The effects of gonadal hormones on the expression of the clock protein PER2 in the central extended amygdala

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ABSTRACT

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Most biological processes exhibit circadian rhythms that are synchronized by the master clock located in the suprachiasmatic nucleus (SCN). Recently, cells in the oval nucleus of the bed nucleus of the stria terminalis (OV-BNST), the central nucleus of the amygdala (CEA), basolateral amygdala (BLA) and dentate gyrus of the hippocampus (DG), have been shown to rhythmically express Period 2 (PER2), a clock protein. It is known that gonadal hormones have an effect on circadian rhythms. However, yet to be determined is what influence they might have on brain circadian oscillators outside of the SCN. The purpose of this series of experiments is to examine PER2 expression; firstly, in intact females over the course of the estrous cycle and, secondly, in gonadectomized females, males, and intact males. Results obtained from the intact females suggest that the pattern of PER2 expression in the OV-BNST and the CEA changes as a function of stage of the estrous cycle. In ovariectomized females peak PER2 expression in the OV-BNST and CEA is at ZT13, the same was also found in gonadectomized and intact males. Together, the data from these studies suggest that gonadal hormones may influence the circadian functioning of these two brain regions.

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Chapter 1: Introduction

Most biological phenomena show a circadian cycle that is synchronized by the internal pacemaker, or biological clock located in the suprachiasmatic nucleus (SCN) (Okamura *et al.*, 2002). Light represents the major synchronizing environmental cue for the SCN and shifts in the light cycle cause phase shifts in the SCN rhythm and consequently in physiological and behavioural activity (Takahashi et al., 2001). The SCN is regarded as the master clock of the circadian system, as lesions to the SCN result in a complete loss of all circadian rhythms, and fetal SCN tissue transplanted to animals whose SCN has been ablated can restore behavioural circadian rhythms (Ralph et al, 1990; Li and Satinoff, 1998). However, considerable evidence suggests that oscillators exist in a number of peripheral tissues as well as in other brain areas.

In the brain, molecular circadian oscillators have been found in the cortex, striatum, olfactory bulbs and paraventricular nucleus (PVN) (Abe et al., 2002; Masubuchi et al, 2000; Shieh, K.R, 2003). Using rhythmic expression of the clock protein Period2 (PER2) as a marker of a circadian oscillator, studies from our laboratory have found oscillators in cells of the oval nucleus of the bed nucleus of the stria terminalis (OV-BNST), central nucleus of the amygdala (CEA), basolateral amygdala (BLA) and the dentate gyrus of the hippocampus (DG) (Amir et al, 2004; Lamont et al, 2005). It has also been shown that although these oscillators were under the control of the SCN, both those in the OV-BNST and the CEA can also be affected by glucocorticoids (Lamont et al., 2005). This raises the intriguing possibility that these oscillators can integrate SCN input with other signals some of which arise from the periphery. The purpose of this thesis is to investigate the ability of gonadal

hormones to modulate the expression of PER2 in the SCN, OV-BNST, CEA, BLA and DG. To do this I investigated patterns of PER2 expression across the rat estrous cycle and its relationship to changes in locomotor activity. In addition, the effects of gonadectomy in both male and female rats on the rhythm of PER2 expression in the SCN and limbic forebrain were examined.

PER2 and the Molecular framework of the internal clock

The clock is comprised of a cell autonomous molecular oscillator made of several rhythmically expressed clock genes that form positive and negative feedback loops of transcription and translation (Duffield, 2003). These clock genes include *Clock*, *Bmal1*, *Cry1*, *Cry2*, *Per1*, *Per2* and *Per3* (Lowrey & Takahashi, 2000; Reppert & Weaver, 2002). *Per2* is commonly used in the identification of novel oscillators (Oishi et al., 1998; Zheng et al., 1999; Shieh, 2003). This is because one of the defining features of the molecular oscillator is the rhythmic expression of PER2, and the analysis of both Per2 mRNA and the protein PER2 have been useful in the identification of clock cells (Oishi et al., 1998; Zheng et al., 1999; Shieh, 2003).

Oscillators outside of the SCN

The SCN does not function alone in the generation of circadian rhythms. Rather, it appears to play the role of the conductor, and synchronizes the oscillations of peripheral clocks (Reppert & Weaver, 2002). Researchers have found peripheral oscillators in many tissues such as the liver, kidney, heart and pancreas (Schibler & Sassone-Corsi, 2002).

Research from our laboratory has shown that neurons in the OV-BNST, a subregion of the BNST, exhibit a daily rhythm in PER2 expression, which like that in

the SCN peaks at ZT13 (Amir et al., 2004). A similar pattern of PER2 expression was also found in the CEA, whereas rhythmic expression of PER2 in the BLA and DG has its peak at ZT1 (Lamont et al., 2005). The circadian pattern of PER2 expression in all these areas is abolished after SCN lesions demonstrating that the oscillators in all these regions are regulated by SCN output (Amir et al, 2004).

The existence of circadian oscillators within these limbic forebrain regions is of particular interest because of the known involvement of these nuclei in behavioural and physiological processes. For example, the BNST modulates neuroendocrine, autonomic and behavioural responses to stress, as well as reproductive and maternal behaviours (Casada & Dafny, 1991; Loewy, 1991; Nijsen et al., 2001; C. D. Walker et al., 2001; D. L. Walker et al., 2003), and the amygdala is implicated in the control of motivational and emotional behaviour (Everitt et al., 2003). The DG has been implicated in emotional memory (Abe, 2001; Akirav & Richter-Levin, 2002).

Recent data suggests that the rhythmic PER2 expression in the OV-BNST and the CEA is influenced by glucocorticoids. Lamont et al. (2005), showed that in these regions the rhythm of PER2 expression is lost after adrenalectomy. Interestingly, adrenalectomy had no effect on the patterns of PER2 expression in DG and BLA or in the SCN itself. Together these data suggest that some oscillators in the limbic forebrain integrate input from the SCN with signals from the periphery.

Gonadal hormones and the circadian system

Gonadal steroids also have profound effects on physiology and behavior and like the adrenal steroids readily gain access to the brain (Nelson, 2000). It seems possible therefore that changes in gonadal steroids might also modulate PER2

expression in the OV-BNST, CEA, BLA and DG. Such effects could be direct because both estrogen receptor (ER) isoforms, ERα and ERβ have been localized in the BNST, CEA and BLA (Perez et al., 2003; Laflamme et al., 1998). Erβ immunoreactivity has also been reported in the DG (Zhang et al., 2002), and androgen receptors (AR) have been observed in the BNST (Zhou et al., 1994).

There is considerable evidence that the circadian and hypothalamic-pituitary-gonadal systems interact. There are direct connections between the SCN and gonadotropin releasing hormone (GnRH) neurons as well as projections from the SCN to ER containing cells in the anteroventral periventricular area (AVPV) (Watson et al., 1995). The AVPV neurons that receive SCN input are critical for the induction of the mid-cycle lutenizing hormone (LH) surge. Moreover, lesions of the SCN results in a state of persistent estrus in female rats (Wiegand & Terasawa, 1982).

Gonadal hormones also influence the circadian system. Testosterone increases the amplitude and shortens the period of circadian activity in male rodents, while in female rodents the onset, amplitude and period of activity vary over the course of the estrous cycle as levels of estrogen and progesterone fluctuate (Albers, 1981). Estrogen appears to increase the amplitude (Gerall et al., 1973) and shorten the period of activity (Morin et al., 1977), while progesterone decreases the amplitude and delays the onset of activity (Rodier, 1971).

Given the interrelationship between gonadal hormones and the circadian system and the finding that some of the subordinate oscillators in the limbic forebrain are modulated by peripheral hormones, in this set of studies the effect of gonadal hormones on PER2 expression in the SCN, the OV-BNST, CEA, BLA and DG was

examined. In these experiments I took advantage of the natural changes in circulating levels of estrogen and progesterone that occur over the course of the estrous cycle in female rats.

The estrous cycle is generally 4 days in length but can be as short as 3 days or as long as 5 days. It is typically divided up into 4 stages: proestrus, estrus, metestrus and diestrus, each associated with a particular level of circulating hormones and a characteristic vaginal cytology (Nelson, 2000). Peak estrogen and progesterone levels as well as the lutenizing hormone (LH) and follicle stimulating hormone (FSH) surges occur on the afternoon of proestrus (Barbacka-Surowiak *et al.*, 2003). Ovulation, behavioural receptivity and proceptivity occur in the early hours of the next day, estrus (Barbacka-Surowiak et al., 2003). Metestrus is characterized by low levels of estrogen and progesterone (Barbacka-Surowiak et al., 2003), and finally during diestrus, estrogen levels begin to rise (Nelson, 2000).

In the first study changes in the pattern of locomotor activity across the estrous cycle were examined, as well as the pattern of PER2 expression in the limbic forebrain and SCN during the metestrus phase of the cycle. The second study investigated PER2 expression in intact females over the course of the estrus cycle. The final experiment compared the pattern of PER2 expression in the SCN and limbic forebrain of gonadectomized females, males and intact males.

Chapter 2: Study1

Methods

Animals

12 female Wistar rats (Charles River Laboratories, St-Constant, Quebec) weighing between 150-175g on arrival were used in this study. These rats were housed individually in clear plastic cages (50 cm X 26.8 cm X 36.4 cm) each equipped with a running wheel (34.5 cm in diameter). The rats had ad libitum access to both food and water for the duration of the experiment.

Apparatus

All of the cages were individually housed within sound and lightproof chambers (66 cm X 66cm X 44 cm) equipped with a 4W white fluorescent light, a computer-controlled lighting system (VitalView MiniMitter Co. Inc., Sunriver, OR), and a ventilation system. The running wheels each had a magnetic microswitch, which was connected to the computer. Running-wheel activity data was recorded with VitalView software (MiniMitter Co.) and analyzed using Actiview (v1.2) (MiniMitter Co.).

Procedure

Each animal was entrained to a 12h:12h light/dark cycle (LD cycle) for 2 weeks before any manipulation occurred. The rats were randomly divided into groups based on the time of day when they would be perfused, which were ZT1, ZT7, ZT13, and ZT19 (ZT0 being when the lights would turn on).

After the initial entrainment period, vaginal swabs were taken daily. The resultant vaginal smears were examined under a light microscope and classified according to cell type. Proestrus was identified by the prevalence of large nucleated

epithelial cells, estrus by the prevalence of cornified cells usually seen along with large sheathings, metestrus by the presence of a mixture of cells types, with some cornified cells, and leukocytes, and finally diestrus by the presence of mainly leukocytes (Rubinow *et al.*,2004). Only rats showing regular cycles were included in the study. For 16 consecutive days, the running wheel data obtained from VitalView (MiniMitter Co.) was used to calculate the number of wheel revolutions for each dark period. A 1-way analysis of variance (ANOVA) and Bonferroni post-hocs were conducted to determine whether the day of the estrous cycle influenced the number of wheel revolutions per dark period.

The rats were perfused on metestrus, when both estrogen and progesterone levels are low.

Perfusions

The rats were deeply anesthetized with sodium pentobarbital (Somnotol™, MTC Bimeda) (100 mg/kg) and perfused intra-cardially with 300 ml of cold saline solution (0.9% NaCl), followed by 300ml of cold 4% paraformaldehyde in a 0.1M phosphate buffer. Following the perfusions, brains were removed and postfixed overnight in 4% paraformaldehyde solution at 4C. The next morning the brains were sliced on a vibratome (Vibratome, St. Louis, MO, USA) into 50 μm thick coronal sections. These sections were then stored at -20C in Watson's cryoprotectant (Watson et al., 1986), composed of 0.1 M Sodium phosphate buffer, sucrose, Polyvinylpyrrolidone (PVP-40, Sigma) and Ethylene Glycol (Sigma).

Immunocytochemistry

Brain sections were removed from the Watson's cryoprotectant, and washed six times for ten minutes in cold 50 mM Trisma buffered saline (TBS) to remove the cryoprotectant. Sections were rinsed three times in cold TBS between incubations in each of the following solutions, except between the pre-block solution and the primary antibody, when no rinses occurred. The free floating brain sections were incubated in a quench solution, which consisted of 90%TBS and 10%H_2O_2, for 30 minutes at room temperature, then a pre-block solution, which consisted of 0.3% Triton X in TBS (Tx), and 5% normal goat serum, for 1 hour at 4°C. After incubation in the pre-block, sections were transferred directly into a primary antibody solution for 48 hours at 4°C, consisting of an affinity purified rabbit polyclonal antibody raised against PER2 (ADI, San Antonio, TX) diluted to 1:400 in Tx (0.30%), and 2% normal goat serum. The brain sections were then incubated in a secondary antibody solution, consisting of 2% normal goat serum, and a goat derived biotinylated anti-rabbit IgG, (Vector Labs, Burlingame, CA) and diluted to 1:200 in Tx for an hour at 4C. The brain sections were then transferred into the ABC solution, which consisted of an avidin-biotin-peroxidase complex (Vectastain Elite ABC kit, Vector Labs), where they remained for 2 hours at 4°C. The sections were then rinsed in cold TBS, then rinsed again with cold 50 mM Tris-HCl, and finally rinsed with 0.05% 3,3'-diaminobenzidine (DAB) in 50 mM Tris-HCl. The brain sections then underwent DAB staining via incubation on an orbital shaker for 10 minutes in a solution of DAB in 50 mM Tris-HCl, 0.01% H_2O_2, and 8% $NiCl_2$. Finally the sections were rinsed

with cold TBS, and then mounted onto gel-coated slides, dehydrated through a series of alcohols, soaked in Citrosol and coverslipped with Permount (Fisher).

Data Analysis

Evidence of PER2 immunoreactivity (PER2-ir) was examined under a light microscope and images from OV-BNST, SCN, CEA, were captured using a Sony XC-77 video camera, a Scion LG-3 frame grabber (Scion, Frederick, MD) and NIH Image software (v1.63). A 400X400 μm frame was used to capture images. The number of cells expressing PER2 immunoreactivity were then counted using Image SXM software (v1.75). For each brain region of interest a mean number of PER2 immunoreactive cells was calculated for each animal by quantifying the average of the 6 images that showed the largest number of stained cells. For all analyses the alpha level was set at .05. Group differences were investigated via 1-way analysis of variance (ANOVA) and, where appropriate, were followed with the Bonferroni post-hoc test.

Results - Activity

Figures 1 and 2 illustrate that the female rats show a large increase in activity on the night of proestrus. The results of the ANOVA (Table A2) carried out on these data indicated that there was a significant effect of day of the estrous cycle on the number of wheel revolutions ($F(3, 44) = 5.886$, $p<.01$). Post-hoc paired comparisons revealed that the number of wheel revolutions on the night of proestrus was significantly higher than all other days of the estrous cycle ($p<.05$).

Results – PER2 expression

PER2 expression at each of the four timepoints for the metestrus stage of the cycle is shown in Figure 3. The 1-way ANOVA revealed a significant effect of ZT on PER2-ir in the SCN, $F(3, 8) = 6.790$, $p<.05$ (Table A3). Bonferroni post-hoc tests demonstrated that there was a significant difference between ZT1 and ZT13 ($p<.05$). In regards to the OV-BNST and the CEA, there was no significant effect of ZT time on PER2-ir expression. However, there was a trend towards peak expression at ZT19 (Tables A4 and A5).

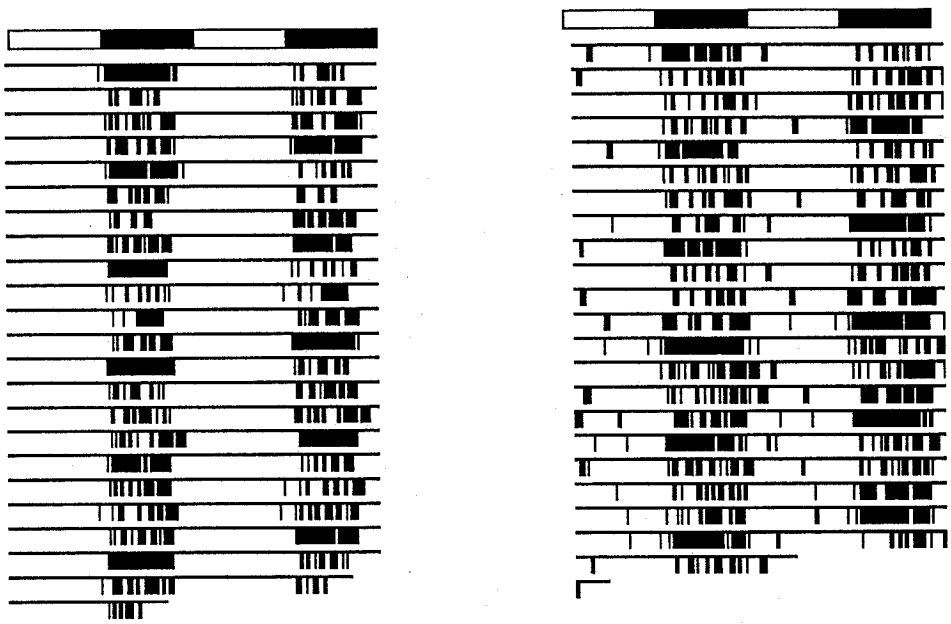

Figure 1: Actograms obtained from intact females demonstrating the increase in running wheel activity every fourth night. The above are double-plotted actograms with each column representing a day. In each actogram the vertical marks indicate periods of activity of at least 10 wheel revolutions/10 min. Successive days are plotted from top to bottom.

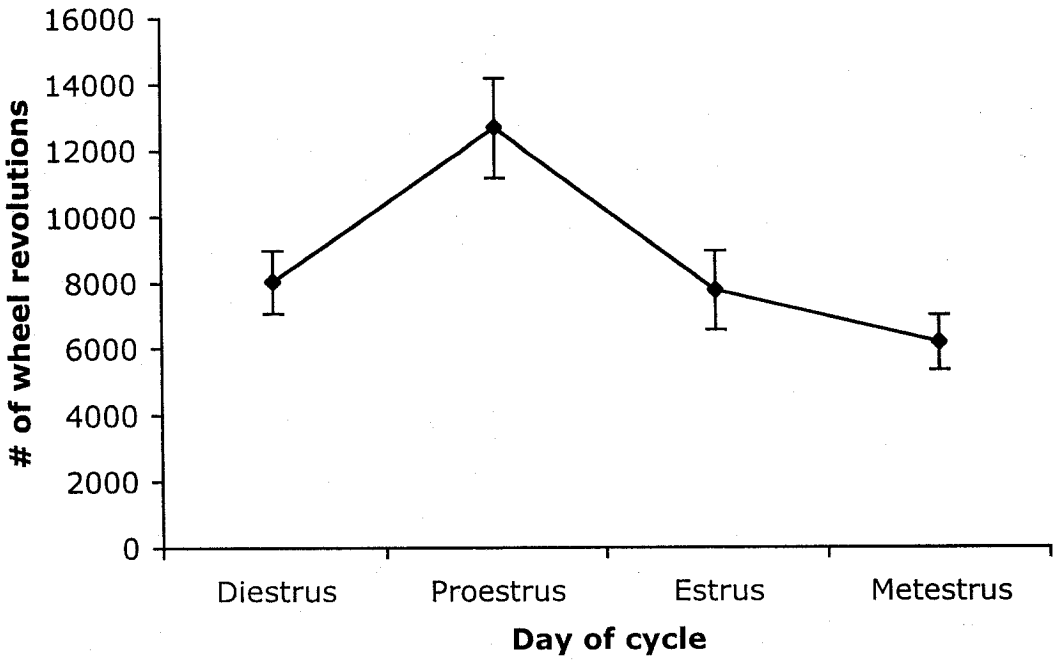

Figure 2: Number of wheel revolutions (means ± SEM) per night over the course of the estrous cycle.

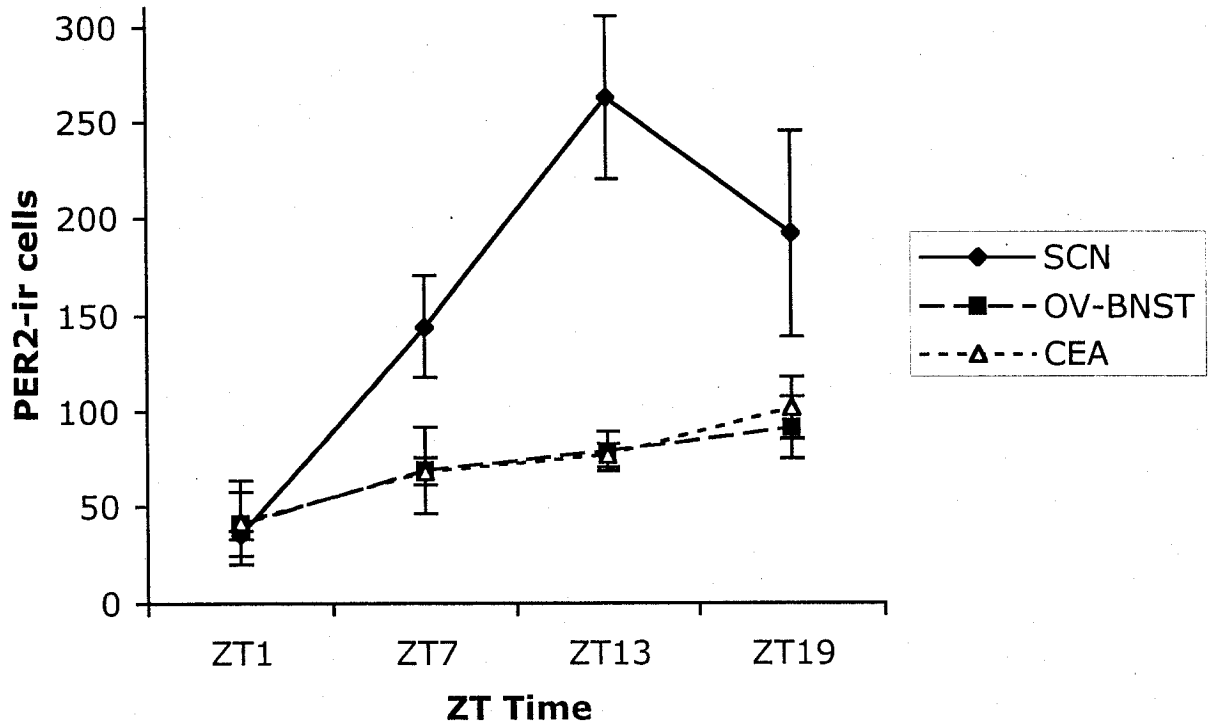

Figure 3: PER2-ir expression (means ± SEM) in the SCN, OV-BNST and CEA during metestrus.

Discussion

These results replicate those of Slonaker (1924), by showing that intact female rats have a large increase in running activity on the night of proestrus. The obtained pattern of PER2 expression in the SCN was similar to that obtained previously in males, and showed peak expression at ZT13. Interestingly, however, the pattern of PER2 expression in the OV-BNST and CEA did show a trend towards a peak at ZT19, rather than at ZT13. These data suggest that patterns of PER2 expression in these regions may vary as a function of the estrous cycle. This possibility was investigated in the next experiment.

Chapter 3: Study 2

Methods

Animals

Two cohorts of rats were used in this study. The first consisted of 64 female Wistar rats, the second 32 female Wistar rats. All were obtained from Charles River Laboratories, St-Constant, Quebec and weighed between 150-175g on arrival. The rats were housed four per cage and were given ad libitum access to food and water. All rats were entrained to a 12h:12h LD cycle (for half lights on from 8:00 to 20:00, and for the remainder lights were on at 20:00, off at 8:00). The rats were given an initial two-week period to entrain to their LD cycle. Daily vaginal smears were obtained for 8 days to confirm that they were cycling with a normal 4-5 day cycle.

Procedure

Rats from the first cohort were randomly assigned to one of 16 groups with 4 animals per group. Groups differed in time of day of perfusion (ZT1, ZT7, ZT13 and ZT19) and day of the cycle that they were perfused at the conclusion of the experiment. The second cohort was also randomly assigned to one of the 16 groups, with 2 animals per group. The rats were divided into groups as follows in the table below:

Table 1: Female rats from both cohorts were divided into groups according to ZT and day of the cycle. This table shows how the first cohort was divided up.

	ZT1	ZT7	ZT13	ZT19
Diestrus	4	4	4	4
Proestrus	4	4	4	4
Estrus	4	4	4	4
Metestrus	4	4	4	4

Vaginal smears were obtained for 4 days prior to their perfusion and again just before perfusion to confirm group classification.

The rats were perfused, and the obtained brains were removed and processed for PER immunocytochemistry according to the protocols described in Study 1. However, images from the BLA and BG were also taken and the PER2-ir cells were counted as per Study 1. A 200X400 μm frame was used to capture images from the

DG. Means and standard deviations were calculated for each group of intact females, and 2-way ANOVAs were conducted in order to determine the effects of both day of the cycle and ZT on the number of PER2-ir cells in each region of interest. 1-way ANOVAs and post-hoc tests were conducted as appropriate.

Results

As Figure 4 shows in the SCN the peak expression of PER2-ir cells remained consistent over the course of the estrous cycle, peaking at ZT13. The 2-way ANOVA conducted for the SCN (Table B6) revealed a significant main effect of ZT, $F(3, 34) = 51.546$, $p<.01$, but no main effect of day of the estrous cycle nor a significant time X day interaction. Bonferroni post-hoc analysis revealed that PER2 expression was higher at ZT13 than any other time point ($p<.01$).

In the OV-BNST the peak expression of PER2-ir cells was not consistent for all days of the estrous cycle, with peaks at ZT13 on the days of proestrus and estrus, a trend towards a peak at ZT19 on the day of metestrus and a peak of ZT7 on the day of diestrus (Figure 5). Statistical analysis of the OV-BNST data (Table B7) revealed a significant main effects of day of the cycle, $F(3, 81) = 3.66$, $p<.05$, and ZT, $F(3, 81) = 25.725$, $p<.01$, as well as a significant interaction of day of the cycle and ZT $F(9, 81) = 4.45$, $p<.01$. 1-way ANOVAs investigating the effects of ZT on PER2 expression during each day of the estrous cycle were conducted. For diestrus, there was a significant effect of ZT $F(3, 20) = 10.215$, $p<.01$, with ZT7 differing significantly from ZT1 ($p<.05$) (Table B8). There was also a significant main effect of ZT time for metestrus, $F(3, 22) = 6.898$, $p<.01$ (Table B9), with ZT19 differing from ZT1 ($p<.01$). For proestrus, there was a main effect of ZT, $F(3, 19) = 11.151$, $p<.01$

(Table B10), with ZT13 differing from both ZT1 and ZT19 ($p<.01$). And finally, for estrus there was a main effect of ZT, $F(3, 20) = 11.604$, $p<.01$ (Table B11), with ZT13 differing significantly from ZT1 ($p<.01$).

Peak expression of PER2-ir cells in the CEA, like the OV-BNST, was not consistent over the course of the estrous cycle, and had the same pattern of expression as found in the OV-BNST (Figure 6). The 2-way ANOVA (Table B12) revealed a significant main effect of ZT, $F(3, 79) = 13.042$, $p<.01$ as well as a significant time X cycle interaction $F(9, 79) = 3.153$, $p<.01$, there was also a main effect of day of the cycle that tended towards significance. Subsequent 1-way ANOVAs revealed a significant effect of ZT during diestrus, $F(3, 20) = 3.503$, $p<.05$ (Table B13), with ZT7 differing from ZT1 ($p<.05$). However, the 1-way ANOVA conducted on the metestrus data did not reveal a significant main effect of time (Table B14). For proestrus there was a significant effect of ZT, $F(3, 18) = 14.052$, $p<.01$ (Table B15), with ZT13 differing from both ZT1 and ZT19 ($p<.01$). And finally for estrus, there was also a significant effect of ZT, $F(3, 24) = 7.775$, $p<.01$ (Table B16), with ZT13 differing significantly from ZT1 ($p<.01$).

In both the BLA and DG peak expression of PER2-ir cells was consistent over the course of the estrous cycle, with peak expression occurring at ZT1 (Figures 7 and 8). In regards to the BLA, the results of the 2-way ANOVA (Table B17) showed only a significant main effect of ZT $F(3, 70) = 37.261$, $p<.01$. Bonferroni post-hoc analysis revealed that the highest expression of PER2 was at ZT1($p<.01$). Finally for the DG (Table B18), again there was only a significant main effect of ZT time $F(3,$

67) = 65.879, $p<.01$. Post-hoc analysis via the Bonferroni revealed again that ZT1 had the highest expression of PER2 ($p<.01$).

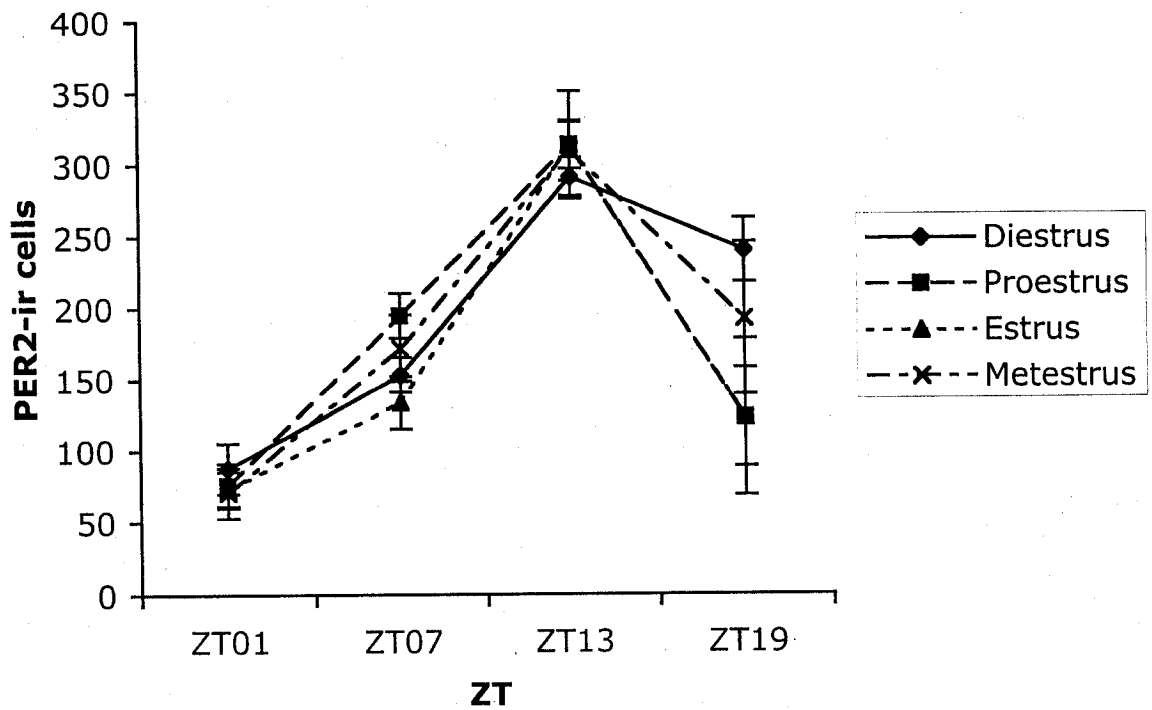

Figure 4: PER2-ir expression (means ± SEM) in the SCN over the course of the estrous cycle. The peak of expression is at ZT13 for all days of the cycle.

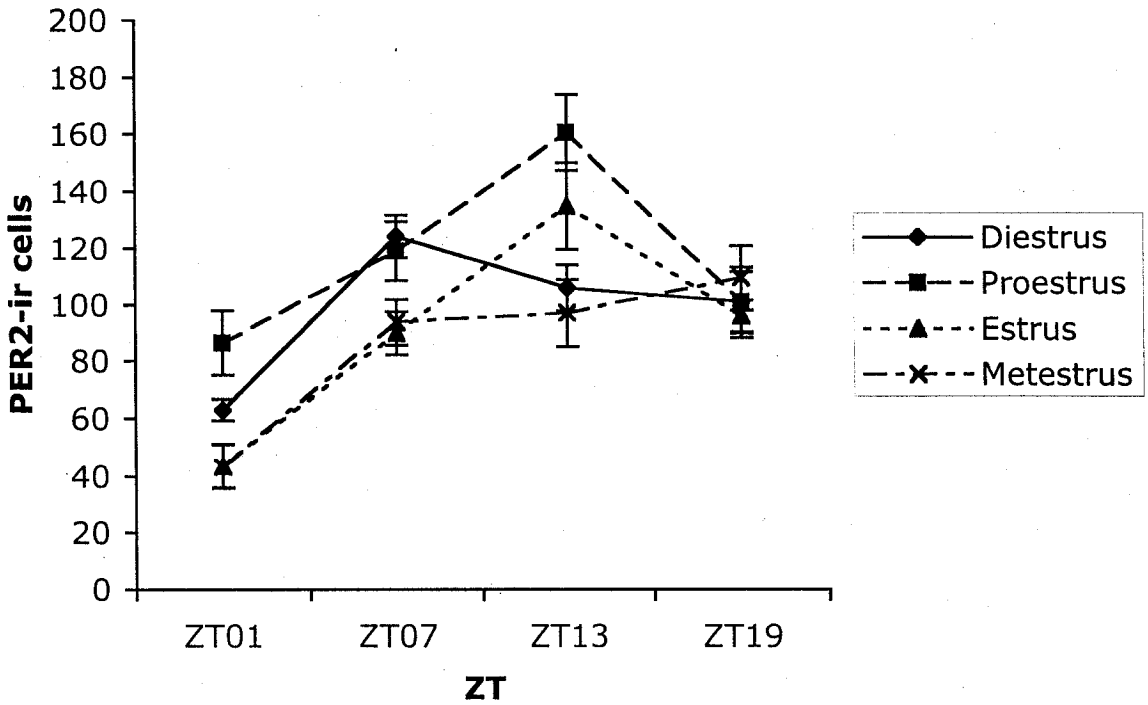

Figure 5: PER2-IR expression (means ± SEM) in the OV-BNST over the course of the estrous cycle. The peak of expression is at ZT13 during proestrus and estrus. However, the peak shifts to ZT19 and ZT7 during metestrus and diestrus respectively.

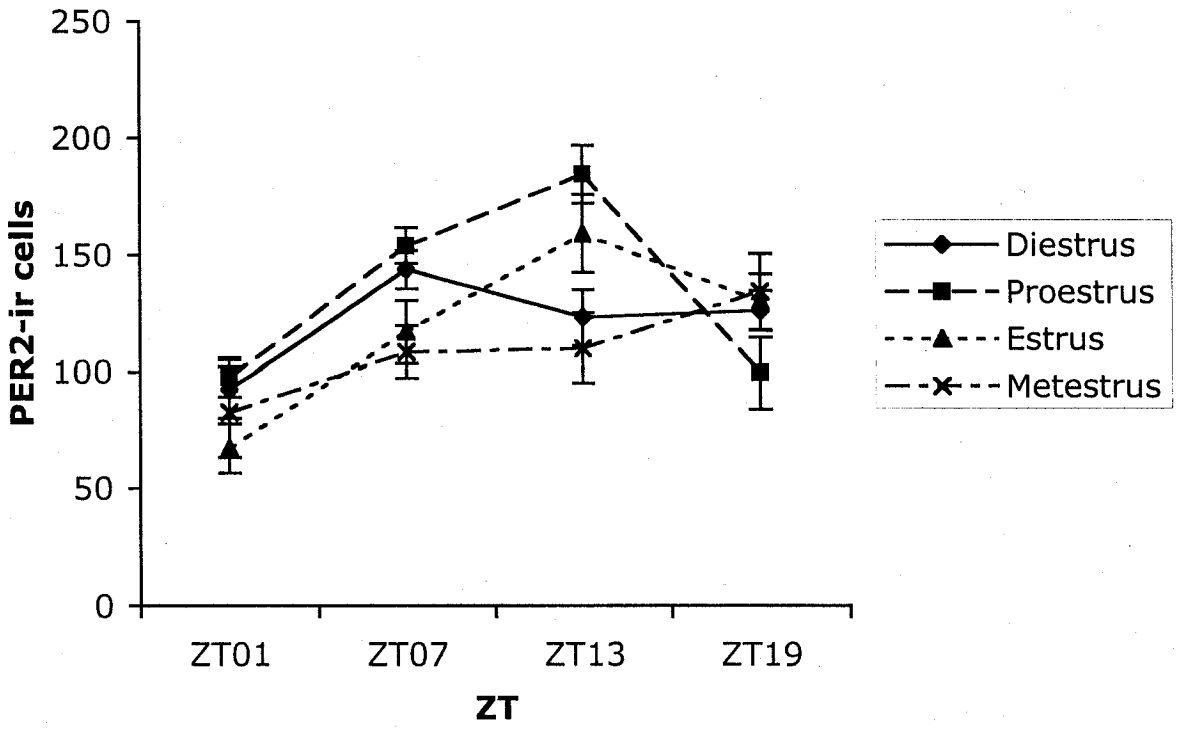

Figure 6: PER2-ir expression (means ± SEM) in the CEA over the course of the estrous cycle. Note the same pattern of PER2-ir expression as in the OV-BNST.

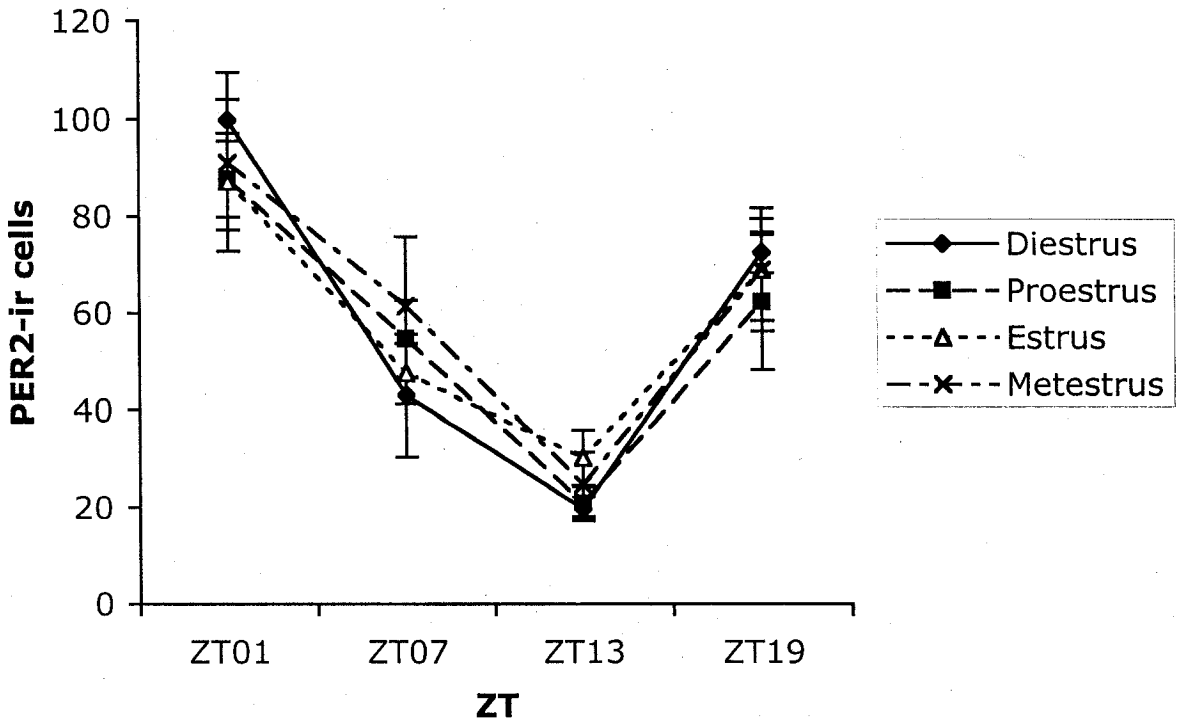

Figure 7: PER2-ir expression (means ± SEM) in the BLA over the course of the estrous cycle. Peak expression of PER2 is at ZT1 irrespective of day of the estrous cycle.

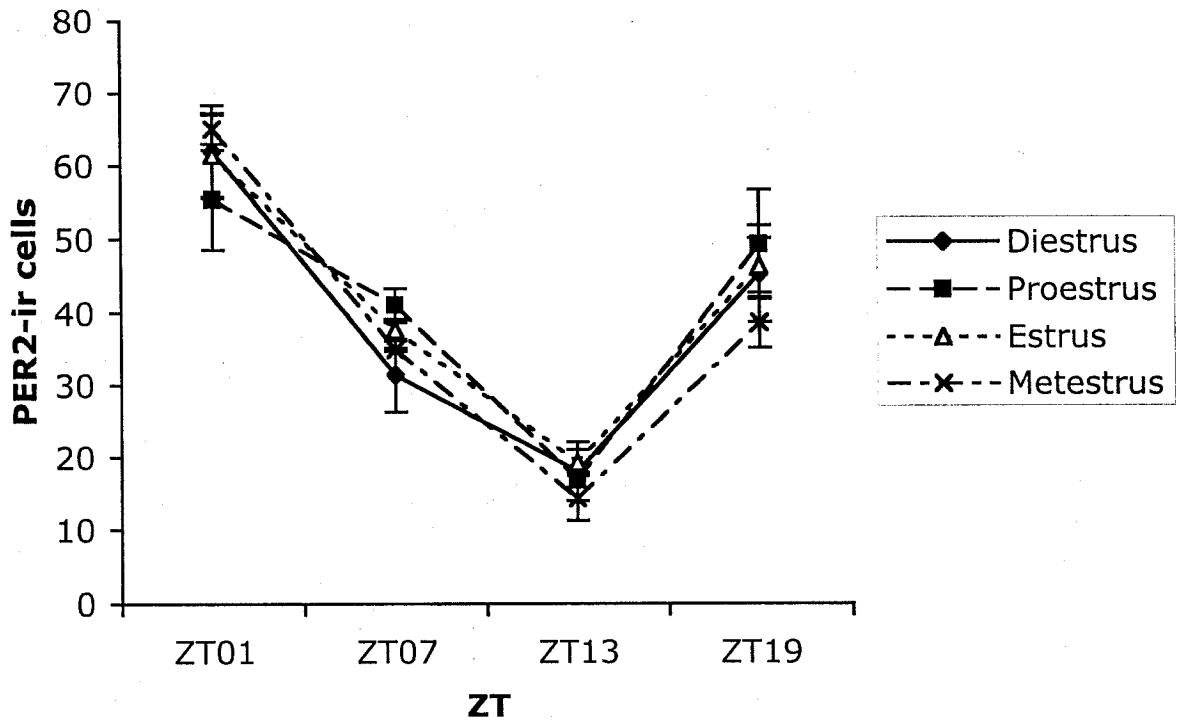

Figure 8: PER2-ir expression (means ± SEM) in the DG over the course of the estrous cycle. The peak of PER2 expression is at ZT1 throughout the estrous cycle.

Discussion

The results from this experiment indicate that stage of the estrous cycle influences the pattern of PER2 expression within the OV-BNST and the CEA. However, the pattern of PER2 expression does not appear to change over the course of the estrous cycle in the SCN, BLA or DG. These results suggest that gonadal hormones influence the expression of PER2 within the OV-BNST and the CEA. To further examine this issue the next set of experiments examined patterns of PER2 expression in ovariectomized females, as well as in gonadectomized and intact males.

Chapter 4: Study 3

Methods

Animals

12 female and 24 male Wistar rats (Charles River Laboratories, St-Constant, Quebec) weighing 175-200g on arrival were used for this experiment. The rats had ad libitum access to food and water throughout the course of the experiment and were housed as described in the first study. The rats were initially divided into three groups; ovariectomized females, gonadectomized males and intact males, and then randomly assigned to subgroups based on the time of day that they would ultimately be perfused, which were ZT1, ZT7, ZT13, and ZT19.

Procedure

The rats were all given 2 weeks to entrain to a 12h:12h LD cycle, after which the female rats and 12 of the male rats underwent gonadectomy. The remaining 12 intact males were perfused after the initial entrainment period. During this

entrainment period daily vaginal smears were obtained from the female rats for 5 days to ensure that they were cycling normally.

The following are flowcharts that outline the course of the experiment for the female rats and the male rats that underwent gonadectomy.

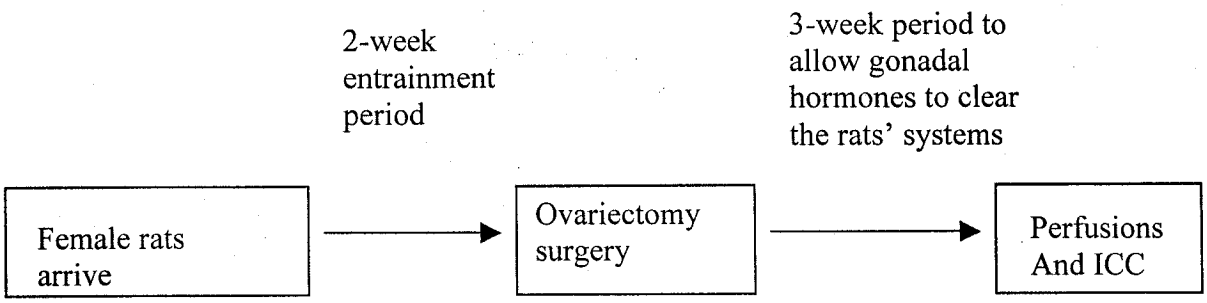

Figure 9: Course of the experiment for ovariectomized females.

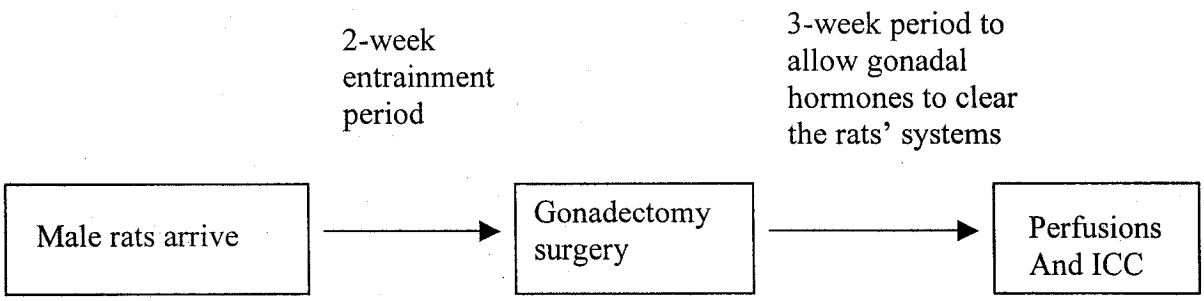

Figure 10: The course of the experiment for gonadectomized male rats.

Surgeries

Ovariectomy Surgery

The female rats were weighed and anesthetized with a solution of Ketamine (100 mg/ml)-Xylazine (20 mg/ml) given intraperitoneally (0.1 ml/100g). Ovaries were removed through bilateral dorsolateral incisions of the skin and peritoneum. The muscle was sutured and the skin was stapled with wound clips. Immediately following the surgery the rats were given an intramuscular injection of Procillin™(MTC Bimeda, Cambridge, ON), an antibiotic (0.1 ml) and Anafen™ (Ketoprofen injectable, Merial, QC) (10mg/ml) for pain relief (0.5 ml/100g).

After surgery the animals were given about 3 weeks to ensure entrainment to the 12h:12h LD cycle and to allow the gonadal hormones to clear from their system. The inspection of actograms was used to confirm that the rats were entrained. After this 3-week period the animals were perfused intra-cardially, and brains processed for PER2 immunocytochemistry as per the procedure described in the first study.

Gonadectomy Surgery

The male rats were anesthetized using a solution of Ketamine (100mg/ml)-Xylazine (20mg/ml) as the anesthetic, given intraperitoneally (0.15ml/100gm). The surgery consisted of one ventral incision above the bladder. The testes were then tied off and removed. The muscle and skin were sutured and the animals were given an intramuscular injection of Procillin™(MTC Bimeda), an antibiotic (0.3 ml, 0.15ml per leg). Once the animals had awoken they were placed in individual cages with running wheels as per the females described above. At this point the males were

randomly assigned to one of four groups, based on the time of day that they would be perfused, which were ZT1, ZT7, ZT13 and ZT19.

The males were left for 3 weeks to allow them to entrain to a 12h:12h LD cycle and to allow gonadal hormones to clear their system. They were then perfused intra-cardially, and their brain sections underwent immunocytochemistry for PER2 as described in study 1.

Data Analysis

Data analysis for Study 3 was conducted in the same manner as Study 2. Means and standard deviations were calculated for each of the three groups for each of the regions of interest. Additionally, 2-way ANOVAs were used to statistically analyze whether there were effects of group and ZT time on PER2 expression. Again, Bonferroni post-hoc tests were conducted where appropriate, and the alpha level was set to .05.

Results

Figure 11 shows that for each of the three groups peak PER2 expression appeared to be at ZT13 in the SCN. There were significant effects of both group $F(2, 24) = 7.951$, $p<.01$, and ZT $F(3, 24) = 62.177$, $p<.01$, as well as a significant group X time interaction, $F(6, 24) = 2.552$, $p<.05$ (Table C19). Further analysis via 1-way ANOVAs revealed that at ZT19 there was a main effect of group, $F(2, 9) = 12.301$, $p<.01$, with normal males having the highest number of PER2-ir cells ($p<.05$). At no other time point was there a significant effect of group.

Figure 12 illustrates that the pattern of PER2 expression in the OV-BNST for each of the three groups had a peak at ZT13. Statistical analysis revealed significant

effects of both group $F(2, 24) = 14.298$, $p<.01$ and ZT time $F(3, 24) = 24.380$, $p<.01$, however, there was not a significant group X time interaction (Table C20). Post-hoc analysis revealed that gonadectomized males had lower PER2 expression than both the ovariectomized females and intact males ($p<.01$), and that ZT13 had the highest level of PER expression ($p<.01$).

Figure 13 illustrates the pattern of PER2 expression with a peak at ZT13 in the CEA of all three groups. A 2-way ANOVA revealed that there were significant main effects of both group $F(2, 24) = 19.521$, $p<.01$ and ZT $F(3, 24) = 21.271$, $p<.01$, but no significant group X time interaction (Table C21). Bonferroni post-hoc analysis again revealed that the gonadectomized males had the lowest PER2 expression compared to the other two groups ($p<.05$), and that ZT13 had the highest level of PER2 expression ($p<.01$).

Figures 14 and 15 depict the pattern of PER2 expression in the BLA and DG respectively. In both these regions, peak PER2 expression occurred at ZT1. In both these regions, 2-way ANOVAs revealed that there were only significant effects of ZT (BLA, $F(3, 24) = 32.109$, $p<.01$) (DG, $F(3, 24) = 23.468$, $p<.01$) (Tables C22 and C23). For both the BLA and DG, post-hoc analysis revealed that ZT1 had the highest level of PER2 ($p<.01$).

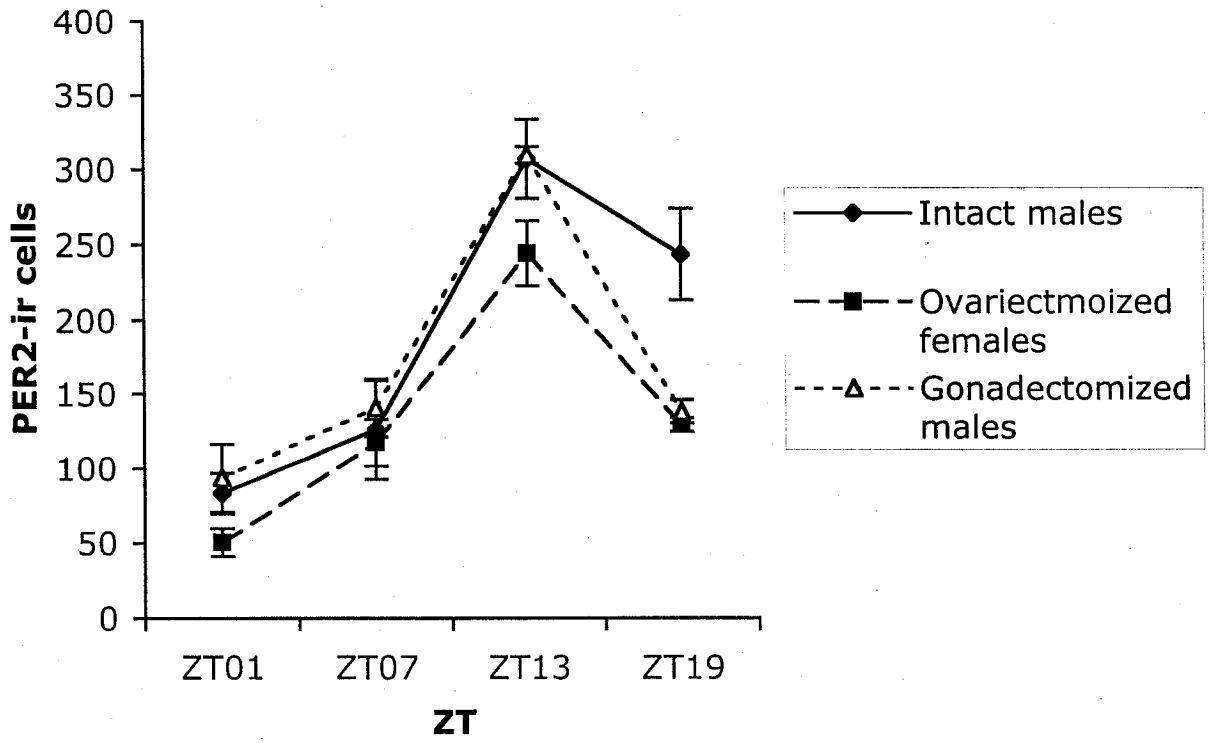

Figure 11: PER2-ir expression (means ± SEM) in the SCN of intact males and gonadectomized males and females.

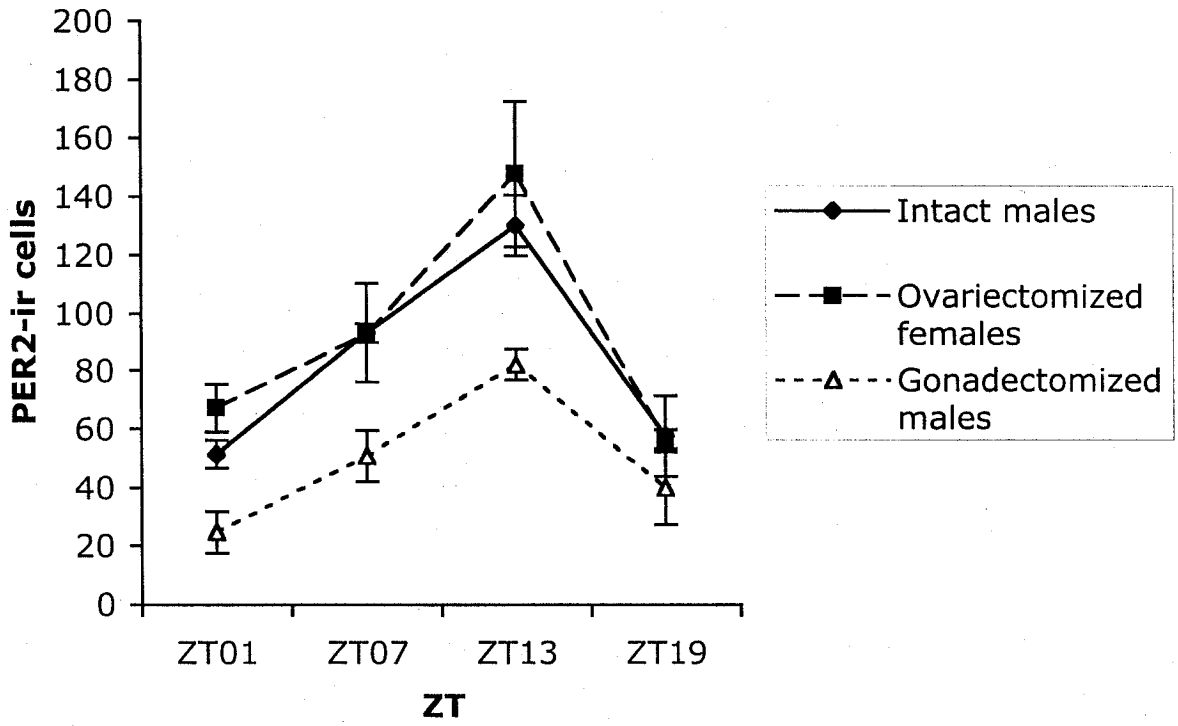

Figure 12: PER2-ir expression (means ± SEM) in the OV-BNST of intact males and gonadectomized males and females.

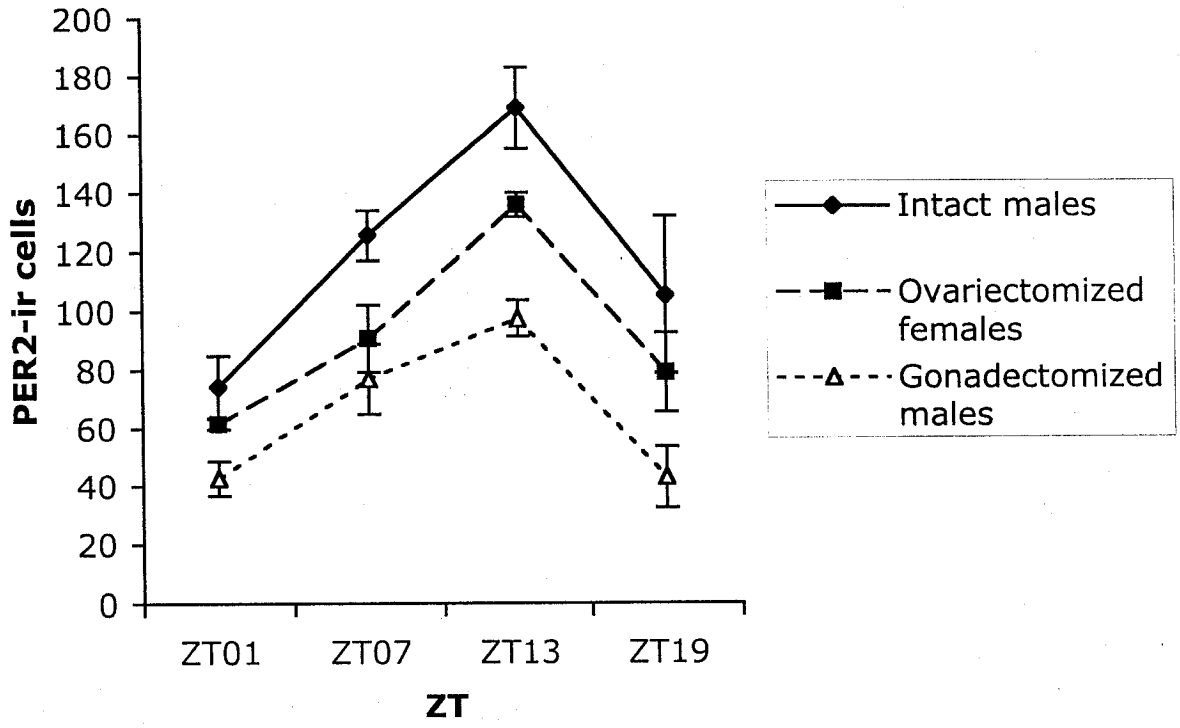

Figure 13: PER2-ir expression (means ± SEM) in the CEA of intact males and gonadectomized males and females.

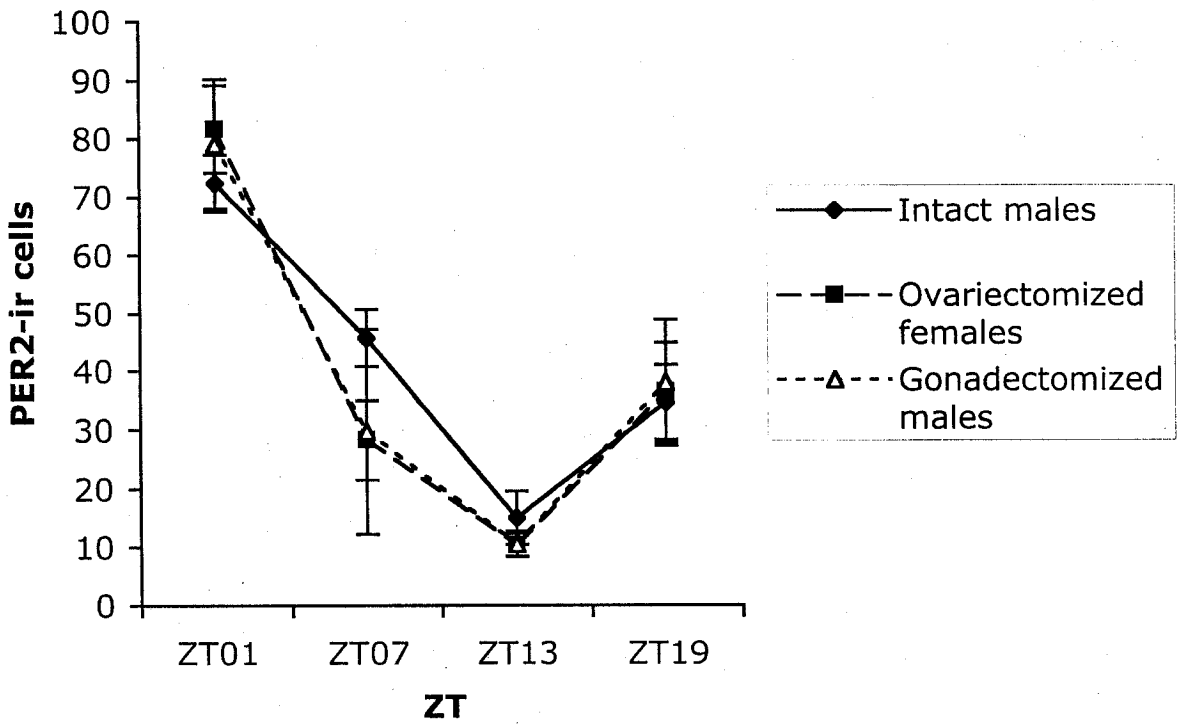

Figure 14: PER2-ir expression (means ± SEM) in the BLA of intact males and gonadectomized males and females.

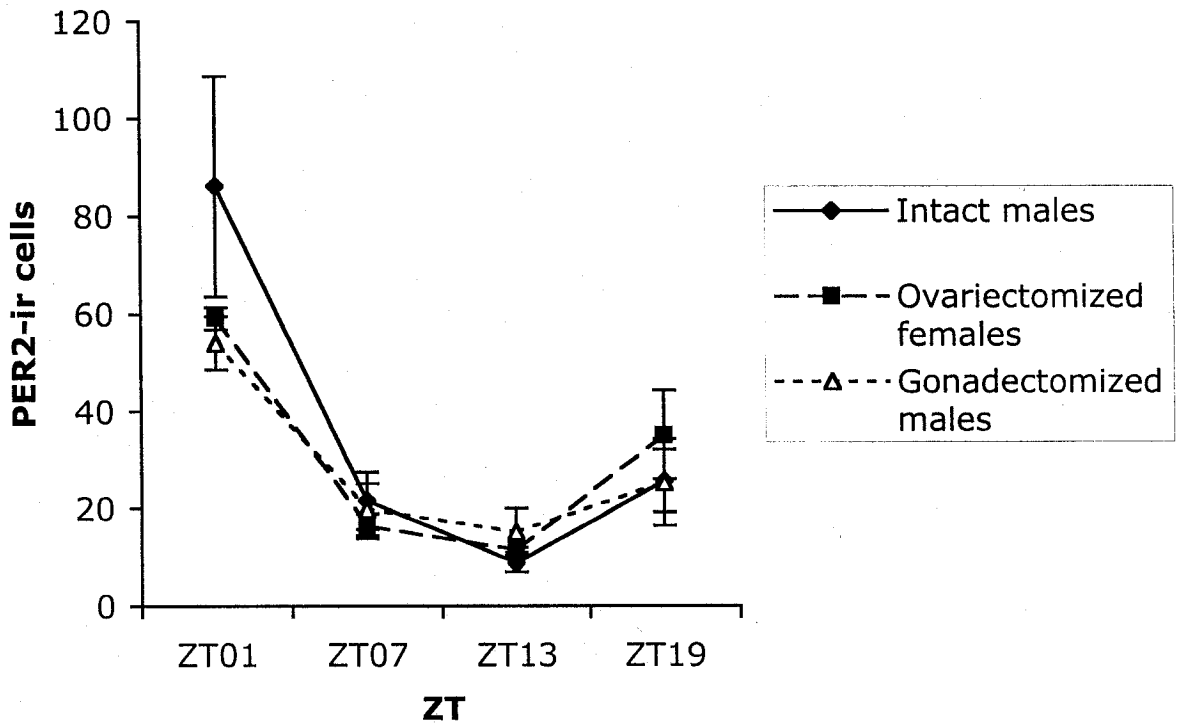

Figure 15: PER2-ir expression (means ± SEM) in the DG of intact males and gonadectomized males and females.

Chapter 4: General Discussion

The results from the first study demonstrated an increase in running wheel activity on the night of proestrus, as first reported by Slonaker (1924). The results also indicated that during metestrus there is a trend towards peak PER2 expression at ZT19 in the OV-BNST and CEA, while peak expression was at ZT13 in the SCN. The results from the second study indicate that in both the OV-BNST and the CEA the pattern of expression of PER2 changes as a function of stage of the estrous cycle. PER2 expression peaks at ZT13 on the days of proestrus and estrus as seen in males, but peaks at ZT19 on metestrus and ZT7 on diestrus. Interestingly, the SCN, BLA and DG do not demonstrate shifts in expression as a function of the estrous cycle, as the peak of PER2 expression remains consistent at ZT13 and ZT1 respectively over the 4-day estrous cycle. In the third study it was found that patterns of PER2 expression in ovariectomized females was similar to that seen in males: a peak at ZT13 in the OV-BNST, CEA and SCN, and a peak at ZT1 in the BLA and DG. Although the ovariectomized females had the same pattern of PER2 expression as intact females on the day of proestrus, cross experiment comparisons of ovariectomized and intact female rats indicate that the ovariectomized females had lower numbers of PER2-ir cells at each time point, in each of the five regions examined. This latter comparison should be treated with caution, however, because it is possible that this difference in number of PER2-ir cells is due to inter-assay variability.

Overall the results of these studies suggest that in the absence of cyclic changes in hormones in the OV-BNST and CEA peak expression of PER2 is at ZT13,

and that it is the gonadal hormones that are linked to shifts in peak expression over the course of the estrous cycle. These results are of particular interest as the brain areas affected by gonadal hormones that are also affected by adrenalectomy (Lamont et al., 2005). This adds support to the theory that some oscillators can integrate signals from the SCN with signals from the periphery.

Which specific aspect of the estrous cycle that is causing this shift in peak expression of PER2 is not yet known. It is possible that estrogen has a direct effect on the OV-BNST and CEA to induce the shifts in peak PER2 expression because both regions contain ERs. On the other hand it is possible that estrogen induced progesterone receptors (PRs) play a key role in this shift of expression.

In the AVPV, a brain region integral to the LH surge, PR mRNA is highest during diestrus and lowest on the morning of proestrus (Simerly et al., 1996). Studies have shown that there are PRs in the BNST (Auger & De Vries, 2002). Therefore, it would be of interest to determine whether the number of PRs in the BNST fluctuate over the course of the estrous cycle, as well as to determine whether there are PRs specifically in the OV-BNST and CEA.

Also unknown is whether the gonadal hormones have a direct effect on PER2 expression, or whether the change in PER2 expression is indirect and due to the gonadal hormones interacting with another aspect of the molecular clock. It is also possible that the changes in PER2 expression seen across the estrous cycle are due to differences in the amount of *Per2* mRNA being produced or due to the speed at which PER2 is degraded.

The potential influence of the LH surge on PER2 expression in intact females

The answer to why these shifts occur may be tied to the LH surge that takes place on the afternoon of proestrus. Studies have shown that progesterone surges on the day of proestrus greatly amplifies the LH surge (Levine, 1997). Meanwhile, downregulation of PRs renders neurons incapable of further transmission of the daily neuronal signal (Levine, 1997). Thus preventing LH surges on subsequent days of the estrous cycle (Chappell & Levine, 2000), as there is not a sufficient number of PRs to generate the surge (Levine, 1997). Research has shown that PRs are necessary for the LH surge to occur, as the PR antagonist RU-486 almost completely blocks LH surges (Levine, 1997). It is possible that the downregulation of PR receptors seen on estrus, metestrus and diestrus could promote the shifts seen in PER2 expression in the OV-BNST and the CEA on the days of metestrus and diestrus. In essence, it could be that the rising levels of estrogen and the increasing number of PRs lead to a peak of expression of PER2 at ZT13 in the OV-BNST and the CEA beginning during proestrus. The downregulation of PRs seen on estrus, metestrus and diestrus could then lead to the shifts in PER2 peak expression seen on metestrus and diestrus in the OV-BNST and the CEA.

Potential studies arising from the results of intact and ovariectomized females

It would be important to ascertain whether the same shifts in the pattern of PER2 expression observed in intact females could be replicated in ovariectomized females receiving hormone replacement. Starting with only estrogen replacement, it would be possible to determine whether estrogen is sufficient for the shift in the pattern of expression. If estrogen replacement does not reproduce the shift in PER2

expression, the next step would be to try a hormone replacement schedule including the administration of both estrogen and progesterone, in order to determine whether progesterone is necessary for this shift in PER2 peak expression.

Secondly, it would be of interest to investigate PER2-ir expression in ovariectomized females immediately after the removal of the ovaries as well as in older females that are no longer cycling. In the current set of experiments the ovariectomized females used were left for quite some time after their surgery. However, one week post ovariectomy surgery of rats aged 2-6 months there are already low or undetectable levels of circulating ovarian steroid hormones, as well as maximal concentrations of circulating gonadotropins in response to the loss of steroid negative feedback (Chakraborty & Gore, 2004). Therefore, it would be of interest to examine whether the same results would be found in ovariectomized females that are perfused less than a week after surgery, before there is an increase in circulating gonadotropins, compared to those perfused weeks after the surgery. The investigation into older females would also be of interest as their estrous cycle has ceased naturally. Once the estrous cycle has terminated, females enter persistent diestrus, where estrogen levels are low and progesterone levels are lower than young cycling females (Chakraborty & Gore, 2004). It is possible that the pattern of PER2 expression could look the same as seen in ovariectomized females, or it could be blunted, or it may look similar to one of the patterns of expressions seen on either diestrus or metestrus.

Thirdly, progesterone and estrogen receptor quantification in the OV-BNST and CEA with additional PER2 labeling would allow for a more comprehensive

analysis of PER2-gonadal hormone interactions. It would be important to carry out this experiment with intact females as well as ovariectomized females to investigate whether there are changes in the number of receptors over the course of the estrous cycle, as well as when the ovaries are removed. This could be accomplished by double-labeling. As PER2 and hormone receptors are nuclear proteins, it might be useful to use a fluorescent stain for one and a regular nuclear stain for the other.

Fourthly, to further elucidate the theory that PRs are playing an important role in the shift of peak PER2 expression, the use of a PR antagonist would be useful. Direct administration of a progesterone antagonist into the OV-BNST and the CEA of intact females could potentially eliminate shifts in PER2 peak expression. Additionally, progesterone antagonists would allow for the investigation into when the peak expression of PER2 would occur when PRs are no longer available.

The potential role of progesterone-glucocorticoid interactions on PER2 expression

Progesterone-glucocorticoid interactions may also be a determining factor in shifts of peak PER2 expression. Surges of progesterone on the day of proestrus may also act on glucocorticoid receptors as well as PRs, as the AR, PR and glucocorticoid receptor (GR) are closely related ligand-responsive transcriptional factors (Nelson et al., 1999). Previous studies have also demonstrated that during proestrus there are increases in peak corticosterone levels compared to other days of the cycle (Atkinson & Waddell, 1997). It has also been found that once ovariectomized the basal corticosterone profiles of these rats resemble those found in intact males (Seale et al., 2004). In addition, both the OV-BNST and the CEA are susceptible to glucocorticoids, as both these regions lose their rhythm of PER2 expression after

adrenalectomy, while the BLA and DG are unaffected (Lamont et al., 2005). Interestingly, the regions that do not appear to be affected by the estrous cycle are the ones that are not affected by adrenalectomy. And finally, both the OV-BNST and the CEA contain high densities of GRs (Honkaniemi, et al., 1992; Rosenfeld, P. et al., 1993; Makino, S. et al., 1994). Therefore, the progesterone-glucocorticoid receptor interaction could influence the shifts in peak PER2 expression over the course of the estrous cycle, as well as the return to a peak in expression at ZT13 seen in ovariectomized females.

In order to investigate this theory further, it would be important to study females that have been adrenalectomized. This would allow investigation into whether there would be a loss of the PER2 rhythm in the OV-BNST and the CEA, as seen in males (Lamont et al., 2005). Also it would be possible to observe whether the same would be seen on all days of the estrous cycle.

Potential sexually dimorphic influences of glucocorticoids on PER2 expression

In male rats gonadectomy does not lead to a change in the timing of the peak expression of PER2-ir cells compared to intact males. Rather, there is a decrease in absolute number of PER2-ir cells. This demonstrates that gonadal hormones must have an influence on the level of PER2 expression in males. Such an influence could be due to a lack of testosterone, which could then in turn affect the ARs. Conversely, it could be the lack of testosterone being aromatized to estradiol that in turn affects ERs that is then causing this drop in PER2-ir expression. The decrease in PER2-ir cell levels found in gonadectomized males, and the return to a peak of ZT13 in ovariectomized females may too be influenced by corticosterone. For instance, it has

been shown that after gonadectomy, subsequent corticosterone secretion in gonadectomized males is similar to that of intact females, while in ovariectomized females this secretion is similar to that of intact males (Seale et al., 2004). It is most likely that male and female gonadal hormones influence hypothalamic-pituitary-adrenal (HPA) axis activity via discrete receptors. This is due to the finding that nonaromatisable dihydrotestosterone (DHT) replacement reverses the effect of gonadectomy, which demonstrates a direct effect of testosterone on androgen receptors (Seale et al., 2004). Therefore, the finding that gonadal hormones influence the HPA via different receptors may help to explain why there is a shift of peak PER2 expression over the course of the estrous cycle, and why ovariectomy leads to a return to peak expression at ZT13, while in males the peak expression of PER2 remains at ZT13 despite gonadectomy.

Sexual dimorphism in gonadal hormones effects on PER2 expression

It is also possible that due to sex differences in some of the regions examined, gonadal hormones may have a different effect on PER2 expression depending on the gender of the rat. Overall, the volume of the BNST does not show sexual dimorphism (Segovia *et al.*, 1999). However, males have greater volume than females in the posterior region of the medial BNST (BNSTMP) (Segovia *et al.*, 1999). Yet females have a larger volume in the anterior region of the medial BNST (Segovia *et al.*, 1999), a region that encompasses the OV-BNST, as well as in the lateral BNST (BNSTLA) and AVPV. Sex differences in the amygdala have also been found in humans and in mice (Koshibu *et al.*, 2004). In humans, the amygdala between childhood and young adulthood enlarges more in males than females, leading to a

larger amygdala in adult males than in females (Caviness *et al.*, 1996). In mice, the volume of the amygdala in females is smaller compared to that found in males (Koshibu et al., 2004).

It is feasible to investigate this hypothesis with the use of adult rats. Giving gonadectomized male rats estrogen and progesterone would allow one to ascertain whether the same pattern of PER2 expression seen in intact females would be seen in these feminized males. Conversely ovariectomized females given testosterone would allow one to examine whether masculinized females would exhibit the same PER2 expression pattern as seen in intact males. Therefore, if the sexual dimorphism of the brain region leads to gonadal hormones having a different effect, then the males given female gonadal hormones should not have the same pattern of PER2 expression as seen in intact females.

Functional significance of changes in PER2 expression

The results from this series of experiments also lead to the question of what is the functional significance of these changes in PER2 expression over the course of the estrous cycle. It is already known that in the OV-BNST and CEA those cells that express PER2 are also immunoreactive to enkephalin (Lamont et al., 2005), hence it is possible that enkephalin may be affected by these changes in PER2 expression. Alternatively, there may be an effect on the electrical output of these regions. Research involving the recording of multiple unit neural activity (MUA) has shown that MUA recorded from the BNST is in phase with the SCN (Yamazaki et al., 1998). Therefore it is possible that the shifts in PER2 expression over the course of the estrous cycle might correspond to changes in MUA in the OV-BNST and CEA.

Conclusions

Overall, the results of this series of experiments reveal that gonadal hormones have an important influence on the expression of PER2 in the OV-BNST and the CEA, two homologous brain structures (Dong & Swanson, 2004). Their influence include playing a role in the shifts of peak expression of PER2, as seen in the intact female, as well as in the decrease in number of PER2-ir cells, as seen in gonadectomized males. These experiments also open the door to many possible future experiments in order to further understand the relationship between gonadal hormones and the circadian system.

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Appendix A

Table 2: Analysis of Variance of number of wheel revolutions over course of the estrous cycle

Source	*df*	*F*	η^2	*p*
Day of Cycle	3	5.886**	.286	.002
Error	44	(15981819.09)		

Note: mean in parentheses represents mean square error

**$p<.01$

Table 3: Analysis of Variance of PER2-ir cells in the SCN during metestrus.

Source	*df*	*F*	η^2	*p*
ZT	3	6.790*	.718	.014
Error	8	(4056.174)		

Note: mean in parentheses represents mean square error

*$p<.05$

Table 4: Analysis of Variance of PER2-ir cells in the OV-BNST during metestrus

Source	*df*	*F*	η^2	*p*
ZT	3	1.568	.370	.271
Error	8	(869.997)		

Note: mean in parentheses represents mean square error

Table 5: Analysis of Variance of PER2-ir cells in the CEA during metestrus

Source	*df*	*F*	η^2	*p*
ZT	3	2.956	.526	.098
Error	8	(613.624)		

Note: mean in parentheses represents mean square error

Appendix B

Table 6: Analysis of Variance for PER2-ir in the SCN of intact females

Source	*df*	*F*	η^2	*p*
Day of cyc (D)	3	0.257	.022	.856
ZT (Z)	3	51.546**	.820	.000
D X Z	9	1.592	.296	.157
Error	34	(2245.580)		

Note: mean in parentheses represents mean square error

*p<.05 **p<.01

Table 7: Analysis of Variance for PER2-ir in the OV-BNST of intact females

Source	*df*	*F*	η^2	*p*
Day of cyc (D)	3	3.66*	.120	.016
ZT (Z)	3	25.725**	.488	.000
D X Z	9	4.446**	.332	.000
Error	81	(673.336)		

Note: mean in parentheses represents mean square error

*p<.05 **p<.01

Table 8: Analysis of Variance for PER2-ir cells in the OV-BNST of females during diestrus

Source	*df*	*F*	η^2	*p*
ZT	3	10.215**	.605	.000
Error	20	(313.973)		

Note: mean in parentheses represents mean square error

*p<.05 **p<.01

Table 9: Analysis of Variance for PER2-ir cells in the OV-BNST of females during metestrus

Source	*df*	*F*	η^2	*p*
ZT	3	6.898**	.485	.002
Error	22	(678.269)		

Note: mean in parentheses represents mean square error

*p<.05 **p<.01

Table 10: Analysis of Variance for PER2-ir cells in the OV-BNST of females during proestrus

Source	*df*	*F*	η^2	*p*
ZT	3	11.151**	.638	.000
Error	19	(888.790)		

Note: mean in parentheses represents mean square error

*p<.05 **p<.01

Table 11: Analysis of Variance for PER2-ir cells in the OV-BNST of female rats during estrus

Source	*df*	*F*	η^2	*p*
ZT	3	11.604**	.635	.000
Error	20	(822.590)		

Note: mean in parentheses represents mean square error

*p<.05 **p<.01

Table 12: Analysis of Variance for PER2-ir in the CEA of intact females

Source	*df*	*F*	η^2	*p*
Dayofcyc (D)	3	2.282	.080	.086
ZT (Z)	3	13.042**	.331	.000
D X Z	9	3.153**	.264	.003
Error	79	(1044.752)		

Note: mean in parentheses represents mean square error

*p<.05 **p<.01

Table 13: Analysis of Variance for PER2-ir cells in the CEA of females during diestrus

Source	*df*	*F*	η^2	*p*
ZT	3	3.503*	.344	.034
Error	20	(638.537)		

Note: mean in parentheses represents mean square error

*p<.05 **p<.01

Table 14: Analysis of Variance for PER2-ir cells in the CEA of females during metestrus

Source	*df*	*F*	η^2	*p*
ZT	3	1.594	.185	.221
Error	21	(1469.655)		

Note: mean in parentheses represents mean square error

Table 15: Analysis of Variance for PER2-ir cells in the CEA of female rats during proestrus

Source	*df*	*F*	η^2	*p*
ZT	3	14.052**	.701	.000
Error	18	(757.832)		

Note: mean in parentheses represents mean square error

*p<.05 **p<.01

Table 16: Analysis of Variance of PER2-ir cells in the CEA of female rats during estrus.

Source	*df*	*F*	η^2	*p*
ZT	3	7.775**	.538	.001
Error	20	(1263.048)		

Note: mean in parentheses represents mean square error

*p<.05 **p<.01

Table 17: Analysis of Variance of PER2-ir in the BLA of intact females

Source	*df*	*F*	η^2	*p*
Dayofcyc (D)	3	0.170	.007	.916
ZT (Z)	3	37.261**	.615	.000
D X Z	9	0.471	.057	.890
Error	70	(459.852)		

Note: mean in parentheses represents mean square error

*p<.05 **p<.01

Table 18: Analysis of Variance of PER2-ir cells in the DG of intact females

Source	*df*	*F*	η^2	*p*
Dayofcyc(D)	3	0.315	.014	.814
ZT (Z)	3	65.879**	.747	.000
D X Z	9	0.736	.090	.674
Error	67	(105.494)		

Note: mean in parentheses represents mean square error

*p<.05 **p<.01

Appendix C

Table 19: Analysis of Variance for PER2-ir cells in the SCN of gonadectomized males and females and intact males

Source	*df*	*F*	η^2	*p*
ZT (Z)	3	62.177**	.886	.000
Group (G)	2	7.951**	.339	.002
Z X G	6	2.552*	.390	.047
Error	24	(1168.078)		

Note: mean in parentheses represents mean square error

*p<.05 **p<.01

Table 20: Analysis of Variance for PER2-ir cells in the OV-BNST of gonadectomized males and females and intact males

Source	*df*	*F*	η^2	*p*
ZT (Z)	3	24.380**	.753	.000
Group (G)	2	14.298**	.544	.000
Z X G	6	.850	.175	.544
Error	24	(411.113)		

Note: mean in parentheses represents mean square error

*p<.05 **p<.01

Table 21: Analysis of Variance for PER2-ir cells in the CEA of gonadectomized males and females and intact males.

Source	*df*	*F*	η^2	*p*
ZT (Z)	3	21.271**	.727	.000
Group (G)	2	19.521**	.619	.000
Z X G	6	.632	.136	.704
Error	24	(441.213)		

Note: mean in parentheses represents mean square error

*p<.05 **p<.01

Table 22: Analysis of Variance for PER2-ir cells in the BLA of gonadectomized males and females and intact males

Source	*df*	*F*	η^2	*p*
ZT (Z)	3	32.109**	.801	.000
Group (G)	2	.134	.011	.876
Z X G	6	.555	.122	.762
Error	24	(210.329)		

Note: mean in parentheses represents mean square error

*p<.05 **p<.01

Table 23: Analysis of Variance for PER2-ir cells in the DG of gonadectomized males and females and intact males.

Source	*df*	*F*	η^2	*p*
ZT (Z)	3	23.468	.754	.000
Group (G)	2	.682	.056	.516
Z X G	6	1.315	.255	.290
Error	24	(218.783)		

Note: mean in parentheses represents mean square error

*p<.05 **p<.01