Regulation of Suprachiasmatic Nucleus and Hippocampal Cellular Activity as a Function of Circadian Signaling

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

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2017

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ABSTRACT

Circadian rhythms are defined as oscillations in biological processes with a period similar to the 24 hour period of the earth's rotation. In mammals, circadian rhythms are controlled by the suprachiasmatic nucleus (SCN), a hypothalamic structure working as a central pacemaker that drives the circadian rhythms that are expressed in other brain regions, as well as throughout the rest of the organism. The manifestation of circadian rhythms is structured by the presence in nearly all cells of a molecular clock, a transcriptional and translational feedback loop that directs the timing of numerous cellular processes. Although endogenous circadian rhythms are self-sustained, entrainment of the SCN circadian clock is induced by diverse environmental factors in order to maintain a synchrony with external geophysical cycles, with light being recognized as one of the most robust entrainment signals. Here, I first present a study aimed at describing genome-wide expression changes in the SCN triggered by light inputs in the SCN **(Chapter2).** In this respect, previous studies have shown that the effects of light in the SCN are time-of-day specific, resulting from differential responses in specific neuronal signaling pathways. In particular, activation of the *mitogen-activated protein kinases/ extracellular signal–regulated kinases* (MAPK/ERK) signaling pathway in the SCN couples photic stimuli with circadian entrainment through changes in gene expression. Using microarray analysis and pharmacological interventions, I describe here

the number and nature of transcripts induced by light at multiple points of the circadian cycle, with a particular emphasis on the subgroup of light-induced genes whose expression is modulated by the activation of the MAPK/ERK signaling pathway. This examination is followed by studies aimed to determine the influence of circadian rhythms on the function of the hippocampus. First, I assess the role of circadian rhythms in the formation and retrieval of contextual memories, a task mediated by the hippocampus **(Chapter 3)**. There is a known relationship between circadian rhythms and memory formation, but the cellular and molecular mechanisms linking them are not fully understood. Here, the recruitment and activation of particular neuronal ensembles, essential for the encoding and retrieval of contextual memories, is correlated with specific phases of the circadian clock. Finally, hippocampal function is also examined here by the modulation of adult neural stem/progenitor cell function **(Chapter 4)**. The circadian clock is expressed in embryonic and mature tissues, and regulates the proliferation and differentiation rates of tissue-specific stem cells. Here, I identify the expression and circadian oscillation of the molecular clock in this particular population of stem cells in the adult hippocampus, and then evaluate the changes observed in proliferation and differentiation rates of the stem/progenitor cell population as a consequence of a genetic disruption of the molecular clock.

DEDICATION

To Andrea

Mi princesa valiente.

It is your braveness and love what keeps me moving forward.

ACKNOWLEDGMENTS

My sincere thanks to my advisors Dr. Kari Hoyt and Dr. Karl Obrietan. Working under your guidance throughout the years help to shape my life as a brain researcher, it is your patience and hard work that guide me in this journey and help me continue despite my flaws. I deeply appreciate your generosity in time and resources dedicated to my formation. I feel really fortunate to have you as my mentors.

In addition, I thank Dr. Nam Lee, as a member of my candidacy and dissertation committee, his advice, feedback and recommendations were invaluable during all these years. I also acknowledge Dr. John Oberdick for the science talks and the constant feedback and encouragement.

My deepest thanks belong also to my fellow lab members during all these years, especially Dr. Katelin Hansen and Dr. Kate Karelina. As a young student, I could not ask for better models of scientific success. I thank as well other members of the lab in particular Heather Dziema-Schutzler, Dr. Kensuke Sakamoto, Kelin Wheaton, Kaitlin Snider, Kyle Sullivan, Paul Horning, Sydney Aten, Ashley Garcia, and all the undergraduate and rotating students involved in the lab during all these years. I can gladly say that I learn a lot from all of you.

Infinite thanks to my wife, you gave me the strength I needed to complete this stage of my career, you complete me. And I give thanks to my family, to my dad and my mom that showed me how love and hard work pay off. Also thanks to my sister, her dedication and support are invaluable for my success. To all my family because I always feel the love that emanates from all of you despite the distance.

Special thanks to all my friends that support me and stand by me in every situation, good times are better and bad times are more bearable next to you. .

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Choi, Yun-Sik, Kate Karelina, Diego Alzate-Correa, Kari R. Hoyt, Soren Impey, J. Simon Arthur, and Karl Obrietan. 2012. "Mitogen- and Stress-Activated Kinases Regulate Progenitor Cell Proliferation and Neuron Development in the Adult Dentate Gyrus." Journal of Neurochemistry 123 (5): 676–88.

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FIELDS OF STUDY

Major Field: Pharmacology

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CHAPTER 1

Introduction

The earth as a dynamic system is characterized by geophysical cycles such as light, temperature, precipitation and pressure. These cycles have a profound effect on the evolutionary development and ongoing sustainability of life on earth. In this respect, daily oscillations in light, temperature and humidity generated a set of selection forces for which life forms developed time-keeping mechanism as evolutionary adaptation (Vaze and Sharma 2013). These biological clocks are expressed in nearly all species, and are believed to improve their adaptability by increasing the ability to anticipate periodic changes in the external environment (Paranjpe and Sharma 2005). Given that the environmental cycles that time-keeping mechanisms in most organisms are aligned with have periods of around 24 hours, 29 days, or one year, the terms circadian, circatidal, and circannual were coined respectively, in reference to endogenous rhythms generated by biological clocks in living systems (Roenneberg and Merrow 2005).

The Study of Biological Clocks Across Time

The presence of physiological, and behavioral cycles in most organisms has been recognized for centuries, but remained unexplored due to the general belief that such oscillations correspond to passive responses to light, temperature, and other geophysical cycles (Roenneberg and Merrow 2005). However, early observations of leaf movements led to the identification of an endogenous periodicity intrinsic to living organisms. In 1729, the astronomer Jean-Jacques d'Ortous deMairan reported for the first time the existence of endogenous rhythms in the touch-me-not plant (*Mimosa pudica*). deMairan observed that daily leaf movements continued when the plants were moved to constant darkness conditions, thus concluding that the folding and unfolding of the leaves did not depend on light-dark cycles (de Mairan 1729). In 1751, Carl von Linné described a '*flower clock'* that estimated the time of the day based on the timing of opening and closing of flowers (Chandrashekaran 1998). The observation made by deMairan was later confirmed separately by Du Monceau and Zinn in 1759, both reporting the persistence of the periodic leaf movements under constant light and temperature when moving and studying the plants inside caves (Bünning 1960). Further advancements were made in 1832 by De Candolle with experiments that demonstrated that legumes grown in altered light-dark conditions showed persistent periodic leaf movements (Bünning 1960). A century later (1932 and 1935) the German biologist Erwin Bünning studied the heritability of daily rhythms. In his experiments, Bünning described how, in both plants and insects, rhythms with a period close to 24h, that persisted even after the subjects were raised and kept in constant light or darkness for several generations (Bünning 1960). In

1922, Curt Paul Richter presented the first evidence of endogenous periodicity in rodents when studying the locomotor activity of rats kept in the dark and at constant temperature (Richter 1922). By the mid-twentieth century daily rhythms were studied systematically, and descriptions of endogenous rhythms comprised a high diversity of organisms including monkeys, birds, and unicellular *Dinoflagellates (Kuhlman, Mackey, and Duffy 2007).* It was widely accepted that biological clocks were expressed in nearly all eukaryotes. This growing understanding resulted in the need of a unifying theory capable of supporting and strengthening the relatively new discipline. With that need in mind, the first international symposium under the topic 'Biological Clocks' was held at the Cold Spring Harbor Laboratory in 1960, with a recorded attendance of 150 scientists. After the symposium Colin Pittendrigh published one of the most influential papers in the field in which he summarized in 16 qualities the basic properties of ***circadian*** (circa = approximately; dies = a day) ***clocks*** (Pittendrigh 1960). A couple of years after the Cold Spring Harbor symposium, another important description in the circadian biology field was made by Jürgen Ashoff and Rütger Wever, reporting the presence of endogenous daily rhythms in humans (Kuhlman, Mackey, and Duffy 2007; Jürgen von Aschoff and Wever 1962). In 1971, Seymour Benzer and his student Ronald Konopka moved the field forward with regard to the genetic bases of circadian clocks. The paper described clock mutants in the fruit fly *Drosophila melanogaster* as a result of a forward genetic screen. Three mutants were defined when evaluating both eclosion and locomotor rhythms: arrhythmic, short-period and long-period phenotypes. Lastly, the study mapped the probable responsible gene to bands 3A6 and 3C2 of the X chromosome (Konopka and

Benzer 1971). Around the same time, several efforts were dedicated to the identification of the anatomical location of the central circadian rhythm controller. Successful descriptions were made for the optical lobes in cockroaches (Nishiitsutsuji-Uwo and Pittendrigh 1968) and the pineal gland in sparrows (Gaston and Menaker 1968). Following the same question, two seminal papers identified the Suprachiasmatic Nucleus (SCN) in the rat hypothalamus as the central clock pacemaker. One of the papers, published by Robert Moore and Victor Eichler, documented the loss of daily rhythms of corticosterone levels in the adrenal gland of female rats after surgical lesion of the SCN (R. Y. Moore and Eichler 1972). The second paper followed a similar approach and described the loss of locomotor and drinking circadian rhythms caused by electrolytic lesions of the SCN (F. K. Stephan and Zucker 1972). After more than a decade, Seymour Benzer and his students contributed additional advances in the identification of the molecular agents involved in the generation of circadian rhythms. In 1984, Pranhitha Reddy and collaborators described the cloning of the *period* gene in *Drosophila melanogaster* (Reddy et al. 1984).

In the 1980's another piece of vital information was added to the biological clock field. For centuries it was believed that the existence of a time-keeping mechanism was restricted to eukaryotes, but in 1986 the presence of daily rhythms separating nitrogen fixation processes from photosynthesis in the prokaryotic organism *Synechococcus spp,* a group of cyanobacteria, was described (Mitsui et al. 1986). In the following decades, the tremendous expansion in the number of groups studying biological clocks led to an increase in the pace at which new discoveries were reported. Among others, some of the

featured studies include the formulation of the first model explaining how clock genes could generate a circadian rhythm. The model specified by Hardin, Hall and Rosbash in 1990 formulate the existence of a transcriptional and translational feedback loop, in which the transcription of a gene is followed by the synthesis of the respective protein; this protein in turn will inhibit its own transcription. Consequently, after degradation of the messenger RNA (mRNA) and the decay of the protein, the cycle can be restarted (Hardin, Hall, and Rosbash 1990). In the same decade, other clock genes in addition to *period* were identified in key species: *timeless* also in *Drosophila (Sehgal et al. 1994)*, *frequency* in *Neurospora crassa* (Feldman and Hoyle 1973), *clock* (Antoch et al. 1997) and *Mop3/bmal1*in mice (Ikeda and Nomura 1997), and *toc1*(Timing of CAB expression 1) in *Arabidopsis thaliana (Somers et al. 1998)*. Finally, between the years 2000 and 2009 the identification of diverse clock genes together with the implementation of gene expression techniques allowed the detection of clock genes in mostly all cells in the organisms, moving the field towards the study of circadian peripheral clocks in diverse organs. This was amplified by the development of high-throughput screenings of gene expression (Panda et al. 2002).

Throughout recent history, the advancement of the field has been constant and has produced outstanding depictions of the features of endogenous time-keeping mechanism in living organism. However, a full understanding of the role of circadian rhythms is far from completed. The association between the central pacemaker's function and the coordination of the rest of the physiological and behavioral systems still needs to be clarified.

Characteristics of Circadian Rhythms in Mammals

Circadian rhythms are defined as endogenous and self-sustained biological rhythms whose free-running period is equal to or approximately equal to the period of the earth's rotation (~24h). These rhythms are innate and can be found in almost all organisms on earth, in multicellular organisms circadian rhythms are expressed in almost all cell types. Additionally, the intrinsic period length of circadian rhythms is very precise with a minimum cycle-to-cycle variance. The intrinsic period length and phase of circadian rhythms are subject to modifications and can be entrained to match the period of distinct environmental variables (Pittendrigh 1960; Roenneberg and Merrow 2005).

The main features of circadian rhythms described above are expressed in the mammalian circadian system as a result of a hierarchical regulation in which a central pacemaker directs and synchronizes subordinate peripheral oscillators found throughout the entire body (Ko and Takahashi 2006). The mammalian central circadian clock has been identified in the brain hypothalamus, more specifically in a distinctive region known as the Suprachiasmatic Nucleus (SCN) (R. Y. Moore and Eichler 1972; F. K. Stephan and Zucker 1972). Based on anatomical location and the expression of key neuropeptides, the SCN has been classically divided in two main regions, a dorsomedial SCN or 'shell' and a ventrolateral SCN or 'core'(Robert Y. Moore 1996). The core SCN is characterized by the expression of the vasoactive intestinal polypeptide (VIP), calretinin, neurotensin (NT), and gastrin releasing peptide (GRP). The SCN shell is characterized by the

expression of arginine vasopressin (AVP), angiotensin II, and met-enkephalin(Abrahamson and Moore 2001). From the core to the shell, there are profuse afferent projections, however just few projections travel from the shell to the core(Leak, Card, and Moore 1999). At the functional level, this observation is significant because the core SCN is viewed as a signal integrator, receiving main inputs from the retina, the thalamus, and the raphe nuclei to then relay this information to the shell portion of the SCN (see review (Morin and Allen 2006) for a comprehensive review). Together, both SCN regions constitute a group of around 20,000 neurons, the majority of which form synapses that release the inhibitory neurotransmitter gamma-aminobutyric acid (GABA), along with VIP in the core cells or AVP in the shell cells(R. Y. Moore and Speh 1993). Individual SCN neurons can generate independent oscillations(Yamaguchi et al. 2003), but coordinated signaling between local circuits within the SCN (especially between the core and the shell) is where robust rhythms are generated (Herzog et al. 2004). Outputs from the SCN are received by other hypothalamic regions like the subparaventricular zone (sPVZ)(Abrahamson and Moore 2001) and the dorsal medial hypothalamus that in turn send modulatory projections to the rest of the nervous system and to hormone control centers where synchronizing information is subsequently transmitted to peripheral clocks distributed through the body (Watts and Swanson 1987). Intrinsic oscillations in the SCN as a central pacemaker allow the generation of circadian rhythms with a precise period and phase even in the absence environmental cues (R. Y. Moore 1983). However, another important characteristic of the SCN central clock is its ability to adjust the period of the circadian rhythms to external variables (known as *Zeitgebers)* such as light-dark

cycles, food, and temperature. This alignment process is known as circadian entrainment (see reviews by (S. Daan 2000; Golombek and Rosenstein 2010). Anatomical and functional studies have shown that photic modulatory signals reach the SCN via the retinohypothalamic tract (RHT), originating from melanopsin-containing retinal ganglion cells. In contrast, non-photic signals reach the SCN via the geniculohypothalamic tract (GHT) originating in the intergeniculate leaflet (IGL), and the raphe-hypothalamic tract (Morin and Allen 2006).

The Mammalian Molecular Clock

At the cellular level, the timing of circadian rhythms is sustained by a molecular clock (for review see (Dunlap 1999; Partch, Green, and Takahashi 2014), essentially a transcriptional and translational feedback loop program involving a distinctive group of 'clock genes'(Shearman et al. 2000) . Built around this core circadian clock there is a group of 'clock controlled genes' that in turn guide the specialized circadian rhythms in each type of cell, subsequently reflected in tissue and organ-specific circadian rhythms(Bozek et al. 2009). The core molecular clock includes three basic helix-loop-helix transcriptional factors, *circadian locomotor output cycle kaput* ***(CLOCK)****(Vitaterna et al. 1994)*, *neuronal PAS domain protein 2****(NPAS2)****(DeBruyne, Weaver, and Reppert 2007)* and *brain and muscle aryl hydrocarbon receptor nuclear translocator (Arnt)-like protein* ***(BMAL1)****(Bunger et al. 2000).* In the segment of the loop occurring during the day, BMAL1 forms a heterodimer with either CLOCK or NPAS2, this heterodimer in

turn binds primarily to consensus E-box DNA motifs (Gekakis et al. 1998; DeBruyne, Weaver, and Reppert 2007) facilitating the transcription of 3 *period* genes **mPer1** (Gekakis et al. 1998)**, mPer2** (Shearman et al. 1997) and **mPer3** *(*mouse orthologs of the *Drosophila period* gene)(Zylka et al. 1998), 2 *cryptochrome* genes **Cry1** and **Cry2**(Griffin, Staknis, and Weitz 1999)*,* the **Rev-erbα gene**(Guillaumond et al. 2005), the *retinoic acid receptor-related orphan receptor alpha* **(Rorα)** gene(Sato et al. 2004), and also in multiple 'clock controlled genes.' In the second segment of the feedback loop, advancing towards the early night, the levels of PERIOD and CRY proteins start accumulating, form heterodimers and are translocated into the nucleus(Yagita et al. 2000) where they repress their own transcription by binding and inhibiting BMAL1:CLOCK/NPAS2 mediated-transcription(Sangoram et al. 1998; Sato et al. 2006). PERIOD and CRY proteins are subsequently degraded, allowing the binding of BMAL1:CLOCK/NPAS2 to initiate another transcriptional cycle. A complementary component of the core circadian clock is constituted by the oscillatory expression of the *bmal1* gene, RORα and REV-ERBα proteins, which bind the *ROR response element* (RRE) and induce and repress transcription of the *bmal1* gene respectively(Guillaumond et al. 2005). Corresponding with the generation of circadian rhythms, the period of the core autoregulatory feedback loop is approximately 24 hours. Of note, the rhythmicity of the core molecular clock is modulated at the level of transcriptional rate, protein stability, and nuclear trafficking, all of which can be modulated by posttranslational modifications of the clock proteins. Protein phosphorylation constitutes one of the main modifications involved in the timing of circadian rhythms(Takano, Isojima, and Nagai 2004; Miyazaki

et al. 2004). The first observations suggesting a role of protein phosphorylation in the timing of the molecular clock began with the identification of the *tau* mutant in hamsters, characterized by a reduction in the circadian rhythm period to ~20 hours (Ralph and Menaker 1988). Posterior genetic analysis establish that the tau mutation occur in the protein *casein kinase I epsilon* **(CKIε)**(Lowrey et al. 2000)**.** CKIε is a key protein kinase for the regulation of timing oscillations through the phosphorylation of BMAL1, CRY and PER proteins. PER phosphorylation by CKIε(Vielhaber et al. 2000) and *Glycogen Synthase Kinase 3 Beta* **(GSK3β)**(Iitaka et al. 2005) modulate its nuclear translocation, and therefore inhibitory activity over the BMAL1:CLOCK/NPAS2 transcription dimer. CKIε also regulates the phosphorylation of CRY and BMAL1proteins before their translocation to the nucleus (Eide et al. 2002). CKIε/δ also regulate the required turnover or PER proteins, phosphorylation at specific residues facilitates the ubiquitinylation by *beta-transducin repeat containing E3 Ubiquitin Protein Ligase* **(β-TRCP),** and subsequent degradation by the proteasome (Eide et al. 2005). CRY protein degradation in turn is initiated by phosphorylation by *5′ adenosine monophosphate-activated protein kinase* **(AMPK),** followed by ubiquitinylation mediated by the E3 ligases *F-box and leucine rich repeat protein3 or 21* **(FBXL3/21)** and proteasome degradation (Lamia et al. 2009). Additional levels of regulation converge acts on the BMAL1 protein, where the *mitogen-activated protein kinases* (MAPK) pathway mediates inhibitory phosphorylation in BMAL1(Sanada, Okano, and Fukada 2002), and rhythmic SUMOylation of BMAL1 regulates its turnover (Cardone et al. 2005). The interdependent molecular mechanisms just described are central to maintain robust oscillations of cell-autonomous circadian

rhythms even in the absence of environmental cues. However, the period and phase of circadian rhythms can be entrained by these external cues inducing a reset of the molecular clock.

Circadian Entrainment

The period of free-running circadian rhythms is close to, but not perfectly aligned with, the 24 h rotation of the earth (Pittendrigh 1960). Furthermore, in high latitudes there are seasonal variations in the period of certain external cycles. For example, the length of the daylight (photoperiod) in the summer differs from daylight length in the winter (Johnston 2005). If not synchronized properly, circadian rhythms may drift away from the actual period of environmental oscillations, and consequently the organism will lose the capacity to anticipate and respond to natural cycles (Vaze and Sharma 2013). Therefore, the period and phase of endogenous oscillations of circadian rhythms are constantly entrained to match the period of environmental oscillations. Notably, entrainment to environmental cues is not merely a passive response of the circadian clock, but rather entrainment results from an active interaction between signals generated by external cues and the timing of the molecular clock (Roenneberg, Daan, and Merrow 2003). Consequently, entrainment only occurs when environmental cues or Zeitgebers are presented during the appropriate phase of the circadian cycle. This is exemplified in the construction of Phase Response Curves (PRC), a graphical representation of how similar stimuli produce different effects across the day(Serge Daan and Pittendrigh 1976).

Oscillations of circadian rhythms are characterized by an endogenous period defined as the length of one full cycle, and a circadian phase, defined as the displacement in the oscillatory cycle relative to an environmental reference point (Pittendrigh 1960). In the constant present of Zeitgebers the period and phase of circadian rhythms are aligned to the environmental cycle. Light as a Zeitgeber is used as reference point of day and night; therefore circadian rhythms period and phase are adjusted to match the illuminated segment of the cycle, and the dark period of the cycle. In the free running state, when light as Zeitgeber is no longer present, the endogenous cycles will still correspond with the previously illuminated segment of the cycle, which constitute the subjective day, and also correspond with the dark segment of the cycle, which constitute the subjective night. PRCs are plots that depict the magnitude of phase shifts, caused by Zeitgebers presented at different times of the day (Johnson 1999). In mammals, light constitutes one of the strongest entrainment signals, and thus light-induced entrainment has been widely studied in nocturnal rodents. PRC constructed from these studies show that in free-running conditions photic input given during the subjective day, cause minimal phase shifting, but the same inputs during early subjective night cause shift delays, modifying the phase of the circadian rhythms to start the new cycle later than the usual reference point. Light pulses given during the late subjective night cause phase advances, modifying the phase of the circadian rhythms to start the new cycle before the usual reference point (Serge Daan and Pittendrigh 1976). During light-induced entrainment, photic input reach the retina and activate melanopsin-containing ganglion cells(Provencio et al. 1998) that in turn send direct projections to the SCN that comprising the retinohypothalamic tract

(RTH)(Gooley et al. 2001), one of the main afferents to the SCN. Terminals of the RTH release glutamate(Castel et al. 1993; de Vries et al. 1993) and *pituitary adenylate cyclase-activating peptide* (PACAP) principally in the ventrolateral SCN (J. Hannibal et al. 1997). In postsynaptic neurons of the SCN, glutamate acts upon ionotropic receptors like the *N-methyl- D-aspartate receptor* (NMDA)(Mintz et al. 1999) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Michel, Itri, and Colwell 2002), glutamate can also act upon metabotropic receptors(Meeker, Greenwood, and Hayward 1994). PACAP in turn acts upon *PACAP- type I receptor* (PAC1) a G-protein coupled receptor (GPCR)(Michel et al. 2006), and also upon the VIP receptor(Kalamatianos et al. 2004). The glutamate activation of NMDA receptors together with the consequently activation of L-type voltage-dependent calcium channels induces transient increases in the intracellular Ca^{2+} concentration(Colwell 2001; Dziema and Obrietan 2002). Similarly, the activation of GPCRs induce the accumulation of *3',5'-cyclic adenosine monophosphate* (cAMP)(Tischkau et al. 2000). These two second messengers in turn activate several signaling cascades mediated by protein kinases including *protein kinase A* (PKA) (Prosser and Gillette 1989), *protein kinase G* (PKG)(Ferreyra and Golombek 2001); *calcium-calmodulin kinase* (CamK)(Agostino et al. 2004), the MAPK/ERK pathway (Obrietan, Impey, and Storm 1998) and the effector kinases *Nuclear Mitogen- And Stress-Activated Protein Kinase* (MSK) (Butcher et al. 2005)and *Ribosomal S6 Kinase* (RSK)(Butcher et al. 2004). All of these signaling cascades principally converge on the activation of two transcription factors, *cAMP response element (CRE) binding protein* (CREB)(Obrietan et al. 1999) and *ETS*

transcription factor (ELK-1) (Coogan and Piggins 2003). The ultimate effect of light on the SCN is the transcriptional induction of thousands of genes. Included in this group are the immediate early genes *FBJ Murine Osteosarcoma Viral Oncogene Homolog* (c-Fos), Jun-B and *early growth response 1* (EGR1)(Dziema et al. 2003), and the core clock genes mPer1 and mPer2. Changes in the levels of these two clock genes, together with numerous clock-controlled genes, are responsible for the appropriate shifts in the circadian clock phase (Yan and Silver 2004).

Central and Peripheral Clocks

Cell-autonomous molecular clock expressed in almost all cell types of the organism (Yoo et al. 2004). Within each cell type, oscillations of specific clock-controlled genes reflect the circadian regulation of multiple biological processes, including but not limited to sleep-wake cycles(Saper, Scammell, and Lu 2005), temperature(Brown et al. 2002), hormonal signaling (Kaneko et al. 1980; Oster et al. 2006) and metabolism(Kalsbeek et al. 2007). At the systems level, circadian rhythm synchrony is maintained by a hierarchical organization entailing a constant communication and feedback between a central clock in the SCN and several self-sustained peripheral clocks distributed within the brain and across the entire body (see review by (Dibner, Schibler, and Albrecht 2010)). Thus, loss of the SCN central clock does not eliminate oscillation in peripheral tissues, instead it cause a phase desynchronization of the peripheral clocks (Yoo et al. 2004). Outputs from the SCN

support synchronization and reach peripheral clocks either by direct neural transmission(Leak and Moore 2001) or by hormonal signals(Balsalobre et al. 2000; Le Minh et al. 2001). Peripheral clocks have been described in other brain regions using molecular clock expression markers(Abe et al. 2002). Self-sustained oscillation is present in the olfactory bulb and the retina, whereas secondary SCN-dependent clocks have been described in almost 27 different brain regions including the cerebral cortex, hippocampus, central and basal nucleus of the amygdala, pituitary, and the pineal gland. Genetic and pharmacological interventions modifying clock genes in these regions induce major changes in the main biophysical, physiological, and behavioral parameters related with each region (Guilding and Piggins 2007). Circadian oscillations mediated by the molecular clock have been also described in peripheral organs constituting an important variable controlling tissue-specific processes(Dibner, Schibler, and Albrecht 2010). Process regulated by oscillation of the molecular clock include wound healing(Cable, Onishi, and Prendergast 2017) and stem cell differentiation in the skin(Janich et al. 2013), metabolism and toxic clearance in the liver(Gachon et al. 2006), urine filtration in the kidneys(Kamperis et al. 2004), cell division in the small intestine(Matsu-Ura et al. 2016), metabolism in muscle (Lefta, Wolff, and Esser 2011) and adipose tissue(Le Martelot et al. 2009), blood pressure in the circulatory system (Ohashi et al. 2016), and immune responses(Scheiermann, Kunisaki, and Frenette 2013). Remarkably, besides the light-induced entrainment mediated by the SCN central pacemaker, mammalian peripheral clocks can also be entrained by other Zeitgebers, food availability being one of the most effective(Damiola et al. 2000). Consequently, when food is restricted to specific times of

day, metabolic and behavioral responses are adjusted to be consistent with variations in food availability(Stokkan et al. 2001).

Regulation of Suprachiasmatic Nucleus and Hippocampal Cellular Activity as a Function of Circadian Signaling

Given the impact of circadian rhythms over a broad number of physiological, metabolic, and behavioral processes, elucidation of clock-gated processes in different tissues and cell types constitute a major endeavor in the field biological rhythms. Genome-wide expression analyses have estimated that the around 10 % of each tissue-specific transcriptome is controlled by the circadian clock. However, only 1% of the clock controlled transcriptome overlaps across multiple tissues, reflecting the numerous and diverse clock-gated biological processes (see (Mohawk, Green, and Takahashi 2012)). The work presented here describes the role played by the circadian clock in key biological processes of two brain regions, the SCN and the hippocampus. First, I identify the significant changes in the SCN transcriptome underlying light-induced entrainment, focusing in particular on the changes mediated by the MAPK/ERK signaling pathway **(Chapter 2)**. Using a comprehensive mouse gene expression array assay covering both coding and non-coding transcripts, the effects of light in the SCN transcriptome at different circadian times was evaluated. In addition, by pharmacological inhibition, transcripts responding specifically to the differential light-induced activation of the MAPK/ERK pathway were identified.

Also examined here is the role of circadian clocks in two fundamental hippocampal biological processes. I begin with the characterization of the relationship between the molecular clock and the encoding and retrieval of contextual fear memories **(Chapter 3).** First, I report that the ability of mice to retrieve contextual fear conditioning memories differs along the day, specifically when comparing retrieval at day versus early night. Also, using the mPeriod1-Venus mouse line as a circadian clock reporter, it was found that oscillations of the molecular clock in hippocampal neurons are heterogeneous, exhibiting cells at diverse phases of the circadian clock within the CA1 hippocampal region. Subsequent analysis demonstrates that memory ensemble neurons recognized by the induction of the immediate early gene c-Fos showed relatively higher levels of the reporter mPeriod1-Venus. Finally, I demonstrate the importance of the circadian clock in the regulation of proliferation and differentiation of adult neural stem/progenitor cells localized in the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) **(Chapter 4).** Using the clock marker mouse line mPer1-Venus, it was determined that stem/progenitor cells in the rodent SGZ express a functional circadian clock. By disruption of the molecular clock in the bmal1 knockout line, an abnormal increase in the proliferation and differentiation capacities of these stem/progenitor cell population was observed.

CHAPTER 2

Light-induced Changes in the Suprachiasmatic Nucleus Transcriptome Regulated by the MAPK/ERK Pathway

Mammals exhibit evolutionary conserved circadian (24 hour) oscillations in physiological, metabolic, and behavioral processes. These self-sustained rhythmic oscillations are generated by approximately 20,000 neurons organized in the suprachiasmatic nucleus (SCN) of the hypothalamus (Herzog 2007). The SCN thus constitutes a biological master clock driving and coordinating intrinsic rhythms of peripheral clocks distributed through the entire organism , whose period matches the phase of oscillations of environmental signals, such as light, temperature, nutrients, and even social interactions(Welsh, Takahashi, and Kay 2010). In order to maintain the synchrony between the phases of circadian and environment oscillations, the timing of the SCN clock is entrained by environmental *Zeitgebers* ('time givers'), the light-dark cycles produced naturally by the 24h earth rotation being one of the most potent (J. Aschoff 1960).

The SCN endogenous timing is mediated by a transcriptional feedback loop of a specific set of *clock genes* that are interlocked with additional transcriptional loops of thousands of *clock-controlled genes* (King and Takahashi 2000). Previous studies have

shown that light-induced entrainment is achieved, at least in part, by transcriptional changes that reset the clock feedback loop in the SCN (Romijn et al. 1996). Specifically, photic stimuli activate retinal photoreceptive ganglion cells that reach the SCN principally through the retinal hypothalamic tract (RHT), then through the release of glutamate and pituitary adenylate cyclase-activating polypeptide (PACAP), different intracellular second-messenger signaling pathways are induced (Jens Hannibal 2002), generating changes in the expression of key genes that reset the SCN clock and modify the phase of the circadian rhythms (Rusak et al. 1990).

Intriguingly, entrainment is not merely a passive response to *Zeitgebers*, instead the internal oscillator responds and adjust its own timing to entrainment signals discriminating the time of day. For example, exposure to brief light pulses in the first and final hours of the subjective generate circadian phase delays and advances respectively; whereas similar photic pulses during the subjective day does not modify the phase(Roenneberg, Daan, and Merrow 2003). Central to this process is the MAPK/ERK signaling pathway, which plays a critical role in the coupling between photic inputs and gene transcription that lead to circadian phase entrainment (Dziema et al. 2003). Corresponding to the time-of-day differences in entrainment, the ERK/MAPK pathway is highly responsive during the subjective night, but shows low responsiveness during the subjective day(Obrietan, Impey, and Storm 1998). Furthermore pharmacological inhibition of this pathway abrogates light-induced phase entrainment (Butcher et al. 2002).

The role of MAPK/ERK in entrainment has been widely described, but the task of identifying the number and nature of the genes whose transcription is regulated by this pathway and that are crucial for entrainment is still unfinished. Particularly, some studies have tried to identify the entire set of genes in the SCN that respond to light (Jagannath et al. 2013; Hatori et al. 2014; Porterfield, Piontkivska, and Mintz 2007), but to our knowledge all of them have been centered on protein coding genes, leaving out a significant group of other of transcripts known as non-coding genes (i.e. microRNAs, long-non-coding RNAs, ribosomal RNAs). Here we assess and report the light-responsive transcriptome at different circadian time points chosen based on the profoundly different effects that light has on clock phase entrainment and MAPK/ERK activation: early subjective day (CT5), early subjective night (CT16), and late subjective night (CT23). Moreover, at the same circadian time points we determine the contribution of the group of transcripts whose light-evoked expression is modulated by the ERK/MAPK pathway by analyzing transcriptomic changes when light pulses were delivered in the presence of the *Mitogen-activated protein kinase kinase 1/2* (MEK1/2) inhibitor U0126. Hence, we present here a transcriptome-wide examination of light- and ERK/MAPK dependent circadian entrainment.

Material and Methods

Animal Subjects

The Ohio State University Animal Care and Use Committee approved the experimental protocols and procedures in which adult wild type C57BL/6J were mice

were used (protocol number: 2008A0227). Mice were grouped in home cages under a 12h light/12h dark (LD) cycle, with a temperature ranging between 65-75°F (~18-23°C); 40-60% of humidity, with water and food provided *ad libitum.*

Cannulation

All surgical procedures were performed during the illuminated portion of the light cycle. Adult C57BL/6J wild type (WT) mice (8 to 14 weeks old) were anesthetized by intraperitoneal injection of Ketamine (95.2 mg/kg)– Xylazine (30.8 mg/kg) and full anesthesia was ensured through hind paw toe pinch. Scalp fur was then removed and mice were placed in stereotaxic apparatus (Cartesian Research) followed by the application of ophthalmic ointment to protect the eyes. Next, with the head level, the skull was exposed by a longitudinal cut and a single hole was drilled to access the dura (coordinates: anterio-posterior -0.22mm. from bregma; and medial-lateral +1.0 mm from the midline). From the dura, using a dorso-ventral coordinate of -2.00 mm.,the tip of a 10 mm 24-gauge guide cannula was positioned in the right lateral ventricle. The cannula were secured to the skull with dental cement and a 10.5 mm 30-gauge dummy cannula was placed to seal the guide cannula. After the surgery mice were individually housed and allowed to recover from anesthesia under a heated pad with access to wet food. Full recovery was permitted for 2 weeks, during which ibuprofen (30 mg/kg body weight) was diluted in the drinking water.

Drug Infusion and Light Pulse

During the two weeks of recovery, mice were maintained under the original LD cycle, where for convention Zeitgeber Time 0 (ZT0) corresponds to the time of lights-ON and ZT12 correspond to the time of lights-OFF. Two days before infusion, mice were moved to 12h dark/12h dark (DD) cycle, where by convention circadian time 0 (CT0) corresponds to the expected beginning of the subjective day, and CT12 correspond to the expected time of the subjective night. Infusions were performed under dim red light (<2 lux) at one of three time points: CT4, CT15 or CT22. For infusion of vehicle (DMSO) or U0126 (10mM, Cell Signaling, #9903), mice were manually restrained and a 10.5 mm 30-gauge injector placed in the guide cannula was used to infuse a total of 3uL at a rate of 0.6uL/min. After the infusion, the injector was kept in the guide cannula for one additional minute to prevent the drug from seeping back out. For each of the three time points mice were divided in four groups: DMSO-No Light Pulse (DMSO-Sham); DMSO-Light Pulse (DMSO-Light); U0126-No Light Pulse (U0126-Sham); and U0126-Light Pulse (U0126-Ligth). 30 minutes after the corresponding infusion, mice were placed in a lightproof chamber and either exposed to a light pulse of 100 lux for 10 minutes (light groups) or kept in the dark for the same amount of time (sham groups). Finally, for immunohistochemistry processing, tissue samples were collected 10 minutes after the end of the light/sham pulse, whereas for RNA isolation, tissue was collected 60 minutes after the end of the light/sham pulse.

Immunohistochemistry

Ten minutes after the light/sham pulse, mice were euthanized by cervical dislocation under dim red light, brain tissue was collected and transferred to ice-cold oxygenation media (NaCl 120mM, KCl 3.5mM, HEPES 10mM, $CaCl_2$ 0.5mM, NaH_2PO_4 1.2mM, $MgSO_4$ 2mM, $NaHCO_3$ 32.3mM, Glucose 10mM), then 0.6 mm coronal tissue sections containing the SCN were obtained by vibratome (Leica Biosystems Inc. - VT1200. Buffalo Grove, IL). Sections were fixed in 4% paraformaldehyde (PFA) for 12 hours at 4°C and transferred to 30% sucrose solution in PBS supplemented with sodium azide (2mM) and sodium fluoride (3mM) for cryopreservation. Free Floating coronal sections (40 μm) spanning the entire SCN were obtained with a freezing microtome. A total of 2 sections were used per condition. Sections were washed in PBS (3 times - 5 minutes), permeabilized in 0.1% Triton X-100 in PBS (PBS-T) for 30 minutes, incubated in 0.3% H_2O_2 in PBST 15 min, blocked in 10% normal goat serum (NGS) in PBS-T for 2h, incubated overnight at 4°C with the rabbit polyclonal primary antibody anti-Phospho-Erk1/2 MAPK (Thr202/Tyr204) (1:500, Cell Signaling, #4370) prepared in 5% NGS in PBS-T. Next, sections were incubated for 2h at room temperature in 5% NGS in PBS-T with goat anti-rabbit biotinylated secondary antibody (1:500, Vector Laboratories) followed by incubation in avidin/biotin horseradish peroxidase complex for 1 h (Vectastain Elite ABC labeling kit, Vector Laboratories). Finally, signal was developed with diaminobenzidine-nickel-intensified substrate (Vector Laboratories), cleared with Xylene solution, and mounted on gelatin-coated slides with Permount media (Fisher Scientific, Houston, TX, USA).

RNA isolation from Tissue

Sixty minutes after the light/sham pulse mice were euthanized by cervical dislocation under dim red light, brain tissue was collected and transferred to ice-cold oxygenation media (NaCl 120mM, KCl 3.5mM, HEPES 10mM, $CaCl_2$ 0.5mM, NaH_2PO_4 1.2mM, $MgSO_4$ 2mM, $NaHCO_3$ 32.3mM, Glucose 10mM), then 0.6 mm coronal tissue block containing the SCN were obtained by vibratome sectioning (Leica Biosystems Inc. - VT1200. Buffalo Grove, IL). Sections were placed on a microscope slide, flash frozen over a block of dry ice, and the SCN was dissected out under a stereomicroscope. SCN from two animals under the same treatment were pooled in conical tubes and stored at -80°C. RNA from each pooled sampled was isolated by pestle disruption in 500µL of Trizol (Invitrogen), followed by the addition and mixing with 125µL of chloroform, and centrifugation at 16,000xg for 15 minutes at 4°C. The aqueous phase was recovered and mixed in a 1:1 proportion with chloroform and centrifuged at 16,000xg for 5 minutes at 4°C, this final aqueous phase was mixed 1:1 with 70% ethanol and applied to RNAesy minikit columns (Qiagen). RNA in the columns was washed successively with buffer RW1, buffer RPE x2, and 80% ethanol. The RNA was eluted and resuspended in 20µL of RNase Free water.

Microarray Hybridization and Data Analysis

For microarray hybridization, a single sample corresponds to SCN tissue from two mice under the same treatment group. A total of 36 samples were collected,

corresponding to 4 groups at 3 time points and n=3. RNA from each sample was quantified using a nanodrop2000 (ThermoFisher) spectrophotometer followed by analysis of RNA integrity in an Agilent 2100 Bioanalyzer System (Agilent). Only RNA samples with a 260/280 ratio ≥ 1.9; 260/230 ratio ≥ 1.0 and a RIN score ≥ 7 were used. Probes were prepared with 100 ng of total RNA as starting material using the GeneChip WT Plus Reagent Kit (Affymetrix, Santa Clara, CA); and hybridized to GeneChip Mouse Transcriptome Assay 1.0 following the manufacturer's guidelines (Affymetrix, Santa Clara, CA).

Intensity CEL files from each sample were normalized, log transformed with the SST-RMA algorithm, followed by quality control implemented in the Affymetrix Expression Console (Affymetrix, Santa Clara, CA). The resulting CHP files were loaded into the Affymetrix Transcriptome Analysis Console 3.0 (Affymetrix, Santa Clara, CA) and differentially regulated genes were obtained by one-way ANOVA analysis, where genes with a p-value ≤ 0.05 and a fold change absolute value ≥1.5 were considered significant; the list of genes obtained was plotted in a hierarchical cluster map of log2 normalized signal using the *clustergram* function from MATLAB software, release 2016b (The Mathworks Inc., Natick, MA).

Functional Clustering Analysis And Enrichment Map

From the differentially regulated gene list, the subgroup of protein-coding genes was analyzed in order to identify enriched functional categories. Using the DAVID

(Database for Annotation, Visualization and Integrated Discovery) web-base annotation tool (Huang, Sherman, and Lempicki 2008) the list of coding-genes was uploaded to the DAVID website and contrasted with a *Mus Musculus* genome background for functional enrichment. Enrichment was considered significant for functional categories under the *Functional Annotation Chart* module with a modified Fisher's Exact test (EASE score) ≤ 0.05. Results were visualized using the *Enrichment Map* plugin develop for *Cytoscape 3.0 (Merico et al. 2010)* following permissive parameters with p-value ≤ 0.05; FDR ≤ 0.25 and overlap coefficient of 0.3. Finally, functional categories were arranged following the DAVID *Functional Annotation Clustering* module with their respective Enrichment Score.

Results

Differential Activation of the ERK/MAPK Signaling Pathway by Photic Stimulation

To validate light-dependent activation of ERK/ signaling cascades in the SCN, mice entrained to standard LD lighting conditions (12h light/ 12h dark) were transferred to DD for two days and exposed to a 10 min light pulse of 100 lux at at one of three time points: CT4 (subjective day), CT15 (early subjective night) or CT22 (early subjective night); previous reports have shown that similar light pulses at early and late subjective night induce circadian phase shifts and activate the ERK/MAPK signaling pathway (Butcher et al. 2002). Ten minutes after the end of the light pulse, SCN tissue was collected and immunostaining was performed against ERK1/2 Thr202 and Tyr204

phosphorylation (p-ERK1/2), a marker of ERK1/2 activation. Immunolabeling in coronal and sagittal sections containing the SCN show time-of-day dependent increases in activated p-ERK1/2 levels. Specifically, there is no increase in p-ERK1/2 levels when the light pulse is delivered at CT4 (subjective day) (Figure 2.1C compared to 2.1F and 2.1I compared to 2.1L), whereas a light pulse delivered at CT15 (early subjective day) induced a robust increase in the levels of p-ERK1/2 in the SCN when compared to sham light pulse controls (Figure 2.1 compare A with B; also Figure 2.1 compare Figure D with G and J with M). A similar increase is observed when the light pulse is delivered at CT22 (late subjective night) (Figure 2.1 compare E with H, K with N). It should be noted that this pattern of ERK1/2 activation parallels, at the functional level, where light-induced phase delays at CT15 and phase advances at CT22 (Takahashi et al. 1984).

Pharmacological Inhibition of light-dependent ERK1/2 activation

U0126 is a Mitogen-activated protein kinase kinase 1/2 (MEK1/2) inhibitor previously shown to abolish the effect of light on both circadian phase shifts and ERK1/2 activation in *vivo(Butcher et al. 2002)*. To validate the latter outcome, levels of pERK1/2 were evaluated by delivering light pulses in the presence of U0126. The process followed is described in Figure 2.2A. Briefly, mice were entrained to standard 12h light/12h dark (LD) conditions immediately after weaning, once they develop into adulthood (8 weeks old) cannulas were surgically placed and secured to the skull targeting the lateral ventricles (Figure 2.2B) and used to deliver U0126 to the SCN. Effective delivery of

U0126 to the SCN can be observed by the complete absence of p-ERK1/2 signal around the third ventricle (Figure 2.2C). Mice recovered from surgery for two weeks under the original LD conditions and then were changed to DD conditions for two days. On day three, at specified time points (CT4, CT15 or CT22), U0126 (10mM) was infused into the ventricles; 30 minutes later a phase shifting light pulse (100 lux, 10 min) was delivered, and the SCN containing tissue collected 10 minutes after the end of the pulse. Qualitative analysis of immunolabeling in coronal and sagittal sections at CT4 shows, due to the absence of light-dependent increases in p-ERK1/2, a reduction only in the basal levels of p-ERK1/2 in the SCN (compare Figure 2.2 G with D and Figure 2.2 M with J); U0126 eliminates light-induced increases of p-ERK1/2 observed when DMSO vehicle is infused in the SCN at CT15 (compare Figure 2.2 H with E and Figure 2.2 N with K) and CT22, (compare Figure 2.2 I with F and Figure 2.2 O with L).

Identification of Light-Dependent Changes in the SCN Transcriptome

Following the protocol described in Figure 2.2A, total RNA from the SCN was collected at one of three time points: CT5, CT16 or CT23. These time points correspond to tissue collection 60 min after the sham/light pulse exposure at CT4, CT15 or CT22, respectively. In summary, four treatment groups were established at each of this three time points: DMSO-Sham, DMSO-Light, U0126-Sham and U0126 Light. Consequently, vehicle or U0126 was infused 30 min before the sham or light pulse (100x, 10 min) and SCN tissue was collected for total RNA isolation 60 minutes after the end of the pulse.

As a starting point, microarray hybridization analysis was used to identify transcriptomic changes in the SCN that were induced by light exposure at each time point (Figure 2.3). Specifically, we identified genes with significant changes in expression values by comparing the DMSO-Light group versus the DMSO-Sham group. When comparing the transcriptome changes between DMSO-Light and the DMSO-Sham groups at CT5 we discovered a total of 428 genes showing a significant change induced by light (Figure 2.3A, Table 1_Sheet1). From this group, 55 genes were up-regulated by light, whereas 373 genes were down-regulated by light (Figure 2.3D, E). Fifty-four of the transcripts corresponded to protein-coding genes and 374 to non-coding genes (Figure 2.3D). When we applied the same type of analysis to compare DMSO-Light and DMSO-Sham groups at CT16, we identified 351 genes whose expression in the SCN were changed by light (Figure 2.3B, Table 1_Sheet2); out of these 351 genes, 254 genes were up regulated and 97 down-regulated (Figure 2.3D,E); 128 were coding genes and 223 non-coding genes (Figure 2.3D). Comparisons at CT23 of DMSO-Light and DMSO-Sham resulted in the identification of 705 differentially expressed genes (Figure 2.3C, Table 1_Sheet3), with 282 up-regulated genes, 413 down-regulated genes (Figure 2.3D, E), and 413 and 292 coding and non-coding genes respectively (Figure 2.3D). Finally, an intersectional analysis of up-regulated genes identified 2 genes that responded to light at both CT5 and CT16, five genes that responded to light and CT5 and CT23, and 10 genes that were up-regulated by light at CT16 and CT23 (Figure 2.3E, Table 2_Sheet1). With similar intersectional analysis for down-regulated genes, it was observed that 13 genes were-down regulated by light at both CT16 and CT23 (Figure 2.3E, Table 2_Sheet2).

Gene Functional Ontology Analysis

In order to explore the biological functions in the SCN that potentially change with light, ontological enrichment analysis was performed in the web-based database DAVID (Huang, Sherman, and Lempicki 2008) using the protein-coding genes differentially regulated by light at each time point. Given the small number of coding-genes changing with light at CT5, just 5 functional categories were found with significant enrichment (Figure 2.4A). In contrast to this, the expression of a larger group genes changed in the SCN with light exposure at CT16 (128 genes), or at CT23 (413 genes); as a consequence, more functional categories were enriched at these two time points. For CT16, 48 non-redundant functional categories were identified (Figure 2.4B), whereas for CT23 a total of 102 functional categories were recognized (Figure 2.4C). Since there is a large amount of functional categories that may be enriched under this analysis, visualization and interpretation of the full list of functional categories can be challenging. To overcome this issue, visual representation of functional categories interaction networks were prepared with the *Enrichment Map* plugin for *Cytoscape 3.0* software(Merico et al. 2010). Interaction networks at CT5 identified just 3 clusters corresponding with cluster enrichments given by the comprehensive DAVID database analysis, including Transcriptional Activation, Chromatin Modification, and Cell Membrane proteins (Figure 2.5A). At CT16 a total of 7 clusters were generated and included among them clusters for Circadian Rhythms related genes, Plasma Membrane proteins, Neurotransmitter and Neuropeptide, Inflammatory Responses and Hormone Mediated Signaling (Figure 2.5B). Correspondingly, 16 functional clusters were

generated at CT23, including clusters such as Receptor Proteins, Antigen Processing/Presentation, Complement Pathway, Defense Response to Virus, Innate Immunity, and Neuropeptide Hormones (Figure 2.5C).

Light-dependent Transcriptome Changes Regulated by the ERK/MAPK pathway

To further understand the role of the ERK/MAPK signaling pathway in light-induced changes to the transcriptome, a line of analysis was followed to identify the transcriptomic subgroup whose expression is modulated by the activation of the ERK/MAPK signaling cascade. First, the changes in expressed genes between the U0126-Light and DMSO-Light at each time point was evaluated (Figure 2.6), which corresponded to genes whose expression is modified by the presence of U0126 after a light pulse. With this assessment we detected changes in expression levels of 237 genes at CT5 (Figure 2.6A, Table 3_Sheet1), which was comprised of 86 up-regulated genes and 151 down-regulated genes (Figure 2.6D,E) and encompassed 82 coding genes and 155 non-coding genes (Figure 2.6D). Analysis at CT16 resulted in 840 differentially expressed genes (Figure 2.6B, Table 3_Sheet2), composed of 656 up-regulated genes and 184 down-regulated genes (Figure 2.6D,E), and included 178 coding genes and 662 non-coding genes (Figure 2.6D). Lastly, 464 genes with a significantly different expression were found at CT23 (Figure 2.6C, Table 3_Sheet3), representing 83 up-regulated genes and 381 down-regulated genes (Figure 2.6D,E), and 59 coding genes and 405 non-coding genes (Figure 2.6D). Intersectional analysis pinpointed 5 genes that were up-regulated at

CT5 and CT16; and 5 genes that were up-regulated at CT16 and CT22 (Figure 2.6E, Table 4_Sheet1). Thirty five genes were down-regulated at both CT5 and CT16, 14 genes at CT5 and CT23, 26 genes at CT16 and CT23, and 11 genes whose expression was down-regulated at the three time points (Figure 2.6E, Table 4_Sheet2).

With this data set, two main groups of genes at each circadian time were discovered; the first group corresponds to genes whose expression in the SCN is modified by light (Figure 2.3); whereas the second group corresponds to genes whose expression in the SCN changed when exposed to light in the presence of U0126 compared to light exposure in the absence of U0126 (Figure 2.6). The second step in the line of analysis was to intersect these two main groups in order to isolate those genes that fulfill two conditions: 1) its expression changed in response to light, and 2) its expression in response to light changed when light was given in the presence of U0126. The genes isolated by this analysis constitute the portion of the SCN transcriptome regulated by the light-induced activation of the ERK/MAPK pathway. Accordingly, this specific transcriptome included 26 genes at CT5, where it should be noted that 8 out 26 (31%) were protein-coding genes, 14 (54%) were non-coding genes, 3 (11%) were microRNA precursors and 1 (4%) was a small non-coding RNA (Figure 2.7A, Table 5_Sheet1). For CT16, there were 67 genes, comprised of 39 (58%) protein-coding genes, 24 (36%) non-coding genes, 1 (2%) microRNA precursor, 1 (2%) small non-coding RNA and 2 (3%) pseudogenes (Figure 2.7B, Table 5_Sheet2). For CT23, there were 52 genes, distributed as 4 (7%) coding genes, 43 (83%) non-coding genes, 4 (7%) microRNAs precursor and 1 (3%) Ribosomal RNA (Figure 2.7C, Table 5_Sheet3).

Discussion

Light inputs to the SCN from the retina are transduced intracellularly by several cellular signaling pathways, resulting in the fast modifications in genomic expression required for circadian entrainment including CamKII, PKA, PKG and the MAPK/ERK pathways(reviewed by (Golombek and Rosenstein 2010)). Here, by pharmacological inhibition and immunohistochemical assays, we corroborate previous findings describing the ERK/MAPK pathways as one of the central second-messenger signals coupling photic inputs with alterations in SCN transcriptional activity (Dziema et al. 2003). Furthermore, microarray assay analysis allowed us to cover the portion of the SCN transcriptome that responded to light across defined time points with unprecedented detail by using a gene chip assay that covers a larger number of both coding and non-coding genes described so far. In contrast, previous efforts to identify the light-responsive transcriptome were limited to the protein-coding portion of the transcriptome or restricted to a single timepoint (Jagannath et al. 2013; Hatori et al. 2014; Porterfield, Piontkivska, and Mintz 2007). It should be noted that we limited our study to transcripts that were altered 60 min after the light pulse; therefore analysis of temporal dynamics of transcription was not included. In our analysis, comparisons made between timepoints along the circadian cycle revealed profound differences in the number and nature of transcripts that show a significant change induced by light. Additionally, ontological analysis of the protein-coding transcriptome at each time point, reflect profound

differences in the biological functions affected by light at each time point. Taken together, the data presented here expand the transcriptional landscape that is known to respond to light in the SCN, a key step to fully understanding phenotypical characteristic of circadian clock entrainment.

Several of the transcripts highlighted in our dataset were previously recognized to change in response to light, validating our experimental approach. Within our gene set, we note the presence of key entrainment genes during the subjective night, where light effects are more notable. As expected, there are several genes with a conserved CRE element in their promoters regions, implicating the transcription factor CREB, a recognized central player in light-induced phase entrainment (von Gall et al. 1998; Gau et al. 2002). The immediate-early gene c-Fos is one such example. Several studies have shown that both *cfos* mRNA and c-Fos protein are dramatically increased after light exposure at the subjective night (Rusak et al. 1990; Dziema et al. 2003; Porterfield, Piontkivska, and Mintz 2007; Kornhauser et al. 1990) and blocking its expression disrupts the circadian entrainment produced by light (Wollnik et al. 1995) The CRE element is also present in the promoter region of Per1 and Per 2, two key genes part of the circadian clock transcription loop, whose expression is dually regulated, first by the circadian clock machinery itself, as well as by light-induced inputs to the SCN at night, thus entraining the circadian clock to the novel light-dark cycle (U. Albrecht et al. 1997; Schwartz et al. 2011). In addition, our data validate the light- induction at night of Egr2 and Egr3, two immediately early genes belonging to the zinc finger transcription factor family, whose promoters also contain a CRE element in their promoter regions, but for

which a clear role in entrainment is not established yet (Morris et al. 1998; Porterfield, Piontkivska, and Mintz 2007; Porterfield and Mintz 2009). The microRNA precursor of miR-212/132, was also identified in our analysis. Light signaling to the SCN at night prompts CRE-CREB-dependent expression, processing, and maturation of this precursor miRNA resulting in the generation of miR-132. miR-132 in turn, is able to reduce the entrainment effects exerted by light (H.-Y. M. Cheng et al. 2007) This study also revealed the light-induced expression of Sik1 during the early subjective night. Sik1 is a negative regulator of CREB-mediated transcription and plays a role as repressor of light entrainment (Jagannath et al. 2013).Similarly, we corroborate light induction of Dusp4, a dual specificity Serine/Threonine and Tyrosine phosphatase that modulates MAPK signaling by dephosphorylating ERK1/2, a process widely described in cancer. Dusp4 expression in the SCN may contribute to the termination of the MAPK/ERK activation but its specific role in phase entrainment still needs to be fully resolved (Jagannath et al. 2013; Butcher, Lee, and Obrietan 2003)). The gene Rrad also show increased expression after light stimulation at both at early and late night, this gene has GTPase and calmodulin binding activity which make it suitable for a role in calcium signal transduction initiated by light in the SCN (Porterfield and Mintz 2009) Our analysis revealed members of the family of hormonal nuclear receptors Nr4a1and Nr4a2, two transcription factors linking metabolism with circadian control (Xiaoyong Yang et al. 2006), for which increased levels were detected by our array at both early day and early night (Jagannath et al. 2013; Hatori et al. 2014; Araki et al. 2006) Lhx1 is a gene down-regulated by light that facilitates cell-to-cell synchronization of circadian oscillations in

the SCN (Hatori et al. 2014). Rasd1a is positive regulator of photic responses in the SCN through the activation of ERK (H.-Y. M. Cheng et al. 2004). Finally, our analysis also revealed two genes involved in the SCN control of circadian rhythms such as Prok2 (M. Y. Cheng et al. 2005) and Fbxl21 (Dardente et al. 2008).

Besides the validation of light-dependent genes, we identified new genes with potentially meaningful roles in the generation of circadian rhythms or in the process of light entrainment. We focused our attention on those genes whose light-dependent expression depends on the MAPK/ERK pathway. Noteworthy is the gene Sprouty 4, a negative regulator of ERK signaling mediated by the binding to Raf protein, making it a good candidate to modulate light-induced activation of the MAPK/ERK signaling cascade (Sasaki et al. 2003) Another such gene is Rgs16, a known regulator of G-protein signaling (Hayasaka et al. 2011), as well as Plk2, which place a critical role in synaptic homeostasis (Evers et al. 2010) . Following the analysis presented here, examination of the role of these genes will comprise first validation of changes in the expression of these genes both at transcriptional and translational level in the SCN, determining whether this changes reflect basal circadian oscillations or are induced by light, complemented by validation and identification of the factors that regulate this expression changes. Afterwards individual roles may be explored at different points of the signaling process that couples light with changes in gene expression. Effects of Rgs16 and Plk2 may be analyzed at the synaptic level, where previous studies have indicated play major role either modulating G-protein activity (Rgs16) (Hayasaka et al. 2011) or in synaptic homeostasis mediated by synaptic scaling (Plk2)(Evers et al. 2010). The effect of

Sprouty4 may be analyzed in terms of the regulation of the MAPK/ERK pathway and the role that this pathway plays in light-induced entrainment.

Of particular interest are the set of non-coding transcripts that are shown to be both light regulated and MAPK/ERK dependent. Of note, each time point we examined revealed a substantial number of non-coding transcripts whose functions still need to be elucidated. Given our ever-increasing understanding of the crucial role of non-coding genes in the regulation of neuronal function, this subgroup of non-coding genes modulated by light-induced activation of the MAPK/ERK signaling cascade is of particular interest going forward. Additional investigation will be necessary to clarify their respective roles in circadian entrainment. In order to complement the analysis presented here, efforts should be focused in the identification and selection of candidate transcripts to further validate the effects of light on their expression. Selection of the candidates must be based in the magnitude of induction by light in addition to the magnitude of change induced by the presence of U0126 described here. Also given the insufficient information available for some transcripts describing expression and/or function, especially for non-coding genes, effort must be made to establish potential transcription factors binding to regulatory DNA motifs of this genes, in parallel with analysis of structural conformations and interactions that characterize these transcripts. The information obtained with this analysis can be used to define biological pathways or mechanism that explain the induction initiated by light and the potential role on physiological mechanism underlying entrainment.

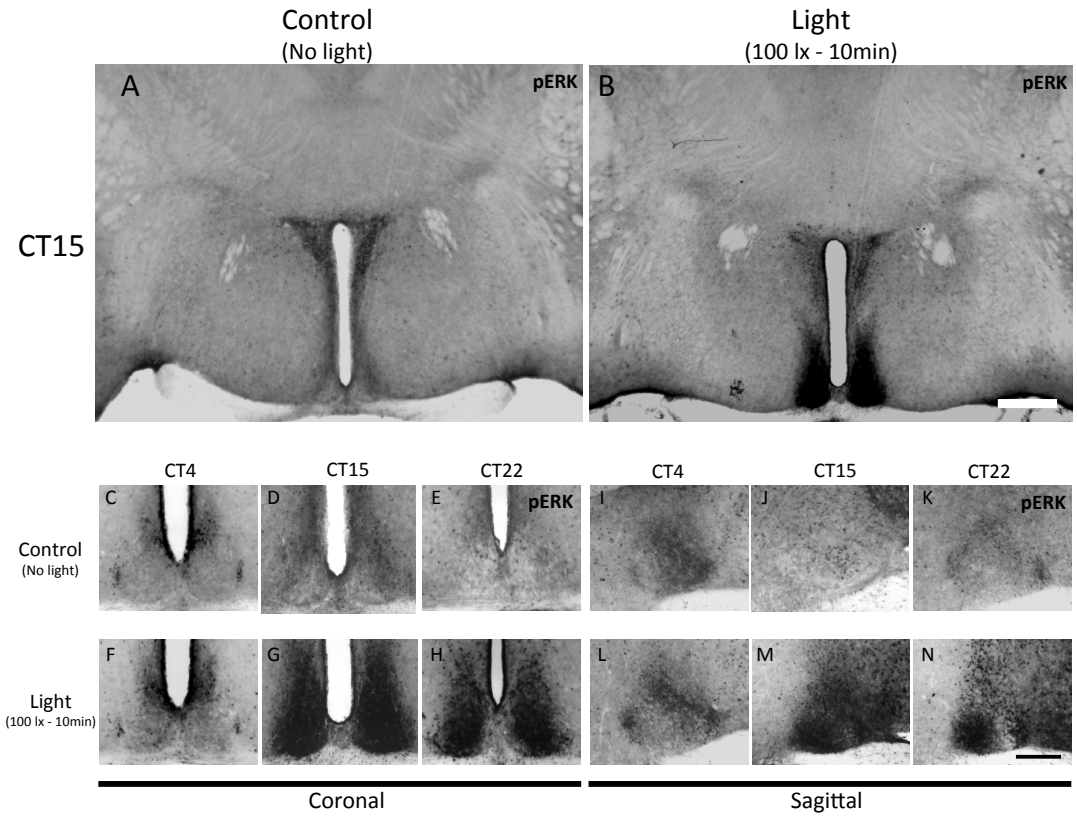

Figure 2.1. Immunolabeling showing time-of-day differences in pERK levels in the SCN as a response to light. (A, B) Representative SCN coronal sections from tissue collected at CT15 showing basal pERK levels (A) and increased levels after a light pulse (B). (C - H) Higher magnification of SCN coronal sections showing pERK basal levels at CT4 (C), CT15 (D) and CT22 (E), and light induced levels at CT4 (F), CT15 (G) and CT22 (H). Note the increase of pERK levels after the light pulse was delivered at CT15 (D) and CT22 (E), whereas no increase was observed at CT4 (C). (I-N) Higher magnification of SCN sagittal sections showing pERK basal levels at CT4 (I), CT15 (J) and CT22 (K), and light induced levels at CT4 (L), CT15 (M) and CT22 (N). Continued.

Figure 2.1 continued.

A similar patter of pERK induction was observed at CT15 and CT22, whereas no increase is observed at CT4. Scale bar 200μm.

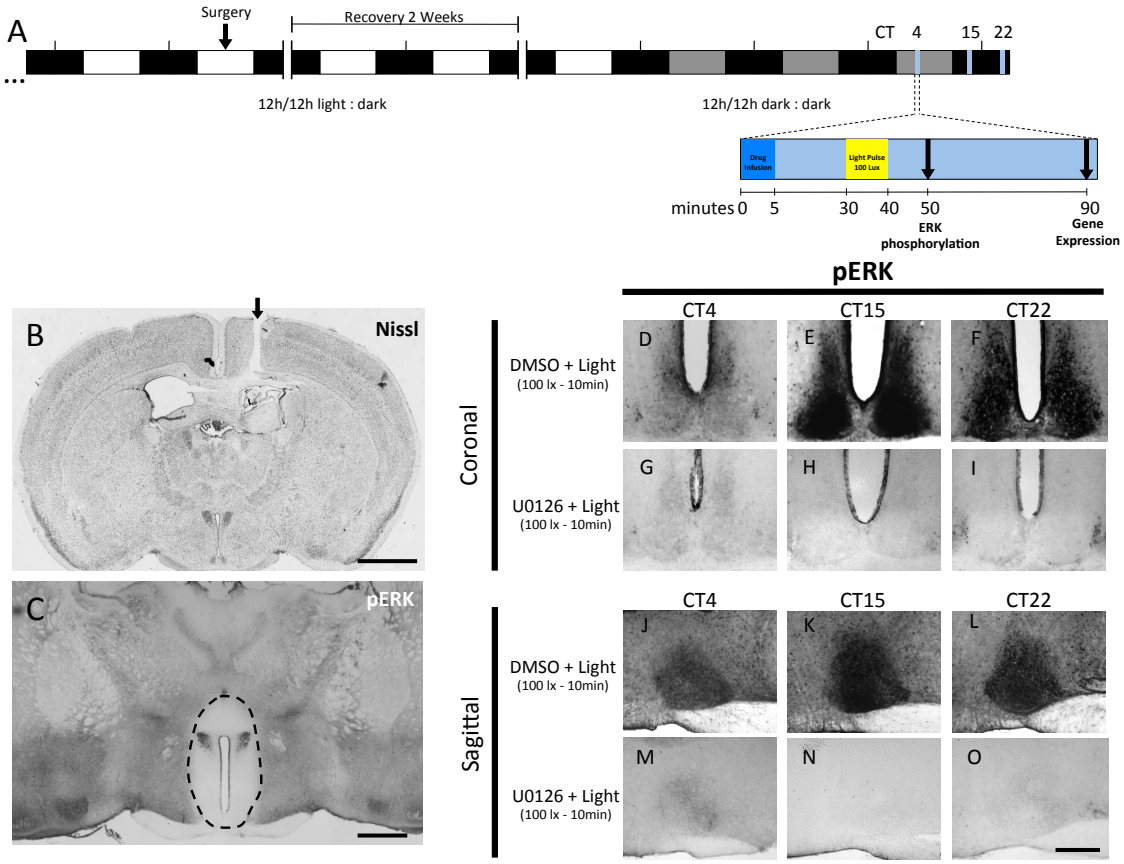

Figure 2.2. Effect of U0126 in SCN pERK levels as a response to light.

(A) Experimental design describing stereotaxic surgery performed to implant a guide cannula targeting the lateral ventricles (LV). Mice were dark adapted for two days prior to the experimental procedures. 30 minutes before the experimental time points U0126 or DMSO (vehicle) was infused into the LV, then at each time point mice were exposed to a sham or actual light pulse (10 min, 100 lux). Finally mice were sacrificed 10 min after the light pulse for Immunolabeling or 60 min after the light pulse for RNA isolation.

(B) Nissl staining for a coronal brain section of a experimental mice depicting the placement of the guide cannula into the LV. Continued.

Figure 2.2 continued.

The arrow indicates the scar left in the cortex by the guide cannula. **(C)** Coronal brain section immunolabeled for Phospho-ERK1/2 (Thr202/Tyr204) shows the inhibitory effect of U0126 in the area of diffusion of the drug from the third ventricle. **(D-F)** Representative coronal SCN images showing pERK levels in the SCN after vehicle infusion and a light pulse at 3 time points: CT4 **(D)**, CT15 **(E)** and CT22 **(F).** pERK levels in the SCN increased in response to a light pulse given after vehicle infusion at CT15 **(E)** and CT22 **(F)**, whereas pERK levels do not change when light pulse is delivered at CT4 **(D)**. **(G-I)** Previous infusion of the MEK1/2 inhibitor U0126 abolished the light-dependent increases in pERK levels at CT15 **(H),** and CT22 **(I),** pERK levels at CT4 remain unchanged **(G). (J-L)** Similar patterns of light induction of pERK are observed in SCN sagittal sections at CT4 **(J)**, CT15 **(K)** and CT22 **(L)**. **(M-O)** Similarly in SCN sagittal sections, the effect of U0126 in light-induced pERK was also replicated at each time point.

Scale bar 1.5mm for **(B)**; 500μm for **(C)**; 200 μm for **(D-O).**

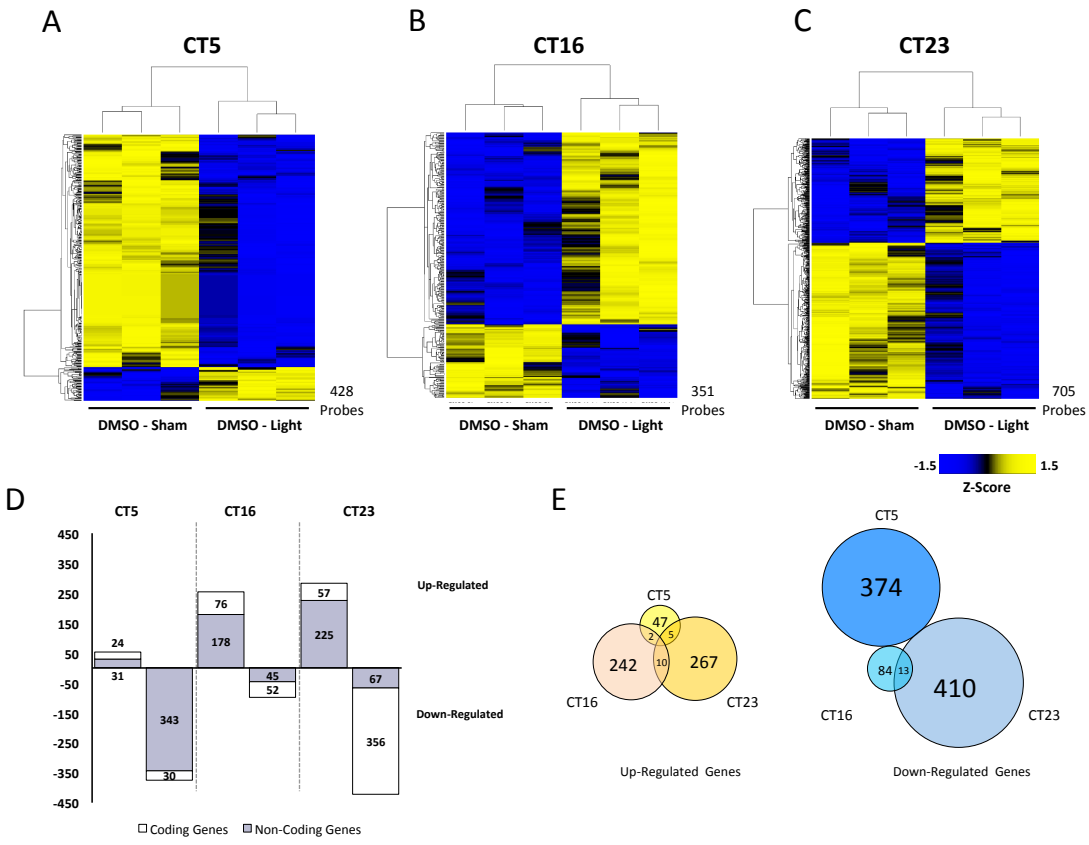

Figure 2.3. Bioinformatic analysis identifies transcripts that change in response to light in the SCN at each time point. **(A-C)** Heat map clustering generated from microarray data describing light-induced changes in gene expression at CT5 **(A),** CT16 **(B),** and CT23 **(C). (D)** The total number of up and down-regulated genes is summarized for each time point. Also the distribution of coding and non-coding genes is shown.

(E) Intersectional analysis help identify genes that respond to light at more than one time point.

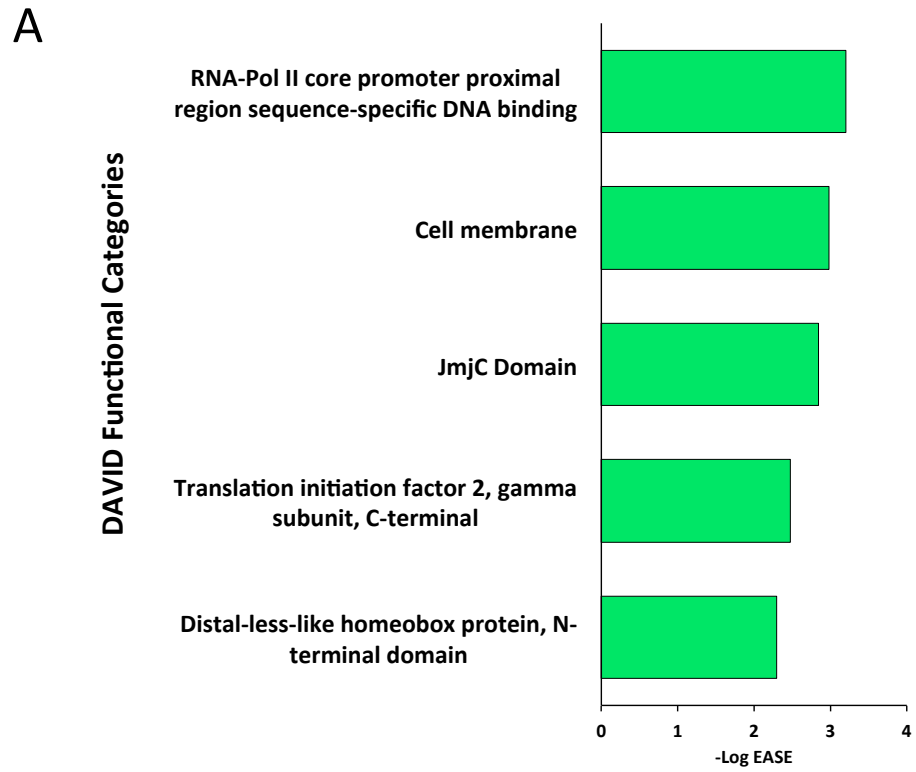

Figure 2.4. Ontological categories enriched after DAVID database analysis**.** Functional categories significantly enriched (as determined by analysis with DAVID database) are sorted based in the –Log of the EASE Score, a modified Fisher Exact P-Value implemented in DAVID for gene-enrichment analysis. **(A)** Seven enriched functional categories were obtained from the list of coding genes with significant changes at CT5 in green. **(B)** Forty eight enriched functional categories were obtained at CT16 in purple. **(C)** One hundred and two enriched functional categories were obtained at CT22 in red.

Continued

Figure 2.4 continued.

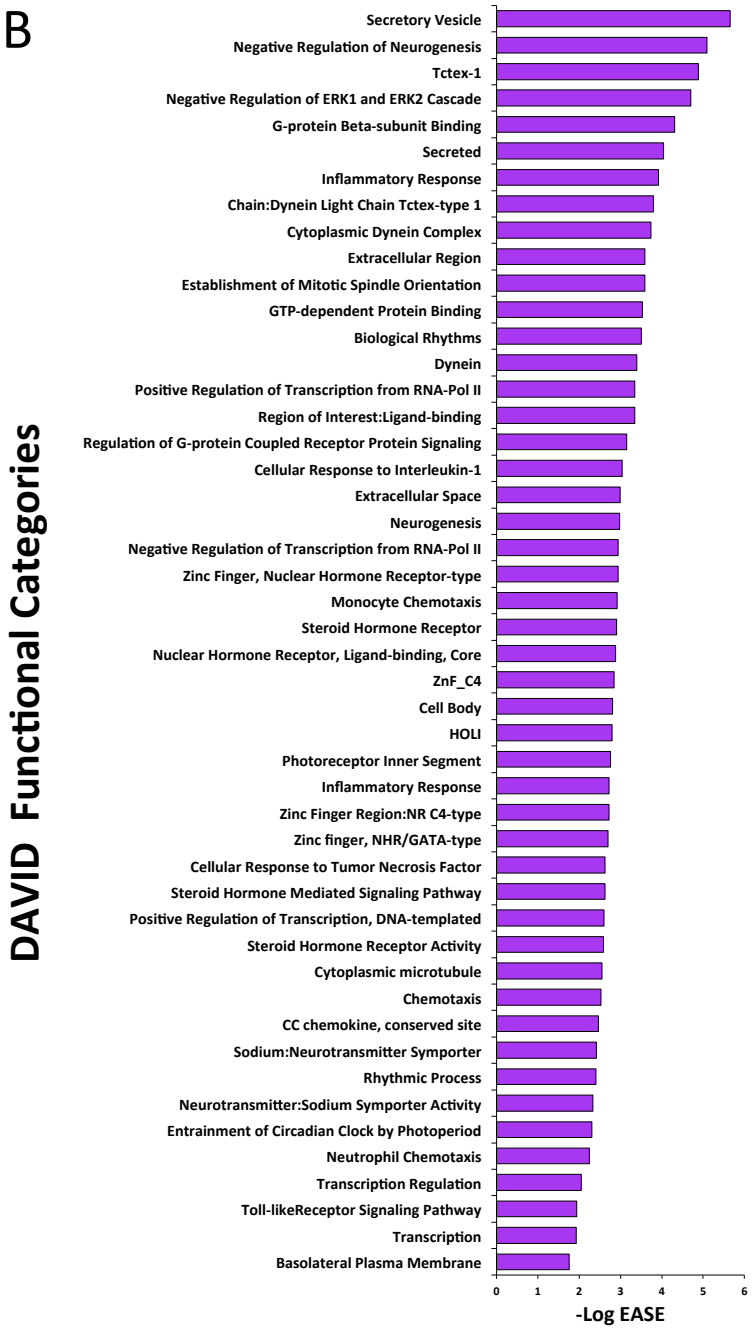

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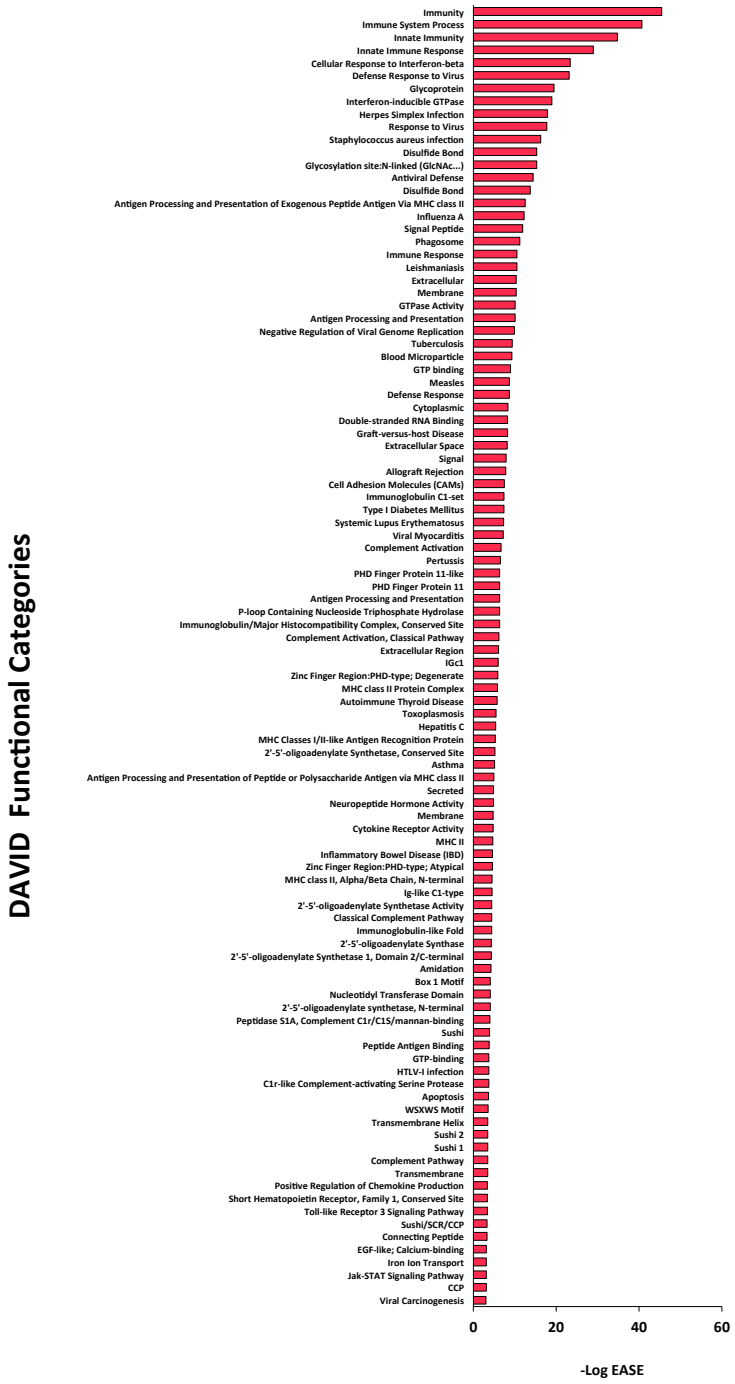
Figure 2.4 continued.

C



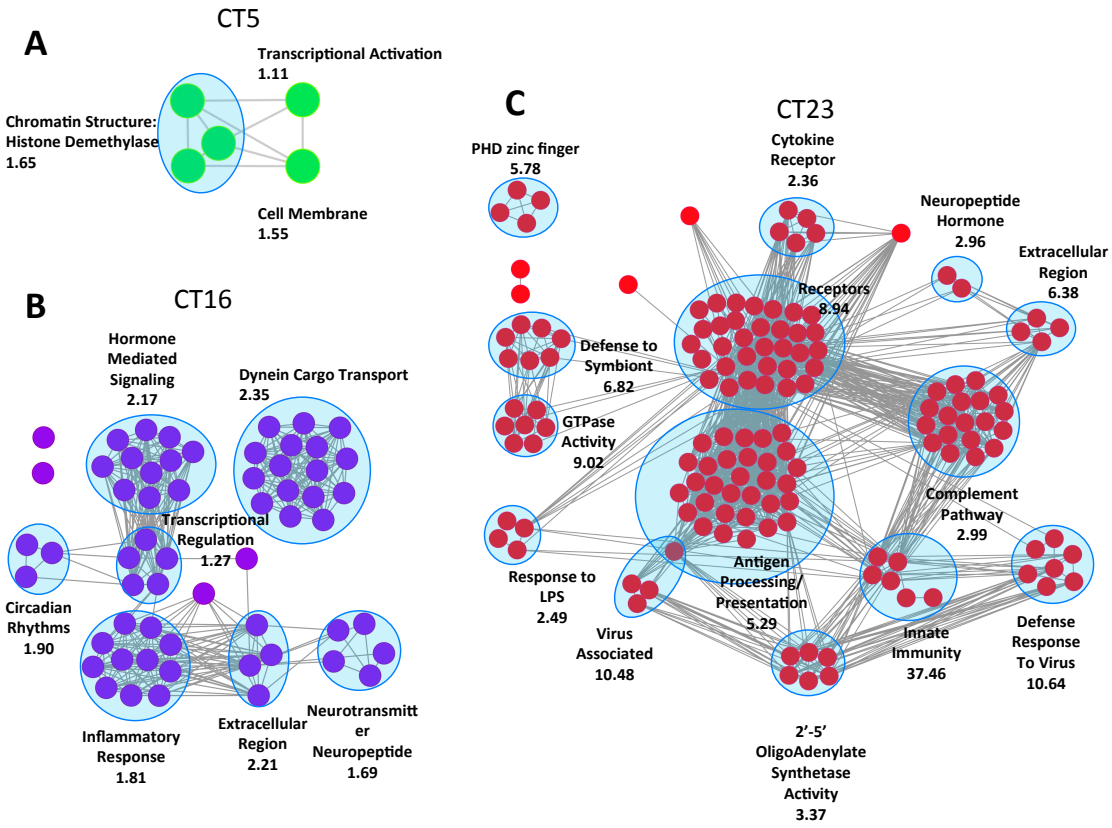


Figure 2.5. Functional cluster enrichment after DAVID database analysis. Ontological category clusters significantly enriched (as determined by analysis with DAVID database) are visualized using the Enrichment Map plug-in for Cytoscape Software. **(A)** Enriched clusters for CT5 in green. **(B)** Enriched clusters for CT16 in purple. **(C)** Enriched clusters for CT22 in red.

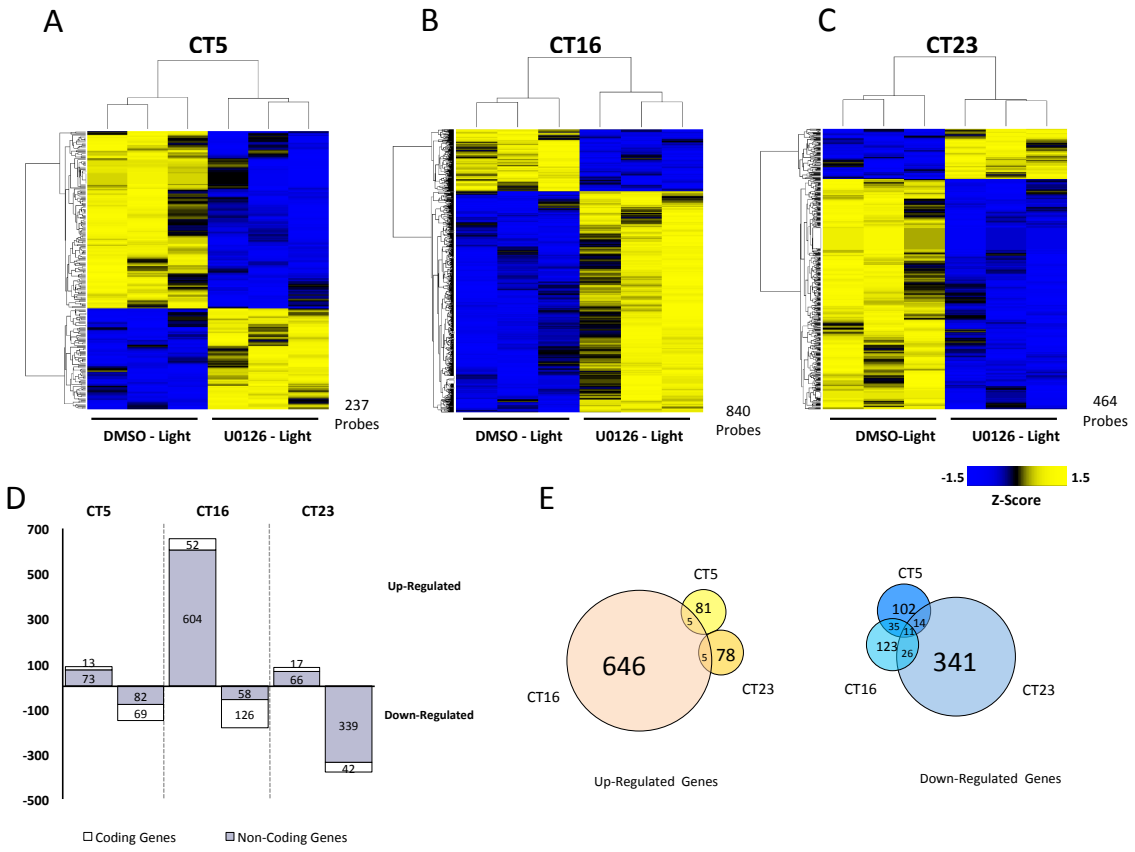


Figure 2.6. Bioinformatic analysis identifies light induced transcripts that change after U0126 infusion in the lateral ventricles. **(A-C)** Heat map clustering generated from microarray data describing the effect of U0126 for genes that show light-induced changes at CT5 **(A)**, CT16 **(B)**, and CT23 **(C)**. **(D)** The total number of up and down-regulated genes is summarized for each time point. Also the distribution of coding and non-coding genes is shown. **(E)** Intersectional analysis help identify genes whose light induction change with U0126 at more than one time point.

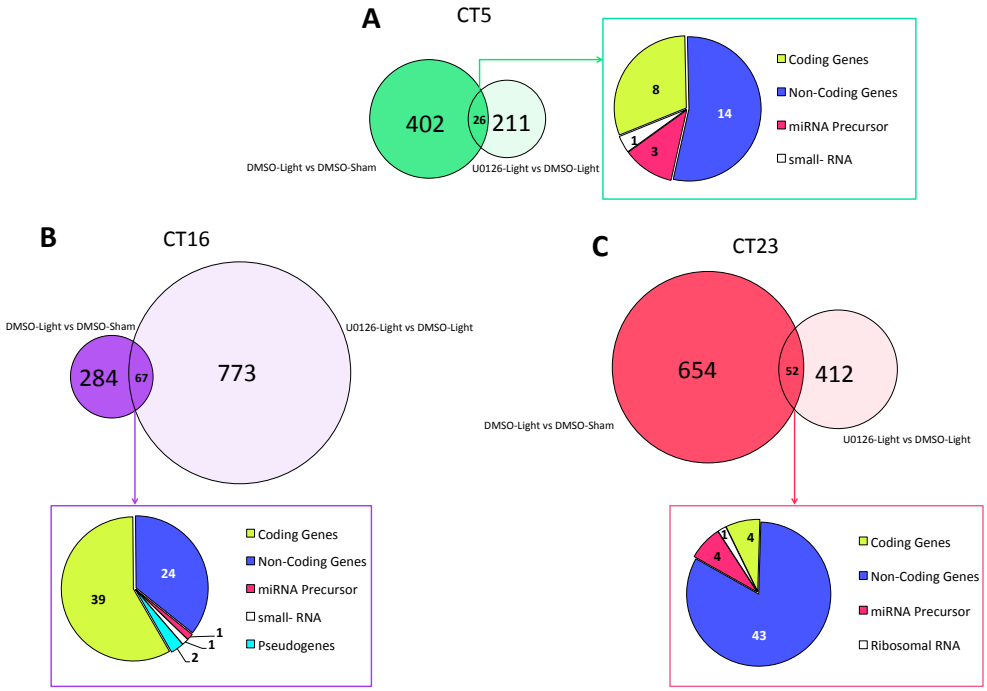


Figure 2.7. Analysis to identify transcripts whose light-dependent expression is modulated by MAPK/ERK pathway activation. **(A-C)** Intersectional analysis identifies genes whose expression is changed by light inputs to the SCN and are dependent on the activation of the MAPK/ERK pathway. 26, 67 and 52 genes are recognized at CT5 **(A),** CT16 **(B**), and CT23 **(C)** respectively.

CHAPTER 3

Circadian Timing in the Forebrain and the Modulation of Memory Formation

Circadian oscillations in physiological and behavioral processes are present in most organisms and help them anticipate and respond to environmental changes. In complex organisms such as mammals, survival is closely related with the manifestation of appropriate behavioral responses that match the environmental variables presented over a 24-hour period (Takahashi et al. 2008). The significance of this relationship is emphasized by the observation that disruption of circadian rhythms has a negative outcome in human cognition (Schmidt et al. 2007) and animal behavior (Urs Albrecht 2013). In recent years, it has been observed that circadian rhythms impact the ability to form and retrieve new memories (Gerstner et al. 2009). In mammals, the SCN functions as a master clock, where endogenous circadian rhythms are generated and synchronized with the environment (Weaver 1998). Comparably, in brain regions associated with memory formation and recall, endogenous ancillary circadian oscillations and expression of clock genes are also observed, although these are still modulated by the SCN central pacemaker (Gerstner and Yin 2010). In both the SCN and peripheral regions, these endogenous rhythms are generated by a transcriptional and translational feedback loop that dictates the timing of the molecular clock (Buhr and Takahashi 2013). However, the

interaction between the SCN master clock and the circadian timing of the circuits that are important for memory formation in different brain regions is not completely clear. Moreover, the exact cellular and molecular mechanisms underlying such interaction are likewise not fully understood.

Crucial observations relating circadian rhythms and memory formation show differences in memory retrieval when comparing performance at different times of the day. Specifically, in wild type (WT) mice day versus night differences in multiple memory paradigms such as Novel Object Location(Snider et al. 2016), Spatial Working Memory in the Radial arm maze (Rawashdeh et al. 2014), Novel Object Recognition (Shimizu et al. 2016) and Cued and Contextual Fear Conditioning (Chaudhury and Colwell 2002) were observed. Additionally, studies aiming to clarify the molecular relationship between the circadian clock and memory have used clock genes knockout mouse models to evaluate memory performance. For example, mice in which the clock gene Per2 is knocked out, exhibit impaired traced fear conditioning, a measure of associative memory (Wang et al. 2009). Also, Bmal1 knockout mice present deficits in contextual fear conditioning and in the Morris Water Maze spatial memory assays, in addition to impairment of Long Term Potentiation (LTP) induction (Wardlaw et al. 2014). Likewise, working memory deficits and the elimination of time-of-day differences in memory performance are observed in the Per1 knock out mouse line (Rawashdeh et al. 2014). Finally, it has been observed that contextual fear conditioning memory formation and consolidation, a hippocampal-mediated task, requires the transcriptional activation of key memory genes mediated by the cAMP/MAPK/CREB pathway (Atkins et al. 1998).

Interestingly, time-of-day differences have been described in the activation of the cAMP/MAPK/CREB pathway that correlate with differences in memory capacity (Eckel-Mahan et al. 2008). Despite the mounting evidence linking memory and circadian biology, the exact functional relationship is far from being fully resolved.

In order to increase our understanding of the circadian modulation of memory, it is necessary to take advantage of new hypothesis formulated to explain how the brain stores information. Several studies support the notion that memories are stored in the brain as engrams: learning-induced enduring physical changes in the brain that involve the activation and strengthening of synaptic connections between a specific group of neurons (**neuronal ensemble**) (Josselyn, Köhler, and Frankland 2015). It has only recently been possible to identify and manipulate such memory neuronal ensembles using the induction of the immediate-early genes c-Fos observed in engram cells as a reporter (Reijmers et al. 2007). Consequently, novel approaches designed to unveil the effect of circadian rhythms on memory mechanism can be brought to bear on an analysis of the effect of the molecular clock timing on memory engram cells.

Here, we used the mPeriod1-Venus transgenic mouse line (Cheng et al. 2009) as a reporter of molecular clock timing to describe a correlation between the neuronal ensemble cells activated by a contextual fear conditioning paradigm in the CA1 region of the hippocampus, identified by c-Fos induction, and specific phases of the circadian cycle, given by the levels of Venus protein. Specifically, we found and corroborate time-of-day differences in memory retrieval capacities of a contextual fear memory. Remarkably, we also found that both during acquisition and retrieval of fear memories,

the levels of Venus protein are on average higher in memory ensemble cells when compared with those cells that are not part of the memory ensemble. These observations implicate that the circadian clock regulates the recruitment of neurons of the CA1 region to form a memory neuronal ensemble.

Materials and Methods

Ethics Statement

The Ohio State University Animal Care and Use Committee approved the experimental protocols and procedures in which mice were used (protocol number: 2008A0227).

Mice

Wild type C57BL/6J and transgenic mPeriod1-Venus with a C57BL/6J background were used for experiments. The transgenic mouse line mPeriod1-Venus was generated and described by (H.-Y. M. Cheng et al. 2009). Briefly, a cassette with a fusion sequence of the fluorescent protein Venus, a nuclear localization signal (NLS), and the PEST sequence (Venus-NLS-PEST) was inserted by recombination into a Bacterial Artificial Chromosome (BAC) containing the mouse period1 (mPer1) locus (Clone ID RP24-277K16, BACPAC Resources Center. Oakland, CA, USA). Next, mice founders were generated by pronuclear injection of the BAC final construct into oocytes from

FVB/N females. Subsequently, founders were backcrossed to C57BL/6J mice for three generations to generate the transgenic line. WT and transgenic mice were housed in groups of 5 animals maximum per cages under a 12h light/12h dark cycle, maintained under constant temperature ranging between 18-23°C; and 40-60% humidity. Water and food were provided *ad libitum.*

Fear Conditioning Apparatus and Software

Fear conditioning was performed on a modified PACS-30 shuttle box (Columbus Instruments - 1371 PACS-30, Columbus, OH). The box was made of black polycarbonate walls with a dimension of 18cm L x 23cm W x 27cm H. The floor of the box consisted of a stainless steel grid (16 bars, 1 cm apart) connected to an electric shock generator (Columbus Instruments - 1372 PACS-30 Controller. Columbus, OH), controlled with the PACS Shuttle Box Software version 3.30. Conditioning and retrieval trials were performed under red dim light (~ 2 lux) and recorded using a Basler acA1300-60gm GigE camera (Basler Inc – 106200. Exton, PA), placed 90 cm above the box floor. Recordings were used for automated tracking and scoring of behavior associated with fear using the EthoVision XT 11.5 Software (Noldus Information Technology Inc. Leesburg, VA), by optimization the procedures described by (Pham et al. 2009). In summary, detection settings were set to gray scale with the subject darker than the background; erosion and then dilation of 1 pixel; a sample rate of 15 Hz with the camera exposure set to 65.5 ms; and activity threshold of 35 units with the compression artifacts

and background noise filters off. Freezing as a measure of fear was automatically scored under the mobility settings with an average interval of 20 samples, and immobility threshold of 2%.

Contextual Fear Conditioning and Retrieval Protocol

Mature mice between 7 and 14 weeks old were used for fear conditioning. Seven days before conditioning the animals were individually housed and maintained under the standard conditions. During the four days prior to conditioning, mice were handled once a day for one minute. Handling was performed at a different times each day to avoid unintended entrainment to handling time. Two days before conditioning, mice were transferred to a 12h/12h Dark: Dark (DD) schedule and maintained in the same schedule for the remainder of the behavioral test. Contextual fear conditioning was carried out at Circadian Time 4 (CT4). For conditioning, mice were placed in the conditioning box for 3 minutes immediately followed by two electric shocks delivered through the grid floor (0.2mA, for 2 seconds, with a 20 seconds interval between shocks). After the last shock the mice were allowed to recover for 1 minute in the conditioning box, and then transferred to their home-cage. Contextual fear conditioning retrieval was measured at CT4 and/or CT15, starting 24 h after conditioning. For retrieval analysis, mice were re-exposed to the conditioning box for 5 minutes without any shock delivered. At the end of this period mice were returned to the respective home-cages. Automated scoring of freezing as a measure of fear is reported as the percentage of time (in seconds)

that the mice spend immobile over the total time of tracking. Analysis was limited to the 3 minutes before the shock on the conditioning trials (pre-shock freezing); the minute of recovery after the shock (after-shock freezing); and to the 5 minutes of re-exposure on the retrieval trials (retrieval freezing). When specified, control groups include mice that were exposed to the box without receiving any shock (Context Only – CTX); mice that received a shock immediately after being placed on the conditioning box and returned to their cages without delay (Immediate Shock – IMM), and mice that remained in their home cages and were never exposed to the conditioning box (Home Cage – HC). For pairwise comparisons, a statistical Student t-test was used to determine experimental difference. For differences across time a repeated measures ANOVA was employed.

Tissue Harvesting and Immunofluorescence Staining

Sixty minutes after either conditioning or retrieval, mice were euthanized by cervical dislocation under dim red light. Brains were collected and transferred to ice-cold oxygenation media (NaCl 120mM, KCl 3.5mM, HEPES 10mM, CaCl2 0.5mM, NaH2PO4 1.2mM, MgSO4 2mM, NaHCO3 32.3mM, Glucose 10mM), then 0.5 mm coronal sections were obtained with a Vibratome (Leica Biosystems Inc. - VT1200. Buffalo Grove, IL). Sections were fixed in 4% paraformaldehyde (PFA) for 6 hours at 4°C and transferred for cryopreservation to 30% sucrose solution in PBS supplemented with sodium azide (2mM) and sodium fluoride (3mM). Free Floating coronal sections (40 μm) spanning the dorsal hippocampus were obtained with a freezing microtome. One

section from a one-in-four series was selected for immunostaining giving a total of 4 sections per mouse. Sections were washed in PBS (3 times, 5 minutes each), permeabilized in 0.1% Triton X-100 in PBS (PBS-T) for 30 minutes, blocked in 10% normal goat serum (NGS) in PBS-T for 2h, incubated overnight at 4°C with the following primary antibodies prepared in 5% NGS in PBS-T: polyclonal chicken anti-GFP (Abcam 13970 1:5000) to detect Venus protein; and polyclonal rabbit anti-c-Fos (Calbiochem PC38 1:2000). Next sections were incubated for 2 hours in 5% NGS in PBS-T with the appropriate secondary antibodies: goat anti- Rabbit conjugated with Alexa 594® (Life Technologies A11037 1:500) and goat anti-Chicken conjugated with Alexa 488® (Life Technologies A11039 1:2000). Hoechst 33342 (AnaSpec Inc. 83218 1:2000) was used for nuclei counterstaining and brain sections were mounted on glass slides using Fluoromount- G® (Southern Biotech 0100-01) mounting media.

Confocal Imaging and Analysis

Bilateral images of the CA1 region for each brain section were taken with a 20x objective of a Leica TCS SP8 confocal laser microscope controlled by the *LAS X* software. A total of 2 regions were captured per hemisphere. Signal from each channel was quantified using the NCBI free image analysis (Schindelin et al. 2012). Briefly, using the Trainable Weka Segmentation plug-in (Arganda-Carreras et al. 2017), regions of interest (ROIs) were recognized over the Hoechst channel to delineate each cell nuclei, and subsequently superimposed over the corresponding c-Fos and Venus channels. From

this procedure, the total number of cells per regions was established, and for each cell the fluorescence intensity signal for the c-Fos and Venus channel was recorded. Next, cells were assigned as c-Fos positive if the channel signal was ≥ 2x standard deviation over the mean signal intensity. With these measurements, for each mouse the percentage of c-Fos positive cells and c-Fos negative cells was quantified, and for each of the two groups the corresponding average Venus fluorescence signal was also determined. For pairwise comparisons, a statistical Student t-test was used to determine experimental differences. In the comparison of multiple experimental groups an ANOVA was employed followed by Tukey's post hoc comparisons.

Results

Time-of-day Differences in Contextual Fear Conditioning Memory Retrieval

Contextual Fear Conditioning is a hippocampal-dependent task used to evaluate associative memory formation (Fanselow 1980) During the conditioning portion of this behavioral assay, different parameters of a context (the conditioning chamber) are represented in the hippocampus, and subsequently the context is associated with a negative stimulus (electric footshock) that activates the basolateral amygdala, a brain region involved in the generation of fear responses(Fanselow 1980). Later, during the retrieval portion of the assay, a re-exposure to the conditioning chamber induces a fear response even in the absence of the footshock (Fanselow 1990). In order to evaluate if differences exist in contextual memory retrieval at different parts of the day in mice, WT mice were fear conditioned under dim red light at CT4 (CFC, n=7)Two additional control

groups were included: 1) one group exposed to the context for the same amount of time as the experimental group but without receiving any shock (context-only CTX, n=9), and 2) a group of mice that receive a shock without delay after being placed in the conditioning box and removed after 1 minute of recovery (Immediate Shock – IMM, n=7). After being returned to their home-cages, the animals remain undisturbed for 24 hours. Starting the next day, a memory retrieval trial was conducted consecutively at CT4 and CT15 until a final trial was completed 72 hours after conditioning (Figure 3.1A). Analysis of behavior during the retrieval trials showed a significant increase in the levels of freezing in the Contextual Fear Conditioned mice (CFC) compared with both control groups (Figure 3.1B. Repeated measures ANOVA $p<0.05$). Remarkably, a significant difference in freezing levels is also observed when comparing retrieval at CT4 versus retrieval at CT15 in the CFC group (Student t-test $p<0.05$). Such difference was not present in the control groups CTX and IMM (Figure 3.1C.).

mPeriod1-Venus levels in CA1 Neurons Activated After Contextual Fear Conditioning

Encoding of contextual fear conditioning involves the activation of neuronal ensembles in distinct subregions of the hippocampus, like CA1, CA3 and the dentate gyrus (DG), constituting roughly 10% of total cells in each subregion. To establish if a relationship exists between the neurons activated during the encoding of a new fear memory and specific phases of the circadian cycle, mPeriod1-Venus animals were fear conditioned at CT4 and sacrificed 60 minutes after the end of the conditioning trial. Control mice were mPeriod1-Venus that underwent the same handling but were never exposed to the conditioning box (Home Cage – HC, n=4) and were sacrificed at CT4

(Figure 3.2A). This type of control is necessary because exposure of mice to a novel context like the conditioning chamber in the absence of a footshock can also induce activation of CA1 neurons (Deng, Mayford, and Gage 2013). Mobility analysis performed during the recovery period of the conditioning paradigm showed an increase in the percentage of freezing time after the second shock for all animals in the CFC group (n=6) (Figure 3.2B, Student t-test $p<0.05$). These results are consistent with previous learning paradigm reports, suggesting an adequate training of the mice.

Immunolabeling for c-Fos performed on tissue obtained from control (Figure 3.2C) and conditioned mice (Figure 3.2F) reveal an increase in the number of c-Fos positive cells in the conditioned group (CFC) relative to the home cage controls (HC) (Figure 3.2I, Student t-test $p<0.05$), indicating the activation of a higher percentage of CA1 neurons during the conditioned trial (Milanovic et al. 1998). Interestingly, evaluation of the levels of Venus protein in activated neurons (c-Fos positive cells), and neurons that were not activated (c-Fos negative cells) in both HC (figure 3.2D) and CFC (Figure 3.2G) groups, reveals that average levels of Venus protein are higher in the c-Fos positive cells than c-Fos negative cells (Figure 3.2J, ANOVA $p<0.01$).

mPeriod1-Venus levels in CA1 Neurons Activated After Retrieval of Contextual Fear Conditioning.

During the retrieval trials of Contextual Fear Conditioning, the activation of hippocampal CA1 neurons is also induced. Although it is believed that cells activated

during the conditioning trial are also re-activated during retrieval, the degree of reactivation is not 100% complete. Based in the differences found on fear memory retrieval at CT4 and CT15 in WT mice, differences in contextual fear conditioning retrieval were also evaluated in mPeriod1-Venus mice (Figure 3.3A). For this assay, mice from the experimental group (CFC) were conditioned at CT4 (n=10). To evaluate effective learning of the task, 24 hours later (CT4) a retrieval trial was given to all the mice. Subsequently, mice received a retrieval trial 35 hours after conditioning (CT15) and half of the mice were sacrificed 60 minutes after the end of the retrieval trial (n=5). Home-cage control mice (HC) were sacrificed at the same time CT15 (n=4). Finally, another retrieval trial was given to the remaining mice (n=5) 48h after conditioning (CT4). Sixty minutes after the end of the trial mice were sacrificed with home-cage control animals (Figure 3.3A, HC n=4). To analyze differences in memory performance at different parts of the day, freezing percentage as a measure of fear associated with a contextual memory was used. Remarkably, evaluation of freezing levels shows that there was higher percentage of freezing during the CT4 retrieval trials than freezing levels at CT15 retrieval trials (Figure 3.3B, Student t-test $p<0.05$).

Cellular level analysis was done by quantification of the number of active cells with c-Fos positive immunolabeling, and the quantification of the relative levels of Venus protein. Representative images show immunolabeling for c-Fos and Venus protein in HC mice at CT4 (Figure 3.3C-E), and CT15 (Figure 3.3F-H). Comparable immunolabeling images are presented for c-Fos and Venus in CFC mice at CT4 (Figures 3.3K-M) and CT15 (Figure 3.3N-P). Quantification in the number of c-Fos positive cells in CA1

region of the HC control group (n=4), an indication of basal levels of activated cells at the two different time points, shows that the percentage of activated cells at CT15 is higher than the percentage of activated cells in CT4 (Figure 3.3I, Student t-test $p<0.05$). Moreover, consistent with the results obtained after conditioning, levels of Venus protein are on average higher for the c-Fos positive cells relative to Venus protein levels in c-Fos negative cells. This observation remains consistent for CA1 cells from tissue obtained at CT4 and CT15 (Figure 3.3J, ANOVA $p<0.01$). Analogous immunolabeling analysis performed in tissue collected in CFC group (n=5) at CT4 and CT15 showed no differences in the number c-Fos positive activated cells (Figure 3.3Q, Student t-test). Nevertheless, consistent with results obtained with HC mice, in the CFC group; average levels of Venus protein are increased in c-Fos positive cells when compared with c-Fos negative cells at CT4 and CT15 (Figure 3.3R ANOVA $p<0.05$).

Discussion

Circadian rhythms are present in a vast variety of organisms, regulating key biological processes. The capacity to store information as memories is also regulated by circadian rhythms, a phenomenon described in both invertebrate and vertebrate species (Gerstner et al. 2009). Specifically it has been noted that associative and recognition learning and memory, as well as synaptic plasticity, are circadian regulated in sea slugs(R. I. Fernandez et al. 2003), fruit fly(Lyons and Roman 2009), zebrafish (Rawashdeh et al. 2007), rodents(Chaudhury and Colwell 2002; Valentinuzzi, Menna-Barreto, and Xavier 2004) and humans(Wright et al. 2006).

In mammals, previous research efforts focused on the role played by circadian clock genes in the formation of new memories demonstrated that the SCN is a central pacemaker that is required for the proper expression of differences in learning and recall of memory (reviewed by (Smarr et al. 2014) . Indeed, lesions to the SCN in rats induce deficits in a passive avoidance memory task memory (Friedrich K. Stephan and Kovacevic 1978/4). Furthermore, dramatic changes in light schedule, strong enough to induce changes in the circadian phase, generate deficits in the recall of contextual memories in the Morris Water Maze (Devan et al. 2001)). At the same time, alterations of molecular clock genes or clock-controlled genes also impact the capacity to encode and recall different types of memory (Gerstner et al. 2009). Particularly, the expression of Period1 and Period2 (Wang et al. 2009), BMAL1 (Wardlaw et al. 2014) and VIP (Chaudhury et al. 2008), are necessary for an appropriate learning and retrieval of memory and the underlying synaptic plasticity. Unfortunately, until now most studies have been correlational, and a clear picture of how circadian rhythms are engaged in the formation and expression of new memories have been missing.

This may be explained because the brain mechanism essential for the encoding and storage of information are not completely deciphered. Memory is defined as "retention over time of experience-dependent internal representations' of the world (Kandel, Dudai, and Mayford 2014). The latest hypothesis formulated about this subject states that memories are encoded by changes in the strength of the synaptic connections of neuronal ensembles forming an engram(Josselyn, Köhler, and Frankland 2015). Recent studies have been able to identify, manipulate and even artificially generate

memory engrams (Tonegawa et al. 2015). This suggests that neuronal ensembles active during the encoding of new memories are the substrate that underlies the internal representations retained in the brain when memories are formed and recalled. Of note, several lines of evidence point out that memory neuronal ensembles can be recognized by the expression of immediate early genes like c-Fos, Arc or Zif268(Sakaguchi and Hayashi 2012).

We used these premises to establish a novel approach to clarify how the timing of circadian clock in the CA1 region of the hippocampus may modulate the activation of neuronal ensembles fundamental for memory engrams formation. We selected the contextual fear conditioning (CFC) model of associative memory, for which neuron CA1 regions are necessary (Lee and Kesner 2004) and more importantly for which previous studies have demonstrated differences in memory performance across the circadian cycle in mice (Chaudhury and Colwell 2002). Memory performance is quantified in this behavioral task by measuring the percentage of freezing as a representation of fear, which in turn is a manifestation of an association memory between context and the potential delivery of a nocive stimulus(Fanselow 1980) It is believed CA1 neurons are crucial for the internal representation of this context. Here, we demonstrate that the manifestation of contextual memories is different at two distinctive parts of the circadian cycle, namely CT4 and CT15. By evaluating memory performance at CT4 and CT15 over the course of 3 days, we can rule out that the differences observed between CT4 and CT15 as a consequence of memory extinction, and instead as a truly time-of-day differences in memory performance. In addition, the fact that mice are nocturnal animals may constitute

a confounding factor when analyzing mobility levels that may result in increased levels of activity and therefore less freezing during the subjective night (CT15), but by including the context-only (CTX) control group, it can be observed that basal level of activity in these mice inside the conditioning chamber do not present significant differences. (Figure 1B,C). Finally, the inclusion of the immediate shock (IMM) as second control group ratifies that proper fear conditioning in this task requires the formation of an internal representation of the context, for which the hippocampus is essential, and that it is formed during the exploration time given before the shock in our experimental group. The results obtained here, together with previous observations, constitute compelling evidence that time is a variable regulating the manifestations of memory.

As predicted, we observed that following conditioning during the subjective day (CT4) there was an increase in the number of activated cells in the CA1 region, supporting the role played by the hippocampus in formation of contextual memories. Intriguingly, the observation that levels of Venus proteins in activated cells is increased, lead us to think that CA1 neurons under specific phases of the circadian cycle are more likely to be activated and be part of the neuronal ensemble that sustain the encoding of the memory engram. Another interesting finding is the increase of activated cells during subjective night (CT15) in the HC control group (Figure 3I). These data suggest that there is a natural oscillation in the activation of cells within the hippocampus that may be guided by circadian rhythms, a notion that is supported by the fact that activated cells present higher levels of Venus protein (Figure 3J), identifying these cells with a particular

phases of the circadian rhythm. Lastly, it was observed that there are no differences in the percentage of cells activated after conditioning retrieval at CT4 and CT15 (Figure 3Q), but again for both time points activated cells present higher levels of Venus (Figure 3R). The observations described above are consistent with two main concepts. First, previous studies in the field of memory formation suggest that in the amygdala, out of a finite group of neurons that can be activated by the encoding of a new memory, just a small subset of neurons are recruited to be part of the memory engram (Han et al. 2007). Furthermore it has been shown that the likelihood of a neuron to be recruited is increased in neurons that present higher levels of CREB protein and the resulting higher excitability (Yiu et al. 2014). Also, it has been observed that in the SCN, circadian oscillations in the, levels of mPeriod1 protein are correlated with different levels of excitability (Pitts, Ohta, and McMahon 2006)). Our results may indicate that a similar mechanism is involved in the hippocampus. That is, differential timing given by the circadian clock groups cell of the CA1 region under a determined circadian phase, which in turn modifies the excitability parameters of the cells in a way that makes them more likely to be recruited to the memory engram ensemble. The exact molecular and physiological parameters that oscillate and change the excitability of the cells under the circadian clock still need to be determined.

Future experimental approaches will require an extensive profile of the molecular clock phase of CA1 neurons throughout the circadian cycle, this will help identify if there are differences in the overall state of the CA1 molecular clock that might be related with the differences in memory retrieval described here. The results obtained in this work also

indicate that after fear conditioning at CT4 there are differences in memory retrieval capacity at CT4 compared with CT15, it will be really informative to perform the fear conditioning at CT15 and subsequently compare memory retrieval capacity at both CT4 and CT15, and finally complement these behavioral analysis with a quantification of the number of cells activated by the conditioning paradigm together with an analysis of the molecular clock phase at each timepoint. Further experiments addressing the number of cells activated by conditioning and retrieval at different time points and the molecular clock phase of these cells might help identify how the circadian clock modulates throughout the circadian cycle the activation of CA1 cells in response to conditioning or memory retrieval paradigms. Additionally, examination of contextual fear conditioning retrieval capacity in mouse models in which the oscillations of the circadian clock is ablated will support the role of the molecular clock in the regulation of memory formation. Finally, evaluation of electrophysiological parameters of CA1 sorted at different phases of the circadian cycle will help to determine if the difference between neurons recruited to the memory neuronal ensemble are engaged in distinctive physiological dynamics that facility their activation in response to memory inputs to the hippocampus.

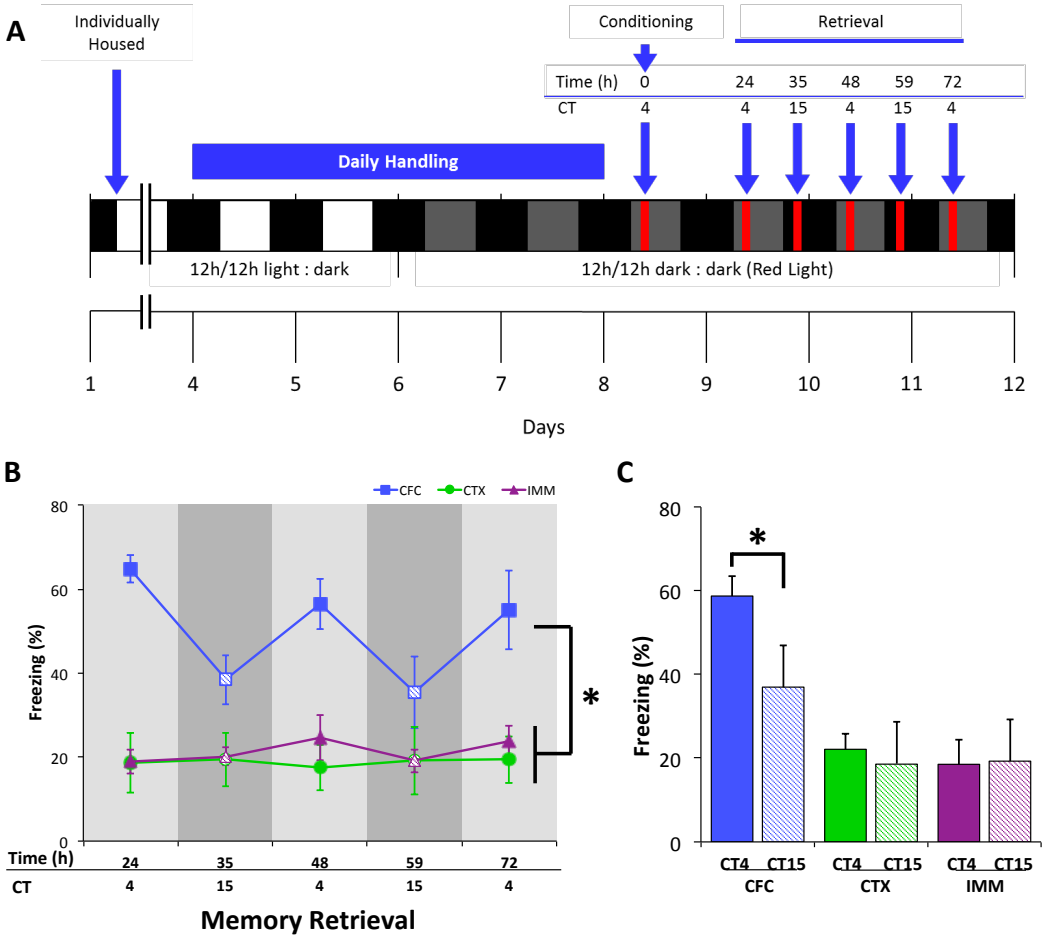


Figure 3.1 Circadian Modulation of Contextual Fear Memory Retrieval

(A) Experimental design. Mice were conditioned at CT4 and subsequently tested at CT4 and CT15 for the next 3 days. Both, conditioning and testing was conducted under dim red light (~2 Lux). White and black blocks represent the 12h periods of light and dark respectively; gray blocks represent 12h dark periods that correspond with the previous 12h light periods.

Continued.

Figure 3.1 continued.

(B) Quantification of freezing behavior during the 5 minutes retrieval test show a significant higher freezing in the contextual fear conditioning (CFC) group, compared with the context exposure (CTX), and immediate shock (IMM) groups. **(C)** Mice of the CFC group show a significant higher freezing at CT4 when compared with CT15, but there are no differences in freezing between CT4 and CT15 in the CTX and IMM groups. (*$p<0.05$).

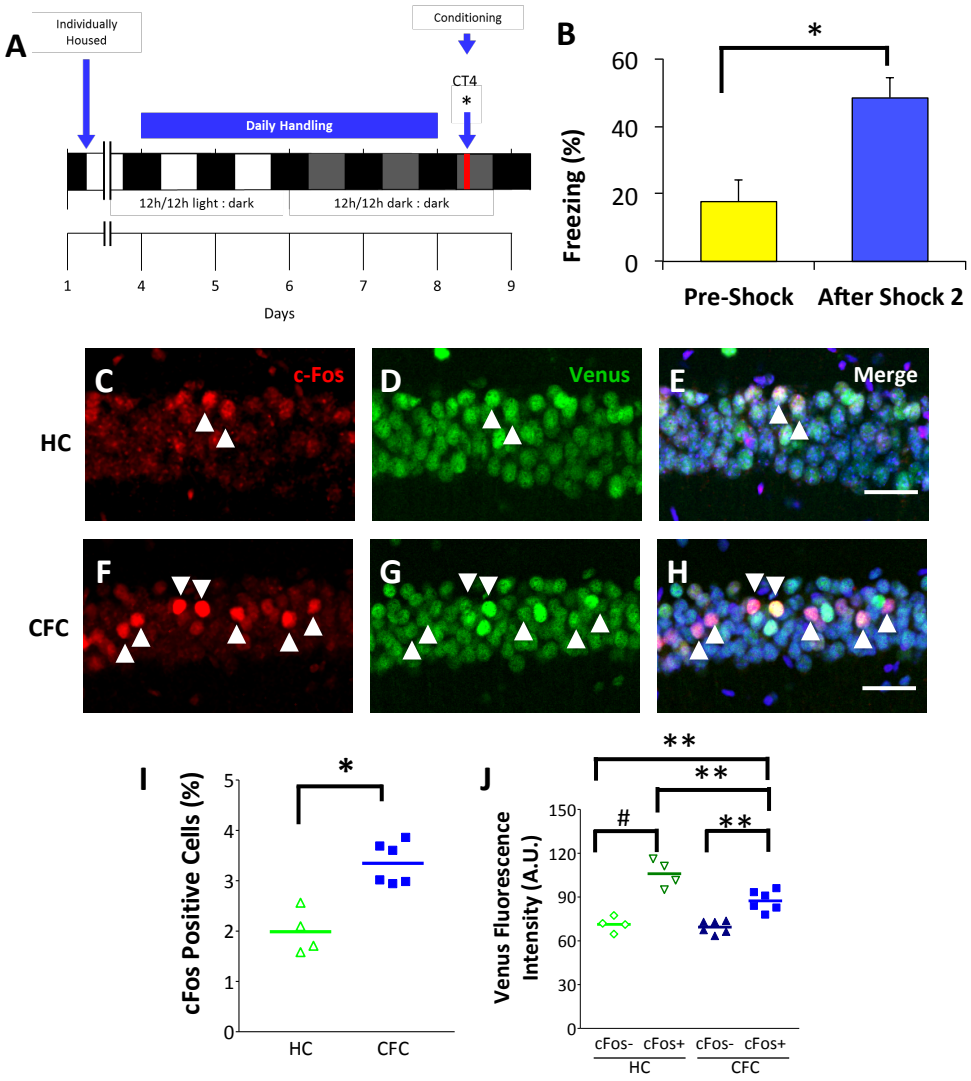


Figure 3.2. mPeriod1-Venus levels in CA1 Neurons Activated After Contextual Fear Conditioning. **(A)** Experimental design. Mice were conditioned at CT4 and 60 minutes later tissue was collected and processed for immunofluorescence. Conditioning was conducted under dim red light (~2 Lux). White and black blocks represent the 12h periods of light and dark respectively; gray blocks represent 12h dark periods that correspond with the previous 12h light periods. Continued.

Figure 3.2 continued.

(B) Quantification of freezing behavior before and after the footshock shows a significant increase of freezing after the footshock conditioning. **(C-H)** Representative immunofluorescence images from home cage (HC) control and conditioning (CFC) mice labeled for c-Fos **(C, F)** and Venus **(D, G).** Arrows indicate c-Fos positive cells. **(I)** Quantification shows a significant increase in the percentage of c-Fos positive cells in mice from the CFC group. **(J)** Quantification of Venus fluorescence intensity sorted between c-Fos positive and c-Fos negative cells show significant higher levels of Venus in c-Fos positive cell in both HC and CFC groups. Scale bar 50 μm. (*$p<0.05$; ** $p<0.01$, # $p<0.001$).

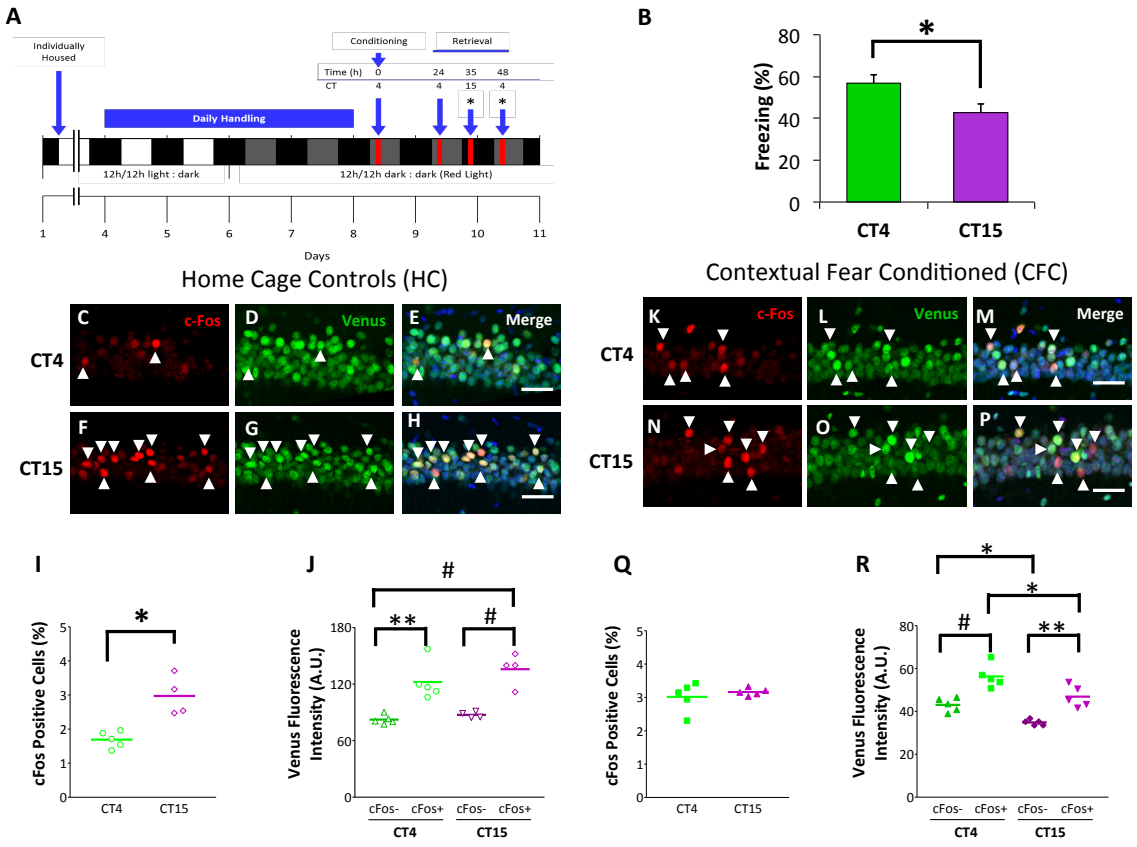


Figure 3.3. mPeriod1-Venus levels in CA1 Neurons Activated After Retrieval of Contextual Fear Conditioning. **(A)** Experimental design. Mice were conditioned at CT4 and underwent a retrieval test 24h later (CT4), followed by a retrieval test 36 hours after conditioning (CT15), and a third retrieval test 48 after conditioning (CT4). Tissue was collected 60 minutes after retrieval at CT15 (36h) and CT4 (48h) tissue was collected and processed for immunofluorescence. Conditioning was conducted under dim red light (~2 Lux). **(B)** Quantification of freezing behavior at CT4 and CT15 shows significant higher levels of freezing at CT4 compared with freezing levels at CT15.

Continued.

Figure 3.3 continued.

(C-H) Representative immunofluorescence images from home cage (HC) control mice showing c-Fos levels at CT4 **(C)** and CT15 **(F),** and levels of Venus at CT4 **(D)** and CT15 **(G).** Arrows indicate c-Fos positive cells. **(I)** Quantification shows a significant increase in the percentage of c-Fos positive cells in HC mice from sacrificed at CT15. **(J)** Quantification of Venus fluorescence intensity in c-Fos positive and c-Fos negative cells show significant higher levels of Venus in c-Fos positive cell in tissue collected at CT4 and CT15. **(K-P)** Representative immunofluorescence images from conditioned (CFC) mice showing c-Fos levels at CT4 **(K)** and CT15 **(N),** and levels of Venus at CT4 **(L)** and CT15 **(O).** Arrows indicate c-Fos positive cells. **(Q)** Quantification shows no significant differences in the percentage of c-Fos positive cells in CFC mice sacrificed at CT4 and CT15. **(R)** Quantification of Venus fluorescence intensity in c-Fos positive and c-Fos negative cells show significant higher levels of Venus in c-Fos positive cell in tissue collected at CT4 and CT15. Scale bar 50 μm. (*$p<0.05$; ** $p<0.01$, # $p<0.001$).

CHAPTER 4

Circadian Clock Modulation of Hippocampal Adult Neural Stem/Progenitor Cell Proliferation and Differentiation

The circadian clock is expressed in most of mammalian cells where it can generate cell-autonomous circadian oscillations and while also transducing entrainment signals derived from the brain's central pacemaker located within the suprachiasmatic nucleus (SCN). Within the tissues of multicellular organisms, the circadian clock is intimately linked with the control of several biochemical processes that reflect oscillation in physiological processes characteristic of the correspondent tissue(Reppert and Weaver 2002). Remarkably, several lines of evidence in multiple cell types have linked the circadian clock with homeostatic response pathways such as cell division and responses to stress(Borgs, Beukelaers, Vandenbosch, Belachew, et al. 2009). Cell division is a process tightly regulated by the coordinated expression of cyclins, cyclin-dependent kinases (CDKs), and their respective inhibitors, creating checkpoints to cell cycle progression (Bloom and Cross 2007).Thus, the link between the molecular clock and the cell cycle is made by the circadian-controlled expression of key cell cycle regulators like

Cyclins D1, B1 and B2((W. S. Yang and Stockwell 2008)); and the CDK inhibitors p21(Gréchez-Cassiau et al. 2008) and wee1(Matsuo et al. 2003). Additionally, in response to DNA damage, checkpoint kinases are activated in order to arrest the progression of the cell cycle, and such activation is regulated by molecular components of the circadian clock (Kondratov and Antoch 2007). Besides the link between molecular components of the circadian clock and the cell cycle, it has been also observed that disturbances in the expression of clock genes generate disorders in cell cycle progression that may result in higher incidence of proliferative disorders like cancer(Gery et al. 2006).

Notably, the circadian clock is involved in regulating the rates of proliferation and timely differentiation in stem cells(Janich et al. 2011; Matsu-Ura et al. 2016), specialized cells with the capacity of self-renewal and to differentiate into multiple tissue-specific cells types. In the adult mammalian brain, the production of new neurons from neural stem/progenitor (NPS) cells or neurogenesis is restricted to at least in two defined regions: 1) the subventricular zone (SVZ) of the lateral ventricle, in which resident NPS cells migrate and differentiate into neurons that will integrate into circuits of the olfactory bulb, and 2) the subgranular zone (SGZ) of the dentate gyrus (DG) that produces new neurons that are integrated to circuits of the hippocampal granule cell layer (GCL) (see review by (Zhao, Deng, and Gage 2008).

Hippocampal neurogenesis is regulated by several extrinsic factors, such as neurotransmitters(Vaidya, Vadodaria, and Jha 2007), growth factors (Kuhn et al. 1997), cytokines (Butovsky et al. 2006) and hormones (Cameron and Gould 1994).

Neurogenesis can also be induced by environmental enrichment(Kempermann, Kuhn, and Gage 1997), voluntary exercise(van Praag, Kempermann, and Gage 1999), and pathological insult(Jessberger et al. 2007), or in response to antidepressant treatment(Malberg et al. 2000). In response to these factors, NPS cell in the SGZ that reside in a quiescent state are activated, and initiating a program of proliferation and differentiation that results in the generation and circuit integration of new neurons in the GCL. The neurogenic capacity of the dentate gyrus has a significant role in learning and memory (see review (Deng, Aimone, and Gage 2010). Considering the important role of adult neurogenesis on behavior, and given that the circadian clock potentially regulates the proliferative and differentiation potential of NPSc, here we examine the relationship between these two biological processes. In this study, we present *in vivo* evidence showing that the circadian clock modulates the proliferation and differentiation capacity of the NPSc population. Significant oscillation of the molecular clock was detected in the SGZ NPSc cell population. Additionally, using a knockout mouse line in which circadian timing is arrested, we demonstrate that both the proliferation and differentiation capacity of the NPSc is increased. Our data reveal an inherent oscillatory capacity of the molecular clock in NPS cells of the SGZ, with the capacity to control the rate of proliferation and differentiation. Together, these findings indicate that the circadian clock exerts very powerful control over the key functional features of NPS cells.

Materials and Methods

Mice

For this study, mPeriod1-Venus and Bmal1$^{-/-}$ (BMAL-KO) mice were used (Bunger et al. 2000). The mPeriod1-Venus mouse line was generated and described by (H.-Y. M. Cheng et al. 2009), and was maintained with homozygous breeders with a C57BL/6J background (). Bmal1$^{-/-}$ mice are reported to be sterile; therefore heterozygous Bmal1$^{-/+}$ founders were obtained from Jackson Laboratories (strain B6.129-Arntl^{tm1Bra}/J, Stock No 009100). The line was maintained with heterozygous breeders from which Bmal1$^{-/-}$ (KO) and Bmal1$^{+/+}$ (WT) littermates were generated and used for experiments. Mice were housed in groups of maximum 5 animals per cages under a 12h light/12h dark cycle, maintained under constant temperature ranging between 18-23°C; and 40-60% humidity. Water and food were provided *ad libitum.* Experimental protocols and procedures were approved by The Ohio State University Animal Care and Use Committee (protocol number: 2008A0227).

Immunofluorescence Profiling of Venus Expression.

Adult (8 - 12 weeks) mice from the molecular clock reporter line mPeriod1-Venus were used to profile clock expression in Neural Stem/Progenitor cells residing in the subgranular zone of the hippocampal dentate gyrus. Mice were transferred from the

standard 12h/12 light: dark conditions to 12h/12h dark:dark conditions. After two days of dark adaptation, groups of mice were sacrificed every 4 hours over the course of an entire subjective day, starting at CT2 with the last group sacrificed at CT22. At each time point, mice were given an overdose of Ketamine-Xylazine. After ensuring full anesthesia, mice were transcardially perfused with ice-cold saline solution followed by 4% paraformaldehyde. Brains were post-fixed in 4% paraformaldehyde for 12h at 4°C and then moved to 30% sucrose for cryopreservation. Using a freezing microtome, 40μm brain sections were obtained by serial sectioning spanning the entire dorsal hippocampus. Sections were washed in PBS, permeabilized for 20 minutes in PBS-T (0.1% Triton X-100 in PBS), and blocked in 10% horse serum in PBS-T for 2h. Sections were incubated overnight at 4°C with a goat polyclonal primary antibody anti-Sox2 at a 1:250 dilution (Santa Cruz Biotech sc-17319) prepared in 5% horse serum in PBS-T. Next, sections were incubated with donkey anti-goat conjugated with Alexa 594® (Life Technologies A-11058) for 2 hours in 5% horse serum in PBS-T. After PBS washes, sections were blocked in 10% normal goat serum (NGS) in PBS-T for 2 hours, then incubated with chicken polyclonal primary antibody anti-GFP at a 1:2500 dilution (Abcam 13970) in 5% NGS - PBS-T to detect Venus protein. Next, sections were incubated with a goat anti-chicken conjugated with Alexa 488® (Life Technologies A11039 1:2000). Nuclei were counterstained with DRAQ5 (1:1000) and brain sections were mounted on glass slides using Fluoromount- G® mounting media (Southern Biotech 0100-01).

BrdU Injection and Tissue Harvesting

Adult (8-12 weeks) WT and BMAL-KO littermates were used for proliferation and differentiation analysis. For proliferation experiments, mice received a mid-day, single intraperitoneal (i.p.) injection of the synthetic thymidine analog 5-bromo-2'-deoxyuridine (BrdU) diluted in saline solution at a dose of 50 mg/kg, and sacrificed 3 hours later. For differentiation experiments, adult mice received mid-day, single daily injections of BrdU (50 mg/kg in saline) for five consecutive days. 10 days after the last injection mice were sacrificed also at midday. At the respective time-points, mice were sacrificed by anesthetic overdose with a Ketamine-Xylazine cocktail followed by transcardial perfusion with saline solution and 4% paraformaldehyde. Brains were dissected out from the skull and post-fixed in 4% paraformaldehyde and then cryopreserved in a 30% sucrose diluted in Phosphate-buffered saline (PBS) solution. Serial brain sections (40μm) through the hippocampus were obtained with a freezing microtome.

BrdU Immunolabeling

Brain sections were washed with PBS and incubated in hydrochloric acid (HCl) 2N for 1h followed by boric acid 0.05M pH 8.0 for 10 min. Sections were washed and incubated for 2 minutes in 0.3% Hydrogen Peroxide in H_2O. After further washing with PBS, sections were permeabilized with PBS-T for 20 minutes, blocked in 10% NGS, and incubated overnight at 4°C with a rat monoclonal primary antibody anti-BrdU (Accurate Chemical & Scientific Corp) at a 1:200 dilution in PBS-T. Next day, section were washed

in PBS-T and incubated for 2h with a biotinylated goat anti-rat secondary antibody (1:500; Vector Labs). Sections were washed and incubated with the ABC staining solution for 1h (Vector Labs) and signal developed with nickel-intensified 3,3'-diaminobenzidine (Vector Labs).

For immunofluorescence co-labeling, brain sections were washed with PBS, sections permeabilized with PBS-T for 20 minutes, blocked in 10% Horse Serum and incubated overnight at 4°C with a goat polyclonal primary antibody anti-doublecortin (DCX) at a 1:500 dilution (Santa Cruz Biotech sc-8066) prepared in 5% horse serum in PBS-T. Next, sections were incubated with donkey anti-goat conjugated with Alexa 594® (Life Technologies A-11058) for 2 hours in 5% horse serum in PBS-T. After PBS washes, sections were treated with in HCl 2N for 1h and boric acid 0.05M pH 8.0 for 10 min. Sections were blocked in 10% normal goat serum (NGS) in PBS-T for 2 hours, then incubated with rat monoclonal primary antibody anti-BrdU (Accurate Chemical & Scientific Corp) at a 1:200 dilution in 5% NGS. Next, sections were incubated with a donkey anti-rat conjugated with Alexa 488® (Life Technologies A21208 1:500). Nuclei were counterstained with DRAQ5 (1:1000) and brain sections were mounted on glass slides using Fluoromount- G® mounting media (Southern Biotech 0100-01).

Imaging Analysis

For each mouse, one section every 6th section in the series spanning the dorsal hippocampus was selected, yielding 4 brain sections per animal. Immunofluorescence and immunohistochemistry microphotographs were analyzed using the ImageJ software

(Schindelin et al. 2012). Following convention for such an analysis, the SGZ was demarcated to a 50μm band underneath the granule cell layer of the dentate gyrus. Immunofluorescence microphotographs were taken using a Zeiss 510 confocal microscope using a 20x objective. For the screening of the circadian clock in the SGZ, Sox2 was used as marker of NPS cells. For each section in the Sox2 channel, circular regions of interest (ROI) were used to manually delineate Sox2 positive cells and to subsequently quantify relative Venus fluorescence in the respective channel. The relative levels of Venus fluorescence were quantified in 60 to 80 Sox2 positive cells at each time point (n=3). To evaluate NPS cell differentiation between WT and BMAL-KO mice for each section, the number of BrdU positive cells and the number of BrdU-DCX double positive cells was counted using ImageJ software. The total number obtained for each section was multiplied by six to extrapolate the total number of positive cells per animal. For immunohistochemistry, 10x bright field microphotographs were acquired on a Leica DMIR microscope using Metamorph software (Universal Imaging, Downingtown PA). ImageJ software was used to define an automatic threshold over background and applied to all the images, next BrdU positive cells were counted for each section summed, and multiplied by 6. For pairwise comparisons, a statistical Student t-test was used to determine experimental differences.

Results

Circadian Clock Reporter Expression in NPS Cells of the SGZ of the Mouse Dentate Gyrus

In order to determine the expression of circadian clock components in the NPS cell population located in the SGZ of the mouse dentate gyrus, the mPeriod1-Venus mouse line was used as reporter. Mice were dark adapted for two days sacrificed every four 4 hours along one circadian cycle (Figure 4.1A). For each time point, relative levels of the fluorescent protein Venus were examined in Sox2 positive cells, a marker that identifies NPS cells. Representative images show Sox2 positive cells recognized along the SGZ of mice sacrificed at CT2 (Figure4.1B) and CT10 Figure (Figure 4.1E). For each of the NPS cells identified the relative levels of Venus fluorescence were quantified (Figure 4.1C,F). Interestingly, besides possessing a robust expression of Venus, there is also a time-of-day regular oscillation of this marker (Figure 4.1H). These results suggest the expression of a functional circadian clock in the NPS cells of the dentate gyrus.

Genetic Ablation of the Circadian Clock Modifies the Proliferation Capacity of NPS Cells

Disruption of the transcriptional and translational feedback loop characteristic of the circadian clock is achieved by ablation of the clock gene Bmal1. Specifically, the BMAL-KO mouse line present arrhythmic locomotor activity, and loss of circadian oscillations in metabolic process concomitant with a loss of oscillations of the molecular

clock. To evaluate the role of the circadian clock in the proliferation capacity of NPS cells we injected adult WT and BMAL-KO littermates with BrdU in order to identify the number of proliferating cells in the SGZ. Injections were applied at midday and 3 hours later animals were sacrificed and brains processed for immunolabeling analysis (Figure 4.2A). Representative images from WT (Figure 4.2B) and BMAL-KO (Figure 4.2C) show the presence of actively proliferating cells in the SGZ. Additionally, quantification of the number of proliferating cells revealed that this population is significantly larger in BMAL-KO (n=6) mice than in WT mice (n=6) (Figure 4.2D, Student t-test $p<0.05$). The short-term changes in the number of BrdU positive cells described for BMAL-KO mice suggest that the expression of the circadian clock is implicated in the control of proliferation of NPS cells in the dentate gyrus SGZ.

Genetic Ablation of the Circadian Clock Modifies the Number of Differentiating NPS Cells

In addition to the increase in the number of proliferating cells, the number of NPS cells in the SGZ differentiating into neurons was also compared between WT and BMAL-KO mice. For this, mice from both genotypes received a single injection of BrdU (50mg/Kg) at midday for five days. 10 days after the final injection, brains were harvested for immunofluorescence analysis (Figure 4.3A). In this manner, co-labeling of BrdU and doublecortin (a marker of immature neurons; DCX) allowed for the identification of a distinctive population of cells at an early stage of neuronal differentiation derived from proliferating adult NPS cells. Representative images showing BrdU and DCX immunolabeling for WT (Figure 4.3B) and BMAL-KO (Figure 4.3C)

reveal the presence of cells that were previously proliferating (BrdU-positive in green) and immature neurons (DCX-positive in red). Consistently with the results obtained in the short-term analysis of proliferation, a higher number of BrdU-positive cells were observed in the BMAL-KO mice (n=8) compared with WT control mice (n=8) (Figure 4.3D Student t-test $p<0.05$). Likewise, there was also an increase in the number of immature neurons derived from proliferating adult NPS cells (BrdU and DCX positive) in BMAL-KO mice (n=8) than in WT mice (n=8) (Figure 4.3E Student t-test $p<0.05$). The above observations suggest that, in addition to an increase in the number of proliferating cells, the disruption of the molecular clock induces an increase in the percentage of cells derived from the proliferating population that can start a differentiation program to generate new neurons.

Discussion

In multicellular organisms, the expression the molecular clock in multiple cells types helps to establish a hierarchical organization for the expression and entrainment of circadian rhythms through the entire body (Partch, Green, and Takahashi 2014). Given this organization, cell autonomous oscillations inside many cell types constitute local pacemakers modulating the adequate timing of numerous cellular processes(Dibner, Schibler, and Albrecht 2010). Previous studies have linked components of the molecular clock with the regulation of cell cycle progression in mammalian cells, a connection with profound implications for tissue homeostasis (Borgs, Beukelaers, Vandenbosch,

Belachew, et al. 2009). For example, loss of key clock genes like Period1 or Period2 is a feature in some types of malignancies (Gery et al. 2007), (Xiaoming Yang et al. 2009) In addition, downregulation of the transcription factor Bmal1 is correlated with induced premature aging (Kondratov et al. 2006) The connection between circadian oscillations given by the molecular clock and cell cycle progression are also exemplified in the circadian control of proliferation and differentiation of certain stem cell populations in the epidermis (Janich et al. 2013; Janich et al. 2011)()(), the intestines(Matsu-Ura et al. 2016) and also inside neurogenic niches of the brain (Bouchard-Cannon et al. 2013).

The hippocampal SGZ is one of two main brain regions in which new neurons are constantly generated throughout adulthood (Ming and Song 2011). This capacity is mediated by a distinctive NPS cell population that originates during embryonic development (Li et al. 2013). These cells retain self-renewal and neuronal differentiation capacity. In the SGZ, there are two types of NPS cells: 1) a pool of cells in a quiescent state (in the G_0 phase of the cell cycle) with a radial-like morphology and 2) transiently amplifying progenitors engaged in cell cycle progression, with morphology featuring short processes. Both of types of NPS cells express the Sry-related HMG box transcription factor, Sox2, but can be discriminated by the expression of the glial fibrillary acidic protein (GFAP) in the quiescent progenitors (Bouchard-Cannon et al. 2013). The current understanding of neurogenesis proposes that quiescent NPS cells are induced to transiently re-enter the cell cycle to self-renew. After a limited number of divisions a program of differentiation towards the neuronal lineage is started, generating DCX-positive immature neuroblasts after ~2 weeks, and mature granule neurons after ~6

weeks (Zhao, Deng, and Gage 2008). Given the precise control of the cell cycle required for the neurogenesis process, it stands to reason that regulation via the molecular clock is also involved.

Here using the clock reporter mPeriod1-Venus, we observed oscillations along the circadian cycle of the Venus fluorescent protein reporter in dentate gyrus of Sox2 positive NPS cells, suggesting the existence of a functional circadian clock in this population of cells. Furthermore, in a mouse model in which oscillation of the molecular clock are disrupted, we observed an increase in the number of BrdU-incorporating cells in the hippocampal SGZ in both short-term and long-term evaluations, suggesting that the number of proliferating cell in this region increases. Similarly, we also observed an increase in the number of BrdU-DCX double positive cells, implying an increase in the number of immature neurons derived from adult NPS cells in the SGZ. The results presented here support previous studies that report the existence of a functional circadian clock in adult NPS cells, and increased number of proliferating cells and DCX positive newborn neurons in the SGZ of BMAL-KO mice (Bouchard-Cannon et al. 2013). Similarly, genetic disruption of clock gene Period2 (Per2), another key component of the molecular clock, induced an increase in the percentage of proliferating cells and newborn neurons in the rodent SGZ (Borgs, Beukelaers, Vandenbosch, Nguyen, et al. 2009). Together, these *in vivo* approaches indicate that the adult NPS cell population displays an inherent circadian oscillatory capacity, and that the circadian clock to cell cycle connection plays a significant role in determining the proliferative and neurogenic capacity of these progenitor cells.

It should be noted, however, that in a study published by (Rakai et al. 2014), it was reported that in the same BMAL-KO mouse model, the number of proliferating cells in the SGZ did not present a significant change. Detailed observation of the results obtained by Rakai et al. indicate an increased variation in the number of proliferating cells in the SGZ of BMAL-KO mice, particularly, the low number of animals used in this report such variations may conceal significant differences in cell proliferation. Even though our analysis show a significant difference in the number of proliferating cell, an increased variation in the number of proliferating cells in BMAL-KO mice is also observed.

Our phenotypic description is in agreement with more detailed studies indicating that the molecular clocks exert a restrictive force that limits the entrance and progression of the cell cycle. For example, (Bouchard-Cannon et al. 2013) report in the BMAL-KO mouse line an increased number of cells progressing into the cell cycle, concomitant with a reduced number of cells entering a quiescent phase. Likewise downregulation of Per2 is related with an increase in proliferation in some mammalian cell lines(Fu et al. 2002)

Continuation of this work may be focused in the evaluation of proliferative and differentiation capacity of NPS cell from wild-type and BMAL-KO mice *in vitro*. This approach will help to identify cell-autonomous properties of the molecular clock in these cells. Furthermore, profiling *in vivo* specific nature of proliferating cells using stage-specific makers will help to establish if increased number of proliferating cells observed is the result of a increased in the gross number of NPS cells or if it's the result of a higher proportion of NPS cells engaged in active proliferation without alteration in the gross

number of NPS cells. In addition, analysis of cell death rates in the SGZ of wild-type and BMAL-KO mice will help establish if the increased number of immature neurons observed is the consequence of an increased proliferation or a reduction in the cell death rate.

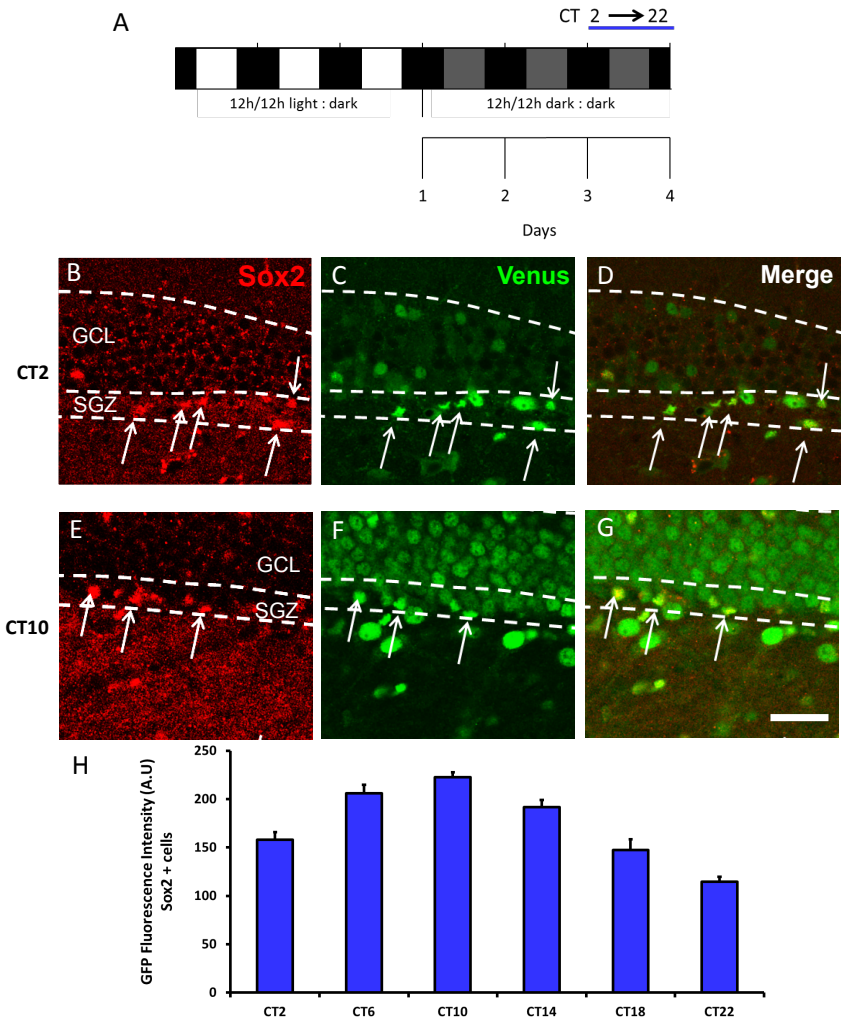


Figure 4.1. Circadian clock oscillation in adult NPS cells. **(A)** Experimental design, mice were dark adapted for two days and on the third day mice were sacrificed every 4 hours across the circadian cycle. Immunolabeling showing the differential expression levels of GFP CT2 **(B-D)** and CT10 **(E-G)** in the Sox2 positive NPS cell population of the SGZ. Arrows indicate Sox2 positive cells. **(H)** Quantification of the GFP intensity in Sox2+ cells shows the oscillatory behavior of the circadian clock in the NPS cells of the SGZ. Scale bar 50µm. GCL (Granular Cell Layer). SGZ (Subgranular zone).

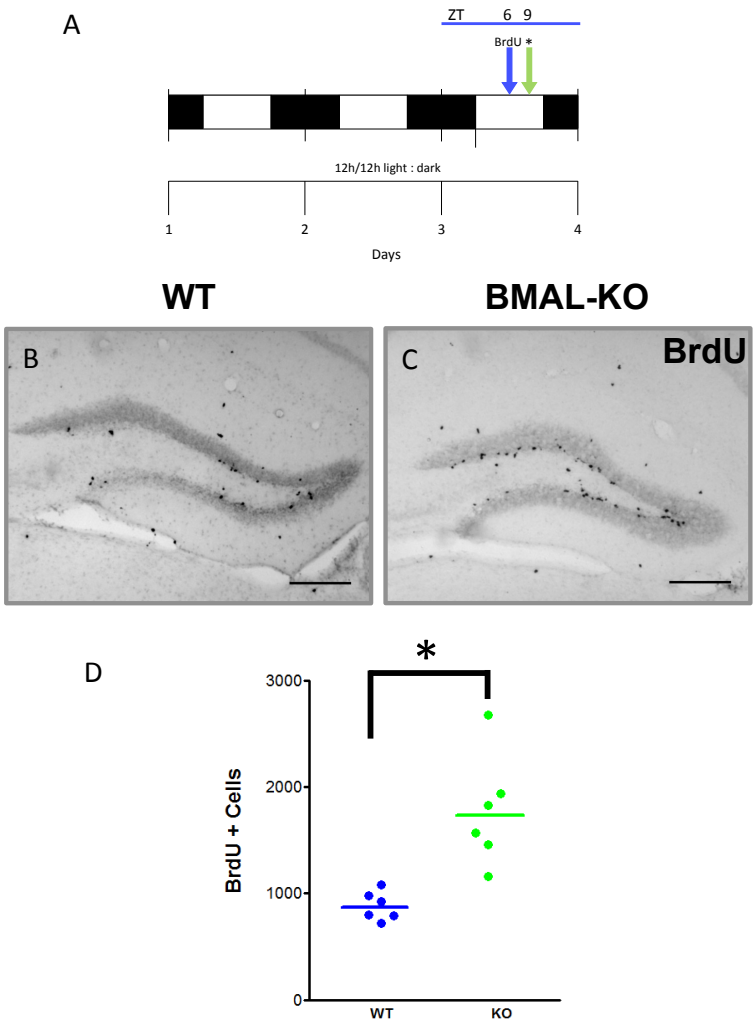


Figure 4.2. Ablation of molecular oscillations increases the number of proliferating cells in the SGZ. **(A)** Experimental design. Mice were injected with *5-bromo-2'-deoxyuridine* (BrdU) (50mg/Kg) at midday (ZT6), 3 hours later (ZT9) tissue was collected for immunolabeling. Immunohistochemistry microphotographs show BrdU incorporation in the SGZ of WT **(B)** and BMAL-KO **(C)** mice. Quantification of the number of BrdU positive cells **(D)** show a significant increased in the number of proliferating cells in the SGZ of BMAL-KO mice. (* $p<0.05$) Scale bar = 200µm.

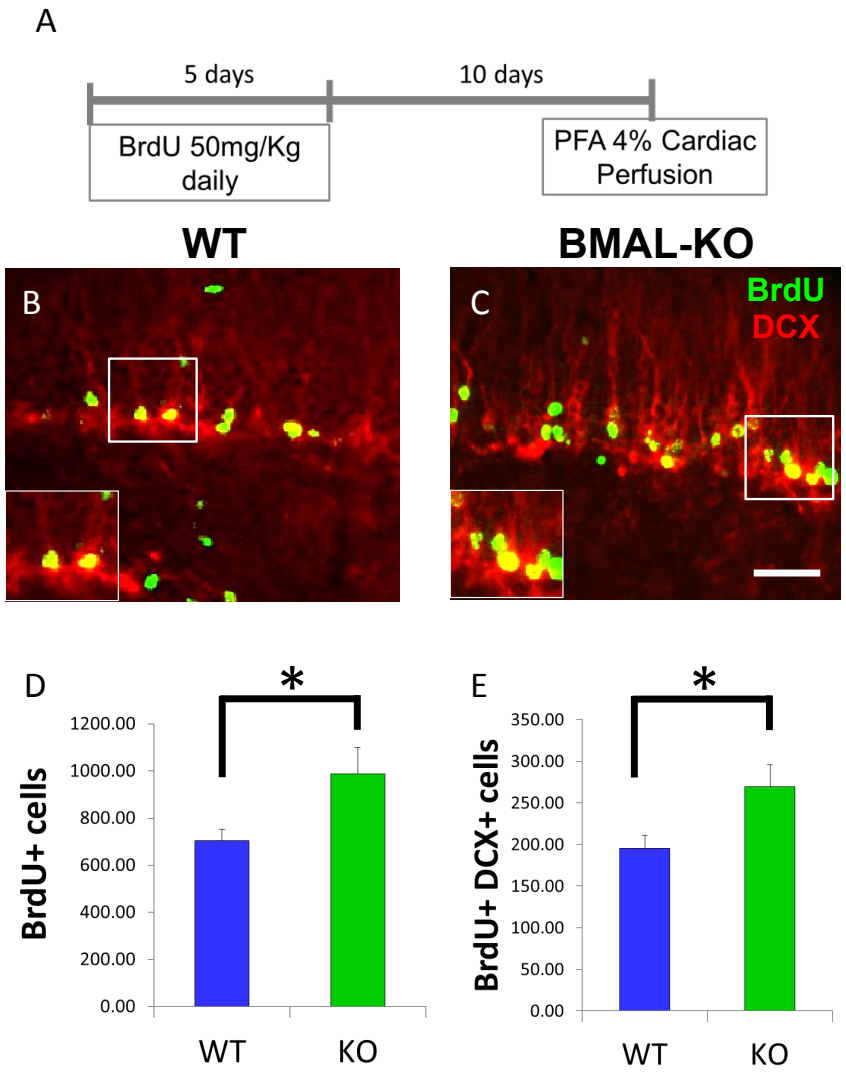


Figure 4.3. Ablation of molecular oscillations increases the number of proliferating cells differentiating into neuroblast in the SGZ. **(A)** Experimental design. Mice were injected with *5-bromo-2'-deoxyuridine* (BrdU) (50mg/Kg) at midday for five consecutive days. 10 days after the last injection tissue was collected for immunolabeling. Immunofluorescence microphotographs show BrdU incorporation (BrdU- Green) and Doublecortin expression (DCX- red) in the SGZ of WT **(B)** and BMAL-KO **(C)** mice; insets show a higher magnification. Continued.

Figure 4.3 continued.

Quantification of the number of BrdU positive cells **(D)** and BrdU - DCX double positive cells **(**E**),** show a significant increased in the number of proliferating cells and neuroblast in the SGZ of BMAL-KO mice. (* $p<0.05$) Scale bar = 50 μm SGZ (Subgranular zone).

CHAPTER 5

Conclusions and Future Directions

Circadian oscillations in physiological and biochemical process in mammalian cells are orchestrated by an endogenous molecular clock (Welsh, Takahashi, and Kay 2010). This molecular clock is built around a group of 'clock genes' that give shape to a self-sustained transcriptional and translational feedback loop (Partch, Green, and Takahashi 2014). Oscillations of the molecular clock in turn modulate the expression of a clock-controlled transcriptome, fundamental for the manifestation of circadian regulated biological processes (Bozek et al. 2009). In multicellular organisms, numerous molecular clocks distributed in different cells must be synchronized to maintain coherence at the systems-level of diverse circadian rhythms (Dibner, Schibler, and Albrecht 2010). The mammalian suprachiasmatic nucleus (SCN), localized in the brain hypothalamus, has been identified as the central pacemaker regulating the timing and synchronization of circadian rhythms throughout the body (R. Y. Moore 1983). In fact, ablation of the SCN causes desynchronization of circadian rhythms distributed throughout peripheral tissues(Mohawk, Green, and Takahashi 2012).

The existence of circadian rhythms and biological clocks provides adaptive mechanism that allow organisms to adjust physiological and behavioral processes to anticipate oscillations in environmental variables such as light-dark cycles, temperature, photoperiod, social interactions, and food availability. Therefore, the phase of the central pacemaker must be aligned, or entrained, to the phase of environmental cues or *zeitgebers* to generate effective responses (Vaze and Sharma 2013). Central to entrainment of circadian rhythms in the SCN are three main anatomical pathways. First, the retinohypothalamic track (RTH), derived from the optic nerve, relays information concerning the incidence of light from the retina to the SCN (Jens Hannibal 2002). Second, the raphe-hypothalamic tract originates in the raphe nucleus and conveys non-photic information to the SCN from patterns of locomotor activity that serve as an entrainment signal to the SCN circadian phase. Both the RTH and the raphe-hypothalamic tract have synaptic terminals. The third input pathway to the SCN is originated in the intergeniculate leaflet (IGL). Both the retina and the raphe nucleus send collateral tracks to the IGL, that in turn sends direct projections to the SCN via the geniculohypothalamic tract (GHT), transmitting principally non-photic entrainment information to the SCN (Robert Y. Moore 1996). Entrainment signals are integrated in SCN circuits by modulation of the molecular clock phase, primary through changes in the expression of clock and clock-controlled genes (Yan and Silver 2004) . Outputs of the SCN are distributed to local brain regions and peripheral molecular clocks through hormonal signals together with neural transmission through the autonomic nervous system (Kuhlman, Mackey, and Duffy 2007).

Thus, the circadian system in mammals is composed of a central clock that integrates self-sustained oscillations with oscillations in environmental factors and generates outputs signals that synchronize peripheral (Dibner, Schibler, and Albrecht 2010). The present dissertation is centered on the role of circadian rhythms on local clocks in the mouse brain. Specifically, it focuses on the circadian modulation of entrainment in the SCN itself, where changes in the SCN transcriptome in response to photic entrainment signals at different points of the circadian cycle were profiled. In addition, I examine the circadian modulation of cellular activity in two defined cell populations of the mouse hippocampus: 1) activation of pyramidal neurons of the CA1 region in the formation of spatial memories and 2) in the proliferation and differentiation of adult progenitor/stem cells in the subgranular zone of the dentate gyrus. Together, the results presented here highlight the significant effect of circadian rhythms in physiological and behavioral processes that are modulated by the activation of cellular mechanism in defined brain regions, and that ultimately guide effective adaptation to oscillations of environmental conditions.

Summary

Circadian rhythms are entrained by multiple zeitgebers, but photic inputs given by regular light dark cycles constitute one of the more direct inputs to the SCN (and hence serves as a potent circadian entrainer) (Golombek and Rosenstein 2010). In mice, the circadian rhythm phase is persistently aligned with the duration of the

photoperiod(Johnston 2005). However, adjustments can occur at part of the cycles that are most susceptible to modification. Incidence of photic inputs during the subjective night have a greater impact on the phase modification of the circadian rhythm than photic inputs occurring during the subjective day. Characterization of this processes at the molecular level reflects a significant activation of signaling pathways induced by light delivered at the subjective night, whereas negligible activation of the same pathways is observed during the subjective day (Obrietan, Impey, and Storm 1998). Additionally, analysis performed at a more detailed time resolution using phase response curves (PRC) reveals that light-induced changes in the phase of circadian rhythms is not homogeneous during the subjective night, resulting in phase delays when photic inputs reach the SCN in the first hours of the subjective night, and phase advances when photic inputs reach the SCN in the final hours of the subjective night (Roenneberg, Daan, and Merrow 2003). Downstream of the activation of singling pathways by light, circadian rhythms entrainment is mediated by changes in gene expression, and induction of the clock genes mPer1 and mPer2 seems to be part of the molecular clock reset process (Yan and Silver 2004). Therefore, it is postulated that mechanisms involved in the transduction of entrainment signals are the same, but the difference between phase delays and phase advances are given by the phase at which entrainment signals reach the molecular clock (Golombek and Rosenstein 2010).

Consistent with this idea, photic inputs resetting the molecular clock are associated with genome-wide changes in gene expression, able to mediate the observed change in the circadian phase (Panda et al. 2002). The identification and profiling of

genes whose expression is modified by light in the SCN began just recently and requires ongoing investigation. Previous endeavors in this matter fail to cover the entire diversity of transcripts encoded in the mouse genome (i.e coding and non-coding genes). Studies profiling protein-coding genes in response to light have been completed in the past (Jagannath et al. 2013; Hatori et al. 2014; Porterfield, Piontkivska, and Mintz 2007), but the work presented here **(Chapter 2)** expands the list of candidate genes involved in the expression of phase changes. Here, genome-wide profiling incorporated the profiling of protein coding genes together with a diverse group of non-coding genes such as microRNA precursors, long non-coding RNAs (lncRNA), small RNAs, ribosomal RNAs and pseudogenes. Previous studies develop by (Butcher et al. 2002) have demonstrated that the infusion of U0126 in the lateral ventricles reduces the phosphorylation of ERK causing an uncoupling of photic signals from the entrainment of the circadian clock. Accompanying the profile of light-regulated genes, the role played by the MAPK/ERK signaling cascade in coupling light with gene expression was also assessed. By the intraventricular infusion of the MEK1/2 inhibitor U0126 before the light pulse, the analysis performed here allowed for the identification of transcripts whose expression is regulated by photic activation of the MAPK/ERK pathway in SCN neurons.

Outputs from the central pacemaker regulate peripheral clocks both in different brain regions as well as to the different organs of the body. Within each region, the molecular clock responds to these signals, adjusting its own phase and timing (Brown and Azzi 2013). Hence, the data presented here also approached the circadian modulation of activity of hippocampal cells. The hippocampus is a central brain region principally

associated with the formation and consolidation of semantic and episodic memories, as well as with the generation of spatial representations and navigation (Buzsáki and Moser 2013). The expression of core clock genes in multiple subregions supports and modulates different properties of hippocampal cells that are reflected at the cognitive and behavioral level (Wang et al. 2009). Even though SCN projections do not extend directly to the hippocampus, some evidence indicates indirect connections between the SCN and the hippocampus mediated by GABAergic connections with the septum (Ruby et al. 2008).

To understand how circadian rhythms modulate memory formation in the hippocampus, efforts to this point have focused on establishing to what extent endogenous timing in local memory circuits are autonomous, and the relationship between the molecular clock and the cellular/molecular mechanisms underlying memory. Initial approaches addressing the first of these two problems included the evaluation of learning and memory phenotypes induced by either lesioning the SCN (Friedrich K. Stephan and Kovacevic 1978/4) or by loss-of-function mutations of clock genes in the SCN to evaluate its effect in learning and memory (Wardlaw et al. 2014), As expected, such manipulations impair memory, establishing a functional dependence between circadian rhythms and cognition. Through these and similar studies, modifications to the light exposure period or even chronic light exposure (known to cause disruption in circadian rhythms) also produced deficits in formation and retrieval of memories (Craig and McDonald 2008). More precise studies evaluated the performance on a memory task in a rodent model in which circadian oscillation were made arrhythmic using a paradigm consisting of a single light pulse followed by the modification of the light schedule (Ruby et al. 2008). In this

model, the SCN is intact but its pace making function is impaired. Behavioral analysis of recognition memory show decreased performance (Ruby et al. 2013). However, surgical ablation of the arrhythmic SCN in these animals restored recognition memory capacity (F. Fernandez et al. 2014), suggesting that intact function of the SCN pacemaker plays a major role in timing the oscillations seen in memory performance, and that impaired timing may interfere with the normal performance in this task. Another approach used conditional mutation of clock genes to demonstrate spatial memory deficits in a mouse model in which the clock gene BMAL1 was knocked-out in excitatory forebrain neurons, (including key brain regions like the hippocampus), while its expression was maintained in the SCN (Snider et al. 2016). In this animal model, the molecular clock in the SCN was intact, whereas in the forebrain is compromised. Spatial memory analysis in these animals showed significant deficits, implying the need of a functioning peripheral clock for the proper formation and recall of spatial memories. The aforementioned observations point towards an integrated function in which the coordinated timing of central and peripheral clocks jointly regulate memory mechanisms.

Regarding the association between the molecular clock and the cellular and molecular mechanisms underlying memory, evidence collected so far suggests the existence of time-of-day effects in the manifestation of synaptic plasticity, the physiological mechanism underlying the formation of new memories. Induction of Long Term Potentiation (LTP), an electrophysiological phenomenon associated with an increase in the strength of the synaptic connections in the hippocampus of rodents, is circadian regulated, showing higher magnitudes during the subjective day compared with

the subjective night in the CA1 region of the hippocampus (Chaudhury, Wang, and Colwell 2005). Several studies also point out that mutations in core clock genes alter the formation and retrieval of memories mediated by the activity of the hippocampus (Wardlaw et al. 2014). Together, both lines of evidence provide a strong association between the timing of the molecular clock in the hippocampus and oscillations in memory formation and retrieval.

Here, a novel approach was used to clarify the cellular mechanism behind the association between the molecular clock and memory formation **(Chapter 3)**. Contextual fear conditioning (CFC) is a hippocampal-dependent behavioral paradigm designed to evaluate associative memory. Time-of-day differences in the capacity of wild type animals to retrieve fear memories that are associated with a context were presented in this work (see also (Chaudhury and Colwell 2002; Eckel-Mahan et al. 2008)). These results demonstrate better retrieval performance during the subjective day (CT4) than during the subjective night (CT15), suggesting that a circadian component may be modulating the formation and retrieval of contextual memories. Furthermore, in the hippocampus it is believed that formation of spatial memories induces the coordinated activation of a small percentage of neurons (a neuronal ensemble) that are reactivated when the memory is retrieved (Reijmers et al. 2007). In the work presented here, neuronal ensembles activated during CFC training and retrieval were identified by the expression of the immediate early gene c-Fos in the hippocampus of a mouse line expressing the circadian clock reporter mPeriod1-Venus. This approach allowed an assessment of the circadian clock phase from individual neurons constituting the memory neuronal ensemble. By

evaluating Venus protein levels in cells expressing c-Fos versus cells that do not express c-Fos after CFC, a correlation was found revealing increased levels of Venus protein in c-Fos positive cells. This correlation was consistent across different groups. Control animals that never underwent fear conditioning and remained in their home cages (HC) showed a significantly reduced number of c-Fos positive cells, however levels of Venus protein were still higher in these cells when compared with Venus levels in c-Fos negative cells. Similar results were obtained after the evaluation of fear memory retrieval, where it was shown that retrieval of fear memories was enhanced at CT4 compared with retrieval at CT15. Again, at both time points, HC and CFC mice presented increased Venus levels in c-Fos positive cells. Of note, differences in retrieval capacity at CT4 and CT15 do not show a correlation with the percentage of activated neurons in the memory ensemble.

An additional aspect of circadian modulation of hippocampal activity corresponds to the regulation of proliferation and differentiation capacity of adult neural stem (NPS) cells present in the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus (Bouchard-Cannon et al. 2013; Rakai et al. 2014). Just proximate to the layer of granule cells in the DG is a population of cells capable of self-regeneration, as well as the capacity to differentiate into neurons that later mature and integrate in hippocampal circuits (a process known as neurogenesis) (Zhao, Deng, and Gage 2008). In recent decades, the mechanism of adult neurogenesis has gained increasing clarity. Adult NPS cells can be found in a quiescent state, in which the cells acquire a ramified morphology and are described as *radial glia*. These cells respond to multiple stimuli that

activate the cell cycle and thus undergo cell division. Cells derived from radial glia enter a proliferative stage know as *transiently amplifying progenitor cells* in which the pool of precursor cells is expanded. The first step in the differentiation of precursors cells begin with generation of *neuroblast* that later exit the cell cycle and mature into post-mitotic neurons that are integrated into dentate gyrus circuits (Ming and Song 2011). Multiple factors modulate the proliferation and differentiation of SGZ precursors cells. Among them, are neurotransmitters, neurotrophic and growth factors that transduce the effects of systemic inducer of NPS cell proliferation and differentiation (Gonçalves, Schafer, and Gage 2016). For example, voluntary running (van Praag, Kempermann, and Gage 1999) or access to enriched environments (Kempermann, Kuhn, and Gage 1997) induce an increase in the proliferation of precursor cells. This increase is meditated by brain-derived neurotrophic factor (BDNF)(Zigova et al. 1998). Similarly, the fibroblast growth factor-2 (FGF-2), vascular endothelial growth factor (VEGF), and insulin-like growth factor-1 (IGF-1) have been reported to modulate the adult neurogenesis (Kang and Hébert 2015). In contrast, factors such as cortisol (Heine et al. 2005), induced by stress or inflammatory signals released by the immune system, are known to reduce the neurogenic process(Ekdahl, Kokaia, and Lindvall 2009). At the functional level, adult neurogenesis has been implicated in several cognitive tasks including the encoding of temporal patterns in memory formation, cognitive flexibility during learning, memory consolidation, and pattern separation (see review by Gonçalves, Schafer, and Gage 2016).

Control of the cell cycle progression is key for the regulation of adult NPS cells proliferation and differentiation. Since the molecular clock is modulated cell cycle

progression in multiple tissues and in stem cells in other organs, part of the work described here was focused on the establishment of a relationship between the molecular clock and the proliferation of adult NPS cells **(Chapter 4)**. Mice from the mPer1-Venus reporter line were used to demonstrate the oscillation of the molecular clock in adult NPS cells from the SGZ, identified by the expression of the stem cell marker *SRY-related high-mobility group box* (Sox2). Additionally, a genetic mouse model in which clock oscillation is ablated was used to determine the role of the circadian clock in NPS cell proliferation, using a short-term injection protocol of the thymidine analog *5-bromo-2'-deoxyuridine* (BrdU), which is incorporated in the DNA by the replication process in the S phase of cell cycle. This study revealed that ablation of clock oscillations deregulated NPS cell proliferation, inducing an increase in the number of proliferating cells in the SGZ compared with mice in which the clock was not altered. Moreover, when a long-term BrdU injection protocol was used in the same clock ablation model, an increase of both NPS cell proliferation (by BrdU incorporation analysis) and differentiation (by BrdU incorporation and expression of the neuroblast marker Doublecortin) was found.

Implications and Future Directions

Given the function of the suprachiasmatic nucleus (SCN) as a central pacemaker (Weaver 1998)and the hippocampus as a peripheral clock (Abe et al. 2002), the evidence in this dissertation describes multiple aspects of circadian regulation of physiological and behavioral traits displayed in mammals. Beginning with the profile of changes in the

SCN transcriptome in response to light at different time points of the circadian cycle, together with the pharmacological inhibition of signaling cascades that couple photic inputs with behavioral entrainment, the work described here constitutes an essential step in the clarification of the numerous molecular mechanisms that participate in the regulation of behavioral the molecular clock and its synchronization with geophysical variables. To this point, the role of individual protein coding genes in the maintenance and entrainment of circadian rhythms has been established, but these studies only describe partial mechanisms and fail to incorporate the emerging roles of noncoding RNA. The gene profile presented here complements previous publications of genome wide analysis of light-induced changes in gene expression(Jagannath et al. 2013; Hatori et al. 2014; Porterfield, Piontkivska, and Mintz 2007), and adds another level of regulation (that of non-coding RNAs) that has not been explored before. It should be noted that the role of some non-coding RNAs such as the microRNA miR-132 in circadian entrainment has been widely studied (H.-Y. M. Cheng et al. 2007), however the first comprehensive list of candidate non-coding RNAs with potential roles in circadian entrainment is given here. Establishing the role of these candidate genes constitutes the next step towards a better understanding of photic entrainment. However, traditional methods of analysis make this endeavor a slow process, in which the function of just a handful of targets is achieved. New technologies allowing for the profiling and manipulation of multiple transcripts *in situ* will be crucial to expedite the discovery of new cellular mechanisms regulating entrainment.

Previous behavioral entrainment studies suggest that robust cellular and molecular mechanisms underlie the capacity of organisms to synchronize oscillations of biological processes with oscillation of environmental cues (Roenneberg, Daan, and Merrow 2003). Failure to respond properly to these variables may have detrimental consequences for optimal physiological and cognitive responses (Vaze and Sharma 2013). Important examples are extracted from the study of circadian rhythms in humans. Sleep-wake cycles are closely regulated by circadian rhythm (Saper, Scammell, and Lu 2005). In industrialized societies, challenges to human sleep and circadian rhythms (such as shift work, jet lag and high levels of illumination at night) are associated with mood disorders, cardiovascular disease, cancer incidence, metabolic disorders, dysfunction of the immune system and cognitive impairment (Navara and Nelson 2007). Additionally, some neurodegenerative diseases and even normal aging present disturbances in the maintenance of regular sleep-wake cycles and circadian rhythms(Musiek 2015). A full illumination of the mechanism and molecules that generate and modulate circadian rhythms can guide the discovery of new targets and pharmacological agents that help mitigate the desynchronization of circadian rhythms caused either by disease or by modern human social interactions.

The hippocampus has a major role in the formation, consolidation and retrieval of some types of memory. Studies addressing hippocampal-dependent memory tasks described a marked influence of circadian rhythms on memory acquisition and recall. For a complete understanding of the mechanisms by which the timing of the circadian clock affects memory performance, analysis at multiple levels are required. At the system and

behavioral level, it has been observed that disruption in the synchronization between the SCN central clock and the local clock in the hippocampus disrupts memory performance (Smarr et al. 2014). At the cellular level, the induction of synaptic plasticity is circadian-regulated, exibiting significant differences across the day (Chaudhury, Wang, and Colwell 2005). Similarly, at the molecular level, the activation of signaling cascades underlying the formation of new memories (e.g. MAPK) show circadian oscillations(Eckel-Mahan et al. 2008). Furthermore, mutations in clock genes generate significant effects in the capacity to form and retrieve new memories (Wardlaw et al. 2014).

New levels of circadian regulation of memory were directly and indirectly addressed in the work presented here. By examining the molecular clock phase in the cells that are part of the memory circuit (or ensemble) in the CA1 region of the hippocampus, it was found that the cells recruited to form a memory ensemble where at a similar circadian phase. Thus, it is possible to infer that clock-gated molecular processes may dictate which cells are recruited to the memory ensemble. However, these inferences must be complemented with further assessments of the how the molecular clock phase modifies neuronal mechanisms, such as excitability or synaptic properties that allow the recruitment of different populations of neurons to the memory ensemble. For example, when the formation of two different spatial memories are close in time, the cells and circuits recruited are shared, but if the two memories are formed far apart in time, the circuits recruited are completely independent (Cai et al. 2016). This suggests that defined groups of cells are available to sustain memory acquisition at specific time points, the

delimitation of these groups may be given by the circadian phase. On the other hand, it was observed here that the rate of proliferation and differentiation in the adult hippocampus was modulated by the circadian clock, and elimination of oscillation of the molecular clock was associated with increased rates of proliferation and differentiation. Since the rate of neurogenesis has been related with the regulation of memory mechanism (Deng, Aimone, and Gage 2010), this observation comprises indirect evidence of how circadian rhythms modulate memory formation.

Although the effects of circadian rhythms in memory formation are widely accepted, the mechanisms underlying these effects are not fully clear. Diverse studies have approached the problem from different perspectives and using different behavioral paradigms. Further studies must create a common framework to integrate the findings at different levels under a single hypothesis that dissects the mechanism that relates circadian rhythms with memory formation. This endeavor remains highly relevant given the increasing variables that disrupt normal light-dark and sleep-wake cycles, and the subsequent association with cognitive impairment(Schmidt et al. 2007). Thus, future work assessing time-of-day effects in memory formation and cognitive capacity will aid in the development of therapeutic agents for neurological disorders.

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APPENDIX

LIST OF ONLINE TABLES

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Table 3. List of Transcripts Modulated by Light in the Presence of U0126 Per Time Point.

Table 4. List of Transcripts Modulated by Light in the Presence of U0126 Intersected Between Time Points.

Table 5. List of Transcripts Modulated by Light Activation of the MAPK/ERK Pathway

Please visit https://goo.gl/xxPxYX to view tables Online.