ESTROUS CYCLICITY MODULATES CIRCADIAN RHYTHMS IN FEMALE SYRIAN HAMSTERS

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LIST OF SYMBOLS AND ABBREVIATIONS

17-β-E₂, 17-Beta Estradiol
ASCN, Anterior SCN
cDNA, complementary DNA
CT, Circadian Time
Ct, threshold cycles
DD, Constant Dark
DNA, Deoxyribonucleic acid
E₂, Estradiol
ER-α, Estrogen Receptor Alpha
ER, Estrogen Receptor
ER-β, Estrogen Receptor Beta
FSH, Follicle Stimulating Hormone
GnRH, Gonadotropin Releasing Hormone
L:D, Hours of Light to Hours of Darkness
LCM, Laser Capture Microscopy
LH, Luteinizing Hormone
LL, Constant Light
MPO, Medial Preoptic Area
mRNA, messenger RNA
MSCN, Median SCN
OC, Optic Chiasm
ON, Optic Nerve
PCR, Polymerase Chain Reaction
PRC, Phase Response Curve
PSCN, Posterior SCN
RHT, Retinohypothalamic Tract
RNA, Ribonucleic acid
RT, Reverse Transcription
RT-PCR, Real Time Polymerase Chain Reaction
SCN, Suprachiasmatic Nucleus
Tau (τ), Free Running Period
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INTRODUCTION

From unicellular to complex multicellular organisms, the ability to synchronize biological processes with rhythmic environmental events is essential to preserving life. Organisms must optimize their chances of survival by coordinating internal functions with the environment surrounding them. For example, it would not be beneficial to an animal to search for food when predation is at its worst. Nor would it be ideal for seasonal breeders to mate during winter because the chances of the offspring perishing during the cold months would be immense.

It is advantageous for organisms to be able to anticipate predictable events that occur on a daily basis. These events can be classified as daily, or circadian rhythms. Daily rhythms, also referred to as nychthemeral, are patterns that are present only when there is an environmental cycle to drive them. Nychthemeral is derived from the Greek word nychthémeron, meaning “duration of a day”. These rhythms are acquired and cease to exist when the environmental cycle stops. Circadian rhythms are not acquired, but are genetically-linked, endogenous daily rhythms that continue to oscillate even under constant environmental conditions. The term circadian is derived from Latin circa meaning “approximately” and dies meaning “a day”, and has a period close to, but not exactly equal to, twenty-four hours. Circadian rhythms are but one type of biological rhythm which include rhythms with other periods such as ultradian rhythms, which have periods shorter than 18 hours, and infradian rhythms, which have periods longer than
30 hours. There are also rhythms that are associated with seasonal cycles, and these are referred to as circannual. In this document the focus is on the key characteristics and unique properties of circadian rhythms.

Three vital traits must be present in order to define a rhythmic oscillation as circadian. The first is the persistence of a robust pattern of behavior or physiology that repeats at a consistent interval that is close to the length of a day, and it must continue to do so even at slightly different ambient temperatures or in different metabolic states, as long as the environment is unvarying. In other words, the rhythm must carry on with a period of approximately 24 hours in constant temperature, light, or dark conditions. The second trait necessitates temperature compensation; that is that the period length of the rhythm must be constant even if the tissue generating the rhythm changes temperature. The third requirement is that endogenous rhythm must be able to entrain to acute environmental cues, such as sunrise and sunset. If a rhythm possesses these characteristics, then it is considered to be a circadian rhythm.

A circadian rhythm can be described, measured, quantified, and compared using a set of parameters that have become accepted in the field. These parameters include amplitude, period, frequency, and phase. Amplitude details the maximum extent of the oscillation. Period is the amount of time it takes for a distinct reference point to be expressed in consecutive cycles. It is signified as the unit of time per cycle and is best computed by analyzing at least 5 waveform cycles. Frequency is the inverse of periodicity and is the number of cycles per unit of time. The phase of a rhythm represents the temporal location of a specific marker within a cycle, for example, the time
of onset of wheel-running activity. One can calculate a phase angle difference between two rhythms, or a change in the predicted phase of a rhythm (called a phase shift) using long-term behavioral recordings such as an actogram (see Fig. 1).

**Figure 1**: Representative actogram of a male Syrian hamster’s (*Mesocricetus auratus*) rate of activity running on a wheel. Each row represents one day of activity (24 hrs).

When an animal is housed in constant conditions it expresses its endogenous circadian rhythm and is said to “free-run”. These rhythms are usually close to, but not exactly 24 hrs in period. The rhythms are considered to “free-run” because environmental stimuli are not resetting the rhythms each day. This free running period is referred to as tau (τ).

The most common, and most important, synchronizer of circadian rhythms is the 24 hour solar cycle of light and darkness. Almost all circadian rhythms can be entrained to light-dark cycles; the rhythms are entrained to environmental photic stimuli.
variation in light intensity and duration, is generally considered to be one of the most powerful synchronizers. Thus, lighting conditions and schedules are crucial when studying circadian rhythms. Lighting conditions are often abbreviated according to the ratio of the amount of light to the amount of darkness (L:D) in a 24 hour time frame. For example, a lighting schedule of 12 hours of light and 12 hours of darkness, or 14 hours of light and 10 hours of darkness, would be abbreviated as (12:12) or (14:10), respectively. Constant conditions are symbolized as LL for light and DD for darkness.

Exposure to light causes phase shifts when given at specific times during the circadian day. Circadian time (CT) is the endogenous time of the internal organism and is used as the reference time scale in circadian data analysis. CT 12 is generally defined as the onset of an animal’s subjective night in research studies. Exposure to a brief period of light (a light pulse) during the early subjective night induces a phase-delay, (a shift in the onset of activity to later in the circadian day), while a light pulse given during the late subjective night will induce a phase-advance, (a shift in the onset of activity to earlier in the circadian day). Light pulses given during subjective day have no effect on the phase of the circadian rhythm.

In the absence of photic cues, other non-photic stimuli are able to shift or synchronize the circadian clock. These include temperature pulses (Rensing & Ruoff, 2002), drugs and chemicals like gamma-aminobutyric acid (Ehlen et al., 2006) & (Mintz et al., 2002) and N-methyl-D-aspartic acid (Mintz et al., 1999), induced activity (Myazaki, et al., 2001), food availability (Strubbe et al., 1986) and locomotor activity associated with refeeding after deprivation (Mistleberger et al., 1997). Feedback loops
also affect entrainment. These include humoral feedback loops (Strother, et al., 1998), transcriptional feedback loops (Shirai et al., 2006), and the circadian clock controlling humoral signals that can entrain peripheral clocks (Tsuchiya et al., 2005).

In order to calculate the effect that an entraining agent has on a stable, free-running rhythm, one must formulate a phase response curve (PRC). First introduced by DeCoursey in 1960, a PRC illustrates the relationship between the timing and the effect of a treatment designed to affect a circadian rhythm. It is calculated by comparing the phase angle difference after a stable activity pattern is re-established following a stimulus, and thereby determines if a phase has been shifted. A PRC is a graph showing time of the subject's subjective day along the x-axis and the amplitude of the phase shift along the y-axis. By referring to a PRC, an investigator can determine the effect of a stimulus at any given circadian time on the circadian clock phase.

Most organisms express circadian rhythms. Unicellular organisms, prokaryotic cyanobacteria, plants, insects, fish, reptiles, amphibians, mollusks, birds, and mammals all express circadian rhythms. In mammals the main oscillator responsible for generating circadian rhythms is the suprachiasmatic nuclei (SCN), which is referred to as the circadian pacemaker; the clock that sets the pace of all circadian rhythms within the organism. It is a paired structure within the hypothalamus that lies dorsal to the optic chiasm and lateral to the 3rd ventricle (see Fig. 2).
Figure 2: Syrian Hamster 12 µm SCN cross section at 40X and stained with Gills Hematoxylin; 3V, third ventricle; OC, optic chiasm.

In mammals, the SCN is responsible for coupling photic information with the generation and regulation of its endogenous rhythms involved in physiology, behavior, and hormonal secretion (Stephan and Zucker, 1972) and (Meijer and Rietveld, 1989). There are many proposed pathways by which cues, both external and internal, modulate the SCN. One of the best characterized is the retinohypothalamic tract (RHT), a neural projection from the retina of the eyes to the SCN via the optic nerve (Moore and Lenn, 1972). It is well documented as a necessity for circadian photoreception, and thus the major pathway involved in the photic entrainment of circadian rhythms (Moore and Card, 1985). Although studying SCN afferents and efferents is one mechanism to correlate endocrine regulation with circadian rhythmicity, another mechanism involving endocrine feed back signals as modulators into and out of the SCN has been studied extensively as well.
The complex relationship between the SCN and steroid hormones has been under investigation since the field of circadian biology originated. The SCN somehow synchronizes reproduction in seasonal breeders with length of photoperiod to ensure that ovulation, finding a mate, and sexual receptivity occur around the same time, and thus maximize chances for reproductive success (Goldman BD, 1999). The prime example of endocrine feedback loops associated with the SCN is the estrous cycle. The general mechanism entails photic input into the SCN so that the SCN can regulate endocrine and reproductive systems to allow gonadal activation and therefore regulate ovulation. When adult male or female hamsters are transferred from a long to a short photoperiod (less than 12.5 hours of light per day), gonadal function is suppressed and the reproductive tract involutes (Hoffman and Reiter, 1965) and (Gaston and Menaker, 1967). Other examples of how hormones modulate the SCN is by estradiol shortening the period of circadian rhythms (Morin, Fitzgerald, Zucker, 1977), the interaction of estradiol and progesterone in modulating the circadian activity of female hamsters (Takahashi and Menaker, 1980), and the ability of progesterone to delay ovulation in proestrus hamsters when given 24 hours before ovulation (Reuter, Ciaccio and Lisk, 1970).

The estrous cycle is the result of interactions among components of the hypothalamus-hypophysis-ovary axis in which estradiol (E\textsubscript{2}) is a major regulator of ovulation. E\textsubscript{2} controls the mammalian reproductive cycle by both negative and positive feedback actions on the secretion of gonadotropin releasing hormone (GnRH) and the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). GnRH is produced by the hypothalamus and stimulates release of LH and FSH from the anterior
pituitary. LH and FSH act on the ovary to produce estrogen and progesterone. When $E_2$ is in high concentrations, it acts through feedback inhibition to suppress the release of FSH and LH by suppressing the trigger GnRH. This is referred to as negative feedback. Positive feedback occurs when lowering levels of $E_2$ trigger the hypothalamus to release GnRH, however $E_2$ is still required for induction and maintenance of the gonadotropin-releasing hormone surge (Evans et al., 1997). The GnRH surge is critical to facilitate the LH surge (Karsch et al., 1997), which stimulates the ovaries, which in turn produce high levels of estrogen. Thus, the varying concentration of $E_2$ is either inhibitory or stimulatory to other hormones involved in the generation of the estrous cycle.

The estrous cycle is homologous to the menstrual cycle in humans and its duration is approximately 29 days. In rodents, the estrous cycle is approximately 4 days or 96 hours, with hamsters having one of the most precisely timed reproductive cycles of all spontaneous ovulators. This cycle persists in both normal (circadian periodicity ~ 24 h) and tau mutant (circadian periodicity ~ 20 h) hamsters for at least a month in the absence of external time cues (Refinetti and Menaker, 1992). The days of the estrous cycle are diestrus 1, or also referred to as metestrus, diestrus 2, or simply diestrous, proestrus, and estrus. Diestrus 1 is the corpus luteal phase, diestrus 2 length varies and can even be skipped, proestrus has the LH surge and the highest $E_2$ levels, and estrus has high activity levels and is the day of ovulation.

The exact mechanism detailing endocrine feedback and the SCN is not understood despite the established relationship between hormone concentrations, amount of light per day, and the SCN. One hypothesis is that neural projections to the SCN area
may relay hormonal signals and communicate directly with the SCN. Van der Beek et al., 1997 determined that GnRH-containing axons are pre-synaptic input to the SCN and the peri-SCN, and together with an anterograde tracer demonstrated a direct SCN-GnRH connection. They concluded that a monosynaptic pathway from the SCN to the GnRH system may play a role in the circadian regulation of the estrous cycle in the female rat. Poletini et al., 2007 determined that SCN afferents and efferents regulate the release of prolactin, a prohormone that stimulates mammary gland development and milk production, secretory surges during parturition and lactation.

Another hypothesis is that humoral factors released and taken in by the SCN cells drive circadian rhythmicity. Silver et al., 1996 discovered that locomotor activity rhythms are restored in arrhythmic, SCN-lesioned animals by transplantation of SCN tissue encased in semipermeable capsules, thereby demonstrating that SCN neural projections are not essential for the generation of circadian rhythms. Others have found that melatonin, a hormone involved in regulation of the sleep-wake circadian cycle by chemically causing drowsiness and lowering the body temperature in preparation for sleep, feeds back on SCN rhythmicity to modulate circadian patterns of activity (Cassone et al., 1993). Still others have hypothesized that the SCN controls the circadian rhythm of melatonin synthesis in the mammalian pineal gland by a multisynaptic pathway and that ablation of the SCN terminates the circadian regulation of melatonin synthesis (Perreau-Lenz et al., 2003).

At the center of all these hypotheses is the fact that the SCN is necessary for and helps regulate the estrous cycle (Alleva et al., 1971). Are there estrogenic pathways
between the SCN and other brain regions? Does estrogen act directly on SCN cells to modulate the estrous cycle? Does estradiol have acute effects on activity rhythms controlled by the SCN? These and other questions were considered in the design of experiments for this thesis.

The SCN is most easily studied in rodent models, such as rats, mice, and hamsters. Hamsters, specifically Syrian hamsters (*Mesocricetus auratus*), are a preferred model to study circadian rhythmicity because of their well defined temporal patterns of locomotor activity and the behavior of their activity bouts (Davis and Menaker, 1980), their sensitivity to photoperiod (Steger, Bartke, and Goldman, 1982), and their precise four day estrous cyclicity (Refinetti and Menaker, 1992). For these reasons, the Syrian hamster is the chosen model studied in the experiments that compose this thesis (see Fig. 3).

**Figure 3**: Picture of a Syrian hamster, the model species used in this thesis.
Experiment 1: Sex Differences in Circadian Locomotor Rhythm Regulation in Syrian Hamsters

Introduction:

Locomotor activity is one of the best studied circadian rhythms. It is persistent despite changing variables, easy to manipulate in a laboratory setting, associated with many other rhythms, and has been made easier to monitor and analyze with modern day computers. Some of the other rhythms associated with it include body temperature (Refinetti R., 1999) and most essential to this thesis, estrous cyclicity. Studies have demonstrated the link between locomotor activity and the estrous cycle. Loss of activity rhythms coincides with loss of reproductive rhythms (Fitzgerald and Zucker, 1976) & (Stetson and Watson-Whitmyre, 1976) and as demonstrated by Morin et al. in 1977, the circadian rhythm system that regulates temporal characteristics of running by female hamsters can be modified by estrogen.

Studies have also shown that locomotor, specifically wheel-running, activity displays a sexual dichotomy proposed to be a result of differentiation of the circadian system during development, specifically that the system is sexually differentiated in its response to E₂. Sexual differences in locomotor activity include males having significantly greater ability to entrain and a more consistent activity pattern; while intact females possess less capability to entrain and whose activity onset varies significantly
with day of the estrous cycle, specifically that elevated estradiol is correlated with an earlier onset of activity (Davis, Darrow and Menaker, 1983). Estradiol is involved in the regulation of circadian periodicity (Morin et al., 1977) and the circadian pacemaker of female hamsters, but not that of males, is sensitive to the period shortening effects of \( \text{E}_2 \) (Zucker et al., 1980). \( \text{E}_2 \) is responsible for the significant phase advances and the increased running time characterized with shorter bouts of activity (Morin and Cummings, 1982) that are observed during periods of high circulating levels of estradiol (Pittendrigh and Daan, 1976). Wheel-running activities fluctuate in female rats showing 4-day rhythmicity with activities increased in estrus and proestrus stages of the estrous cycle, and these fluctuations are not present in male rats (Shinodo et al., 1988). See Fig. 4. These differences in wheel-running activity of rats are thought to be a consequence of sexual differences in the organization and maturation of the circadian system (Díez-Noguera A, and Cambras T., 1990).

**Figure 4:** Representative actograms from a male and a female hamster. **A.** Male hamster displaying a stable activity pattern. **B.** Female showing a distinctive variation in activity as a function of estrous cycle phase.
Estrous and circadian cycles are clearly capable of influencing each other. The estrous cycle modulates circadian activity as evidenced by the observation that the periodicity of the estrous cycle is displayed in the amount and temporal organization of running-wheel activity (Richards, MPM, 1966); (Carter, SB, 1972); (Takahashi and Menaker, 1980); (Albers, Gerall, and Axelson, 1981); and (Thomas and Armstrong, 1989), and that estradiol shortens the period of hamster circadian rhythms (Morin, Fitzgerald, Zucker, 1977). However, studies have also demonstrated that photic and non-photic cues of the circadian system can phase shift the estrous cycle.

Photic and non-photic phase shifting significantly impacts ovulation in female rodents. Environmental LD cycles entrain the circadian system which, in turn, provides temporal information to rhythms of estrous cyclicity and locomotor activity (Moline et al., 1981). The timing of ovulation can be altered with phase-shifting of the circadian clock. It is altered by adding or deleting light from the beginning or end of the photoperiod. Light pulses given near the onset of running cause a delay in ovulation whereas light pulses given near the end of running cause an advance in the CT of running and also in ovulation (Sridaran and McCormack, 1980). In Syrian hamsters, activity onset phase delayed more quickly than they advanced and higher estradiol levels were correlated with more rapid advances of activity onset (Moline and Albers, 1988). Sexual differences also are expressed in reentrainment rates of male and female *Octodon degus*. After a 6 hour phase advance, activity rhythms of males housed alone in their cages reentrained significantly faster than those of females (Goel and Lee, 1995). Non-photic phase shifting is also known to shift the estrous cycle. In the Syrian hamster, stimulation
(a cage change) on the day of proestrus often resulted in a one day delay of the estrous cycle (Young-Janik and Janik, 2003); thereby causing the usual four day cycle to become five days instead.

These experiments were designed to analyze the differences in the expression of male and female locomotor activity rhythms in regards to the amount of wheel-running activity, the activity patterns associated with sex and day of the estrous cycle, and if sexual differences are evident in re-entrainment rates of Syrian hamsters.

**Methodology:**

*Animals:* For all experiments, adult male and female Syrian hamsters (*Mesocricetus auratus*) were bred at Kent State University from stock purchased from Harlan Sprague-Dawley, Inc. Hamsters were group housed in a 14:10 L:D cycle with food and water available *ad libitum* until maturation (12 – 20 weeks old), when they were moved into individual cages equipped with running wheels.

*Experiment A:* This study was designed to test for differences in wheel-running activity and also of reentrainment rates of male and female Syrian hamsters. The animals were maintained in a Aschoff type II protocol (14:10 L:D cycle) and profiled to determine the differences in activity and total wheel revolutions per day until the day of treatment. The day of treatment consisted of the onset of darkness being delayed by 8 hours. The animals were then reentrained to another 14:10 light:dark cycle and analyzed for re-entrainment rates.
Actogram Analysis: Wheel-running activity was monitored and wheel counts averaged into 6-minute bins. Each wheel revolution activated a microswitch on the outside of the cage that was monitored continuously by a Dell computer using ClockLab software. For each animal, an activity profile of counts/bin was generated across an 8 to 10-day period. Counts for each male were averaged across the 8-day period. Counts for each female were divided into 4 groups according to the phase of the estrous cycle and average across the 2 days measured for each cycle. Mean activity profiles for males and females were generated, with females subdivided by the phase of the estrous cycle. Total wheel revolutions per day were also calculated. This data was analyzed using 1-way ANOVA, \( P < 0.0001 \) and Tukey-Kramer test for pairwise comparisons, \( P < 0.05 \). The percentage of females as compared to males able to reentrain was calculated and the data was analyzed using \( P < 0.05 \), Fisher’s Exact Test.

Experiment B: The animals were maintained in a Aschoff type II protocol (14:10 L:D cycle) until the day of treatment. The animals were given a 30 minute light pulse at CT 19 to induce a maximum phase advance response. After the light pulse, the animals remained in constant darkness for at least 10 days, wheel-running activity monitored, and PRC were analyzed to calculate phase-shifts.

Phase Shift Analysis: The objective was to determine if females have a phase response curve (PRC) to light that is reduced in amplitude relative to males, or have a
“compressed” PRC with shorter phase delay and/or phase advance regions. This may be why females have more difficulty reentraining to a shift of the LD cycle than males. PRCs were analyzed using MatLab software. Estimates of the circadian periods were made by measuring the angle of a line visually fitted through the onsets of activity that occurred on the 7 days preceding the light pulse. A second line was fitted to activity onsets that occurred 4–11 days after the light pulse. Days 1–3 post-light pulse were not used in the data analysis to avoid including transients or unstable onsets. Phase shifts were determined by the difference between the predicted onset of activity before treatment (as predicted if no treatment were to be given) and the actual line of activity onset after the light pulse. The resulting angle was then converted to hours to calculate the exact shift. This was performed on each hamster’s activity pattern.

**Results:**

*Experiment A: The differences between male and female Syrian hamsters’ circadian locomotor activity and re-entrainment rates after an 8-hour phase delay.*

The activity profiles of males and females were distinctly different. In female hamsters, the onset of activity is slightly advanced on diestrus 2 and proestrus relative to estrus, diestrus 1, and the male profile. The mean activity profile is distinctly bimodal during estrus (see Fig. 5), and the pattern of activity differs from other days of the cycle. Peak activity levels are much higher during diestrus 2 and proestrus than during diestrus
1 and estrus, demonstrating that the pattern of daily wheel-running varies across the estrous cycle.

**Figure 5:** The mean activity profile for male and female hamsters.

![Activity Profile Graph]

The total wheel revolutions per day were overall significantly greater in males; however in females, the total wheel running was significantly greater during proestrus and diestrus 2 (see Fig. 6). These results suggest that the quantity of wheel-running varies across the estrous cycle.
**Figure 6:** Total wheel-running is significantly reduced on estrus and diestrus 1 as compared to diestrus 2, proestrus, and in males (1-way ANOVA, $P < 0.0001$, Tukey-Kramer test for pairwise comparisons, $P < 0.05$).

Reentrainment rates after a large shift of the L:D cycle in female hamsters were significantly slower than those of male Syrian hamsters. Males tended to reentrain more readily while the females could not or were doing so very slowly (see Fig. 7).
Figure 7: Actograms of male and female Syrian hamsters reentraining to an 8-hour phase delay of a 14:10 L:D cycle. Each record is double plotted. A. A male successfully phase delayed and reentraining. B. A female hamster illustrating a delayed phase shift. C. A female appears as if not affected by the phase delay. D. A female displaying an irradic activity plot.

Also, the percentage of females able to reentrain was grossly decreased when compared to male hamsters (see Fig. 8).
Figure 8: Females reentrained to a shift of the LD cycle at a much slower rate than did males. \((P < 0.05, \text{ Fisher’s Exact Test})\).

Experiment B: The difference between male and female phase advance response to a light pulse at CT 19.

There was no significant difference in the amplitude of a light-induced phase advance between males and females when a 30 minute light pulse was administered at CT 19 (see Fig. 9).

Figure 9: Phase response curves of male and female Syrian hamsters.

A. Male, phase shift: +1.99 hrs

B. Female, light pulse on diestrus 2, phase shift: +1.67 hrs
In addition, excluding the diestrus 2 group due to small sample size, there was no significant difference across the estrous cycle (see Fig. 10).

**Figure 10:** Sexual differences in amount of phase shifts to 30 minute light pulse.

A. Males compared to females

B. Females shifts with day of the estrus cycle
Conclusions:

In conclusion, there is a distinct difference in circadian locomotor activity patterns in Syrian hamsters between the sexes and by day of estrous cycle in females. Males have a stable, steady activity pattern whereas females have varying patterns and quantity of daily wheel-running across the estrous cycle. Females have difficulty entraining to large shifts of the LD cycle, suggesting, that the PRC to light in females is either qualitatively (shape) or quantitatively (amplitude) different than that in males. These data extend the findings of Moline & Albers, 1988 that the rate of phase shifts of the locomotor activity rhythm are correlated with changes in circulating estradiol concentrations. This further demonstrates that phase shifts induced by photic stimuli are modulated by the estrous cycle.
EXPERIMENT 2: DISTRIBUTION OF ESTROGEN RECEPTORS ALPHA AND BETA IN MALE AND FEMALE SYRIAN HAMSTER BRAINS

Introduction:

Estrous cyclicity and the circadian system are intimately linked and hypothesized to be regulated by the same multi-oscillator system (Schwann and Turek, 1985). Research has shown that the SCN is necessary for and helps regulate the estrous cycle (Alleva et al., 1971). Horizontal knife cuts in the SCN area prevent hamster gonadal responses to photoperiod (Eskes and Rusak, 1985). SCN lesions abolish the LH surge and induce irregular estrous cyclicity (Refinetti et al., 1994) and fetal SCN transplants do not restore normal estrous cycles (Meyer-Bernstein et al., 1999) when they restore other circadian rhythms.

Circadian physiology is significantly impacted by the estrous cycle. Estradiol is involved in the regulation of circadian periodicity (Morin et al., 1977) and peak gonadotropin release has a 2 – 3 hour positive phase angle to the circadian rhythm of locomotor activity (Stetson and Gibson, 1977). Phase shifting the free running period by shifting the timing of onset of light also shifts the timing of ovulation (Sridaran and McCormack, 1980). The interaction between estrogen and progesterone modulates the circadian activity in female hamsters on a day-to-day basis (Takahashi and Menaker, 1980).
Controversy exists over the mechanism by which estradiol modulates circadian rhythms. Some theories propose that increased levels of E₂, during proestrus, bind to estrogen receptors (ERs) in the preoptic and mediobasal areas of the hypothalamus and send an estrogen signal that interacts with a precisely timed neural impulse originating from the SCN, resulting in the induction of the GnRH surge from the hypothalamus which is followed by the LH and FSH surge from the pituitary which stimulates growth of ovarian follicles.

Others have proposed that E₂ acts on other areas of the hypothalamus and communicates back to the SCN through an “estradiol signal” during a specific time frame (2 and 4 p.m.) and then the SCN triggers the LH surge (Barbacka-Surowiak et al., 2003). This theory has been proposed in the rat but remains questionable in the hamster and other animals.

Another theory is that ER-immunoreactive (-ir) neurons, or neurons expressing ER mRNA, send information to the SCN which, in turn, conveys signal to GnRH neurons to stimulate GnRH and, in consequence, the LH surge. It has been hypothesized that the SCN regulates the timing of the LH surge via inputs to GnRH neurons and via inputs to ER expressing neurons (de la Iglesia et al., 1995 and 1999). Another study has documented the expression of ER-beta (ER-β) mRNA in single cell reverse transcription-polymerase chain reaction of GnRH neurons (Herbison et al., 2001), providing evidence that suggests estrogen may be acting on the GnRH neurons themselves.

One proposed theory is that estrogen has direct effects on the SCN. SCN neurons in the human hypothalamus appear to contain estrogen receptor-alpha (ER- α) and ER-β
and indicate for the first time that estrogen and progesterone may act directly on neurons of the human biological clock (Kruijver and Swaab, 2002). Immunoreactivity for either ER-α or ER-β, with predominance of ER-β, is localized in not only neurons but also astrocytes in cultured cells of the SCN in neonatal rats. Co-expression of both ER-α and ER-β was also observed in the same neuron suggest that estrogen's effect on the SCN may be mediated at least in part by its ER-containing neurons (Su et al., 2001). This would be made possible through the interaction of estrogen with specific types of receptors, either classical or membrane receptors.

Classical receptors are located in the cytosolic and/or nuclear compartments of cells within the brain and result in genomic changes via transcription and translation. Circulating estrogen is lipid soluble and can diffuse from blood serum through the cell membrane, and if an ER is present, it binds to a specific ER to induce a conformational change in the ER, leading to dissociation of co-repressors from the ER and allowing recruitment of “co-activators” (McKenna et al., 1999) and (McKenna and O’Malley, 2002). Through interaction with specific DNA target sequences, called estrogen response elements (ERE), the active ER modulates the rate of transcription by acting like a ligand-activated transcription factor, in which the estrogen binding initiates changes in gene transcription, resulting in changes in levels of target gene mRNA and translated protein (Shupnik, 2002).

Membrane receptors bind specific messenger molecules on the exterior surface of the cell and may also mediate the effects of estradiol. Two types of responses can occur when a membrane receptor is activated; a direct response and a second messenger
response which involves the production of an intracellular mediator to ultimately cause
the effect. E\textsubscript{2} can affect second messenger systems including calcium mobilization and a
plethora of kinases to alter cell signaling (Kelly and Ronnekleiv, 2008). Recently
discovered is E\textsubscript{2}’s ability to interact with a novel estrogen receptor-interacting protein,
PELP1, which has been implicated to be important for the mediation of both genomic and
nongenomic signaling of 17-β-estradiol (Brann DW et al., 2008). Also E\textsubscript{2} has rapid,
membrane-initiated actions that are due to surface expression of classical estrogen
receptors (Micevych PE and Mermelstein PG, 2008).

Classical estrogen receptors are members of the nuclear receptor family of
transcriptional modulators. Two receptor molecules have been identified, ER-α and ER-
β. ER-α is found in all reproductive tissues, is the most abundant and was the first ER to
be identified. ER-β is the product of a different gene (Kuiper et al., 1996; Tremblay et
al., 1997) and has little similarity to ER-α. ER-α is the receptor necessary to modulate
normal function of the ovaries (Hewitt and Korach, 2003) and appears to be the ER that
mediates the negative feedback regulation of LH (Couse and Korach, 1999). It is
expressed in different brain regions than ER-β and has in particular been documented to
be expressed in the neurons projecting to the female hamster SCN (de la Iglesia, 1999).

ER-β mRNA has been demonstrated through in situ hybridization to be expressed in the
male rat SCN (Shugrue et al., 1997), the SCN of ovariectomized female rats treated with
E\textsubscript{2} (Shima, Yamaguchi, and Yuri, 2003), and in rat LHRH neurons (Hrabovszky et al.,
2000). ER-β protein has also been localized to rat LHRH neurons (Hrabovszky et al.,
2001).
Current research is ambiguous on the question of whether ER-α and/or ER-β are present or absent in the SCN of Syrian hamsters. If ERs do exist in the SCN, this could suggest that estrogen may possibly act directly on the cells of the SCN to impact the organism’s reactions to estrogentic effects on circadian rhythms. Therefore, this study was designed to determine whether ER-α and/or ER-β are expressed within the SCN of male and female Syrian hamsters and if so, whether is there a distinct regional organization of that expression within the SCN.

**Methodology:**

**Animals:** For all experiments, adult male and female Syrian hamsters (Mesocricetus auratus) were bred at Kent State University from stock purchased from Harlan Sprague-Dawley, Inc. Hamsters were group housed in a 14:10 L:D cycle with food and water available ad libitum until maturation (12 – 20 weeks old).

**Preparation:** Eight Syrian Hamsters, four males and four females, were randomly selected for this experiment. Animals were euthanized by decapitation and the brains, with the optic nerves still attached, were removed within 3 minutes of the decapitation. The entire brains were embedded in Tissue-Tek OCT and flash frozen on dry ice flakes. All brains were then stored at -70°C for no longer than 3 weeks. The brains were then sectioned in twelve micrometer thick, coronal slices beginning at the medial preoptic area
(MPO; Bregma 0.9 mm) and continued caudally through the SCN to the retrochiasmatic area (RCh; Bregma -1.2 mm) on a Leica cryostat at -20°C. All sections were mounted on glass slides and returned to -70°C.

**Laser Capture Microdissection:** The slides were individually defrosted for no longer than 30 seconds in order to stain the tissue. The slides were stained with Hematoxylin Gill’s Formula, dehydrated through a series of ethanol rinses, and placed in xylenes for 3 – 5 minutes. Microdissection of target SCN cells was performed using the Autopix® Automated laser-capture microscope (LCM) according to the protocol provided by the manufacturer. Extra drops of xylenes placed on the SCN sections allowed cells to be visualized for microdissection (see Fig. 11).

Figure 11: Laser Capture Microscopy Images;

A. Syrian Hamster 12 μm SCN cross sections at 40X and stained with Gill’s Hematoxylin.
B. The Microdissected SCN Tissue Sample as viewed on the cap at 100X after LCM.

Twelve different samples were microdissected from within each animal’s SCN; four anterior, four median, and four posterior sections were collected and assigned individual PCR tubes so each was considered a different sample until data analysis was performed (see Fig. 12). Total cellular extract from each sample was stored at -70°C until purification.

**Figure 12:** Organization of SCN Samples; samples were ran individually for analysis.
RNA extraction:

_PicoPure RNA Isolation Process:_ The PicoPure RNA isolation kit is capable of isolating extremely small amounts of RNA that can be used directly in reverse transcription protocols. See protocol in Appendix A. Samples were stored at -70°C for no longer than 3 months until use.

_Picking the Primer Sequence:_ Forward and reverse primers were designed using Primer Express software. Primers were diluted with ethylenediaminetetraacetic acid (EDTA) to yield 50, 300, and 900 nM concentrations. They were used to determine optimal concentrations for the PCR reaction and then analyzed in Step 2 of RT-PCR. The primer sequences for ERα were forward: AATGCTGAAACACAAGCGCC and reverse: ATGTCGTTCTGCCTTCCAA. ERβ forward sequence was CTTTGTGGAGCTCAGCCTGTT and reverse was CATCCAGCAGCTTTCCAAGAG. β-Actin was used as a control gene, and forward was ATCCTGTGGCATCCACGAA and reverse was CGACGTCACACTTCATGATGG.
Two-step RT-PCR Gene Expression Quantification Assay:

Step 1: TaqMan® Reverse Transcription Reagents: The eluted RNA was reverse-transcribed to cDNA using TaqMan® Reverse Transcription (RT) Reagents. RT was performed as described in the manufacturer’s protocol (see Appendix A). cDNA was stored at -20°C no longer than 3 months until use. Total cDNA was analyzed using two-step RT-PCR with the TaqMan ABI 7000 sequence detection system according to the manufacturer’s protocol. Each sample was used to amplify ERα, ERβ, and the housekeeping gene, β-Actin, which served as a control for total RNA concentration during PicoPure or cDNA synthesis during TaqMan sample preparation.

Step 2: There was 2.5 μL each of the forward and reverse primers along with 5 μL cDNA template, and 10 μL 10X SYBR® Green PCR Buffer in 0.2 μL PCR optical tubes or in 96-well optical reaction plates. SYBR Green acts as a fluorescent reporter dye to bond with the double-stranded DNA to detect the PCR product. A Passive Reference dye is in the SYBR Green PCR Buffer to act as an internal reference for normalization. It corrects fluctuations in the fluorescent reading which can be caused by changes in DNA concentration or total volume. A 96-well reaction plate was set up so that each sample was run in triplicates in order to measure and record variance. Each real-time quantification of the target transcript was determined based on the threshold cycles (Cts) of the target and the Passive Reference. The triplicate Ct values were considered acceptable when they were within 1 Ct of each other, had solid amplification plots,
specific melting points. The mean triplicate Ct values of each target transcript was calculated and then subtracted from the control transcript triplicate mean Ct value and expressed as a fold change in expression. The fold changes in expression were then analyzed for significance by a two-way ANOVA with significance attributed to $\rho < 0.05$.

**Results:**

*Sex differences in expression of ER-$\alpha$ and ER-$\beta$ Syrian hamster SCN samples:* ER-$\beta$ was expressed in both male and female SCN samples. There was not a significant difference between the male and female samples as both had equal quantities of ER-$\beta$ mRNA present. Before DNAase treatment was incorporated into the protocol, the samples expressed ER-$\alpha$ amplification in both male and female SCN samples. There was not a significant difference between the male and female samples. We evaluated the specificity of the ER-$\alpha$ primer by doing the fore-mentioned protocol to the medial preoptic area of the female Syrian hamster, where ER-$\alpha$-ir cells have been identified using immunocytochemistry (Li H-Y, et al., 1993) and we found that the ER-$\alpha$ primer was successful in identifying existing ER-$\alpha$ mRNA. In order to confirm that the amplification was not from genomic DNA, DNAse treatment of a subset of samples was performed in addition to the RNA purification step. DNAse treatment eliminated PCR amplification of ER-$\alpha$ in male and female SCN samples. This suggests that no significant amounts of ER-$\alpha$ mRNA were present within the SCN samples.
Organization of expression of ER-α and ER-β within the Syrian hamster SCN before DNA-ase Treatment: There appeared to be a significant difference in the male median SCN expression of ER-α (see Fig. 13), but further evaluation showed that ER-α was not expressed and thus, voided this.

Figure 13: Male and Female Hamster Ct Fold Difference Graphs

Organization of expression of ER-β within the Syrian hamster SCN: As analyzed using a repeated measures ANOVA with significance attributed to p<0.05, there was no significant differences in the expression of ER-β mRNA in male and female hamsters throughout the SCN or by the location of the sample.
Conclusions:

ER-β mRNA was expressed in both male and female SCN cells in equal quantities whereas ER-α mRNA was expressed consistently in all samples that were not DNase treated and was eliminated in DNase treated samples. There is no significant difference in the expression of ER-β between the sexes or by location of the sample. However, we did establish the novel technique of using laser capture microscopy as an effective technique for quantifying gene expression in discrete brain regions and that RT-PCR is a more sensitive technique in identifying genetic material than in previous other studies.
EXPERIMENT 3: ACUTE EFFECTS OF ESTRADIOL MICROINJECTIONS INTO THE SCN REGION

Introduction:

The timing of the master circadian clock can be shifted by a variety of stimuli, including estrogen feedback. Since ERs are expressed in the SCN, there is a possibility that estrogen can exert a direct effect on clock phase through direct action on SCN neurons. In vitro studies have examined SCN neuronal responses to neurotransmitters and found that a heterogeneous population with different neuronal firing patterns and resting firing rates exists. These firing patterns were significantly increased in estrogen-treated SCN neurons in brain tissue slices from ovariectomized rats, suggesting that estrogen has a specific action on specific types of SCN neurons (Kow and Pfaff, 1984). E2 increased the spontaneous firing frequency and depolarized the cell membranes of rat SCN neurons significantly, which implicates estrogen in neuronal cell excitability and neurotransmission in the SCN; and thus, in possibly regulating circadian rhythms (Fatehi and Fatehi-Hassanabad, 2008).
Studies reveal that E_2 has some effects in vitro, but not much has been done to study its effects in vivo. The only study published that attempted to administer E_2 to intact adult males in order to observe any physiological effects found that E_2 did not alter the periodicity of Syrian hamster locomotor activity rhythms (Zucker, Fitzgerald, and Morin, 1980). But the study did not discuss if E_2 has the ability to phase shift an adult, intact male.

Recent studies have examined the hypothalamic neuroendocrine feedback loop, and again confirmed that the SCN regulates circadian rhythms in gonadal hormone secretion, and in turn, hormones feed back to influence SCN functions. However, scientists are now identifying possible mechanisms of how hormones modulate circadian rhythms. Abizaid et al., 2004 found that E_2 enhanced light-induced expression of the transcription factors Fos and pCREB in the SCN of female rats, supporting the hypothesis that steroid hormones play an important role in fine tuning the master circadian clock. Karatsoreos et al., 2007 found that a dense population of androgen receptors exists in the core of the SCN and that androgens act on their receptors within the SCN to alter circadian function. Iwahana et al., 2008 found that these androgen receptors are more highly expressed in males than females. They also noticed that when testosterone is administered to gonadectomized mice, that the distinct male locomotor activity patterns returns and eliminates sex differences in behavioral responses. They conclude that androgenic hormones regulate circadian responses and suggest the SCN as the site of action.
This experiment was designed to investigate whether acute administration of estrogen into the SCN region could induce a phase shift of locomotor activity rhythms. Our hypothesis was that since estradiol is an activator of locomotor activity in hamsters, estradiol would act as a non-photic type of stimulus and induce circadian phase advances when administered in the middle of the subjective day.

**Methodology:**

*Animals:* Twenty-eight adult male Syrian Hamsters (*Mesocricetus auratus*) were bred and housed at Kent State University from stock purchased from Harlan Sprague-Dawley, Inc. Hamsters were group housed in a 14:10 L:D cycle until maturation (12 – 20 weeks old). Food and water were available *ad libitum.*

*Surgical Procedure:* Syrian hamsters were deeply anesthetized with an anesthesia cocktail (110 mg/kg ketamine, 22 mg/kg xylazine and 1.83 mg/kg acepromazine). The skulls were then exposed via small midline incisions and bregma and lambda were identified so that the skulls could be leveled and the stereotaxic could be zeroed. Stereotaxic coordinates were 1.1 mm anterior and 1.7 mm lateral to bregma in order to implant a 4-mm, 26-gauge guide cannula aimed at the SCN. The cannulae were implanted at a 10° angle from the midline. With a 32-gauge injection needle inserted
into the cannula, the final depth was 7.2 mm below dura. The cannulae were held in position by epoxy cement mix.

After surgery, hamsters were individually housed in clean Plexiglas cages (24 x 45.5 x 21cm) and given 24 hours to recover in their original colony room. Hamsters were then placed in a clean animal chamber under DD conditions, given a running wheel, food and water *ad libitum* and allowed to free run until consistent activity rhythms were established (~ 10 days). Each wheel revolution activated a microswitch on the outside of the cage that was monitored continuously by a Dell computer using ClockLab software.

*Microinjections:* After 7-10 days of stable free running activity, microinjections were given at CT 6 with a 32-gage needle. The needle was attached by polyethylene tubing, which was filled with treatment drug or control, to a 1 µL Hamilton syringe. Wearing night vision goggles, the hamsters were gently restrained by hand and administered 0.5 µL injections over a period of 10 seconds, being careful to allow the microinjection needle to remain in position for 10 – 15 seconds to prevent leakage, all while in DD conditions.

Microinjections included 17β-Estradiol with 2-hydroxypropyl-β-Cyclodextrin (0.5µg in 200 nL), muscimol (0.5µg in 200 nL), and vehicle (Cyclodextrin). Hamsters were immediately and cautiously returned to their home cages and monitored for an additional 10 days in order to analyze and/or calculate shifts.
**Histology:** After completion of the experiment, hamsters were deeply anesthetized with sodium pentobarbital (200mg/kg) and microinjected with 0.5 nL India ink diluted 1:1000. The animals were then decapitated and the brains were extracted and post fixed in 4% paraformaldehyde for 48 hours. A vibrotome was used to slice 100 µm thick coronal sections that were mounted on gel subbed slides and analyzed under light microscopy to verify injection site. Only animals with injection sites that did not penetrate the third ventricle and were within 300 µm of the SCN were used in this study.

**Data Analysis:** Phase shifts in the circadian locomotor activity were quantified using the linear regression method of Daan and Pittendrigh (1976). A line was fitted to activity onsets that occurred on the 7 days preceding the microinjection. A second line was fitted to activity onsets that occurred 4–11 days after microinjection. Days 1–3 post-microinjection were not used in the data analysis to avoid including transients or unstable onsets. Phase shifts were determined by the difference between the predicted onset of activity before treatment (as predicted if no treatment were to be given) and the actual line of activity onset after treatment. Paired t-tests were used to test statistical significance with significant attributed to p < 0.05.

**Results:**

Microinjection of neither 17-β-E\textsubscript{2} nor vehicle (cyclodextrin), into the SCN region induced phase advances at CT 6. However, muscimol induced large phase advances (see Fig. 16).
Figure 14: Microinjection Phase Shift Results

Phase Shifts in the Circadian Locomotor Activity of Microinjections into the SCN

Conclusions:

These data demonstrate that acutely administered estradiol does not induce phase shifts of circadian rhythms when injected at CT 6. The positive phase shifting response to muscimol shows that the failure of estradiol to cause phase shifts can not be attributed to poorly sited injections. However, these data do not rule out the possibility that estradiol acutely can alter circadian phase. It remains possible that estradiol could have direct effects on clock phase at other phases of the circadian cycle, or that the dose of estradiol was insufficient to alter clock phase. Finally, it is possible that estradiol is not capable of altering clock phase through direct action on the SCN.
DISCUSSION

It is evident that circadian rhythms are driven by the SCN; however the mechanism detailing endocrine feedback and the SCN is not understood and the role of direct estradiol reception in the SCN remains a mystery. Our data suggests that while it is possible that estradiol’s effects on rhythms are mediated by estrogen receptors in the SCN, it is likely that the SCN is influenced as well by other brain regions that contain estrogen receptors.

In Experiment 1, we found that sex differences do exist in Syrian hamster locomotor activity and that the pattern and quantity of daily wheel-running varies across the estrous cycle in female Syrian Hamsters. We also observed how phase shifts induced by photic stimuli are modulated by the estrous cycle, specifically how the rate of reentrainment of the locomotor activity rhythm was significantly slower in females as compared to males. These results extended the existing theory that the rate of phase shifts in females is correlated with changes in circulating estradiol concentrations (Moline and Albers, 1988).

In Experiment 2, we discovered the expression of ER-β mRNA in male and female SCN samples using a novel technique consisting of LCM and RT-PCR. We deepened the pool of knowledge that supports the theory that ERs are present within the SCN by finding that ER message is present within the cells of the SCN. Interestingly, ER-β mRNA levels did not differ between males and females, suggesting that ER-β
expression is insensitive to steroid feedback or is differentially regulated in males and females to allow for the equivalent expression in the SCN.

In addition, this experiment was the first to successfully apply the combination of laser capture microscopy and RT-PCR to hamster brain tissue. However, there were a number of limitations caused by the difficulty of designing primers for hamsters, for which genomic sequence information is sparse. Another interesting area of investigation would be to measure female ER expression on different days of the estrous cycle, in order to determine whether estrogen feedback in females regulates ER expression.

In Experiment 3, administration of 17-β-E₂ into the SCN at CT 6 did not induce a phase shift as we had hypothesized. This could be a result of several things. We might have used too weak of concentration, not been at a sensitive time or in the correct window of time necessary to induce a phase shift, or it may be that intact males do not display a PRC to estradiol. In the future, researchers should use gonadectomized animals, or follow the estrous cycle in intact females using vaginal smears, to evaluate the effects of acute administration of estradiol. Another idea may be to pair the microinjection with a light pulse to see if the phase shift is attenuated by the administration of E₂ to the cells of the SCN.

Although we answered some of our questions, many remain. In particular, the mechanism by which estrogen shortens circadian period is unresolved. In addition, our results from Experiment 1 showed that female hamsters are deficient in the ability to reentrain to a shifted light-dark cycle as compared to males. The mechanism for this
difference is also unknown. However, our data is consistent with the idea that $E_2$ acts directly on the SCN or through SCN afferent regions to modulate circadian physiology.
APPENDIX A: PROTOCOLS

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Protocol for Making Cage Tops

Materials: 7” Steel running wheel, new steel cage top, big plastic cage, 1 magnet, 1 switch, 2 zip ties, ruler, scissors, epoxy, old pen/pencil, marker, heavy book(s), long q-tip, and small piece aluminum foil (about 3” x 3”).

1. Put cage top up-side-down, with food/water area farthest away from you and water circle on your right, on to table top.

2. Align continuous top bar of wheel with depression ridge closest to you (opposite of food/water area). Measure 2” from your left-hand side and attach left side of wheel to the nearest wire with a zip tie. Then attach other side of wheel to nearest wire with second zip tie. Pull both zip ties as tight as you possibly can!

3. Turn cage top right-side-up, with food/water area closest to you and water circle on the right, and set on table top.

4. Put old pen/pencil between wheel and cage top, so the cage top is resting on the pen/pencil instead of the wheel.

5. Put on top of a big plastic cage and spin wheel to ensure that the wheel is not sticking out too far from back of cage top and rubbing against back of plastic cage. With a marker, mark place on wires where you should glue both ends of wheel so no rubbing occurs.

6. Put cage top on counter again, with pen/pencil still in place and top of cage resting on shelf. Get switch (pull backing off if present) and position the middle of it on top of cage 4” from right side of cage top and in middle area of wheel’s length (about 3.25”-3.5” from zip ties). Mark location so can epoxy later.

7. Get out magnet and position directly under where switch is going to be. This way the current between the magnet and switch will be dead on together.

8. Get ready heavy book(s) and epoxy by first putting on gloves, squeezing epoxy onto the aluminum foil, and mixing it with the q-tip.

9. Epoxy both wheel ends to wires on cage top, then the switch to top. Move cage top to ridge beneath counter shelf and balance by putting the heavy book(s) on top of switch to keep in contact with cage top.

10. Then epoxy magnet directly below the switch in between 2 wires on the wheel. Ensure that is does not stick out so far that it hits the cage tops when the wheel revolves. Let dry. DO NOT MOVE! LABEL FIXED/DRYING! Then check in A-102.
Quick Nissl Staining & Microdissection

A. Cryostat – Cut 12um sections at -20° C. Store the frozen sections in –70ºC freezer, thaw around edges for 15-30 sec.

B. Fixation – Fix immediately
   1. Place in 75% ethanol (30 sec)
   2. Place in distilled water (15-30sec) (*Do a few dips to clear the alcohol)
   3. Proceed to staining

C. Staining –
   1. Stain with Hematoxylin for 1 minute.
   2. Rinse with distilled water (Dip a few times).
   3. 75 % Ethanol (30 seconds)
   4. 95% Ethanol (30 seconds)
   5. 100% Ethanol (30 seconds)
   6. Xylene (5 minutes)
   7. Air dry in hood
   8. *To preserve the highest quality of RNA, Wear clean disposable gloves throughout the procedure. Use clean, RNase-free instruments. Wipe all areas down with RNase Away.

D. Laser Capture Microscope –
   1. Set up scope with slides and caps
   2. Click and drag Red view indicator to desired area on road map image
   3. Right click on RMI to magnify, choose 400%
   4. Enable visualizer
   5. Click and drag on live image to get desired area
   6. Focus image
      i. Select objective
      ii. Select light intensity
      iii. Focus
      iv. Select camera (B&W: Fluorescence, Color: all other)
      v. Select Filter (Open for white light)
   7. Focus Laser
      i. Click on LI
      ii. Drag cap onto slide
      iii. Enter cap name
      iv. On LI, go to open area and search for laser spot (adjust light intensity if need to)
      v. Focus laser on 10 x objective (20 doesn’t work well!)
      vi. Right click center of laser spot, choose Locate Laser Position
vii. Fire

viii. Should have clear center, wide black collar & satellite spot at 6:00

ix. Start with, but can adjust if needed

1. Power:  40 – 60
2. Pulse:  1500 – 1700
3. Intensity:  200
4. Hits:  1

x. Click on spot size and measure inside edges of black collar (5–7.5 um)

8. Microdissection Area Selection (Can also go to 9. Autoscan)

i. Click RMI

ii. Click red rectangle in toolbar = ROI tool

iii. Draw ROI on RMI (region for microdissection)

iv. Click red rectangle to deselect region of interest tool

v. Right click on black ROI box on RMI, choose Acquire Region (= Static image) *if blank, then area too big

vi. Select annotation tool

1. Polygon = Large area: To Draw line around area
2. Line = Single cell layer
3. X = Specific cell

vii. Deselect annotation tool

9. Autoscan

i. Right Click on LI, select Autoscan

ii. Go to Analysis, Choose size.

iii. Mark region of interest by using ◦◦◦◦ and xxxx for background

iv. Click learn

1. Unimode is simplest
2. Multimode picks up to 4 shades
   a. Do this only in brightfield
   b. Increases sources of error b/c increases false positives with increased modes
   c. Too low mode eliminates cell

10. Microdissection

i. Click on Static Image (SI) *Make sure tool has been selected, area has been outlined, tool deselected, laser enabled, and laser located!

ii. Right click. Select “capture selected cells” (250 cells is max!)

iii. A “Captured Areas” window opens in upper left screen, this is the Cap Image (CI). The LI displays a stationary laser spot

iv. Go to Region List and Capture cells

1. up to 250 cells
2. if have too many cells, increase minimum region of interest
11. Cap Removal
   i. Drag cap to QC
   ii. Click on CI to view to take image
   iii. To remove cap: Drag to rectangular removal station
        ****This is the point of no return!
   iv. To retrieve cap: Click Instrument Icon on top toolbar
        1. Click “Open Instrument Door”
        2. Unload Cap Bottom-Up!, so polymer film is facing up!
        3. Making sure cap unload station is positioned properly in the stage, click OK to close door.

12. Extraction Buffer
   i. Place extraction devise (ExtracSure) on cap, teeth down
   ii. Pipette 12 µL Buffer solution into CapSure
   iii. Place 0.5 mL centrifuge tube on top.  *Be careful, break easily!
   iv. Place in alignment tray
        1. Can leave at 25° C until all captures are complete!

13. Archiving Images
   i. Double Click on whatever you want to return original site
   ii. Single click to highlight area in
        1. QC = Cap
        2. SI = Static Image  (Green)
        3. LI = Live Image
        4. RMI = on LI to select ROI  (Red)
        5. ROI = Microdissection area  (Black)
   iii. Right Click, select “acquire SI”
   iv. Right Click, save image. Name. Close.
        ***Do NOT close LI! If do, click “Intrument” on toolbar & select “Activate Live Video”

E. Incubator
   1. Cover with pre-heated (42° C) incubator block
   2. Place in 42° C incubator/oven for 30 minutes
   3. Spin centrifuge tubes to collect buffer
   4. Place centrifuge tube in -80° C freezer immediately, for up to 8 months.
   5. Throw away cap and devise
**PicoPure Protocol w/ DNase Treatment & RT**

Materials: Per Sample: 1 column, 1 microcentrifuge tube, 1 PCR tube and ice tray!

1. 250 µL CB onto column membrane, leave x 5 minutes
2. Get out DNase treatment reagents
3. spin 13.0 x 1 minute
4. 10 µL EtOH into RNA + XB, pipette ↑ and ↓ x 3, place into column
5. spin 2.0 x 2 min
6. 5 µL DNase Inhibitor into 35 µL RDD, invert to mix and keep on ice
7. spin 13.0 x 30 sec
8. 100 µL W1 into column
9. spin 10.0 x 1 min
10. place DNase MM into column, leave 25 C x 15 min
11. Place 40 µL W1 into column
12. Spin 10.0 x 15 sec
13. 100 µL W2 into column
14. Spin 13.0 x 2 min
15. Get out RT Reagents and turn on Thermocycler
16. Spin 13.0 x 1 min
17. Transfer membrane to tube
18. 13 µL EB directly onto membrane, leave x 1 min
19. Spin 3.5 x 1 min and then 13.0 x 1 min
20. Transfer 2 µL into 0.2 µL PCR tube
21. Store on ice while defrosting 10x Taq, MgCl2, dNTPs, Ologo d(T)s.
22. Mix RT MM with above reagents and nuclease-free ice, vortex briefly, ice
23. Add RNase Inhibitor and Multiscribe RT, pipette ↓ and ↑ x 3 and ice.
24. Add 89 µL RT MM to each sample
25. Spin 2.0 x 30 sec
26. Place in thermocycler
27. Put away all samples and reagents. Store cDNA at -15 C to – 25 C
Mixed Primer Concentrations Protocol

Note: Primers must be reconstituted first to a 100 uM stock before diluting into the following concentrations. Do this on idt’s dna website with the reconstitution calculator.

Making Primers with total volume of 300 uL.

Concentration of 50 nM: 1.2 uL of 100 uM stock to 298.8 uL water

Concentration of 300 nM: 7.2 uL of stock to 292.8uL water

Concentration of 900 nM: 21.6 uL of stock to 278.4 uL water

Making Primers of total volume of 100 uL

300 nM: 2.4 uL stock with 97.6 uL water

900 nM: 7.2 uL stock with 92.8 uL water

RT-PCR Protocol

Step 1: Reverse Transcription: Total = 100 μL reactions = 89 μL MM + 11μL RNA

Note: This protocol starts after samples have been purified during PicoPure and, if amplified completely through RiboAmp, i.e.) either eRNA or aRNA. You will use OligodT, not Random Hexamers, and this will be the first reagent to run out.

Suggestions: Continue to this step directly after either of above stated procedures. It is better to stop after you have synthesized cDNA to minimize possible RNA degradation.

Materials:
1. TaqMan Reverse Transcription Reagents located in -20 freezer
2. 1 - 2.0 mL microcentrifuge tube for RT-Master Mix
3. electronic pipettors with tips
4. Centrifuge with adapters
5. Vortex
6. Thermocycler
7. Ice
Method:

1. Decide how many samples you will be running and add 1 extra if you are running more than 2 to account for pipetting errors during the procedure. Whatever number you get, this is needed for the Master Mix (MM) ratios.

2. Start making the RT-MM, according to above number, by adding the first 5 reagents, refer to MM Ratio spreadsheet, in the following manner:
   a. Leave all reagents on ice until needed
   b. Thaw by vortexing and then pipette in immediately after vortexing
   c. Leave MM on ice in between adding reagents
   d. After all 5 reagents are added, vortex MM tube and put back in ice.

3. Then add the enzymes in the following manner:
   a. NEVER VORTEX AN ENZYME!!!! SIMPLY PIPETTE UP AND DOWN SEVERAL TIMES TO MIX!!!! LEAVE ENZYMES ON ICE AT ALL TIMES!
   b. Add designated amount of RNase Inhibitor, pipette up and down
   c. Add designated amount of MultiScribe RT, pipette up and down
   d. Turn entire tube up-side-down several times to mix, centrifuge veru briefly if need to
   e. Now leave MM tube on ice

4. Load/Start thermocycler program, “RT,” by entering in the following info:
   a. 0.5 mL tube
   b. 100 μL Fill. Vol
   c. Then wait til lid heats up and pause program by pushing start/stop key, then select til pause shows up in darkened box, then press enter. Paused should blink in the lower left corner under word, “Incubate”.

5. Add 89 μL of RT-MM to each sample of RNA

6. Remove air bubbles by centrifuging 2.0 x 30 seconds

7. Put in close together in thermocycler and resume program by the same above procedure accept push select until “RESUME” appears in darkened box.

8. Store cDNA in -20 freezer
Step 2: PCR: Total = 20μL reaction = 2.5μl each primer + 5μL cDNA + 10μLSGMM

Note: Have all primers pre-made and alliquotted out and reaction plate set up.

Suggestions: Leave your plate in ice even though the protocol does not call for. Strategically place each ingredient so you know if you accidently skipped/missed a well.

Materials:
1. MicroAmp 96-well reaction plate or ABI Optical Tubes
2. MicroAmp Support Base
3. ABI Optical Adhesive Cover Starter Kit or Optical Caps
4. Primers, Forward and Reverse
5. cDNA samples on ice
6. SYBR Green Master Mix (SGMM) located on fridge’s 2nd shelf
7. electronic pipettors and tips
8. Dr. Wolverton’s centrifuge
9. SDS 7000

1. Design your reaction plate by using the well worksheet. Run triplicates of each.
2. Have primers, cDNA, and SYBR Green MM out and on ice.
3. Pipette 2.5 uL of each primer (forward and reverse) into each well. Place the pipette tip on the bottom of the well for the first primer application and for the second primer, place the drop on the side of the same well.
4. Pipette 5 ul cDNA into each well.
5. Pipetter 10 uL SYBR Green MM into each well
6. Seal reaction plate with adhesive cover or optical tubes with caps and centrifuge at 2.0 for 30 seconds, use Wolverton’s centrifuge for the reaction plates
7. Set up SDS Software for your specific plate by doing the following:
   1. Turn on instrument
   2. Open SDS program
   3. Identify well contents and add detector to plate document and to each well
   4. Under Instrument tab, change reps in third step from 40 to 45, total volume to 20 uL and check the dissociation box. Then save document and click run.
8. Analyze your results.
SCN Guide Cannula Implantation Protocol

Materials:
1 mL Syringes (4)     Iodine     Spatula
2 clamps               Needles     Tape
2 long, teethed forceps Scalpel with Blades Transfer Pipette (2)
Cannulas               Scoopula     Wound Clips
Cotton Swabs           Sharpie Fine Point (2) Zephiran Chloride
Crazy glue             Small surgical scissors (7 mL/1 L H₂O)
Dremel Tips             Small Forceps

Drugs: Code is 30033
Ketamine (amount according to weight chart + 0.01mL, 0.7 mL to kill subject)
Marcaine (topical pain killer- no more than 0.2 mL/subject)
Nembutal (if dying, 0.6 mL to kill subject)

What to record:

<table>
<thead>
<tr>
<th>Subject/Study</th>
<th>Weight (g)</th>
<th>Volume of Ketamine (mL)</th>
<th>Time of Ketamine Injection</th>
<th>Comments</th>
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1. Weigh subject and add 5 g, administer amount of Ketamine by intraperitoneal injection according to chart. If will not go out, or if starts awakening, administer more Ketamine in 0.05 mL injections until ½ of the original dose is reached.

2. Set up the following: Sx tools, cannulas with straight needles, and 3 wound clips/subject submerged in ZCl. Ensure that the arm is at a 10° angle from the midline.

***Optional to shave subject’s head at this point***

3. Position the hamster on the ear bars. How to check for correct position:
   a. check to see if any upward movement when pushing up from below
   b. let subject’s body rest on platform, does it stay on the ear bars
   c. when body is on platform and in ear bars, try to move nose side to side
d. Place Styrofoam underneath when correctly positioned

4. Apply iodine to the top of head going with the hair, then cut skin and connective tissue with scalpel by pushing down firmly against the skull. Ensure the incision is long enough.

5. Administer Marcaine with syringe and needle. Wait about 1 minute and then swab with cotton swab.

6. Push the muscles and connective tissue away from the midline of the skull by beginning at the caudal most portion and continue to superior portions. Absorb any blood with a cotton swab and allow skull to dry some.

7. Identify Bregma by the origin of 2 perpendicular lines and mark with Sharpie. If hard to visualize Bregma, then use small amount of EtOH on the surface of the skull avoiding contact with the muscles.

8. Insert the Guide Cannula into the arm holder, align the needle of cannula with Bregma (the sharpie dot), and zero out all stereotaxic measurements.

9. Go to the following coordinates: 1.1 mm anterior to Bregma, 1.7 mm Lateral to Bregma, and then mark with another Sharpie. Move arm up.

10. Turn Dremel onto setting 6-8 and drill skull using only the weight of the Dremel and not additional force. Stop when you reach the Dura. (Check if you have drilled far enough with a needle)

11. Absorb blood with cotton swab and search for place to drill for wound clip.

12. Drill 2 holes for the wound clip. Do not drill through to the dura, instead drill ½ way through bone.

13. Reposition the dorsal/ventral axis of the guide cannula over original drill hole, ensure proper coordinates. If you need to enlarge the hole, take Dremel and make circular motions with it around the perimeter of the previous hole.

14. Move needle down until you hit the Dura, then zero out the stereotaxic (only dorsal/ventral axis), and slowly insert the needle 2 mm below Dura if using 5.2 mm needle, 2.2 mm below Dura if using 5.0 mm needle. (The SCN is 7.2 mm below Dura at 10 degrees). **Try 2.7 mm below Dura (7.18.06).**

15. Take a wound clip with your forceps, pry open its prongs using a clamp, and position clip for insertion.
16. Slightly bend the clip between the forceps and insert into one of the holes, then slowly manipulate the clip to be inserted into the other hole and then clamp together

17. Loosen the skin around the incision and absorb any more blood with the cotton swabs and make sure field is as dry as possible

18. Using the scoopula pour cement mix around the perimeter of the cannula avoiding any contact with the subject’s skin.

19. Using the transfer pipette dispense cement liquid onto the mix while pulling up on the skin so the skin doesn’t get glued down. Leave sit for a minute or so to harden.

20. Leave a minute or so more and then super glue the skin toward the outer circumference of the cannula and let dry.

21. Slowly loosen the arm clamp holding the cannula. Ensure that the cannula is not stuck to the clamp and then move the arm upwards (slowly) and out of the way.

***Use spatula between clamp sides if cap is glued to the cannula!***

22. Put subject in new cage with few pellets in bottom and cover with bedding.

23. The following day check the animal and then put a dummy cap onto the top of the cannula.

**Site Check With India Ink**

1. Dilute India ink to 1:1000 and vortex to mix
2. get out injection needle, syringe with needle, microtubing with probe on the end, and distilled water
3. Push 2 mL of di water through tubing, leaving a minute amount inside
4. attach injection needle to tubing and pull about 0.5 nL air into it
5. aspirate 8 nL of diluted India ink into injection syringe
6. push out 1 nL to ensure no blockage in the probe
7. Hold animal down x 10 seconds
8. Place probe into cannula
9. inject 5 nL India ink into subject and pause 10 seconds before removing
10. Administer 0.6 mL Nembutal via an i.p. injection.
11. Decap and place brain in 4% paraformaldehyde x 48 hours.
12. Vibratome at 100 um thick sections
13. Mount on gel subbed slides.
14. If you want to save sections, then DPX and Coverslip
**Protocol for Microinjection Solutions**

1. Making 0.9 % Saline Solution:
   1.0 g NaCl in 100mL water = 1.0 % saline solution
   so
   0.9 g NaCl in 100 mL water = 0.9 % saline solution

2. Making β-E2-Water Soluble Solution (Look at Water solubility mg/mL and subtract a little b/c not dissolving in water)…Specs are 25 mg/mL. We are going with…
   20 mg β-E2-encapsulated with cyclodextrin in 1 mL 0.9 % Saline
   (which is actually only 1 mg/mL E2 b/c 52.4 mg E2/1 g solid)
   *Inject 0.5 µL

3. Making Cyclodextrin Solution
   19 mg Cyclodextrin in 1 mL 0.9 % saline
   (ratio from original conc. ratio from the bottle of E2, see above)
   *Inject 0.5 µL

4. Making Muscimol Solution
   11.4 mg Muscimol in 1 mL Saline = 100 mM solution

**Protocol for Estradiol Injections**

1. Insert Guide Cannulas into 12 male hamsters, between 12-20 weeks old.
2. Place in DD and have animals free run until consistent actogram ( ~ 10 days)
3. At CT 6 inject 3 animals with 17-β- E₂ encapsulated with Cyclodextrin (0.5 mg in 0.5 µL) and 3 animals with vehicle (0.5 µL cyclodextrin)
4. Leave injection needle in place for at least 15 seconds
5. Cannot inject same animal again for at least 9 days
6. Inject _ animals with Muscimol (5.0 µg in 440 nL)


de la Iglesia HO, Blaustein JD, Bittman EL. “The suprachiasmatic area in the female hamster projects to neurons containing estrogen receptors and GnRH.” Neuroreport. 1995 Sep 11;6(13):1715-22.


Kruijver FP and Swaab DF. “Sex hormone receptors are present in the human suprachiasmatic nucleus.” Neuroendocrinology. 2002 May;75(5):296-305.


