The Dynamics of Hypothalamic Cell Proliferation in Mice

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The Dynamics of Hypothalamic Cell Proliferation in Mice

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A Thesis in the Field of Biology

for the Degree of Master of Liberal Arts in Extension Studies

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Abstract

Adult neurogenesis is the process by which new neurons are generated by neural stem cells (NSC) and are integrated into existing neural circuits in the adult brain. Contemporary research in adult neurogenesis in mammals has primarily focused on the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus, as these regions have the most robust cell proliferation. However, there also is evidence for neurogenic activity in other areas, including in the hypothalamus, which is a master regulator, maintaining homeostasis in many physiological and behavioral functions. The present studies tested whether the circadian clock influences the rate of cell proliferation in the hypothalamus, and compared the overall rate of proliferation in the median eminence (ME) (a subregion of the hypothalamus) with the rest of the hypothalamus. Mice were injected with the S-phase marker 5-bromo-2'-deoxyuridine (BrdU). A quantitative analysis of the resulting BrdU-labeled cells in the hypothalamus revealed a pronounced diurnal pattern of cell proliferation; the ME maintains a rhythm of proliferation that is distinct from the rest of the hypothalamus. In a 72-hour longitudinal study after a single BrdU pulse, stable numbers of $BrdU^+$ cells were observed in the ME, with a corresponding increase in $BrdU^+$ cells in the rest of the hypothalamus. These data suggest that circadian signals regulate the timing of hypothalamic cell proliferation, and that newly generated cells in the ME either migrate away or die, maintaining the stable numbers of $BrdU^+$ cells in this region.

Dedication

I dedicate this thesis to my friend, my mentor, my make-believe sister, Carol Ann Paul. I will be forever grateful to her for setting me on this path and giving me the confidence to see this journey through to the end. I am saddened that she is not here to celebrate this accomplishment with me; she will forever live in my heart.

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Chapter I

Introduction

Adult Neurogenesis

Adult neurogenesis is the process by which new neurons are generated from precursor cells, and then integrated into existing neural circuits. Until a few decades ago, the concept of adult neurogenesis was met with great skepticism. The prevailing dogma was that following embryonic and early postnatal development, new neurons could not be added into the brain circuitry. It was thought that there was a fixed number of brain cells, and that this supply deteriorates as we age, possibly leading to neurodegenerative disease states. However two landmark studies demonstrated that newborn neurons are generated in the adult rat brain (Altman & Das, 1965) and that newborn neural cells in songbirds were functionally integrated into existing neural circuits (Paton & Nottebohm, 1984). Techniques for labeling and visualizing cells in S phase and the discovery of stem cells eventually changed the prevailing dogma. Today, it is accepted that neurogenesis does indeed occur in the adult brain, a process that has been demonstrated across multiple vertebrate (Altman & Das, 1965) and invertebrate species (Cayre, Malaterre, Scotto-Lomassese, Strambi, & Strambi, 2002), and even in humans (Eriksson et al., 1998).

Neural Stem Cells and the Neurogenic Niche

The current definition of pluripotent stem cells is that they have the capacity to continually self-renew and give rise to a wide array of cell types (<http://stemcells.nih.gov/info/

basics/Pages/Default.aspx>). These cells allow the renewal and replacement of somatic cells such as in our skin, blood, and liver (Goodell, Nguyen, & Shroyer, 2015). In cases of tissue damage or injury, stem cells are activated to generate new tissue for repair or replacement. In the central nervous system (CNS), neural stem cells (NSCs) have this same self-renewal capacity but are specialized to give rise exclusively to new neuronal or glial cell types (Reynolds & Weiss, 1992; Richards, Kilpatrick, & Bartlett, 1992).

In general, stem cells are maintained in microenvironments referred to as niches. The purpose of the niche is to sustain these cells in their quiescent state, and to regulate self-renewal and cell fate when called into action (Arai & Suda, 2008). NSCs, too, reside in neurogenic niches: self-contained neuroanatomical structures that are highly vascularized (Putnam, 2014) and serve to house, maintain, and support the NSC's ability to renew and replenish the brain (Zhao, Deng, & Gage, 2008). In mammals, niche structures have been found in two discrete regions of the adult brain, in the subventricular zone (SVZ) of the lateral ventricle (Reynolds & Weiss, 1992; Richards et al., 1992) and in the subgranular zone (SGZ) of the dentate gyrus of the hippocampus (Gage et al., 1995; Palmer, Takahashi, & Gage, 1997). New cells produced in the SVZ migrate, mature, and disperse throughout the olfactory bulb (Alvarez-Buylla & Garcia-Verdugo, 2002) and are thought to be important for detecting environmental odor changes and supporting olfactory memory (Rochefort, Gheusi, Vincent, & Lledo, 2002). In the hippocampus, the continuous addition and integration of new neurons into the existing circuitry plays a role in pattern separation of memories (Deng, Aimone, & Gage, 2010; Sahay, Wilson, & Hen, 2011).

Crayfish, Neurogenesis and the Immune System

From an evolutionary standpoint, mechanisms of adult neurogenesis are highly conserved (Cayre et al., 2002; Sullivan, Benton, Sandeman, & Beltz, 2007). In mammals, the traditional understanding is that NSCs are self-renewing and give rise to new neurons (<http://stemcells.nih.gov/info/basics/Pages/Default.aspx>). These neural stem cells are programmed to divide asymmetrically such that one daughter cell remains a stem cell (the self-renewal event) while the other is fated to divide, differentiate, and integrate as a new cell into existing neural circuits (Suh et al., 2007). In decapod crustaceans, 1st-generation neuronal precursors (functionally analogous to NSCs) (Sullivan, Sandeman, Benton, & Beltz, 2007) are responsible for the generation of new neurons. These cells reside in a neurogenic niche. New neurons are added in two distinct brain structures in the crayfish brain, local interneuron cluster 9 and projection interneuron cluster 10 (analogous to mitral and tufted cells in the olfactory bulb of mammals) that innervate the olfactory and accessory lobes (Kim, Sandeman, Benton, & Beltz, 2014). These regions are functionally analogous to the mammalian olfactory bulb and higher order-processing centers, respectively (Schmidt & Demuth, 1998; Sullivan & Beltz, 2005).

Interestingly, 1st-generation neural precursors in the crayfish *Procambarus clarkii* are not self-renewing and must be replenished from an extrinsic source (Benton, Chaves da Silva, Sandeman, & Beltz, 2013; Benton, Zhang, Kirkhart, Sandeman, & Beltz, 2011). Benton et al. demonstrated that all neural precursor cells populating the neurogenic niche are derived from the innate immune system and access the niche by way of the circulatory system (Benton et al., 2014). Comparative and genomic analyses reveal remarkable similarities between vertebrate and invertebrate animal models regarding the genetic, cellular and mechanistic activity that underlie brain development (Cayre et al., 2002).

Blood and Neurons: What do they have in common?

Hematopoietic niches (of mesodermal origins) (Anthony & Link, 2014) and neurogenic niches (of ectodermal origins) (Gage, 2000) share many common molecular and regulatory pathways necessary for the maintenance and control of their respective stem cells. One suggestion is that emergence of both of these niches may have involved neural crest derived cells during development (Coste, Neirinckx, Gothot, Wislet, & Rogister, 2015). By drawing parallels between the hematopoietic and the nervous systems and examining commonalities between tissues that share developmental origins, our understanding of the relationship between the immune system and adult neurogenesis will be expanded.

In mammals, the hematopoietic niche is within the bone marrow, the principle blood-manufacturing center (Hoffman & Calvi, 2014). The niche houses hematopoietic stems cells (HSC) and mesenchymal stem cells (MSC) that give rise to all blood cell types (e.g. red cells, white cells, and platelets) that are necessary to sustain life and maintain immune health.

The idea that bone marrow-derived blood cells can give rise to new neurons (transdetermination) is not novel (Brazelton, Rossi, Keshet, & Blau, 2000; Cogle et al., 2004; Mezey & Chandross, 2000; Mezey, Chandross, Harta, Maki, & McKercher, 2000; Mezey et al., 2003; Woodbury, Schwarz, Prockop, & Black, 2000). Mezey et al. demonstrated that when mice deficient in bone marrow cells (animals that die within 48 hours after birth) received transplanted bone marrow cells from healthy mice, these animals survived. These transplanted cells were able to migrate into the brain, differentiate into neurons, integrate into existing neural circuits, and ultimately express neuron-specific markers (Mezey et al., 2000). While this report and others have demonstrated bone marrow as a source of neuronal precursors, the concept has been met with great skepticism. The primary concern drawn from these studies is the lack of evidence that

this phenomenon occurs under natural conditions and that entry of blood cells into the CNS in these studies was made possible only due to a disruption of the blood brain barrier (BBB), thereby making it unnaturally permeable.

Yet in another study, Dennie et al. demonstrated that bone marrow derived cells contribute to increases in neuronal and microglial cell populations in the adult mammalian brain. Using an SV40-derived vector, a recombinant simian virus used for gene delivery to bone marrow progenitor cells, these studies showed that after several months transduced bone marrow progenitors migrated and differentiated into mature neurons in the dentate gyrus of the hippocampus. What sets these studies apart from those mentioned above is that these experiments were conducted in adult animals with an uncompromised (i.e., non-lesioned; non-irradiated) CNS (Dennie, Louboutin, & Strayer, 2016)

The Hypothalamus and its Neurogenic Properties

Contemporary research in adult mammalian neurogenesis has primarily focused on the SVZ and SGZ as these regions demonstrate the most robust and consistent cell proliferation. However, several reports have revealed evidence of neurogenic activity outside of the SVZ and SGZ. The advancement of immunohistochemical labeling techniques, particularly for 5-bromo-2'-deoxyuridine (BrdU), a synthetic analog of thymidine that is incorporated into the cell during DNA replication (Kuhn, Dickinson-Anson, & Gage, 1996), has facilitated studies of many regions of the brain for indications of neurogenesis. Indeed, evidence of adult neurogenesis has been shown in several forebrain regions as well as the visual cortex (Luskin & Shatz, 1985), the striatum (Ernst et al., 2014), and the hypothalamus (Kokoeva, Yin, & Flier, 2005; Lee & Blackshaw, 2012; Migaud et al., 2010). These findings suggest neurogenesis is a more

widespread process than first believed. Neurogenic regions outside the SVZ and SGZ tend to sustain proliferative activity but at markedly lower levels. For instance, unlike the labeling patterns of the SVZ and SGZ that show a dense population of newborn cells along the lateral ventricle and the granular layer of the hippocampus, respectively, the labeled cells of the hypothalamus tend to be widely distributed throughout the parenchyma, decreasing in density the greater the distance from the third ventricle (Pencea, Bingaman, Wiegand, & Luskin, 2001). Despite the apparent diminished neurogenic activity in the hypothalamus as compared to the SVZ and SGZ, its potential significance should not be underestimated.

In relation to potential extrinsic sources of NSCs, hypothalamic neurogenesis is intriguing. The hypothalamus has an intimate relationship with the body; it serves as the link between the central and the peripheral nervous systems, particularly as a critical regulator of neuro-endocrine communication. Anatomically, the hypothalamus sits at the ventral-most part of the brain and has direct access to the periphery via the pituitary gland. As part of the hypothalamus-pituitary-adrenal (HPA) axis, the hypothalamus is vitally important for survival as it maintains homeostasis by acting as a regulator of many physiological and behavioral functions such as heart rate, blood pressure, metabolism, body temperature, hunger, thirst, sleep cycles, circadian rhythms, and mood. Regulation of these functions relies on the secretion of numerous hypothalamic factors, all of which pass through the hypothalamic-pituitary vascular portal system. The neuro-endocrine nexus is a highly vascularized region that is controlled by a complex feedback system (Lanzer & Topol, 2002). Hormones that are targeted for the pituitary gland are released from neuronal terminals of the hypothalamus in response to homeostatic changes. These hormones are able to rapidly reach their target tissues via the rich capillary network of the median eminence (ME). The ME is positioned beneath the third ventricle at the

base of the hypothalamus and connects the hypothalamus to the pituitary gland. A rich vascular supply is crucial for maintaining proper homeostatic function. Indeed, blood flow to the HPA axis, specifically the pituitary gland, is not only remarkably stable, but is the highest of any tissue in the body (Lanzer & Topol, 2002).

Recent studies have identified a population of candidate neural progenitor cells known as tanycytes located in the hypothalamus (Lee & Blackshaw, 2012; Perez-Martin et al., 2010). Interestingly, this hypothalamic region seems to share common features with that of the well-characterized neurogenic niches of the SGZ and SVZ. Much like the SVZ's proximity to the lateral ventricle, this region in the hypothalamus lies adjacent to the lateral walls of the third ventricle (Xu et al., 2005). Other commonalities between the hypothalamic niche and the SVZ and SGZ are that these regions are highly vascularized and exhibit similar cellular and extracellular architectural patterns (Rojczyk-Golebiewska, Palasz, & Wiaderkiewicz, 2014). Furthermore, tanycytes express the neural stem cell marker nestin (Wei et al., 2002) and doublecortin-like protein (DCL) (Saaltink, Havik, Verissimo, Lucassen, & Vreugdenhil, 2012), as well as several neural stem cell-specific genetic markers (Lee & Blackshaw, 2012; Rojczyk-Golebiewska et al., 2014).

Due to the hypothalamus' role as a master regulator, it seems plausible that these newly generated cells are involved in metabolic activity. Indeed, Bless et al. demonstrated a relationship between hypothalamic neurogenesis and energy homeostasis in female mice (Bless, Reddy, Acharya, Beltz, & Tetel, 2014). In another study, dynamic neurogenic activity of tanycytes was observed in mice following dietary changes, further suggesting that hypothalamic neurogenesis contributes to metabolism and energy balance (Lee et al., 2012). While there is

evidence that tanycytes in the hypothalamus have NSC properties (Perez-Martin et al., 2010), the general consensus is that they are not responsible for all hypothalamic adult neurogenesis.

A Low Rate of Neurogenesis in the Hypothalamus

While only a single injection of BrdU is required to label proliferating cell populations in the SGZ or the SVZ, a standard approach for examining adult neurogenesis in the hypothalamus in mice involves infusion of BrdU into the lateral ventricles of the brain for one to two weeks (Kokoeva et al., 2005; Kokoeva, Yin, & Flier, 2007; Pierce & Xu, 2010). The literature suggests a requirement for long-term administration of BrdU because the hypothalamus is resistant to labeling. However, this is surprising because of its highly vascularized nature (Lanzer & Topol, 2002) and proximity to the periphery, and the fact that nucleosides readily cross the BBB (Taupin, 2007). Thus, alternative explanations for the lack of short-term labeling are a circadian influence (e.g., that cells go through S-phase during a restricted window and that the BrdU-labeling opportunity is therefore limited), or a delay in the arrival of labeled precursors from a source outside the hypothalamus. The overarching questions are: (1) does the circadian clock influence the rate of proliferation in the hypothalamus; (2) do neural stem cells responsible for adult neurogenesis in the hypothalamus reside in the brain, as currently proposed, or do these neural precursors come from an extrinsic source?

Does cell proliferation in the hypothalamus operate under circadian control?

Circadian rhythms are essential to an animal's survival as they control many biological functions such as hormone production and release (Weitzman, 1976), sleep-wake cycles (Saper, Lu, Chou, & Gooley, 2005), body temperature regulation (Saper et al., 2005), hunger and thirst

(Van den Pol & Powley, 1979), as well as cell replication and repair (Matsuo et al., 2003). Contemporary studies are now looking at the influence of the circadian clock and light cycles on adult neurogenesis. In one study that observed cell proliferation in the lobster brain, where $BrdU^{+}$ cells can be easily and accurately counted, circadian control of neurogenesis was observed. The greatest level of cell proliferation in these nocturnal animals occurred during the transitional period between light and dark (dusk), while the least occurred at the end the dark phase (dawn) (Goergen, Bagay, Rehm, Benton, & Beltz, 2002) . Other studies in mammals have shown that the circadian clock has an effect on adult neurogenesis and cell survival (Holmes, Galea, Mistlberger, & Kempermann, 2004; Rakai, Chrusch, Spanswick, Dyck, & Antle, 2014), regulation and expression of clock genes (Gilhooley, Pinnock, & Herbert, 2011; Holmes et al., 2004; Malik, Kondratov, Jamasbi, & Geusz, 2015), as well as the cell cycle time (Bouchard-Cannon, Mendoza-Viveros, Yuen, Kaern, & Cheng, 2013). However, in another study, circadian control over the rate of cell proliferation in the dentate gyrus of the hippocampus as a whole was not observed, but a circadian influence was found in the hilus (Kochman, Weber, Fornal, & Jacobs, 2006).

The influence of the circadian clock on cell proliferation in the adult hypothalamus has not been examined, but is of particular interest because this region is a master regulator of physiologies and functions that rely heavily on the circadian clock. To enlighten this issue, a series of experiments were conducted to examine whether the rate of cell proliferation fluctuates over the course of a 24-hour light/dark cycle. Does light (or lack of) effect the rate of cell proliferation in the hypothalamus, as was observed in the lobster brain? Or, does cell proliferation in the hypothalamus operate at a steady rate as was observed in the dentate gyrus of the hippocampus (Kochman et al., 2006)? An understanding of how cell proliferation in the

hypothalamus changes over the circadian cycle allowed for the optimization of BrdU administration for the longitudinal study that looked at hypothalamic cell proliferation over a three-day period of time.

Are adult-born hypothalamic cells supplied from an extrinsic source?

The present study also tested whether newborn cells or their precursors in the hypothalamus might originate in an extrinsic source by conducting a longitudinal study that examined changes in the number of BrdU-labeled in the hypothalamus over 72-hours. The work of Benton et al. demonstrating that 1st-generation neuronal precursors in the crayfish model *P. clarkii* are derived from the immune system, and access the brain by way of the circulatory system (Beltz, Zhang, Benton, & Sandeman, 2011), laid the groundwork for this study. In this crayfish study, a single-pulse of BrdU labeled dividing cells in the neurogenic niche. Five to seven days following BrdU administration, labeled cells were no longer present in the niche because the labeled cells had divided and their daughters had migrated away, and BrdU was no longer available for renewed labeling. Remarkably, however, 8 days after the initial pulse of BrdU and long after the 2 day clearing time for BrdU in crayfish (Benton et al., 2011), labeled cells appeared in the niche once again (Benton et al., 2014). This discontinuity in the appearance of BrdU^{+} cells in the niche is strong evidence for an extrinsic source of precursors, as the second appearance of labeled cells is interpreted as the arrival of cells that were BrdU-labeled while in the source tissue, and whose arrival in the niche was therefore delayed. The purpose of the longitudinal study in mice was to test the idea of an extrinsic source of NSCs in a clinically relevant model. The hypothalamus is the focus of these studies because of its proximity to the

circulation and the low level of neurogenesis compared with the SGZ and SVZ, which allows for direct counts of all cells in this region.

Evidence of constitutive neurogenesis in the hypothalamus has been reported using the standard approach of a single-pulse injection of BrdU (Migaud et al., 2010). Other studies have adopted a more prolonged approach for BrdU administration that requires multiple peripheral injections (Perez-Martin et al., 2010; Xu et al., 2005), or continuous intracerebroventricular infusions (Kokoeva et al., 2005, 2007; Pierce & Xu, 2010) as a more reliable method for obtaining robust proliferative responses in the hypothalamus. The present studies tested whether the apparent resistance to BrdU-labeling is due to a requirement for more BrdU or, alternatively, to a delay between BrdU administration and the appearance of precursor cells in the hypothalamus (i.e., a time-dependent rather than concentration-dependent function).

Summary

The circadian and longitudinal studies that compose this thesis examine basic properties of adult neurogenesis in the hypothalamus, about which very little is known. The goal of these experiments was to provide an explanation for the low rate of neurogenesis observed in the hypothalamus compared to the SVZ and SGZ. These experiments test two hypotheses: (1) that circadian factors influence the rate of proliferation in the hypothalamus, (2) that some neural precursors responsible for adult neurogenesis come from an extrinsic source.

Chapter II

Materials and Methods

Animals

C57BL/6 male mice were bred and raised at the Wellesley College Animal Facility (Wellesley, MA). Adult mice, age 14-15 weeks, were housed three or four animals per standard rodent cage. Animals were maintained in an environmentally controlled room at 22-24°C on a 12/12-hour light/dark cycle. Food and water was provided *ad libitum*. All animals were handled and maintained according to the guidelines established by Wellesley College's Institutional Animal Care and Use Committee (IACUC).

BrdU Treatments and Brain Collection

BrdU treatment was used to test for differences in hypothalamic cell proliferation. There have been concerns that some BrdU incorporation may be the result of DNA repair. However, irradiation studies, which induce DNA repair mechanisms, did not show an increase in the number of BrdU-labeled cells following treatment (Mizumatsu et al., 2003).

BrdU was prepared at a concentration of 15 mg/mL dissolved in sterile saline and administered by intraperitoneal (i.p.) injection to each animal at a dose of 50 mg/kg body weight for the circadian study (Aim 1), and at a dose of 100 mg/kg body weight for the longitudinal study (Aim 2), a dosage range that reliably labels dividing cells in mouse (Pan, Wang, & Xia, 2013). The experimental schedule is outlined in Tables 1 and 2.

Aim 1 tested for circadian influences on adult neurogenesis by asking whether cell

proliferation (i.e., BrdU incorporation) varies at different times during the light-dark cycle. Groups of mice received a single i.p. injection of BrdU at a designated time point (Table 1), and were sacrificed two hours later[1]. This two-hour window accommodated the 0.5-1 hour clearing-time for BrdU in mice (Duque & Rakic, 2011), and allowed sufficient labeling time for SVZ and SGZ cells used as the control. Aim 2 was a longitudinal study testing for a discontinuity or delay in the appearance of labeled cells in the hypothalamus following BrdU treatment. Each mouse received a single injection of BrdU at time zero, followed by sampling at 2, 6, 12, 18, 24, 36, 48, and 72 hours (Table 2)[2]. Injections were administered at the time of day (time zero) when BrdU incorporation was reaching its maximum according to the circadian study (i.e., 3 hrs. before the onset of the dark phase).

Table 1: Aim 1 injection and brain collection schedule. Mice were divided into six groups. Each mouse received a single i.p. injection of BrdU @ 50 mg/kg body weight and was sacrificed 2 hours later. Injection and brain collection sequence proceeded over a 24-hour period. Mice were sacrificed by transcardial perfusion, and brains harvested and sectioned for IHC processing.

Light Cycle												
BrdU Injection												
Time	0:00	2:00	4:00	6:00	8:00	10:00	12:00	14:00	16:00	18:00	20:00	22:00
Brain Collection												

[1] Efforts were made to sample animals at two hours post-BrdU injection. To allow for sufficient time between perfusions, animals in the same test groups received BrdU injections in a staggered manner by up 15 minutes. On occasion, sampling times exceeded 2 hours by ≤ 10 minutes.
[2] Efforts were made to sample animals exactly at their designated test time. On occasion, targeted sampling times were exceeded by ≤ 30 minutes.

Table 2: Aim 2 injection and brain collection schedule. Mice each received a single i.p. injection of BrdU @ 100 mg/kg body weight at time zero. At each of eight time points after injection (2 hours, 6, 12, 18, 24, etc.) over a 72-hour period, mice were sacrificed by transcardial perfusion, and brains harvested and sectioned for IHC.

Hours	0	2	6	12	18	24	36	48	72
Injection & Brain Collection									

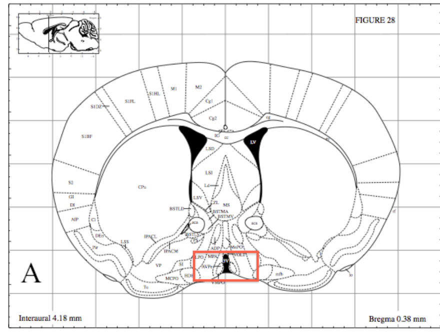
Histological Procedures and Immunocytochemistry

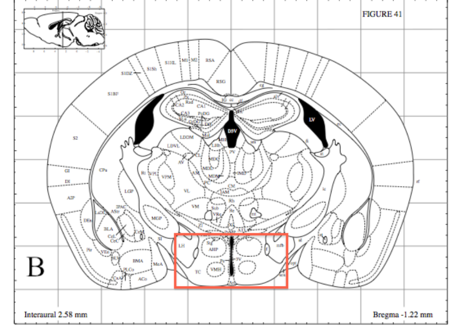
Brains were sectioned (50µm thick) through the hypothalamus (bregma 0.38 to -2.70 mm) using a freezing microtome; sections were collected and stored at -20°C in a cryoprotectant solution. Sections included the SVZ of the lateral ventricle and SGZ of the hippocampus that served as positive controls. Free-floating sections were processed immunohistochemically (IHC) for BrdU using rat monoclonal anti-BrdU (Accurate, Westbury, NY) at a concentration of 1:400. Processing for visualization was done using Alexa Fluor 488 goat anti-rat IgG at a concentration of 1:200 (Life Technologies), followed by nuclear labeling with propidium iodide (PI) (Life Technologies) at a concentration of 1 µg/mL in PBS. Following the IHC protocol, sections were mounted on gel coated frosted slides, air-dried and coverslips applied with Fluoro-Gel mounting medium (Electron Microscopy Science, Hatfield, PA).

Quantification

The number of $BrdU^+$ cells in the hypothalamus of each 50µm thick coronal section was counted using a Nikon 80i fluorescent microscope. In order to eliminate any bias, the quantification process was performed under blinded-conditions. The hypothalamus was defined by three parameters: (1) rostral to caudal, bregma 0.38 to -2.70 mm, (2) ventral to dorsal, the

ventral most region of the brain to the upper most limit of the third ventricle, (3) the lateral boundaries extended to the optic tracts and medially to the walls of the third ventricle (Fig. 1).





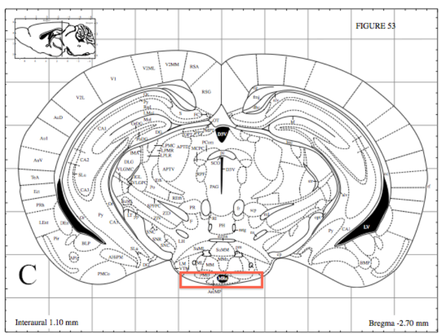


Figure 1: Representative schematic drawings of coronal mouse brain sections spanning anterior (A), medial (B), and posterior (C) regions of the hypothalamus (bregma 0.38 to -2.70 mm); red boxes indicate region of interest. Each 50μm coronal brain section was analyzed throughout the entire hypothalamus and counts of $BrdU^+$ cells recorded. (Images modified from (Paxinos, Franklin, & Franklin, 2001)).

Statistical Analysis

All statistical analyses were performed with JMP Statistical Software (SAS). Comparisons of significant differences in the mean number of $BrdU^+$ hypothalamic cells between groups were done using a one-way analysis of variance (ANOVA). Analysis of covariance (ANCOVA) and regression analyses using fit models were performed as appropriate. Differences were considered statistically significant at $p \leq .05$.

Chapter III

Results

Circadian Effects on Hypothalamic Cell Proliferation

These experiments tested the hypothesis that the circadian clock influences hypothalamic cell proliferation in adult mice maintained in a 12:12 light/dark cycle. Mice were divided into six groups (n = 4-6/group), each group representing one of six zeitgeber times (ZT) (ZT 0, 4, 8, 12, 16 & 20). ZT0 represents the transitional period between the light phase and the dark phase, while ZT12 represents the transitional period between the dark phase and the light phase. Mice were injected with the thymidine analog 5-bromo-2′deoxyuridine (BrdU) and sacrificed two hours later. Brains were processed for BrdU and propidium iodide (PI), and analyzed for cell proliferation within the hypothalamus (HYP). Cell counting was conducted using a Nikon 80i fluorescent microscope. The subventricular zone (SVZ) and subgranular zones (SGZ), which are known for a high rate of constitutive cell proliferation, were used as positive controls for BrdU labeling (Fig. 2A). The hypothalamus also showed reliable labeling by two hours post-BrdU injection (Fig. 2C).

The number of $BrdU^+$ cells in the hypothalamus showed a diurnal pattern with the greatest number of labeled cells observed at the transition between light and dark and continuing through ZT4. The fewest BrdU-labeled cells were labeled at the end of the light period as the animals were transitioning from the light phase into the dark phase, and continued through the first five hours of the dark period (ZT12 and ZT16) (Fig. 3A). A quadratic regression was calculated as a predictor of hypothalamic cell proliferation based on ZT resulting in a quadratic

term of $p = .05$ ($F(2, 30) = 3.30$) (Fig. 3B), suggesting that the rate of hypothalamic cell birth is influenced by circadian factors.

The mean number of BrdU-labeled cells per section was also calculated in order to correct for any damaged sections or loss of hypothalamic tissue. The same circadian pattern of $BrdU^+$ cells was observed supporting a strong diurnal pattern of cell birth in the hypothalamus (Fig. 4).

Does the median eminence have a circadian rhythm distinct from the rest of the hypothalamus?

The median eminence (ME) is a highly proliferative sub-region of the hypothalamus (Lee et al., 2012). The ME lies in the ventral-most region of the hypothalamus and its volume occupies approximately 1/200 the entire hypothalamus (Fig 5).

To correct for any loss or tissue damage to the ME, the mean number of $BrdU^+$ cells per section was calculated (Fig 6A). A quadratic regression showed that changes in the rate of cell proliferation in the ME across the light-dark cycle were not significantly different ($F(2, 30) = 0.71$, $p = .50$). Nevertheless, the proliferation of new cells in the ME across the 24-hour period showed an overall pattern contrasting with that of all other regions of the hypothalamus. At ZT0, the period when the animals were transitioning from the dark to the light phase, the ME showed the lowest number of $BrdU^+$ cells per section, the time when the greatest number of $BrdU^+$ cells was observed in the remaining regions of the hypothalamus. At ZT16 the mean number of $BrdU^+$ ME cells per section showed the highest rate of cell birth, again opposite to the pattern observed in all other regions of the hypothalamus (Figs. 6A & B). The rate of BrdU labeling in the ME maintained a steady state throughout the remainder of the light cycle and into the beginning of the dark phase, gradually declining through the remainder of the dark cycle (Fig. 6A). If the

mean number of $BrdU^+$ ME cells is subtracted from the mean number of $BrdU^+$ cells in the entire hypothalamus, the diurnal pattern observed in the rest of the hypothalamus resembles the overall pattern shown previously (compare Figs. 6B & 3). Overall, these data show a strong circadian influence on cell proliferation in hypothalamic areas outside of the ME, as well as a possible contrasting circadian rhythm within the ME (Figs. 6A & B).

As stated above, the ME occupies a relatively small volume of the hypothalamus (~1/200), yet exhibits the most robust level of cell birth. To normalize the labeled cell counts per section of tissue, the percentage of $BrdU^+$ cells in the ME relative to that in the remainder of the hypothalamus was calculated and graphed. The mean number of labeled cells in the ME accounts for between a minimum of 33% (ZT0) and a maximum of 57% (ZT16) of hypothalamic cell labeling, demonstrating that the ME is the most proliferative region per volume of hypothalamic tissue (Fig. 7).

Longitudinal Study: Quantification of BrdU-labeled Cells in the Hypothalamus Over a 3-day Period

This experiment tested the hypothesis that newborn cells or their precursors in the hypothalamus might originate in an extrinsic source and that over a 72-hour test window an increase in BrdU-labeled cells would be observed in the hypothalamus, due to ongoing cell proliferation and the addition of new cells. Therefore, in order to observe the dynamics of cell proliferation in the hypothalamus over a 72-hour period, a group of mice (n = 32) was administered a single intraperitoneal (i.p.) injection of BrdU (time zero) and sampled at 2, 6, 12, 18, 24, 36, 48 and 72 hours post-injection (n = 4/time point) (Fig. 8A). Injections were conducted at the end of the dark phase (ZT20), the time of day when BrdU incorporation was reaching its

maximum according to the circadian study (Fig. 3A). Because all mice were labeled at the same time and the clearing time for BrdU is 0.5-1 hour (Duque & Rakic, 2011), the resulting BrdU incorporation observed over 72 hours takes into account cell divisions during this period, as well as factors that may influence the labeled cell populations (e.g., cell migration, cell death, incorporation of cells from an extrinsic source).

Mean $BrdU^+$ cell counts in the hypothalamus for the eight sampling times ranged from 401 to 652; there were no statistical differences between the mean values for the various sampling times. A one-way ANOVA showed that differences in the number of BrdU-labeled cells in the hypothalamus among the groups of mice across the eight sampling times was not significant ($F(7,24) = 0.50$, $p = .83$) (Fig. 8A). To correct for any loss or tissue damage to the hypothalamus, the mean number of $BrdU^+$ cells per section was calculated; nevertheless, the same pattern of $BrdU^+$ cell labeling across the 72-hour period persisted (Fig. 8B).

It was perhaps surprising that at the last sampling time (72-hrs post-injection), the mean number of BrdU-labeled cells in the hypothalamus was not significantly different from the number of labeled cells observed at 2 hours post-injection ($t(6) = 0.85$, $p = .43$). An approximate doubling in cell counts in the hypothalamus was expected, due to S-phase labeled cells completing mitosis and exiting the cell cycle. Cells in various phases of mitosis (Figs. 9A; 10A & 10C) and newly divided cells (Figs. 9B; 10A & 10B) were observed as early as 12 hours post-BrdU injection in widespread regions of the hypothalamus, including the ME.

Our circadian experiment reported above suggests that cell proliferation in the median eminence operates on a circadian rhythm that contrasts with that of the hypothalamus. To test whether the patterns of labeling were also distinct in the longitudinal study, independent cell counts of $BrdU^+$ cells in the ME were also conducted. To correct for any loss or tissue damage to

the ME, the mean number of $BrdU^+$ cells per ME section was calculated (Fig. 11A) and counts were not statistically different over the 3-day period ($F(7,24) = 0.85$, $p = .91$). The mean number of $BrdU^+$ ME cells per section remained fairly stable with cell counts ranging between 3.1-4.0 cells per section of tissue over the first seven sampling times (2, 6, 12, 18, 24, 36 & 48 hours). And although these values are not statistically different, at 72-hours post-injection the mean number of $BrdU^+$ ME cells per section decreased to approximately 70% of the value at two hours post-BrdU injection (Fig. 11A). To reveal the number of labeled cells in the hypothalamus independent of those contributed by the ME, the mean number of $BrdU^+$ cells in the hypothalamus per section minus those in the ME per section (HYP minus ME) was calculated and graphed (Fig. 11B).

Divergent BrdU cell-labeling patterns over the 72-hour period were observed when the mean number of $BrdU^+$ ME cells per section was compared with the mean number of labeled cells per section in the rest of the hypothalamus (HYP minus ME) (Fig. 12). A one-way ANCOVA was conducted and a significant interaction was found between the average number $BrdU^+$ cells in the ME per section and those in the hypothalamus minus the ME (HYP minus ME) based on sampling time ($F(1, 60) = 4.84$, $p = .03$). The mean number of $BrdU^+$ cells in the ME per section remained relatively unchanged across the 72-hours testing period. In contrast, the hypothalamus not including the ME (HYP minus ME), the mean number of $BrdU^+$ cells per section shows an upward trend line as the average number of labeled cells increased over the 72-hour experiment, although there were no statistically significant differences among these groups (HYP-ME: ($F(7, 24) = 0.65$, $p = .71$); ME: ($F(7, 24) = 0.85$, $p = .91$)) (Fig. 12).

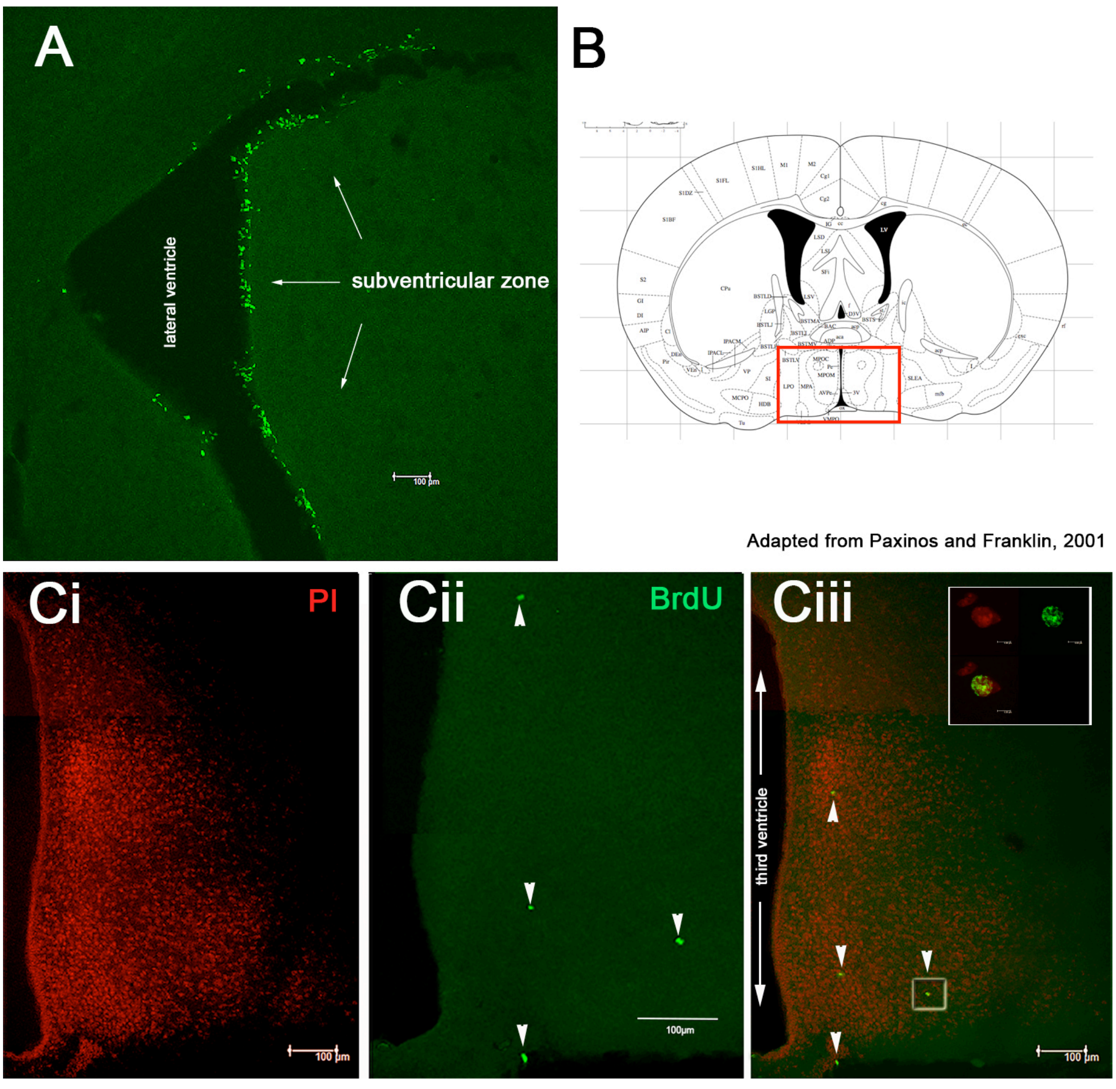


Fig. 2. Representative confocal images depicting regions of interest 2 hours post-BrdU injection. (A) BrdU-labeling of the SVZ of the lateral ventricle. (B) Schematic drawing of a coronal mouse brain section showing the hypothalamus (region of interest; red box). (C) Coronal hemi-brain section through the hypothalamus (bregma -2.00mm) showing a dispersed $BrdU^{+}$ cell-labeling pattern. Ci: propidium iodide (PI) (red); Cii: BrdU (green) (imaged magnified by 60% to highlight labeled cells); Ciii: Composite with inset showing an enlargement of a $BrdU^{+}$/PI co-labeled cell. White arrowheads denote $BrdU^{+}$ cells; A & C scale bars = 100μm; inset scale bar (Ciii) = 6μm.

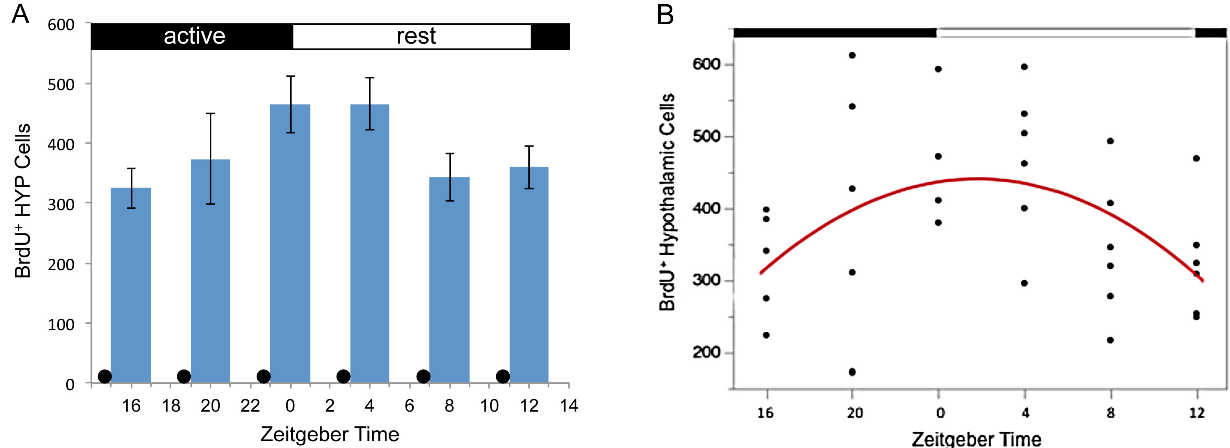


Fig. 3. (A) Hypothalamic cell proliferation showed a pronounced diurnal pattern across the 24-hour light-dark cycle (zeitgeber time). Adult mice (n = 4-6/group) were injected with BrdU (black dot) and sacrificed two hours later. Each bar represents the mean (± SEM) number of BrdU$^+$ cells at each of six evenly spaced 2-hr time periods. Horizontal black bars represent the dark phase (physically active period); white bar represent the light phase (rest period). (B) A quadratic regression was calculated as a predictor of hypothalamic cell proliferation based on zeitgeber time and was found to be significant ($F(2, 30) = 3.30$, $p = .05$), with an R^2 of .18.

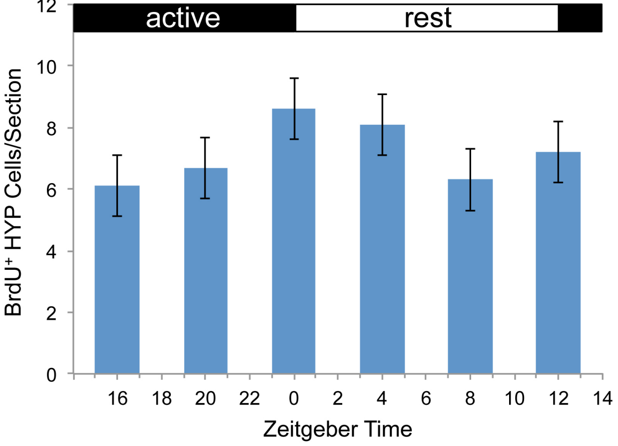


Fig. 4. A diurnal pattern of hypothalamic cell proliferation is maintained when the number of BrdU$^+$ cells is calculated per section. Horizontal black bars represent the dark phase (active period); white bar represent the light phase (rest period); error bars ± SEM.

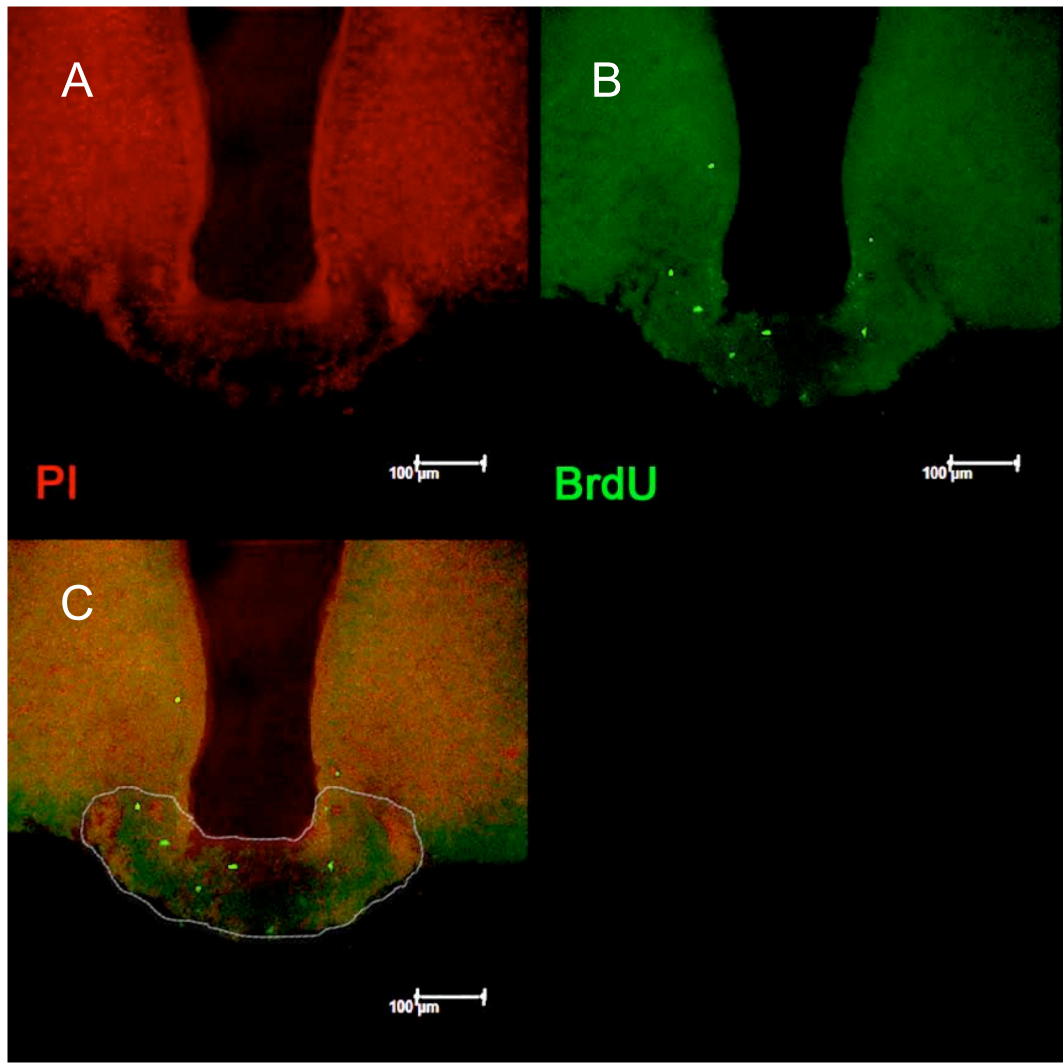


Fig. 5. Representative confocal image of a coronal section through the hypothalamus at the level of the median eminence (approx. Bregma -2.00 mm). Note the relatively high density of $BrdU^+$ cells within the ME (outlined in C), as compared to the rest of the hypothalamus. A: propidium iodide (PI) (red); B: BrdU (green); Scale bars = 100μm.

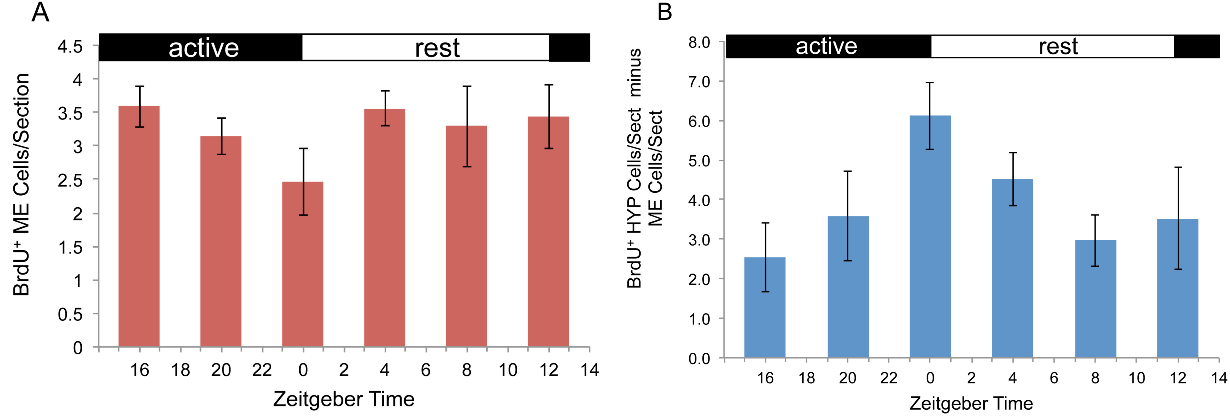


Fig. 6. Cell proliferation in the ME (A) and all other hypothalamic regions (ME minus HYP) (B) show opposing diurnal patterns across the 24-hour light-dark cycle (zeitgeber time). A: The mean number of $BrdU^+$ ME cells per brain section. B: The mean number of $BrdU^+$ cells per section in the hypothalamus minus those observed in the ME. Horizontal black bars represent dark phase (active period); white bar represent the light phase (rest period); n = 4-6 mice/group; error bars ± SEM.

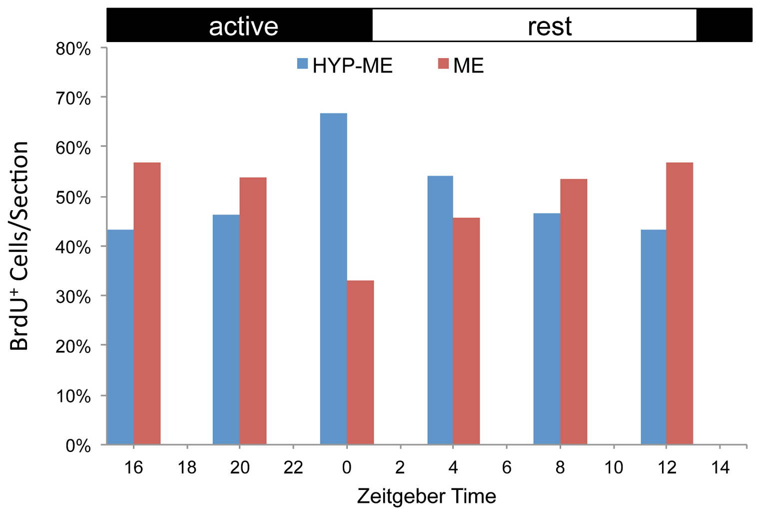


Fig. 7. BrdU labeling in the ME accounts for between 33% and 57% of all cell labeling in the entire hypothalamus. Each bar represents the average percentage of $BrdU^+$ hypothalamic cells per section in the hypothalamus less the ME (HYP-ME) (blue) and in the ME (red) across the 24-hour light-dark cycle (zeitgeber time). Horizontal black bars represent dark phase (active period); white bar represent the light phase (rest period); n = 4-6 mice/group.

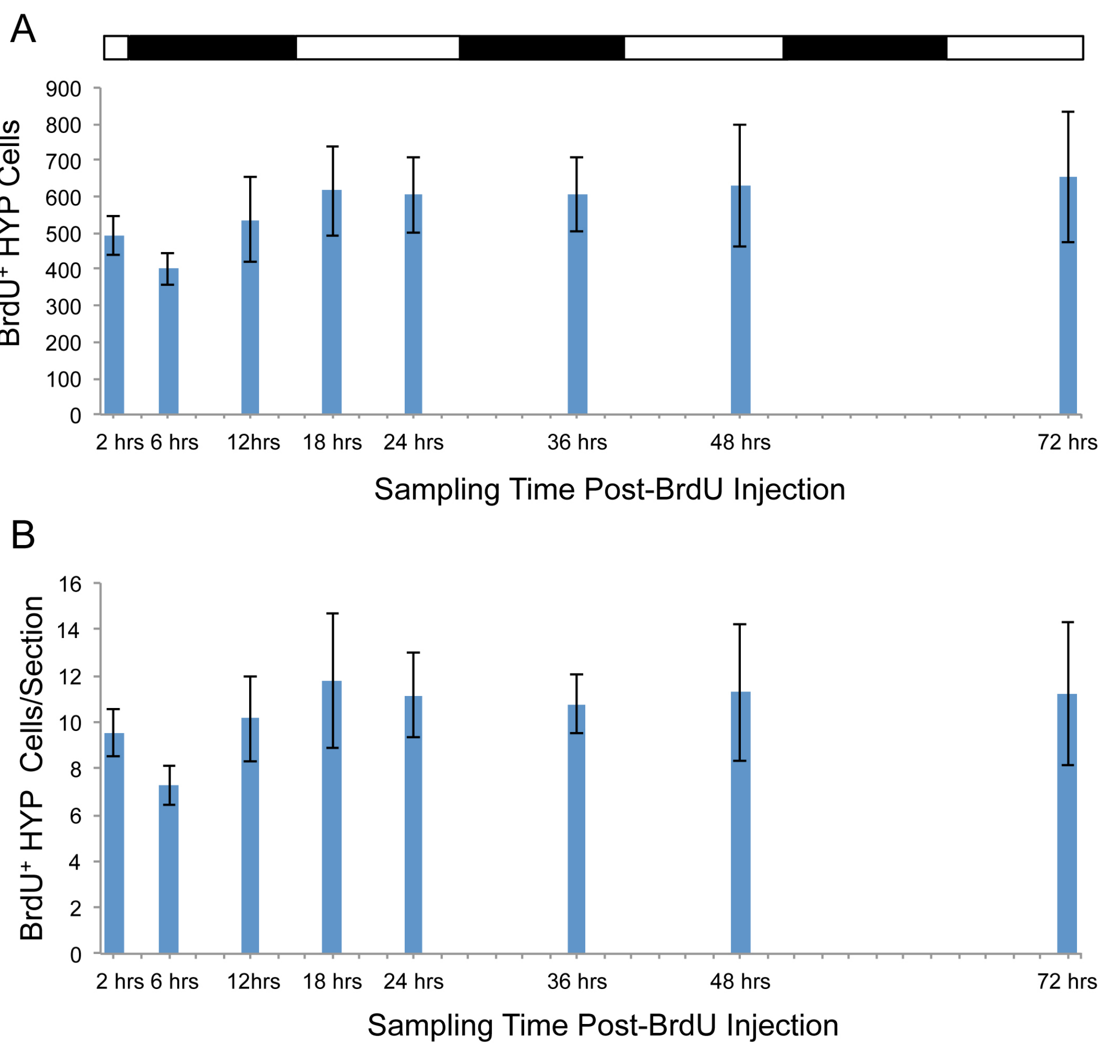


Fig. 8. (A) Hypothalamic cell proliferation showed a gradual rise over the first 4 sampling times, plateauing at 18 hours. Adult mice (n=32) were injected with BrdU at time zero and sampled at eight time points (n=4 per sample time). Each bar represents the mean $BrdU^+$ cell counts (B) The same pattern of hypothalamic cell proliferation is maintained when the number of $BrdU^+$ cells is calculated per section. Horizontal black bars represent dark phase (physically active period); white bars represent the light phase (rest period). Error bars ± SEM.

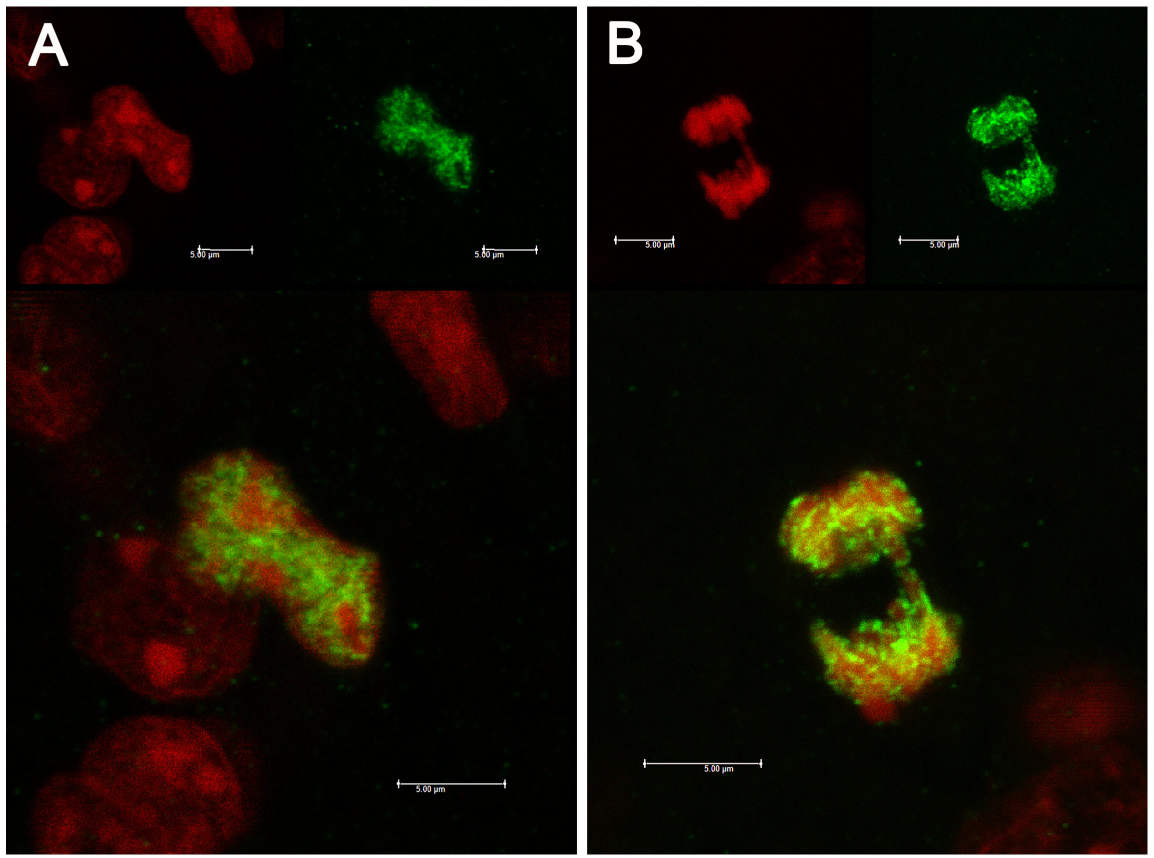


Fig. 9. Representative confocal images depicting mitotic and newly divided cells in the hypothalamus 12-hours post-BrdU injection. (A) Composite image of a BrdU^{+}/PI co-labeled cell undergoing mitosis at approximately telophase. (B) Composite image of a BrdU^{+}/PI co-labeled cell completing the final stages of mitosis. BrdU (green); propidium iodide (PI) (red); scale bars = 5μm.

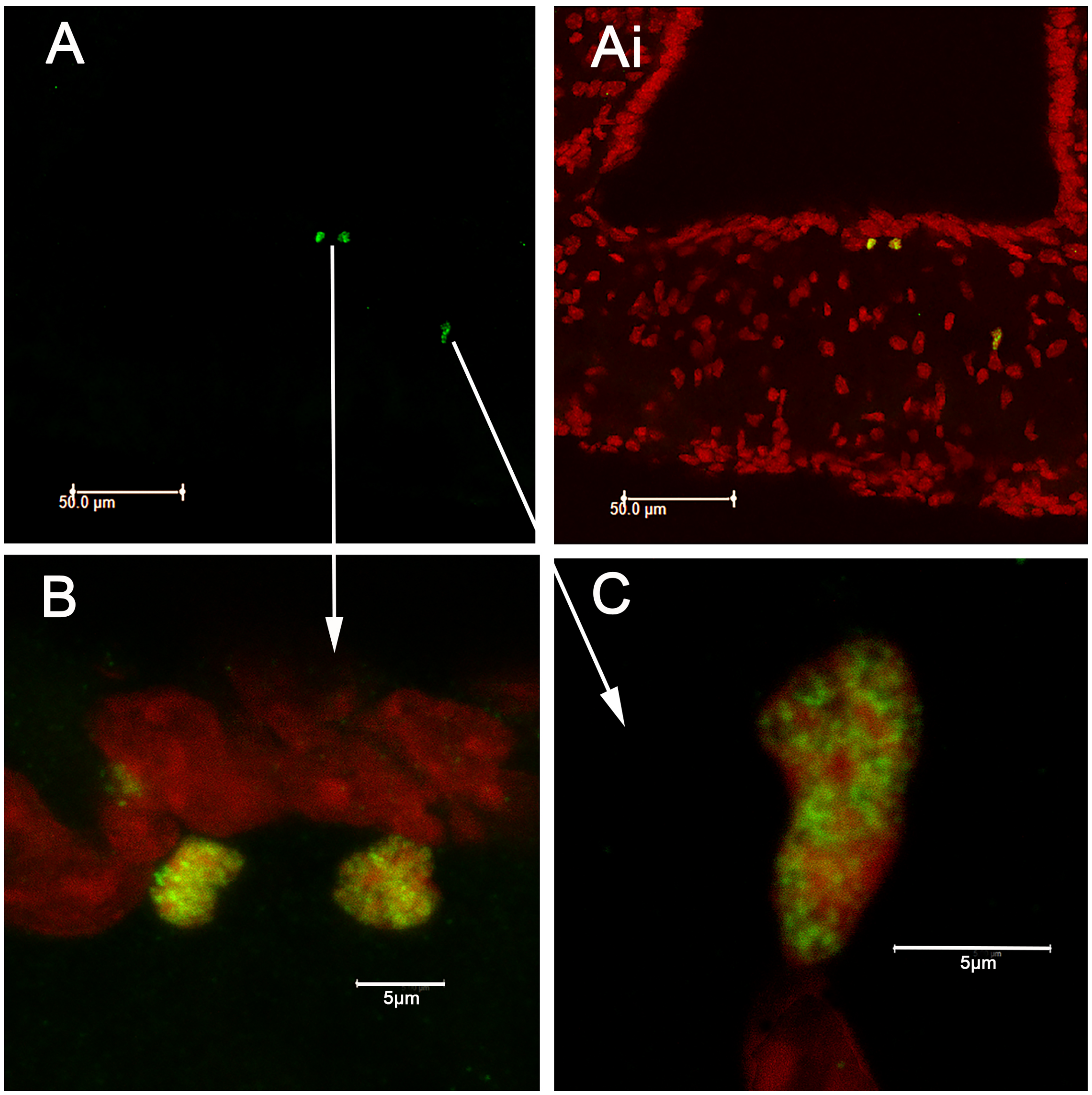


Fig. 10. Representative confocal images of newly divided and mitotic cells in the ME region of the hypothalamus 12-hours post-BrdU injection. (A) $BrdU^+$ cells and (Ai) $BrdU^+$/PI co-labeled cells. (B) Cell pair suggesting that these are newly divided $BrdU^+$/PI co-labeled cells. (C) $BrdU^+$/PI co-labeled cell undergoing mitosis at approximately telophase. BrdU (green); propidium iodide (PI) (red); A & Ai scale bars = 50µm; B & C scale bars = 5µm.

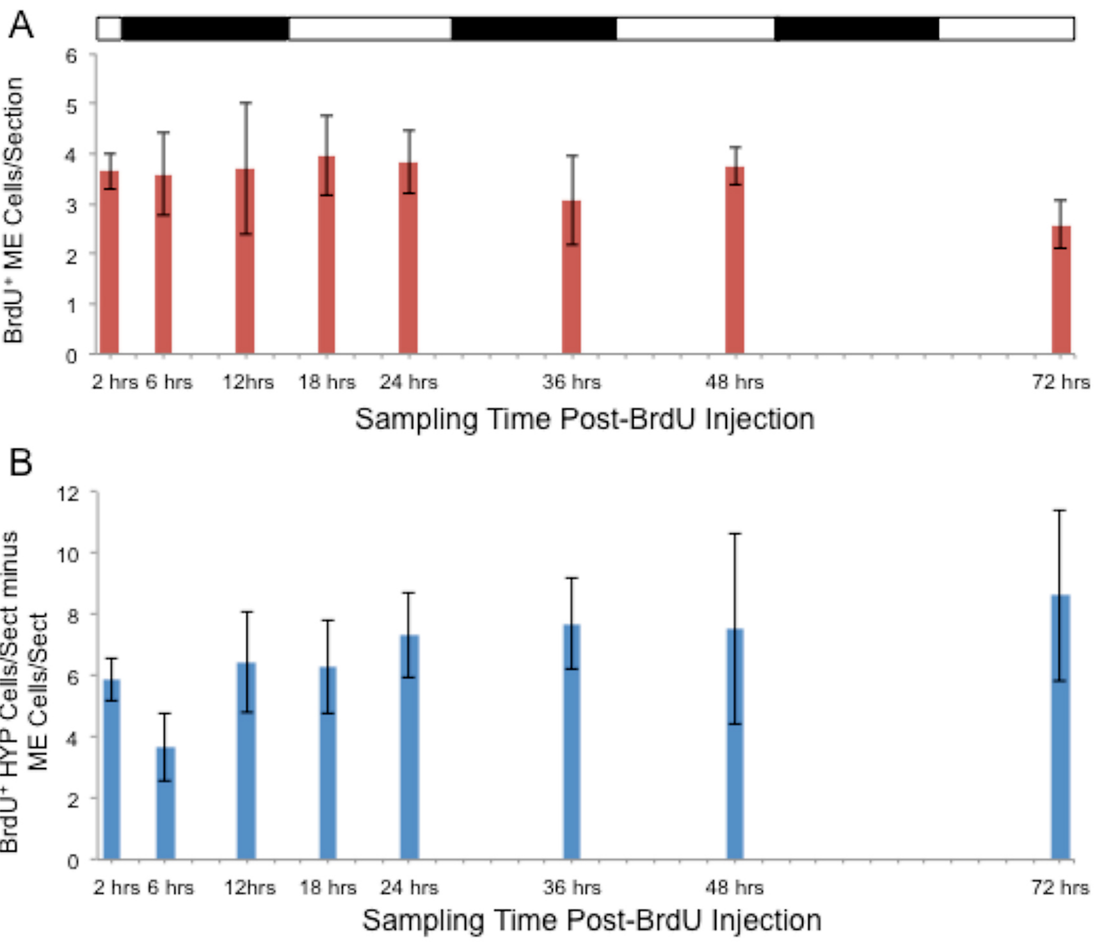


Fig. 11. BrdU-labeling in the ME (A) and all other hypothalamic regions (HYP minus ME) (B) across a 72-hour period. A: The mean number of $BrdU^{+}$ ME cells per section. B: The mean number of $BrdU^{+}$ cells per section in the hypothalamus minus those observed in the ME. Horizontal black bars represent dark phase (active period); white bar represent the light phase (rest period); n = 4 mice/time point; error bars ± SEM.

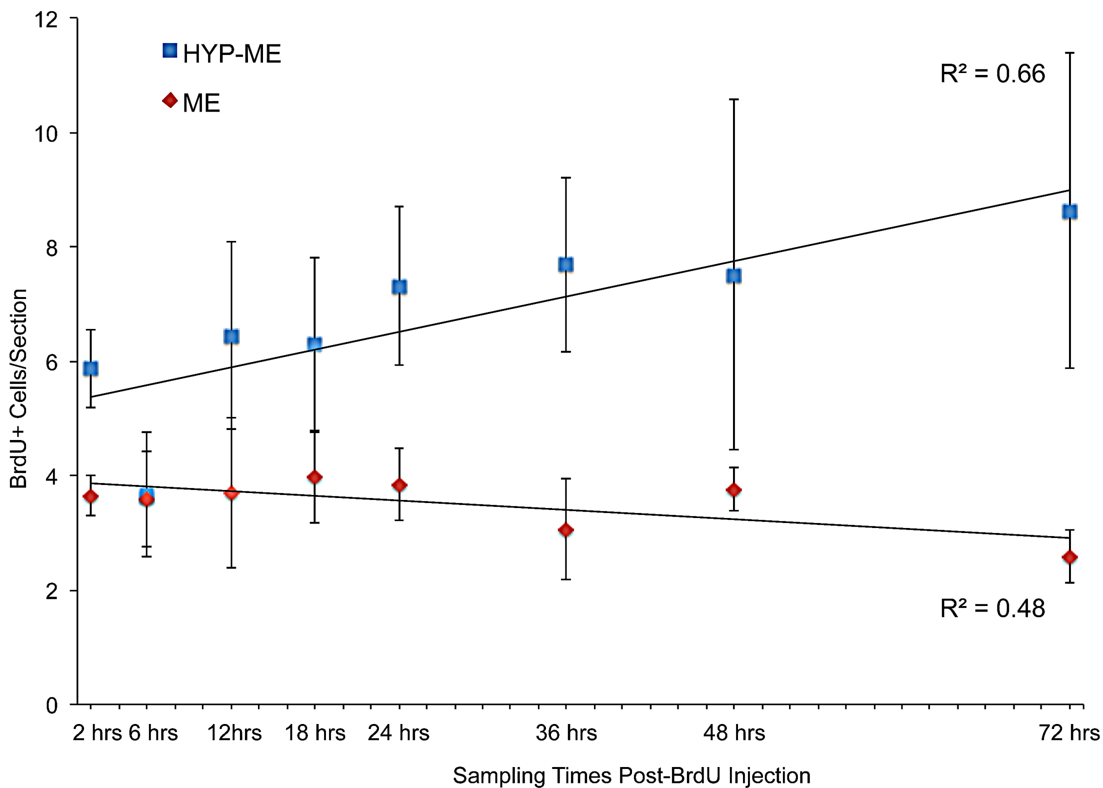


Fig. 12. Contrasting rates of cell proliferation in the ME vs. the rest of the hypothalamus over three days. Adult mice were injected with BrdU at time zero and sampled (n = 4) at eight points over 72-hours. The slope of the lines represent that the average number of $BrdU^+$ cell per section to sampling time significantly depends on the ME (red diamonds) vs. the rest of the hypothalamus (HYP minus ME) (blue squares) (p = .03). Error bars ± SEM.

Chapter IV

Discussion

Circadian Control of Hypothalamic Cell Proliferation

The circadian clock in mammals is a highly regulated neural system entrained to the 24-hour light-dark cycle. Circadian rhythms manifest through physical, behavioral and molecular changes in response to internal signals that are driven by photic cues. Most of these biological functions come under the control of a “master clock”, the suprachiasmatic nucleus (SCN), located in the hypothalamus (Yamazaki et al., 2000). Proliferating tissues throughout the body adhere strongly to the timing of the circadian clock (Bjarnason & Jordan, 2000); however, influences on neurogenic activity are less well understood.

In the present study, we asked whether circadian factors influence cell proliferation in the hypothalamus. Results indicate that the circadian clock does indeed affect the cell cycle in the hypothalamus as a whole (Figs. 3 & 4). The number of $BrdU^+$ cells in the hypothalamus showed a diurnal pattern of cell proliferation with the greatest addition of newly labeled cells occurring as the animals transitioned from the dark phase (physically active period) to the light phase (rest period). The fewest number $BrdU^+$ cells were observed also at a time of transition as the animals shifted from the light phase to the dark.

Cell proliferation was independently assessed in the median eminence (ME), a region within the hypothalamus that is of particular interest because of its high rate of BrdU incorporation relative to the rest of the hypothalamus (Fig. 7). The ME sits at the ventral-most aspect of the hypothalamus and serves as a neuro-hormonal link between the brain and the body.

Its rich capillary network allows the passage of releasing and inhibiting regulatory hormones (e.g., gonadotropin-releasing hormone, thyrotropin-releasing hormone, corticotropin-releasing factor (CRF), etc.) from hypothalamic neuron terminals to the pituitary gland (Yin & Gore, 2010). The ME also exhibits morphological similarities to the well-characterized neurogenic niches of the SVZ and SGZ in that it sits in close proximity to both the ventricular and the circulatory systems. Interestingly, the ME is one of several regions of the CNS that lacks a proper BBB and therefore is considered "leaky" (Yin & Gore, 2010).

Tanycytes are a specialized group of cells that reside in the ependymal layer that lines the third ventricle, including in the ME. Studies have suggested the neurogenic niche responsible for adult neurogenesis in the hypothalamus may lie along the third vertical wall and that these tanycytes are the NPCs of the hypothalamus (Kokoeva et al., 2005; Perez-Martin et al., 2010). Four subtypes of tanycytes have been identified and are classified as alpha-type (α1 and α2) and beta-type (β1 and β2). The β2 tanycytes, which reside within the ME, have been proposed to be the neural progenitors based on the finding that they express molecular markers specific to neural stem and progenitor cells (e.g., Hes1 and Hes5 (Lee et al., 2012)).

In the present study, the ME contained the greatest density of BrdU^{+} cells (Fig. 5) relative to the dispersed labeling pattern observed in the parenchyma of the remainder of the hypothalamus (Fig. 2C). In addition, BrdU labeling in the ME accounted for between 33% and 57% of all hypothalamic cell labeling (Fig. 7), even though this region accounts for only a small proportion of the hypothalamus. This was not surprising as a previous study showed that the ME exhibits relatively higher levels of neurogenic activity (~5 times greater) than that of any other area of the hypothalamus (Lee et al., 2012). Others have described the same dramatic variations in patterns of labeled cells throughout in the hypothalamus with the greatest concentration of

labeled cells observed adjacent to ependymal layer of the third vertical (Xu et al., 2005), decreasing in density the greater the distance from the ventricle wall (Pencea et al., 2001). Yet other studies have suggested that neurogenic activity extends beyond the boundaries of the third ventricle wall as $BrdU^+$ cell populations have been observed scattered throughout the parenchyma of the hypothalamus (Migaud et al., 2010; Pencea et al., 2001), as we observed in this study as early as 2 hours following a pulse of BrdU.

The circadian experiments suggest that the ME operates on a circadian rhythm that is distinct from the rest of the hypothalamus (Figs. 6 & 7). Although the 24 hr. data points are not statistically different, these findings nevertheless suggest an opposing diurnal pattern of BrdU labeling with the fewest number of $BrdU^+$ cells observed in the ME at time point ZT0, the same time point the greatest number of $BrdU^+$ cells were observed in remainder of the hypothalamus. In contrast, at time point ZT16, the greatest number of $BrdU^+$ cells were observed in the ME, while the fewest number of $BrdU^+$ cell were observed in the rest of the hypothalamus. Given the suggestion that the ME is not the only region in the hypothalamus that exhibits proliferative activity, and that others have been reported neurogenic activity in the subependymal layer (Perez-Martin et al., 2010) and in the nearby parenchyma (Migaud et al., 2010; Pencea et al., 2001; Xu et al., 2005), more studies are necessary to gain a greater understanding of the identity, location, and role of other potential progenitor cell populations that may supply the hypothalamus.

Functional Significance of Hypothalamic Circadian Rhythm

What might be the functional significance of the opposing circadian rhythms observed within the hypothalamus? Opposing processes of neural communication (e.g., arousal and

relaxation; excitation and inhibition; homeostatic feedback loops) are fundamental to the function and control of the CNS. The ME serves as a direct conduit from the hypothalamus to the body. The hypothalamus is a vitally important region that functions to maintain homeostasis and reproductive behaviors. The permeability of the BBB and its vascularized structure allow for the rapid and direct release of signaling factors (e.g., hormones) to the body via the pituitary, and may also allow freedom of movement into the hypothalamus.

Mice are nocturnal animals and therefore are most active during the dark phase, and generally inactive during the light phase. Consequently, certain hormones such as corticosterone (the equivalent to cortisol in humans) are at peak levels at the onset of the dark phase and at the lowest levels during periods of rest (light phase) (De Boer & Van der Gugten, 1987). Corticosterone is an important hormone that exerts various effects on the body, including glucose regulation, lipid metabolism, immune activation, and stress response. In times of stress, CRF is secreted by the paraventricular nucleus (PVN) of the hypothalamus and accesses the circulatory system at the ME, ultimately acting on the pituitary gland. The pituitary responds by releasing adrenocorticotropic hormone (ACTH) that in turn acts on the adrenal glands for the release of corticosterone. Corticosterone levels are regulated by a complex negative feedback loop controlled by HPA axis; as corticosterone levels rise, CRF release is inhibited, stopping the release of ACTH from the pituitary.

As a metabolic “highway”, the ME experiences its high volume of “traffic” during the dark phase (active period) in mice. Throughout this active period energy demands are placed on the body that are accompanied by increases in cardiovascular demands, gastrointestinal tract activity, and food and water requirements. These biologic systems are regulated by the hypothalamus to maintain homeostasis. Increases in cell proliferation were observed in the ME at

the onset of the dark phase, a time the fewest number of $BrdU^+$ cells were observed in all other regions of the hypothalamus (Figs. 6A & 6B). While these differences were not significant among either group (ME: ($F(5, 27) = 0.78$, $p = .57$); HYP-ME: ($F(5, 27) = 1.42$, $p = .25$)) this observation is nonetheless interesting. Possibly, cell divisions are taking place in the ME (whether originating from tanycyte populations or other NPC populations) during this active period, and then these cells migrate to the hypothalamus to complete their lineage to ultimately integrate into existing neural circuits. Interestingly, twelve hours after the greatest number of $BrdU^+$ cells were observed in the ME, the number of $BrdU^+$ cells observed in the hypothalamus (minus the ME) reached their peak. While it is possible that the hypothalamic regions other than the ME operate with independent, contrasting circadian rhythms, this "rhythm" may also be a result of the proliferative activity in the ME. For example, if cells are migrating from the ME into the rest of the hypothalamus, the contrasting "rhythm" in BrdU labeled cells in the rest of the hypothalamus could simply be the observation of a delay due to the migration time required for cells to travel from the ME into these other regions. Ultimately, additional studies will be necessary to increase the 'n' to determine whether differences in the number of labeled cells in the hypothalamus over time is significant.

Regulation of Cell Proliferation in the Hypothalamus

The circadian influence on cell proliferation in the hypothalamus suggested by these studies is among the first regulatory factors that have been demonstrated for adult-born hypothalamic cells. The future demonstration that these labeled cells differentiate into neurons is critical. In addition, the functional significance of adult neurogenesis in the hypothalamus must be resolved in order to understand how these new cells impact the role of the hypothalamus as

the master endocrine gland and its role in maintaining homeostasis. Studies that have linked hypothalamic neurogenesis and feeding (Kokoeva et al., 2005), and energy regulation and balance (Bless et al., 2014), provide interesting insights into the functional significance of adult neurogenesis in the hypothalamus. Moreover, Lee et al. (2012) demonstrated an upregulation in neurogenic activity in ME in response to a high fat diet (Lee et al., 2012), while others showed that the same diet inhibited hypothalamic neurogenesis in the arcuate nucleus, a region in close proximity to the ME (McNay, Briancon, Kokoeva, Maratos-Flier, & Flier, 2012).

Further Testing the Circadian Rhythm

Studies that manipulate the light cycle will reveal how strongly cell proliferation is entrained to the zietgeber. If the light cycle were reversed, does the neurogenic rhythm follow the light change? And is proliferation sustained in constant light conditions, demonstrating that cell proliferation is supported by an endogenous rhythm? Do all circadian cycles support cell proliferation equally well, or does prolonged light or night alter cell generation? Does light intensity and/or duration influence the rhythm of cell proliferation? Is the role of the circadian clock in the proliferations of new cells in the hypothalamus affected by age? These and other questions will continue to shed light on adult neurogenesis in this under-studied brain region.

The Longitudinal Study of BrdU Labeling in the Hypothalamus

Experiments also tested the hypothesis that newborn cells or their precursors in the hypothalamus might originate in an extrinsic source and that within three days following a single pulse of BrdU an increase in BrdU-labeled cells would be observed in the hypothalamus, due to ongoing cell proliferation and the addition of new cells. Specifically, the goal was to test for a

delay in the arrival of $BrdU^+$ cell in the hypothalamus, and if given enough time, the addition of new cells to the hypothalamus would increase to numbers that could not be accounted for by cell division.

Examination of adult neurogenic activity in the hypothalamus in mice often involves infusion of BrdU into the lateral ventricles of the brain for up to two weeks (Kokoeva et al., 2005, 2007), a BrdU administration protocol that has been justified by the low levels of BrdU labeling after the more standard single BrdU pulse (Kokoeva et al., 2007). An alternative explanation for the lack of short-term labeling would be a circadian influence on the cell cycle or a delay in arrival of labeled precursor cells from a source outside the hypothalamus, as has been found in crayfish (Benton et al., 2014). The present study was designed to test both of these possibilities.

Surprisingly, the mean number of $BrdU^+$ cells in the hypothalamus (as a whole) showed no significant differences throughout the 3-day data collection period ($F(7, 24) = 0.50$, $p = .83$); neither did the number of BrdU-labeled cells per section in the ME ($F(7, 24) = 0.85$, $p = .91$). A comparison of the first (2 hr.) and last (72 hr.) time points suggests only a 60% increase in $BrdU^+$ cell counts in the hypothalamus during this period, while a 2-fold increase in $BrdU^+$ cell counts might be predicted based on a single cell division of the BrdU-labeled population. The fact that a doubling in $BrdU^+$ cells did not occur could be explained if only a portion of the originally-labeled cell population went through mitosis. In addition, these findings could result from a slow cell cycle time (>3 days) among some of the $BrdU^+$ cells, cell death in the proliferating population, or cell migration away from the hypothalamus.

To our knowledge, the cell cycle time for proliferative cells in the adult hypothalamus has not been defined. However, as early as 12 hours post-BrdU injection, mitotic cells and

"doublets" were observed throughout the hypothalamus (Figs. 9 & 10). This observation suggests that at least some hypothalamic cell populations have a cell cycle time that approximately aligns with those of progenitor cell populations in other areas of the brain (e.g., SVZ, ~18-25 hours; SGZ, ~25 hours) (Cameron & McKay, 2001; Ponti et al., 2013). It is also possible that there are multiple neurogenic regions, some with relatively slower cell cycle times. While cell death could account for the modest increase in labeled cell counts observed in the present study, other experiments have shown that newly generated granule cells in the hippocampus typically die between one and two weeks after birth (Gould, Beylin, Tanapat, Reeves, & Shors, 1999). This may suggest that our results are not be due to cell death given that the last sampling time was only three days post-BrdU incorporation. Further, circadian phenomena cannot explain the small increases in BrdU labeling, because the earlier experiment demonstrated labeling at all times of day; that is, there were no "dead spots" when BrdU was not incorporated into cells.

The most prominent finding in this study is the contrasting rates of cell proliferation in the ME and the rest of the hypothalamus (Fig. 12). When graphed over time, the number of $BrdU^+$ cells in the hypothalamus (minus the ME) vs. those in the ME showed diverging patterns. While the average number of $BrdU^+$ cells in the hypothalamus (minus the ME) increased over time, those in the ME remained flat. A one-way ANCOVA was conducted to compare the interaction between $BrdU^+$ cells in the ME and those in the hypothalamus minus the ME (HYP minus ME) based on sampling times, and a significant interaction was found ($F(1, 60) = 4.84$, $p = .03$). This suggests that the mean numbers of labeled cells within each region (HYP minus ME and ME) do not exhibit the same rate of change over the 72-hour post-injection period.

This finding is particularly interesting considering that on average the number of BrdU$^+$ cells in the ME, across all sampling times, accounted for between 26-52% of all labeled cells in the entire hypothalamus, and that ME BrdU$^+$ cells were observed in different stages of mitosis as early as 12 hours post-BrdU injection (Fig. 10A & C). This suggests that at least some cell types in the ME have a cell cycle time inside the 72-hour window of this study. In addition, within the ME, pairs of BrdU$^+$ cells, or "doublets", were observed in close proximity to each other (Fig. 10A & B). While we cannot determine whether these "doublet" cells were indeed newborn "sister" cells without further testing, from a qualitative standpoint, paired cells like these were not observed prior to the time mitotic cells were first observed in this region.

Considering these findings, it was surprising that the average number of labeled cells in the hypothalamus as a whole or in the ME did not show a statistically significant increase over the 72-hour period (Fig. 11). A natural question is how the numbers of labeled cells could be stable, in spite of the observation of BrdU labeling and cell division. Do new cells migrate away from the ME, so that BrdU-labeled cell numbers remain stable in the ME? Do newly divided ME cells migrate into other regions in the hypothalamus, accounting for the small (though statistically insignificant) increases in BrdU$^+$ cells observed in that region? Does cell death among the BrdU$^+$ cells mask potential increases due to cell division, indicating that hypothalamic adult neurogenesis is part of a neuronal turnover mechanism?

As described above, tanycytes are a population of cells concentrated in the ependymal layer lining the floor and the walls of the third ventricle. While several studies have reported tanycytes as the potential neural progenitor cells responsible for adult born neurons in the hypothalamus, and that these cells migrate from the wall of the third ventricle into the hypothalamic parenchyma (Matsuzaki et al., 2009; Xu et al., 2005), other studies have suggested

that neurogenic activity extends beyond the boundaries of the third ventricle wall as $BrdU^+$ cell populations have been observed scattered throughout the parenchyma of the hypothalamus (Migaud et al., 2010; Pencea et al., 2001), as was confirmed in this study.

Since the discovery of adult neurogenesis and the broad acceptance of its existence, research today focuses mainly on the highly proliferative regions of the SVZ and SGZ. Although there is evidence that several other brain regions (e.g., visual cortex (Luskin & Shatz, 1985), striatum (Ernst et al., 2014), and hypothalamus (Kokoeva et al., 2005; Lee & Blackshaw, 2012; Migaud et al., 2010)) have adult neurogenic activity, the scientific community has not readily accepted these findings, mainly because these other regions tend to show lower levels of neurogenic activity. Nonetheless, future studies will be necessary to unravel the functional role of proliferative activity in the hypothalamus, whether adult-born cells are incorporated into existing (or new) networks, and what factors regulate proliferative activity in this region.

Broader Implications

The neurogenic theory of depression suggests that this disease results from impaired adult neurogenesis in the dentate gyrus of the hippocampus, and that if neurogenesis is restored, recovery will follow (Jacobs, van Praag, & Gage, 2000). Although this theory is controversial (Eisch & Petrik, 2012; Hanson, Owens, Boss-Williams, Weiss, & Nemeroff, 2011; Miller & Hen, 2015), it is generally agreed that there is an important link between neurogenesis and depression (David et al., 2009; Jacobs et al., 2000; Kempermann & Kronenberg, 2003). Pharmacological treatments for depression often include the use of selective serotonin reuptake inhibitors (SSRIs) as they have been shown to upregulate neurogenesis (Banasr, Hery, Printemps, & Daszuta, 2004; Paizanis, Hamon, & Lanfumey, 2007; Santarelli et al., 2003).

However, antidepressant treatment is often accompanied by adverse side effects such as weight gain, sleep disturbances, and sexual dysfunction (Ferguson, 2001). Interestingly, these side effects are consistent with imbalances in homeostatic functions that are regulated by the hypothalamus. Studies have suggested hypothalamic neurogenesis contributes to metabolism and energy homeostasis (Bless et al., 2014; Lee et al., 2012), and that when mice are treated with ciliary neurotrophic factor (CNTF) (a hormone known to cause weight loss and anorexia), they showed an increase in hypothalamic neurogenesis (Gloaguen et al., 1997; Kokoeva et al., 2005). Therefore, although adult neurogenesis in the hypothalamus may not be as robust as in the hippocampus or olfactory bulb, the addition of new cells to this region may have profound implications for healthy and diseased states.

Future Directions

The brain is comprised of multiple cell types (e.g., neurons, astrocytes, oligodendrocytes, ependymal cells, and microglia). This study looked strictly at cell proliferation in the hypothalamus and changes observed throughout the dark/light cycle and over time. Determining whether the $BrdU^+$ cells differentiate into neurons and if there is evidence for cell migration, will require further studies. Testing for neuronal and stem cell markers and for apoptosis will help define the fate(s) of the BrdU-labeled hypothalamic cell populations.

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