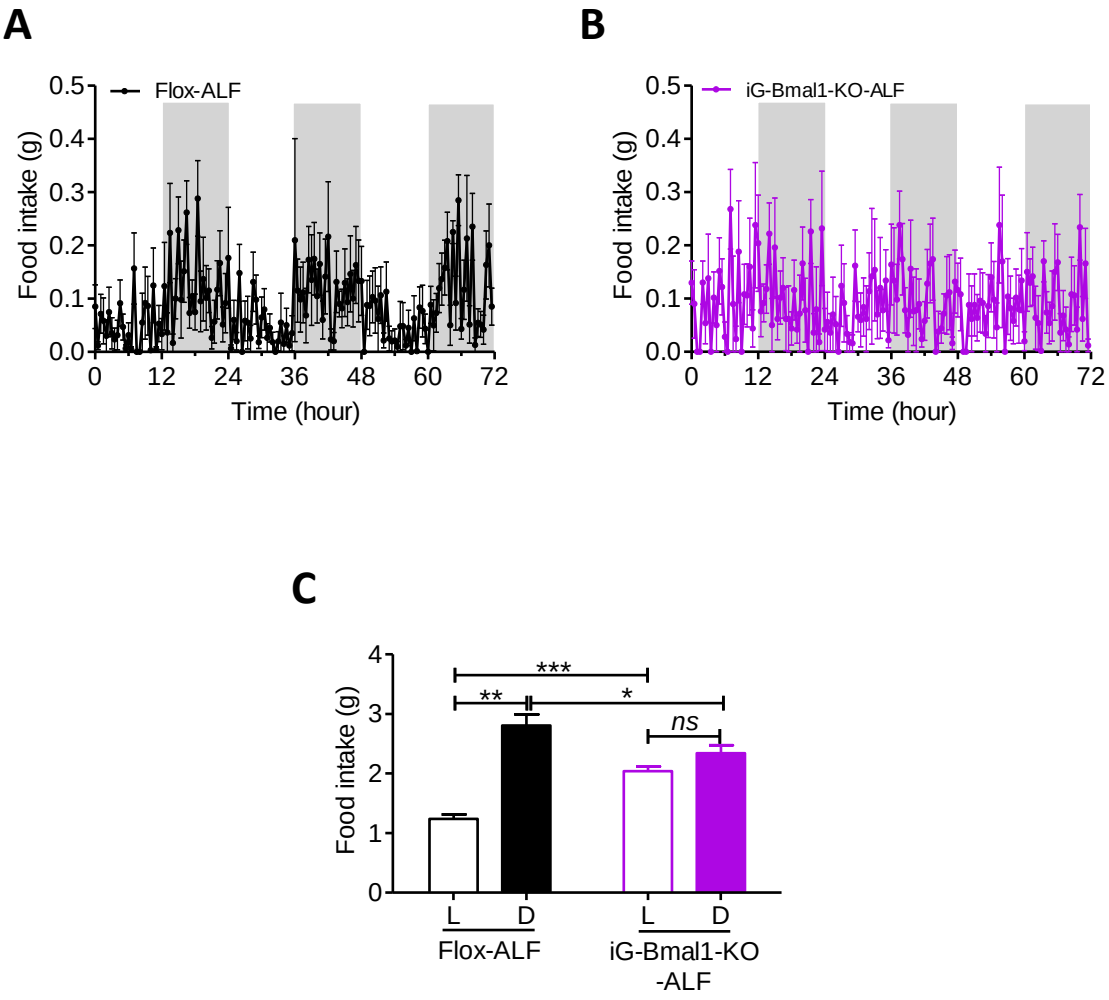

Figure 3.2.4.7 The daily rhythm in the food intake was disrupted in the *iG-Bmal1-KO* mice. **(A and B)** The daily rhythm of food intake measured using indirect calorimetry in the Flox-ALF **(A)** and *iG-Bmal1-KO*-ALF **(B)** mice. The grey box indicates the dark-phase. **(C)** 12-hour average food intake during the light- (L) and dark-phase (D). n=5-6. *, p<0.05; **, p<0.01; ***, p<0.001; ns, not significant.

Figure 3.2.4.8

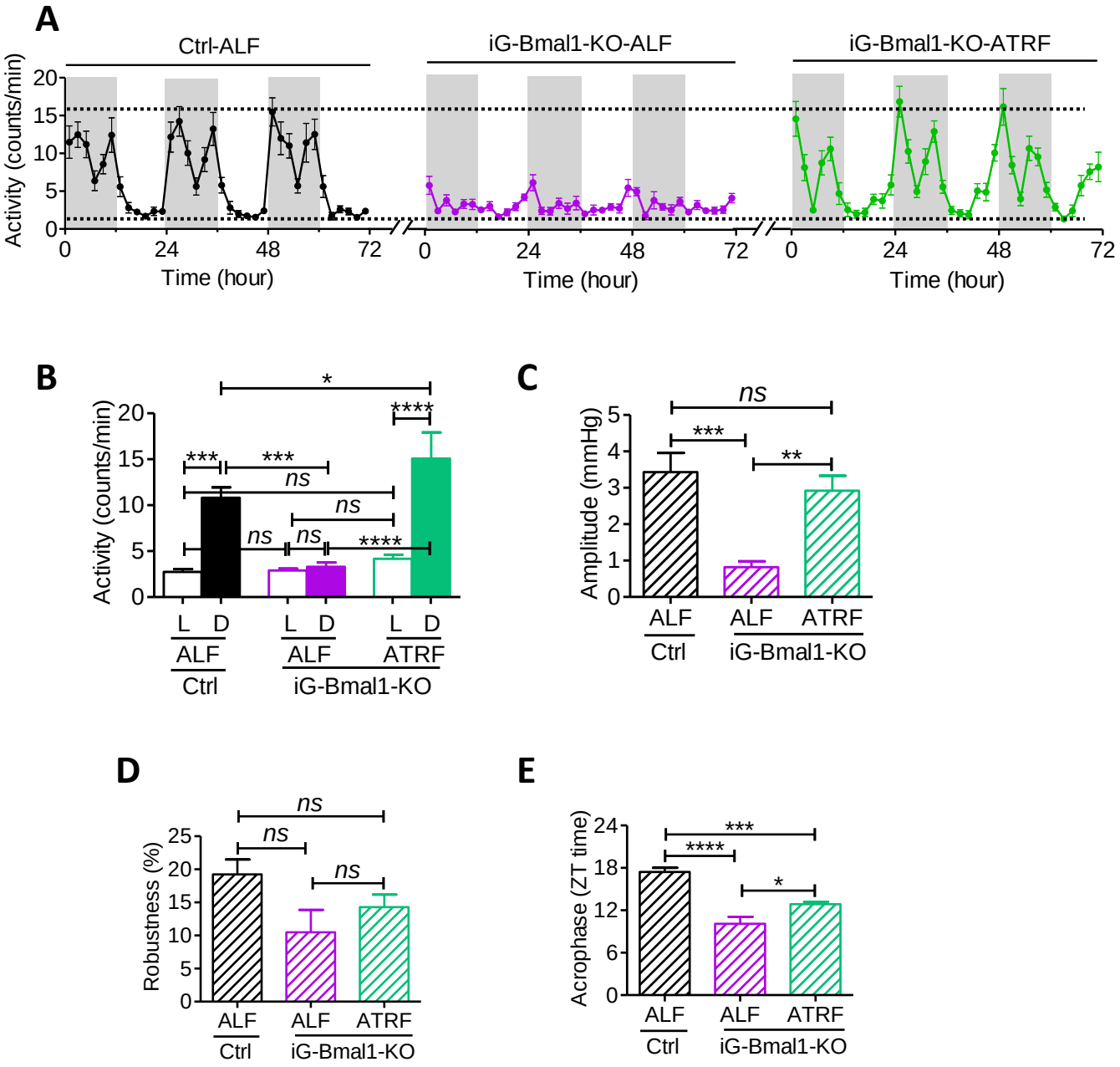

Figure 3.2.4.8 ATRF completely restored the abolished locomotor activity oscillation in the *iG-Bmal1-KO* mice. **(A)** 2-hour average locomotor activity in the Flox-ALF, *iG-Bmal1-KO*-ALF and *iG-Bmal1-KO*-ATRF mice. **(B)** 12-hour average locomotor activity during the light-phase (L) and dark-phase (D). **(C-E)** The amplitude **(C)**, robustness **(D)** and acrophase **(E)** of locomotor activity oscillation. n=12. *, p<0.05; **, p<0.01; ***, p<0.001; ****, P<0.0001; ns, not significant.

Figure 3.2.4.9

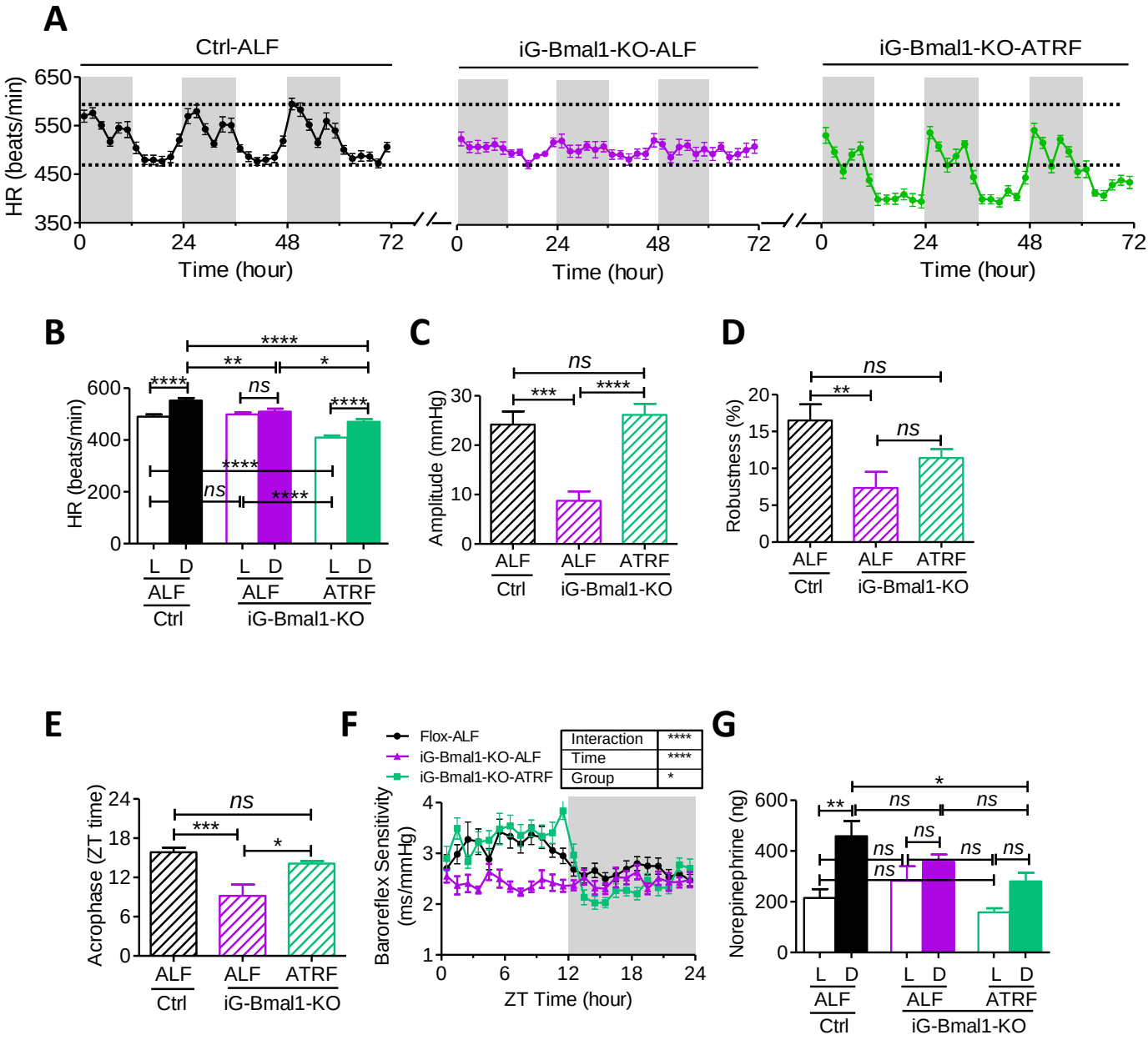

Figure 3.2.4.9 The effects of ATRF on the autonomic function in the *iG-Bmal1-KO* mice. **(A)** 2-hour average HR in the Flox-ALF, *iG-Bmal1-KO*-ALF and *iG-Bmal1-KO*-ATRF mice. **(B)** 12-hour average HR during the light-phase (L) and dark-phase (D). **(C-E)** The amplitude **(C)**, robustness **(D)** and acrophase **(E)** of HR oscillation. **(F)** The hourly BRS. **(G)** The urinary NE excretion during the light- (L) and dark-phase (D). n=6-12. *, p<0.05; **, p<0.01; ***, p<0.001; ****, P<0.0001; ns, not significant.

3.2.4.3 ATRF improves daily oscillations of RER and EE in mice lacking *Bmal1*.

We have demonstrated that the oscillations of RER and EE in the *db/db* mice were improved with ATRF. To determine if the improvements were dependent upon the presence of require Bmal1, we monitored the RER and EE using indirect calorimetry system in Flox and *iG-Bmal1-KO* mice before and after ATRF. As shown in Fig. 3.2.4.10A, the RER daily oscillation was altered in the *iG-Bmal1-KO*-ALFmice with an increased light-phase RER (Fig. 3.2.4.10B). ATRF significantly improved the oscillation of RER in both the Flox-ATRF and *iG-Bmal1-KO*-ATRF mice (Fig. 3.2.4.10B). Interestingly, the oscillation of RER in *iG-Bmal1-KO*-ATRF mice is even more robust than the Flox-ATRF mice (Fig. 3.2.4.10B), which was reflected by greater amplitude in the iG-*Bmal1-KO*-ATRF than Flox-ATRF mice (Fig. 3.2.4.10E). The advanced acrophase of RER oscillation in the *iG-Bmal1-KO*-ALF mice was corrected (Fig. 3.2.4.10G). Of note, the 24-h RER in the *iG-Bmal1-KO* mice was higher than the Flox mice in both feeding regimens (Fig. 3.2.4.10D). Regarding EE, the deletion of *Bmal1* altered the EE oscillation (Fig. 3.2.4.11A), characterized by increased light-phase EE (Fig. 3.2.4.11C). ATRF increased EE oscillation in both the Flox and *iG-Bmal1-KO* mice (Fig. 3.2.4.11B). Cosinor analysis revealed the amplitude and robustness of EE oscillation was enhanced in both strains of mice after ATRF compared to ALF; however, the amplitude and robustness was still significantly lower in the i*G-Bmal1-KO*-ATRF mice than that of the Flox-ATRF mice (Fig. 3.2.4.10E and F). The advanced acrophase was corrected in the *iG-Bmal1-KO*-ALF mice (Fig. 3.2.4.10G). No differences were found in 24-h EE between Flox and *iG-Bmal1-KO* mice with either ALF or ATRF (Fig. 3.2.4.10D).

Figure 3.2.4.10

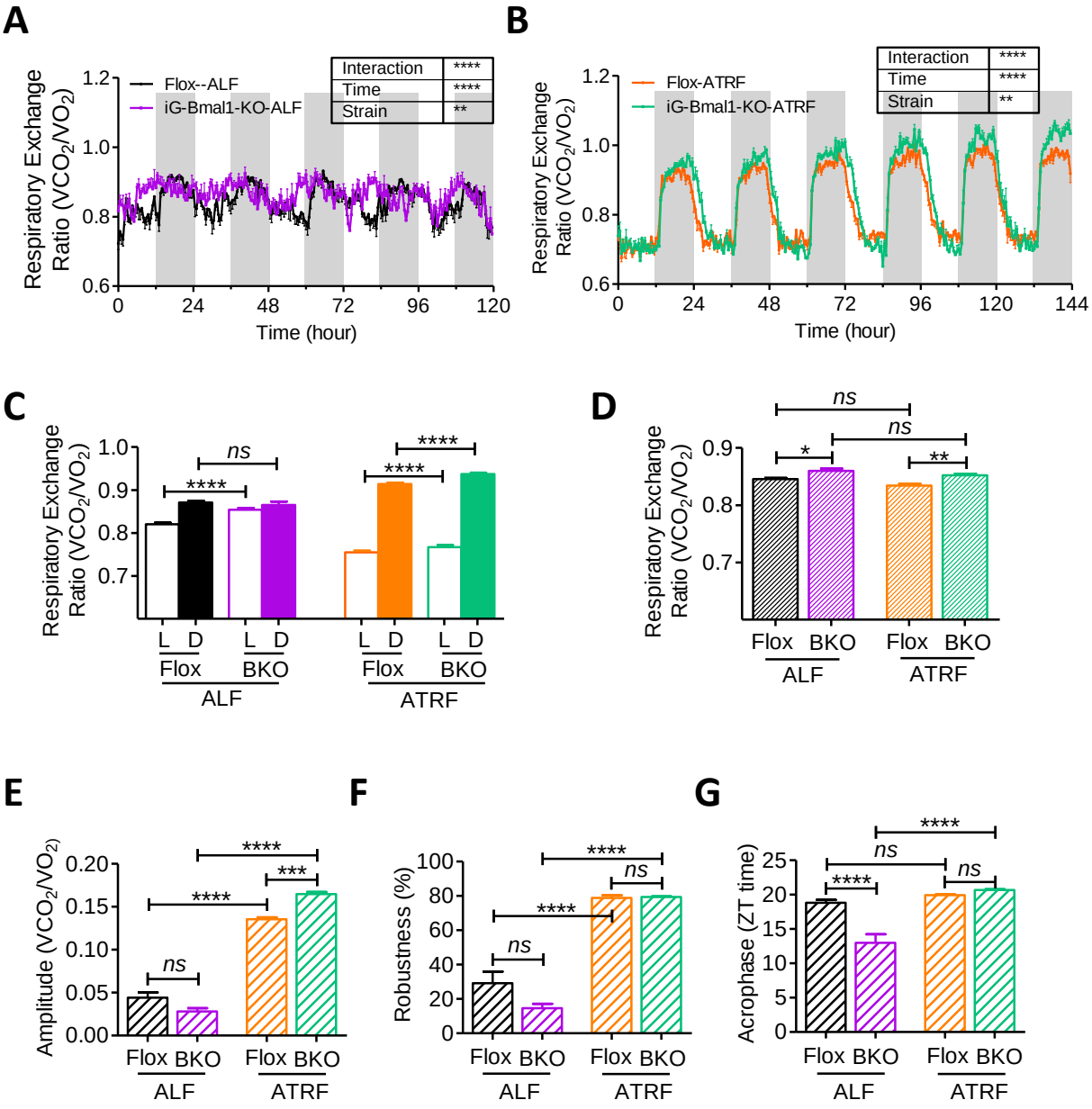

Figure 3.2.4.10 ATRF drives robust daily rhythm of respiratory exchange ratio (RER) in the *iG-Bmal1-KO* mice. RER was recorded by indirect calorimetry. (**A** and **B**) The 72-hour recording of the RER in the Flox and *iG-Bmal1-KO* mice with ALF (**A**) or ATRF (**B**). The light grey box indicates the dark-phase. **(C)** 12-hour average RER during the light-phase (L) and dark-phase (D). **(D)** Daily RER. **(E-G)** The amplitude **(E)**, robustness **(F)** and acrophase **(G)** of RER oscillation; Flox groups: n=6; *iG-Bmal1-KO* groups: n=5. *, p<0.05; **, p<0.01; ***, p<0.001; ****, P<0.0001; ns, not significant.

Figure 3.2.4.11

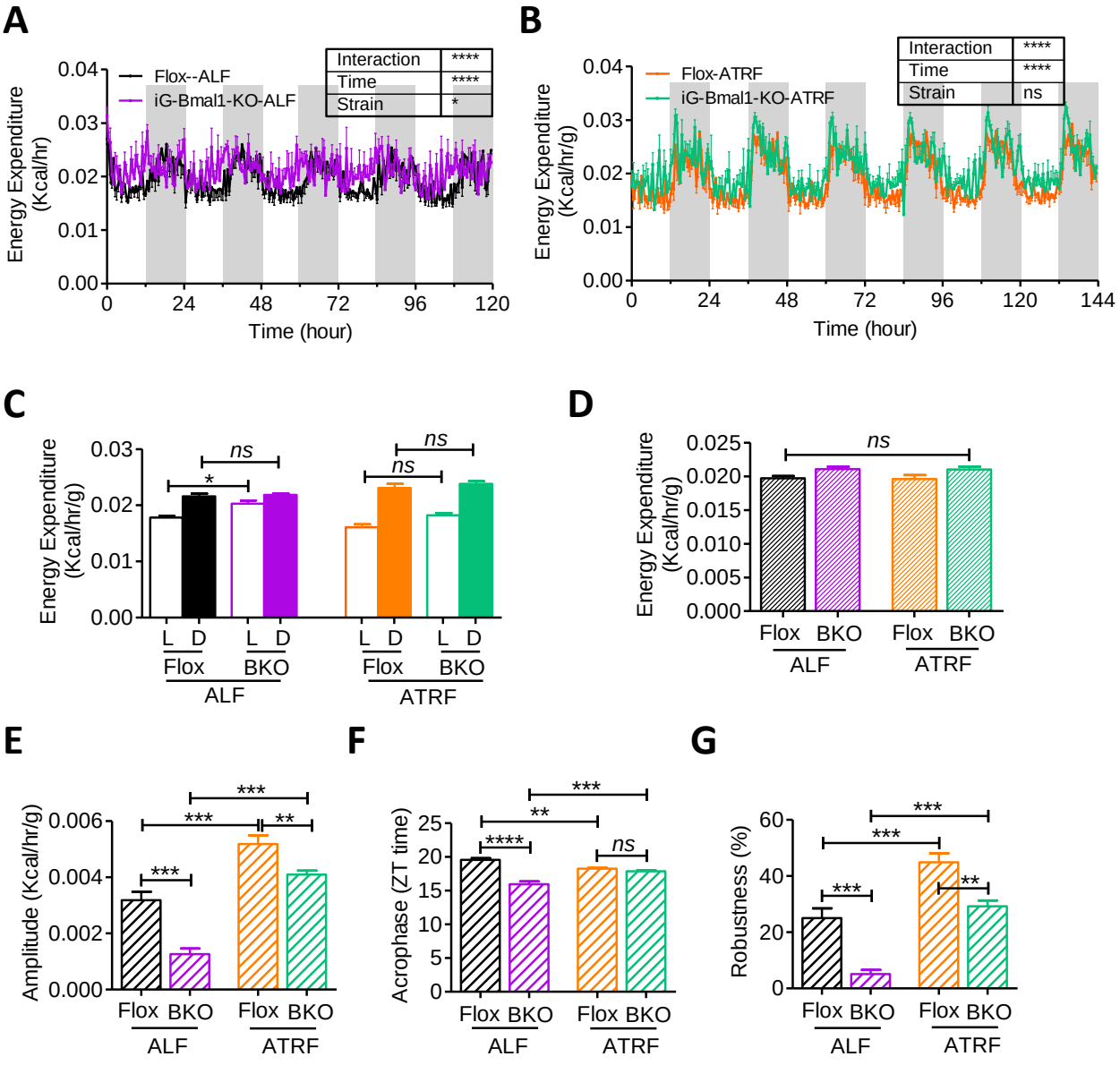

Figure 3.2.4.11 ATRF improves the daily rhythm of energy expenditure (EE) in the *iG-Bmal1-KO* mice. EE was recorded by indirect calorimetry. The plotted EE is normalized to lean mass in each mouse. (**A** and **B**) The 72-hour recording of the RER in the Flox and *iG-Bmal1-KO* mice with ALF (**A**) or ATRF (**B**). The light grey box indicates the dark-phase. **(C)** 12-hour average EE during the light-phase (L) and dark-phase (D). **(D)** Daily EE. **(E-G)** The amplitude **(E)**, robustness **(F)** and acrophase **(G)** of EE oscillation; Flox groups: n=6; *iG-Bmal1-KO* groups: n=5. *, p<0.05; **, p<0.01; ***, p<0.001; ****, P<0.0001; ns, not significant.

3.2.5 Determination of whether the 8-hour ATRF induced protection of BP daily rhythm is attributable to time-restriction or calorie-restriction.

3.2.5.1 8-hour ATRF decreased total calorie intake in the *db/db* mice.

When calculating the total food intake in the control and *db/db* mice on the ALF or ATRF regimens, we found the *db/db*-ALF mice consumed a significant greater amount of food than the Ctrl-ALF mice (Fig 3.2.5.1A). The 8-hour ATRF had no effect on the amount of daily food intake in the control mice (Ctrl-ALF vs. Ctrl-ATRF, Fig 3.2.5.1A); however, it significantly decreased the daily food consumption in the *db/db* mice (*db/db*-ALF vs. *db/db*-ATRF, Fig 3.2.5.1A). Since calorie restriction is known to be beneficial to the health (Heilbronn and Ravussin 2003), it is important to understand whether time-restriction itself is able to induce the protective effects.

3.2.5.2 The 12-hour ATRF restored the BP daily rhythm in the *db/db* mice without reducing calorie intake.

In order to test whether time-restriction alone restores the BP daily rhythm in the *db/db* mice, we increased the food available time from 8-hour to 12-hour (this experiment was done by Wen Su). As shown in Fig. 3.2.5.1B, the food intake in the *db/db* mice with 12-hour ATRF was comparable to that on the ALF regimen. Importantly, we found the disrupted BP daily rhythm in the *db/db* mice can be recovered with 12-hour ATRF (Fig. 3.2.5.2, this experiment was done by Wen Su), indicating the time-restriction without reducing calorie intake is able to restore BP daily rhythm.

Figure 3.2.5.1

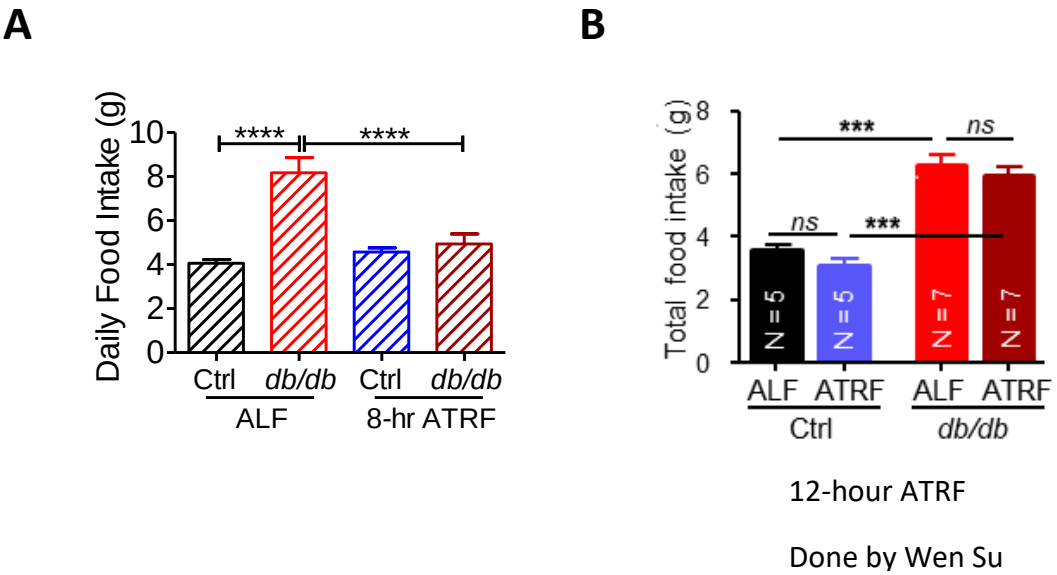

Figure 3.2.5.1 The daily food intake in the control and *db/db* mice with ALF or ATRF. **(A)** Daily food intake in the control and *db/db* mice with ALF or 8-hour ATRF. **(B)** Daily food intake in the control and *db/db* mice with ALF or 12-hour ATRF (done by Wen Su). n=5-10. ***, p<0.001; ****, P<0.0001; ns, not significant.

Figure 3.2.5.2

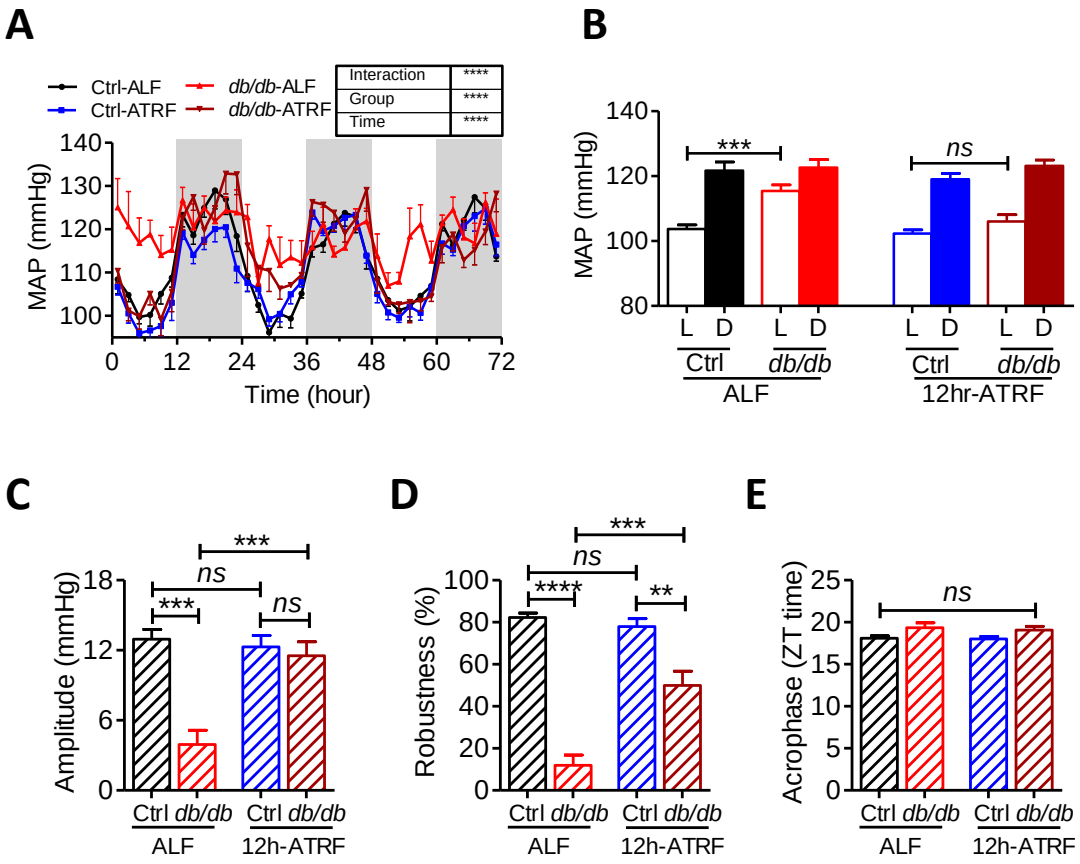

Figure 3.2.5.2 The 12-hour ATRF restored the BP daily rhythm in the *db/db* mice (done by Wen Su). (A) 2-hour average MAP in the control and *db/db* mice with ALF or 12hr-ATRF. **(C)** 12-hour average MAP during the light-phase (L) and dark-phase (D). **(D-F)** The amplitude **(D)**, robustness **(E)** and acrophase **(F)** of MAP oscillation. n=5; *db/db*-ATRF: n=5. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001; ns, not significant.

3.2.6 Investigation of the effects of aldosterone and phenylephrine on the clock gene expression.

3.2.6 Aldosterone induced time-dependent phase shift in Per2::luc expression in the aorta and mesentery artery explants.

We have shown that the altered time-of-day expressions of clock genes mRNA were restored in the *db/db* mice with ATRF. However, the underlying mechanisms for this restoration are not clear. Although the ATRF improved the time-of-day variations in the urinary excretion of glucocorticoid (corticosterone), mineralocorticoid (aldosterone) and sympathetic transmitter (NE) in the *db/db* mice; and although glucocorticoid is a known factor to induce the phase shift of clock genes, it is not known whether the mineralocorticoid and sympathetic neurotransmitter also induce the phase shift of clock gene. To determine the effect of mineralocorticoid and sympathetic neurotransmitter on the clock gene, we treated the explants of aorta and mesentery arteries (MA) isolated from the *mPer2*^{Luc} mice with 100nM aldosterone and 100nM phenylephrine (PE) at 3-hour interval across 24 hours and generated a phase response curve (PRC). We also treated the aorta and MA with 1nM dexamethasone as positive controls. As expected, dexamethasone induced a time-dependent phase shift of Per2::luc in the aorta and MA (Fig. 3.2.6A and 3.2.6B). In both the aorta and MA, the phase of Per2::luc advanced when the dexamethasone was added at 0-hour, 3-hour, and 21-hour relative to the Per2::luc peak, delayed at 6-hour, 9-hour and 12-hour and shifted relative less at 15-hour and 18-hour (Fig. 3.2.6A and 3.2.6B). To our surprise, aldosterone also induced

a similar time-dependent phase shift as dexamethasone in the aorta and MA (Fig. 3.2.6C and 3.2.6D). However, the PE did not induce a clear PRC in either the aorta or MA (Fig. 3.2.6E and 3.2.6F).

Figure 3.2.6

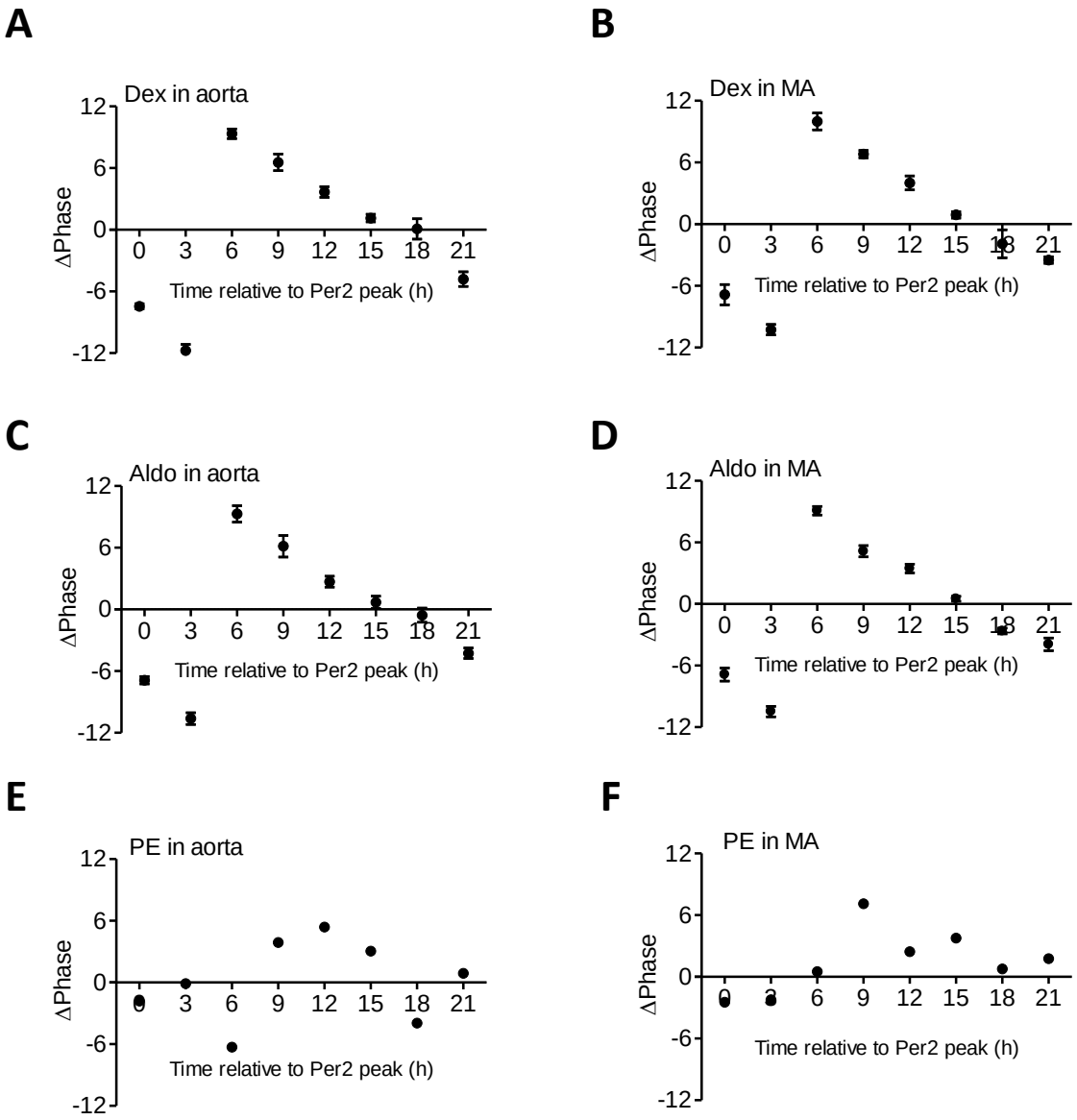

Figure 3.2.6 The phase response curve (PRC) of dexamethasone, aldosterone and phenylephrine on the Per2::luc expression in the aorta and mesentery arteries (MA) explants. 1nM dexamethasone (Dex), 100Nm aldosterone (aldo) and phenylephrine (PE) was added at indicated times relative to the Per2::luc peak. **(A and B)** The PRC of dex on the aorta and MA. **(C and D)** The PRC of aldo on the aorta and MA. **(E and F)** The PRC of PE on the aorta and MA.

CHAPTER IV. GENERAL DISCUSSION

4.1 Summary

In project 1, we generated a novel type 2 diabetic *db/db-mPer2*^{Luc} mouse model to explore the role of clock genes in the disruption of BP circadian rhythm in diabetes. Using this model, we found: 1) the *db/db-mPer2*^{Luc} mice are obese, hyperglycemic, and glucose-intolerant and thus resemble type 2 diabetic patients; 2) the *db/db-mPer2*^{Luc} mice are normotensive but exhibite a compromised BP daily rhythm, which is associated with the disruption of daily rhythms in baroreflex sensitivity, locomotor activity, and metabolism, but not heart rate or food and water intake; 3) a desynchrony of peripheral tissue oscillation is caused by various extents of phase advances of the mPer2 oscillation *ex vivo* of many tissues except the central SCN pacemarker; 4) the similar desynchrony of mPer2 phase is also observed *in vivo* in the kidney, liver, and submandibular gland.

In project 2, we tested the effects of active-time restricted feeding on BP circadian rhythm to explore a potential theraputic strategy for the disrupted BP circadian rhythm in diabetes. The major findings are: 1) the disruption of BP daily rhythm is associated with altered food intake rhythm in the *db/db* mice; 2) inactive-time restricted feeding (ITRF) alters BP daily rhythm in the healthy C57BL/6J mice; 3) active-time restricted feeding (ATRF) prevents and restores the disruption of BP daily rhythm in the *db/db* mice; 4) ATRF has minimal effects on body weight, body composition, blood glucose, plasma insulin and fatty acid, and insulin sensitivity in the *db/db* mice; 5) ATRF improves the rhythms of energy metabolism, sleep-wake cycle, BP-regulatory hormones

and of the autonomic nervous system in the *db/db* mice; 6) ATRF improves the time-of-day variations in the mRNA expression of clock genes, especially *Bmal1*; 7) Bmal1 is partially required for ATRF to protect the BP circadian rhythm.

4.2 The *db/db-mPer2^{Luc}* mouse models

The *db/db* mice have been used extensively for studying the pathogenesis of obesity and diabetes. Interestingly, the diabetic phenotype of *db/db* mice varies depending on the genetic background. The hyperglycemia is more severe when the leptin receptor mutation is expressed on a C57BL/KsJ background than on a C57BL/6J background (Leiter, Coleman et al. 1981). Probably because of its severe diabetic phenotype, the *C57BL/KsJ-db/db* mice are most commonly used. Interestingly, the *db/db-mPer2^{Luc}* mice have significantly higher body weight than the age-matched *C57BL/KsJ-db/db* mice (65.72 ± 1.38 g *vs.* 47.07 ± 1.05 g; n=12; P<0.001). However, the hyperglycemia in the *db/db-mPer2^{Luc}* mice is much less severe than that in the *C57BL/KsJ-db/db* mice (320.3 ± 18.46 mg/dl *vs.* 585.9 ± 9.163 mg/dl; n=12; P<0.001). These results suggest that while the *C57BL/KsJ-db/db* mice are more like the maturity-onset condition of diabetic patients with obesity and marked hyperinsulinemia, while the *db/db-mPer2^{Luc}* mice more closely resemble the *C57BL/6J-db/db* mice (Hummel, Coleman et al. 1972) and more closely mimic diabetic patients with obesity, moderate hyperglycemia, and glucose intolerance.

In agreement with the severity of the diabetic phenotypes, the *db/db-mPer2^{Luc}* mice are normotensive, which contrasts with the hypertensive *C57BL/KsJ-db/db* mice

(Park, Bivona et al. 2008, Su, Guo et al. 2008, Goncalves, Tank et al. 2009, Senador, Kanakamedala et al. 2009). Despite this difference, the *db/db-mPer2*^{Luc} mice also exhibite non-dipping BP, similar to the *C57BL/KsJ-db/db* mice (Park, Bivona et al. 2008, Su, Guo et al. 2008, Goncalves, Tank et al. 2009, Senador, Kanakamedala et al. 2009), which is typified by a lack of BP fall during the inactive light phase.

4.3 Mechanisms underlying diabetes induced BP circadian rhythm disruption

As described in Chapter 1.2.4, the BP circadian rhythm is regulated by multiple factors. However, the mechanisms by which diabetes induces BP circadian rhythm disruption are not well understood. Using the *db/db-mPer2*^{Luc} mice, we have demonstrated that, in the absence of any change in the phase of SCN, the phase of the mPer2 protein daily oscillation was advanced to various extents in a tissue-specific manner in peripheral tissues. This finding was revealed by monitoring *mPer2* protein oscillation in real-time in our novel *db/db-mPer2*^{Luc} mice. In agreement with the important role of *Bmal1* in the renal, smooth muscle, and fat tissues in the regulation of BP rhythm under physiological conditions (Tokonami, Mordasini et al. 2014, Xie, Su et al. 2015, Chang, Xiong et al. 2018), we found that the phase of mPer2 protein oscillation was advanced in the WAT, kidney, MA, and aorta from the *db/db-mPer2*^{Luc} mice to 4.6, 2.21, 1.71, and 0.99 hours, respectively. These results are also consistent with previous studies reporting mPer2 mRNA daily oscillation was altered in these tissues from *db/db* mice (Su, Guo et al. 2008, Caton, Kieswich et al. 2011, Su, Xie et al. 2012, Nernpermpisooth, Qiu et al. 2015). In contrast, it was surprising that the phase of mPer2 protein daily oscillation in the adrenal gland, an important source of hormones that

regulate the BP circadian rhythm, was not significantly changed in the *db/db-mPer2*^{Luc} mice relative to control mice. It was also surprising that the phase of mPer2 protein daily oscillation in the thymus, an important organ that produces T lymphocytes, was advanced up to 4.23 hours. This result is consistent with a previous report that T lymphocytes play a critical role in angiotensin II-induced hypertension (Guzik, Hoch et al. 2007), and suggests that clock genes in T lymphocytes may be crucially involved in the disruption of the BP circadian rhythm in diabetes.

It has been long believed that the BP circadian rhythm, just like other physiological and behavioral circadian rhythms, is mostly controlled by the master pacemaker in the SCN. However, the current study demonstrated that the phase of mPer2 protein daily oscillation was not significantly altered in the SCN tissue from the *db/db-mPer2*^{Luc} mice compared to controls. These results confirm previous reports that there is little or no change of the SCN mPer2 mRNA daily oscillation in *db/db* mice (Kudo, Akiyama et al. 2004, Nernpermpisooth, Qiu et al. 2015, Grosbellet, Dumont et al. 2016). These results are also consistent with previous reports that peripheral clock gene oscillations are altered in some tissues from diabetic patients (Ando, Takamura et al. 2009, Pappa, Gazouli et al. 2013) and *db/db* mice (Kudo, Akiyama et al. 2004, Caton, Kieswich et al. 2011, Su, Xie et al. 2012, Nernpermpisooth, Qiu et al. 2015). In addition, in *db/db* mice, the alternations of peripheral clock expression occur as early as 6-8 weeks of age (Kudo, Akiyama et al. 2004, Caton, Kieswich et al. 2011) whereas the disruption of BP circadian rhythm is not detectable in *db/db* mice until 11-weeks or older, indicating that peripheral clock impairment precedes the disruption of the BP

circadian rhythm. Taken together, these results suggest that the peripheral oscillators, in contrast to the master SCN pacemaker, are strongly affected by diabetes and may ultimately be responsible for the disruption of BP circadian rhythm.

Besides the desynchrony of peripheral clocks, we also examined other factors that may contribute to diabetes associated BP circadian rhythm disruption. The results from both the *db/db-mPer2*^{Luc} and *db/db* mice demonstrate for the first time that the disrupted BP daily rhythm in the diabetic mice is associated with loss of daily rhythm in spontaneous baroreflex sensitivity. The baroreflex is a critical mechanism for maintaining BP homeostasis, and baroreflex sensitivity exhibits daily variations in humans (Hossmann, Fitzgerald et al. 1980, Di Rienzo, Parati et al. 2001). Interestingly, the observed loss of daily variation in baroreflex sensitivity resembles the loss of baroreflex sensitivity daily variation we reported in the smooth muscle *Bmal1* knockout mice (Xie, Su et al. 2015); this indicates that loss of baroreflex sensitivity daily variation in the diabetic mice may contribute to the decreased nocturnal BP decline phenotype.

The sleep-wake cycle is critical to BP circadian rhythm. In this dissertation, we have demonstrated that *db/db* mice have decreased daytime sleep and increased nighttime sleep compared to control mice. The sleep bout lengths are significantly shortened during the daytime. Previous study also revealed an altered diurnal rhythm of sleep time and increased sleep fragmentation in *db/db* mice (Laposky, Bradley et al. 2008). This earlier study demonstrated that the NREM sleep is increased during the nighttime; the REM sleep is increased during the daytime and decreased during the

night. Although the sleep stages cannot be distinguished in the present dissertation, the overall decreased daytime sleep may contribute to the increase of BP during this period. In addition, the shortened sleep bout length during the daytime suggests increased sleep fragmentation. In humans, sleep fragmentation index is associated with increased awake arterial BP (Morrell, Finn et al. 2000). Although these are no investigations on sleep fragmentation and BP circadian rhythm, it is possible that sleep fragmentation is associated with abnormal BP circadian rhythm since sleep fragmentation is a major characterization of obstructive sleep apnea (OSA) (Kimoff 1996). Previous study has demonstrated that the severity of OSA is an independent predictor of BP circadian rhythm (Nabe, Lies et al. 1995). Therefore, changes in sleep fragmentation may contribute to the non-dipping BP in *db/db* mice.

The daily rhythm of locomotor activity is abolished in both the *db/db-mPer2*^{Luc} and *db/db* mice. This finding is consistent with previous reports that the daily locomotor activity rhythm is lost in the *C57BL/KsJ-db/db* mice (Su, Guo et al. 2008). We speculate that the loss of locomotor activity rhythm in both strains of *db/db* mice results from their severe obesity, i.e., that they are too heavy to move around. Although the loss of locomotor activity rhythm may potentially contribute to the loss of the BP daily rhythm, the loss of locomotor activity mainly occurred during the night in the *db/db-mPer2*^{Luc} mice, whereas the loss of BP dipping occurred during the day. Therefore, it is unlikely that the loss of locomotor activity accounts for the disrupted BP daily rhythm in the diabetic mice.

The neuroendocrine system is prominently involved in the generation of the BP circadian rhythm. We have determined the mRNA expression of renin-angiotensin-system (RAS) at four time points in control and *db/db* mice. We found no apparent time-of-day variations in the mRNA expression of RAS in either control or *db/db* mice, suggesting that the variation of BP may not be regulated at transcription level of RAS. Numerous studies have demonstrated that plasma renin activity (PRA) exhibits circadian variation (Gordon, Wolfe et al. 1966, KATZ, ROMFH et al. 1975, Modlinger, Sharif-Zadeh et al. 1976, Cugini, Manconi et al. 1980, Beilin, Deacon et al. 1983, Cugini, Salandi et al. 1983, Kawasaki, Uezono et al. 1983, Stern, Sowers et al. 1986, Kawasaki, Cugini et al. 1990, Portaluppi, Bagni et al. 1990, Brandenberger, Follenius et al. 1994). Therefore, it is possible the BP circadian rhythm is achieved by the oscillation of PRA. Further experimentation on the oscillation of PRA in *db/db* mice is needed. We observed apparent daily variations in urinary aldosterone and corticosterone excretion in *db/db* mice. However, due to limited sample collection in control mice, we were not able to measure aldosterone and corticosterone in control mice. Consequently, it is not known whether there is a difference between control and *db/db* mice regarding the variations of urinary aldosterone and corticosterone excretion. For the autonomic nervous system (ANS), we have reported indirect, but compelling evidence that *db/db* mice have increased sympathetic nervous activity and decreased parasympathetic nervous activity compared to control mice. These findings are consistent with previous studies and confirmed the critical role of ANS in BP circadian rhythm.

The *db/db* mice have a leptin receptor mutation. One may speculate that impaired leptin signalling contributes to the disruption of BP circadian rhythm in *db/db* mice, particularly given the fact that leptin signaling is implicated in obesity associated hypertension (Simonds, Pryor et al. 2014). However, several considerations suggest that it is unlikely that the abnormal BP daily rhythm in the *db/db* mice is directly mediated by the loss-of-function mutation in the leptin receptor. First, the leptin is demonstrated to increase arterial BP. In the human, the level of plasma leptin is positively associated with the BP level (Barba, Russo et al. 2003), and the loss-of-function mutations in the gene encoding leptin are associated with low BP (Simonds, Pryor et al. 2014). In diet-induced obese (DIO) mice, increasing the level of leptin significantly elevates BP, while blocking the actions of leptin reverses the effect (Simonds, Pryor et al. 2014). However, the loss-of-function mutation of leptin receptor in *db/db* mice does not lower BP, but rather increases BP, indicating the change in the BP of the *db/db* mice is not due to the leptin signaling deficiency. Second, the circulating leptin levels in humans have a 24-hour oscillation that is higher during the night and lower during the day (Sinha, Ohannesian et al. 1996). This pattern is opposite to the BP circadian rhythm. Since leptin is positively associated with BP, it is unlikely that the circadian rhythm of BP is mediated by the oscillation of leptin. Third, while the leptin receptor mutation is present thoughout the life of the *db/db* mice, the disruption of BP circadian rhythm in the *db/db* mice is only detectable in mice older than 11-weeks (Senador, Kanakamedala et al. 2009). These indicate the loss of BP circadian rhythm in diabetes is not due to deficiency of leptin signialling.

4.4 Mechanisms of diabetes induced clock gene disruption

In this dissertation, we found the shifts of mPer2 phase observed *in vitro* from tissues explanted from the *db/db-mPer2*^{Luc} mice reflect phase shifts observed *in vivo*. This raises the question as to whether the mPer2 phase shifts in the *db/db* mice might be caused directly by leptin receptor mutation within cells or indirectly by hyperphagia, obesity, and diabetes. Although it is currently uncertain, there is some evidence to support all possibilities. First, leptin is an adipocyte-derived hormone that binds to the leptin receptor and promotes weight loss by reducing appetite and food intake and by increasing energy expenditure (Kelesidis, Kelesidis et al. 2010). Circulating leptin levels display diurnal variations in both humans and rodents (Sinha, Ohannesian et al. 1996, Ahren 2000, Cha, Chou et al. 2000). There is also evidence that leptin can directly regulate clock gene oscillations. For example, leptin can phase advance the electrical activity rhythm in the rat SCN *in vitro* (Prosser and Bergeron 2003). Thus, leptin receptor mutation in various tissues may directly advance the mPer2 oscillations *in vivo*. Second, consistent with a previous report (Ktorza, Bernard et al. 1997), the current study demonstrated that both the *db/db-mPer2*^{Luc} and *db/db* mice consumed more food than the control mice. It is possible that increased food intake due to impaired leptin signaling alters circadian rhythms. In fact, evidence that increased food intake affects behavioral, metabolic, and molecular circadian rhythms has been demonstrated in HF diet-fed mice (Kohsaka, Laposky et al. 2007, Hatori, Vollmers et al. 2012, Pendergast, Branecky et al. 2013, Branecky, Niswender et al. 2015) and *db/db* mice (Kennedy, Ellacott et al. 2010). Thus, hyperphagia may mediate leptin receptor mutation-

associated phase advance of the mPer2 oscillation *in vivo*. Third, the current dissertation has demonstrated hyperglycemia, hyperinsulinemia, and glucose intolerance in the *db/db-mPer2*^{Luc} mice. Both glucose and insulin have been shown to alter clock gene expression rhythms *in vitro* and *in vivo*. For example, glucose down-regulates *Per1* and *Per2* mRNA expression in rat-1 fibroblasts (Hirota, Okano et al. 2002). The amplitude of circadian expression of REV-ERBα and DBP was enhanced with 0.5mM glucose compared to 25mM glucose in fibroblasts (Lamia, Sachdeva et al. 2009). Decreasing the amount of glucose in the medium significantly increased the circadian period length and decreased the circadian amplitude of U2OS cells stably expressing Bmal1-luciferase (Lamia, Sachdeva et al. 2009) and also increased the circadian period of Per2-luciferase expression in mouse fibroblasts cells (Putker, Crosby et al. 2018). 5.5mM glucose significantly shortened the period and delayed the phase of *Per2* mRNA levels compared to 0.5mM glucose in cultured mHypoE-37 neurons (Oosterman and Belsham 2016). Insulin is able to induce phase-dependent bi-directional phase shifts in diabetic rat livers (Yamajuku, Inagaki et al. 2012). In addition, insulin suppresses *Bmal1* transcriptional activity by promoting postprandial Akt-mediated Ser42-phosphorylation of *Bmal1*, which affects its intracellular localization in mouse liver (Dang, Sun et al. 2016). Therefore, it is likely that diabetes resulting from leptin receptor mutation may also have an indirect effect on the mPer2 phase advances in peripheral tissues *in vivo*. Nevertheless, future studies are required to distinguish these potential mechanisms.

Another interesting finding is that there are some similarities with respect to the effects of high-fat diet and leptin receptor mutation (*db/db-mPer2*^{Luc} mice) on the

mPer2 rhythm. For example, using *ex vivo* bioluminescent analyses, Pendergast *et al* demonstrated that mice fed a high-fat diet for 1 week display about a 4 hour phase advance in the mPer2 rhythm of the liver but not in the SCN or lung (Pendergast, Branecky et al. 2013). Using the same *ex vivo* assay, the current study demonstrated the *db/db-mPer2*^{Luc} mice also exhibit more than 3 hours phase advance in the mPer2 rhythm in the liver, while no phase shift is found in the SCN or lung. These results suggest that the liver clock is sensitive whereas the SCN and lung are resistance to both high-fat diet and leptin receptor mutation. Both the high-fat diet and leptin receptor mutation are associated with alternations in food intake. In particular, a high-fat diet changes the food intake pattern such that more food is consumed during the light-phase when fed high-fat diet than when fed the chow diet (Pendergast, Branecky et al. 2013). Leptin receptor mutation in the *db/db-mPer2*^{Luc} mice leads to increased total food intake throughout the day and night without changing the food intake pattern. Therefore, increased food intake during the light-phase may be a primary determinate of the liver clock. Despite these similarities, there were some differences with respect to the effects of high-fat diet and leptin receptor mutation on the mPer2 rhythm. For example, the mPer2 rhythm in the white adipose tissue (WAT) and aorta explants show more than 4 hour and 1 hour phase advance, respectively, in the *db/db-mPer2*^{Luc} mice, but no change is seen in the high-fat diet fed mice (Pendergast, Branecky et al. 2013). These results indicate that either the high-fat diet has no effect on Per2 expression in WAT and aorta or that one week of high-fat feeding is not long enough to change Per2 expression in WAT and aorta. It is probably the latter possibility since attenuated Per2

mRNA expression in WAT is observed after 6 weeks of high-fat diet (Kohsaka, Laposky et al. 2007). However, due to the limitation of sampling, the phase of Per2 was not calculated (Kohsaka, Laposky et al. 2007).

4.5 Effects of ATRF on BP

Multiple studies have investigated the effects of time-restricted feeding on metabolic health. However, no study has examined the relationship between food intake pattern and BP circadian rhythm. In this dissertation, we first monitored food intake pattern and BP circadian rhythm simultaneously in free-moving mice and demonstrated that non-dipping *db/db* mice have an altered food intake pattern. In addition, when imposing the abnormal feeding rhythm (i.e. the inactive-time restricted feeding; ITRF) on healthy mice, their BP daily rhythm is significantly altered. Importantly, active-time restricted feeding (ATRF) significantly decreased the light-phase (i.e., resting phase) BP, resulting in the restoration of BP daily rhythm in the *db/db* mice. These results demonstrate that the altered food intake pattern is associated with the loss of BP circadian rhythm. However, whether there is a cause-effect of this food intake pattern and the BP circadian rhythm is not known. Interestingly, the *db/db-mPer2*^{Luc} mice have similar non-dipping BP as the *db/db* mice. However, the food intake rhythm of *db/db-mPer2*^{Luc} mice remains normal, that they eat similar percents of food during both the light- and dark-phase compared to the control mice. However, other regulatory factors of the BP circadian rhythm, such as the daily rhythms of locomotor activity and baroreflex sensitivity and the time-of-day variations of clock genes, are similarly affected in both the *db/db-mPer2*^{Luc} and *db/db* mice. Therefore, it is possible the diabetes-

associated circadian rhythm disruptions in other aspects rather than the food intake rhythm contribute to the abnormal BP circadian rhythm.

Despite the difference in the food intake rhythm between the *db/db-mPer2*^{Luc} and *db/db* mice, both strains of the mice consume significantly greater amounts of food compared to their controls. In addition, the ATRF regimen not only reduces food intake time, but also decreases total amount of food intake. These results raise the possibility that change in calorie intake (overfeeding or fasting) may contribute to the BP circadian rhythm disruption, especially given the results that calorie restriction achieved by decreasing the calorie intake every day or intermittent fasting is able to reduce BP level in humans and laboratory animals (Nakano, Oshima et al. 2001, Wan, Camandola et al. 2003, Mager, Wan et al. 2006, Harvie, Pegington et al. 2011). However, in human studies, since both the daytime and nighttime SBP is reduced after calorie restriction, it is not known whether the circadian variation of BP is also changed (Nakano, Oshima et al. 2001, Harvie, Pegington et al. 2011). In animal studies, the calorie restriction is always accompanied with time restriction as the food was given at a certain time of the day (Wan, Camandola et al. 2003, Mager, Wan et al. 2006). And again, the circadian variation of BP has not been examined in the calorie-restricted animals (Wan, Camandola et al. 2003, Mager, Wan et al. 2006). In this dissertation, we performed time restriction without calorie restriction by prolonging the duration of food available time. We found the *db/db* mice consumed similar amounts of food with 12-h ATRF compared to ALF. Importantly, the BP daily rhythm can also be restored. These data indicate time

restriction without calorie restriction is able to restore BP circadian rhythm in *db/db* mice.

We further explored the mechanisms underlying ATRF induced BP circadian rhythm restoration in *db/db* mice. We found ATRF significantly improves the rhythm of the sleep-wake cycle and increases the sleep bout length during the light-phase. Few studies have examined the effects of food intake pattern on sleep. Afaghi *et al* found the meal given 4-h before bedtime significantly shortened sleep onset latency (SOL) compared to the same meal given 1-h before bedtime (Afaghi, O'Connor et al. 2007). Another study demonstrated that caloric intake at night in women (represented as percent of total caloric intake, %) positively correlates with sleep latency and negatively correlates with sleep efficiency (Crispim, Zimberg et al. 2011). These results are consistent with the present study and indicate that an early dinner time and less calorie intake during dinner are associated with increased sleep quality. The daily variations of urinary aldosterone and corticosterone excretion are also improved with ATRF. The effects of time-restricted feeding on aldosterone rhythm have not been reported previously. However, the rhythm of corticosterone in response to time-restricted feeding was documented as early as 1970s. The peak of corticosterone shifts in response to food available time: when feeding only occurs during the light-phase of rat, the peak of corticosterone has a 12-h shift (KRIEGER 1974). The most dramatic change with ATRF is time-of-day variation in autonomic nervous system (ANS) activity. We have demonstrated indirect evidence that ATRF significantly decreases sympathetic nervous system (SNS) activity and increases parasympathetic nervous system (PNS) activity

during the fasting period compared to ALF, resulting in restored ANS daily variation in *db/db* mice. ATRF is a repeated switching between feeding and fasting state every 24 h. Although there is no study of the effects of time-restricted feeding on ANS rhythm, various studies have demonstrated that fasting is able to suppress the sympathetic nervous system (SNS) (Young and Landsberg 1977, Prinz, Halter et al. 1979, Young, Saville et al. 1982, Young, Rosa et al. 1984) and the suppression is reversed by feeding (Young and Landsberg 1977). However, the effect of fasting on SNS in previous studies was tested after 2 days of fasting, which is impractical in any clinical application. In this dissertation, we have demonstrated that 16-h of fasting is able to suppress the SNS and subsequently lower BP during the fasting period, resulting in the restoration of BP circadian rhythm in *db/db* mice. Previous studies proposed that the mechanism underlying dietary mediated modulation of SNS might be through the alternation of insulin secondary to changes in carbohydrate intake (Landsberg 1986). For example: alterations in carbohydrate and fat enhance SNS activity without increasing total calorie intake (Young and Landsberg 1977, Schwartz, Young et al. 1983, Walgren, Young et al. 1987); and a diet with low carbohydrate decreases SNS (Jung, Shetty et al. 1979, DeHaven, Sherwin et al. 1980). In addition, insulin infusion increases SNS activity in the absence of a glucose change (Rowe, Young et al. 1981). In this dissertation, we found ATRF decreases SNS activity during the fasting period without changing plasma insulin level, indicating that other mechanisms may have participated. Using the *iG-Bmal1-KO* mice, we have demonstrated that the urinary norepinephrine (NE) excretion is not decreased during the fasting period after ATRF compared to ALF, and importantly, that

ATRF cannot completely restore the BP in *iG-Bmal1-KO* mice. In addition, study from the embryonic *Bmal1-KO* mice showed that the sympathoadrenal function is impaired as the plasma NE and epinephrine (Epi) levels are significantly reduced in the embryonic *Bmal1-KO* mice at both ZT2 and ZT14 compared to control mice (Curtis, Cheng et al. 2007). These data indicate that Bmal1 may play an important role in dietary induced modulation of SNS as well as of BP. Studies of the effects of time-restricted feeding on parasympathetic nervous system (PNS) function are rare. Results from dietary restriction, achieved by intermittent fasting or calorie restriction, have been shown to increase PNS activity in rats (Mager, Wan et al. 2006). In humans, 6-months of calorie restriction also increases PNS activity (de Jonge, Moreira et al. 2010). In this dissertation, we first demonstrated that the PNS activity is increased during the fasting period of ATRF. The mechanisms underlying dietary induced PNS modulation need further investigation.

4.6 Effects of ATRF on metabolism

Results from previous studies demonstrate that ATRF is beneficial to metabolic health in high-fat fed mice, including decreased body weight, total cholesterol, triglyceride, glucose intolerance, insulin and insulin resistance (Hatori, Vollmers et al. 2012, Sherman, Genzer et al. 2012, Tsai, Villegas-Montoya et al. 2013, Chaix, Zarrinpar et al. 2014, Yasumoto, Hashimoto et al. 2016). The mechanisms of the beneficial effects of ATRF on metabolism are implicated in multiple pathways. Hatori *et al* and Chaix *et al* from the Panda group revealed that ATRF have profound effects on the enzymes and proteins involved in the regulation of nutrient hemostasis (Hatori, Vollmers et al. 2012,

Chaix, Zarrinpar et al. 2014). In the liver, the mRNA expression of fatty acid synthase (*Fasn*), stearoyl coA desaturase1 (*Scd1*) and elongation of very long chain fatty acids protein 5 (*Elovl5*) are reduced upon ATRF. The *Fasn*, *Scd1* and *Elovl5* genes encode enzymes involved in lipid synthesis, desaturation and elongation respectively. Conversely, the expression of hepatic lipase (*Lipc*) gene, which encodes the enzyme that catalyzes the hydrolysis of triglyceride, is enhanced, along with increased expression of 3-hydroxybutyrate (BHBA), one of the end products of β-oxidation. Therefore, ATRF shifts lipid toward degradation. In addition, the expression of peroxisome proliferator-activated receptor gamma (*Pparγ*), a key regulator of lipid storage, is significantly repressed in the liver and brown adipose tissues (BAT), which may explain reduced hepatic steatosis and BAT lipid droplets. Since hepatic lipid homeostasis contributes to cholesterol and bile acid metabolism, Hatori *et al* and Chaix *et al* determined the mRNA expressions of squalene epoxidase (*Sqle*) and 7-dehydrocholesterol reductase (*Dhcr7*), two key enzymes involved in de novo cholesterol biosynthesis. They found both the *Sqle* and *Dhcr7* mRNA expressions are enhanced, along with increased protein levels of sterol regulatory element-binding proteins (SREBPs), suggesting increased cholesterol biosynthesis. On the other hand, the peak expression of cholesterol 7 alpha-hydroxylase (Cyp7a1), a gene that encodes the rate limiting enzyme in cholesterol breakdown to bile acid, is also elevated. Consequently, the overall levels of cholesterol are decreased. For glucose hemostasis, the mRNA expressions of pyruvate carboxylase (*Pcx*) and glucose-6-phosphatase (*G6pc*), genes that encode enzymes that mediate the committing step in gluconeogenesis, are reduced. On the contrary, the expressions of glucose-6-phosphate

dehydrogenase (*G6pdx*), a gene that encodes the rate-limiting enzyme of the pentose phosphate cycle (PPC) and the levels of its substrate, glucose-6-phosphate (G6-P), are elevated, suggesting increased activity of the PPC. Since PPC pathway is in parallel with glycolysis, the elevated activity of the PPC indicates increased glycolysis. Therefore, ATRF reprograms glucose metabolism away from gluconeogenesis toward glycolysis. ATRF also improves protein homeostasis in the liver. In high-fat fed mice under *ad libitum*, the oscillation of phospho-ribosomal protein S6 (pS6), an indicator of protein synthesis, is blunted while ATRF restores the oscillation. Energy homeostasis is also improved with ATRF. The levels of 5' adenosine monophosphate-activated protein (AMP) and phospho- acetyl CoA carboxylase (ACC) (pACC, relative to total ACC) are increased, which reflects increased activity of AMP-kinase (AMPK), a key regulator of energy metabolism. AMPK also plays an important role in diabetes as the net effects of AMPK activation inhibit lipogenesis, stimulate fatty acid oxidation, lipolysis and glucose uptake, and modulate insulin secretion from pancreatic beta-cells (Winder and Hardie 1999). In addition, the expressions of uncoupling protein 1/2/3 (*UCP1/2/3*), which encodes proteins participate in thermogenesis, are increased in the BAT, suggesting increased energy expenditure. In summary, the alternations in nutrient and energy homeostasis participate in the beneficial effects of ATRF on high-fat fed mice.

In this dissertation, however, we did not observe significant improvements of ATRF on nutrient hemostasis of the *db/db* mice, including fat body mass, non-fasting blood glucose, insulin sensitivity, plasma insulin, NEFA or cholesterol. These results are in contrast to the effects of ATRF on high-fat fed mice. A possible explanation for the

discrepancy between *db/db* and high-fat fed mice is that effective impacts of ATRF on metabolism may require intact leptin signaling, given the consideration that the *db/db* mice have a loss-of-function mutation of the leptin receptor. Actually, in high-fat fed mice under ATRF, the leptin levels are significantly decreased compared to ad libitum feeding (Hatori, Vollmers et al. 2012, Chaix, Zarrinpar et al. 2014). As reviewed by Stern et al (Stern, Rutkowski et al. 2016), leptin interacts with glucose and lipid metabolism in multiple tissues. For example, leptin increases glucose uptake and glycogen synthesis *in vivo* (Barzilai, She et al. 1999) and *in vitro* in soleus muscle (Ceddia, William Jr et al. 1999), cultured L6 muscle cells (Bates, Gardiner et al. 2002), C2C12 myotubes (Kellerer, Koch et al. 1997) and adipocytes (Müller, Ertl et al. 1997). It also inhibits glycogenolysis and gluconeogenesis in hepatocytes (Ceddia, Lopes et al. 1999). In addition, leptin modulates function of the pancreas by inhibiting insulin secretion in the pancreatic β Cell (Ceddia, William Jr et al. 1999, Seufert, Kieffer et al. 1999, Laubner, Kieffer et al. 2005) and glucagon secretion in the α Cell (Soedling, Hodson et al. 2015) (Tudurí, Marroquí et al. 2009). For lipid metabolism, leptin has been shown to inhibit lipogenesis and increase fatty acid oxidation and triglyceride hydrolysis in adipocytes (William, Ceddia et al. 2002), liver (Huang, Dedousis et al. 2006, Huynh, Neumann et al. 2013) and skeletal muscles (Minokoshi, Kim et al. 2002). Reduced body weight with ATRF in high-fat fed mice has consistently been observed in other studies (Arble, Bass et al. 2009, Hatori, Vollmers et al. 2012, Sherman, Genzer et al. 2012, Tsai, Villegas-Montoya et al. 2013, Chaix, Zarrinpar et al. 2014, Yasumoto, Hashimoto et al. 2016). However, the body weight of the *db/db* mice is not significantly decreased following ATRF, despite less food

consumption vs. ALF. One explanation for the unchanged body weight is that ATRF decreases energy expenditure (EE) in the *db/db* mice. Therefore, we monitored the EE and found that ATRF significantly decreases the 12-h light-phase EE, but leaves the 12-h dark-phase EE unchanged in the *db/db* mice; therefore, the 24-h daily EE is reduced. Lowered light period EE is also observed in dark-phase high-fat fed mice; however, dark-phase high-fat feeding also significantly increases their EE during the dark period, therefore resulting in unaltered 24-h EE (Bray, Ratcliffe et al. 2013). Interestingly, in the current study, there is a trend of decrease in the light-phase EE and increase in the dark-phase EE in chow-fed control mice with ATRF, while the 24-h daily EE is unchanged, which is consistent with high-fat fed mice. Therefore, the different EE response to ATRF between control and *db/db* mice may be attributable to impaired leptin signaling due to leptin receptor mutation in the *db/db* mice. Actually, leptin is known to stimulate EE through its action on the hypothalamus (Meier and Gressner 2004), and plays a critical role in the regulation of body weight (Halaas, Gajiwala et al. 1995, Friedman and Halaas 1998). However, whether the improved nutrient homeostasis and increased EE during the dark-phase with ATRF in high-fat fed mice is dependent upon leptin signaling need further investigations.

Another intriguing finding of this dissertation is that both the *db/db-mPer2*^{Luc} and *db/db* mice lose their rhythms in RER, and ATRF partially restores the RER oscillation in the *db/db* mice. In normal mice, there are apparent time-of-day variations in RER, with higher values during the active-phase indicating the preferential use of carbohydrates and lower values during the inactive-phase indicating the preferential use

of fats. This is consistent with previous observations in humans (van Moorsel, Hansen et al. 2016) and rodents (Bray, Ratcliffe et al. 2013, Oosterman, Foppen et al. 2015, Sun, Wang et al. 2015). Previous studies also demonstrated RER in the *db/db* mice is decreased at one specific time of the day (Osborn, Sanchez-Alavez et al. 2010, Choi, Kim et al. 2015). However, it is surprising that it has not been reported whether the daily rhythm of RER is disrupted in the *db/db* mice. The current study first demonstrated that the leptin receptor mutant mice, regardless of gene background, lose the RER time-of-day variations. These results suggest that the flexibility to use different sources of fuel is compromised in diabetic mice. Importantly, imposing ATRF partially restores the RER oscillation in the *db/db* mice. However, it is not clear whether the attenuated RER daily rhythm is associated with compromised BP dipping and whether the recovery of RER oscillation after ATRF contributes to the restoration of BP rhythm.

We further investigated whether the improved oscillation of RER and EE upon ATRF is Bmal1-depenent. In contrast to the partial recovery of BP daily rhythm with ATRF in *iG-Bmal1-KO* mice, ATRF dramatically improves the RER oscillation that is even more robust than the flox mice. These results are comparable with the results of a recent published paper that ATRF drives the rhythms of RER in mice lacking clock genes (Chaix, Lin et al. 2018). In that paper, the authors monitored the RER in three different strains of clock gene knockout mice, the liver-specific *Bmal1* knockout mice (*Bmal1-LKO*), liver-specific *Rev-erbα/β* double knockout mice (*Rev-erb α/β LDKO*) and *Cry1/2* double knockout (*CDKO*) mice. The oscillations of RER are blunted in all the three strains of mice and ATRF restores the rhythms, suggesting the feeding and fasting cycle is able

to drive the rhythm of RER in mice lacking clock genes. Regarding EE, ATRF also significantly increased the EE oscillation in both the iG-Bmal1-KO and Flox mice; however, the amplitude and robustness were still lower in the *iG-Bmal1-KO* mice. In addition, the 24-h EE in the *iG-Bmal1-KO* mice are not significantly different between ATRF and ALF. These results are in contrast to the results of the Chaix et al study, which showed increased EE upon ATRF in high-fat fed *Bmal1-LKO*, *Rev-erb α/β LDKO* and *CDKO* mice (Chaix, Lin et al. 2018). One possible explanation for this difference is the mice in Chaix et al study were fed with high-fat diet while the *iG-Bmal1-KO* mice were fed with chow diet. It is possible that the EE responses to ATRF are different between different diets, especially considering the fact that high-fat diet itself has been shown to decrease EE (Choi, Kim et al. 2015). In addition, the body weight in the mice with ATRF is lower than the mice fed ad libitum; therefore when the EE is normalized to body weight, the resulting EE will be higher in the mice with smaller body weight if assuming the EE before normalization are equal between ALF and ATRF. However, the body weights between ATRF and ALF in the *iG-Bmal1-KO* mice fed chow diet are not significantly different; hence the normalized EE with ATRF is the same as the mice with ALF. An alternate explanation is that the deletion of *Bmal1* in this dissertation is global, in both SCN and peripheral tissues, while the *Bmal1 KO* mice in Chaix *et al* study is liver-specific. The SCN has been shown to control EE (Coomans, van den Berg et al. 2012). Therefore, the EE responses to ATRF might be different in mice with and without intact central clock.

4.7 Limitations and Future directions

The major limitation of this dissertation is the diabetic mouse model. The diabetic phenotypes of the *db/db* mice are caused by the leptin receptor mutation. However, the leptin receptor mutation in humans is rare, and it is not known whether the protective effect of ATRF on the BP circadian rhythm in the *db/db* mice also applicable to the diabetic patients whose the diabetic symptoms are often induced by a change of lifestyle. Therefore, testing the effect of ATRF on the BP circadian rhythm is needed in different diabetic animal models, such as the high-fat diet induced diabetic mouse.

The second limitation of this dissertation is all the experiments were done in male mice. It is not known whether there is a gender difference in the mechanisms of diabetes-associated BP circadian rhythm disruption and whether ATRF is equally applicable to female diabetic animals.

We have demonstrated that the ANS participates, in part, in the protection of ATRF on the BP circadian rhythm; however, we did not find any changes with ATRF in the time-of-day mRNA expressions of the norepinephrine biosynthesis/ disposition enzymes or the adrenergic receptors in the vascular. It is possible that the effects of ATRF on the enzymes and receptors are at translational, or post-translational, levels. It could also be that the ATRF affects the activities of the enzymes, rather than on their expressions. In addition, the effects of ATRF on the autonomic nervous system may act

in the brain. Therefore, further experiments are needed to understand the underlying mechanisms of the ATRF on the ANS.

Using the *iG-Bmal-KO* mice we have demonstrated that Bmal1 partially participates in the protection of ATRF on the BP circadian rhythm. It is not known whether other clock genes, such as Cry1, also play a role. Previous studies have reported that *Cry1/2* double knockout mice exhibit abnormal BP circadian rhythm (Masuki, Todo et al. 2005, Doi, Takahashi et al. 2010). Importantly, the time-of-day expression of *Cry1* is also altered in the *db/db* mice and recovered with ATRF. Therefore, it is interesting to test the role of *Cry1* in the protection of ATRF on the BP circadian rhythm.

We have demonstrated that ATRF effectively prevented and restored the disrupted BP circadian rhythm in diabetic mice. It would be interesting to test whether ATRF is also able to prevent or restore the BP circadian rhythm in diabetic patients.

4.8 Concluding remarks

Findings from this dissertation demonstrate that the desynchronization of peripheral tissues participates in the compromised BP circadian rhythm in diabetes. Moreover, the active-time restricted feeding may serve as a novel and effective therapy against the disruption of BP circadian rhythm in diabetes.

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