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The Role of the Circadian System in Reproductive, Neural, and Immune Health

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The Role of the Circadian System in Reproductive, Neural, and Immune Health

By

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by Erin Marie Gibson

Abstract

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In mammals, the suprachiasmatic nucleus (SCN) acts as the master circadian pacemaker of the body. The SCN coordinate the thousands of individual oscillators throughout the body to maintain optimal health and homeostatic functioning. The importance of maintaining circadian rhythmicity is exemplified by its role in reproduction, as disruption of the circadian clock inhibits reproduction. Experiment 1 shows that the proper temporal coordination by the SCN of two hypothalamic neuropeptides, GnRH and RFRP-3, is imperative to initiation of the luteinizing hormone (LH) surge that stimulates ovulation in the Syrian hamster. The maintenance of temporal homeostasis in reproductive, neural, and immune health was further studied using a model of circadian disruption similar to chronic jet lag that resulted in an incongruence between the endogenous ~24-hour rhythm generated by the SCN and the environmental 24-hour day. In Experiment 2, jet-lagged hamsters exposed to 6-hour shifts in the light:dark cycle every 3 days for 25 days exhibit a dysregulation in the temporal activation of the GnRH and kisspeptin systems, two neuropeptide populations that stimulate the LH surge, and in the temporal deactivation of the RFRP-3 system that inhibits the reproductive axis, around the time of the LH surge. The importance of temporal homeostasis was further explored in Experiment 3, in which jet-lagged hamsters exhibit decreases in hippocampal cell proliferation and neurogenesis compared to non-jet-lagged controls. Interestingly, jet lag hamsters not only exhibit deficits in learning and memory during the period of repeated phase shifts, but also one month following the cessation of chronic jet lag, suggesting that the jet-lag-induced decrease in neurogenesis has long lasting effects on hippocampal function. Similarly, as jet lag influences hippocampal cell proliferation, it is also shown to impact immune function by dysregulating splenocyte proliferation in Experiment 4. Additionally, both innate and humoral immune functions, including complement system activity, total antibody production, and bacterial killing, are suppressed in jet-lagged hamsters compared to controls. Taken together, the previous studies strongly implicate the necessity of coordination between an intact circadian system and environmental cues to maintain proper homeostatic function.

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Chapter 1

Introduction

Nearly all physiological and behavioral processes follow a 24-hour cycle that evolved to coincide with environmental 24-hour rhythms (Sharma, 2003). Everything from the sleep-wake cycle, reproduction and body temperature, to immune function is precisely orchestrated on a daily schedule by the suprachiasmatic nucleus (SCN), the master brain clock. At the cellular level, this ~24-hr rhythm is generated by interacting autoregulatory transcriptional/translational loops (Reppert and Weaver, 2002). Endogenous circadian rhythms are synchronized to environmental cues, with light being the main zeitgeber (German for time giver) for mammals. A subset of retinal ganglion cells is directly responsive to light and convey this environmental cue to the SCN clock through a direct, retino-hypothalamic tract (Berson et al., 2002). These rhythms enable an organism to anticipate events in the environment, and facilitate optimal timing of cellular and system-wide processes. Critically, the daily pattern of a vast array of physiological and molecular processes (Reppert and Weaver, 2002; Reddy et al., 2005) must be coordinated with each other to maintain normal health, and this phase relationship among systems is compromised during abrupt changes in external time (e.g., jet lag, shift work) (Yamazaki et al., 2000). The goal of the present body of work was to determine the impact, and neuroendocrine mechanisms responsible, of disruptions in circadian timing on health and functioning.

The Role of Endogenous Timing Systems in Reproduction

Several lines of evidence suggest an important role for the circadian system in reproductive functioning. For example, destruction of the circadian clock through SCN lesions or clock gene disruption results in marked alterations in ovulation and fecundity, especially in rodents (Nunez and Stephan, 1977; Wiegand and Terasawa, 1982; Miller et al., 2004). In many spontaneously-ovulating rodents, including mice, rats, and hamsters, ovulation is triggered when threshold values of estradiol coincide with a daily circadian signal originating from the SCN on the day of proestrus. During most of the estrous cycle, estrogen exhibits a negative feedback effect on the hypothalamo-pituitary-gonadal (HPG) axis, but on the day of proestrus, increased levels of estradiol serve a permissive role, stimulating the luteinizing hormone (LH) surge that initiates ovulation (de la Iglesia et al., 2003; Petersen et al., 2003). This mechanism allows for optimization of fertility by coordinating estrous behavior with ovulation. Unlike rodents, the sexual behavior of primates is not limited to the time of ovulation, allowing for less stringent control of the reproductive axis by the circadian system (Yamaji et al., 1971).

As indicated previously, in both rodents and primates, estradiol negatively feeds back to the hypothalamus to inhibit the reproductive axis during most of the estrous/menstrual cycle. Pulsatile secretion of gonadotropin-releasing hormone (GnRH) into the hypophysial portal system stimulates the synthesis and secretion of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), in the anterior pituitary that then act on the gonads to release sex steroid hormones. At the time of the LH surge, estradiol switches from suppressing

reproduction to stimulating the axis (de la Iglesia et al., 2003; Petersen et al., 2003). The neuroendocrine mechanisms that permit this transition in the actions of estradiol are not well understood. GnRH neurons do not express ERα, the critical receptor in negative and positive feedback regulation of GnRH by estradiol (Dorling et al., 2003; Herbison, 2006), indicating that the negative feedback effects of this hormone occur upstream from the GnRH system.

RFamide Peptide Control of the Reproductive Axis

Kisspeptin

Two recently identified populations of hypothalamic RFamide peptides (Arg-Phe-NH2), kisspeptin and RFamide-related peptide 3 (RFRP-3), express ERα and have been shown to positively and negatively regulate the HPG axis, respectively (Kriegsfeld et al., 2006; Smith et al., 2006; Adachi, 2007). Kisspeptin mRNA and protein are localized to hypothalamic areas associated with reproduction, including the anteroventral periventricular (AVPV) and arcuate (ARC) nuclei in mice, rat, and hamster (Gottsch et al., 2004; Mason et al., 2007; Simonneaux et al., 2009), the preoptic area (POA) and ARC in sheep (Franceschini et al., 2006; Smith et al., 2007), and the infundibular nucleus (homologue to ARC) in humans and monkeys (Rometo et al., 2007). Administration of kisspeptin stimulates GnRH activity and gonadotropin secretion in rodents (Castellano et al., 2005; Messager et al., 2005), monkeys, and humans (Dhillo et al., 2005; Shahab et al., 2005; Dhillo et al., 2007b; Dhillo et al., 2007a). Kisspeptinergic neurons project to GnRH neurons that express GPR54, the orphan G-protein coupled receptor for the kisspeptin ligand (Irwig et al., 2004; Han et al., 2005; Clarkson and Herbison, 2006; Smith, 2008). Recent work suggests that kisspeptin most likely modulates the reproductive axis upstream of GnRH activity by stimulating the release of GnRH into the hypophysial portal blood system (Irwig et al., 2004; Han et al., 2005; Shahab et al., 2005; Williams et al., 2011). Whereas kisspeptin stimulates the reproductive axis in all species examined thus far, the role of specific populations of kisspeptin neurons varies between species.

In female rodents, *Kiss1* mRNA is downregulated following ovariectomy and upregulated by estradiol treatment in the AVPV, implicating this population of kisspeptin cells in the positive feedback of effects of estradiol. Opposing the actions of estradiol in the AVPV, ovariectomy results in decreased *Kiss1* expression in the ARC, suggesting estradiol-induced negative feedback may be regulated by the ARC kisspeptin (Smith et al., 2005). A similar result is seen in kisspeptin expression in the ARC of ewes, monkeys and most likely, humans (Smith et al., 2007; Smith, 2008; Dhillo et al., 2007b; Rometo et al., 2007). Unlike rodents, the positive and negative feedback actions of estradiol regulation in ewes are mediated within the same nucleus, the ARC, with the negative feedback effects of estradiol mediated by the rostral ARC (Blache et al., 1991; Caraty et al., 1998) and the caudal ARC mediating estradiol-induced positive feedback (Estrada et al., 2006). In women, exogenous kisspeptin-54 stimulates the HPG axis (Dhillo et al., 2007b) and menopause is associated with hypertrophy of neurons within the infundibular nucleus (ARC) that co-express *Kiss1* mRNA (Rance and Uswandi, 1996; Rometo et al., 2007). The number of *Kiss1* mRNA expressing neurons increases in postmenopausal women and ovariectomized monkeys compared to premenopausal women and intact monkeys, presumably due to removal of estradiol negative feedback (Rometo et al., 2007). The role of kisspeptin in mediating the positive feedback of estradiol in primates is still unspecified.

That GnRH antagonists block the stimulatory effects of kisspeptin led to the supposition that this peptide regulates the HPG axis at the level of the GnRH neurons in the hypothalamus rather than at the pituitary level (Gottsch et al., 2004; Irwig et al., 2004; Williams et al., 2011). Studies, in which peripheral injections of kisspeptin elicit increases in LH and FSH concentrations in mice, rats, ewes, monkeys, and humans (Gottsch et al., 2004; Matsui et al., 2004; Shahab et al., 2005; Dhillo et al., 2007b), suggested actions at the pituitary level, without crossing the blood-brain-barrier. Additionally, pituitary *Kiss1* mRNA and *gpr-54* mRNA have been localized to a subset of LHβ subunit expressing cells, and these cells may be directly regulated by estrogen (Kotani et al., 2001; Muir et al., 2001; Richard et al., 2008). In some studies of pituitary cell cultures, kisspeptin has been shown to stimulate LH release (Smith et al., 2008a; Gutierrez-Pascual et al., 2007), whereas others show no effect of kisspeptin on rat pituitary cells (Matsui et al., 2004). The lack of coherent data from *in vivo* and *in vitro* studies makes it difficult at this time to precisely ascertain the role of kisspeptin at the level of the pituitary.

RFamide-Related Peptide-3

Whereas the role for kisspeptins in the mediation of the positive feedback arm modulating reproductive timing has ample support, the neural loci regulating the estradiol-induced negative feedback is less well understood. The recent identification and characterization of a closely related RFamide peptide, RFRP-3, is positioned to be a major negative regulator of the reproductive axis by estrogen. This RFamide peptide that inhibits the secretion of gonadotropins was first identified in the avian brain and termed gonadotropin-inhibitory hormone (GnIH) (Tsutsui et al., 2000; Satake et al., 2001; Bentley et al., 2003). GnIH has been shown to inhibit gonadotropin synthesis (Ciccone et al., 2004), secretion (Tsutsui et al., 2000; Osugi et al., 2004), and gonadal development and maintenance in birds (Ubuka et al., 2006) as well as sexual behavior in song sparrows (Osugi et al., 2004) and quail (Ubuka et al., 2006). Avian GnIH-ir cell bodies project to GnRH neurons that express the receptor for the GnIH ligand (Bentley et al., 2006b; Ubuka et al., 2008). Our lab identified a mammalian ortholog, RFRP-3, in rodents that negatively modulates the reproductive axis (Kriegsfeld et al., 2006). Mammalian RFRP-3-ir cells are localized to the dorsomedial hypothalamus (DMH) in hamsters, rats, and mice (Kriegsfeld et al., 2006; Johnson et al., 2007). *In vivo* administration of RFRP-3 greatly attenuates LH secretion and inhibits sex behavior in male rodents (Kriegsfeld et al., 2006; Johnson et al., 2007). Direct application of RFRP-3 has also been shown to decrease the excitability of GnRH cells in 41% of GnRH neurons in male and female mice (Ducret et al., 2009).

In mammals, RFRP-3-ir fibers project extensively throughout the brain, including the hypothalamic preoptic area (POA), the AVPV, and limbic structures. RFRP-3-ir fibers form close appositions with GnRH cell bodies in the POA (Kriegsfeld et al., 2006; Johnson et al., 2007). All evidence to date points to a direct control of GnRH neurons by RFRP-3-ir cells (Ducret et al., 2009; Ubuka et al., 2008; Kriegsfeld et al., 2006; Gibson et al., 2008). However, RFRP-3 also putatively modulates the reproductive axis at the level of the pituitary. The RFRP receptor (GPR147) has been localized to the pituitary in mammals (Hinuma et al., 2000) and RFRP-3-ir fibers have been reported to extend heavily in to the median eminence (Ubuka et al., 2006). In contrast, Rizwan and colleagues showed RFRP-1/3-ir cells projected to hypothalamic regions including the POA, midbrain, brainstem, and hippocampus but not the median eminence. Intraperitoneal injections of the retrograde tracer Fluorogold labeled GnRH neurons in the POA

but did not label RFRP cell bodies, suggesting that RFRP cells do not project to areas not protected by the blood–brain barrier, including the pituitary (Rizwan et al., 2009). Further supporting the notion that RFRP-3 does not act at the pituitary level comes from work where administration of an intravenous bolus of RFRP-3 to ovariectomized rats did not result in changes in basal concentrations of LH (Rizwan et al., 2009). Whereas this finding suggests that RFRP is not a hypophysiotropic neurochemical, a second group showed that i.c.v. injections of the same peptide moderately inhibited basal secretion and frequency of pulses of LH in intact and gonadectomized male and female rats. The inhibitory effect of RFRP-3 was blocked by RFRP receptor antagonists (Pineda et al., 2010b). Likewise, cultured pituitary cells cultured in the absence of GnRH did not exhibit a decrease in LH concentration when exposed to RFRP-3, but when GnRH was present, RFRP-3 administration resulted in a decrease in LH secretion (Murakami et al., 2008). This finding suggests that RFRP-3 does not affect LH via GnRH release but may act directly at the level of the pituitary. The contradictory findings related to the role of RFRP-3 at the level of the pituitary may be a result of variations in technique as different antiserums may be used to identify RFRP-3-ir fibers in the median eminence or different RFRP-3 peptides may be used in i.c.v. or i.p. injections. Similarly, interspecies variations may exist as the previous studies were conducted using mouse, ewe, and hamster models. The locus of RFRP actions in regulating the reproductive axis is still enigmatic especially due to the lack of coherent findings regarding RFRP actions at the level of the pituitary.

Circadian Control of the Preovulatory LH Surge

The SCN, the locus of the mammalian circadian clock, projects to the GnRH system to convey timing information to the HPG axis. Circadian initiation of the LH surge occurs four hours prior to the onset of the activity period in Syrian hamsters, with a cessation of the surge occurring two hours later (Stetson, 1978). GnRH neurons exhibit robust increases in immediate-early gene expression one hour after the peak in the LH surge (Lee et al., 1990; Lee et al., 1992; Hoffman et al., 1993). Using a behavioral and physiological paradigm known as splitting, de la Iglesia and others were able to eloquently demonstrate the tight circadian control over the LH surge (de la Iglesia et al., 2003). Two-thirds of Syrian hamsters maintained in constant light (LL) for 8 weeks exhibit a divergence in wheel-running behavior. Instead of exhibiting one running bout every 24-hours, these animals will maintain two bouts of activity spaced 12 hours apart (de la Iglesia et al., 2000; Yan et al., 2005). If split animals are ovariectomized and given proestrus estradiol concentrations, two daily LH surges are observed, whereas animals maintained on a normal 14:10 light/dark (LD) schedule exhibit only one (Swann and Turek, 1985). In non-split animals, the one activity bout in hamsters housed in LD is associated with bilateral and symmetrical activation of the SCN and GnRH systems at the time of the LH surge. Split hamsters maintained in LL, however, display antiphase activation of the right and left SCN, with each unilaterally activated nucleus associated with the initiation of one of the activity bouts and LH surges. Interestingly, asymmetrical activation of the GnRH system is found in split hamsters, with increased activation on the side ipsilateral to the activated SCN (de la Iglesia et al., 2000; de la Iglesia et al., 2003). This study suggests that the SCN neurally controls the GnRH system in order to time the LH surge. The purpose of Experiment 1 was to investigate the role of the RFRP system in regulation of the LH surge, as well as to determine if or how the circadian system coordinates the activation of the RFRP system in addition to the GnRH system.

Numerous studies indicate that circadian disruption, such as shift work or jet lag, is a risk factor for reproductive dysfunction, including preterm delivery, low birth weight, and spontaneous abortion in women (Nurminen, 1998; Mozurkewich et al., 2000; Scott, 2000). Women who work rotating or fixed night shifts have lower pregnancy rates compared to women working only daytime shifts (Ahlborg et al., 1996), and women with rotating shift work conditions have an increased risk of subfecundity (Uehata and Sasakawa, 1982; Bisanti et al., 1996). Studies using models of temporally disrupted animals via genetic or surgical approaches have provided additional insight into the importance of circadian control of the reproductive axis for successful reproduction. Studies in rats have shown that disruption of communication between SCN and GnRH neurons, either by ablating the SCN or by severing the neuronal pathway between the SCN and the GnRH cells, results in extended, irregular estrous cycles and infertility (Brown-Grant and Raisman, 1977; Wiegand et al., 1980). Female mice with a mutation in the core *Clock* gene that regulates circadian rhythms at the cellular level show analogous reproductive deficits as in human studies, including estrous acyclicity, lack of a coordinated LH surge on the day of proestrus, and high pregnancy failure (Kennaway, 2005; Dolatshad et al., 2006; Hoshino et al., 2006). *Clock* mutant mice lack the appropriate circadian daily-timing signal required to coordinate hypothalamic hormone secretion, in spite of the fact that hypothalamic levels of GnRH, pituitary release of LH, and serum levels of estrogen are all normal (Miller et al., 2004). Presently, all of the studies investigating the adaptive significance of clock function in reproduction have explored these questions by either using organisms whose circadian clock is disrupted at the cellular level or whose master circadian clock has been destroyed. In Experiment 2, we aimed to investigate the impact of temporal disruptions on the reproductive axis by implementing a non-invasive disruption of circadian homeostasis similar to jet lag and examining the subsequent impact of this disruption on the temporal control of the GnRH, RFRP, and kisspeptin systems.

Circadian Implications for CNS Health

Frequent transmeridian travel, chronic shift work, and poor sleep hygiene have become routine in society. Despite the deleterious impact on millions of individuals, few controlled studies have investigated the effects of circadian disturbances on psychological and physical health. Retrospective studies indicate a strong link between not only shift work and fertility, but also cognitive deficits. Female flight attendants experiencing frequent transmeridian travel exhibit marked deficits in working memory and decreased temporal lobe volume associated with elevated concentrations of the stress hormone, cortisol, relative to non-jet-lagged controls (Cho et al., 2000; Cho, 2001). Despite these observations, a causal link between disrupted internal biological timing and brain health has not been explored. Whereas most studies supporting a link between the circadian system and regulation of the cell cycle involve genetically disrupting the circadian clock, global temporal disruptions, such as those seen in jet lag, have been shown to dysregulate the core clock mechanisms without permanently altering molecular pathways (Davidson et al., 2009). In Experiment 3, we aimed to use this model in order to surmise the impact of global circadian insult on hippocampal cell proliferation and neurogenesis without molecularly eliminating the core circadian clock, as well as controlling for potential alterations in the stress and reproductive axes.

Adult neurogenesis was first documented in mammals by Altman and Das in 1965 (Altman and Das, 1965; Altman, 1969). Prior to this discovery, it was believed that adult cell proliferation

and neurogenesis did not occur. Adult neurogenesis has been documented in the subgranule zone of the dentate gyrus in the hippocampus (SGZ), the subventricular zone, and the rostral migratory stream, although some evidence exists that it may be more ubiquitous than previously thought (Altman and Das, 1965; Eriksson et al., 1998; Huang et al., 1998; Gould et al., 1999a; Kempermann et al., 2003). Within the SGZ, approximately 9000 new cells are born each day from hippocampal neural precursor cells (NPC), with half of these cells surviving past one month and 80-90% differentiating into new neurons (Cameron and McKay, 2001). Two of the strongest mediators of NPC activity in the hippocampus are estrogen, the output of the reproductive axis, and glucocorticoids, the output of the stress axis (Tanapat et al., 2005; Tanapat et al., 1999; Ormerod et al., 2003; Fuchs et al., 2006; Izquierdo et al., 2006).

Estrogens have a marked impact on learning and memory processes with estrogens enhancing performance on working memory tasks, such as the radial arm maze, in female rats (Daniel et al., 1997). Much of the impact of estrogens on learning and memory is believed to be mediated by alterations to hippocampal granule cell morphology and physiology. Estrogen-receptor β (ERβ) mediates hippocampal synaptic plasticity via increases in synaptic proteins, including PSD-95, synaptophysin, and GluR1. Activation of ERβ enhances long-term potentiation (LTP), dendritic branching, and mushroom-type spines, all indicators of glutamatergic synapses. Similarly, ERβ agonists improve memory on hippocampal-dependent tasks (Liu et al., 2008). Much of the impact of estrogen on synaptic plasticity within the dentate gyrus of the hippocampus may result from direct effects of local estrogen synthesis, as blocking of the local synthesis of estrogen at hippocampal synapses decreases the production of spinophilin, a marker of dendritic spines, and synaptophysin, a marker of presynaptic vesicles (Kretz et al., 2004).

Local synthesis of estrogen in the hippocampus also impacts cell proliferation and neurogenesis. Blocking of estrogen production using letrozole, an aromatase inhibitor, suppresses the number of newly proliferated cells while enhancing the number of apoptotic cells (Fester et al., 2006). Not surprisingly, newly born adult neurons in female rats are susceptible to changes in estrogen concentrations seen during the estrous cycle, pregnancy, and aging (reviewed in (Pawluski et al., 2009), and distinctive estrogens (17α estradiol, 17β estradiol, estrone) can differentially impacts on neurogenesis (Barha et al., 2009). However, the impact of estrogen on adult cell proliferation and neurogenesis may be species specific because fluctuations in endogenous estrogen do not change adult neurogenesis levels in mice (Lagace et al., 2007). Much like the role ERβ plays in hippocampal-dependent learning, estrogen receptors are involved in estrogen-mediated increases in cell proliferation but activating both ERα and ERβ simultaneously decreases cell proliferation, suggesting that ERα and ERβ reciprocally regulate each other (Mazzucco et al., 2006). The impact of estrogens on adult-born neurons is further complicated by the new cells susceptibility to estrogen in a dose- and time-dependent manner, with cells decreasing their responsiveness to estrogen with increased exposure (Tanapat et al., 2005). Further complicating the role of estrogens in adult cell proliferation and neurogenesis is that estrogenic effects on cell proliferation are partially mediated by glucocorticoids, as estrogen initially increases cell proliferation but then subsequently decreases it via glucocorticoids (Tanapat et al., 1999; Ormerod et al., 2003).

Centrally, glucocorticoids act in various locations of the brain. Glucocorticoids mediate slow genomic responses through the activation of two receptor types; mineralcorticoid (MR) and

glucocorticoid receptors (GR). MR have a high affinity for glucocorticoids, are occupied at rest and basal levels, are expressed primarily in the hippocampus, lateral septum and amygdala, and display a circadian rhythm of expression. GR have a 10 fold lower affinity for glucocorticoids, are occupied only during stressful situations and during the circadian peak which usually occurs prior the activity period, are expressed more ubiquitously throughout the brain, including the hippocampus, the paraventricular nucleus, and the pituitary, and do not follow a circadian rhythm in expression (De Kloet et al., 1998). Within 7 days of birth one-fourth to one-half of new cells in the hippocampus express GR and not MR, but within one month virtually all cells express both. Because of the high expression of GR receptors in the hippocampus, stress has been shown to have many effects on this region of the brain. Stress or glucocorticoids have been shown to result in changes in excitability and LTP of hippocampal neurons, a 10% decrease in hippocampus volume, atrophy of apical dendrites in the dentate gyrus, CA1, and CA3 regions of the hippocampus (Watanabe et al., 1992; Donohue et al., 2006; Fuchs et al., 2006), atrophy of CA3 neurons (Magarinos et al., 1997; Tata et al., 2006), changes in synaptogenesis and mossy fiber terminals (Sandi, 2004; Izquierdo et al., 2006), including decreases in vesicles, as well as deficits in hippocampal-dependent memory (Kempermann and Gage, 2002; de Kloet et al., 2005).

Some of the deficits in hippocampal-dependent memory may be a direct impact of glucocorticoids on cell proliferation and neurogenesis. Two types of stress/glucocorticoid administration have been shown to impact neural precursor cells (NPC) in the hippocampus in both *in vivo* and *in vitro* models in numerous animal models: acute and chronic stress (Gould et al., 1998; Tanapat et al., 2001; Joels et al., 2004; Koo and Duman, 2008). Acute situations, including short-term restraint stress (Koo and Duman, 2008) and exposure to predator odor (Tanapat et al., 2001), result in a decrease in cell proliferation for 24 hours. Adrenalectomy reverses this outcome (Tanapat et al., 2001). Cultures of NPCs exposed to acute glucocorticoids exhibit a decrease in cell proliferation for 24 hours, that is mediated in a dose-dependent manner (unpublished results, Kaufer lab). Whereas the effects of stress/glucocorticoids on cell proliferation are removed after 24 hours, the total number of new cells in the hippocampus is still decreased 1 week post-stressor (Heine et al., 2004b). However, 3 weeks after the cessation of the stressor, there is no difference between the number of new cells in the stressed versus non-stressed conditions, indicating that there is a cap in the total number of cells that survive or that the cells exposed to the stress/glucocorticoids are more resilient to cell death and survive better (Tanapat et al., 2001). In chronic stress/glucocorticoids situations (i.e. 2-3 weeks of restraint stress or forced cold swim), a decrease in cell proliferation in the hippocampus is still present even 3 weeks after the cessation of the stressor and the return of glucocorticoids to basal levels, indicating that in chronic stress, glucocorticoids may be necessary for the initial suppression of cell proliferation but the maintenance of this suppression (up to 3 weeks) is independent of elevated concentrations of glucocorticoids (Heine et al., 2004b; Ostrander et al., 2006). Those cells born prior to a stressor, as indicated by 5-bromo-deoxyuridine (BrdU), a thymadine analogue that gets incorporated into new cells during DNA synthesis, labeling show a decrease in survival related to stress and glucocorticoids concentrations. Interestingly, if during this time, animals are allowed to participate in a hippocampally-dependent memory task they exhibit an increase in cell survival (Gould et al., 1999b). Stress and glucocorticoid exposure also impact the differentiation of these NPCs. One study showed that there was no difference in mature adult-born neurons and immature adult born neurons between animals exposed to stress and those not

exposed to stress (Wong and Herbert, 2006). However, *in vitro* models using NPCs have shown that exposure to glucocorticoids results in shifting of fate choice away from the neuronal phenotype and towards oligodendrocytes (unpublished results, Kaufer lab).

Adult-born neurons have been shown to function differently than neurons born younger in life. Adult-born neurons are more numerous, mature more rapidly, and are more integrated into the circuitry mediating hippocampal-dependent behaviors, but this difference is only seen in rats not mice (Snyder et al., 2009). Learning directly improves survival of adult-born neurons and this impact lasts well beyond the time when the hippocampus is necessary for memory (Gould et al., 1999b; Leuner et al., 2004). While hippocampal-dependent learning promotes the survival of adult-born neurons, this enhancement occurs only when the learning takes place during a critical period in cell maturation (Epp et al., 2007). Contrary to this, some evidence suggests that learning may also have a negative impact on neurogenesis (Ambrogini et al., 2004). Some of the strongest evidence implicating neurogenesis in learning and memory processes is found in studies inhibiting neurogenesis. Using irradiation to inhibit adult neurogenesis during a critical period of training resulted in deficits in long-term memory formation, as tested using the Morris water maze, a hippocampal-dependent task (Snyder et al., 2005). Similarly, decreasing neurogenesis impacts hippocampal-dependent tasks, such as trace memory tasks, but not hippocampal-independent tasks (Shors et al., 2001). The previous studies point to a strong involvement of adult-born neurons in hippocampal-dependent learning and memory tasks, however how factors, such as glucocorticoids, mediate granule NPC cell proliferation and neurogenesis remains incomplete.

Two theories could explain the decreases in NPC cell proliferation seen in both acute and chronic stress/glucocorticoid situations. One hypothesis is that stress/glucocorticoids result in an increase in cells leaving the cell cycle while the other hypothesis suggests that there is an increase in cell cycle arrest. The latter hypothesis appears to be more fully supported. The rapidity of the decrease in cell proliferation seen in acute stress as well as the rapidity of recovery (24 hours) in association with a subsequent increase in p21, a CDK cell cycle inhibitor, suggests that cell cycle arrest may be mediating the decrease in cell proliferation. In chronic stress situations, there is also an increase in hippocampal p27Kip1, another CDK cell cycle inhibitor, coincidently with the decrease in cell proliferation (Heine et al., 2004a).

Interestingly, just as the circadian clock is based on a series of interconnected autoregulatory transcriptional/translational loops and post-translational modifications, such as phosphorylation, methylation, and degradation, the same mechanisms drive the cell cycle (Hunt and Sassone-Corsi, 2007). In fact, many cell cycle genes, including *Wee-1, c-myc,* and *Cyclin-D1* are all regulated in a circadian manner (Walisser and Bradfield, 2006). Fibroblasts derived from *Clock* mutant embryos exhibit inhibition in both cell proliferation and cell growth, implicating the circadian clock in both proliferation and maintenance of new cells (Miller et al., 2007). The previous studies implicate a role for the circadian system in regulating cell proliferation not only in the hippocampus, but also potentially throughout the brain and body.

Circadian Implications for Immune Function

The immune system evolved to not only fight pathogens during the time of infection, but also to 'remember' the pathogens in order to better respond during future attacks. In order to

accomplish this task, two separate systems formed. The innate immune system is the initial defense to pathogen exposure and initiates a non-specific defense consisting of physical and chemical barriers, phagocytic cells, natural killer (NK) cells, cytokines and activation of the complement system. For more long-term protection, the adaptive immune system, an antigen-specific system that is stimulated by exposure to infectious agents with increased defensive capabilities with each successive exposure to microbes, developed. The adaptive immune system consists of two main groups of lymphocytes: B-lymphocytes secrete antibodies, such as immunoglobulin A (IgA), IgM, IgD, that recognize extracellular antigens while T-lymphocytes consist of helper T-cells that secrete cytokines that stimulate proliferation and differentiation of T-cells and activate B-cells and macrophages, and cytotoxic T-cells that kill cells that produce foreign antigens.

Complicating the already convoluted nature of the immune system is the influence of steroid hormones on immune function. One of the strongest modulators of the immune response is glucocorticoids. Traditionally, glucocorticoids were believed to be anti-inflammatory, with chronic exposure resulting in immuno-suppression. The stress response is associated with induction of lymphocyte/leukocyte apoptosis, specifically T-cell apoptosis, and a shift from an abundance of T-cells that mediate cellular immunity (Th1) to humoral immunity (Th2) (Yoshimura et al., 2001). Similarly, glucocorticoids suppress chemoattractants and pro-inflammatory cytokine expression (reviewed in (Sorrells and Sapolsky, 2007)). However, recently, stress hormones have been implicated in pro-inflammatory responses as well (reviewed in (Sorrells and Sapolsky, 2007); Cox, 1995; Liles et al., 1995). Surprisingly, expression of some pro-inflammatory cytokines, such as IL-1β, is enhanced following an acute stressor in both rodents and humans (O'Connor et al., 2003; Deinzer et al., 2004). While glucocorticoids are known for their suppressive tendencies regarding immune cell proliferation and survival, some aspects of the immune system, including neutrophils, are enhanced by glucocorticoids (Yoshimura et al., 2001). One of the leading theories on the adaptive role of glucocorticoids in immune function stemmed from the belief that glucocorticoid-mediated suppression of the immune system was beneficial, shuttling resources to immediately necessary processes, such as muscle metabolism, in order to survive. Interestingly, early in the stress response, the immune system is activated, not suppressed by glucocorticoids (Herbert and Cohen, 1993). These data suggest that initially, lower concentrations of glucocorticoids stimulate immunity, while increasing concentrations suppress immunity, potentially to prevent autoimmune damage (reviewed in (Sorrells and Sapolsky, 2007)).

The role of HPA activation in the immune response is more complicated than that of the HPG axis. Estrogens, specifically, are pro-inflammatory, which supports the higher incidence of autoimmune diseases in females compared to males (Cunningham and Gilkeson, 2011). Across the ovulatory cycle, varying concentrations of estrogen differentially impact immune markers, including a suppression of interferon-related and other immune genes in blood leukocytes and a shift to Th2 type immune responses (i.e. increases in interleukin-4 (IL-4)) during the luteal phase (Faas et al., 2000; Dosiou et al., 2004). Estrogens robustly affect the proliferation and function of lymphocytes (Lu et al., 2002), with estrogen altering the survival and activation of B-cells (Grimaldi et al., 2002). ERα plays a role in estrogen-induced thymic atrophy and T-cell phenotype, specifically CD4 T-cells (Li and McMurray, 2006; Pernis, 2007). *In vitro* studies

show that estrogen directly impacts B- and T-cells by inducing rapid signaling events, such as ERK, Akt phosphorylation and NFkappaB activation (Adori et al.).

Whereas estrogens may modulate lymphocytes throughout the ovulatory cycle, the circadian system regulates B- and T-cells and other immune markers throughout the day. Total T- and B-lymphocytes, and their respective subsets, peak in the blood serum during the rest phase while NK cells peak during the active phase in rats (Pelegri et al., 2003). The main source of mature lymphocytes is the spleen, in which one-half of the body's lymphocyte population travels every 24 hours. Rhythms in T-cells persist in constant conditions, suggesting an independent molecular clock (Depres-Brummer et al., 1997). The role of the circadian system in regulating immune function is further supported by the presence of clock genes in numerous immune factors. Spleens, lymph nodes, and peritoneal macrophages in mice contain the circadian clockwork, as exemplified by the fact that individual spleen cells harvested and stimulated *in vivo* with endotoxin at different times of day display circadian rhythms in the secretion of cytokines, including TNF-α and IL-6 (Keller et al., 2009). 24-hour oscillation in *Per1/2, Clock, Bmal1,* cytokines, and cytolytic factors also exist in the NK cells (Arjona and Sarkar, 2006). The maintenance of circadian homeostasis is critical for the progression of many diseases, including cancer. *Per1* suppresses cancer cell proliferation and breast tumor growth, and this impact is time-dependent (Yang et al., 2009). Similarly, overexpression of *Per1* in prostate cancer cells reduces tumor growth and enhances apoptosis (Cao et al., 2009). *Per2* also exhibits anti-tumor effects through cell cycle arrest and apoptosis induction, specifically in *Per2* knockouts cell cycle mediators *p53* were downregulated while *cyclin B* and *c-myc* were upregulated (Sun et al.). Mice lacking the *MOP3/Bmal1* gene had disrupted B-cell development, with a disturbance in the cell's ability to mature from pre-B-cells to mature B-cells (Sun et al., 2006) and accelerated growth of Glasgow osteosarcomas (Filipski et al., 2004). The previous studies point to a significant role for the circadian system in proper immune functioning.

In humans, the importance of circadian homeostasis in fighting disease is highlighted in the susceptibility of chemotherapeutic agents to circadian time of day and the presence of a functional circadian system (Gorbacheva et al., 2005). Disruptions of the circadian system, such as those seen from rotating shift work or chronic transmeridian flights, are associated with higher incidences of cancer, psychological pathologies, cardiovascular disease, and diabetes (reviewed in (Navara and Nelson, 2007); Filipski et al., 2006). The former findings led The World Health Organization to include shift work as a carcinogen in their 2007 report (Stevens et al., 2007). Many studies attempt to discern the impact of the circadian system on disease presence through molecular knockdown of the clock or SCN lesions. Recently, animal models of temporal disruption similar to the human experience of jet lag have been devised. Mice exposed to chronic jet lag (4 weeks of 6-hour phase shifts) had changes in the innate immune response with jet lag increasing endotoxic shock induced by LPS, as well as higher incidences of hypothermia and death. Interestingly, these results are not a result of sleep deprivation or stress but more likely related to the altered or abolished rhythms in clock gene expression in the SCN, liver, thymus, and peritoneal macrophages (Castanon-Cervantes et al., 2010). Similar shifts in the light:dark cycle resulted in a decrease in immune response to sheep red blood cells and thymus-dependent antigen and total number of splenic plaque forming cells (Hayashi and Kikuchi, 1985). Mice exposed to chronic phase shifts for 6 weeks exhibited suppression of the rest/activity cycle, body temperature rhythms and acceleration of liver carcinogenesis, as well as

alterations in clock gene expression in the liver and tumor. This acceleration was most likely mediated by the downregulation of *p53* and subsequent upregulation of *c-myc* (Filipski et al., 2009). Interestingly, more robust phase shifts (12-hours/5 days) for 3 months were shown to exacerbate inflammation with an increase in colitis and a decrease in body weight in mice challenged with dextran sodium sulfur (Preuss et al., 2008). While the previous studies highlight the importance of the circadian system in proper activation of the immune system, in Experiment 4 we aimed to investigate the impact of temporal dysregulation on baseline immune function, specifically controlling for potential disruptions in the HPA and HPG axes which are associated with chronic jet lag (Ahlborg et al., 1996; Cho, 2001; Zhu et al., 2004).

Maintaining circadian homeostasis is imperative to optimal brain and body functioning. The purpose of the following studies was to investigate how circadian disruptions impact homeostatic processes from the molecular/cellular level to behavior using a model of jet lag instead of gene knockouts or lesion models. Specifically, we aimed to investigate the role of the circadian system in control of the HPG axis and the subsequent impact of circadian disruption on proper circadian and neuroendocrine regulation of this axis. Similarly, we investigated how temporal dysregulation altered hippocampal cell proliferation and neurogenesis and its effect on hippocampal-dependent memory. Lastly, the role of the circadian system in regulating cell proliferation and the immune system was further explored, highlighting the impact of circadian disturbances on the immune system independent of an immune challenge.

Chapter 2: The Effects of Temporal Disruption on the Reproductive Axis

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Experiment 1: Alterations in RFamide-Related Peptide Expression Are Coordinated with the Preovulatory Luteinizing Hormone Surge

Abstract

The preovulatory luteinizing hormone (LH) surge is triggered when the circadian pacemaker, the bilateral suprachiasmatic nucleus (SCN), stimulates the gonadotropin-releasing hormone (GnRH) system in the presence of high estrogen concentrations (positive feedback). Importantly, during the remainder of the estrous cycle, estradiol inhibits LH release via negative feedback. We have recently documented the presence of a novel mammalian RFamide-related peptide (RFRP), a putative gonadotropin-inhibitory hormone (GnIH), that presumably acts upstream of GnRH to modulate the negative feedback effects of estrogen. The present series of studies utilized female Syrian hamsters to examine the possibility that, in addition to driving the LH surge positively, the SCN concomitantly coordinates the removal of steroid-mediated RFRP inhibition of the gonadotropic axis to permit the surge. We found that the SCN forms close appositions with RFRP cells, suggesting the possibility for direct temporal control of RFRP activity. During the time of the LH surge, immediate early gene expression is reduced in RFRP cells, and this temporal regulation is estrogen-dependent. To determine whether projections from the SCN regulate the timed reduction in activation of the RFRP system, we exploited the phenomenon of 'splitting'. In split animals in which the SCN are active in antiphase, activation of the RFRP system is asymmetrical. Importantly, this asymmetry is opposite to the state of the GnRH system. Together, these findings point to novel circadian control of the RFRP system and potential participation in the circuitry controlling ovulatory function.

Introduction

A master biological pacemaker located in the suprachiasmatic nucleus (SCN) coordinates daily rhythms in behavior and physiology (Moore and Eichler, 1972; Stephan and Zucker, 1972). The circadian system manages daily patterns of hormone secretion required for normal health and reproductive functioning (Kriegsfeld and Silver, 2006). Destruction of the SCN, its output, or the genes regulating cellular clock function lead to pronounced abnormalities in ovulation and fecundity (Nunez and Stephan, 1977; Wiegand and Terasawa, 1982; Miller et al., 2004). Despite the well-established role of the circadian system in regulating ovulatory function across species, the precise mechanisms by which the SCN coordinates the hormonal timing required to initiate this process remain unspecified.

The gonadotropin-releasing hormone (GnRH) neuronal system represents the final common pathway in the neural regulation of the reproductive axis. (Silverman and Witkin, 1994; Herbison, 2006). GnRH neurons project to the median eminence to regulate the synthesis and secretion of the pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). In some species of rodents, including hamsters, the LH surge is initiated by interactions between the circadian and GnRH systems, with estrogen serving a permissive role

(de la Iglesia et al., 2003). Throughout most of the ovulatory cycle, estrogen acts through negative feedback to restrain gonadotropic axis activity (Petersen et al., 2003). However, during a limited time window on the day of ovulation, estrogen ceases to inhibit the reproductive axis and high estrogen concentrations are required for the initiation of the preovulatory LH surge (i.e., positive feedback) (Seegal and Goldman, 1975; Karsch et al., 1997; Levine, 1997). This mechanism is different from that of primates, where a specific *pattern* of estrogen secretion is necessary to stimulate the LH surge at the level of the pituitary(Yamaji et al., 1971; Karsch et al., 1973; Knobil, 1974). In primate species, the inhibitory (negative feedback) effects of low estrogen concentrations are circumvented when estradiol rises above a threshold value and remains elevated for at least 36 hours (Karsch et al., 1973), with limited circadian dependence. In our rodent model system, the positive feedback effects of estrogen are contingent upon a diurnal signal from the circadian clock. In fact, a daily LH surge will occur in ovariectomized animals implanted with capsules containing estradiol levels equivalent to those found on the day of proestrus (Legan et al., 1975; Legan and Karsch, 1975), indicating that the coincidence of both threshold values of estrogen and a circadian signal are necessary to stimulate the release of surge levels of GnRH. In regularly cycling rodents, although the SCN is signaling the reproductive axis once daily, estradiol concentrations are permissive only on the day of proestrus. The mechanism(s) mediating this transient change in the effects of estrogen on the reproductive axis of rodents remain undetermined.

A great deal of research has focused on the stimulatory role of the SCN in triggering the preovulatory LH surge when estrogen levels are high (van der Beek et al., 1997; Horvath et al., 1998), whereas less is known regarding the neural locus on which estrogen acts to suppress reproductive axis function during other stages of the cycle. We have recently described a novel RFamide (Arg–Phe–NH_2)-related peptide (RFRP) in rodents that putatively modulates the negative feedback effects of estrogen (Kriegsfeld et al., 2006) and is orthologous to gonadotropin-inhibitory hormone (GnIH), an avian hypothalamic dodecapeptide shown to inhibit gonadotropin release (Osugi et al., 2004; Tsutsui et al., 2007), synthesis (Ciccone et al., 2004), and gonadal development and maintenance (Ubuka et al., 2006). The RFRP gene gives rise to the two biologically active peptides, RFRP-1 and RFRP-3, with administration of RFRP-3 shown to suppress LH secretion in several mammalian species, including hamsters (Kriegsfeld et al., 2006; Johnson et al., 2007).

In the present series of studies, we explored the possibility that the SCN serves a dual role in ovulatory function, both applying positive drive to the GnRH system to induce the LH surge while concomitantly coordinating the removal of inhibitory influences by downregulation of RFRP activity. We began by examining whether or not the SCN projects upon RFRP cells as a mechanism of neural control. To determine if RFRP activity varies across the estrous cycle, we examined the activational state of the RFRP system throughout the day of proestrus and on the day of diestrus. Additionally, we used a 'splitting' model to confirm SCN neural management over this system. Hamsters housed in a light/dark schedule exhibit one activity bout every 24 hours. This activity bout is associated with symmetrical, bilateral activation of the SCN and GnRH system. When housed in constant light, some hamsters exhibit a 'splitting' in behavior, with two daily activity bouts, each reflecting an antiphase oscillation of the left and right sides of the bilateral SCN (de la Iglesia et al., 2000; Tavakoli-Nezhad and Schwartz, 2005; Yan et al., 2005). Notably, split animals exhibit two daily LH surges (Swann and Turek, 1985), and

asymmetrical activation of the GnRH system (de la Iglesia et al., 2000; de la Iglesia et al., 2003). If the RFRP system is regulated by SCN efferents and is coordinated with the LH surge, then the pattern of RFRP cell activity should also be asymmetrical and opposite to GnRH in split animals.

Materials and Methods

Animals. Adult, female LVG Syrian hamsters (*Mesocricetus auratus*) (n=133) were used. All animals were purchased from Charles River (Wilmington, MA) at 4-5 weeks of age. Animals were housed in translucent propylene cages (48 x 27 x 20 cm) and provided with *ad libitum* access to food and water for the duration of the study. Animals were maintained in a colony room at 23 ± 1°C with a 24 hr light/dark cycle (14/10 hr light/dark) with lights on at 07:00 and lights off at 21:00. All experimental protocols conformed to the Institutional Animal Care and Use Committee guidelines of the University of California, Berkeley.

Tract Tracing. Hamsters were deeply anesthetized with 60 mg/kg ketamine and 5 mg/kg xylazine. The head of the animal was shaved and positioned in a stereotaxic apparatus (David Kopf Instruments, Tujanga, CA), and the animal was prepared for aseptic surgery.

SCN: To examine whether or not the SCN projects to RFRP cells, small iontophoretic injections of the anterograde tracer biotinylated dextran amine (BDA) were aimed at the SCN in hamsters (n=10). A glass micropipette (tip diameter 10-12 μm) was filled with 10% BDA (10,000 Mol. Wt., Molecular Probes, Eugene OR) in 0.01 M phosphate-buffered saline (PBS; pH 7.4). Injections were aimed at the following coordinates: 0.8 mm anterior to bregma, 0.1 mm lateral to midline, and 7.9 mm below the dura. Iontophoretic injections were made using 5-mA positive current pulses (7 seconds on, 7 seconds off) for 7 minutes. Three minutes after the injection, the pipette was removed under negative current in order to prevent leakage of the tracer along the tract. Animals were perfused and brains were collected one week after surgery during the middle of the LD cycle (14:00) on the day of diestrus to maximize the numbers of RFRP neurons visualized as described below.

DMH: To establish whether the DMH projects primarily ipsilaterally or contralaterally to hypothalamic brain sites where GnRH cells are located, pressure injections of BDA were aimed at the DMH in Syrian hamsters (n = 7). A 0.5 μl syringe (Hamilton, Reno, NV**)** was filled with BDA conjugated to Texas Red (1:10; Vector). Each animal was prepared as described above. Injections were aimed at the following coordinates: 0.5 mm posterior to bregma, 0.4 mm lateral to midline, and 8.3 mm below the dura. 300 nl of BDA was injected over a 10 minute period. One week after stereotaxic surgery, animals were perfused and brains were collected as described below.

Serum Collection. Animals were anesthetized using isoflurane, and blood samples were collected from the retro-orbital sinus. Samples were collected at 13:00, 16:00, 17:00, 18:00, 19:00, 20:00, and 23:00 (n=8/time point) relative to lights out on the day of the LH surge and -8 h relative to lights out on the day prior to the surge (i.e. diestrus).

Examination of RFRP Cellular Activity Around the Time of the LH Surge. Estrous cyclicity was monitored by daily examination of vaginal discharge from all hamsters. There is a reliable release of discharge every 4 days, on the day of proestrus (Orsini, 1961). Animals were monitored for at least 3 weeks for estrus regularity. Only hamsters with regular, 4-day estrous cycles were used. The day on which vaginal discharge was observed was designated as the day

of proestrus. Brains were collected at 13:00, 16:00, 17:00, 18:00, 19:00, 20:00, and 23:00 (n=8/time point) on the day of the LH surge. One additional group was examined at 13:00 on the day prior to the LH surge (i.e., diestrus) to examine the state of RFRP cell activation when LH is maximally inhibited by estrogen feedback.

Examination of the Role of Estrogen in RFRP Control. Because estrogen concentrations are elevated for an extended period on the day of proestrus, it is possible that any changes in RFRP expression require the presence of this sex steroid. To determine if inhibitory RFRP cells require estrogen to alter their activity during the LH surge, additional groups of animals were bilaterally ovariectomized (n=16) under isoflurane anesthetic. Subsequently, animals were either implanted with a SILASTIC capsule (Dow Corning Corp.; 10-mm length, 1.45-mm id, 1.93-mm od) containing powdered 17-estradiol (n=8) or an empty capsule (n=8). In estrogen-implanted animals, this treatment provides estradiol concentrations comparable to those seen on the day of proestrus (Meyer-Bernstein et al., 1999) and results in daily LH surges at the same time of day that the LH surge would normally occur (Norman et al., 1973). Animals were perfused as described below just before the LH surge (20:00) or following the surge (23:00).

Examination of RFRP Receptor Expression in the Pituitary. Because a significant number of GnRH cells receive RFRP input across species (Bentley et al., 2006b), the present study was principally concerned with gonadotropin regulation via this mechanism. However, reports in other species indicate actions of RFRP at the level of the pituitary (Tsutsui et al., 2006; Tsutsui et al., 2007). As a result, we examined whether or not RFRP receptor expression is seen in the pituitary of female Syrian hamsters, as a potential additional mechanism of regulatory control using reverse transcription PCR (RT-PCR). Pituitaries from Syrian hamsters (n=2) were examined, whereas liver tissue from rat (n=1) was used as a negative control (Hinuma et al., 2000). Tissue was collected, its total RNA was extracted (RNeasy Mini Kit, Qiagen Inc., Valencia, CA), and 3'-RACE cDNA was then synthesized (SMART cDNA synthesis kit, Clontech Laboratories Inc., Palo Alto, CA). Touch-down PCR was conducted using specific primers for hamster GPR147, the cognate receptor for RFRP (Sense: TCTCG GGCCA GGCCT CCCAG CA and Antisense: TCTCG GGCCA GGCCT CCCAG CA). Initial denaturing occurred for 3 minutes at 95°C followed by 16 touchdown cycles from 68°C to 60°C (annealing temperature, decrease 0.5°C every cycle) and continued for another 25 cycles with 60°C annealing temperature. PCR products were detected on a 1% agarose gel.

'Splitting' Induction. After one week in a 14/10 light/dark cycle (lights on 07:00) an additional group of Syrian hamsters (n=30) was transferred to constant light (LL) conditions. Wheel running activity was recorded continuously using VitalView (MiniMitter Co., Inc., Sunriver, Oregon) for 12 weeks. Accumulated counts were rescored every 10 min and stored to the attached computer. After eight weeks of exposure to constant light, animals were ovariectomized and subcutaneously implanted with a SILASTIC capsule (Dow Corning Corp.; 10-mm length, 1.45-mm id, 1.93-mm od) filled with powdered 17-estradiol (Sigma, St. Louis, MO). Animals were perfused as described below 1 hour prior to one activity bout in split animals (n = 9) and either 1 hour (n = 6) or 13 hours (n = 5) prior to the activity bout in non-split animals. Ten animals did not exhibit regular activity patterns and were not used in this study.

Perfusion and Histology.

Tract Tracing. Hamsters were deeply anesthetized with sodium pentobarbital (200 mg/kg) and perfused intracardially with 150 ml of 0.9% saline, followed by 300-400 ml of 4% paraformaldehyde in 0.1 M PBS, pH 7.3. Brains were postfixed for 2-3 hr at 4°C and cryoprotected in 30% sucrose in 0.1 M PBS overnight. For visualization of SCN-injected BDA and RFRP, every 4th 35-µm coronal section was washed in PBS, incubated in 1% H_2O_2, and then incubated in 0.4% Triton X-100 (PBT) for 1 hour**.** Sections were then incubated in avidin-biotin complex (ABC Elite Kit, Vector, Burlingame, CA; 1:1,000 in PBT) for 1 hour at room temperature. In order to amplify the BDA signal, sections were then incubated in biotinylated tyramide (0.6%) for 30 minutes at room temperature prior to incubation in CY-2 conjugated streptavidin (1:200) for 60 minutes. Following labeling for BDA, sections were labeled using an antibody directed against RFRP (1:10,000; PAC 123/124) as previously described and validated (Kriegsfeld et al., 2006) with CY-3 donkey anti-rabbit (1:200; Jackson, West Grove, PA) as the secondary antibody/fluorophore. For visualization of BDA in the DMH, the above procedure was followed but BDA was visualized using CY-3 conjugated streptavidin (1:200; Jackson) and RFRP was visualized using CY-2 donkey anti-rabbit (1:200; Jackson).

RFRP and GnRH Cellular Activity. For simultaneous visualization of RFRP and FOS across the estrous cycle, every fourth 40-µm coronal section from the mediobasal hypothalamus was washed in 0.1M PBS, incubated in 0.5% H_2O_2, and incubated in normal goat serum (NGS, 1:50; Jackson) in 0.1% Triton X-100 (PBT) for 1 hour. Sections were then incubated for 48 hours at 4ºC with a rabbit anti-FOS Ab diluted at 1: 50,000 (Santa Cruz, Santa Cruz, CA) and NGS diluted at 1:1000 with 0.1% PBT for 48 hours. After incubation in anti-FOS, brains were incubated for 1 hour in biotinylated goat anti-rabbit (1:300; Vector Laboratories, Burlingame, CA) and then in ABC for 1 hour. The FOS signal was amplified with biotinylated tyramide solution (0.6%) for 30 min as previously described (Kriegsfeld et al., 2006). Cells were then labeled by using CY-2 conjugated streptavidin (1:200; Jackson) as the fluorophore. This protocol allowed for the amplification of the highly diluted anti-FOS required for double-labeling with two antibodies generated in the same species (rabbit). After labeling for FOS, sections were incubated in anti-RFRP antibody (1;10,000; PAC 123/124) in 0.1% PBT for 48 h (Kriegsfeld et al., 2006). RFRP cells were labeled with CY-3 donkey anti-rabbit (1:200; Jackson) as the secondary/fluorophore. For simultaneous visualization of RFRP and FOS in split animals (and their unsplit controls), this same procedure was applied using an anti-RFRP antibody directed against the Syrian hamster form of this peptide (1:10,000; PAC 1365). Double-label immunohistochemical studies using PAC 123/124 and PAC 1365 confirmed that these two antibodies label the same population of cells in the DMH. The prior double-labeling protocol without the addition of the RFRP primary antibody was performed to control for the possibility of two primary antibodies generated in the same species cross-reacting. This control confirmed the specificity of this procedure with robust FOS expression and the absence of signal when excited using the standard wavelength for CY-3.

The previous FOS/RFRP protocol was followed for visualization of GnRH and FOS with the RFRP antibody replaced with an antibody directed against GnRH (mouse; 1:2000; Abcam Cambridge, MA). Cells were visualized using CY-3 donkey anti-mouse (1:200; Jackson). All sections immunohistochemically treated were mounted onto gelatin-coated slides, dehydrated in a graded series of alcohols, and coverslips were applied.

SCN Cellular Activity. To identify split and non-split SCN, FOS activation in the SCN was visualized fluorescently using an antibody directed against FOS as described above and with 3,3'-diaminobenzidine (DAB; Vector). Sections were mounted, dehydrated, and coverslipped as described previously.

Control Procedures. Several control procedures were implemented to ensure the specificity of immunohistochemical labeling. PAC123/124 RFRP antibody raised against the white-crown sparrow form of the peptide was preadsorbed with avian and rat RFRP ligand for 24 hours prior to tissue application previously (Kriegsfeld et al., 2006) and was shown to eliminate staining. Additional controls with PAC 123/124 and PAC 1365, raised against the Syrian hamster form of the peptide, preadsorbed with Syrian RFRP ligand were performed. This procedure eliminated staining in both cases (**Figure 1B, E**). Because kisspeptin is an RFamide peptide with a C-terminus structure similar to that of RFRP, RFRP antibodies (both PAC 123/124 and PAC 1365) were preadsorbed with kisspeptin peptide to examine potential cross-reactivity. This procedure did not result in a change in the pattern or intensity of RFRP cell body labeling, indicating that the antibody does not exhibit cross reactivity with this peptide (**Figure 1C, F**). Finally, double-label studies with PAC123/124 and PAC1365 confirm that both antibodies label identical cell populations (**Figure 1G-I**). Previously, a competitive ELISA was used to confirm the specificity of PAC 123/124 RFRP antibody. Cross-reactivity was not observed for any of the closely related peptides (Carp, Mollusc, Bovine, or chicken RFa) (Tsutsui et al., 2007). Additional studies using a competitive ELISA were recently conducted indicating the absence of cross reactivity with LPLRFa, NPFF, PrRP, and FMRFamide peptides (Bentley et al., in review).

Light microscopy. Sections were investigated using a Zeiss Z1 microscope. Sections were examined using the standard wavelengths for CY-2 (488 nm) and CY-3 (568 nm). Every 4th section through the dorsomedial hypothalamus (DMH) or 12 sections per animal and the medial preoptic area (MPOA) or 10 sections per animal were assessed, and those areas expressing RFRP-ir and GnRH-ir were investigated for coexpression with FOS protein using confocal microscopy (see below). For light microscopy, areas identified as having double-labeled cells were digitally captured at 200x in 8 bit greyscale using a cooled CCD camera (Zeiss). Each label was captured as a single image without moving the position of the stage or plane of focus between captures. Images were superimposed digitally. Brain areas were examined for double-labeling using Photoshop software in which CY-2 and CY-3 channels could be turned on and off independently. Only those RFRP and GnRH cells with a visible nucleus in which FOS expression was localized were counted as double-labeled cells. The total numbers of RFRP cells and GnRH cells and the percentage of cells expressing FOS were recorded by two independent observers blind to the experimental conditions.

Confocal microscopy. Brain sections used for light microscopy were also used for the confocal scans in order to confirm results at the conventional microscopy level. RFRP cells with putative SCN contacts were scanned through the extent of each cell in 0.5 μm increments. Only those cells in which the BDA labeled fiber contacted an RFRP cell in the same 0.5 μm scan were counted as close contacts. Cells characterized as being double-labeled with FOS/RFRP or FOS/GnRH at the conventional microscopy level were confirmed in the same manner to ensure that FOS was expressed within the cells rather than in overlapping cells in the same field of view. Likewise, cells classified as single-labeled were assessed to ensure that the conventional

microscopy strategy did not result in false negatives. At least 10% of those cells quantified using conventional microscopy were assessed in confocal scans. The cells assessed using confocal microscopy were chosen randomly across groups. Regions of the brain with putative double-labels identified at the light level were scanned at 400x using confocal microscopy. Cells were observed under a Zeiss Axiovert 100TV fluorescence microscope (Carl Zeiss, Thornwood, NY) with a Zeiss LSM 510 laser scanning confocal attachment. The sections were excited with an Argon-Krypton laser using the standard excitation wavelengths for CY-2 and CY-3. Stacked images were collected as 1.0 μm multitract optical sections. Using the LSM 3.95 software (Zeiss), red and green images of the sections were superimposed. RFRP or GnRH cells in a given brain region were examined through their entirety in 1.0 μm steps. Captured images were examined for double-labeling using Photoshop.

LH Radioimmunoassays. Serum LH concentrations were measured in duplicate in a single RIA with reagents obtained from the National Institutes of Health as previously described (Greives et al., 2007). The antiserum was rLH-S-11 and the standard was rLH-RP3. The sensitivity was 0.01 ng/tube and the intra-assay coefficient of variation was 2.8% for the low pool and 8.4% for the high pool. The antisera were highly specific for the hormones measured, with low cross-reactivity with other hormones.

Statistics. Data for RFRP cell counts FOS expression in RFRP cells, and LH results were analyzed using one-way analyses of variance (ANOVA) for those studies relating to the change in activation over the estrous cycle. Total cell counts and FOS expression in RFRP and GnRH cells in split and non-split (NS) animals were analyzed using two-way analyses of variance (Condition (split or NS) × Lateralization). Group differences were evaluated using Tukey tests when sample sizes were equal and Tukey-Kramer tests when sample sizes were unequal (e.g. split data). Differences were considered significant if $p<0.05$.

Results

The SCN Projects Extensively to RFRP-ir Cells in the DMH. Of the 10 hamsters injected with BDA, 5 injections were localized to the SCN and 5 misses were centered in the adjacent hypothalamus and retrochiasmatic area. Three injections filled the SCN unilaterally, whereas one injection was centered in the dorsomedial SCN and another in the ventrolateral SCN (**Figure 2A**). All five injections resulted in extensive projections and terminal fields in the DMH (**Figure 2B**). These smaller, localized injections in the SCN provided the opportunity to investigate the subregions from which identified projections originate. As in our previous work (Kriegsfeld et al., 2006), RFRP-ir cells were confined to the DMH (**Figure 2C**). For the three whole SCN injections, contacts upon RFRP perikarya ranged from 56.4-65.2% (**Figure 2D, E)**. A dorsomedial injection resulted in 20.4% of RFRP cells receiving SCN contacts, whereas 48.3% of RFRP cells were labeled with a comparably-sized ventrolateral injection. Despite marked projections from the SCN to the RFRP system, RFRP cells do not project back to the SCN at any time point investigated (**Figure 2F).** In all cases, RFRP fiber labeling was absent in the SCN, with dense projections targeting the adjacent sub-paraventricular zone and retro-chiasmatic area (Kriegsfeld et al., 2006).

RFRP-ir Cell Numbers are Reduced During the LH Surge and Reinstated Thereafter. All females exhibited regular 4-day estrous cycles. The preovulatory LH surge began at -4 h prior to the light offset on the day of proestrus and was complete within two hours (**Figure 3**). On the day of diestrus II, RFRP cell numbers were maximal in the DMH. In Syrian hamsters held in a 14/10 LD cycle, LH concentrations peak 4 h prior to lights out on the day of proestrus, with an initial increase seen 6 h before dark and cessation of the surge ~2 h prior to darkness (Stetson, 1978). On the day of proestrus, RFRP-ir cell numbers were reduced relative to the day of diestrus II for all time points ($p<0.05$ in each case) excluding 20:00 and 23:00 ($p>0.05$ in each case) (**Figure 4**). In all cases, quantification of FOS positive RFRP-ir and FOS negative RFRP-ir cells at the confocal level agreed with assessment at the conventional microscopy level.

Activational State of RFRP Cells is Reduced During the LH Surge and Reinstated Thereafter. As with RFRP cell numbers, the percentage of RFRP-ir cells expressing FOS was greatest during diestrus II when estrogen is maximally inhibiting LH production ($p<0.05$ relative to all other time points). Relative to diestrus II, on the day of proestrus, RFRP cell activity was reduced at all time points ($p<0.05$; **Figure 5**). Importantly, maximal reductions in FOS expression are observed 3 h prior to lights out, with sustained reductions in activity until 20:00 ($p<0.05$; **Figure 5**). Generally, FOS expression represents cellular activity about 1 h prior to sacrifice (Hoffman and Lyo, 2002), suggesting that maximal reductions in RFRP cellular activity occurred between 4 and 2 h prior to lights out. Following the time of the LH surge in hamsters (23:00), RFRP cellular activity was increased ($p<0.05$; **Figure 5**). In all cases, quantification of FOS positive RFRP-ir and FOS negative RFRP-ir cells at the confocal level agreed with assessment at the conventional microscopy level.

Daily Changes in RFRP Cellular Activity Require Estrogen. Because estradiol levels are maximal on the day of proestrus, we examined whether changes in the state of RFRP cells require the presence of this hormone. Based on the results above, we examined estradiol-treated and control hamsters at 20:00 (low RFRP cellular activity on the day of proestrus) and 23:00 (high RFRP activity). Estradiol-implanted hamsters exhibited low RFRP cell FOS expression at 20:00 with a marked increase at 23:00 ($p<0.05$). In contrast, hamsters without estradiol replacement had low RFRP cell FOS expression at both time points ($p>0.05$) (**Figure 6**). In all cases, quantification of FOS positive RFRP-ir and FOS negative RFRP-ir cells at the confocal level agreed with assessment at the conventional microscopy level.

The DMH Projects Ipsilaterally to Hypothalamic Regions Containing GnRH Cells. Of the 7 hamsters injected with BDA, 3 were localized to the DMH. For all three injections, projections from the DMH to all forebrain and hypothalamic sites expressing GnRH neurons including, the tenia tectum, medial and lateral septum, diagonal band of Broca, preoptic area, and anterior hypothalamus, were principally ipsilateral (for POA see **Figure 7**).

RFRP Activity is Lateralized and Associated with the LH Surge in 'Split' Hamsters. When held in constant light, 11 hamsters maintained one bout of activity, whereas 9 animals split their activity into two antiphase bouts (**Figure 8A, B**). In non-split controls, as expected, GnRH neurons exhibited maximal FOS expression 1 h (NS1) prior to their activity bout compared with

animals killed 13 h (NS13) prior to activity ($p<0.05$; **Figure 8C, E**); activity was symmetrical for both hemispheres ($p>0.05$). In contrast, RFRP cell activity showed the opposite pattern, with minimal expression in NS1 hamsters compared to NS13 animals ($p<0.05$; **Figure 8G, I**). When hamsters with split behavior were examined, GnRH FOS expression was asymmetrical, with maximal activity on the side of the brain ipsilateral to the activated SCN ($p>0.05$; **Figure 8D, F**). The opposing pattern of activity was seen in the RFRP system, with higher FOS expression in RFRP cells contralateral to the activated SCN ($p<0.05$; **Figure 8H, J**). In all cases, quantification of FOS positive RFRP/GnRH-ir and FOS negative RFRP/GnRH-ir cells at the confocal level agreed with assessment at the conventional microscopy level.

RFRP Cells Project to the Median Eminence and Hamster Pituitary Expresses RFRP Receptors. RFRP-ir cells in the DMH project extensively to the median eminence in hamster tissue, indicating RFRP may mediate the LH surge at the level of the median eminence and pituitary **(Figure 9B).** Using RT-PCR, we investigated the existence of RFRP receptor in the pituitary of Syrian hamsters by examining pituitaries from two different hamsters as well as liver tissue from rat as a control, which has previously been shown not to express RFRP receptors (Hinuma et al., 2000). As expected, pituitary tissue exhibited robust expression of RFRP receptors while rat liver tissue and a control sample did not express RFRP receptors **(Figure 9A)**.

Discussion

A host of neurochemicals suppresses GnRH secretion, with the inhibitory transmitters GABA (Demling et al., 1985; Adler and Crowley, 1986; Hartman et al., 1990) and the endogenous opioids (Barkan et al., 1983; Gopalan et al., 1989; Kumru et al., 2001), receiving the most attention. However, a mechanism coordinating disinhibition from these suppressive factors during stimulation of the LH surge of the estrous cycle has not been identified. Here we show a novel neural locus and neuropeptidergic system that displays properties consistent with such a role. The present findings point to interactions between the circadian and RFRP systems in the control of the preovulatory LH surge. Anterograde tracing shows pronounced projections from the SCN to the RFRP neuronal system, with close contacts of presumptive SCN terminals on RFRP cell perikarya. The RFRP system exhibits reduced expression and activity on the day of proestrus, with activity markedly suppressed around the time window in which the LH surge occurs. These findings suggest alterations in RFRP peptide synthesis, secretion, and/or transport throughout the ovulatory cycle. The system returns to pre-surge activity levels during early evening on the day of ovulation, after the surge has ceased. Additionally, we observed an asymmetry in the activational state of the RFRP system at the time of the surge that was opposite to the state of the GnRH system in split animals. Together, these findings suggest a novel network of ovulatory control with the possibility that the circadian system serves a dual role, both stimulating GnRH cell activity to initiate the LH surge while coordinating disinhibition from the influences of estrogen.

At the time of ovulation, GnRH neurons exhibit a robust increase in immediate early gene expression, with activation lagging behind the initiation of the LH surge by ~1 h (Lee et al., 1990; Berriman et al., 1992; Lee et al., 1992; Hoffman et al., 1993; Wang et al., 1995). In ovariectomized Syrian hamsters held in a 14/10 LD cycle, LH concentrations peak 4 h prior to lights out on the day of proestrus, with cessation of the surge ~2 h prior to darkness (Stetson,

1978) (present study, **Figure 3**). The present findings indicate a pattern of FOS expression in RFRP cells opposite to that observed for GnRH, further suggesting complementary circadian control of both systems. The SCN projects extensively to RFRP-ir cells, with both dorsomedial and ventrolateral subregions of the SCN projecting to the DMH. Whereas only one animal is assessed for anterograde injections into both subregions of the SCN, these results provide a possible avenue for future investigation of SCN control of the RFRP system. At the conventional and confocal microscope levels, we observe close appositions between SCN terminals and RFRP cells. Although laser scanning confocal microscopy is not adequate to identify true synapses onto RFRP neurons, high-power confocal scans showing a presumptive bouton contacting a cell body in the same optical plane provide strong support for a functional relationship between the fiber and the cell body it is contacting.

Our analysis of split animals suggests neural circadian control of the RFRP system. RFRP cells exhibit reduced activity on the side of the brain bearing the activated SCN, a pattern opposite to GnRH cell activation (**Figure 8**). These findings, along with anterograde tracing results showing that the DMH projects to ipsilateral brain regions containing GnRH cells, are consistent with the notion that the SCN coordinates disinhibition of the RFRP system with the initiation of the surge (**Figures 7 and 10**). Overall, FOS expression in RFRP-ir cells in split and non-split animals was reduced relative to animals housed in a LD cycle. This finding is likely due to constant light conditions, as a similar reduction has been observed previously under these conditions (de la Iglesia et al., 2003). It remains possible that the RFRP system also modulates the surge at the level of the pituitary, as the RFRP receptor, GPR147 (Hinuma et al., 2000; Kriegsfeld et al., 2006; Tsutsui and Ukena, 2006), is present in hamster (present study) and quail (Ubuka et al., 2006) pituitary, with RFRP fibers extending into the median eminence ((Ubuka et al., 2006), **Figures 9 and 10**). We previously reported sparse RFRP fibers extending into the external layer of the median eminence (Kriegsfeld et al., 2006), but refinements of our amplified immunohistochemical procedures in the present report indicate more pronounced innervation than previously suggested, in agreement with reports in other species.

In rats, mice, and hamsters RFRP cells project to GnRH cells directly, providing a potential pathway for its suppressive actions (Kriegsfeld et al., 2006). Likewise, in avian species, RFRP cells also project to GnRH cells (Bentley et al., 2006b), and GnRH neurons express GnIH, the RFRP ortholog found in birds, receptor mRNA (Ubuka et al., 2008). Although the pattern of transcriptional activation of RFRP cells is consistent with a release of the reproductive axis from RFRP inhibition at the time of the surge, it is possible that RFRP cellular activity is unrelated to timed changes in reproductive axis activity. RFRP cells project widely in mammalian brain throughout midline hypothalamic regions and limbic structures, suggesting actions in addition to GnRH control (Kriegsfeld et al., 2006). However, recent observations indicate that injections of RFRP-3 inhibit GnRH neuronal activation during the estradiol-induced LH surge (Anderson, 2007) suggesting a need for the removal of RFRP influences at this time. Whether such removal of RFRP inhibition to the reproductive axis occurs normally during ovulation requires further study.

RFRP-ir cells exhibit changes in FOS expression on the day of proestrus, when estrogen concentrations are chronically elevated. Given the design of the present series of experiments, we cannot discern the specific contribution of the circadian system versus stage of cycle (i.e.,

proestrus) to changes in RFRP cell FOS expression. Several lines of evidence point to a role for both factors in this daily regulation. First, when estrogen concentrations are clamped at proestrus levels, the RFRP system exhibits the same timed pattern of activation, with timed changes abolished in ovariectomized animals given empty implants (**Figure 6**). Additionally, the abolition of this daily pattern in RFRP activity in animals implanted with empty capsules, suggests a circadian mechanism requiring estrogen. Further studies examining the daily pattern of RFRP cellular function during other stages of the cycle are necessary to fully explore this possibility. Collectively, these findings suggest the possibility that RFRP cells exhibit daily changes in responsiveness to estrogen, with reduced sensitivity around the time of the LH surge. Alternatively, because only a subset of these cells express ERα (Kriegsfeld et al., 2006), it is possible that cells expressing temporal changes in activity are not estrogen-responsive, but instead, are controlled by an estrogen-responsive clock mechanism. Whether estrogen acts upstream of RFRP cells to modulate their activity remains to be determined.

Whereas circadian behavior, such as wheel running, can be supported via diffusible signals (Silver et al., 1996), all findings to date indicate that neural communication from the SCN is required for initiation of the LH surge (Nunez and Stephan, 1977; Hakim et al., 1991; Meyer-Bernstein et al., 1999; de la Iglesia et al., 2003; Kriegsfeld et al., 2006). In addition to direct connections to GnRH neurons, the SCN projects extensively to the anteroventral periventricular nucleus (AVPV), a brain region associated with the induction of the preovulatory LH surge and changes in progesterone receptor expression (Le et al., 1997; Levine, 1997). The role of progesterone in the regulation of the RFRP system is unknown and may provide insight into an additional mechanism of ovulatory control. The cells to which the SCN projects are estrogen-responsive (Watson et al., 1995), suggesting that the AVPV may be an important integration point for circadian signals and positive feedback effects of estrogen. Conversely, ER-expressing cells in the preoptic area project to the SCN (de la Iglesia et al., 1999), providing a mechanism for steroidal feedback to the circadian system. The stimulatory peptide, kisspeptin, is robustly expressed in the AVPV, with kisspeptin-ir cells expressing ERα, and showing a pattern of activation consistent with a role in driving the LH surge (Smith et al., 2006; Adachi, 2007; Greives et al., 2007). It remains to be established whether the SCN projects to kisspeptin cells in the AVPV or to cells of an unidentified phenotype. Furthermore, RFRP cells project extensively to the AVPV (Kriegsfeld et al., 2006), suggesting potential interactions between the RFRP and kisspeptin systems in this nucleus, and opportunity for further exploration (**Figure 10**).

The present studies provide evidence for incorporating the RFRP system into the conceptual framework for the ovulatory machinery of some rodents, and perhaps other species. These data suggest a neural route of communication from the SCN clock to an inhibitory peptidergic pathway mediating the negative feedback effects of estradiol. Additionally, the RFRP system is in a position to amalgamate with the well-established pathways and mechanisms responsible for initiating the LH surge (**Figure 10**). Together, these findings suggest the possibility that the DMH and the RFRP system play a significant role in modulating the LH surge and ovulation.

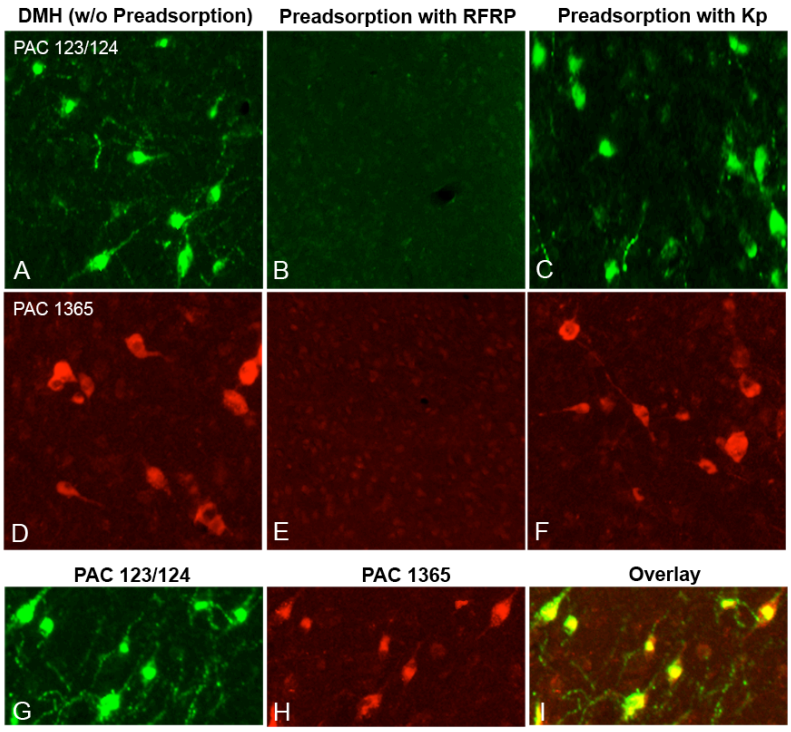

Figure 1. **RFRP-r Labeling is Abolished with Hamster RFRP Peptide Preadsorption.** Photomicrographs of RFRP-ir cell body labeling in the DMH using PAC 123/124 (A) and PAC 1365 (D). (B, E) Preadsorption with hamster RFRP peptide abolishes RFRP staining (C, F). Staining of RFRP is not affected by preadsorption with kisspeptin peptide (Kp). (G-I) RFRP antibodies PAC 123/124 (G) and PAC 1365 (H) label the same cell populations (I).

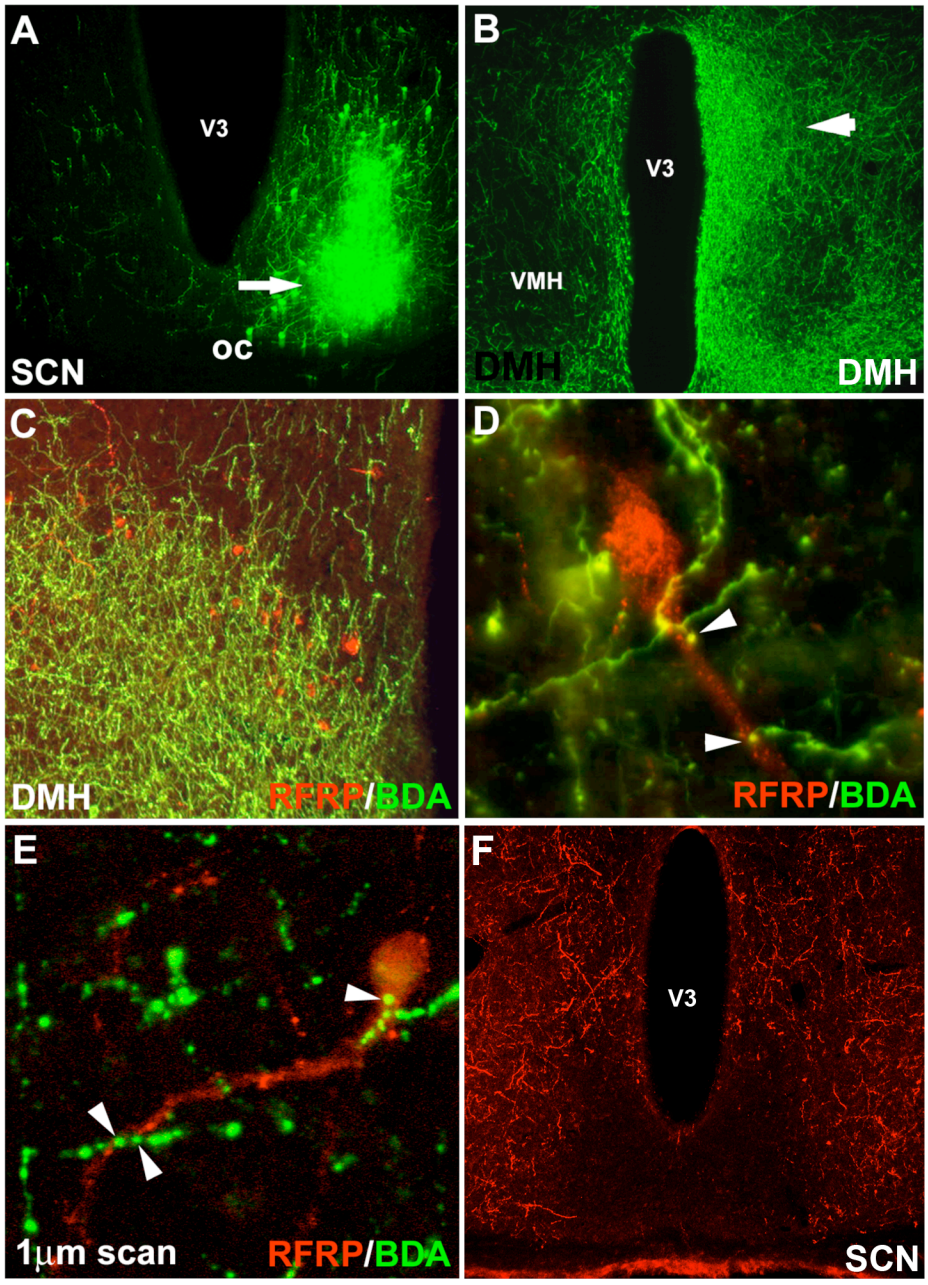

Figure 2. **SCN Fibers Project to RFRP-ir Cells in the Dorsomedial Hypothalamus.** (A) Example injection site from an injection of BDA that filled the ventrolateral aspect of the SCN. (B) Low-power photomicrograph indicating terminal fibers from the SCN project to the DMH, principally ipsilaterally. (C-E) Examples of SCN projections in close apposition to RFRP-ir cells in the DMH at the light (low-power; C, high-power; D) and confocal levels (E). (F) RFRP fibers target the sub-paraventricular zone and retrochiasmatic area, but do not terminate in the SCN.

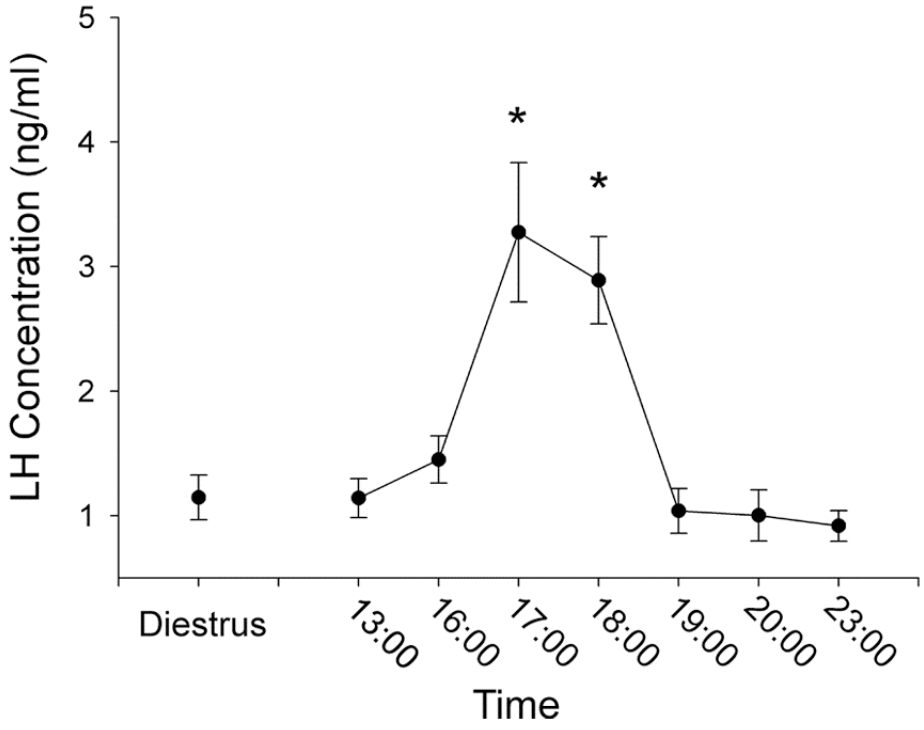

Figure 3. **Luteinizing Hormone Concentrations in Syrian Hamsters.** Mean (±SEM) luteinizing hormone (LH) concentrations on the day of diestrus and during various times on the day of proestrus. *=significantly different from diestrus values and all other proestrus values, excluding 16:00, $p<0.05$.

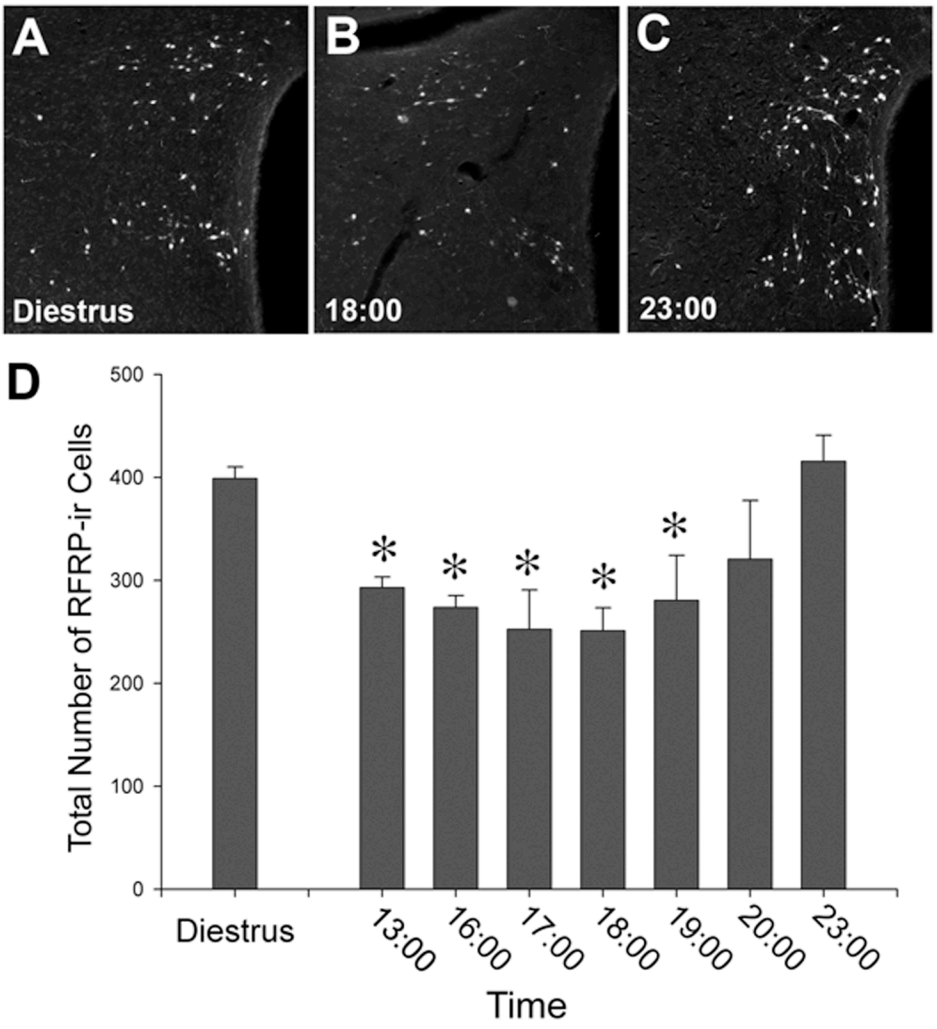

Figure 4. **RFRP-ir Cell Numbers are Reduced during the LH Surge and Reinstated Thereafter.** (A) Low-power photomicrograph of RFRP-ir cell body labeling in the DMH on the day of diestrus. (B-C) Low-power photomicrographs of RFRP-ir cell body labeling in the DMH on the day of proestrus during the trough (18:00) and peak (23:00) of expression respectively. (D) Mean (±SEM) RFRP-ir cell counts on the day of diestrus and throughout the day of proestrus. * = significantly different from diestrus values and 23:00 values on the day of proestrus, $p<0.05$.

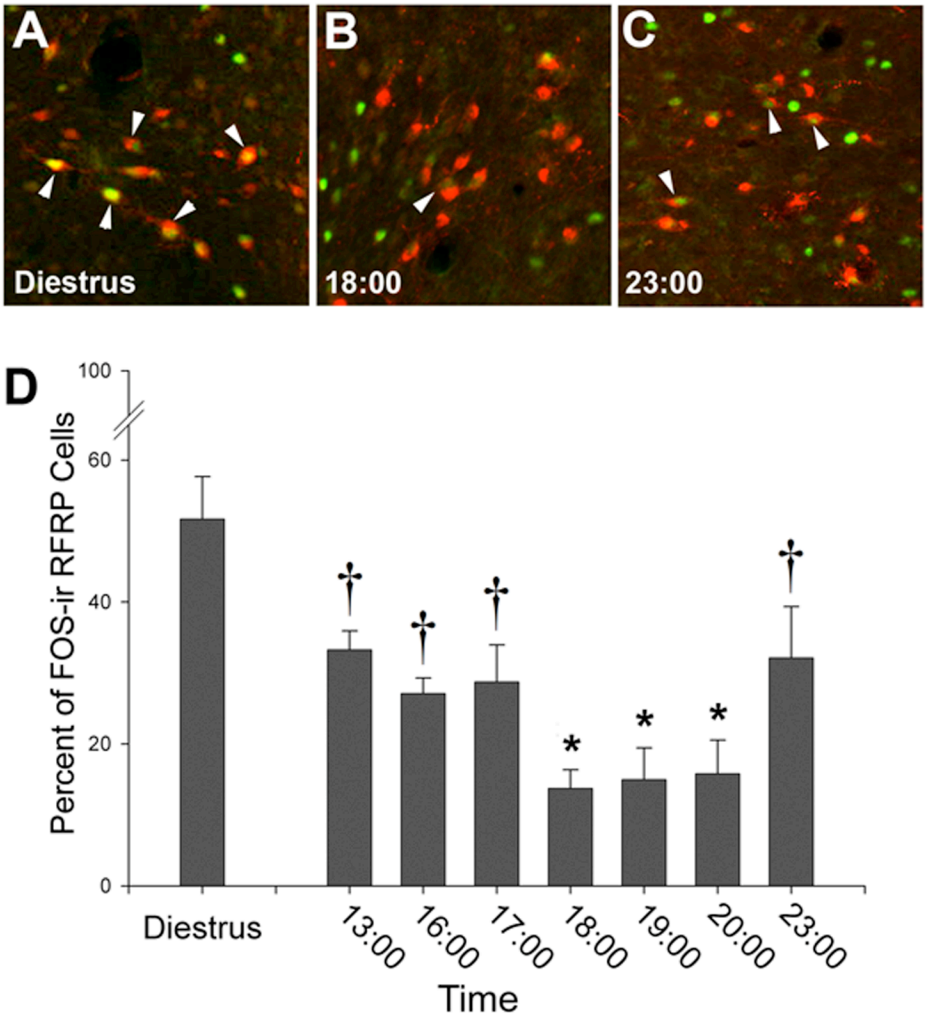

Figure 5. **Activation of RFRP-ir Cells is Reduced during the LH Surge and Reinstated Thereafter.** (A-C) The percent of RFRP-ir cells expressing FOS is reduced on the day of proestrus, around the time of the LH surge. Low-power photomicrographs of RFRP-ir cells expressing FOS on the day of diestrus (A) and during the trough (B) and peak (C) of expression on the day of proestrus. (D) Mean (±SEM) percentage of RFRP-ir cells expressing FOS on the day of diestrus and throughout the day of proestrus. All bars not sharing a symbol differ significantly from each other, $p<0.05$.

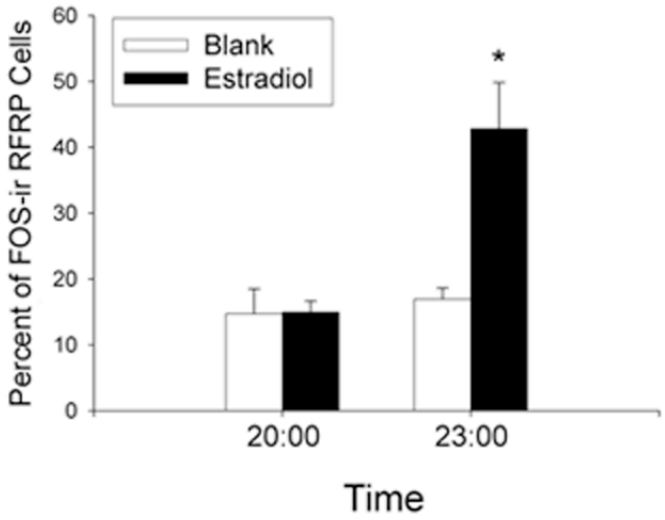

Figure 6. **Estrogen is Required for Daily Changes in Activational State of RFRP-ir Cells.** Mean (±SEM) percentage of RFRP-ir cells expressing FOS in ovariectomized hamsters implanted with estradiol or a blank capsule during the LH surge (20:00) or following the LH surge (23:00). * = significantly different from all other values, $p<0.05$.

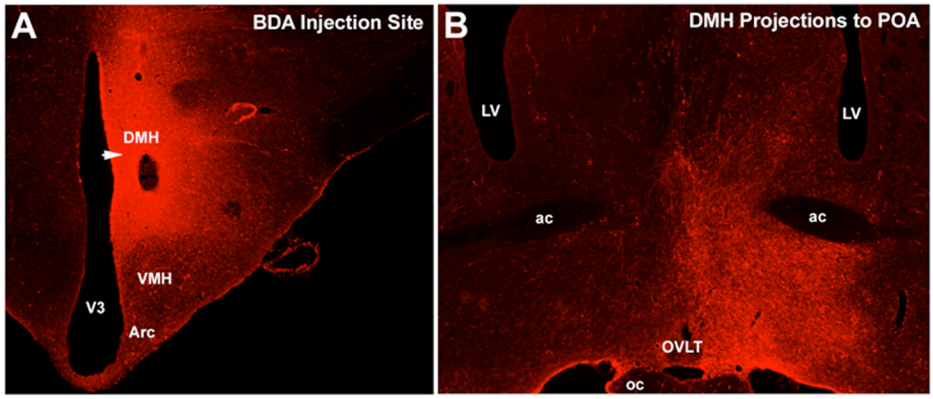

Figure 7. **The DMH Projects Ipsilaterally to Hypothalamic Regions Containing GnRH-ir Cells.** (A) A representative injection of the anterograde tracer, BDA, localized to the DMH. (B) DMH projections were localized to numerous hypothalamic regions containing GnRH-ir cells, including the preoptic area. Projections were principally ipsilateral.

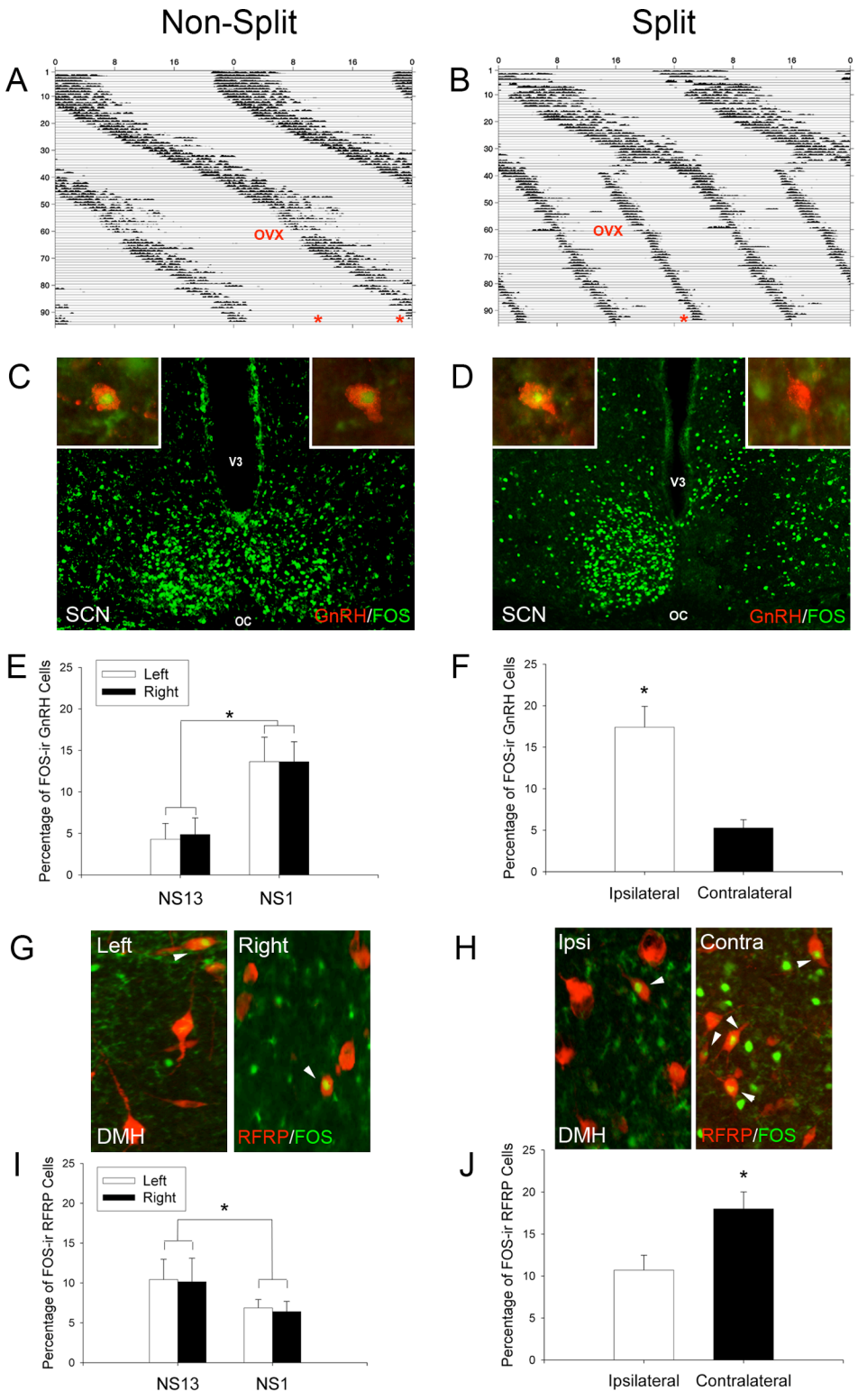
Non-Split
Split
A
B
OVX
OVX
C
D
V3
V3
SCN
OC
GnRH/FOS
SCN
OC
GnRH/FOS
E
F
Left
Right
Percentage of FOS-ir GnRH Cells
NS13
NS1
Percentage of FOS-ir GnRH Cells
Ipsilateral
Contralateral
G
H
Left
Right
DMH
RFRP/FOS
Ipsi
Contra
DMH
RFRP/FOS
I
J
Left
Right
Percentage of FOS-ir RFRP Cells
NS13
NS1
Percentage of FOS-ir RFRP Cells
Ipsilateral
Contralateral

Figure 8. **Lateralization of GnRH and RFRP Activational Patterns in 'Split' Hamsters is Associated with the LH Surge.** (A-B) Actograms of wheel running activity in estradiol-implanted, ovariectomized hamsters kept in constant light conditions (LL). (A) Non-split (NS) hamsters were sacrificed (*) 1 hour or 13 hours prior to the onset of the activity bout. (B) Split hamsters were sacrificed (*) 1 hour prior to the onset of one of the two activity bouts. (C-D) Photomicrographs of FOS activation in SCN and GnRH cells (insets) of NS and split hamsters. (E-F) Mean (±SEM) percentage of FOS-ir GnRH cells in non-split hamsters sacrificed 1 hour (NS1) or 13 hours (before the surge; NS13) prior to their activity bout and split hamsters sacrificed 1 hour prior to one of their activity bouts. (G-H) Photomicrographs of FOS-ir RFRP cells in the DMH of NS and split hamsters. (I-J) Mean (±SEM) percentage of FOS-ir RFRP cells in NS1, NS13, and split hamsters. * = significantly different from all other values, $p<0.05$.

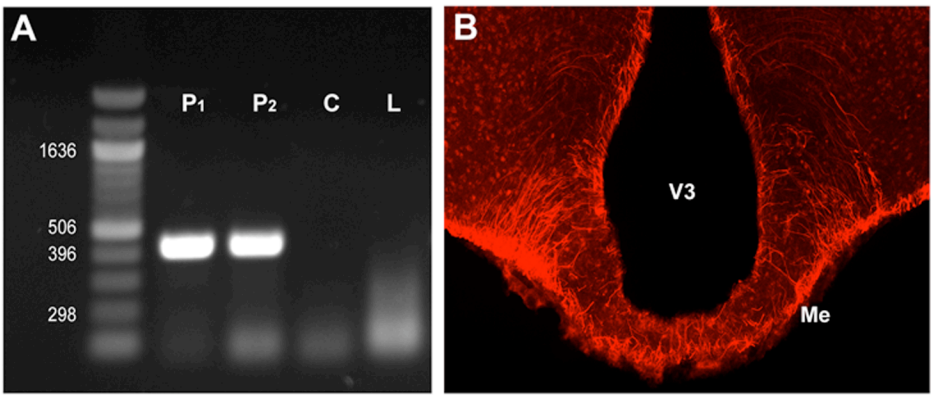

Figure 9. **Hamster Pituitary Expresses RFRP Receptors and RFRP-ir Cells Project to Median Eminence.** (A) Using reverse transcription PCR, pituitaries from 2 hamsters (P1 and P2) were shown to express the RFRP receptor, GRP147. RFRP receptor expression was not detected in a control sample (C), water, or in tissue from rat liver (L) which was previously shown not to express RFRP receptors (Hinuma et al., 2000). (B) RFRP fibers from the DMH project to the median eminence (Me), suggesting control of the LH surge may also occur at the level of the Me and the pituitary.

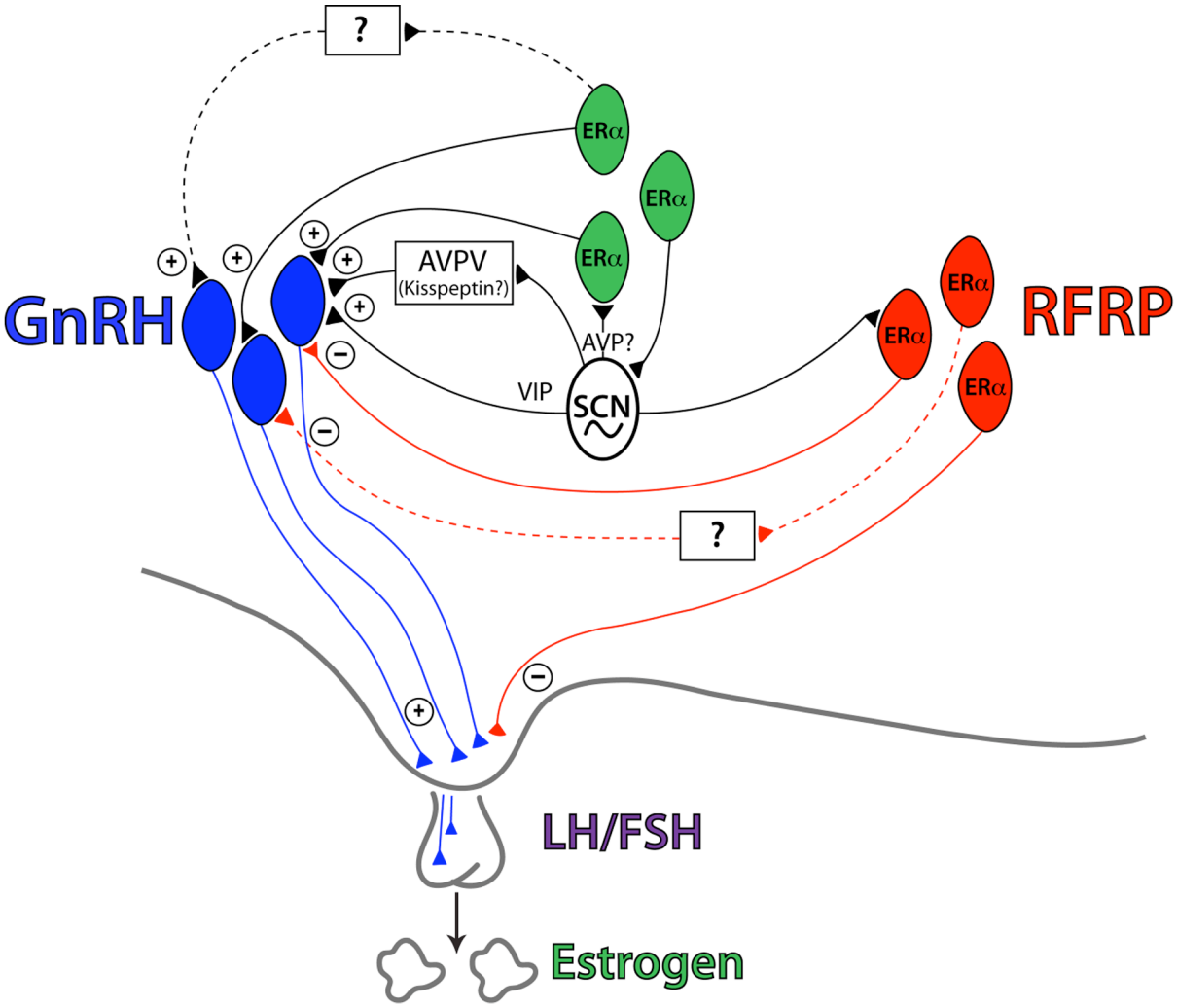

Figure 10. **Integration of RFRP in Regulating Induction of the LH Surge.** A proposed model incorporating a role for the RFRP system in the ovulatory circuitry. Black lines depict projections from the circadian system to GnRH neurons and neurons containing estrogen receptors (de la Iglesia et al., 1995) as well as to the RFRP system (present study) and the AVPV (Watson et al., 1995) reported in rats, mice, and hamsters. Kisspeptin cells in the AVPV are active at the time of the LH surge (Irwig et al., 2004; Kauffman et al., 2007). Neurons containing ER-α in the preoptic area and elsewhere are known to project to the SCN (de la Iglesia et al., 1999) and to GnRH neurons (Simonian et al., 1999) and may play a role in mediating the circadian signal to GnRH neurons directly and/or indirectly. Red lines depict interactions between the RFRP and GnRH systems described in the present study and our previous work (Kriegsfeld et al., 2006). These lines indicate a putative role for RFRP in modulating the negative feedback effects of estrogen (Kriegsfeld et al., 2006), with SCN communication allowing for removal of negative feedback on the reproductive axis during the time of the LH surge. Dashed lines indicate indirect connections between systems which have not yet been explored. According to the model generated from the present findings, RFRP cells respond to estradiol with HPG axis inhibition during most of the estrous cycle. At the time of

the LH surge, however, the SCN signals the RFRP system to ignore estradiol input and remove negative feedback influences on the HPG axis, allowing activation of the GnRH system directly or indirectly via the circadian system. (AVP = arginine vasopressin; VIP = vasoactive intestinal polypeptide)

Chapter 2: The Effects of Temporal Disruption on the Reproductive Axis

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Experiment 2: Chronic Jet Lag Disrupts the Pattern of Temporal Activation in Hypothalamic Neuropeptides Mediating the Preovulatory Luteinizing Hormone Surge in Female Syrian Hamsters

Abstract

Chronic rotating shift work and transmeridian travel is associated with an increased incidence of reproductive dysfunction. One potential mechanism mediating such deficits is the resulting incongruence between internal biological rhythms and external environmental cues. The master circadian clock, the SCN, coordinates precise timing between hypothalamic peptides in order to maintain reproductive function. SCN stimulation of the GnRH and kisspeptin systems concomitant with SCN inhibition of the RFRP system is associated with the onset of the LH surge and ovulation. In the present study, we investigated whether or not reproductive deficits associated with chronic circadian disruption are associated with alterations in the time-sensitive activation of the GnRH, kisspeptin, and RFRP systems. In control hamsters, the LH surge was associated with an increase in activation of the GnRH and kisspeptin systems, as well as a deactivation of the RFRP system. In contrast, the precise temporal pattern in activation/deactivation of these three neuropeptide populations was abolished with a tonic level of neuronal activation in all three populations throughout the day. These data suggest that deficits in reproductive function and fecundity may be a result of dysregulation of the brain nuclei mediating the hypothalamo-pituitary-gonadal (HPG) axis.

Introduction

Precise coordination of molecular and cellular rhythms to maintain 24-hour oscillations in physiology and behavior is imperative to optimal functioning on a planet with a 24-hour day (Maywood et al., 2006). Briefly, in mammals, the core clock genes *Clock* and *Bmal1* drive the transcription of the clock genes *Period (Per1, Per2, Per3)* and *Cryptochrome (Cry1, Cry2)*. The protein products of the *Per* and *Cry* genes then dimerize and feedback into the nucleus of the cell to inhibit their own transcription, resulting in an autoregulatory feedback loop that takes approximately 24 hours to complete (Reppert and Weaver, 2002; Van Gelder et al., 2003; Chen et al., 2009). Expression of clock genes can be found throughout the body, including in cells mediating the HPG axis. In most species, including many mammals and birds, coordination between the environment and the reproductive axis is necessary both physiologically and behaviorally to synchronize those behaviors that mediate reproductive success with an active reproductive axis. The GnRH system, located in the rodent anterior hypothalamus, serves as the final common pathway that functions to regulate peripheral gonadal events in most species (Silverman and Witkin, 1994; Herbison, 2006). GnRH neurons project to the median eminence to regulate the synthesis and secretion of the gonadotropins, luteinizing hormone (LH) that modulates steroidogenesis and follicle-stimulating hormone (FSH) that modulates gametogenesis. Numerous studies have shown that the circadian molecular machinery is not only present in GnRH cells, but also plays an important role in GnRH function. In female mice, *Per2* and *Bmal1* cycle with an approximate 24-hour rhythm in GnRH neurons, with timing of the

peak expression consistent over the estrous cycle (Hickok and Tischkau, 2009). Since heterogeneity exists among the GnRH population, an immortalized GnRH cell line derived from fragments of GnRH genes inserted into embryos (GT1-7 cells), has been developed to further study the role of GnRH in stimulation of the HPG axis (Mellon et al., 1990). GT1-7 cells transfected with dominant-negative *Clock* disrupts GnRH secretion (Chappell et al., 2003). Similarly, GT1-7 cells exhibit daily changes in GnRH secretion in response to kisspeptin, a modulator of the HPG axis upstream of the GnRH system, without changes in GnRH transcription (Zhao and Kriegsfeld, 2009).

Kisspeptin is a member of the RFamide peptide family expressed in the anteroventral periventricular (AVPV) nucleus across species and is a potent stimulator of the reproductive axis (Gottsch et al., 2004; Mason et al., 2007). In the GT1-7 immortalized GnRH cell line, co-treatment with kisspeptin and 17β-estradiol increases GnRH secretion markedly over estradiol treatment alone (Novaira et al., 2009). Administration of kisspeptin antagonists not only delay the onset of puberty in female rats, including delayed vaginal opening and decreased uterine and ovarian weights, but also suppresses the magnitude of the LH and FSH surges necessary for ovulation (Pineda et al., 2010a). Whereas blocking kisspeptin can delay puberty, restoration of the kisspeptin system in middle-aged rats can restore the LH surge (Neal-Perry et al., 2009). Kisspeptinergic activation of the HPG axis appears to be regulated at the level of GnRH cells with GnRH cells only sensitive to kisspeptin stimulation in the afternoon when the LH surge can occur (Williams et al., 2011). In order for the GnRH-induced LH surge to occur on the afternoon of proestrus, the HPG access must be freed from the inhibitory effects of estrogen that suppress activation of the reproductive access during most of the estrous cycle, and estrogen must retain a permissive role in activating the LH surge (Karsch et al., 1997; Levine, 1997; Petersen et al., 2003). Whereas it has been hypothesized that estrogen stimulates the positive drive on the reproductive axis through the kisspeptin neuronal system (Williams et al., 2011), the disinhibition of estrogenic-negative feedback effects may be mediated through the RFRP system.

Located in the dorsomedial nucleus of the hypothalamus (DMH) in mice, rats, and hamster, the RFRP system, the ortholog to the avian GnIH system, has been implicated in regulation of the inhibitory effects of estrogen on the HPG axis during most of the estrous cycle (Kriegsfeld et al., 2006; Gibson et al., 2008). The suppressive actions of GnIH on reproduction in birds (Ciccone et al., 2004) includes suppression of gonadal development and gonadotropin synthesis and release (Ubuka et al., 2006) and decreases in LH and copulatory behavior in white-crown sparrows (Bentley et al., 2006a). In mammals, RFRP-3 administration reduces LH concentrations in hamsters (Kriegsfeld et al., 2006) and suppresses LH secretion and male sex behavior in rats (Johnson et al., 2007; Pineda et al., 2010b). Recently, the presence of an RFRP system has been identified in humans (Ubuka et al., 2009). RFRP-3 alters the HPG axis *in vitro* by directly inhibiting the firing rate in GnRH neurons in mouse cultures (Ducret et al., 2009). In the presence of GnRH, RFRP-3 suppresses LH secretion in cultured female rat pituitary cells (Murakami et al., 2008). *In vivo* GnIH/RFRP directly interacts with GnRH neurons in European starlings (Ubuka et al., 2008) and in hamsters (Kriegsfeld et al., 2006; Gibson et al., 2008). At the time of the LH surge in female Syrian hamsters, cellular activation of the RFRP system is downregulated, allowing for a disinhibition of the HPG axis (Gibson et al., 2008). This time-dependent disinhibition of the reproductive axis is concomitant with a stimulation of the GnRH

system, most likely by estrogenic effects on the kisspeptin system (Gibson et al., 2008; Williams et al., 2011).

The precise temporal control of the LH surge is mediated by the suprachiasmatic nucleus (SCN), located in the anterior hypothalamus. The master circadian pacemaker regulates the LH surge, with lesions of the SCN abolishing it (Everett and Sawyer, 1950; Legan and Karsch, 1975; Wiegand and Terasawa, 1982). The LH surge not only requires a daily temporal signal from the SCN but coincidence of this signal in the presence of a permissive gonadal hormone milieu to stimulate GnRH secretion into the hypophysial portal system (Petersen et al., 2003; Chappell, 2005). The simultaneous circadian signal and high concentrations of estradiol necessary for induction of the LH surge is further supported by the finding that ovariectomized hamsters provided with proestrous concentrations of estradiol will exhibit daily LH surges (Abraham et al., 2003). Whereas the SCN neurally controls the GnRH system (de la Iglesia et al., 2003; Gibson et al., 2008), it also targets kisspeptin neurons via vasopressinergic connections (Vida et al., 2010; Williams et al., 2011), and the RFRP system (Gibson et al., 2008), pointing to a strong circadian control of the reproductive axis at multiple levels.

Based on the redundant interactions of the circadian system and the reproductive axis, it is not surprising that temporal disruptions negatively impact reproduction. Temporal disruptions, such as jet lag or rotating shift work, occur when endogenous rhythms become incongruent with the environment. *mPer* expression in the mouse SCN rapidly adjusts to phase shifts but *mCry* lags behind, suggesting that phase shifts can impact the molecular clockwork mediating circadian rhythms as well as the master clock (Reddy et al., 2002). Mice with a mutation of the circadian *Clock* gene show irregular estrous cycles, even though GnRH concentrations, pituitary release of LH, and estrogen/progesterone concentrations are normal. *Clock* mutants also have increased rates of fetal re-absorption and decreased rates of full-term pregnancies, potentially as a result of sudden suppression of progesterone mid-pregnancy (Miller et al., 2004). Female shift workers in Denmark exhibit increased duration of pregnancy and decreased fetal growth compared to day workers (Zhu et al., 2004). Similarly, shift work in Swedish midwives is associated with subfecundity (Ahlborg et al., 1996). The previous studies point to a potential role for disruptions in circadian homeostasis in reproductive failures. The purpose of the present study is to investigate whether the deficits in reproductive function associated with circadian disruptions are a result of dysregulation between the precise temporal control over the neuropeptidergic systems regulating the LH surge and ovulation: GnRH, RFRP, and kisspeptin.

Materials and Methods

Animals. Adult, female LVG Syrian hamsters (*Mesocricetus auratus*) (n=40) were used. All animals were purchased from Charles River (Wilmington, MA) at 4-5 weeks of age. Animals were housed in translucent propylene cages (48 x 27 x 20 cm) and provided with *ad libitum* access to food and water for the duration of the study. Animals were maintained in a colony room at 23 ± 1°C with a 24 hr light/dark cycle (14/10 hr light/dark) with lights on at 07:00 and lights off at 21:00. All experimental protocols conformed to the Institutional Animal Care and Use Committee guidelines of the University of California, Berkeley. After 2 weeks in LD, animals were bilaterally ovariectomized under isoflurane anesthetic. Subsequently, animals were implanted with a SILASTIC capsule (Dow Corning Corp.; 10-mm length, 1.45-mm id, 1.93-mm

od) containing powdered 17-estradiol. In estrogen-implanted animals, this treatment provides estradiol concentrations comparable to those seen on the day of proestrus (Meyer-Bernstein et al., 1999) and results in daily LH surges at the same time of day that the LH surge would normally occur (Norman et al., 1973).

Jet Lag Procedure/Behavioral Monitoring. Two weeks after surgery, half (n=10) of the animals were exposed to a 6-hr phase advance every 3 days for 25 days (Jet Lag) while the other half remained in a 14:10 LD (lights on at 0700 hr) cycle for the same duration as jet-lagged animals (Control). Locomotor behavior was monitored for all animals using an infrared monitoring system (Data Sciences; St. Paul, MN) mounted to the wire lids on each cage. All movement in the cage was detected by interruptions in the infrared beam and relayed to a computer. Cumulative counts were recorded every 10 min and analyzed using Dataquest 3 software (Data Sciences; St. Paul, MN). In ovariectomized Syrian hamsters held in a 14/10 LD cycle, LH concentrations peak 4 h prior to lights out on the day of proestrus, with cessation of the surge ~2 h prior to darkness (Stetson, 1978). In order to assess neuropeptide activation around the time of the LH surge, animals (n=5/time point) were sacrificed 7 hr, 4 hr, or 2 hr prior to activity onset or 2 hr after activity onset.

Perfusion and Histology.
Hamsters were deeply anesthetized with sodium pentobarbital (200 mg/kg) and perfused intracardially with 150 ml of 0.9% saline, followed by 300-400 ml of 4% paraformaldehyde in 0.1 M PBS, pH 7.3. Brains were postfixed for 2-3 hr at 4°C and cryoprotected in 30% sucrose in 0.1 M PBS overnight. For simultaneous visualization of RFRP and FOS, every fourth 40-µm coronal section from the mediobasal hypothalamus was washed in 0.1M PBS, incubated in 0.5% H_2O_2, and incubated in normal goat serum (NGS, 1:50; Jackson) in 0.1% Triton X-100 (PBT) for 1 hour. Sections were then incubated for 48 hours at 4ºC with a rabbit anti-FOS Ab diluted at 1: 50,000 (Santa Cruz, Santa Cruz, CA) and NGS diluted at 1:1000 with 0.1% PBT for 48 hours. After incubation in anti-FOS, brains were incubated for 1 hour in biotinylated goat anti-rabbit (1:300; Vector Laboratories, Burlingame, CA) and then in ABC for 1 hour. The FOS signal was amplified with biotinylated tyramide solution (0.6%) for 30 min as previously described (Kriegsfeld et al., 2006). Cells were then labeled by using CY-2 conjugated streptavidin (1:200; Jackson) as the fluorophore. This protocol allowed for the amplification of the highly diluted anti-FOS required for double-labeling with two antibodies generated in the same species (rabbit). After labeling for FOS, sections were incubated in anti-RFRP antibody (1;10,000; PAC 123/124) in 0.1% PBT for 48 h (Kriegsfeld et al., 2006). RFRP cells were labeled with CY-3 donkey anti-rabbit (1:200; Jackson) as the secondary/fluorophore. The previous FOS/RFRP protocol was followed for visualization of GnRH and FOS with the RFRP antibody replaced with an antibody directed against GnRH (LR-5; 1:20,000; generous gift from Dr. R. Benoit; McGill University Health Centre, Montreal, Quebec). For kisspeptin/FOS labeling, the same procedure was followed but with kisspeptin (rabbit polyclonal anti-kisspeptin-10 anti-serum; 1:2000; generated by Dr. J.D. Mikkelsen; Copenhagen University Hospital, Copenhagen, Denmark) incubated as the first primary antibody visualized by CY-2 conjugated streptavidin and FOS (1:5000) as the second primary antibody visualized with CY-3 donkey anti-rabbit. Cells were visualized using CY-3 donkey anti-mouse (1:200; Jackson). All sections immunohistochemically treated were mounted onto gelatin-coated slides, dehydrated in a graded series of alcohols, and coverslips were applied.

Light microscopy. Sections were investigated using a Zeiss Z1 microscope. Sections were examined using the standard wavelengths for CY-2 (488 nm) and CY-3 (568 nm). Every 4th section through the dorsomedial hypothalamus (DMH), the medial preoptic area (MPOA), or the anteroventral periventricular (AVPV) were assessed, and those areas expressing RFRP-ir, GnRH-ir, and kisspeptin-ir were investigated for coexpression with FOS protein. For light microscopy, areas identified as having double-labeled cells were digitally captured at 200x in 8 bit greyscale using a cooled CCD camera (Zeiss). Each label was captured as a single image without moving the position of the stage or plane of focus between captures. Images were superimposed digitally. Brain areas were examined for double-labeling using Photoshop software in which CY-2 and CY-3 channels could be turned on and off independently. Only those RFRP, GnRH, or kisspeptin cells with a visible nucleus in which FOS expression was localized were counted as double-labeled cells. The total numbers of RFRP, GnRH, and kisspeptin cells and the percentage of cells expressing FOS were recorded by two independent observers blind to the experimental conditions.

Statistics. FOS expression in RFRP, GnRH, and kisspeptin cells in Jet Lag and Control animals were analyzed using two-way analyses of variance (Condition (Jet Lag or Control) × Time). Group differences were evaluated using Tukey tests when sample sizes were equal and Tukey-Kramer tests when sample sizes were unequal. Differences were considered significant if $p<0.05$.

Results.

Rhythms in GnRH cellular activity around the time of the LH surge are abolished in jet-lagged animals. In hamsters held in a static LD cycle, the initiation of the LH surge occurs 4 hours prior to activity onset, with the cessation of the surge within 2 hours (Stetson, 1978). GnRH cells exhibit an increase in FOS expression that lags ~1 hours behind the LH surge (Lee et al., 1990; Berriman et al., 1992; Lee et al., 1992; Hoffman et al., 1993; Wang et al., 1995). In control hamsters, GnRH activation was increased during the time of the LH surge (-2 hours relative to activity onset) compared to all other time points (-7 hrs, -4 hrs, +2 hrs; $p<0.05$ for all cases). In contrast, the temporal pattern of activation in the GnRH population was abolished in the jet-lagged animals, with similar percentages of GnRH neurons expressing FOS at all time points around the onset of activity ($p>0.05$; **Figure 1A**).

Temporal activation of the kisspeptin system is eliminated with jet lag. In control hamsters, the percentage of kisspeptin cells expressing nuclear FOS exhibit a significant daily pattern with increased expression 2 hours prior to activity onset, suggesting a temporal control over kisspeptin activation ($p<0.05$ compared to all other time points within condition). In jet-lagged hamsters, this temporal pattern was eliminated with equal percentages of kisspeptin-ir cells expressing FOS at all time points ($p>0.05$). 2 hours after the onset of activity, activation of kisspeptin neurons in control animals is significantly decreased compared to all other time points ($p<0.05$), but jet lag results in a maintenance of increased kisspeptin/FOS expression, suggesting a potential disruption in deactivation of the kisspeptin system following the LH surge ($p>0.05$; **Figure 1B**).

Jet-lagged hamsters lack the temporal deactivation of the RFRP system during the LH surge compared to non-jet-lagged controls. The RFRP system, implicated in the negative

feedback arm controlling the reproductive axis, exhibits decreased activation during the end of the LH surge (-2 hours) compared to all other time points ($p<0.05$), with a subsequent increase in activation following the cessation of the surge (+2 hours; $p<0.05$ compared to all other times points within control condition). Similar to the abolishment of the circadian-mediated pattern in activation seen in both the GnRH and kisspeptin systems, the temporal pattern of activation in the RFRP system is eliminated, with an equivalent percentage of RFRP cells expressing FOS at all time points ($p>0.05$). The subsequent increase in RFRP expression following the onset of activity is eliminated in the jet-lagged animals, with a significant suppression of activation in the jet lag group compared to controls at +2 hrs ($p<0.05$; **Figure 1C**).

Discussion

The purpose of the present study was to investigate whether such deficits in reproduction may be a result of temporal dysregulation in the necessary SCN-controlled activation of the stimulatory arm of the reproductive axis and suppression of the inhibitory arm during the LH surge. Animals exposed to the bi-weekly phase advances in the light:dark cycle remain rhythmic with ~24 hour rhythms in activity, but their behavior is no long synchronized with the light:dark cycle (**see Chapter 3, Experiment 3**). Whereas in control animals, the GnRH and kisspeptin system exhibited timed increases in activation during the time of the LH surge and onset of activity period (-2 hours), the RFRP system subsequently was deactivated (**Figure 1A-C**). In contrast, around the time of the LH surge, the circadian-mediated increases in percentages of GnRH and kisspeptin cells expressing FOS and decreases in RFRP/FOS cells was abolished, with a tonic level of FOS expression maintained throughout the day (**Figure 1A-C**). These data suggest that one potential mechanism mediating the dysregulation of the reproductive axis in female shift workers may be a lack of coordination between activation and deactivation in the neuropeptide populations regulating the HPG axis.

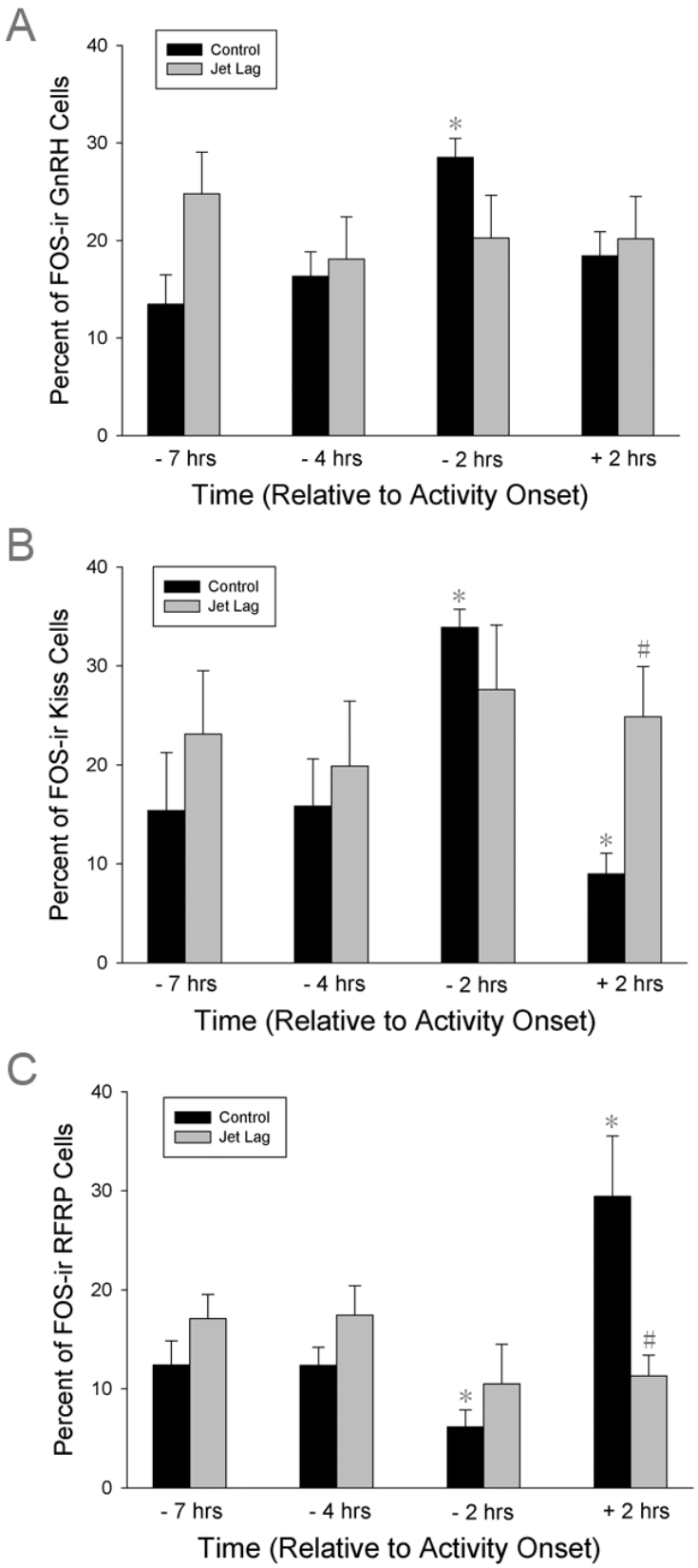
Precise coordination between the stimulatory GnRH and kisspeptin systems and inhibitory RFRP temporally control the onset of the LH surge and ovulation in Syrian hamsters. Disturbances to this timed-orchestration between these hypothalamic neuropeptides may play a role in the subsequent dysregulation seen in individuals with disruptions in circadian homeostasis. Whereas *Clock* mutants, that exhibit deficits in circadian function at the cellular and behavioral levels, have severe disabilities in maintaining the proper gonadotropin milieu to initiate LH surges and fecundity (Miller et al., 2004), more subtle alterations to the circadian system through behavioral shifts that result in an incongruence between endogenous rhythms and environmental cues also exhibit deficits in fertility (Ahlborg et al., 1996; Zhu et al., 2004).

Studies utilizing splitting, *in vitro* cell cultures (i.e. GT1-7 cell lines), and *in vivo* pharmacology as models of circadian control of reproduction point to a dual role for the SCN in the timing of ovulation by both supplying a positive drive to the GnRH system to initiate the LH surge while concomitantly removing the negative influence of the RFRP system (de la Iglesia et al., 2003; Gibson et al., 2008). Recently, the SCN has been shown to regulate the positive arm of the reproductive axis not only through the GnRH system, but also upstream through the kisspeptin system (Williams et al., 2011). In estradiol implanted ovariectomized ewes, upregulation of kisspeptin protein and mRNA in the ARC and downregulation of RFRP protein in the DMH during the breeding season were associated with increases in kisspeptin contacts on GnRH neurons and decreases in RFRP contacts (Smith et al., 2008b). Thus, the closely related ERα-

expressing RFamide peptides, kisspeptin and RFRP, appear to have opposing roles in the estrogen-dependent control of mammalian reproduction.

The circadian system projects to systems associated with positive control of the reproductive axis, including the GnRH system as well as to the AVPV and ERα rich areas in the anterior hypothalamus (de la Iglesia et al., 1995; Watson et al., 1995). SCN fibers also form close appositions to RFRP-ir cells in the DMH which negatively modulate ovulatory functions (Gibson et al., 2008). Kisspeptin-ir cells in the AVPV are activated at the time of the LH surge, along with GnRH neurons, while RFRP cells exhibit reduced activity (Irwig et al., 2004; Kauffman et al., 2007) (Gibson et al., 2008). RFRP-ir fibers contact GnRH cell bodies and may or may not project to the median eminence, indicating that RFRP control of the reproduction occurs at the level of GnRH and possibly the pituitary (Kriegsfeld et al., 2006; Murakami et al., 2008; Rizwan et al., 2009). Kisspeptin-ir cell bodies also project to GnRH neurons that express GPR54, the receptor for the kisspeptin ligand (Han et al., 2005; Clarkson and Herbison, 2006; Smith et al., 2008b). Thus, during most of the estrous cycle RFRP cells respond to estradiol by inhibiting the HPG axis. At the time of ovulation, the SCN signals the RFRP system to ignore the estradiol signal, releasing the GnRH system from inhibition. Concomitantly, the circadian system signals the GnRH system directly or indirectly through the kisspeptin system in the AVPV, to respond to the increased levels of estradiol and initiate the LH surge. Together, the two neuropeptides, kisspeptin and RFRP, work in controlled opposition to finely regulate the activity of the GnRH system and synthesis and secretion of the gonadotropins. These new additions to the reproductive axis repertoire of peptides provide novel avenues of research into the timing of reproduction.

The present findings suggest that circadian control over the HPG axis is imperative to reproductive success. The means by which these disturbances in the orchestration among the GnRH, kisspeptin, and RFRP systems during the LH surge and ovulation impact fertility are an interesting area for further enquiry. Future studies in which the LH profile of animals exposed to chronic jet lag is precisely monitored with high temporal resolution will be an important first step in identifying any abnormalities in the surge profile or its coordination with sexual behavior and motivation. The coincidence of the LH surge with rodent female sexual behavior is a result of the evolutionary co-opting of the same hormones that mediate the induction of the LH surge, estrogen and progesterone, with the onset of estrous behaviors. Finally, previous findings suggest that circadian disruptions on the molecular level negatively impact pregnancy (Miller et al., 2004), indicating that more mild disturbances to the circadian system, as those seen in phase shifts, may have similar impact requiring further investigation.


A
Control
Jet Lag
Percent of FOS-ir GnRH Cells
40
30
20
10
0
*
- 7 hrs
- 4 hrs
- 2 hrs
+ 2 hrs
Time (Relative to Activity Onset)
B
Control
Jet Lag
Percent of FOS-ir Kiss Cells
40
30
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- 7 hrs
- 4 hrs
- 2 hrs
+ 2 hrs
Time (Relative to Activity Onset)
C
Control
Jet Lag
Percent of FOS-ir RFRP Cells
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- 7 hrs
- 4 hrs
- 2 hrs
+ 2 hrs
Time (Relative to Activity Onset)

Figure 1. **Circadian Disruptions Abolish the Temporal Pattern in Activation of the GnRH, Kisspeptin, and RFRP Systems.** In control animals, GnRH (A) and kisspeptin (B) cells exhibit increased FOS expression at the time of the LH surge (-2 hrs), with subsequent decreases in activation following the surge (+2 hrs). Jet lag abolishes this rhythm in both neuropeptide populations (A,B), with tonic levels of FOS expression at all time points, including a maintenance of high expression of kisspeptin/FOS following the cessation of the surge. (C) Simultaneous to the increased activation of the GnRH and kisspeptin neuron populations during the surge, control animals exhibit a deactivation of RFRP cells. In jet-lagged hamsters this temporal decrease of FOS expression in RFRP cells during the LH surge is eliminated. * = significantly different from all other time points within condition, $p<0.05$; # = significantly different from other condition within same time point, $p<0.05$.

Chapter 3: The Effects of Temporal Disruption on Hippocampal Neurogenesis and Learning and Memory

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Experiment 3: Experimental 'Jet Lag' Inhibits Adult Neurogenesis and Produces Long-Term Cognitive Deficits in Female Hamsters

Abstract

Circadian disruptions through frequent transmeridian travel, rotating shift work, and poor sleep hygiene are associated with an array of physical and mental health maladies, including marked deficits in human cognitive function. Despite anecdotal and correlational reports suggesting a negative impact of circadian disruptions on brain function, this possibility has not been experimentally examined. In the present study, we investigated whether experimental 'jet lag' (i.e., phase advances of the light:dark cycle) negatively impacts learning and memory and whether any deficits observed are associated with reductions in hippocampal cell proliferation and neurogenesis. Because insults to circadian timing alter circulating glucocorticoid and sex steroid concentrations, both of which influence neurogenesis and learning/memory, we assessed the contribution of these endocrine factors to any observed alterations. Circadian disruption resulted in pronounced deficits in learning and memory paralleled by marked reductions in hippocampal cell proliferation and neurogenesis. Significantly, deficits in hippocampal-dependent learning and memory were not only seen during the period of the circadian disruption, but also persisted well after the cessation of jet lag, suggesting long-lasting negative consequences on brain function. Together, these findings support the view that circadian disruptions suppress hippocampal neurogenesis via a glucocorticoid-independent mechanism, imposing pronounced and persistent impairments on learning and memory.

Introduction

Frequent transmeridian travel, rotating shift work schedules, and irregular sleep patterns result in an incongruence between the endogenous circadian timing system and the external environment (Reddy et al., 2002; Maywood et al., 2006; Wittmann et al., 2006; Davidson et al., 2009). This loss of synchrony is associated with a number of clinical pathologies, including a higher incidence of cancer (Hansen, 2006; Conlon et al., 2007), diabetes (Poole et al., 1992), hypertension and cardiovascular disease (Ha and Park, 2005; Kivimaki et al., 2006), reduced fertility and fecundity (Ahlborg et al., 1996; Zhu et al., 2004), and an exacerbation in a number of pre-existing psychological pathologies (Skipper et al., 1990; Bildt and Michelsen, 2002) relative to individuals with consistent schedules. Most relevant to the present series of studies, several lines of investigation using human and animal models suggest a pronounced influence of circadian timekeeping on learning and memory (Tapp and Holloway, 1981; Cho et al., 2000; Cho, 2001; Ralph et al., 2002).

In mammals, the master circadian pacemaker is located in the suprachiasmatic nucleus (SCN) in the anterior hypothalamus (Moore and Eichler, 1972; Stephan and Zucker, 1972). The SCN generates endogenous oscillations with a period of approximately, but not precisely, 24 hours,

resulting in a desynchrony between internal and environmental time in the absence of an external synchronizing cue. This desynchrony is prevented through entrainment, with light being the primary zeitgeber (time giver; ZT) in mammals (Dibner et al., 2010). At the cellular level, circadian rhythms are generated by 24-hour autoregulatory transcriptional/translational feedback loops consisting of 'clock' genes and their protein products (Reppert and Weaver, 2002; Van Gelder et al., 2003; Maywood et al., 2007; Chen et al., 2009). Importantly, clock gene expression is ubiquitous and allows the CNS and periphery to exhibit system-specific rhythms in daily activity, a necessity for optimal health and functioning.

Several correlational studies suggest an association between circadian disruptions and impaired cognitive function in humans (Maywood et al., 2006). For example, learning and memory deficits and reduced temporal lobe volume are observed in chronically jet-lagged female flight attendants relative to controls (Cho et al., 2000; Cho, 2001). These cognitive deficits are associated with elevated circulating cortisol concentrations relative to flight attendants permitted recovery following transmeridian travel (Cho et al., 2000). However, in order to establish a cause-effect relationship between alterations in circadian timing and learning and memory deficits, experimental studies in which circadian perturbations are controlled and applied to a homogenous population are required.

In addition to the effects of circadian timing, numerous lines of evidence point to a strong association between neurogenesis and learning and memory, suggesting that new cell birth/maturation might be negatively affected by disruption of daily rhythms. For example, newly born hippocampal cells markedly increase following a hippocampus-dependent learning task (Gould et al., 1999b; Ambrogini et al., 2000; Leuner et al., 2006). Importantly, learning tasks that are hippocampus-*independent* do not result in increased dentate gyrus neurogenesis (Gould et al., 1999b; Van der Borght et al., 2005). More recent studies using antimitoic and DNA alkylating agents, irradiation, targeted viral vector, and genetic approaches to more specifically disrupt neurogenesis provide further support for the importance of new neuron proliferation/maturation in learning and memory (Shors et al., 2001; Madsen et al., 2003a; Madsen et al., 2003b; Clelland et al., 2009).

Given the impact of circadian perturbations on the stress and reproductive axes (Ahlborg et al., 1996; Zhu et al., 2004), and established effects of glucocorticoids and estrogen on hippocampal cell proliferation/ neurogenesis (Mirescu and Gould, 2006; Galea, 2008) and learning and memory (Daniel et al., 1997; Gould and Tanapat, 1999; Shors, 2006; Galea et al., 2008; Dagyte et al., 2009), it is possible that disruptions in circadian timing negatively impact cognitive function through glucocorticoid- and/or ovarian hormone-dependent changes in neurogenesis. Alternatively, circadian disruption may impact brain function more directly, as mice lacking one of the core clock genes, *Period2,* that drives circadian rhythms at the cellular level, exhibit alterations in neural/progenitor cell proliferation in the hippocampus (Borgs et al., 2009b). This finding suggests that the state of the circadian system may directly affect the cell cycle and cell proliferation (Borgs et al., 2009a).

In the present series of studies, we sought to establish whether or not disruptions in circadian timing impact learning and memory. Additionally, given the association between adult neurogenesis and learning and memory, we examined the possibility that hippocampal cell

proliferation and neurogenesis are affected by disturbances in circadian timing. We examined dentate gyrus cell proliferation and neurogenesis in female Syrian hamsters exposed to 4 weeks of twice weekly phase advances in the LD cycle (i.e., a 6-hr experimental 'jet lag'). Similar manipulations have been previously used to assess the impact of experimental jet lag on mortality (Davidson et al., 2006) and tumor progression (Filipski et al., 2004). We intentionally chose repeated phase advances for this initial characterization because these manipulations require significantly more time for behavioral and physiological re-entrainment than phase delays (Takamure et al., 1991; Yamazaki et al., 2000; Reddy et al., 2002; Davidson et al., 2006; Loh et al., 2010). Likewise, these behavioral manipulations allow for the study of circadian disruption on variables of interest without invasive surgical manipulations or global disruption of the molecular circadian clockwork. The relative contribution of alterations in the stress and reproductive axes to any observed deficits were controlled through adrenalectomy or ovariectomy and hormone replacement (corticosterone or estrogen, respectively). Hippocampal-dependent memory was assessed using a conditioned place preference (CPP) paradigm during the time of circadian perturbations, and well after the cessation of jet lag, to explore whether or not any impact on cognitive functioning persists following re-entrainment.

Materials and Methods

Animals. Adult (>60 days of age) female LVG hamsters (*Mesocricetus auratus*; Charles River, Wilmington, MA) were maintained on a 14:10 light:dark (LD) cycle (lights on at 0700 h) prior to the onset of all experiments, with a light intensity ranging from 100-300 lux at the level of each cage. All animals were maintained in a colony room at 23 ± 1°C and provided with *ad libitum* access to water and food. Estrous cyclicity was monitored for all animals by daily inspection for preovulatory vaginal discharge (Orsini, 1961). Only animals with regular, 4-day estrous cycles were used in the experiments. The first cohort of hamsters either remained intact or was ovariectomized or adrenalectomized to assess the influence of estrogen and glucocorticoids on cell proliferation and neurogenesis (n=27). All surgeries were conducted under isoflurane anesthesia. Ovariectomized hamsters received a SILASTIC brand capsule (Downing Corning Corp., Midland, MI; 10 mm length, 1.45 inner diameter, 1.93 out diameter) containing powdered 17-β estradiol (OVX + E_2). These capsules result in proestrous concentrations of plasma estradiol (Meyer-Bernstein et al., 1999). Adrenalectomized hamsters were given a solution of 0.9% saline, 5% sucrose and corticosterone (25 μg per ml of 0.9% saline; Sigma) to mimic basal glucocorticoid concentrations and maintain electrolytes (ADX). This treatment results in basal levels of corticosterone, the dominant hamster glucocorticoid in non-stressed animals (Ottenweller et al., 1985; Meyer-Bernstein et al., 1999; Mirescu et al., 2004). Two weeks after surgery, hamsters were placed into their respective lighting conditions. A second cohort was used to investigate the impact of jet lag on learning and memory (n=20). The final cohort of hamsters was used to assess the impact of jet lag on behavior and the stress axis (n = 14).

Jet Lag and Hippocampal Cell Proliferation/Neurogenesis. Hamsters either remained intact or were adrenalectomized and provided with basal corticosterone concentrations or ovariectomized and provided with proestrous estradiol concentrations. Half (n=4-5/group) of the animals from each condition were exposed to a 6-hr phase advance every 3 days for 25 days (Jet Lag) while the other half remained in a 14:10 LD (lights on at 0700 hr) cycle for the same duration as jet-lagged animals (Control). All animals were injected with the thymidine analog,

bromodeoxyuridine (BrdU), to label the dividing cell population. BrdU (50 mg/kg body weight; Sigma) was injected intraperitoneally (i.p.) 7 hours after lights on, one day after every second phase advance (i.e., every 6 days) for the jet lag condition or at the same time and day for control hamsters (**Figures S1 and S2**). Multiple injections of BrdU were used to estimate the total population of newly-generated cells throughout the 25-day temporal disruption, as well as to maximize the number of cells surviving until differentiation (Kempermann et al., 1997; Glenn et al., 2007).

Hamsters were then anesthetized with sodium pentobarbital (200 mg/kg) and perfused transcardially with 150 ml of 0.9% saline followed by 300 ml of 4% paraformaldehyde in 0.1M PBS (pH 7.4) 24 hours after the last BrdU injection. Brains were postfixed in 4% paraformaldehyde for 3 hours at 4°C and cryoprotected in 30% sucrose in 0.1M PBS overnight (**Figure S2)**.

Histological Procedures, Microscopy, and Quantification. Brains were sectioned in the coronal plane at 40 μm thickness using a cryostat (Leica, CM3050-S, Leica Microsystems Inc., Bannockburn, IL). For BrdU immunofluorescence, sections were rinsed in 0.4% Triton X-100 (PBT) followed by 10 min in 0.9% saline. Sections were then denatured in 2M HCl for 30 min at 37°C, rinsed in PBT, and incubated in normal donkey serum (1:50; Jackson ImmunoResearch) in PBT for 1 hr. Sections were then co-incubated for 48 hr at 4°C in rat anti-BrdU (1:1000; Accurate Chemical), guinea pig anti-glial fibrillary acidic protein (GFAP) (1:1000; Advanced Immunochemical), and mouse anti-neuronal nuclei protein (NeuN) (1:1000; Chemicon). Following incubation in the primary antibodies, sections were rinsed in PBT and incubated in the dark for 1 hr with DAPI which binds strongly to DNA and labels cellular nuclei (1:1000: Sigma), CY2 donkey anti-rat (1:500; Jackson ImmunoResearch), CY5 donkey anti-guinea pig (1:500; Jackson ImmunoResearch), and CY3 donkey anti-mouse (1:500; Jackson ImmunoResearch) to visualize BrdU, GFAP, and NeuN, respectively.

GFAP was used to assess gliogenesis while NeuN, a vertebrate nervous system nuclear protein ubiquitous in the CNS, was used to label mature neurons. This same protocol was followed for proliferating cell nuclear antigen (PCNA), a co-factor for DNA polymerase and a convenient endogenous marker for newly proliferated cells, immunofluorescence using mouse anti-PCNA (1:4000; Santa Cruz) as the primary antibody. PCNA was visualized with CY3 donkey anti-mouse (1:500; Jackson ImmunoResearch). Sections were then rinsed, mounted on gelatin-coated slides, dehydrated with a graded series of alcohols, and coverslips were applied.

All cell counting was performed by individuals blind to the experimental conditions. All sections were counted using a Zeiss Z1 microscope (Carl Zeiss, Thornwood, NY) at 400× using the standard wavelengths for FITC (485 nm), CY3 (546 nm), CY5 (640 nm), and DAPI (359 nm). For BrdU-positive cells or PCNA-positive cells, every 12^{th} unilateral section throughout the extent of the dentate gyrus (including the subgranule zone, the granule zone and the hilus) was counted, excluding those cells in the outermost field of focus. Volume reconstruction was conducted by multiplying the number of BrdU-positive or PCNA-positive cells per dentate gyrus by 24 to estimate the total number of labeled cells per brain (Mirescu et al., 2004). The volume of the analyzed region was determined using Cavalieri's principle with NIH ImageJ software (Gundersen and Jensen, 1987). 25 BrdU-labeled cells from at least 4 sections/animal were

randomly chosen and assessed for double-labeling with NeuN or GFAP (Epp et al., 2007). Images were digitally captured at 400× in 8-bit greyscale using a cooled CCD camera (Zeiss). At least 20% of those BrdU-labeled cells assessed for double-labeling were analyzed in confocal scans to ensure that counts using conventional microscopy did not result in false positives. In all cases, those cells identified as double-labeled at the conventional microscopy level were identified as double-labeled in the confocal microscopy analysis.

Assessment of Learning and Memory: Conditioned Place Preference. Intact, adult, female LVG hamsters (4-5 weeks of age) were used to assess hippocampal-dependent learning and memory using the conditioned place preference (CPP) paradigm (n = 20), considered an ideal hippocampal-dependent memory test in Syrian hamsters. Hamsters do not perform well on other established hippocampal-dependent memory tests, including the Morris water maze or the Olton radial arm maze (Ferbinteanu and McDonald, 2001; Ralph et al., 2002). In the CPP paradigm animals learn an association between a specific context and a rewarding stimulus (a running wheel in the present case) (Ralph et al., 2002; Cain et al., 2004). Learning is indicated by an increase in dwell time - the total time spent in the context previously paired with the rewarding stimulus compared to the non-rewarded. The animal was considered to enter a chamber when both forepaws were within the chamber. Estrous cyclicity was monitored daily for 2 wks prior to testing to ensure that pre-testing occurred on the same day of the estrous cycle for all hamsters to control for estrogenic effects on learning and memory (Frick and Berger-Sweeney, 2001). Stainless steel wheels, 17.5 cm diameter, were placed in the home cages of all hamsters 2 wks prior to onset of the testing to acclimate the animals to wheel running. All testing occurred in a dark room illuminated by dim red light to encourage exploration, particularly of the white compartment.

The CPP apparatus included two boxes (60 × 45 × 40 cm), one white and one black, connected by a clear pathway (30 × 25 × 18 cm). Sliding partitions that matched the color of the compartments were used to isolate animals in one of the chambers during training. To further distinguish the boxes, a unique odor was placed into a wall-mounted plastic container matching the color of each chamber. Before each session a cotton ball saturated with either 0.5% isoamyl acetate or eucalyptus oil was added to each of the chambers. Each box was associated with one of the odors for the duration of the experiment. Between tests the compartments were cleaned with 70% ETOH. Tests for context preferences were determined by recording the total amount of dwell time in each context. The CPP consisted of three phases: Pretest, Training and Probe trials.

Pretests commenced during estrus with Probe trials occurring on diestrus. Hamsters were exposed to the CPP apparatus at the same time of day for the entire behavioral protocol, with all Pretest, Training, and Probe trials occurring within 4 hrs of lights off. Because Syrian hamsters show a place preference for a reward-paired context only when the training and probe trials occur at the same time of day (ZT) or the same circadian time (CT) (Ralph et al., 2002), we elected to train hamsters at the same ZT (i.e., within 4 hrs of lights off). Pretest 1 occurred on Days 13 through 16 of the jet lag paradigm, depending on the estrous state of the animals. Probe 1 occurred on Days 21 through 25 **(Figure S2)**. After Probe 1, jet-lagged animals were returned to a static light:dark cycle (14:10 LD). Pretest 2 was conducted after *all* hamsters were maintained in a *static* LD cycle for 28 days to determine if jet lag had lingering effects on

learning and memory long after cessation of the temporal disruption. Control animals were housed in 14:10 LD for the duration of the experiment. Specific procedural details are below:

CPP test 1. *Pretest 1* - Animals were placed into the clear center partition and allowed to explore the entire apparatus for 10 min. Videos were scored to determine the total amount of time spent in each compartment. If the hamster exhibited a preference for one of the boxes (white or black), the wheel was assigned to the box opposite their preference to remove the possibility of bias for a particular box for each individual animal. Video recordings were also examined to calculate the total amount of time the animals were active or ambulating to ensure that control and jet-lagged hamsters were equally motivated to explore the apparatus. *Initial Training* – Hamsters were trained for 25 min/day with the wheel placed in the compartment assigned to each animal based on Pretest preferences. Each animal received 4 training sessions in which it was confined to the box containing the wheel and 4 training sessions in which it was confined to the box without the wheel (alternating days). *Probe 1* – On the test day, the wheel was not present in the apparatus. Hamsters were tested to determine if they retained a memory for the chamber paired with the wheel by placing the hamsters into the clear center partition and permitting them to freely explore the entire apparatus for 10 min. Hamsters were videotaped and the total amount of time spent in either the black or white compartment was recorded as in Pretest 1.

CPP test 2. *Pretest 2* – One month after all hamsters were placed into a static LD cycle, they were tested to determine if they maintained a memory for the previous learning task. Hamsters were placed into the center partition and permitted to freely explore the apparatus for 10 min. During this test, no wheel was present to assess whether animals recalled the location of the wheel during CPP Test 1. Videos were assessed and the amount of time spent in each chamber was recorded as in Pretest 1. *Reversal Training* – For this training experience, the wheel was now placed into the opposite chamber from that which each individual hamster experienced during the first training session. All other training conditions were implemented as in the Initial CPP Test 1 Training Session. *Reversal Probe* – To determine if hamsters learned the new location of the wheel, a probe trial was conducted in which the wheel was removed. As in Probe 1, hamsters were released into the apparatus and allowed to freely explore for 10 min. Video recordings were analyzed and the total amount of time spent in each compartment was recorded as in CPP Test 1, Probe 1.

Jet Lag Treatment, Behavioral Monitoring, and Hypothalamo-Pituitary Adrenal (HPA) Axis Activation. Hamsters were either exposed to the jet lag condition (n = 7) or to a fixed LD cycle (n = 7). Locomotor behavior was monitored for all animals using an infrared monitoring system (Data Sciences; St. Paul, MN) mounted to the wire lids on each cage. All movement in the cage was detected by interruptions in the infrared beam and relayed to a computer. Cumulative counts were recorded every 10 min and analyzed using Dataquest 3 software (Data Sciences; St. Paul, MN). The power of all rhythms was assessed using Fourier analysis (Clocklab) in which an animal was considered rhythmic when its highest peak occurred approximately 1 cycle/day. Clocklab software was also used to determine the nocturnality index and *alpha* for the jet-lagged animals prior to the onset of the phase advancements (fixed LD), as well as during the jet lag paradigm on days 2-4, 15-17, and 21-23. The nocturnality index is the ratio of the time active during the dark phase compared to the total time active over 24 hrs. Animals that are more active during the dark phase will have a higher nocturnality index. *Alpha*

is defined as the difference between activity onset and activity offset. Activity onset was defined as the first bout of sustained activity after a period of 2 hrs with less than 20 min of activity. Activity offset was defined as the final bout of activity before a period of 2 hrs with less than 20 min of activity. In addition to monitoring the activity rhythms of the hamsters, cortisol concentrations were assessed throughout the 25-day jet lag schedule from blood samples collected through the retroorbital sinus. Animals were anesthetized using isoflurane, and blood samples were collected 7 hrs into the rest phase based on their activity profile for all hamsters on days 2, 8, 15, and 25 of the jet lag paradigm **(Figure S2)**. Cortisol was measured in 25 µl aliquots of serum using RIA kits from ICN Biomedicals, Inc., Diagnostic Division (Costa Mesa, CA). The cortisol assay has been validated previously for use in Siberian hamsters (Anand et al., 2002). The intraassay coefficient of variation for cortisol was 1.39%, and the interassay coefficient of variance was 12.5%. The minimum detectable cortisol concentration was 0.25 µg/dl (Anand et al., 2004).

Statistics. Group mean differences in cell counts were analyzed using analyses of variance (ANOVA) with Tukey or Tukey-Kramer post hoc tests to examine pairwise differences. Cortisol data were analyzed using a two-way repeated measures ANOVA while activity data were analyzed using a one-way repeated measures ANOVA. Student's *t*-tests were performed to assess chamber preference, total duration of time active, and learning and memory for the paired context in the Conditioned Place Paradigm. A Levene test for homogeneity of variance was performed to assess differences in variability between groups during Probe 1. A Pearson Product Moment Correlation was used to assess the correlation between amount of light exposure prior to blood sampling and cortisol concentrations. Findings were considered significant when $P < 0.05$.

Results

Jet Lag Decreases Hippocampal Cell Proliferation and Neurogenesis. In intact hamsters, jet lag markedly suppressed cell proliferation, reducing the number of cells by approximately 50% ($p = 0.007$; **Figure 1A**). Because jet lag and shift work are associated with elevated cortisol (Cho, 2001), and disruptions of the reproductive axis in human populations (Ahlborg et al., 1996), and these alterations may influence cell proliferation and neurogenesis (Cameron and Gould, 1994; Daniel et al., 1997; Barker and Galea, 2008), we compared the effects of jet lag in intact and adrenalectomized hamsters given low, basal corticosterone replacement (Ottenweller et al., 1985; Tanapat et al., 2001) and ovariectomized females administered proestrous levels of estradiol (OVX + E_2). The effect of jet lag on cell proliferation was abolished in adrenalectomized hamsters treated with corticosterone ($p = 0.80$), suggesting that jet lag suppressed cell proliferation through activation of the HPA axis. As expected, ovariectomy and estrogen replacement increased the number of PCNA-labeled cells ($p < 0.05$) (Barker and Galea, 2008). However, jet lag decreased cell proliferation by the same magnitude as observed in intact animals ($P < 0.05$). Together, these findings reveal a pronounced effect of jet lag on hippocampal cell proliferation, likely mediated by the HPA axis.

The consequences of jet lag on hippocampal cell survival and maturation were examined by quantifying BrdU expression in combination with NeuN to assess neurogenesis (**Figure 1B-F**) and GFAP to assess gliogenesis (**Figure 2 and Table 1**). In all three conditions, jet lag reduced neurogenesis by >50% (**Figure 1B-F**). The magnitude of the suppression was not affected by adrenalectomy/ovariectomy and hormone replacement ($p > 0.05$ in both cases). The same pattern

of results was detected in the total number of BrdU-labeled cells (NeuN-positive *and* NeuN-negative) ($F_{1, 21}$ = 18.094, $p < 0.001$; data not shown). Jet lag-induced suppression in neurogenesis did not impact the total volume of the granule cell layer (**Table 1**; $F_{1, 21}$ = 0.0126, $p = 0.91$). Thus, as with cell proliferation, jet lag negatively impacted neurogenesis, although this effect *was not* mediated by glucocorticoids, indicating that the jet lag-induced decrease in neurogenesis is independent of the 'stress' associated with experimental jet lag. This finding is not surprising as stress can differentially affect cell proliferation and survival (Lee et al., 2006). There was no effect of jet lag ($F_{1, 21} = 0.136$, $p = 0.72$) or hormone condition ($F_{2, 21}$ = 0.318, $p = 0.73$) on gliogenesis and less than 3% of BrdU-labeled cells were glia (2.2 ± 0.89%) (**Figure 2 and Table 1**).

Jet Lag Results in Long-Term Deficits in Hippocampal-dependent Learning and Memory. Because reductions in neurogenesis are associated with deficits in learning and memory(Shors et al., 2001; Clelland et al., 2009; Jessberger et al., 2009), we assessed the potential impact of jet lag-induced changes in neurogenesis on hippocampal-dependent memory. Using a conditioned place preference (CPP) paradigm (Ferbinteanu and McDonald, 2001; Ralph et al., 2002) in intact animals, the first learning and memory test commenced during the final 10 days of jet lag. The total duration of time the animals were actively exploring the apparatus during Pretest 1 did not differ between control (527.3 ± 21.48 sec) and jet-lagged hamsters (541.8 ± 18.71 sec)(t_{18} = 0.509; $p = 0.617$), suggesting that any differences observed are not due to differences in alertness or motivation to explore. As expected, when initially exposed to the apparatus, control animals preferred the black compartment (Pretest 1; $t_{18} = 4.193$, $p < 0.001$), whereas jet-lagged animals exhibited no preference ($t_{18} = 0.805$, $p = 0.43$)(**Figure 3A**). If any animal exhibited a significant preference for one compartment during the Pretest, the running wheel was placed into the non-preferred compartment during the training sessions. To assess learning and memory, a probe trial was conducted in which the wheel was removed from the apparatus and the total time hamsters explored each compartment was recorded. The control animals developed a clear preference for the chamber previously containing the wheel, dwelling approximately 3 times longer in this chamber (Probe 1; $t_{16} = 4.620$, $p < 0.001$; **Figure 3B**). Despite identical training, jet-lagged animals were unable to perform the task, spending equal amounts of time in both chambers during the Probe trial ($t_{16} = 0.673$, $p = 0.51$). To examine the possibility that variable incongruence between CT and ZT in jet-lagged animals contributed to the deficits observed, we conducted a Levene test to determine if the variance differed between jet-lagged and control animals. Had the disparity between ZT (time of testing) and CT (time of activity) contributed to the deficits observed, then the variance should be greater in the jet-lagged group. The Levene test confirms that the variance did not differ between control and jet lag animals during Probe 1 ($P = 0.903$), suggesting that the disparity between ZT and CT did not contribute to the deficits uncovered.

It was anticipated that performance would be impaired in the midst of chronic temporal disruption, when rhythms in internal physiology and brain function are incongruous with external time. Given the suppressive actions of jet lag on hippocampal neurogenesis, we assessed whether the negative consequences of repeated circadian disruptions persist long after hamsters have re-synchronized their circadian rhythms to environmental time. Because hamsters recover from a 6-hr phase advance in approximately one week (Golombek and Cardinali, 1993; Davidson et al., 2009), we retested the same hamsters used in the first CPP test 4 weeks after the

cessation of the jet lag treatment to ensure that the animals were re-synchronized to the fixed LD cycle as activity monitoring was not logistically possible during this phase of testing. In Pretest 2, animals were placed into the empty apparatus to determine if they exhibited a preference for either chamber. The retention of the previous training would be reflected if hamsters exhibited a preference for the compartment that previously contained the activity wheel. Control hamsters showed a significant preference for the previously paired chamber (t_{18} = 2.2664, p = 0.02), whereas those that had been jet-lagged continued to show no preference (t_{18} = 1.113, p = 0.28; **Figure 3C**). This was expected, as the jet-lagged animals did not acquire the task during the first CPP session (**Figure 3B**). For the Reversal Experiment, the wheel was placed into the chamber opposite to that initially trained for each hamster during the first CPP test. By placing the wheel in the chamber opposite to the initial training, animals were required to acquire a new preference. Because control hamsters had acquired a preference in the initial CPP test, learning the association between the new chamber and wheel required that the animals override the previous memory. In contrast, because the jet-lagged hamsters did not acquire the initial preference, they might more readily acquire the new preference (Anderson, 2003). After training, control animals spent the majority of their time in the newly-trained chamber (t_{12} = 2.692, p = 0.02), but the previously jet-lagged hamsters did not develop a preference for either chamber (t_{14} = 1.532, p = 0.15; **Figure 3D**). This finding suggests that repeated phase advances negatively impact learning and memory well past the point of readjustment to the current time photoperiod.

Repeated Jet Lag Transiently Activates the Stress Axis. The impact of jet lag on *cell proliferation* was abolished by adrenalectomy and basal corticosterone replacement, suggesting that activation of the HPA axis may contribute to the impact of jet lag on this measure. To examine this possibility, a separate group of hamsters were exposed to the jet lag paradigm (n = 7) or to a fixed LD cycle (n = 7) and blood samples were collected throughout the treatment. There were no significant differences in glucocorticoid concentrations between control and jet-lagged animals on the day after the first 6-hr phase advance (Day 2; p = 0.13), with both groups exhibiting cortisol concentrations equivalent to non-stressed Syrian hamsters (Jasnow et al., 2001). However, at subsequent time points, glucocorticoid concentrations of jet-lagged hamsters were higher than those of control animals ($F_{1,36}$ = 19.786, p < 0.001; **Figure 4A**), with highest concentrations on Day 8 (Jasnow et al., 2001). The increase in cortisol concentrations was attenuated during the second half of the phase advance treatment, with cortisol concentrations at the final time point significantly reduced from initial measurements (p = 0.03; **Figure 4A**), indicating that hamsters habituate to the repeated temporal adjustments. The duration of light exposure prior to sampling was not correlated with cortisol concentrations (R^2 = 0.00000185, p = 0.995), suggesting that light exposure did not impact the cortisol measures differentially in jet-lagged and control animals.

Jet Lag Leads to a Desynchrony Between Internal and External Time. Because a functional circadian system is critical for normal hippocampal memory (Ruby et al., 2008), we monitored the state of the circadian system throughout the jet lag treatment using an infrared monitoring system. In contrast to studies using *acute* phase adjustments, where animals re-synchronize their rhythms to the adjusted LD cycle, jet-lagged animals 'ignored' phase alterations in environmental time and exhibited non-entrained, circadian (~24-hr) rhythms (**Figure 4B-E**). This finding suggests that deficits observed following repeated temporal disruptions result from desynchrony between internal and external time, rather than the absence of circadian

organization. Had the jet lag treatment disrupted circadian functioning, locomotor behavior would have either become arrhythmic, exhibited a change in rhythm amplitude, or shown a rhythm period outside the normal circadian range.

Jet-lagged animals exhibited equivalent durations of activity (*alpha*) throughout the phase-advance treatment compared to activity while housed in a fixed LD cycle (**Table 2**; $F_{3,18} = 1.559$, $p = 0.234$). During fixed LD cycle and days 21-23 of the jet lag paradigm, the majority of activity was confined to the dark phase. In contrast, jet-lagged animals were significantly less active during the dark phase following the first phase-advancement (days 2-4; $p < 0.001$) and mid-way through the jet lag treatment (days 15-17; $p < 0.001$) compared to the fixed LD schedule (**Table 2**). This latter finding further indicates that jet-lagged animals did not entrain their activity to the LD cycle.

Discussion

The present findings show, for the first time, that circadian disruptions lead to marked suppression of hippocampal cell proliferation and neurogenesis, associated with notable deficits in learning and memory. Adrenalectomy abolished the effects of jet lag on cell proliferation, suggesting that circadian disruptions impact this measure, in part, via HPA axis activation. In contrast, the pronounced suppression of neurogenesis is independent of jet lag-induced alterations in circulating glucocorticoid and sex steroid concentrations. Jet-lagged animals exhibited ~24-hr rhythms, not synchronized with external time, suggesting that the observed deficits result from a desynchrony between internal physiology and external time, not from gross disruptions in internal rhythmicity. Additionally, the duration and amplitude of the activity/rest cycle was not impacted by the treatment, suggesting that the results are not a consequence of sleep deprivation as has been shown previously (Mirescu et al., 2006; Mueller et al., 2008). Together, these results underscore the importance of circadian entrainment in maintaining optimal neural and cognitive functioning.

As indicated previously, the circadian system is an organized hierarchy with a master circadian pacemaker, the SCN, coordinating the timing of thousands of subordinate oscillators in the central nervous system (CNS) and periphery (Maywood et al., 2006; Dibner et al., 2010). Neural precursor cells (NPCs) from the dentate gyrus express circadian clock genes and disruption of the cellular clock results in abnormal NPC division and maturation (Borgs et al., 2009b). Whereas most studies supporting a link between the circadian system and regulation of the cell cycle involve genetically disrupting the circadian clock, acute global temporal disruptions (e.g., jet lag) dysregulate the core clock mechanism without permanently altering molecular pathways (Davidson et al., 2009). As a result, we elected to use this model to explore the impact of circadian disruption without genetically altering the core molecular circadian clockwork. Whereas the present studies used phase advances to determine whether or not disrupting circadian organization impacted hippocampal physiology and function because this behavioral manipulation results in maximal circadian desynchrony, future studies should take into account alternating phase advances and phase delays in circadian rhythms to more accurately mimic the shift work and jet lag schedules experienced by human populations. Importantly, the results indicate that cell proliferation and neurogenesis can be suppressed by these temporal changes without genetic modifications of the circadian system, indicating that this phenomenon is worthy of further exploration.

In the present experiments, cortisol concentrations were elevated in jet-lagged hamsters throughout the course of the treatment, with recovery seen near the conclusion of the phase shifting (**Figure 4A**). On Day 8 of the jet lag paradigm, jet lag hamsters exhibited cortisol concentrations comparable to stress-induced values in this species (Jasnow et al., 2001). On subsequent days, cortisol concentrations in the jet-lagged hamsters were lower than those seen in stressed animals but greater than daily maximum values (Meyer-Bernstein et al., 1999). This finding is consistent with the association between jet lag and glucocorticoid concentrations seen in women (Cho et al., 2000; Cho, 2001). Because cortisol should be lowest during the rest phase and may be impacted by light exposure, we assessed whether the duration of light exposure prior to sampling correlated with cortisol measures. Control hamsters were consistently sampled 7 hrs after lights on while sampling of jet-lagged hamsters occurred at CT7 and was variable relative to the LD cycle. There was no relationship between the two variables, suggesting that the duration of light preceding blood sampling, and the variable light exposure relative to the active phase in jet-lagged hamsters, was unlikely to impact cortisol concentrations. Additionally, the fact that the phase relationship between sampling and light differed throughout the one-month examination in jet-lagged animals, yet the increase in cortisol was maintained relative to controls until Day 25, further suggests that light was unlikely to impact interpretation of these findings.

Whereas jet lag reduced cell proliferation in the dentate gyrus of intact animals, this effect was abolished when circulating glucocorticoids were controlled through adrenalectomy and glucocorticoid replacement, suggesting that the jet lag-induced reduction in cell proliferation is mediated via activation of the HPA axis (**Figure 1A**). Because estradiol increases cell proliferation (Barha et al., 2009), it was not surprising that estradiol treatment increased PCNA cell labeling (**Figure 1A**), but it is noteworthy that the magnitude of PCNA suppression by jet lag was maintained in these animals and identical to that observed in intact animals.

Although these findings suggest that jet lag-induced suppression of hippocampal cell *proliferation* is mediated, at least in part, by increased glucocorticoid concentrations, other variables may contribute to this phenomenon. Neurotrophic factors, including BDNF and NGF, have been implicated in cell proliferation and/or survival (Cameron et al., 1998; Frielingsdorf et al., 2007; Glenn et al., 2007). In one study of acute jet lag, a single, 8-hr phase shift increased BDNF levels in the hippocampus (Sei et al., 2003). Likewise, intracerebroventricular injections of NGF phase shift activity rhythms of Syrian hamsters (Pizzio et al., 2005) and increase survival of new cells (Frielingsdorf et al., 2007). Whereas both acute treatments increased these neurotrophic factors, it is possible that more chronic circadian disruptions suppress their expression. The extent to which different jet lag treatments impact neurotrophic factors has yet to be explored.

Unlike the impact of glucocorticoids on cell proliferation, circadian disruptions reduced neurogenesis by >50% in all groups, regardless of adrenal/glucocorticoid and ovary/estradiol status, indicating that the effect of jet lag on cell maturation is independent of increased HPA axis activity or alterations in gonadal steroids **(Figure 1B**). Reductions in the maturation of new neurons may reflect a decrease in production of new progenitor cells that differentiate into neurons, or a decrease in cell survival. Our data suggests that decreased hippocampal neurogenesis resulting from jet lag is a consequence of decreased cell survival, as adrenalectomy

abolishes the effects of jet lag on cell proliferation, whereas reductions in neurogenesis persist. These findings are consistent with the notion that circadian cellular timing can directly impact cell survival (Hunt and Sassone-Corsi, 2007). Indeed, cell cycle genes, including Wee-1, c-myc, and Cyclin-D1, are regulated in a circadian manner (Walisser and Bradfield, 2006), further suggesting that the regular timing of these genes contributes to normal cell functioning. As a result, disruption of proper circadian function may lead to alterations of the cell cycle, including modifications to cell survival and fate.

Reductions in newly-generated hippocampal neurons are associated with impairments in hippocampal-dependent learning and memory tasks (Shors et al., 2001). While it is difficult to provide a direct cause-effect relationship between neurogenesis and learning and memory, as mentioned previously, many studies point to an association between the production of new hippocampal neurons and hippocampal-dependent cognitive processes (Madsen et al., 2003a; Raber et al., 2004a; Raber et al., 2004b; Snyder et al., 2005; Clelland et al., 2009). Despite the fact that the inhibition of neurogenesis following a learning task consistently results in learning deficits, these findings must be interpreted cautiously as it is possible that the procedures used may not be restricted only to those cells born following a learning task or to hippocampal cell populations. However, these findings, combined with the fact that newly born hippocampal cells markedly increase following a hippocampal-dependent learning task (Gould et al., 1999b; Ambrogini et al., 2000; Leuner et al., 2006) while learning tasks that are hippocampus *independent* do not (Gould et al., 1999b; Van der Borght et al., 2005), provides strong evidence for a functional link between these two measures.

In the current study, when tested during the jet lag treatment, jet-lagged hamsters did not learn the conditioned preference task that control hamsters readily acquired (**Figure 3B**). Importantly, when trained one month following placement into a fixed LD cycle, jet-lagged animals were still unable to perform the CPP task (**Figure 3C and D**), suggesting that the impact of jet lag on learning and memory persists well after endogenous processes are re-synchronized to external time. Previous research indicates that both ZT and CT do not affect acquisition of the CPP as long as training and probe trials occur at the same ZT or CT (Ralph et al., 2002). Whereas all animals were trained and tested at the same ZT, the fact that jet-lagged animals did not entrain to the LD cycle resulted in an incongruence between CT and ZT in jet-lagged hamsters. Because we could not control for both ZT and CT, all training and probe trials were conducted at the same time of the light:dark cycle (ZT). This procedure resulted in jet-lagged animals being trained during periods of their activity/rest cycle that varied relative to controls as well as training/testing trials occurring at non-24 hour intervals, potentially contributing to the deficits observed in the former group during the jet lag treatment (Loh et al., 2010). Several points argue against this possibility. First, control and jet-lagged animals spent equal amounts of time actively exploring the apparatus during Pretest 1, indicating that all animals were equally motivated. Furthermore, had circadian phase impacted learning in the jet lag group, the variance in dwell time across animals during Probe 1 should be greater in jet lag compared with control animals, and this was not the case. Finally, jet-lagged animals remained unable to acquire the learning task one month after cessation of the jet lag when ZT and CT should be consistent between jet lag and control conditions.

It is possible that the cognitive impairment seen during phase advancements may result from increased cortisol production in jet-lagged animals (**Figure 4A**) (Shors, 2006). The fact that the same deficits in learning and memory persist one month following maintenance in a static LD cycle, argues against this possibility. Whether or not reductions in neurogenesis persist one month after recovery from repeated phase shifts, suggesting a contribution to these continued deficits, represents an important question for future investigation. Notably, previous work has shown that repeated phase shifting *following* the acquisition of a passive avoidance task impairs retention, suggesting that circadian disruption can also retrogressively impair memory consolidation (Tapp and Holloway, 1981). We now show that phase shifts at least one month *prior* to learning can also impair learning and memory. In agreement with these findings, one recent study found that more mild circadian manipulations, acute phase shifts either before or after contextual fear conditioning, attenuate recall of fear conditioned behavior without inducing sleep deprivation (Loh et al., 2010). Together, these findings reveal that repeated temporal insults grossly impact learning and memory and suggest that resulting changes in hippocampal structure may have long-lasting consequences on cognitive function.

While the mammalian circadian clock can adjust to acute phase shifts in the light:dark cycle, this adjustment requires several cycles to re-establish the relationship between the environment and the internal clock (Yamazaki et al., 2000). Thus, repeated phase advances, such as those seen in experimental jet lag, may result in more pronounced deficits in the ability to re-establish the appropriate phase relationship between the environment and internal physiology. The phase adjustments used in the present experiments result in circadian (~24-hr) rhythms of activity that are not coordinated with external time (**Figure 4B-E; Table 2**). Throughout the jet lag treatment, animals exhibited equivalent bouts of activity but did not confine the majority of their activity to the dark phase of the LD cycle (**Table 2)**. In control animals, nocturnality index and alpha are highly correlated, with the majority of the activity bout being confined to the dark phase of the LD cycle. Because the phase-shifted animals were not synchronized to the light cycle, the duration of the activity/rest cycle was equal to control animals, but the percentage of activity confined to the dark phase was decreased. The former observation suggests that the learning and memory impairments observed in jet-lagged animals did not result from perturbations in circadian rhythmicity, but from desynchrony between internal physiology and external time. Although rhythmic locomotor behavior is a reliable indicator of SCN functioning, it is possible that extra-SCN oscillators (e.g., those in hippocampal cells) behave differently in response to jet lag than those in the master clock (Dibner et al., 2010). Although this possibility is unlikely, given the important role of clock genes in cell cycle regulation (Walisser and Bradfield, 2006), this alternative hypothesis is worthy of exploration. It is noteworthy that sleep deprivation has also been shown to disrupt neurogenesis in both a glucocorticoid-dependent and independent manner (Mirescu et al., 2006; Mueller et al., 2008). In the present studies, it is unlikely that the effects of jet lag on hippocampal structure and function are mediated by disruptions in the activity/rest cycle, as the total duration of the active and inactive phases of the circadian cycle were not different between the fixed LD cycle and jet lag treatment (**Table 2**). However, future studies in which sleep architecture is monitored throughout the jet lag period are necessary to determine whether alterations in sleep contribute to the learning and memory deficits observed.

Together, our findings indicate that experimental jet lag has a pronounced, negative impact on cell proliferation and survival associated with significant deficits in hippocampal–dependent

learning and memory. Importantly, the alterations in the circadian cycle were relatively minor in the present work compared to studies eliminating circadian function through lesions or genetic manipulations. These findings underscore the importance of considering the health consequences for individuals throughout the world engaging in rotating shift work or flexible schedules (e.g., medical residents, airline pilots, security personnel), maintaining poor sleep hygiene, or flying repeatedly across time zones, as the impact of these temporal insults may last well beyond the chronobiological challenges.

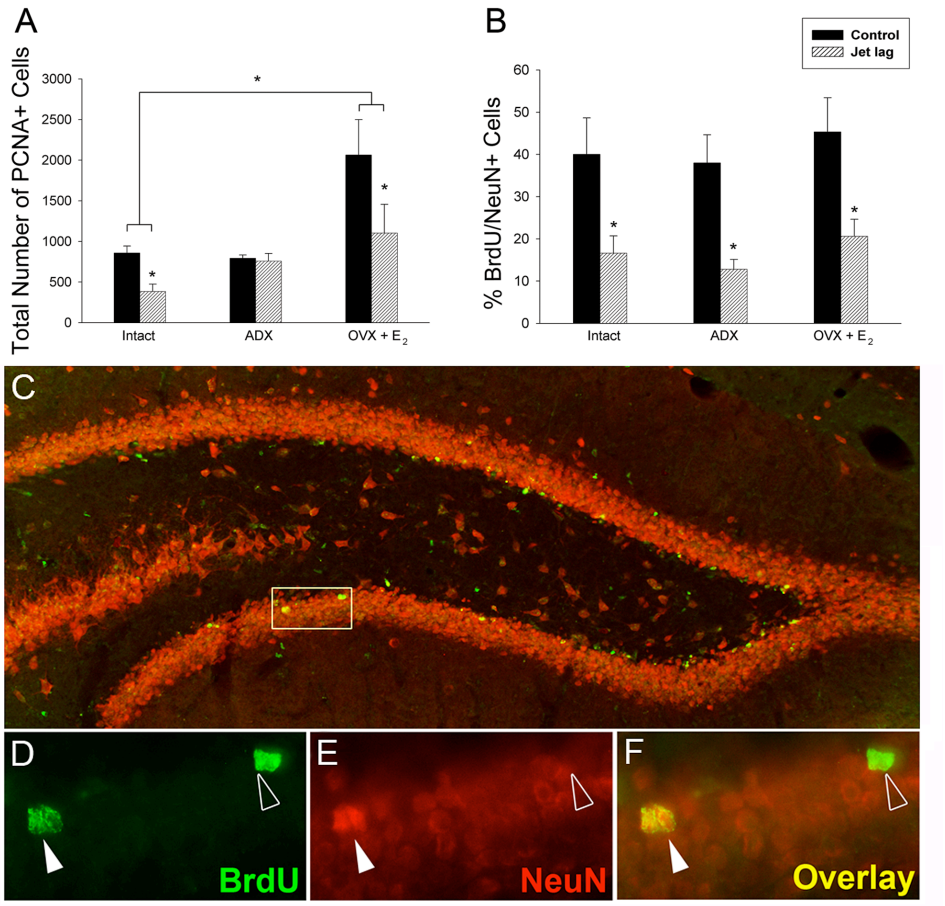

Figure 1. **Jet lag adversely impacts PCNA immunostaining and neurogenesis in the dentate gyrus.** (A) The number of PCNA-labeled cells in the granule cell layer was affected by the hormonal condition of the animal ($F_{2,20} = 4.014$, $p = 0.03$), with ovariectomy and estradiol replacement significantly increasing the number of labeled cells as compared to intact hamsters ($p = 0.04$). Jet lag resulted in a significant decrease in the number of PCNA-labeled cells in both intact and OVX + E_2 hamsters ($p = 0.007$ and $p = 0.05$, respectively by planned comparisons) while the number of PCNA-labeled cells in adrenalectomized animals was not affected by chronic temporal disruption ($p = 0.80$). (B) Neurogenesis was decreased by jet lag ($F_{1,21} = 20.147$, $p < 0.001$), but there was no significant effect of hormone condition ($F_{1,21} = 0.228$, $p = 0.80$) and no interaction ($F_{2,21} = 0.231$, $p = 0.80$). Chronic jet lag resulted in a decrease in neurogenesis by >50% in intact, ADX, and OVX + E_2 hamsters ($p = 0.01$, $p = 0.007$, and $p = 0.05$, respectively; *$p < 0.05$, n = 4/5 animals/group). (C-F) Sections were processed for double-label BrdU (green) and NeuN (red), a marker for mature neurons, and quantified at 400×. (C) Photomicrograph of the dorsal and ventral blades of the dentate gyrus. Cells were considered double-labeled when BrdU (D) and NeuN (E) co-localized in the same focal plane (F; yellow).

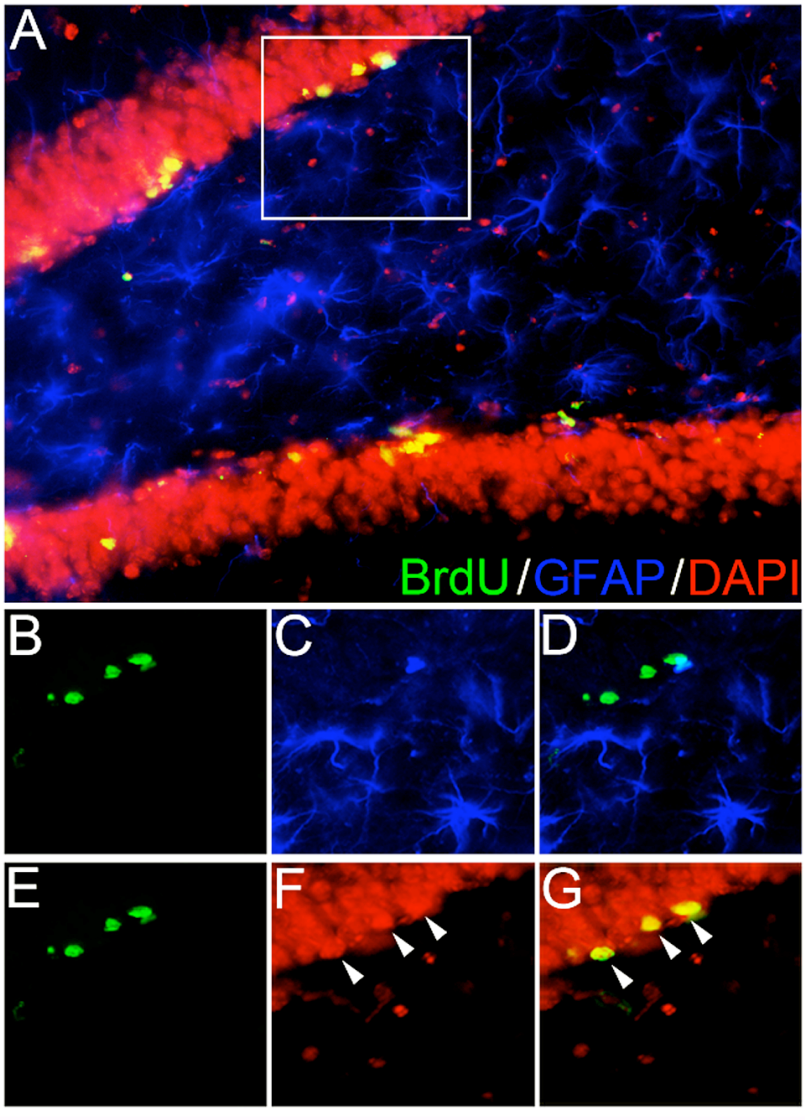

Figure 2. **Gliogenesis is minimal in adult hippocampus.** Hippocampal sections were immunostained for BrdU (green), GFAP (blue), and DAPI (red; color changed to red for purposes of visibility) to assess specificity of BrdU labeling and gliogenesis. (A) Representative photomicrograph (400×) of the dentate gyrus of the hippocampus expressing all three labels. Tissue was double-labeled immunofluorescently with antibodies against BrdU (B) and GFAP (C) to determine whether BrdU-labeled cells were glia (D). Approximately 2% of all BrdU-positive cells co-labeled with GFAP. In the triple-labeled image (D), three cells are labeled for BrdU, but do not co-express GFAP. Tissue was also labeled with fluorescent antibodies against BrdU (E) and DAPI (F) to ensure the specificity of BrdU labeling in mature neurons (G).

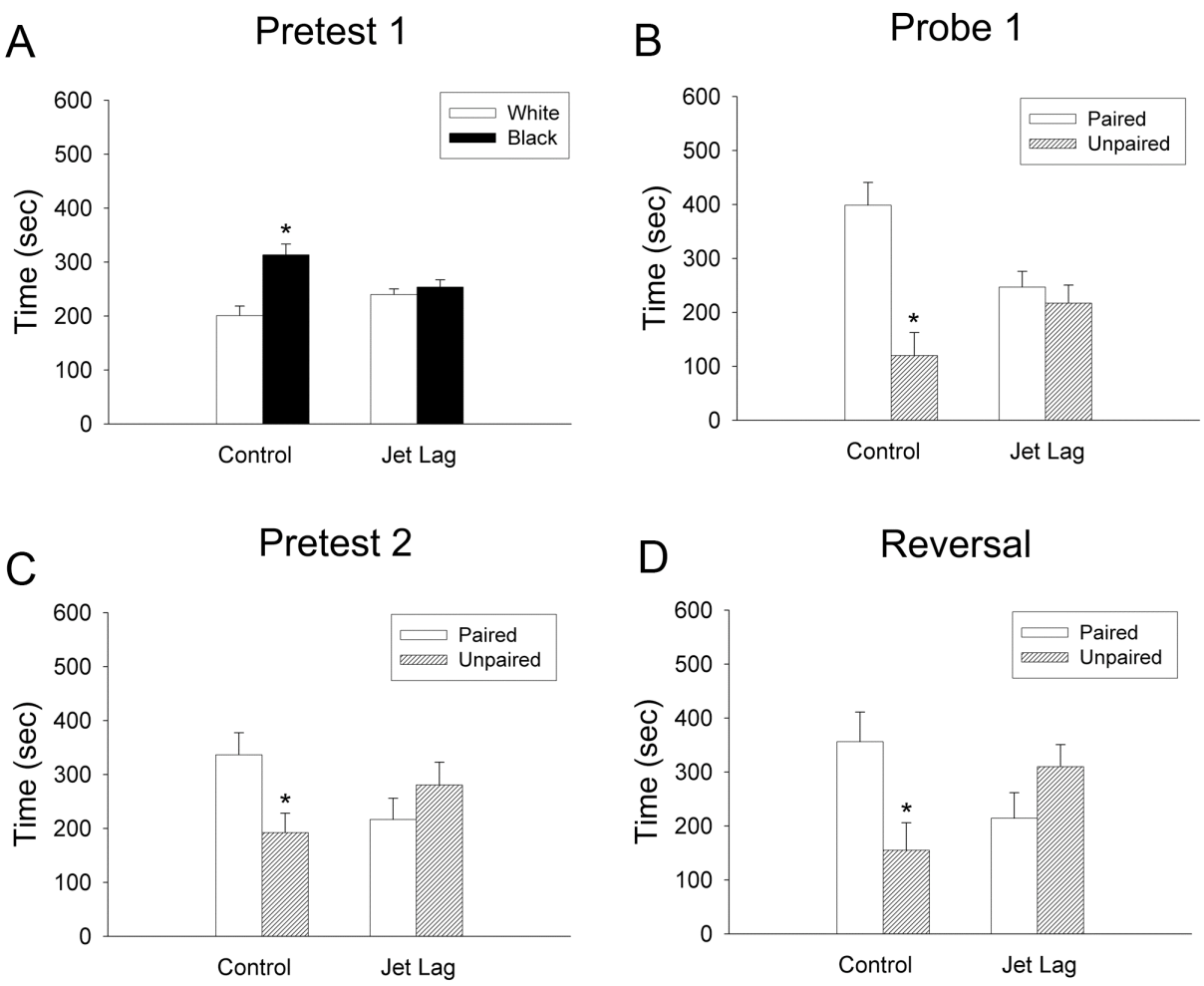

Figure 3. **Jet lag disrupts hippocampal-dependent learning and memory.** Control (n=10) and Jet Lag (n=10) hamsters were introduced to the conditioned place preference (CPP) paradigm at the same time of day throughout the experiment to control for time of day effects on learning and memory (Ralph et al., 2002). (A) Control hamsters exhibited a significant bias for the black chamber during initial exposure to the apparatus (Pretest 1) ($t_{18} = 4.193$, $p < 0.001$) whereas jet-lagged hamsters displayed no preference ($t_{18} = 0.805$, $p = 0.43$). (B) After training, control animals exhibited a significant preference for the chamber previously paired with the rewarding stimulus (Probe 1; $t_{16} = 4.620$, $p < 0.001$), whereas animals undergoing jet lag during training did not learn the task and showed an equal preference for both chambers ($t_{16} = 0.673$, $p = 0.51$). Jet-lagged hamsters were then returned to a static LD cycle for 28 days in order to re-establish entrainment of biological rhythms with the LD cycle. (C) Several weeks after re-entrainment, control animals maintained a preference for the previously paired chamber ($t_{18} = 2.2664$, $p = 0.02$) whereas jet-lagged hamsters continued to show no preference ($t_{18} = 1.113$, $p = 0.28$). All animals were then trained with the wheel being placed in the chamber opposite to that used in the first behavioral test. (D) Even after recovering from chronic temporal disruption, jet-lagged animals did not learn the task ($t_{14} = 1.532$, $p = 0.15$), whereas control animals learned to prefer the new chamber ($t_{12} = 2.692$, $P = 0.02$). * $p < 0.05$.

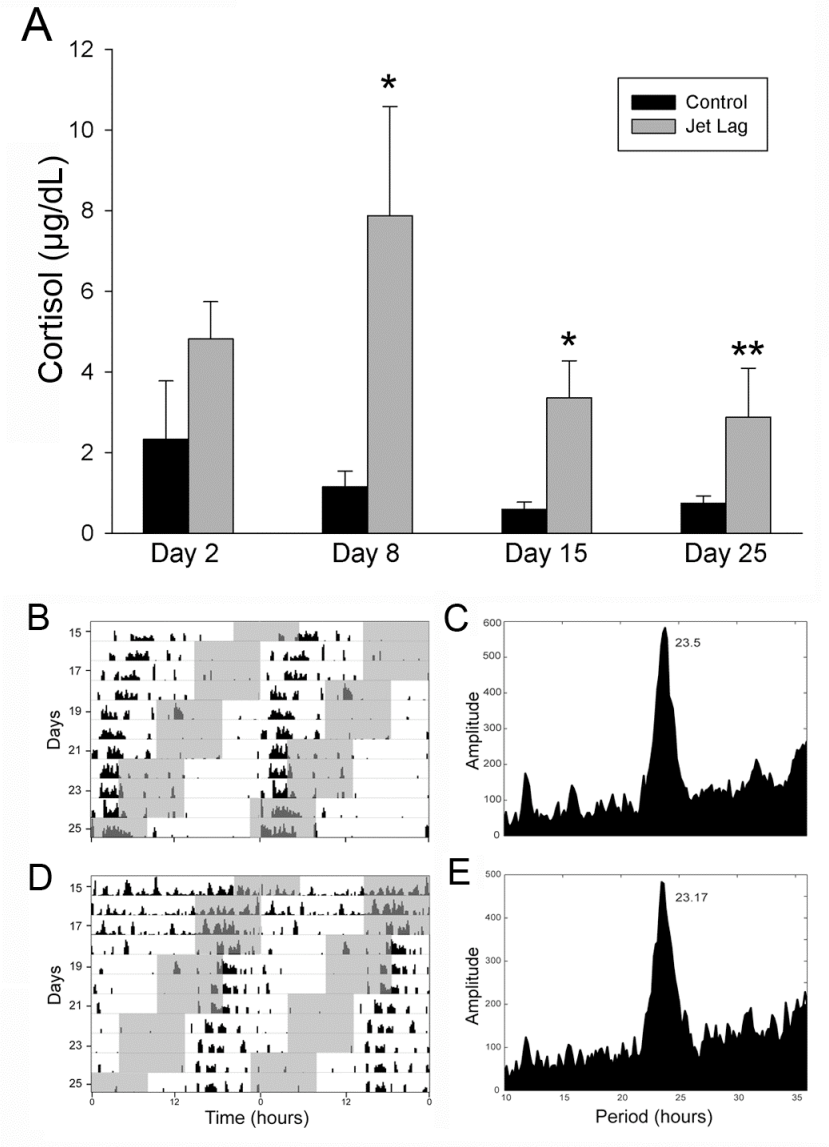

Figure 4. **Jet lag transiently activates the HPA axis.** (A) Jet-lagged animals (n = 7) exhibited increased concentrations of cortisol on days 8 and 15 ($p < 0.001$ and $p = 0.03$) of the 25-day phase advance paradigm but not on the day following the first phase advance ($p = 0.13$) or the final day of the treatment ($p = 0.11$). The stress response in jet lag animals was attenuated throughout the course of the treatment with significantly lower concentration on day 25 compared to day 8 ($p = 0.03$). * Significantly greater than control animals (n = 7) at each time point, $p < 0.05$. ** Significantly less than jet-lagged animals on Day 8 of sampling, $p < 0.05$. (B-E) Jet-lagged animals ignore environmental light cues while maintaining rhythmic behavior. (B, D) Double-plotted actograms and (C, E) Fourier analysis of period length/rhythm amplitude of jet-lagged animals exposed to a chronic phase-advanced LD schedule. Records indicate that jet-lagged animals remain rhythmic (B, D), running with a period ~24 hrs (C, E; $p < 0.01$). Grey bars depict the dark phase of the LD cycle on these double-plotted activity records. Had animals been entrained to the LD cycle, activity would have been confined to these dark periods.

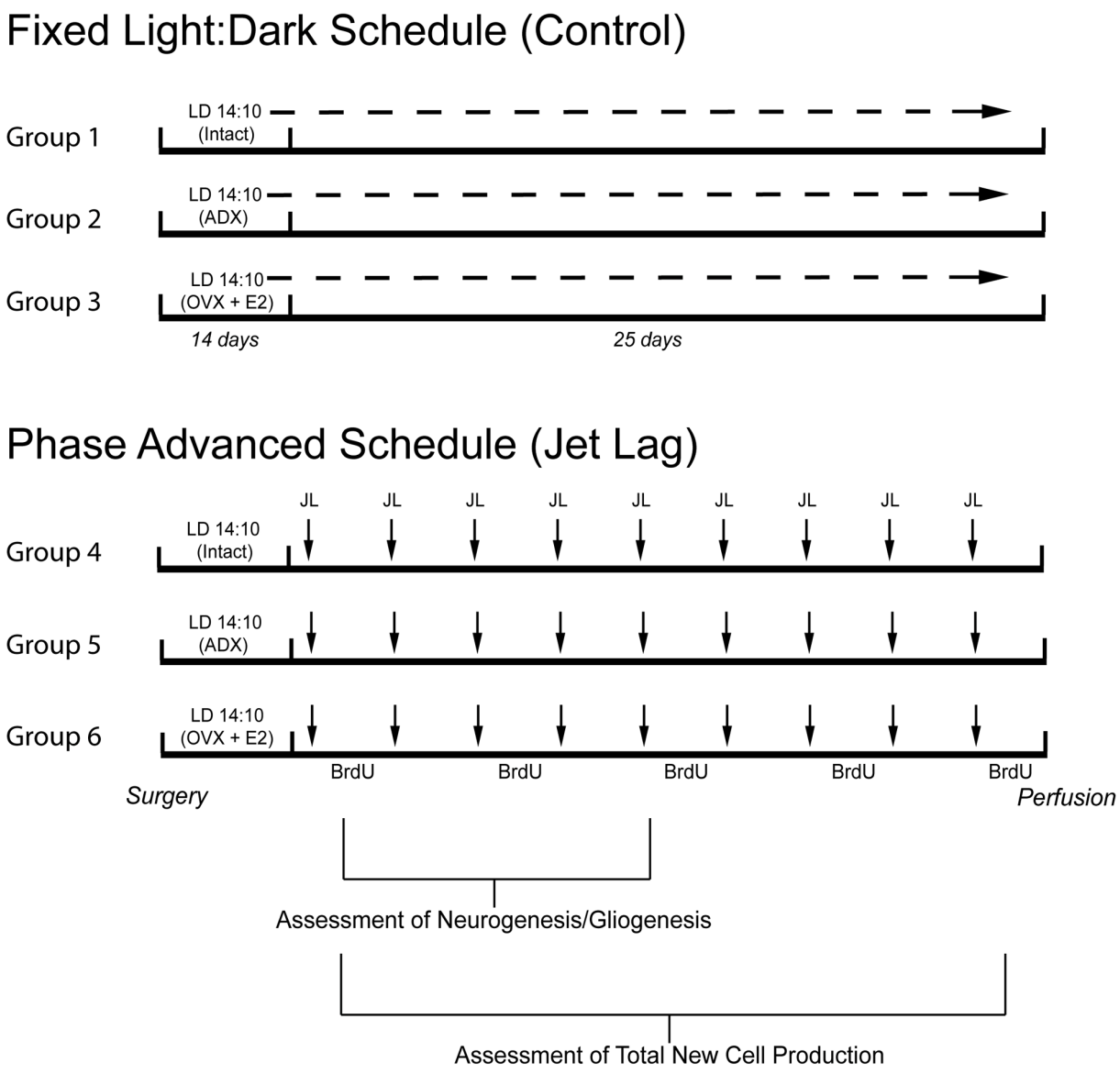

Figure S1. **BrdU Experimental Design.** Two weeks after recovery from adrenalectomy or ovariectomy, female hamsters were maintained in a fixed 14:10 LD cycle (Control) or an LD cycle that was phase advanced by 6 hrs (Jet Lag), every three days, for 25 days. All animals were injected with BrdU the day following every other jet lag (JL) and were perfused 24 hrs after the last injection. The first three injections were implemented early in the experiment to assess neurogenesis (i.e., enough time for cells to mature into neurons) whereas injections were continued past this point to assess total cell proliferation during the experiment.

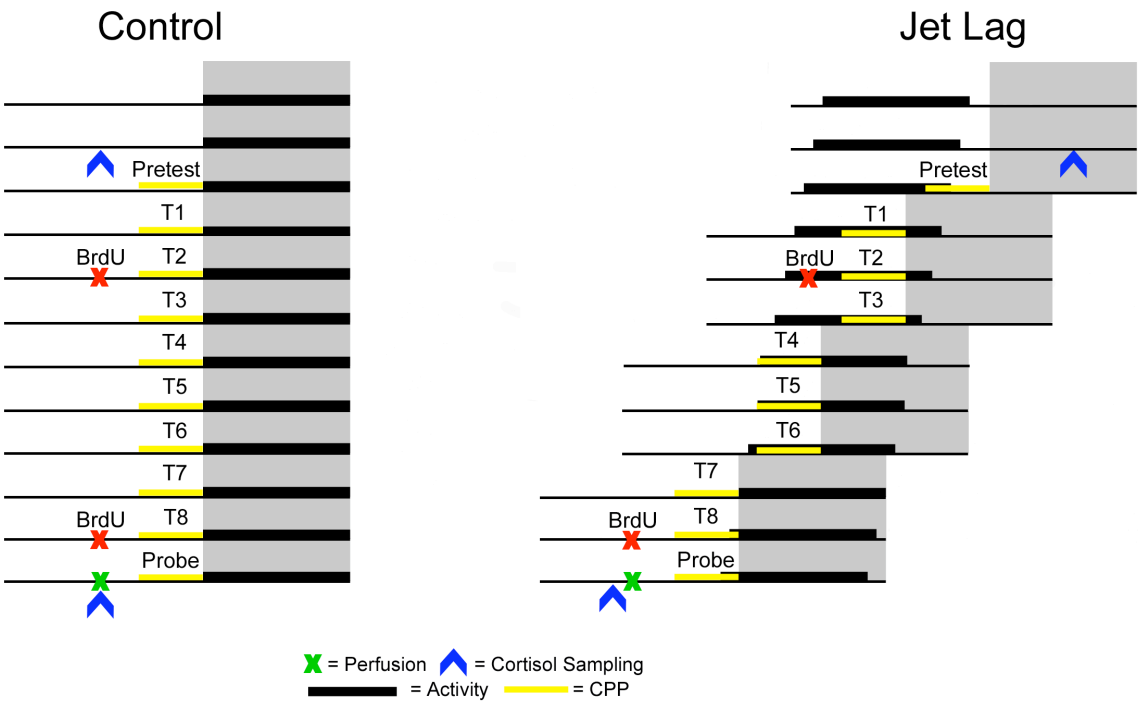

Figure S2. **Hypothetical Procedural Time Course.** Hypothetical activity records and procedural timelines for a control animal exhibiting ~24-hr rhythms in activity (black bars) that were confined to the dark phase (grey bars) of the light:dark cycle and a jet-lagged hamster exhibiting < 24-hr rhythm in behavior. For both control and jet lag animals, all CPP pretest, training and probe trials occurred 4 hrs prior to lights off (yellow bars; ZT10-14;T1 = training day 1). BrdU injections (red X) occurred the day after every other phase advance at ZT7 for all animals, with perfusions (green X) occurring 24 hrs after the final injection. Cortisol samples were acquired on Days 2, 8, 15, and 25 of the jet lag paradigm (blue arrow). For all animals, blood samples were collected at CT7 based on the individual animal's activity profile.

Table 1. **Jet lag does not affect gliogenesis and hippocampal volume.**

	Intact		**ADX**		**OVX + E_2**	
	Control	**Jet Lag**	**Control**	**Jet Lag**	**Control**	**Jet Lag**
%BrdU/GFAP+ Cells	3.0 ± 3.0	3.2 ± 3.2	0.0 ± 0.0	2.4 ± 2.4	2.0 ± 1.2	1.6 ± 1.6
Granule Cell Layer Volume (mm^3)	13.26 ± 0.96	14.48 ± 0.75	14.73 ± 1.10	14.19 ± 0.71	15.25 ± 0.67	14.11 ± 0.56

Table 2. **Circadian analysis of hamsters maintained in a static light:dark (LD) cycle and during days 2-4, 15-17, and 21-23 of the jet lag paradigm.**

	Fixed LD	**Days 2-4**	**Days 15-17**	**Days 21-23**
Alpha (hrs)	6.881 ± 0.678	5.150 ± 1.369	6.384 ± 0.876	7.103 ± 0.657
Nocturnality index	0.647 ± 0.038	0.138 ± 0.027*	0.169 ± 0.058*	0.542 ± 0.075

* = significantly different from Fixed LD and Days 21-23 groups, $p < 0.05$

Chapter 4: The Effects of Temporal Disruption on Immune Function

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Experiment 4: Repeated Jet Lag is Associated with Alterations in Innate and Adaptive Immunity in Female Syrian Hamsters

Abstract

The advent of industrialization and artificial lighting has led to pronounced disruptions to adaptive circadian functioning. Many epidemiological studies have shown that chronic disruption of circadian homeostasis is associated with a higher incidence of many psychological and physiological maladies. Many of these conditions may be a result of deficits in immune functioning, as the circadian clock plays an intricate role in immunity. In this study, we show that chronic jet lag alters both innate and adaptive immune responses. Total IgG, bacterial killing, and complement system activity are suppressed in jet-lagged animals compared to controls, with jet-lag-induced suppression of total IgG and bacterial killing abolished by controlling for activation of the stress axis. In contrast, jet lag enhanced splenocyte proliferation following addition of mitogens, with jet lag impacting B-cell proliferation more than T-cell proliferation. We conclude that chronic circadian perturbation is associated with disruption of both the innate and adaptive immune responses that may lead to increased susceptibility of disease.

Introduction

Circadian dysfunction, through molecular disturbances (i.e. gene knockout models) or behavioral disruptions (i.e. repeated transmeridian flight, rotating shift work, or sleep disturbances) are associated with the prevalence and severity of a whole host of clinical and pathological maladies, including hypertension and heart disease, diabetes, learning and memory deficits, reproductive deficits, and higher incidence of cancer (Poole et al., 1992; Ahlborg et al., 1996; Cho et al., 2000; Cho, 2001; Ha and Park, 2005; Hansen, 2006; Kivimaki et al., 2006; Conlon et al., 2007; Gibson et al., 2010). For example, careers involving chronic temporal disruptions are associated with increased incidences of breast and colorectal cancer (Schernhammer et al., 2001; Schernhammer et al., 2003), contributing to The World Health Organization classification of shift work as a carcinogen (Stevens et al., 2007).

At the molecular level, circadian rhythms are generated ubiquitously throughout the body by a transcriptional/post-translational negative feedback loop that has a period of approximately 24 hours. Briefly, the products of the core clock genes *Clock* and *Bmal1* form a heterodimer and drive the transcription of the clock genes *Period* (*Per1, Per2, Per3*) and *Cryptochrome* (*Cry1, Cry2*). Accumulated levels of PER and CRY proteins within the cytoplasm feedback into the nucleus, suppressing the expression of Clock and Bmal1 (Reddy et al., 2005). Immune factors, such as spleen cells, lymph nodes, and peritoneal macrophages, all rhythmically express clock genes (Kusanagi et al., 2004; Keller et al., 2009). Disruption of the molecular clock results in decreased levels of B-cells in peripheral blood, spleen, and bone marrow that are consequences of alterations in the maturation and differentiation of pre-B-cells to mature B-cells (Sun et al., 2006). Similarly, the immune response in $Per2^{-/-}$ mice is severely suppressed, so that the

induction of proinflammatory cytokines after a mitogen challenge is decreased (Liu et al., 2006). The role of the circadian system in systemic control of both the innate and adaptive immune responses is further supported by the presence of circadian rhythms in circulating T- and B-lymphocytes, natural killer (NK) activity, absolute and relative number of circulating white blood cells, and cytokine levels (Esquifino et al., 1996; Arjona and Sarkar, 2005; Abo et al., 1981; Kawate et al., 1981). Specifically, total T- and B-cells and their various subsets, including cytotoxic and helper T-cells, are highest during the rest phase while circulating levels of NK cells are highest during the active phase (Depres-Brummer et al., 1997). Interestingly, daytime increases in total lymphocytes are suppressed and phase-delayed in clock mutants (Oishi et al., 2006).

The 24-hour changes in lymphocyte populations and their function may be associated with time of day changes in cell proliferation in immunocompetent tissue or changes in lymphocyte trafficking, possibly as a result of interactions between endocrine and immune factors (Fabris, 1994). This is especially true regarding the interactions between the circadian system and the stress and reproductive axes. Repeated transmeridian flights are associated with increased concentrations of circulating glucocorticoids and alterations in the reproductive axis (Ahlborg et al., 1996; Cho et al., 2000; Cho, 2001; Gibson et al., 2010). Glucocorticoids can inhibit both innate and adaptive immune responses on the cellular level, including suppression of lymphocyte proliferation and functioning, induction of lymphocyte apoptosis, reduction in pro-inflammatory cytokines and enhancement of anti-inflammatory cytokines (reviewed in (Sorrells and Sapolsky, 2007)). However, pro-inflammatory effects of stress have been documented outside the nervous system, such as the enhancement of neutrophil proliferation and survival following glucocorticoid administration (Cox, 1995; Liles et al., 1995). Estrogen influences the differentiation, maturation and emigration of lymphocytes, as well as cytokine secretion and antibody production (McMurray, 2001; Li and McMurray, 2006). Estradiol treatment to ovariectomized mice results in a decrease in the percentage of splenic mature B-cells and an increase in pro-inflammatory cytokines (Li and McMurray, 2006). Like humans, mice undergoing repeated phase shifts had heightened responses to subsequent inflammatory challenges (Preuss et al., 2008), however the potential impact of these phase shifts on unchallenged immune parameters and on the stress and reproductive axes was not assessed.

Despite these and other findings, the impact of circadian disruptions on immune function, independent of alterations in the stress and reproductive axes, has not been fully elucidated. Because temporal insults involve alterations in many physiological processes that have reciprocal impact on one another, including the hypothalamo-pituitary-adrenal (HPA) axis, the hypothalamo-pituitary-gonadal (HPG) axis, and the immune system, it remains unclear which system (or combination of systems) is responsible for the increased incidence of disease associated with circadian disruption. The primary goal of the present study was to use a model of jet lag to assess the impact of circadian disruption on both the innate and adaptive immune systems, independent of potential dysregulation of the HPA and HPG axes and a mitogen immune challenge.

Materials and Methods

Animals. Adult (>60 days of age) female LVG hamsters (Mesocricetus auratus; Charles River, Wilmington, MA) were maintained on a 14:10 light:dark (LD) cycle prior to the onset of all

experiments and provided with ad libitum access to water and food throughout the duration of the study. All animals were housed in translucent propylene cages (48 x 27 x 20 cm) and maintained in a colony room at 23 ±1°C. Estrous cyclicity was monitored for all animals by daily inspection for preovulatory vaginal discharge for two weeks (Orsini, 1961). Only animals with regular, 4-day estrous cycles were used in the experiments. For the first study, hamsters either remained intact (n=9), or were surgically manipulated to control for endocrine effects on immune function. To control for HPA axis influences on immune function, hamsters (n=8) were adrenalectamized (ADX/Cort) and supplemented with a drinking solution of 0.9% saline, 5% sucrose and corticosterone (25 μg per ml of 0.9% saline; Sigma) to mimic basal glucocorticoid concentrations and maintain electrolytes (Ottenweller et al., 1985; Meyer-Bernstein et al., 1999; Mirescu et al., 2004). To control for cycling levels of estrogen and any alterations in estrogen levels that may occur as a result of jet lag, another group of hamsters (n=8) was ovariectomized and implanted with SILASTIC brand capsules (Downing Corning Corp., Midland, MI; 10 mm length, 1.45 mm inner diameter, 1.93 mm out diameter) containing powdered 17-β estradiol (OVX/E_2) (Meyer-Bernstein et al., 1999). For the second study, a separate cohort of hamsters (n=20) remained intact. Locomotor behavior was monitored for all animals using an infrared monitoring system (Data Sciences; St. Paul, MN) mounted to the wire lids on each cage. All movement in the cage was detected by interruptions in the infrared beam and relayed to a computer. Cumulative counts were recorded every 10 mins and analyzed using Dataquest 3 software (Data Sciences; St. Paul, MN). On the last day of the jet lag treatment, animals' spleens were removed for the splenocyte proliferation assay. All animals were treated in accordance with the Institutional Animal Care and Use Committee guidelines of the University of California, Berkeley.

Jet Lag Procedure. For the first study, the jet lag paradigm lasted for a total of 25 days, during which animals were subjected to a 6-hr phase advance in the light:dark cycle every 3 days, which is equivalent to a transmeridian flight from New York to Paris (Jet Lag n=4/hormone condition). Control animals (n=4/hormone condition) were maintained in a fixed light:dark cycle (14:10; lights on at 0700 and lights off at 2100). On the last day of the jet lag paradigm, all hamsters were anesthetized using isoflurane, and 2 ml of blood was obtained from each animal via the retroorbital sinus 7 hours into the rest phase. For the second study, the previous jet lag paradigm was implemented for a total of 60 days with intact Control (n=10) and Jet Lag (n=10) hamsters. On the final day of the study, half of the hamsters were sacrificed by cervical dislocation 5 hours into the rest (n=5/condition) or 5 hours into the active phase (n=5/condition). Spleens were removed under aseptic conditions and immediately suspended in culture medium (RPMI-1640/Hepes).

Bacterial Killing Assay. In order to evaluate the ability of an animal to kill a bacterial infection, we used an ex vivo bacterial killing assay. This assay measures the capacity of fresh whole blood to kill bacteria and allows for characterization of a functionally relevant, integrative immune response, including the activities of phagocytes (macrophages, heterophils, and thrombocytes), opsonizing proteins (complement and acute phase proteins) and natural antibodies (predominantly IgM and IgA). Bacterial stock solution was prepared by adding E. coli ($E^{powderTM}$ Microorganisms #0393E7, MicroBioLogics, St. Cloud, MN) containing 10^7 cells to 40 ml of 1M sterile phosphate buffer solution (PBS) warmed to approximately 35-37°C. The stock solution was activated by incubating in a warm water bath (37°C) for 30 minutes before

being vortexed to create a bacterial stock solution. 20 ml of plasma from each animal was added to 180 ml of media along with 20µl of bacterial stock solution. Tubes were incubated at 41°C for 30 minutes. All samples were vortexed and 75 µl was added to Petri plates in duplicate, then covered and incubated overnight at 27°C. Control plates were coated with 75 µl of media only. The next day, the number of viable colonies was counted and duplicates were averaged to determine the proportion of colonies killed in comparison to control plates.

Total Immunoglobulin G Assay. A sandwich ELISA was performed to determine the total immunoglobulin G levels in serum. Plates were prepared by coating individual wells of the well plate with a carbonate-bicarbonate coating buffer for binding the capture antibody mixture. Plates were incubated at 4°C overnight. Plates were then rinsed with PBS wash buffer, and serum was serially diluted using PBS wash buffer to concentrations of 10 µg/ml, 1µg/ml, 0.1µg/ml, 0.01µg/ml, 0.001µg/ml in order to establish a standard curve. Standards were added to the plates in triplicate. Serum samples were then diluted in assay diluent to a 1:200 concentration and 100 µl of the diluted samples were added to plates in duplicate, and incubated overnight at 4°C. The plates were then rinsed with PBS and 100 µl of labeling antibody AP-conjugated goat anti-anti-hamster diluted in assay diluent to a concentration of 1:2500 was added to each well and incubated overnight at 4°C. The next day plates were rinsed in PBS and 100 µl of the buffer was added to each of the microplates along with 0.1M diethanolamine-pNPP substrate buffer, which was then read at multiple time points at 405 nm.

Hemolytic Complement Activity Assay. To measure hemolytic complement activity, a component of the innate immune system, serum was diluted to a 1:40 concentration in dextrose-gelatin veronal buffer (BioWhittaker, Walkersville, MD), and added in duplicate to 96-well round-bottomed microplates. Veronal buffer (VB) was then added to remaining empty wells and serum was serially diluted to a final concentration of 1:80. The final amount of sample in each well was 40 µl, to which 25 µl each of a 0.6% suspension of washed sheep red blood cells (in VB) and a 1:40 dilution of rabbit anti-SRBC in VB was added. The plates were slowly vortexed for 5 minutes, incubated for 1.5 hours at 37°C, then centrifuged for 5 min at 500 rpm at room temperature. 60 µl of the supernatant was removed, added to a U-shaped microplate and read at 405 nm. CH_{50} is the reciprocal of the dilution that caused 50% of antibody sensitized SRBCs to lyse. A higher CH_{50} is correlated with greater lytic ability.

Splenocyte Proliferation. The second cohort of animals was then jet-lagged for an additional 35 days (60 days total), during which the activity rhythms of each animal was continually monitored using the infrared activity monitoring system described above. Spleens were removed from the animals 5 hrs into either their activity phase or rest phase, and immediately placed in supplemented RPMI (RPMI + 10% FBS + 1% Pen/Strep + 1% 1-glutamine + 0.1% ME). Extracted splenocytes were supplemented with culture medium, and cell count was determined with a hemacytometer. Percentage of viable cells was verified with tryptan blue. A solution of 50 µl of 6X tryptan blue, 6µl of cell suspension and 244 µl of culture medium was mixed together and incubated at room temperature for 15 minutes before being loaded into the hemacytometer. Samples with less than 5% tryptan blue stained cells were considered viable. The cell concentration was then adjusted to 5×10^6 cells/ml, by adding appropriate amounts of culture medium to the sample. Cell suspensions were added to a 96 well plate in triplicate, and stimulated with either ConA at concentrations of 5µg/ml, 10 µg/ml, 20 µg/ml, or 40 µg/ml or

LPS at concentrations of 25 µg/ml, 50 µg/ml, 100 µg/ml, or 200 µg/ml. Cell suspensions were incubated at 37°C in a 5% CO_2 incubator for 48-72 hours. A 20-µl solution of phenazine methosulfate (MTS/PMS) was added to each well as part of the colorimetric proliferation assay, and plates were read at 490 nm using an ELISA plate reader at 4 hours post MTS/PMS addition.

Statistical Analyses. Group mean differences in bacteriocidal rates, total IgG levels, and complement system activity were analyzed using analyses of variance (ANOVA). Splenocyte proliferation rates were analyzed using a two-way repeated measures ANOVA. For all analyses, Tukey or Tukey-Kramer post hoc tests were used to examine pairwise differences.

Results

Bacterial Killing Assay. Jet-lagged animals had significantly decreased ability to kill bacteria ($F_{1.25} = 5.440$, $p = 0.028$), with the impact of jet lag dependent upon hormone condition ($F_{2.25} = 3.520$, $p = 0.045$). Hormone condition alone had no effect on bacterial killing ($F_{2.25} = 0.248$, $p = 0.782$). In intact hamsters, there was a trend for a reduction in proportion of bacteria killed in jet-lagged hamsters compared to controls ($p = 0.055$), while jet-lagged animals in the OVX + E_2 group exhibited significantly lower proportions of bacteriocidal rates ($p = 0.008$). This reductions was abolished when glucocorticoid levels were controlled for in ADX + Cort animals ($p = 0.506$) (**Figure 1A)**.

Total Immunoglobulin G concentrations. Total IgG concentrations were significantly affected by jet lag ($F_{1.25} = 20.591$, $p < 0.001$) and the impact of jet lag was dependent upon the hormonal condition of the animal ($F_{2.25} = 41.662$, $p = 0.006$). The hormone condition of the hamster alone did not significantly impact total IgG values ($F_{2.25} = 0.475$, $p = 0.628$). Within intact and OVX + E_2 hamsters, jet lag significantly reduced serum IgG concentrations (both $p < 0.001$), but this effect was eliminated in ADX + Cort hamsters ($p = 0.779$), suggesting a role for glucocorticoids in the jet-lagged-induced suppression of total IgG. In control hamsters, OVX + E_2 animals exhibited increased IgG concentrations compared to ADX + Cort females ($p = 0.039$) (**Figure 1B**).

Complement Assay. Jet lag had a main effect on CH50 concentrations ($F_{1.15} = 8.984$, $p = 0.009$), but there was no significant effect of hormone condition ($F_{2.15} = 0.005$, $p = 0.995$) or interaction between jet lag and hormone condition ($F_{2.15} = 0.134$, $p = 0.876$). Comparisons of jet lag condition within intact females suggest a trend toward a jet lag-induced suppression of the complement system ($p = 0.053$), but OVX + E_2 and ADX + Cort hamsters did not exhibit any significant differences between jet lag and control conditions ($p = 0.210$ and $p = 0.090$, respectively; **Figure 1C**).

Splenocyte Proliferation. Four hours after the addition of ConA, a mitogen that stimulates T-cell proliferation, spleen cultures taken from jet-lagged hamsters (Jet Lag-Active Phase and Jet Lag-Rest Phase) exhibited higher splenocyte proliferation rates than control hamsters (Control-Active Phase and Control-Rest Phase; $F_{3,11} = 4.058$, $p = 0.036$). There was no significant main effect of mitogen ($F_{1,11} = 0.188$, $p = 0.090$) or interaction between jet lag condition and mitogen ($F_{3,11} = 1.590$, $p = 0.248$). The addition of ConA to spleens harvested from jet-lagged animals during their active phase significantly increased proliferation ($p = 0.039$), and this value was

significantly greater than proliferation rates after mitogen exposure in the Control-Active Phase animals ($p = 0.036$) (**Figure 2A)**.

Addition of LPS, a mitogen that stimulates B-cell proliferation, differentially impacted proliferation rates based on condition (Jet Lag-Active Phase, Jet Lag-Rest Phase, Control-Active Phase, Control-Rest Phase), with jet-lagged animals exhibiting increases in splenocyte proliferation ($F_{3,11} = 9.130$, $p = 0.003$). Splenocyte proliferation levels were also significantly impacted by mitogen exposure ($F_{1,11} = 23.936$, $p < 0.001$), but the effect of condition did not depend on the presence of the mitogen ($F_{3,11} = 2.129$, $p = 0.155$). Within the rest phase, jet-lagged animals exhibited increased proliferation rates over control animals, regardless of mitogen condition ($p = 0.024$). In control hamsters, time of day (Rest Phase or Active Phase) differentially impacted the effect of mitogen exposure, with control spleens harvested during the active phase having greater proliferation rates compared to those spleens harvested during the rest phase ($p = 0.045$). Specifically, spleens taken from control animals during the active phase exhibited significant increases in splenocyte proliferation after LPS exposure ($p = 0.033$), but a similar increase following mitogen exposure was not seen in control spleens harvested during the rest phase ($p = 0.648$). The addition of LPS to spleens from jet-lagged hamsters taken during the active phase ($p = 0.008$) and the rest phase ($p = 0.006$) resulted in an increase in proliferation compared to no mitogen (**Figure 2B**).

Discussion

In the present study, we investigated the impact of temporal disruptions on baseline immune function, controlling for alterations in the immune-promoting HPG axis and the immuno-suppressing HPA axis. Specifically, animals that underwent 25 days of repeated phase advances exhibited suppression in bacterial killing ability and complement system activity, both components of the innate immune system. Similarly, jet lag resulted in a decrease in total serum IgG, indicating that circadian disruption affects the humoral immune response as well. The jet-lagged-induced suppression of bacterial killing and total IgG was abolished in ADX + Cort animals, suggesting a glucocorticoid-dependent mechanism for suppression. Interestingly, jet lag had the opposite effect on splenocyte proliferation, with an increase in proliferative ability of spleens harvested from jet-lagged animals compared to controls following a mitogen challenge. The findings from the present study suggest that chronic phase-advances can alter immune function by suppression of the innate and humoral immune response, as well as the amplification of lymphocyte proliferation.

The development of an ~24-hour system that coordinates myriad physiological, behavioral, and hormonal processes to coincide with the 24-hour light/dark cycle underscores the necessity of the synchronization of an organism with the environment for optimal health and functioning (Sharma, 2003; Maywood et al., 2006). The circadian system that is responsible for such coordination in mammals is organized into a hierarchical system that originates with a molecular clock consisting of interlocking transcriptional/post-translational loops that are then synchronized by the master circadian clock localized in the suprachiasmatic nucleus (SCN) (Reppert and Weaver, 2002; Dibner et al., 2010). To ensure maximal functioning, the intricate orchestration of the cellular oscillators located throughout the body must happen so that the processes that they coordinate occur at the proper time of day. The development of a 24-hour society through industrialization has resulted in an incongruence between internal physiology

and external environmental cues (Wittmann et al., 2006; Stevens et al., 2007). Individuals who partake in chronic shift work or jet lag industries have higher incidents of many adverse mental and physiological pathologies, including psychological disorders, cancer, diabetes, and cardiovascular diseases (Skipper et al., 1990; Poole et al., 1992; Hansen, 2006; Conlon et al., 2007). One potential cause for the higher incidence of disease in temporally disrupted individuals may be in the subsequent alteration of the inflammatory response, including factors of the innate and adaptive immune systems. Previous studies have shown that chronic disruption of the circadian system (4 consecutive weekly 6-hr phase advances) interrupts normal functioning of the innate immune response, specifically a magnification of LPS-induced endotoxemic shock and subsequent increases in mortality. This effect was independent of alterations to the sleep/wake cycle and stress axis, both modulators of the immune system (Castanon-Cervantes et al., 2010). Additionally, another study using a different animal model of temporal disruption (12-hr phase shift every 5 days for 3 months) showed that chronic shifting had no impact on intestinal physiology in benign conditions, but when the animals were challenged with an induction of colitus, the disease progressed more rapidly in the phase-shifted animals compared to controls (Preuss et al., 2008).

Whereas previous studies indicate that chronic disruption of the circadian system results in adverse effects in immune-challenged animals, the impact of temporal incongruence on an unchallenged immune system remains incomplete. We investigated the impact of such temporal disturbances on the immune system independent of alterations in the reproductive and stress axes and independent of an immune challenge. Many studies point to the role of estrogen as a pro-inflammatory modulator and glucocorticoids as anti-inflammatory factors, although some evidence exists that glucocorticoids may also have pro-inflammatory effects (Cox, 1995; Liles et al., 1995; Li and McMurray, 2006) (reviewed in (Sorrells and Sapolsky, 2007)). Disturbances to the normal fluctuations in estrogen and glucocorticoids, as seen in chronically circadian disrupted individuals, may directly influence inflammatory responses (Cho et al., 2000; Cho, 2001). Previous studies from our lab suggest that twice weekly 6-hr phase advances for 25 days result in an increase in glucocorticoid concentrations, but no alterations in the wake/rest cycle of animals (Gibson et al., 2010). This model of chronic disruption of the circadian system does not result in arrhythmicity of the circadian rhythm but ~24-hr rhythms that are no longer synchronized to the light:dark cycle, indicating an incongruence between internal rhythmicity and external cues (Gibson et al., 2010). Because of the increased secretion of glucocorticoids throughout the jet lag paradigm and the dysregulation of the ovulatory cycle (Gibson et al. unpublished results; See Chapter 1, Experiment 2), and the potential impact of these factors of the immune system, we sought to investigate whether temporal disruptions alone may impact baseline immune measures. Jet lag suppressed bacterial killing ability, total serum IgG and complement system activity, but its effect on bacterial killing and total IgG was dampened in ADX + Cort hamsters, suggesting a glucocorticoid-mediated suppression of these immune measures. Interestingly, the jet lag effect is not driven by disruptions in estrous cycling or changes in estradiol, as OVX + E_2 hamsters exhibited jet lag-suppression trends similar to those seen in the intact animals (**Figure 1A-C**). The assay of hemolytic complement activity depends on the ability of the primary complement pathway to induce hemolysis of red blood cells sensitized with optimal amounts of anti-red blood cell antibody. Jet lag decreased complement system activity, but the impact of temporal disturbances alone or incongruence with the subsequent alterations to the stress and reproductive axes could not be discerned (**Figure 1C**).

Future studies are needed to more fully elucidate the impact of chronic circadian dysregulation on the complement system.

Whereas both bacterial killing and complement system activity are complement-dependent measures, bacterial killing requires the presence of antibodies, such as IgG, IgA, and IgE, in order to kill bacteria. Such antibodies are produced by mature B-cells that are originally produced in bone marrow but reach maturity in the spleen. The dysregulation of the circadian system through molecular knockdown has been shown to influence cell division and proliferation (Matsuo et al., 2003; Hunt and Sassone-Corsi, 2007). Disruption of circadian homeostasis through molecular clock knockdown promotes tumor growth, including higher rates of spontaneous and carcinogen-induced tumors (Hamilton, 1969; Filipski et al., 2005; Lee et al., 2010). Epidemialogical studies indicate that individuals who experience chronic dysregulation of circadian homeostasis through jet lag or rotating shift work have higher incidences of cancers, including breast, prostate, colon, and lung (Davis et al., 2001; Kloog et al., 2009). Interestingly, jet lag in both WT and knockdown mice result in increased incidences of ovarian, kidney, intestinal and pancreatic tumors (Lee et al., 2010). Neural precursor cells (NPCs) express clock genes, and suppression of these genes results in abnormal cell maturation (Borgs et al., 2009b). Similarly, our lab has shown that chronic phase-advances results in decrements in cell maturation and neurogenesis, as well as glucocorticoid-mediated suppression of cell proliferation (Gibson et al., 2010). These data, along with studies implicating the role of the circadian system in cell cycle regulation and cell survival (Walisser and Bradfield, 2006; Hunt and Sassone-Corsi, 2007), provide further evidence for the necessity of the circadian system in normal cell functioning.

In order to assess if disruptions in circadian homeostasis impacted the proliferative ability of immune markers, we investigated the effect of jet lag on splenocyte proliferation. Circulating T- and B-lymphocyte concentrations are highest during the rest phase (Depres-Brummer et al., 1997), but splenocyte proliferation is increased during the active phase compared to the rest phase (Drazen et al., 2001). To control for time of day effects, spleens were harvested during both the active and rest phases. Serial dilutions of mitogen concentration were performed to determine the minimum dosage that resulted in proliferation to avoid a possible ceiling effect. For both LPS and ConA, mitogens that stimulate the proliferation of B- and T-lymphocytes respectively, the lowest concentration was optimal to assess proliferation ability, however this protocol does not provide direct evidence of immune function. Following addition of ConA, spleens from jet-lagged animals had increased levels of proliferation compared to control spleens, but there was no main effect of mitogen. Interestingly, the proliferation of T-cells was not greatly impacted by dysregulation of the circadian clock or time of day (**Figure 2A**). This was not surprising as molecular disruptions of the core clock through knockdowns of MOP3 do not impact T-cell development but does suppress B-cell levels in blood, spleen, and bone marrow (Sun et al., 2006). Similarly, jet lag altered proliferation more robustly after LPS compared to ConA stimulation. There was a main effect of jet lag and mitogen following LPS stimulation. During the rest phase, jet lag spleens had higher levels of proliferation compared to controls, regardless of the presence of mitogen (**Figure 2B**). As previously reported, in control spleens splenocyte proliferation was increased during the active phase compared to the rest phase (Drazen et al., 2001). However, an increase in proliferation following LPS was only seen in the active phase not the rest phase (**Figure 2B**). Because circulating T- and B-cells are highest during the rest phase, we may have reached a ceiling effect of proliferation during the rest phase

(Depres-Brummer et al., 1997). A similar effect was detected following ConA exposure, with an increase in splenocyte proliferation seen in jet-lagged spleens taken during the active phase compared to the rest phase. Unlike controls, jet lag spleens showed an increase in proliferation after LPS during both the active and rest phases.

It is important to note that the increased splenocyte proliferation seen in jet-lagged hamsters does not convey any information about the functional role of this higher rate of proliferation. At the present time, we do not know if the increase in B- and T-cells observed with jet lag indicates an enhancement of immunity. Future studies are needed to discern the functionality of this increase, specifically aimed at investigating pathogen-specific immune responses, as well as the impact of the HPA axis on splenocyte proliferation. It is also interesting to note that jet lag appears to have a dichotomous impact on various immune measures, by inhibiting total IgG, bacterial killing, and complement system activity while enhancing splenocyte proliferation. This contradictory effect is not unprecedented, as the catecholamines can have similarly differential effects on immune responses. Specifically, chemical sympathectomy increases the number of neutrophils, a measure of innate immunity, while decreasing specific immune response measures, such as total leukocyte number and antigen secretion by splenocytes (Rice et al., 2001). Mice lacking the dopamine transporter gene exhibit impaired cellular immunity in the form of mitogen-induced proliferation but enhanced humoral immunity (i.e. higher Th2-dependent IgG anti-OVA antibody) and innate macrophage activity (Kavelaars et al., 2005). Dopamine can also differentially activate T-cells based on their developmental state, with dopamine activating naïve or resting T-cells and inhibiting activated T-cells (Sarkar et al., 2010).

Regardless of the precise mechanism mediating the impact of jet lag on innate and adaptive immunity, the temporal disruption created by chronic phase advances has detrimental effects on the normal functioning of the immune system. Based on the present data, chronic disruption of circadian homeostasis suppresses total antibody production, the ability for the organism to fight off bacterial infections, and diminishes complement system activity while simultaneously increasing splenocyte proliferation, although the functionality of such enhancement remains to be determined. The higher incidence of disease seen in individuals who experience lives of circadian dysregulation may be associated with a subsequent dysregulation of the immune system prior to disease or pathogen exposure. These results suggest an important role for an intact circadian system in regulation of mammalian immunity.

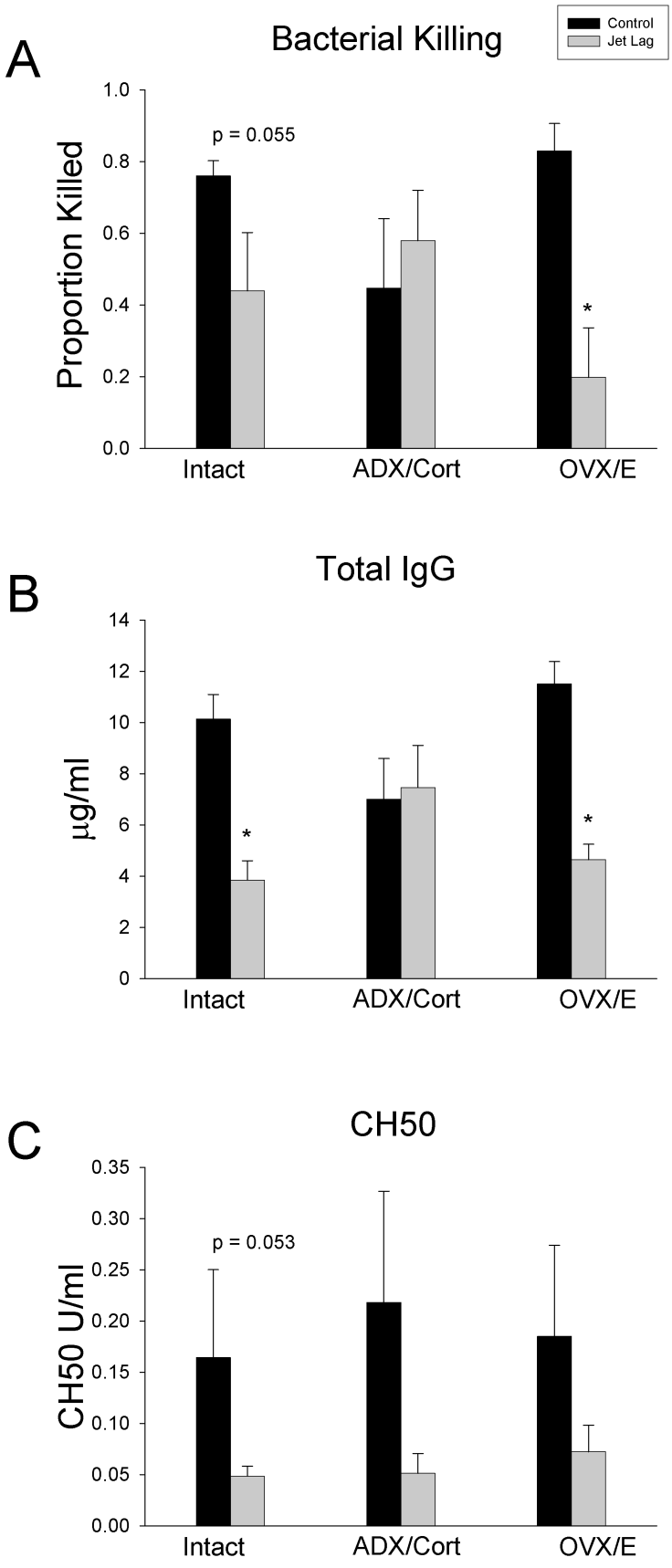
A
Bacterial Killing
Control
Jet Lag
Proportion Killed
1.0
0.8
0.6
0.4
0.2
0.0
p = 0.055
*
Intact
ADX/Cort
OVX/E
B
Total IgG
µg/ml
14
12
10
8
6
4
2
0
*
*
Intact
ADX/Cort
OVX/E
C
CH50
CH50 U/ml
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
p = 0.053
Intact
ADX/Cort
OVX/E

Figure 1. **Temporal Disruptions Alter Innate and Humoral Immune Responses.** Following repeated jet lag, bacterial killing ability (A), total antibody production (B), and complement system activity (C) are suppressed. Controlling for activation of the stress axis abolishes the suppression in bacterial killing ability (A) and total antibody production (B), suggesting that the jet lag-induced reduction in immune function is mediated by an upregulation in the glucocorticoids. * =significantly different than control animals within the same condition, $p<0.05$

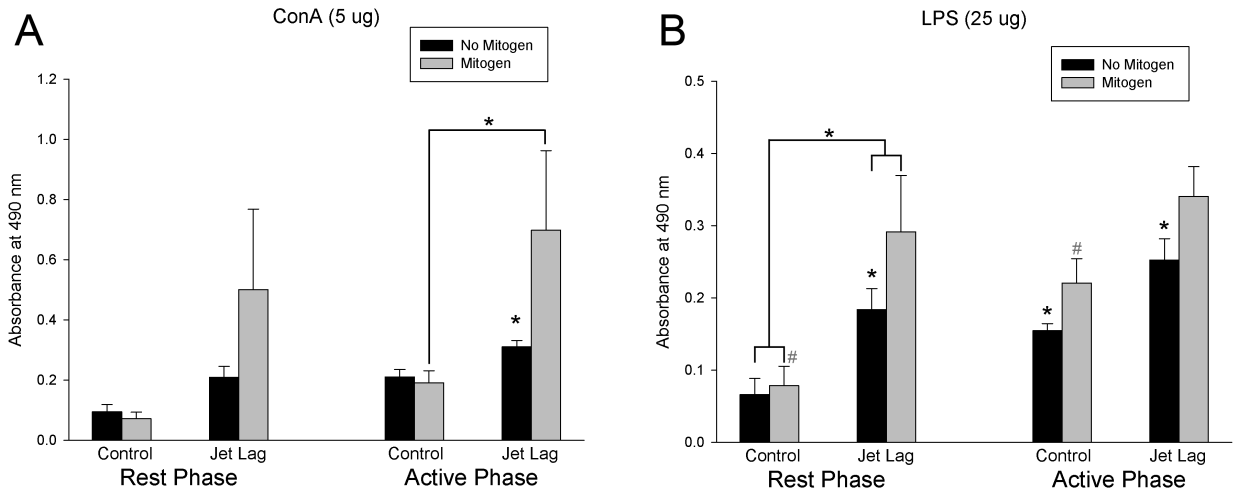

Figure 2. **Temporal Disruption Enhances B- and T-Cell Proliferation Following Mitogen Challenge.** (A) Addition of ConA to splenocytes stimulates proliferation of T-cells. Jet lag induced increased levels of splenocyte proliferation compared to controls, with a greater impact of mitogen exposure occurring in cells harvested during the active phase of the animal's activity/rest cycle. (B) LPS induces B-cell proliferation. There was a main effect of jet lag, with an enhancement of proliferation following phase shifts. Jet lag had a more marked impact on proliferation during the rest phase as compared to the active phase, suggesting that time-of-day may differentially influence how phase shifts impact splenocyte proliferation. Mitogen exposure impacted proliferation to a greater extent in control spleens harvested during the active phase compared to the rest phase # = significantly different from one another, $p<0.05$; * = significantly different, $p<0.05$

Chapter 5

Conclusions

Responses to natural light:dark cycles provide adaptive cues to animals, including humans, that allows for temporal organization of physiology and behavior. With the advent of artificial light and subsequent industrialization, the adaptive synchronizing cue that light formerly provided has become confounded. Societies now function on 24-hour schedules, requiring the rotating shift work schedules seen in doctors, nurses, and factory workers, and the globalization of our lives lends itself to consistent transmeridian travel by businessmen and women, flight attendants, and pilots. As a result, the coincidence of behavior with the light phase of the light:dark cycle in humans is becoming obsolete. This temporal incongruence has manifested as a higher incidence of psychological and physiological maladies, including reproductive deficits, cognitive abnormalities, and immune dysregulation (Ahlborg et al., 1996; Cho et al., 2000; Stevens et al., 2007). While most studies to date, indicate a strong correlation between dysregulation of circadian homeostasis and disruptions in homeostasis in general, the aim of the previous studies was to establish a cause-effect relationship between temporal disruptions and homeostatic deficits.

In Experiment 1, a recently identified putative modulator of the reproductive axis, RFRP (Kriegsfeld et al., 2006) was examined to determine if this factor was regulated by the master circadian clock, the SCN. Previous studies indicated that the SCN neurally controlled the stimulatory arm of the reproductive axis by stimulating the GnRH system that lead to the subsequent secretion of the gonadotropins from the anterior hypothalamus and alterations in the gonadal hormone milieu (de la Iglesia et al., 2003). We showed that not only was there a time-sensitive deactivation of the RFRP system during the same time window as an activation of the GnRH system and the LH surge, but using a model of splitting, we established that this inhibition of the RFRP system was mediated by the SCN. We then proposed that the role of the SCN is to concomitantly remove estrogen-mediated inhibition of the HPG axis through disinhibition of the RFRP system while simultaneously stimulating the positive arm of the reproductive axis through activation of the GnRH system.

Other studies from our lab (Williams et al., 2011), have suggested that the SCN not only regulates the GnRH and RFRP systems during the time of the LH surge, but also the upstream estrogen-sensitive modulator of the GnRH system, kisspeptin. The precise temporal pattern of GnRH and kisspeptin neuron activation and coordination with suppression of the RFRP system is associated with LH surge onset and ovulation. The purpose of Experiment 2 was to determine if disruptions of the circadian system through an animal model of jet lag, that more closely models the human experience of temporal disruptions, would result in a dysregulation in the timing of activation of the hypothalamic neuropeptide populations associated with reproduction. We found that while control animals exhibited the expected pattern of GnRH, kisspeptin, and RFRP activation/deactivation around the time of the LH surge, these patterns were completely abolished in jet-lagged animals. These data suggest that a potential mechanism for the reproductive deficits seen in women living lifestyles that involve shift work or chronic jet lag

may have abnormalities in the activational patterns of the hypothalamic peptides that precisely time ovulation.

In Experiment 3, we used the same experimental model of jet lag to elucidate the impact of circadian disruptions on brain structure and function. Female flight attendants who fly chronic transmeridian flights exhibit atrophy in their temporal lobes and deficits in hippocampal-dependent cognitive tasks (Cho et al., 2000; Cho, 2001). Similarly, hippocampal neural precursor cells harvested from clock knockouts exhibit alterations in cell proliferation and differentiation (Borgs et al., 2009b). In this study, we hypothesized that alterations to the circadian system may disrupt normal hippocampal cell proliferation and neurogenesis with subsequent alterations in learning and memory. In jet-lagged hamsters, cell proliferation was markedly decreased compared to non-jet-lagged controls, but this reduction was eliminated when you controlled for jet lag-induced activation of the stress axis. These data indicated that the decrease in hippocampal cell proliferation associated with jet lag was mediated by increased circulating concentrations of stress hormones. In contrast, the >50% reduction in neurogenesis seen in the jet lag condition was independent of changes in circulating concentrations of stress or gonadal hormones, suggesting that temporal disruptions alone may directly impact cell survival and differentiation. The marked decline in adult neurogenesis was also associated with deficits in learning and memory during the period of the jet lag. More interestingly, deficits in hippocampal learning and memory persisted for up to a month after the animals were returned to a static light:dark cycle, suggesting that temporal disruptions not only adversely impact the brain and brain function during the time of the phase shifts, but also long after its cessation.

Because many retrospective studies suggest an association between temporal incongruence and disease, we examined the impact of these disturbances on immune function. In Experiment 4, we used the same model of chronic jet lag in female Syrian hamsters and looked at the impact of these phase shifts on baseline immune function, independent of mitogen challenge or alterations to the HPA and HPG axes. Jet lag reduced the ability of the serum from these animals to fight bacterial infections and complement system activity, both measures of the innate immune system. Jet lag also impacted the adaptive immune response, with a reduction in total antibody production. However, jet lag-induced suppression of bacterial killing ability and total IgG production was abolished when increases in stress hormones were controlled, suggesting a glucocorticoid-mediated mechanism of suppression. Since we had previously shown that jet lag can impact cell proliferation, we wanted to see if the same model would alter proliferation in immune markers. Since lymphocytes exhibit 24-hour rhythms in secretion (Depres-Brummer et al., 1997) and molecular clock knockdown also alters lymphocyte proliferation (Sun et al., 2006), we investigated whether chronic jet lag would impact splenocyte proliferation. Both T- and B-lymphocyte proliferation was enhanced in jet-lagged hamsters compared to controls. We do not yet know if alterations to the innate and adaptive immune systems associated with chronic jet lag translate into functional difference in a mitogen-challenged immune system.

In conclusion, a mild model of temporal disruption that result in an incongruence between endogenous rhythms and the environment without complete abolishment of the circadian system is associated with alterations in neuronal activation in systems mediating reproductive function, deficits in cell proliferation and neurogenesis in the hippocampus and subsequent hippocampal function, and abnormalities in both innate and adaptive immune function and immune cell

proliferation. These data underscore the importance of further understanding the adverse impact of disruption of circadian homeostasis is imperative to understanding mental and physical health.

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