ANALYSIS OF CIRCADIAN RHYTHM USING A NOVEL SCN-SPECIFIC

CRE TRANSGENIC MOUSE LINE

APPROVED BY SUPERVISORY COMMITTEE

Masashi Yanagisawa, M.D., Ph.D.

Steven L. McKnight, Ph.D.

Yi Liu, Ph.D.

Joel K. Elmquist, D.V.M., Ph.D.

DEDICATION

I dedicate this dissertation to the heroes of September 11th, 2001. For their selfless courage and enthusiasm to do the most good gave me the inspiration and strength to embark on a journey that resulted in this dissertation.

ACKNOWLEDGEMENTS

I would like to thank the members of my dissertation committee for thoughtful comments and kind encouragements. My mentor, Masashi Yanagisawa, thank you for believing in me and constantly assuring me that I am doing something import and interesting.

This dissertation would not have been possible without the tremendous help from the following people:

Histologists – Amber Skach and Clay Williams. Thank you for the best quality technical assistance and teaching me the gold standard methods to harvest, section, and sort mouse brains.

Animal Care – Randal Floyd and Marcus Thorton. Your excellent work in keeping the animals and the facility in top shape at all times was essential for my research.

Lab administration – Shelley Dixon and Stephanie Baldock. Your experience in administration helped me obtain services and reagents in a timely manner.

Over the years Tosh Motoike, Yuichi Ikeda, Hide Kumagai, Taizo Matsuki, Ayako Suzuki, and Masa Funato in the lab have taught me so much from basic and advanced cloning techniques to best approaches to getting results to what it takes to discover. I am able to complete my dissertation because of your teachings, ideas, and encouragements. I thank you.

I could not have as much fun and as many amusing days in the lab without Inik Chang, Allen Tsai, Abdulla Shaito, Makito Sato, and Ivan Lee. Thanks for being my buddies.

I thank my parents for always supportive of my goals. Each of my accomplished goals is fruit bear by the years of sacrifice you made moving from our old home in Taiwan to the new home in the United States.

And most of all, I thank my wife and my son for being the pillars of my life, and the chicken soup for my soul. You make everything “worth it” for me.

ANALYSIS OF CIRCADIAN RHYTHM USING A NOVEL SCN-SPECIFIC CRE TRANSGENIC MOUSE LINE

by

ALEXANDER S. CHANG

DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

June, 2010

ANALYSIS OF CIRCADIAN RHYTHM USING A NOVEL SCN-SPECIFIC CRE TRANSGENIC MOUSE LINE

ALEXANDER S. CHANG

The University of Texas Southwestern Medical Center at Dallas, 2010

Mentor: MASASHI YANAGISAWA, M.D., Ph.D.

ABSTRACT

The neurons that make up the suprachiasmatic nucleus (SCN) temporally organize behavior into circadian cycles of activity and rest. When dissociated, these neurons individually oscillate with various period, phase, and amplitude. These conflicting results can be reconciled if inter-neuronal networking in the SCN is required for a consolidated behavioral circadian rhythm. To test this hypothesis, a novel SCN-specific Cre transgenic mouse line, named NMS-Cre, was developed by inserting a bicistronic Cre expression cassette at the 3'-untranslated region of the Neuromedin S (NMS) gene. By crossing NMS-Cre line to a lox-STOP-lox diphtheria toxin receptor

line, behavioral circadian rhythm was disrupted upon intraperitoneal injection of diphtheria toxin. A histological examination showed that diphtheria toxin injection eliminated ~85% of NMS-Cre containing neurons at the SCN. Next, I generated NMS-Cre mediated Bmal1 conditional knockout animals to study behavioral rhythm output when most of the SCN neurons are without a molecular oscillator. The NMS-Cre(+);Bmal1$^{flox/flox}$ animals have essentially normal circadian rhythm of locomotor activity. Then, I generated NMS-Cre mediated Vesicular GABA Transporter (VGAT) conditional knockout animals because GABA has long been suspected to play a role in behavioral circadian rhythm. The NMS-Cre;VGAT$^{flox/flox}$ animals performed normally in behavioral circadian rhythm parameter such as free-running period, robustness, and phase response curve. These in vivo data demonstrated a model that intra-SCN neuronal network is required for behavioral circadian rhythm, and can be a conduit that mediates molecular clock outputs from a small number of SCN neurons. Despite the fact that virtually all SCN neurons are GABAergic, GABA is an unlikely transmitter for this intra-SCN networking. Finally, NMS knockout animals have a well-consolidated behavioral circadian rhythm. However, when subjected to photic phase advancement, NMS knockout animals shifted their activity onset time quicker than wild type control animals. In situ hybridization results ruled out that an altered response to light stimuli or dampened molecular clock oscillation in the SCN as the cause for the rapid phase shift. NMS knockout animals switching from constant illumination to constant dark environment are unable to return to the typical less than twenty-four hour free-running period. Therefore, NMS is involved in a circadian pacemaker function.

TABLE OF CONTENTS

LIST OF FIGURES

Chapter Three Figures

Chapter Four Figures

Chapter Five Figures

LIST OF TABLES

CHAPTER ONE
Introduction

LITERATURE REVIEW: CIRCADIAN RHYTHM

The earth's rotation around the sun imposes on all organisms a rhythmic selective force. Organisms able to adapt and take advantage of their rhythmic milieu are most likely to thrive against others that do not have such ability. The term circadian (Latin – about a day) was proposed by Franz Halberg in 1959. A circadian rhythm represents the endogenous behavioral and/ or physiological oscillation that persists in constant condition with periods of about twenty-four hours.

There is an urgent need to understand the mechanism of the circadian pacemaker. The globalized human society is living beyond the limitation that our biological circadian pacemaker can adapt to. Unlike biological rhythms such as that of the heart beat, where the fastest pacemaker dictates the beat of the organ, circadian rhythm seem more "stubborn", require longer time to adapt, and have a range that seem limiting in the modern "always-on" environment. Mammalian physiologies that are regulated by a circadian pacemaker include: the sleep/wake cycle, daily onset of activity, body temperature oscillation, and endocrine functions. Therefore, the rise of sleeping disorders, depression, drug abuse, and metabolic disorders can be partially attributed to a distressed circadian pacemaker caused by modern lifestyles that involves frequent trans-meridian jet travel, telecommuting, and shift-work. In clinical practice, it has become essential that a patient's circadian rhythm profile be considered for collection of specimen or drug treatment (Levi and Schibler 2007).

Characteristics of circadian rhythm

There are three essential criteria for a biological rhythm to be classified as a circadian rhythm:

It should continue to oscillate in constant conditions

It should be temperature compensated

It should be adjustable

The earliest work on a persistent daily rhythm of a plant's leaf movement was conducted in 1729 by Jean Jacques d'Ortous de Mairan (Moore-Ede, Sulzman et al. 1982). Over 200 years later, Erwin Bünning paved the foundations for the oscillatory properties of a circadian system in plants and insects and implicated the genetic involvement in the circadian clock. Colin Pittendrigh showed that circadian clocks feature a temperature compensation property, a required feature for any device worthy of time-keeping functions. The German term, zeitgeber, which means "time-giver", was coined by Aschoff in 1951 (Aschoff 1951; Moore-Ede, Sulzman et al. 1982). It is now a common term used to describe time cues imposed on the circadian clock for its adjustments.

The functional framework of the circadian pacemaker

The mammalian circadian pacemaker has the ability to sense light, convey luminance information to its oscillatory machinery, and output a response that results in behavior change.

Light is by far the most powerful entrainment factor on the circadian pacemaker. The rod and cone photoreceptors, together with a subgroup of melanopsin-containing retinal

ganglion cells (mRGC) detect environmental light information (Guler, Ecker et al. 2008; Hatori, Le et al. 2008). The mRGCs relay photic signals through the retinohypothalamic tract (RHT), which then activates neurons in the suprachiasmatic nucleus (SCN) through glutamatergic and peptide pituitary adenylate cyclase activating peptide (PAPCAP) neurotransmission (Morin and Allen 2006).

Using animal experiments, Curt Richter subjected blinded rats to metabolic, endocrinologic, and neurologic challenges that included removal of peripheral organs and hundreds of lesions in the brain. Only lesions of the hypothalamus was found to affect free-running rhythm (Richter 1967). Years later, two independent groups identified that lesion of the SCN results in loss of circadian rhythm of adrenal corticosterone, drinking and locomotor activities. (Moore and Eichler 1972; Stephan and Zucker 1972)

Neuroanatomy and physiology of the SCN

It is now well established that the mammalian circadian pacemaker is located in the SCN (Weaver 1998). The SCN is a small bilateral structure consists of 16,000 – 20,000 neurons straddling the base of the third ventricle. These neurons are 8-12 um in diameter (Van den Pol 1980). The neurons receive direct innervations from the eye via the retinohypothalamic tract (RHT). The SCN neurons innervate within the nucleus and densely innervate subventricular zone (SPZ), nucleus of the paraventricular zone (PVN), and dorsal medial hypothalamus (DMH) (Saper, Lu et al. 2005). These areas relay SCN messages throughout the rest of the brain and peripheral organs that, in turn, manifest rhythm in behavior output such as sleep/wake cycle, body temperature oscillation, and locomotor activity.

The SCN is a heterogeneous structure. Some of the dorsal medial SCN neurons and fibers are strongly stained by arginine vasopressin (AVP) antibody, and some of the ventral lateral SCN neurons are stained by vasoactive intestinal polypeptide (VIP) antibody. Although the in vivo function of γ (gamma)-amino butyric acid (GABA) in the SCN is unknown, nearly all SCN neurons are GABAergic.

SCN is required and sufficient for behavioral circadian rhythm

Lesion studies in the 1970's identified that the SCN bilateral structures are required for behavioral circadian rhythm such as drinking activity, locomotor activity (Stephan and Zucker 1972), and corticosteroid secretion (Moore and Eichler 1972). More than two decades later, a careful analysis of hamster SCN lesions suggested that very few SCN neurons and fibers are required for circadian wheel-running activity. The same study suggested that rostral SCN might be more critical for entrainment and rhythmicity than caudal SCN (Harrington, Rahmani et al. 1993).

Transplantation study showed that fetal SCN tissue is sufficient to drive circadian rhythm behavior (Ralph, Foster et al. 1990; LeSauter, Lehman et al. 1996), even when the tissue is encapsulated in a semi-permeable capsule that permitted transmission of only diffusible factors (Silver, LeSauter et al. 1996). Transforming growth factor α (Kramer, Yang et al. 2001) and Prokineticin 2 (Cheng, Bullock et al. 2002) had been implicated as the two possible diffusible factors that mediate locomotor activity circadian rhythm.

An intracellular oscillator

The neurons of SCN contain a molecular oscillator that is mediated by an autoregulatory feedback loop. The detail components of the molecular oscillator have been extensively reviewed (Reppert and Weaver 2001; Lowrey and Takahashi 2004; Ko and Takahashi 2006). At the core of the oscillator, BMAL1, CLOCK and perhaps other basic helix-loop-helix (bHLH) PAS (or Period-Arnt-Single-minded) domain containing transcription factors such as NPAS2 (Reick, Garcia et al. 2001) serve as positive drivers that up regulate the components of the negative feed-back loop such as Periods and Cryptochromes that heterodimerize and translocate back into the nucleus to down-regulate the action of CLOCK/BMAL1 heterdimers. The core autoregulatory feedback loop takes a full turn in about 24 hours with contributing factors mediated via a range of post-translational modifications.

Each one of the core clock genes has been genetically deleted in mouse (Ko and Takahashi 2006). Among the available core clock gene knockout mice, the only single-gene knockout that confers arrhythmic behavior is the Bmal1 knockout (Bunger, Wilsbacher et al. 2000). Activity rhythm of mice with homozygous dominant-negative Clock mutation become arrhythmic in prolong constant darkness environment (Vitaterna, King et al. 1994). Possibly because of the functional redundancy of NPAS2, Clock null mutants maintain robust free-running rhythm (Debruyne, Noton et al. 2006; DeBruyne, Weaver et al. 2007). Animals carrying Crytochrome 1 (Cry1) or Cry2 null allele maintain free-running activity rhythm; Cry1 and Cry2 double knock-out animals immediately become arrhythmic when released into constant dark condition (van der Horst, Muijtjens et al. 1999).

Soon after the cloning of the molecular oscillatory components, it was realized that cells that make up the peripheral organs also have the necessary intracellular machinery to act as clock cells (Balsalobre, Damiola et al. 1998). However, without a synchronization mechanism, oscillating cells outside of the SCN quickly fall out of phase from each other and are not capable of being pacemakers. It is now well accepted that a coordinated circadian timing structure governs the mammalian physiological behavior (Reppert and Weaver 2002).

SCN clock neurons have to synchronize themselves to each other

Individual SCN neurons oscillate with different period, phase, and amplitude, when dissociated from each other (Welsh, Logothetis et al. 1995). When dispersed neurons form synaptic connection, their circadian rhythm in firing rate is synchronized, while out of phase dispersed neurons do not have detectable neuronal transmission (Shirakawa, Honma et al. 2000). Transgenic animals carrying reporter molecules driven by Period genes enabled examination of SCN in organic slice (Yoo, Yamazaki et al. 2004). These experiments showed that SCN clock neurons oscillate in a wave-like pattern that spread from the dorsal-medial towards the ventral portion of the SCN (Yamazaki, Numano et al. 2000). Brain slices from an animal exposed to constant light condition show desynchronized rhythm in gene expression (Ohta, Yamazaki et al. 2005). Separating SCN explants' dorsal one-third from the ventral region causes individual neurons in the dorsal region to lose their coordinated rhythm in gene expression. The neurons in the ventral portion maintain coordinated oscillation in gene expression; though same as the case for intact slice, some neurons oscillate out of phase of the coordinated

majority (Yamaguchi, Isejima et al. 2003). These data suggest that dorsal SCN clock neurons might require synchronization with ventral SCN clock neurons.

Synchronization of SCN pacemaker

Our understanding of how the mammalian circadian system is physically organized was well established in the mid 90's (Moore 1995; Ralph and Hurd 1995). There are several lines of evidence showing that the molecular oscillator machinery is contained within individual clock cells that oscillate with different phases (Welsh, Logothetis et al. 1995; Liu, Weaver et al. 1997; Shirakawa, Honma et al. 2000; Yamaguchi, Isejima et al. 2003; Aton, Huettner et al. 2006). Yet, behavioral circadian rhythms lock into one phase angle in constant environment. Therefore, animals must have a mechanism to synchronize the multi-phased oscillator. However, little is known about the mechanism(s) that the circadian pacemaker system uses to synchronize multiple oscillators into a unified output signal.

The oscillating properties of dorsal and ventral SCN are normally coordinated, but studies have shown that they can be uncoupled. Hypothalamic organ slices treated with antimitotic agent exhibit diverse phase in secretion of AVP and VIP (Shinohara, Honma et al. 1995). In response to phase changes, the phase of Period1 oscillation is adjusted first in the ventral lateral region, then the dorsal medial region (Nagano, Adachi et al. 2003; Nakamura, Yamazaki et al. 2005). Two separate peaks of spontaneous impulse activity, recorded from ventral and dorsal regions of the SCN harvested after a phase shift, are able to consolidate into a single new peak only if SCN stays intact (Albus, Vansteensel et al. 2005).

SCN pacemaker synchronization factors

Several neurotransmitters have been implicated to have a role in the synchronization of the SCN pacemaker. In a mini review article, Aton and Herzog (2005) proposed a few criteria for the characterization of putative synchronization factors. They suggest that the synchronization molecule should have rhythmic expression at the SCN; and its receptor should be present in the SCN. The daily administration of the factor should entrain rhythm in neurons and behavior. Abolishment of the putative signaling pathway by pharmacologic or genetic manipulation should desynchronize rhythm. The rhythm in signaling activity should be entrained by environmental cues that are known to shift behavior rhythm.

GABA is a candidate synaptic synchronizer because components of the GABA signaling pathway are expressed in nearly all of the SCN neurons (Moore and Speh 1993; Belenky, Yarom et al. 2008). There is a circadian rhythm of GABAergic neurotransmission in the SCN (Itri, Michel et al. 2004). Daily application of GABA synchronizes dispersed SCN neurons' firing rhythm (Liu and Reppert 2000). Furthermore, GABA is suspected to mediate the synchronization of ventral and dorsal regions of SCN as the pacemaker responds to resetting light pulse (Albus, Vansteensel et al. 2005). Yet, GABA antagonism on SCN explants does not reduce amplitude of Period gene expression (Aton, Huettner et al. 2006). Microinjection of GABA inhibitors have modest effects on light-induced phase shift (Gillespie, Mintz et al. 1997). The in vivo investigation of whether GABAergic neurotransmissions in the SCN play a role in

behavioral circadian rhythm remains technically challenging and might be best approached by genetic means.

In a later chapter, a possible new technical breakthrough to enable genetic ablation of GABAergic neurotransmission will be discussed in detail.

Although identified in less than 20% (mainly ventral) of all SCN neurons, VIPergic pathway is crucial for the robust expression of circadian rhythm behavior (Moore and Speh 1993; Abrahamson and Moore 2001; Harmar, Marston et al. 2002; Colwell, Michel et al. 2003). The VIP receptor, VPAC2, is highly expressed in the SCN; VPAC2 knockout mice have a SCN with a disrupted molecular oscillator and exhibit low amplitude behavioral rhythm (Harmar, Marston et al. 2002). Multielectrode array examination of neurons from VIP and VPAC2 knockout animals shows that VIP-VPAC2 signaling pathway is involved in coordinating neuronal firing rhythm in the SCN (Aton, Colwell et al. 2005). In dispersed SCN neurons, VPAC2 agonist-induced rhythm of peak neuronal firing is not affected by GABA inhibitors (Aton, Huettner et al. 2006). Other neuropeptides such as Neuromedin S (Mori, Miyazato et al. 2005), Neuromedin U (Nakahara, Hanada et al. 2004), GRP (Piggins, Antle et al. 1995), and PK2 (Li, Hu et al. 2006; Prosser, Bradley et al. 2007) with similar spatial and temporal expression pattern at the SCN could have their specific functions in pacemaker synchronization.

Gap junctions have been implicated in pacemaker synchronization for the past decade. The connexins had been identified in both ventral and dorsal portions of SCN neurons. Dye coupling, a measure of electrical communication, is high during the day and low at night (Colwell 2000). Furthermore, tetrodotoxin and GABA agonist inhibit dye coupling, suggesting that an activity-dependent mechanism regulate electrical

communication. Electrical synapse is not detected in connexin-36 (Cx36) knockout mice SCN. Cx36 knockout mice have lower amplitude behavioral circadian rhythm and an altered activity onset when transitioned from light/dark to constant dark condition (Long, Jutras et al. 2005).

Taken together, the SCN pacemaker requires multiple factors for the proper adjustment to extrinsic and/or intrinsic zeitgebers. In order to understand how each factor might contribute to the synchronization process, it is crucial to set consistent anatomical borders for the SCN pacemaker which is understudied, uniformly apply or eliminate the factor within the anatomical borders, and be able to provide the system as a biological reagent for multi-disciplinary studies. With this approach, elucidation of the synchronization mechanism could provide a platform for systemic modeling of other coupled biological processes.

Output pathways

Compared to what has been described about the oscillator, very little is known on the mechanism of how SCN transmits the synchronized clock signal for the regulation of physiological and behavioral rhythm (Saper, Lu et al. 2005; Kalsbeek, Perreau-Lenz et al. 2006). Although imprecise and technically challenging, neuroanatomical lesion studies have provided a framework of relay sites that are central to the interpretation of the pacemaker output. Lesions of hypothalamic sites, such as SPZ and DMH, that receive large amounts of either direct or indirect neuronal connection from the SCN causes severely altered circadian rhythm in locomotor activity, feeding, sleep-wake cycle,

temperature oscillation, and hormone secretion (Lu, Zhang et al. 2001; Chou, Scammell et al. 2003).

Through SCN transplant studies, diffusible signaling factors have been implicated to be sufficient in driving circadian locomotor activity (Silver, LeSauter et al. 1996). Three molecules have been implicated to be such factor: Transforming growth factor alpha (TGF-alpha) (Kramer, Yang et al. 2001), Prokineticin-2 (PK-2) (Cheng, Bullock et al. 2002), and cardiotrophin-like cytokine (CLC) (Kraves and Weitz 2006). They all have rhythmic gene expression levels in the SCN, have receptors along the SCN neuronal projection sites, and alter their activity level when injected into the third ventricle.

Although diffusible signals are sufficient in restoring locomotor activity rhythm, neuronal output is required for physiological functions of rhythmic temperature oscillation and hormonal secretion (Saper, Lu et al. 2005). Parabiosis experiments, where SCN-lesioned animals are sutured together with a SCN-intact animal so that their vasculature systems are shared, show that blood-borne factor cannot fully restore circadian rhythm. Therefore, suggests that neuronal network might be required (Guo, Brewer et al. 2005). SCN is in control of the synthesis of melatonin via a multisynaptic pathway. Both inhibitory and stimulatory mechanisms have be implicated for the control of melatonin synthesis (Perreau-Lenz, Kalsbeek et al. 2003). GABA has been implicated as a SCN neuronal output neurotransmitter. Transneuronal tracer shows that SCN is connected to the liver via both branches of the autonomic nervous system and that GABAergic inputs to the paraventricular nucleus (PVN) control plasma glucose level (Kalsbeek, La Fleur et al. 2004).

In summary, SCN neurons are heterogeneous groups of molecular oscillators. They express multiple types of neurotransmitters and oscillate in various phases of a twenty-four hour cycle. When synchronized, these neurons are able to transmit signals that compel circadian rhythm in behavior and physiological functions. The major effort of my dissertation aims to develop a genetic tool that can tease out what it takes for the SCN neurons to achieve pacemaker functionality in the whole animal

Chapter One References

Abrahamson, E. E. and R. Y. Moore (2001). "Suprachiasmatic nucleus in the mouse: retinal innervation, intrinsic organization and efferent projections." Brain Research **916**(1-2): 172-191.

Albus, H., M. J. Vansteensel, et al. (2005). "A GABAergic mechanism is necessary for coupling dissociable ventral and dorsal regional oscillators within the circadian clock." Current Biology **15**(10): 886-893.

Aschoff, J. (1951). "[Determination of loco-motor activity of mice by mechanical rectifier.]." Pflugers Arch **254**(3): 262-266.

Aton, S. J., C. S. Colwell, et al. (2005). "Vasoactive intestinal polypeptide mediates circadian rhythmicity and synchrony in mammalian clock neurons." Nature Neuroscience **8**(4): 476-483.

Aton, S. J. and E. D. Herzog (2005). "Come together, right...now: synchronization of rhythms in a mammalian circadian clock." Neuron **48**(4): 531-534.

Aton, S. J., J. E. Huettner, et al. (2006). "GABA and G(i/o) differentially control circadian rhythms and synchrony in clock neurons." Proceedings of the National Academy of Sciences of the United States of America **103**(50): 19188-19193.

Balsalobre, A., F. Damiola, et al. (1998). "A serum shock induces circadian gene expression in mammalian tissue culture cells." Cell **93**(6): 929-937.

Belenky, M. A., Y. Yarom, et al. (2008). "Heterogeneous expression of gamma-aminobutyric acid and gamma-aminobutyric acid-associated receptors and transporters in the rat suprachiasmatic nucleus." Journal of Comparative Neurology **506**(4): 708-732.

Bunger, M. K., L. D. Wilsbacher, et al. (2000). "Mop3 is an essential component of the master circadian pacemaker in mammals." Cell **103**(7): 1009-1017.

Cheng, M. Y., C. M. Bullock, et al. (2002). "Prokineticin 2 transmits the behavioural circadian rhythm of the suprachiasmatic nucleus." Nature **417**(6887): 405-410.

Chou, T. C., T. E. Scammell, et al. (2003). "Critical role of dorsomedial hypothalamic nucleus in a wide range of behavioral circadian rhythms." J Neurosci **23**(33): 10691-10702.

Colwell, C. S. (2000). "Rhythmic coupling among cells in the suprachiasmatic nucleus." Journal of Neurobiology **43**(4): 379-388.

Colwell, C. S., S. Michel, et al. (2003). "Disrupted circadian rhythms in VIP- and PHI-deficient mice." American Journal of Physiology-Regulatory Integrative and Comparative Physiology **285**(5): R939-R949.

Debruyne, J. P., E. Noton, et al. (2006). "A clock shock: mouse CLOCK is not required for circadian oscillator function." Neuron **50**(3): 465-477.

DeBruyne, J. P., D. R. Weaver, et al. (2007). "CLOCK and NPAS2 have overlapping roles in the suprachiasmatic circadian clock." Nature Neuroscience **10**(5): 543-545.

Gillespie, C. F., E. M. Mintz, et al. (1997). "GABA(A) and GABA(B) agonists and antagonists alter the phase-shifting effects of light when microinjected into the suprachiasmatic region." Brain Research **759**(2): 181-189.

Guler, A. D., J. L. Ecker, et al. (2008). "Melanopsin cells are the principal conduits for rod-cone input to non-image-forming vision." Nature **453**(7191): 102-105.

Guo, H. N., J. M. Brewer, et al. (2005). "Differential control of peripheral circadian rhythms by suprachiasmatic-dependent neural signals." Proceedings of the National Academy of Sciences of the United States of America **102**(8): 3111-3116.

Harmar, A. J., H. M. Marston, et al. (2002). "The VPAC(2) receptor is essential for circadian function in the mouse suprachiasmatic nuclei." Cell **109**(4): 497-508.

Harrington, M. E., T. Rahmani, et al. (1993). "Effects of damage to SCN neurons and efferent pathways on circadian activity rhythms of hamsters." Brain Res Bull **30**(5-6): 655-669.

Hatori, M., H. Le, et al. (2008). "Inducible ablation of melanopsin-expressing retinal ganglion cells reveals their central role in non-image forming visual responses." PLoS One **3**(6): e2451.

Itri, J., S. Michel, et al. (2004). "Circadian rhythm in inhibitory synaptic transmission in the mouse suprachiasmatic nucleus." Journal of Neurophysiology **92**(1): 311-319.

Kalsbeek, A., S. La Fleur, et al. (2004). "Suprachiasmatic GABAergic inputs to the paraventricular nucleus control plasma glucose concentrations in the rat via sympathetic innervation of the liver." Journal of Neuroscience **24**(35): 7604-7613.

Kalsbeek, A., S. Perreau-Lenz, et al. (2006). "A network of (autonomic) clock outputs." Chronobiol Int **23**(3): 521-535.

Ko, C. H. and J. S. Takahashi (2006). "Molecular components of the mammalian circadian clock." Hum Mol Genet **15 Spec No 2**: R271-277.

Ko, C. H. and J. S. Takahashi (2006). "Molecular components of the mammalian circadian clock." Human Molecular Genetics **15**: R271-R277.

Kramer, A., F. C. Yang, et al. (2001). "Regulation of daily locomotor activity and sleep by hypothalamic EGF receptor signaling." Science **294**(5551): 2511-2515.

Kraves, S. and C. J. Weitz (2006). "A role for cardiotrophin-like cytokine in the circadian control of mammalian locomotor activity." Nat Neurosci **9**(2): 212-219.

LeSauter, J., M. N. Lehman, et al. (1996). "Restoration of circadian rhythmicity by transplants of SCN "micropunches"." J Biol Rhythms **11**(2): 163-171.
Levi, F. and U. Schibler (2007). "Circadian rhythms: mechanisms and therapeutic implications." Annu Rev Pharmacol Toxicol **47**: 593-628.
Li, J. D., W. P. Hu, et al. (2006). "Attenuated circadian rhythms in mice lacking the prokineticin 2 gene." J Neurosci **26**(45): 11615-11623.
Liu, C. and S. M. Reppert (2000). "GABA synchronizes clock cells within the suprachiasmatic circadian clock." Neuron **25**(1): 123-128.
Liu, C., D. R. Weaver, et al. (1997). "Cellular construction of a circadian clock: Period determination in the suprachiasmatic nuclei." Cell **91**(6): 855-860.
Long, M. A., M. J. Jutras, et al. (2005). "Electrical synapses coordinate activity in the suprachiasmatic nucleus." Nature Neuroscience **8**(1): 61-66.
Lowrey, P. L. and J. S. Takahashi (2004). "Mammalian circadian biology: elucidating genome-wide levels of temporal organization." Annu Rev Genomics Hum Genet **5**: 407-441.
Lu, J., Y. H. Zhang, et al. (2001). "Contrasting effects of ibotenate lesions of the paraventricular nucleus and subparaventricular zone on sleep-wake cycle and temperature regulation." J Neurosci **21**(13): 4864-4874.
Moore-Ede, M. C., F. M. Sulzman, et al. (1982). The Clocks That Time Us. Cambridege, Harvard University Press.
Moore, R. Y. (1995). "Organization of the mammalian circadian system." Ciba Found Symp **183**: 88-99; discussion 100-106.
Moore, R. Y. and V. B. Eichler (1972). "Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat." Brain Res **42**(1): 201-206.
Moore, R. Y. and J. C. Speh (1993). "GABA is the principal neurotransmitter of the circadian system." Neurosci Lett **150**(1): 112-116.
Mori, K., M. Miyazato, et al. (2005). "Identification of neuromedin S and its possible role in the mammalian circadian oscillator system." Embo J **24**(2): 325-335.
Morin, L. P. and C. N. Allen (2006). "The circadian visual system, 2005." Brain Research Reviews **51**(1): 1-60.
Nagano, M., A. Adachi, et al. (2003). "An abrupt shift in the day/night cycle causes desynchrony in the mammalian circadian center." J Neurosci **23**(14): 6141-6151.
Nakahara, K., R. Hanada, et al. (2004). "The gut-brain peptide neuromedin U is involved in the mammalian circadian oscillator system." Biochem Biophys Res Commun **318**(1): 156-161.
Nakamura, W., S. Yamazaki, et al. (2005). "Differential response of Period 1 expression within the suprachiasmatic nucleus." Journal of Neuroscience **25**(23): 5481-5487.

Ohta, H., S. Yamazaki, et al. (2005). "Constant light desynchronizes mammalian clock neurons." Nature Neuroscience **8**(3): 267-269.

Perreau-Lenz, S., A. Kalsbeek, et al. (2003). "Suprachiasmatic control of melatonin synthesis in rats: inhibitory and stimulatory mechanisms." European Journal of Neuroscience **17**(2): 221-228.

Piggins, H. D., M. C. Antle, et al. (1995). "NEUROPEPTIDES PHASE-SHIFT THE MAMMALIAN CIRCADIAN PACEMAKER." Journal of Neuroscience **15**(8): 5612-5622.

Prosser, H. M., A. Bradley, et al. (2007). "Prokineticin receptor 2 (Prokr2) is essential for the regulation of circadian behavior by the suprachiasmatic nuclei." Proc Natl Acad Sci U S A **104**(2): 648-653.

Ralph, M. R., R. G. Foster, et al. (1990). "Transplanted suprachiasmatic nucleus determines circadian period." Science **247**(4945): 975-978.

Ralph, M. R. and M. W. Hurd (1995). "Circadian pacemakers in vertebrates." Ciba Found Symp **183**: 67-81; discussion 81-67.

Reick, M., J. A. Garcia, et al. (2001). "NPAS2: An analog of clock operative in the mammalian forebrain." Science **293**(5529): 506-509.

Reppert, S. M. and D. R. Weaver (2001). "Molecular analysis of mammalian circadian rhythms." Annu Rev Physiol **63**: 647-676.

Reppert, S. M. and D. R. Weaver (2002). "Coordination of circadian timing in mammals." Nature **418**(6901): 935-941.

Richter, C. P. (1967). "Sleep and activity: their relation to the 24-hour clock." Res Publ Assoc Res Nerv Ment Dis **45**: 8-29.

Saper, C. B., J. Lu, et al. (2005). "The hypothalamic integrator for circadian rhythms." Trends Neurosci **28**(3): 152-157.

Shinohara, K., S. Honma, et al. (1995). "2 DISTINCT OSCILLATORS IN THE RAT SUPRACHIASMATIC NUCLEUS IN-VITRO." Proceedings of the National Academy of Sciences of the United States of America **92**(16): 7396-7400.

Shirakawa, T., S. Honma, et al. (2000). "Synchronization of circadian firing rhythms in cultured rat suprachiasmatic neurons." European Journal of Neuroscience **12**(8): 2833-2838.

Silver, R., J. LeSauter, et al. (1996). "A diffusible coupling signal from the transplanted suprachiasmatic nucleus controlling circadian locomotor rhythms." Nature **382**(6594): 810-813.

Stephan, F. K. and I. Zucker (1972). "Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions." Proc Natl Acad Sci U S A **69**(6): 1583-1586.

Van den Pol, A. N. (1980). "The hypothalamic suprachiasmatic nucleus of rat: intrinsic anatomy." J Comp Neurol **191**(4): 661-702.

van der Horst, G. T., M. Muijtjens, et al. (1999). "Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms." Nature **398**(6728): 627-630.

Vitaterna, M. H., D. P. King, et al. (1994). "Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behavior." Science **264**(5159): 719-725.

Weaver, D. R. (1998). "The suprachiasmatic nucleus: a 25-year retrospective." J Biol Rhythms **13**(2): 100-112.

Welsh, D. K., D. E. Logothetis, et al. (1995). "INDIVIDUAL NEURONS DISSOCIATED FROM RAT SUPRACHIASMATIC NUCLEUS EXPRESS INDEPENDENTLY PHASED CIRCADIAN FIRING RHYTHMS." Neuron **14**(4): 697-706.

Yamaguchi, S., H. Isejima, et al. (2003). "Synchronization of cellular clocks in the suprachiasmatic nucleus." Science **302**(5649): 1408-1412.

Yamazaki, S., R. Numano, et al. (2000). "Resetting central and peripheral circadian oscillators in transgenic rats." Science **288**(5466): 682-685.

Yoo, S. H., S. Yamazaki, et al. (2004). "PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues." Proc Natl Acad Sci U S A **101**(15): 5339-5346.

CHAPTER TWO
Review of the Literature

NEUROMEDIN S: A NEUROPEPTIDE EXCLUSIVELY EXPRESSED IN THE SCN

Neuromedin S (NMS) is a neuropeptide recently identified by Dr. Kangawa's group to be a ligand for G protein-coupled receptors (GPCRs) NMU1R and NMU2R. Since the group also discovered Neuromedin U (NMU) in 1985 as the ligand for these two receptors, they designated the letter S for NMS because of its specific expression in the suprachiamatic nucleus (SCN).

The structure of NMS

After the NMS peptide was isolated from the rat brain using reverse-pharmacological method with the NMU2R receptor, the NMS gene was cloned by polymerase chain reaction. Mori et al determined that NMS is encoded by a separate gene from NMU (Mori, Miyazato et al. 2005). In the mouse, the NMS gene is approximately 11,000 base pair (bp) in length on chromosome 1b. This gene contains ten exons and nine introns. In the 5-prime flanking region that is conserved amongst mouse, rat, and human, this gene contains putative transcription factors binding sites such as AP-1, Sox-5, Oct-1, and GATA-1. However, it does not contain a cAMP-responsive element or an E-box sequence.

The NMS cDNA is approximately 1000 bp. It encodes a 152-amino acid prepropeptide with a characteristic N-terminal signal peptide sequence. The prepropeptide contains four processing sites, which after proprotein convertases cleavage

creates a 36-residue NMS peptide. Interestingly, seven amidated residues at the C-terminus of the NMU peptide is also present at the C-terminus of the NMS peptide. This sequence is thought to be essential for NMU-8 binding to NMU receptors (Minamino, Kangawa et al. 1985). In addition, both NMS and NMU propeptides contain another unknown peptide with 34 and 33 amino acids, respectively. Their functions have not yet been determined.

Central expression patterns of NMS and its receptors

NMS has a very specific expression pattern in rodents. In the brain, NMS expression is largely limited to the hypothalamus. In situ hybridization detected NMS expression exclusively in the suprachiasmatic nucleus (Figure 5- 1). However, the more sensitive quantitative PCR (QPCR) analysis detected low expression in other hypothalamic regions such as lateral hypothalamic area, ventral medial hypothalamus, paraventricular nucleus, and arcuate. In the periphery, NMS is expressed abundantly only in the spleen and testis. In the SCN, NMS does not oscillate in constant darkness. In the light dark cycle, however, it has higher expression during the light period than the dark period.

The NMS receptors, NMU1R and NMU2R, have differential expression pattern throughout the animal. NMU1R is widely expressed in the periphery (Rucinski, Ziolkowska et al. 2007). Its central expression is limited to the hypothalamus (Rucinski, Ziolkowska et al. 2007). NMU2R, in contrast, is mainly expressed in the central nervous system. It has especially high expression in the paraventricular nucleus, the ependymal layer in the wall of the third ventricle (Howard, Wang et al. 2000), the dorsal horn of the

spinal cord (Torres, Croll et al. 2007), and SCN (Mangold, Ksiazek et al. 2008). Both receptors are expressed in the SCN and oscillate with different phases in constant darkness. NMU1R have its peak expression time during the late subjective light period, while NMU2R have its peak expression time in the middle portion of the subjective dark period (Nakahara, Hanada et al. 2004).

NMU1R and NMU2R signaling

Following Howard et al's report on the identification of the NMU receptors in 2000 (Howard, Wang et al. 2000), at least five additional reports were published later that same year with matching results demonstrating the expression patterns of these receptors and that NMU binding to these receptors increase intracellular calcium levels (Fujii, Hosoya et al. 2000; Hosoya, Moriya et al. 2000; Raddatz, Wilson et al. 2000; Shan, Qiao et al. 2000; Szekeres, Muir et al. 2000). Until 2000, the NMU receptors were previously described as orphan G protein-coupled receptors (GPCR) FM-3/GPR66 and FM-4/TGR-1. They are now identified as NMU1R and NMU2R, respectively. Since the activation of NMU receptors mobilize calcium, these two receptors are thought to be Gq-coupled receptors. However, there are recent reports suggesting that NMU2R might also be Gi-coupled (Hsu and Luo 2007; Brighton, Wise et al. 2008).

Physiological functions of NMS

NMS activates the two receptors that NMU also activates. Not surprisingly, they have similar functions in contracting smooth muscle preparations and increase systemic blood pressure in the rat (Mori, Miyazato et al. 2005). Because of the exclusive

expression pattern of NMS in the hypothalamus, its reported physiological functions have mainly been on the possible role in the circadian pacemaker (Mori, Miyazato et al. 2005) and other hypothalamic functions such as feeding suppression (Ida, Mori et al. 2005), HPA-axis activation (Jaszberenyi, Bagasi et al. 2007), LH secretion regulation (Vigo, Roa et al. 2007), increase oxytocin release and milk secretion (Sakamoto, Mori et al. 2008), and urine output reduction (Sakamoto, Mori et al. 2007). One important caveat to note is that all reported experimental design of the NMS physiological functions used exogenous application of synthetic NMS. Therefore, it is currently not known which of these reported functions are specifically mediated by NMS in vivo.

In Chapter Five, the development and characterization of a NMS knockout animal for NMS' role in circadian pacemaker physiology is described.

Chapter Two Reference

Brighton, P. J., A. Wise, et al. (2008). "Paradoxical behavior of neuromedin U in isolated smooth muscle cells and intact tissue." Journal of Pharmacology and Experimental Therapeutics **325**(1): 154-164.

Fujii, R., M. Hosoya, et al. (2000). "Identification of neuromedin U as the cognate ligand of the orphan G protein-coupled receptor FM-3." Journal of Biological Chemistry **275**(28): 21068-21074.

Hosoya, M., T. Moriya, et al. (2000). "Identification and functional characterization of a novel subtype of neuromedin U receptor." Journal of Biological Chemistry **275**(38): 29528-29532.

Howard, A. D., R. Wang, et al. (2000). "Identification of receptors for neuromedin U and its role in feeding." Nature **406**(6791): 70-74.

Hsu, S. H. and C. W. Luo (2007). "Molecular dissection of G protein preference using G(s)alpha chimeras reveals novel ligand signaling of GPCRs." American Journal of Physiology-Endocrinology and Metabolism **293**(4): E1021-E1029.

Ida, T., K. Mori, et al. (2005). "Neuromedin S is a novel anorexigenic hormone." Endocrinology **146**(10): 4217-4223.

Jaszberenyi, M., Z. Bagasi, et al. (2007). "Endocrine and behavioral effects of neuromedin S." Hormones and Behavior **52**(5): 631-639.

Mangold, C., I. Ksiazek, et al. (2008). "Distribution of neuromedin U binding sites in the rat CNS revealed by in vitro receptor autoradiography." Neuropeptides **42**(4): 377-386.

Minamino, N., K. Kangawa, et al. (1985). "NEUROMEDIN-U-8 AND NEUROMEDIN-U-25 - NOVEL UTERUS STIMULATING AND HYPERTENSIVE PEPTIDES IDENTIFIED IN PORCINE SPINAL-CORD." Biochemical and Biophysical Research Communications **130**(3): 1078-1085.

Mori, K., M. Miyazato, et al. (2005). "Identification of neuromedin S and its possible role in the mammalian circadian oscillator system." Embo J **24**(2): 325-335.

Nakahara, K., R. Hanada, et al. (2004). "The gut-brain peptide neuromedin U is involved in the mammalian circadian oscillator system." Biochem Biophys Res Commun **318**(1): 156-161.

Raddatz, R., A. E. Wilson, et al. (2000). "Identification and characterization of two neuromedin U receptors differentially expressed in peripheral tissues and the central nervous system." Journal of Biological Chemistry **275**(42): 32452-32459.

Rucinski, M., A. Ziolkowska, et al. (2007). "Expression of neuromedins S and U and their receptors in the hypothalamus and endocrine glands of the rat." International Journal of Molecular Medicine **20**(2): 255-259.

Sakamoto, T., K. Mori, et al. (2008). "Involvement of neuromedin S in the oxytocin release response to suckling stimulus." Biochemical and Biophysical Research Communications **375**(1): 49-53.

Sakamoto, T., K. Mori, et al. (2007). "Neuromedin S exerts an antidiuretic action in rats." Biochemical and Biophysical Research Communications **361**(2): 457-461.

Shan, L. X., X. D. Qiao, et al. (2000). "Identification of a novel neuromedin U receptor subtype expressed in the central nervous system." Journal of Biological Chemistry **275**(50): 39482-39486.

Szekeres, P. G., A. I. Muir, et al. (2000). "Neuromedin U is a potent agonist at the orphan G protein-coupled receptor FM3." Journal of Biological Chemistry **275**(27): 20247-20250.

Torres, R., S. D. Croll, et al. (2007). "Mice genetically deficient in neuromedin U receptor 2, but not neuromedin U receptor 1, have impaired nociceptive responses." Pain **130**(3): 267-278.

Vigo, E., J. Roa, et al. (2007). "Neuromedin S as novel putative regulator of luteinizing hormone secretion." Endocrinology **148**(2): 813-823.

CHAPTER THREE
Methodology

MATERIALS AND METHODS

Generation of NMS null mouse line

A combined strategy to delete the Neuromedin S (NMS) signal peptide sequence, and insert a Cre recombinase cassette was taken to create a new mouse line with both a null and a Cre knock-in NMS locus.

The NMS-KI targeting vector was constructed with BAC fragments obtained from screening the Children's Hospital Oakland Research Institute (CHORI) BACPAC high density filter by using a 204bp P32-labeled DNA probe targeting the intronic region between exons five and six. The genomic DNA of the BAC library came from a 129/SvEvTac strain.

The NMS-KI vector targets the first exon and deletes a 59bp-sequence corresponding to the signal peptide sequence upon homologous recombination in ES cells. The signal peptide sequence was published by Mori et al (Mori, Miyazato et al. 2005) and confirmed in silico using the SignalP server (Bendtsen, Nielsen et al. 2004). The NMS-KI vector contains a Cre recombinase cassette from pBS185 (Gibco BRL) that replaces the NMS' sequence at ATG, a FRT flanked PGK-NEO cassette in the reversed orientation, and a DTA cassette at the five prime end of the short-arm (Figure 3- 1). A total of 480 neomycin resistant clones were screened by PCR and that resulted in 9 positive clones. Upon confirmation with southern blot analysis, three independent clones were microinjected by UTSW transgenic core into 129/SvEvTac blastocyst (Figure 3- 2).

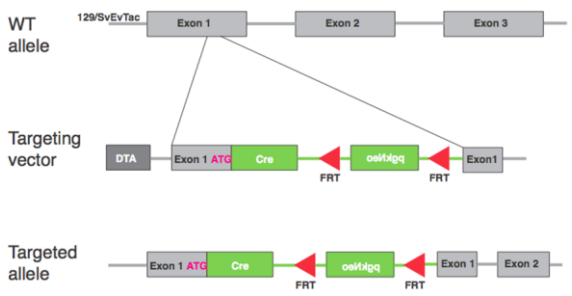

Figure 3- 1 The NMS-KI targeting vector targets the first exon of the NMS gene. Homologous recombination with the targeting vector deletes 59 bp of the signal peptide sequence. The Cre recombinase cassette replaces the NMS gene at the ATG start codon.

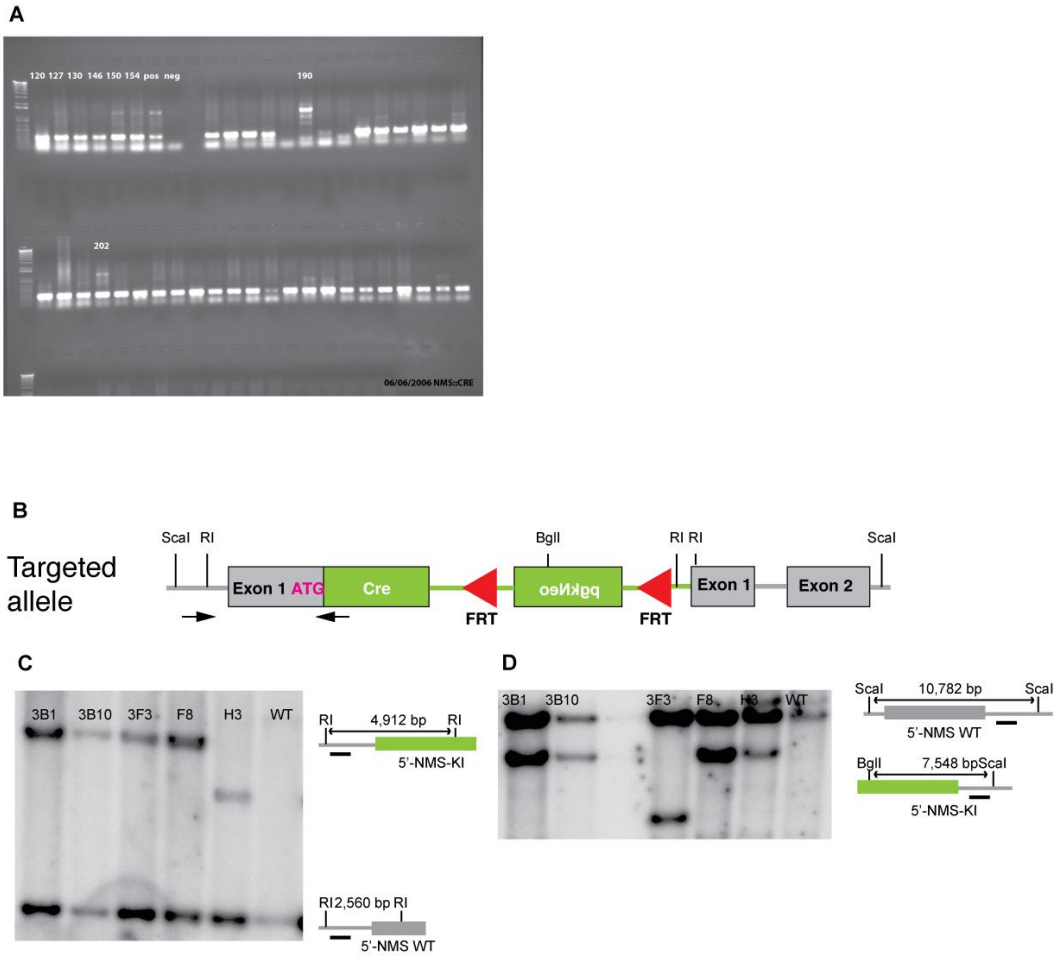

Figure 3- 2 A total of 480 neomycin resistant ES cell clones were screened by PCR and positive clones were confirmed by southern blot analysis. Example gel picture is shown in panel A where the 1400 bp amplicons indicate a positive clone. Primers locations are indicated by black arrows in panel B. PCR positive clones were subjected to southern blot analysis. In panel C and D, positive ES cell genomic DNAs were subjected to indicated restriction enzyme digestions, blotted onto membrane, and hybridized with P32-labeled southern probes. Thicker horizontal grey or green bars indicate wild type or NMS-KI sequence within the vector. Thinner horizontal grey bars indicate sequence outside of the vector sequence. Black horizontal bar indicate probe location. Calculated sizes of the expected fragments are indicated above double arrows. Proportions of the fragment sizes are not to scale. Five prime arm southern is in C and three prime arm is in D. 3B1 and 3B10 clones ultimately were injected and have successful germ-line transmission.

After successful germ-line transmission, Neo cassette was removed by crossing NMS-KI with ROSA26FLPe line. The complete removal of Neo cassette in resulted litter was confirmed by PCR analysis.

NMS-KI animal has been backcross to C57/Bl6j strain for six generations.

Genotyping Assay for NMS-KI allele

A custom multiplex PCR reaction using fluorogenic 5' nuclease chemistry targeting the NMS signal peptide sequence was developed to determine the genotype in one single PRC reaction and without the need to perform gel electrophoresis. A Bio-Rad CFX96 real-time PCR detection system was used to for cycling condition and data collection. Five oligos are used for this assay:

AC141 AGAGAATCCAGAGCTTGAAGATG

AC142 CCTGACGGGTAAAGATCTGC

AC146 CATTTTCCAGGTATGCTCAGAA

AC140 FAM-TCCCCCACTATTCTCCAATCCTG-BHQ

AC149 CAL540-CACCAAAATTTGCCTGCATTACCG-BHQ

Generation of NMS-Cre transgenic mouse line

The IRES2-iCre bicistronic expression cassette was constructed by combining IRES2 sequence (pIRES2-DsRed2, Clontech) and iCre expression cassette (Shimshek, Kim et al. 2002) using traditional restriction enzyme digestion and ligation methods. The functionality of the bicistronic IRES2-iCre cassette was verified by FACS analysis of

pCMV-GRP-IRES2-iCre transfected E19 ROSA26-YFP reporter mouse derived embryonic fibroblasts (Figure 3- 3).

Modification of NMS-BAC was done by recombineering technology (Copeland, Jenkins et al. 2001). BAC clone Rp23-101p2 containing a sequence verified NMS genomic DNA was purchased from CHORI BACPAC. The isolated BAC DNA was electroporated into SW105 E. coli. The targeting vector on the pSP72 backbone included an IRES2-iCre cassette followed by FRT flanked neo/kanamycin cassette with two short NMS homologous arms, 304bp (5-prime) and 205bp (3-prime) surrounding the insert. The neo/kanamycin cassette was removed by arabinose-induced FLPe recombinase.

The NMS-Cre transgenic mouse line was generated by injecting a BAC construct that contains the IRES2-iCre cassette inserted in the 3'-UTR region of the NMS genomic sequence into the pronucleus of a fertilized C57/Bl6J oocyte (Figure 3- 4).

Targeted NMS-BAC DNA was isolated and submitted to UT Southwestern Transgenic Core facility, uncut, for pronucleus injection.

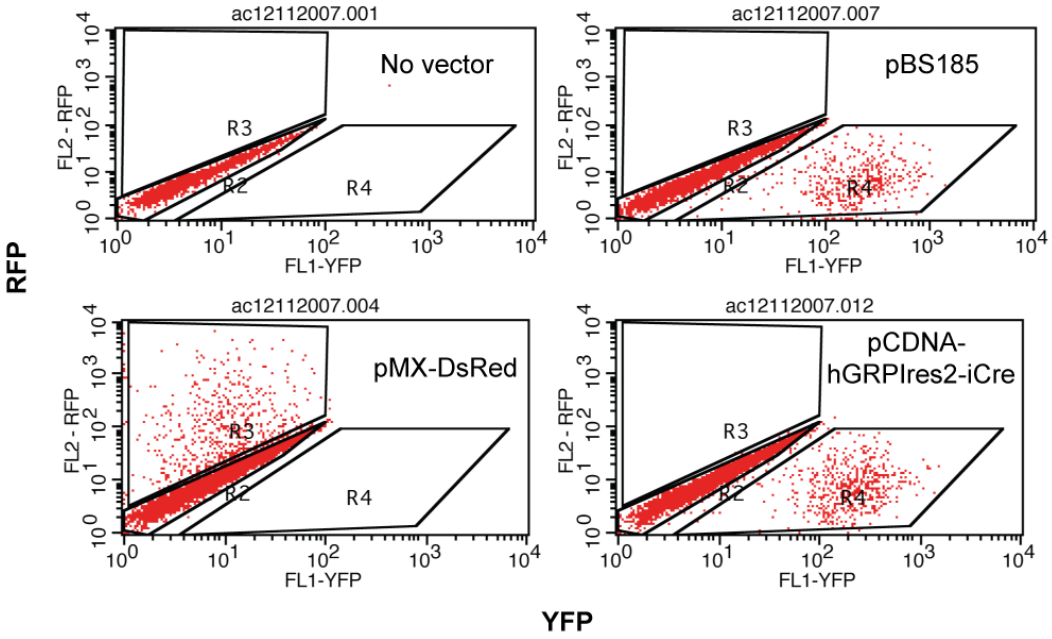

Figure 3- 3 Bicistronic NMS-Cre expression cassette was validated onROSA26-YFP MEFs.

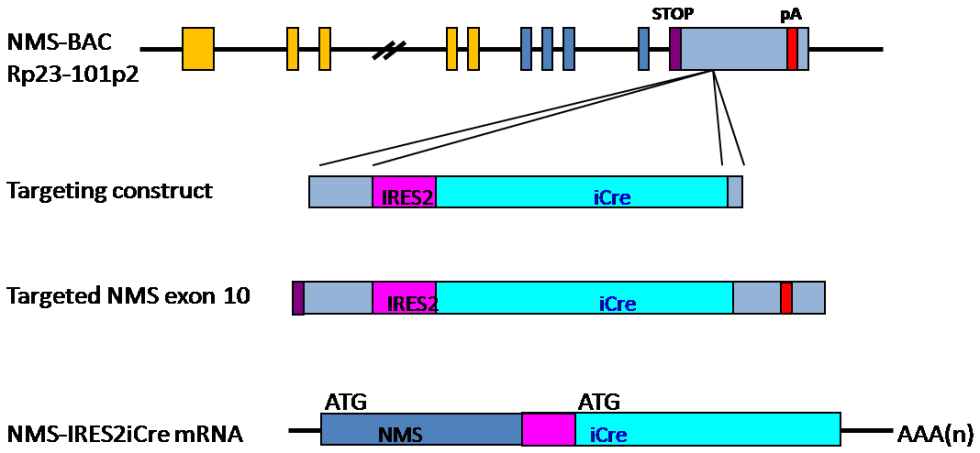

Figure 3- 4 The bicistronic NMS-Cre expression cassette was inserted into the three prime UTR of the NMS gene by recombineering. NMS-containing BAC was purchased from CHORI BACPAC resource and confirm by PCR analysis. Recombineering components were obtained from the Copeland lab from NIH. Successfully recombineered NMS-Cre BAC were confirmed by BAC sequencing.

Establishing NMS-Cre founder lines

The transgenic technology core returned fifty-one pups from ten foster mothers. Eight out of fifty-one (~16%) pups were positive for the IRES2-iCre gene. Genotype diagnostics were carried out using PCR amplification followed by gel electrophoresis with DNA isolated from tail biopsies.

All eight IRES2-iCre positive animals were crossed with ROSA26YFP indicator line to histologically determine the expression pattern of the iCre driver. Three out of the eight positive pups did not have any offspring. One founder did not produce any transgene positive offspring in two litters and was terminated. BAC01-033 line did produce transgene positive litters, but did not have any positive YFP staining in the brain. The remaining three lines showed nearly identical YFP staining in the brain.

NMS-Cre founder lines

Founder ID	IRES2-iCre transgene in offspring	Histological screening results (YFP staining)
BAC01-004	not detected in 2 litters	N/A
BAC01-020	About 50%	SCN; SFO
BAC01-028	About 50%	SCN; SFO
BAC01-033	About 50%	No signal
BAC01-036	100% in first litter	SCN; SFO

SCN, suprachiasmatic nucleus. SFO, subfornical organ

Table 3- 1 NMS-Cre founder lines

Genotyping assay for NMS-Cre

A Taqman® probe real-time PCR assay was developed to detect NMS-Cre transgene. The designed primers are:

AC311 TGAGGGACTACCTCCTGTACC

AC312 ACACAGCATTGGAGTCAGAAG

AC313 FAM-AGCTCAACATGCTGCACAGGAGATCT

Floxed VGAT animals

The floxed VGAT animals used to generate NMS-Cre conditional VGAT knockout were provided by the laboratory of Dr. Brad Lowell (Tong, Ye et al. 2008).

Animal husbandry

Adult house mice (Mus musculs) were group housed in 12 hour light and 12 hour dark cycles with freely available food and water. Cages, water, and chow were replaced every seven days. Breeding pairs were monitored daily and recorded when new litters were born. Litters were weaned between day 17 and day 21 after birth. At time of weaning, ears were punched and tails biopsied for genotyping. Animal records were kept in the Jackson Laboratory Colony Management System database running under the Microsoft Access environment.

Daily activity recording

Telemetry and activity recordings with Mini-Mitter

The E-Mitter telemetry transponder is surgically implanted in the abdomen of a mouse at least 6 weeks old. Recovered animals are single housed in cage furnished with running-wheel, food, and water intake monitoring system for a minimum of 10 days before start of the experiment.

Wheel-running activity, drinking bouts, and Core body temperature and voluntary activity output from the transponder are received by ER-4000 receiver placed underneath the cage. These data are recorded by a PC running VitalView collection program set to collect data every 5 minutes.

Data were transformed to ASCII format individually and analyzed in ClockLab Analysis program by Actimetrics of Wilmette, IL.

Wheel-running behavior recording

Experiments quickly outgrew the eight-channel capacity of the Mini-Mitter system because of the length of the experiments and the number of different lines needing analysis. The majority of the circadian behavior recordings were done in circadian cabinets that were custom built by Phenome Technologies, Inc of Lincolnshire, IL. These cabinets are light-tight, ventilated, and are wired for computer controlled lighting schemes. The animals are individually housed in Techniplast 1144B001 cage body and 1144B116 wire cage lid that are equipped with a four inch running wheel. A magnetic switch is attached to each wheel to enable recording of the number of its revolution. The lighting statuses for each cabinet were indicated via a light sensor placed inside the cabinet and recorded by the ClockLab Data Collection module. A dedicated PC running ClockLab data acquisition software records lighting status and wheel revolution counts in five minute bins. Collected data were transferred to a USB flash drive and analyzed on separate PC running ClockLab Analysis software.

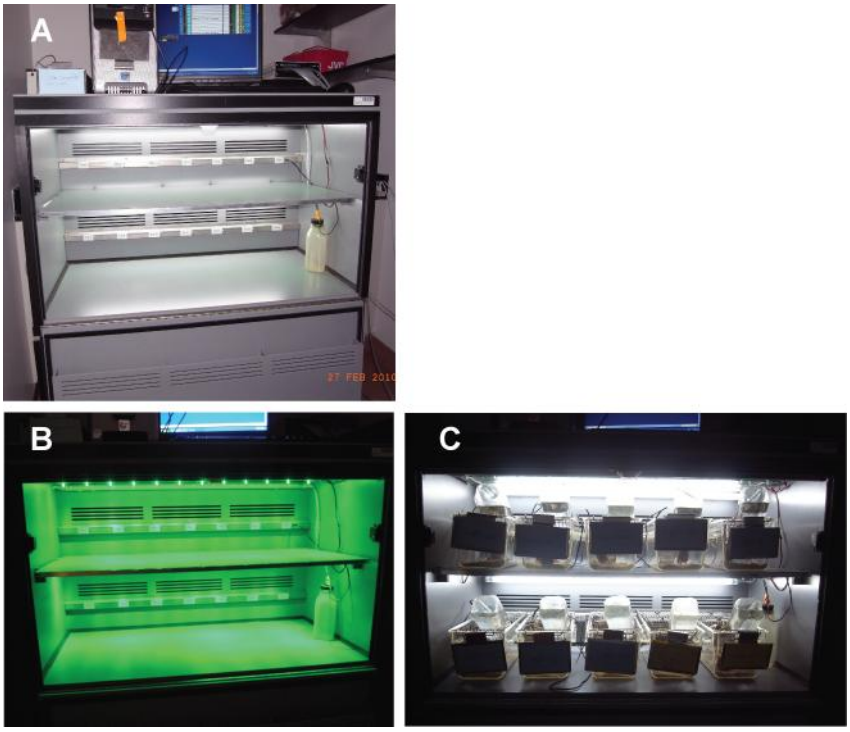

Figure 3- 5 Custom built light box equipped with computer controlled 500 lux fluorescent (A), and green LED lighting system (B). Green LED luminance intensity is adjustable. Computer records lighting status along with up to ten cages per box (C).

ClockLab analysis software settings

Actogram preferences in the ClockLab Analysis software were set as the following:

Onset fit: Off 6 hr; On 6 hr

Quantiles: 5

Bouts: Max gap 10 minutes; Threshold 5 cnt/min

Perfusion and tissue preparation

Mice were anesthetized with a mixture of Xylene and Ketamine followed by intracardiac perfusion with 0.9% DEPC-treated saline until saline flowing through the left atrium became clear. Then, perfusion continues with 4% paraformaldehyde (PFA) solution in DEPC-treated PBS for 3 minutes. Brains with one centimeter of spinal cord attached were removed and placed in 50 mL conical tubes containing ice cold 4% PFA for postfixation in a 4 degree refrigerator on a rocker overnight. A 30% sucrose solution replaces the fixative the next day for cryoprotection. Cryoprotected brains were sliced 35 microns thick on a Lica sliding microtome and collected in four parallel series in PBS. Each series were stored in screw cap tubes with antifreeze solution in minus 20 freezer until use.

IHC Conditions

Experimental conditions for IHC

Antigen	Primary Ab Name	Primary Ab Concentration	2ndary Ab Name	2ndary Ab Concentration	Staining format
AVP-NP	PS41 (Gainer lab)	1:25	Invitrogen Alexa conjugated goat anti-rabbit IgG	1:300	on slide
VIP	ImmunoStar VIP-Ab 20077	1:100	Invitrogen Alexa conjugated goat anti-rabbit IgG	1:300	on slide
GFP/YFP	Invitrogen A6455 Rabbit Anti-GFP AB	1:5000	Invitrogen Alexa conjugated goat anti-rabbit IgG	1:300	on slide
NMS	ABR custom ab	ND 1:1000 - 1:10000	HRP conju-gated goat anti-rabbit IgG	ND 1:600	floating

Blocking solution contains 3% normal goat serum and 0.3% Triton-X100 in PBS.
Primary staining solution contains 3% normal goat serum, 0.3% Tween-20, antibody in PBS
Secondary staining solution contains secondary antibody in PBS. Custom synthesized peptides used for raising custom antibodies against NMS were
NMS-1-1 LPRLLRLDSRMATVDFPKKC
NMS-1-2 LPRLLRLDSRMATVDFPKKDPTTSLGRC Cystein added at C-terminus.
ND, not detected in SCN region

Table 3- 2 Immunohistochemistry conditions

In situ hybridization

Probe cloning

A 400-1100 bp PCR fragment was sub-cloned into pSP72 (Promega, Inc.), pBluescript, or pGEM-Teasy vectors. The T7 promoter was used for anti-sense probe and either T3 or SP6 promoter for sense probe. Sub-cloned probe sequence was confirmed by DNA sequencing.

Ribo-probe labeling

Sixty micrograms of sub-cloned vectors were cut by restriction enzyme digestion, purified by phenol/chloroform extraction, followed by ethanol precipitation. Precipitated cut vectors were resuspended in DNase-free water. Probes were labeled by in vitro transcription (MAXIscript, Ambion, Inc). Unlabeled S35 nucleotides were eliminated using Sephadex column (Roche Applied Science). Purified labeled probes were confirmed by counting in scintillation counter, followed by diagnostic PAGE analysis.

Hybridization procedures were performed by staff of Yanagisawa laboratory histology core.

Hybridized slides were exposed to phosphor screen and read by phosphorimager (Typhoon, GE Diagnostics) before slides were dipped in liquid film emulsion.

Image data collection and analysis

Microscopy images were collected on Nikon (Eclipse 80i) with attached CCD camera to a PC running Nikon NIS-Elements software. Images were analyzed on separate PC running Adobe Photoshop CS4 Extended.

Statistical analysis

Statistical analyses were performed using Graphpad Prism software package.

Chapter Three References

Bendtsen, J. D., H. Nielsen, et al. (2004). "Improved prediction of signal peptides: SignalP 3.0." J Mol Biol **340**(4): 783-795.

Copeland, N. G., N. A. Jenkins, et al. (2001). "Recombineering: A powerful new tool for mouse functional genomics." Nature Reviews Genetics **2**(10): 769-779.

Mori, K., M. Miyazato, et al. (2005). "Identification of neuromedin S and its possible role in the mammalian circadian oscillator system." Embo J **24**(2): 325-335.

Shimshek, D. R., J. Kim, et al. (2002). "Codon-improved Cre recombinase (iCre) expression in the mouse." Genesis **32**(1): 19-26.

Tong, Q., C. P. Ye, et al. (2008). "Synaptic release of GABA by AgRP neurons is required for normal regulation of energy balance." Nat Neurosci **11**(9): 998-1000.

CHAPTER FOUR
Results

THE ROLE OF INTRA-SCN NETWORKING IN BEHAVIORAL CIRCADIAN RHYTHM

The main circadian clock is located in the suprachiasmatic nucleus (SCN) in mammals. Together, the 20,000 neurons that make up the SCN temporally organize behavior into circadian cycles of activity and rest. Animals with large lesions of the brain that eliminate the SCN cannot maintain circadian rhythm (Moore and Eichler 1972; Stephan and Zucker 1972). Conversely, a small portion of the SCN is known to be sufficient to drive behavioral circadian rhythm (Harrington, Rahmani et al. 1993). A cell autonomous circadian oscillation is mediated by a group of core clock genes that form a self regulated transcription-translation feedback loop (Lowrey and Takahashi 2004). Among the available core clock gene knockout mice, the only single-gene knockout that confers arrhythmic behavior is the Bmal1 knockout (Ko and Takahashi 2006).

Most of the SCN neurons have circadian rhythm in gene expression and neuronal activity (Welsh, Logothetis et al. 1995; Honma, Shirakawa et al. 1998; Yamaguchi, Isejima et al. 2003). Even though individual SCN neurons oscillate with different period, phase, and amplitude, when dissociated from each other (Honma, Shirakawa et al. 1998; Aton and Herzog 2005), behavioral circadian rhythm is consolidated and exhibit a period that reflect the mean periods of individual SCN neurons. This is has been demonstrated in clock mutant chimera animals that express a mixture of wild type and clock mutant cells within an intact SCN (Low-Zeddies and Takahashi 2001). Therefore, it has been

speculated that the SCN neurons require a synchronization mechanism in order to send out a united signal and function as the master circadian pacemaker.

The γ-aminobutyric acid (GABA) is a classical inhibitory neurotransmitter widely distributed in the central nervous system. GABAergic neurotransmission is initiated by the release of pre-packed GABA from synaptic vesicles. Vesicular GABA Transporter (VGAT) is the only known transporter for the filling of vesicles at GABAergic synapse (McIntire, Reimer et al. 1997; Sagne, ElMestikawy et al. 1997). Complete loss of VGAT results in embryonic lethality (Wojcik, Katsurabayashi et al. 2006). Essentially all SCN neurons are GABAergic (Moore and Speh 1993; Belenky, Yarom et al. 2008), and GABA has long been a candidate inter-neuronal synchronization factor of the SCN (Ralph and Menaker 1989; Gillespie, Mintz et al. 1997; Wagner, Castel et al. 1997; Liu and Reppert 2000; Albus, Vansteensel et al. 2005).

A genetic approach was carried out to uncover what it takes for SCN neurons to function as a circadian pacemaker. I developed a new transgenic mouse line, named NMS-Cre, that expresses the Cre recombinase in the SCN neurons. By crossing the NMS-Cre line to iDTR, floxed Bmal1, and floxed VGAT, it was demonstrated, respectively: 1) diphtheria toxin (DT)-mediated ablation of a majority of SCN neurons results in an inducible disruption of behavioral circadian rhythm, 2) in contrast, lack of Bmal1 in a majority of SCN does not result in arrhythmic behavior, and 3) removal of GABAergic transmission in a majority of SCN neurons does not alter behavioral circadian rhythm.

Results

Novel NMS-Cre BAC transgenic mouse expresses Cre recombinase in similar pattern compared to endogenous NMS expression

In order to access the expression pattern of functional Cre recombinase in the new NMS-Cre mouse line, NMS-Cre line was crossed to ROSA26-YFP reporter line to produce animals that contain both alleles. Expression of yellow fluorescent protein (YFP) is mediated by the presence of the Cre recombinase that removes the lox-STOP-lox sequence between the ROSA26 promoter and the YFP expression cassette. Animals harboring both alleles are referred to as NMS-Cre(+);R26YFP. Adult NMS-Cre(+);R26YFP animals between the ages of 8 to 16 weeks old were killed and tissues from brain, heart, lung, liver, kidney, adrenal, spleen, large intestine, and small intestine were collected and examined by fluorescent histological analysis for the presence of YFP signals. In peripheral tissues, YFP signals were detected in a part of adrenal medulla, some moncytes in the spleen, and some neuroendocrine cells of intestinal epithelium (Figure 4- 1). No signals were detected in the heart, lung, liver, and kidney (data not shown). In the brain, signals were detected mainly in the SCN (Figure 4- 2) and scarcely in other parts of the hypothalamus such as PVN, DMH, ARC, and AHA (data not shown). The only area detected outside of the hypothalamus is SFO (data not shown). In general, NMS-Cre expression pattern matches that of the endogenous NMS.

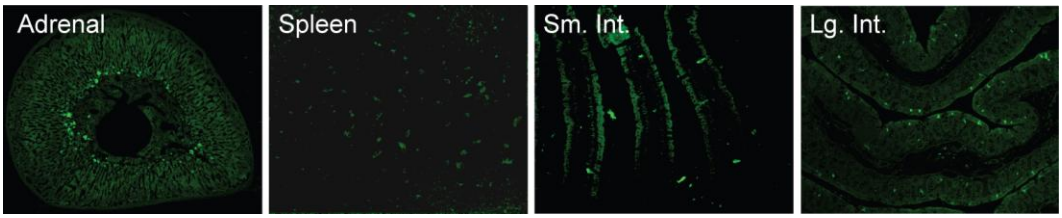

Figure 4- 1 Peripheral YFP fluorescence in NMS-Cre(+);ROSA26-YFP animal was detected in a part of adrenal medulla, some monocytes in the spleen, and some neuroendocrine cells of intestinal epithelium. No signal was detected in the heart, lung, liver, and kidney (data not shown).

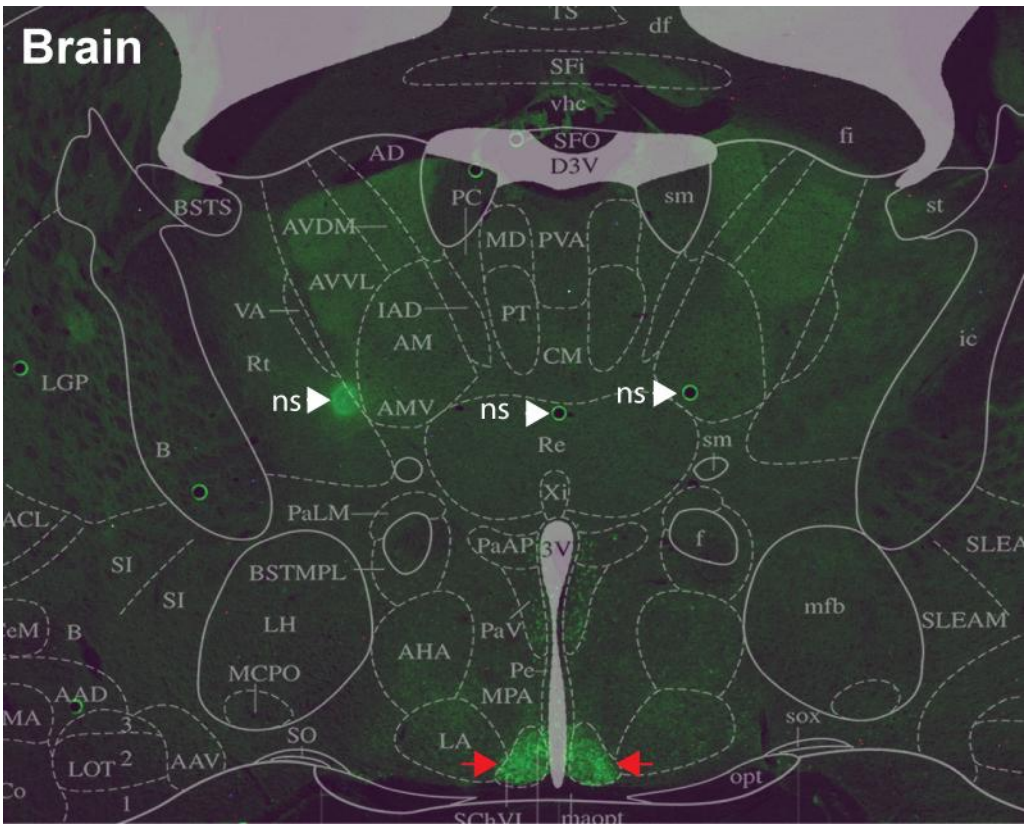

Figure 4- 2 Coronal sections of NMS-Cre(+); R26YFP mouse brains show YFP fluorescence exclusively in the SCN (indicated by red arrows). Ns, non-specific, background fluorescence.

A detailed immunohistochemistry analysis covering rostral to caudal portion of the SCN was performed to assess the cumulative SCN coverage by NMS-Cre because SCN is known to be a heterogeneous structure and very little tissue of the SCN can drive behavioral circadian rhythm. Double immunohistochemistry staining of NMS-Cre(+);R26YFP SCN with YFP/AVP or YFP/VIP show complete coverage of NMs-Cre mediated YFP staining extending from the rostral to caudal portions of the SCN (Figure 4- 3). Detailed cell count shows YFP staining co-localized with 94% of AVP-ir neurons and 98% of VIP-ir neurons in the medial coronal section of the SCN. Over 75% of cells with DAPI stained nuclei were also positive for YFP. There were no YFP positive neurons detected in reporter animals without the NMS-Cre transgene (data not shown).

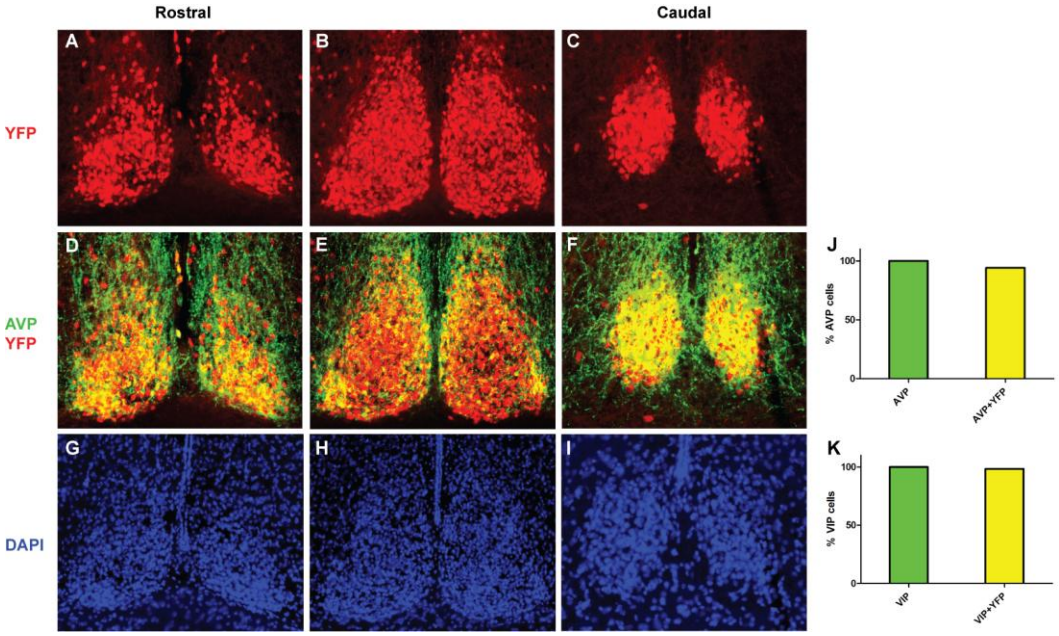

Figure 4- 3 Rostral to caudal SCN regions of the NMS-Cre(+);R26YFP were completely covered by YFP staining (A-C). Double immunohistochemistry show complete co-localization of NMS-Cre mediated YFP expression with established SCN markers AVP (D-F, and J), and VIP (K). DAPI staining of the corresponding regions are shown in panels (G-I).

Inducible ablation of SCN neurons in NMS-Cre(+);iDTR mice results in loss of behavioral circadian rhythm

In order to confirm that a NMS-Cre mediated genetic manipulation is effective, NMS-Cre line was crossed with iDTR, a mouse that carries a Cre inducible diphtheria toxin receptor (DTR) expression cassette at the ROSA26 locus. The wild type house mouse that does not carry DT receptor and can tolerate one thousand fold higher DT concentrations than DTR expressing mice (Cha, Chang et al. 2003). In NMS-Cre(+);iDTR cells that have an activated iDTR cassette, injection of DT inactivates the elongation factor 2 and shuts down cellular translational machinery. Animals were subjected to the experimental scheme illustrated in (Figure 4- 4). Wheel-running activity records indicate that NMS-Cre(+);iDTR animals entrain normally to 12-hour light and 12-hour dark cycles, where consolidated wheel-running period are restricted to the dark cycle. Upon releasing to constant dark condition, NMS-Cre(+);iDTR animals begin to free-run with an expected phase onset, a normal period, and with normal amplitude. When DT is injected, NMS-Cre(+);iDTR animals maintains its total activity level. However, they became active during the next subjective rest period, as measured by the advancement of activity onset time within twenty-four hours post DT injection. Fast Fourier Transform (FFT) power spectrum density analysis shows that NMS-Cre(+);iDTR animals injected with DT has a significant ($P < 0.0004$) decrease in free-running amplitude. Although, Chi square periodogram detected periods within the 22-27 hour range for these animals, their amplitude is significantly ($P < 0.0001$) lower. FFT periodicity detected highest peaks in the ultradian range. These data are compiled in (Table 4- 1).

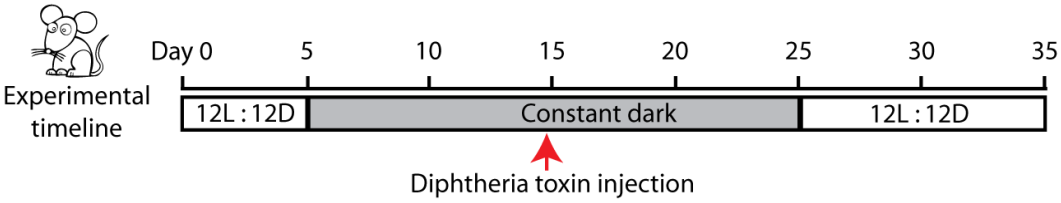

Figure 4- 4 DT experiment timeline

Table 1. Effect of DT on phenotypic characteristics of NMS-Cre;iDTR mice

	NMS-Cre(+);iDTR		NMS-Cre(-);iDTR		
Parameters	Pre DT	Post DT	Pre DT	Post DT	P Value
Free Running period ChiSq (hr)	23.68 ± 0.09	nd	23.76± 0.06	23.73 ± 0.06	ns
Period Amplitude	1406 ± 47.59	686 ± 97.15	1409 ± 62.57	1528 ± 48.25	<0.0001
FFT circadian PSD	184.4 ± 7.4	49.82 ± 17.34	183.6 ± 13.38	222.1 ± 14.17	0.0004
Activity level (Wheel rev cpm)	23.96 ± 0.89	25.21 ± 1.78	24.94 ± 0.78	25.05 ± 0.01	ns
Phase angle of entrainment (hr)	0.299 ± 0.106	4.054 ± 0.887	0.301 ± 0.161	0.609 ± 0.227	0.0007
Phase angle (hr) - 1 day post DT	n/a	5.253 ± 1.428	n/a	0.161 ± 0.069	0.02

Values presented as mean ± SEM; n = 8 for NMS-Cre(-);iDTR, n = 7 for NMS-Cre(+);iDTR. Pre DT, before DT injection. Post DT, intraperitoneal injection of 1ug of diphtheria toxin dissolved in PBS. ChiSq, Chi square periodogram. FFT, fast Fourier transform. PSD, power spectrum density. Cpm, counts per minute. ns, not significant, P >0.05. n/a, not applicable. Statistical analysis performed in Graphpad Prism using Kruskal-Wallis test.
Free running periods before DT injection were similar between WT and NMS-Cre(+) animals.
After DT injections NMS-Cre;iDTR animals began unconsolidated wheel running during the next subjective rest period and were not able to maintain consolidated circadian rhythm. nd, Chi square periodogrma detected period within 22-27 hour range, their amplitudes are significantly lower in the NMS-Cre postive post DT group. FFT periodocity detected highest peaks in the 6-143 hr ultradian range for NMS-Cre positive after DT injection. $WT^{iDTR/+}$ were not affect by DT injection.

Table 4- 1 Effect of DT on phenotypic characteristics of NMS-Cre;iDTR mice

Interestingly, when resubmitted to the 12-hour light and 12-hour dark cycles, the arrhythmic post DT NMS-Cre(+);iDTR animals exhibit consolidated wheel-running activity only in the dark period. Anterograde tracing via intravitreous injection of cholera toxin B fragment indicate that retina innervations to multiple brain centers that are responsible for light-induced activity masking is intact in (data not shown). These data suggests that DT ablation did not affect the light input pathway, and that the activity masking by light is preserved even in animals without a SCN that can maintain

behavioral circadian rhythm. The extent of DT ablation in the SCN and actograms are shown in (Figure 4- 5). The extent of SCN ablation was assessed by immunohistochemistry staining of AVP. The majority of the AVP cell body and fiber staining seen in wild type animals were largely missing in the ablated SCN. AVP staining in the paraventricular nucleus had no noticeable change. DAPI staining of the ablated SCN area shows an identifiable nucleus; however, the structure appeared less defined than wild type animal (data not shown). The effectiveness of DT ablation was further accessed by YFP staining of a DT injected mouse harboring NMS-Cre, iDTR, and R26YFP alleles. Cell counting of remaining YFP expressing cells reveals that about 15% of NMS-Cre positive neurons survive the DT treatment. The surviving neurons do not appear to localize in any specific sub-region of the SCN (Figure 4- 6). Taken together, NMS-Cre dependent DT ablation reveals that an established SCN neuronal network is essential for coherent behavioral circadian rhythm.

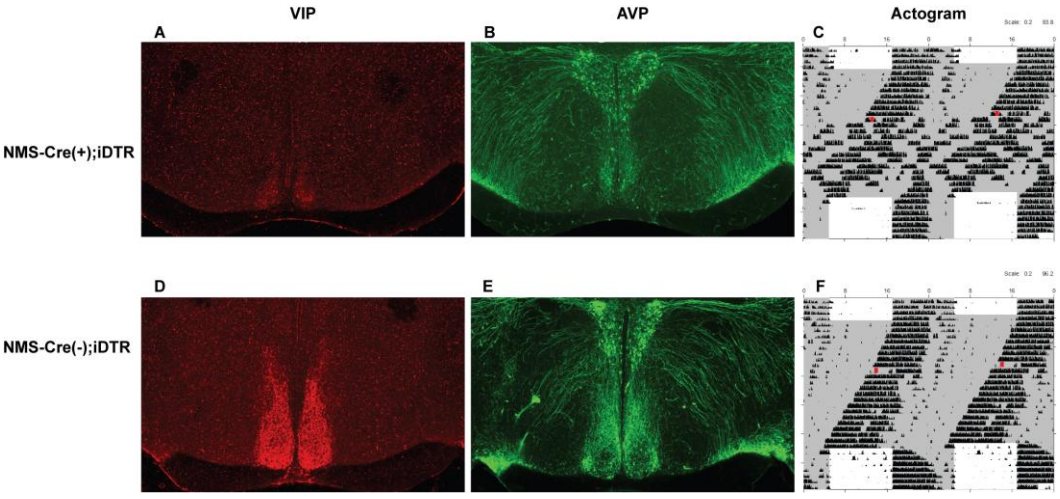

Figure 4- 5 Loss of behavioral circadian rhythm in NMS-Cre(+);iDTR mice upon DT injection. VIP and AVP immunohistochemistry of two DT injected animals showed that DT ablated a majority of VIP and AVP positive neurons only in NMS-Cre positive animal (A, B, D, and E). Brains were collected between ZT6 – ZT10,

peak time for AVP protein expression. Panels C and F are double plotted wheel-running actograms: grey areas indicate light off, and white areas indicate light on. Both genotypes entrained normally to 12 hr light: 12 hr dark cycles and showed normal free run rhythm upon releasing into constant dark condition. Red filled squares indicate the time of DT injection. Upon DT injection, the NMS-Cre(+); iDTR mice became arrhythmic. When LD cycle is reinstated, DT injected NMS-Cre(+);iDTR mice became active only in the dark phase. DT has no effect on the behavioral rhythm of NMS-Cre(-); iDTR animal.

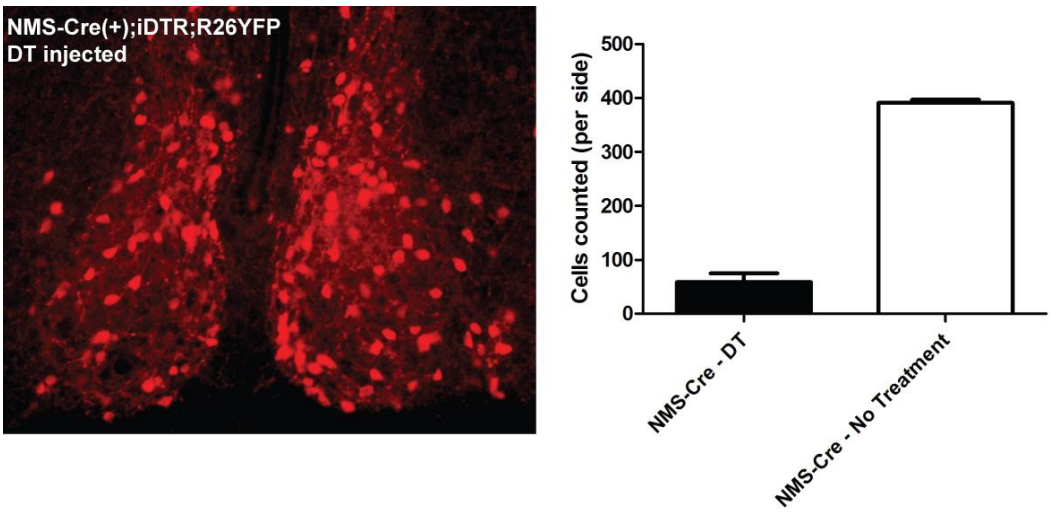

Figure 4- 6 An animal carrying the NMS-Cre, iDTR, and R26YFP transgenes was injected with DT to induce SCN ablation. Compared to animals with NMS-Cre and R26YFP loci, about 15% of the YFP positive SCN neurons were detected in the DT injected animal. Survived YFP neurons appeared to be randomly distributed throughout the SCN.

NMS-Cre(+);Bmal1$^{Flox/Flox}$ animals maintain behavioral circadian rhythm

Among core clock gene knockout animals, Bmal1 (also known as MOP3) knockout is the only single-gene gene knockout animal that results in arrhythmic behavior. Bmal1/Clock heterodimer target genes, mPer1 and mPer2, do not oscillate in SCN or liver of Bmal1 knockout animal (Bunger, Wilsbacher et al. 2000). One well

known caveat of total body Bmal1 knockout animal in behavioral circadian rhythm study is their multiple physiological defects (Rudic, McNamara et al. 2004; Kondratov, Kondratova et al. 2006; Sun, Yang et al. 2006). Since NMS-Cre covers the majority of SCN neurons, a conditional knockout of Bmal1 gene in the majority of SCN would result in a SCN without much of the oscillating core clock, but keep the Bmal1 dependent gene expression pattern in the rest of central and peripheral organs intact. In contrast to the previous DT ablation experiment, NMS-Cre(+);Bmal1$^{flox/flox}$ animals would have a properly networked SCN with functional neurons. Wheel-running experiments showed that NMS-Cre(+);Bmal1$^{flox/flox}$ animals entrain normally to 12-hour light and 12-hour dark cycles. When released into constant dark environment, NMS-Cre(+);Bmal1$^{flox/flox}$ animals exhibit a robust free-running rhythm with a tau that is similar to its littermates with NMS-Cre(-) or Baml1$^{flox/+}$ genotypes (Figure 4- 7and Table 4- 2). NMS-Cre(+);Bmal1$^{flox/flox}$ animals also have wheel-running activity level that is not significantly different from control littermates. Interestingly, NMS-Cre(+);Bmal1$^{flox/flox}$ animals have a slight, but significantly advanced phase onset upon released into constant dark condition. This advancement suggests a possible altered synchronization property among SCN neurons. An ISH experiment has been planned to examine the efficiency of Bmal1 knockdown at the SCN.

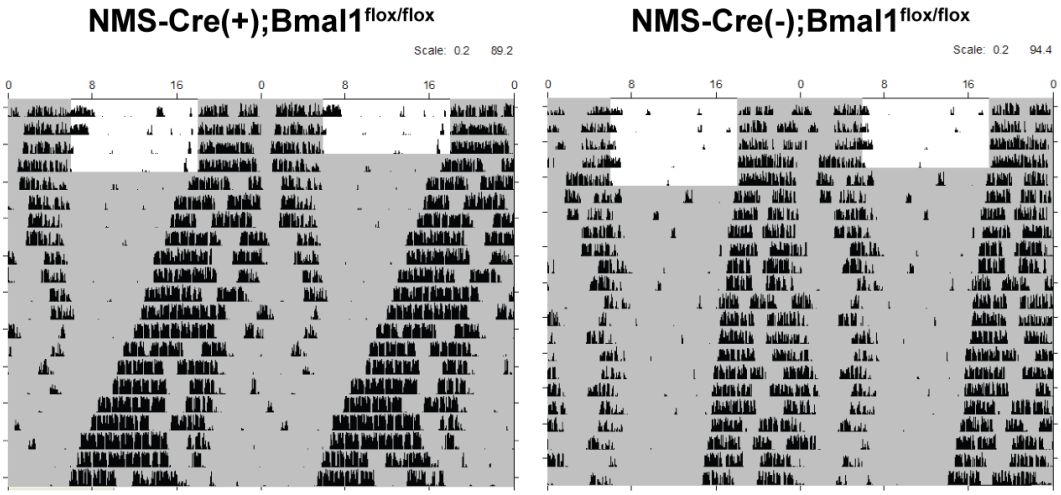

Figure 4- 7 Double plotted wheel-running actograms: grey areas indicate light off and white areas indicate light on. Compiled data analysis of all tested animals is shown in table 2 below. Both genotypes entrain normally to 12 hr light: 12 hr dark cycles. NMS-Cre(+); Bmal1$^{flox/flox}$ animals have slight advancement in initial phase of free-running rhythm upon releasing to constant dark condition.

Table 2. Phenotypic characteristics of NMS-Cre; Bmal1^{flox} mice

Parameters	NMS-Cre(+);Bmal1^{flox}	NMS-Cre(-);Bmal1^{flox}	P Value
Free running period ChiSq (hr)	23.38 ± 0.10	23.73 ± 0.04	ns
Activity level (Wheel rev cpm)	20.11 ± 1.24	12.43 ± 3.48	ns
Phase advancement - DD release (hr)	1.080 ± 0.197	0.252 ± 0.140	0.0283

Values presented as mean ± SEM; In Chisq analysis n = 4 for each genotype. In phase advancement experiment, n = 8 for NMS-Cre(+);Bmal^{flox} and n=4 for NMS-Cre(-);Bmal^{flox}. ChiSq, Chi square periodogram. Cpm, counts per minute. DD, release to constant dark. Statistical analysis by Mann Whitney test. ns, not significant, P<0.05.

Table 4- 2 Phenotypic characteristics of NMS-Cre;Bmal1flox

GABAergic neurotransmission in the NMS-Cre neurons is not necessary for maintenance of behavioral circadian rhythm

Nearly every SCN neuron is GABAergic. GABA signaling has been speculated to be involved in proper pacemaker functions. However, experiments that can directly test the involvement of GABA transmission in behavior are either limited by the precision of microinjection or confounded by off-target effects as the results of widespread GABA receptors. NMS-Cre;VGAT$^{flox/flox}$ animals were generated to test if

behavioral circadian rhythm can be maintained in animals which the majority of its SCN neurons lack GABA in its synaptic vesicles. Coronal sections of NMS-Cre;VGAT$^{flox/flox}$ brain subjected to in situ hybridization confirms that VGAT expression were knockout in a majority of SCN neurons (Figure 4- 8; A-C). NMS-Cre;$^{VGATflox/flox}$ animals entrained normally to 12-hour light and 12-hour dark cycles, free-ran with a normal period, and phase advanced and delayed in response to six hours of light pulse in the same fashion as control littermates (Figure 4- 8; D-F, andTable 4- 3). Every behavioral circadian parameter was normal in NMS-Cre;$^{VGATflox/flox}$ animals. Therefore, GABAergic neurotransmission in the majority SCN neurons is not essential for proper pacemaker functions.

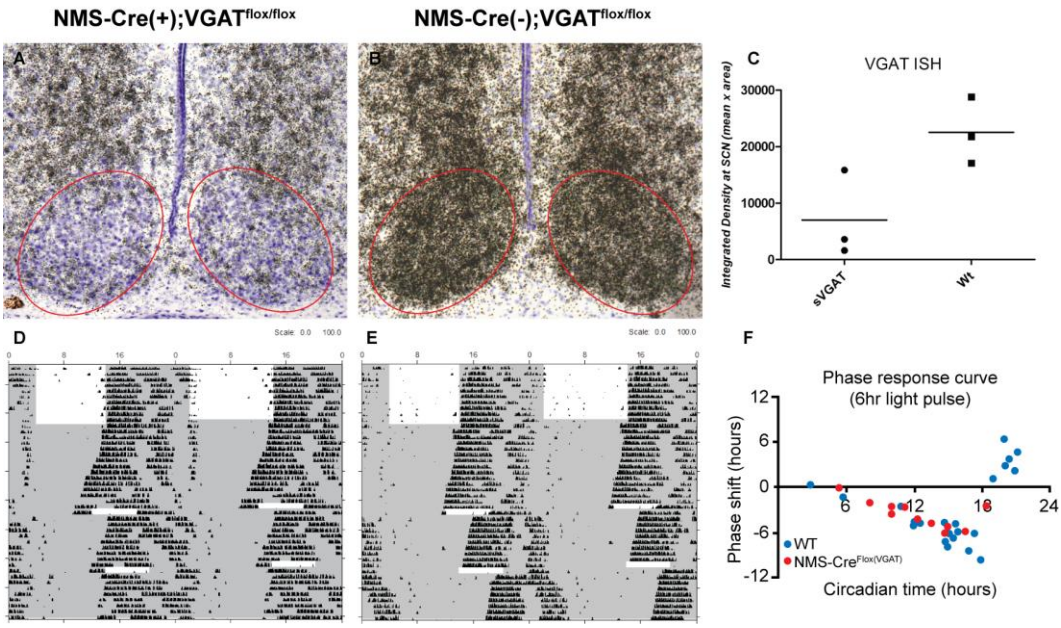

Figure 4- 8 Animals without GABAergic neurotransmission in the SCN have normal behavioral circadian rhythm. In situ hybridization of SCN with 35S-labeled VGAT riboprobe in panels A (NMS-Cre(+); VGAT$^{flox/flox}$) and B (NMS-Cre(-); VGAT$^{flox/flox}$) show dramatic decrease of VGAT expression, specifically in the SCN, indicated by red circles. Densitometry measurements of three consecutive sections

containing the SCN region are graphed in C. Double plotted actograms of animals subjected to 12L:12D, constant darkness, and 6 hour light pulse are presented in D and E. NMS-Cre(+); VGAT$^{flox/flox}$ animal phase-delay normally in response to light pulse. Preliminary PRC of littermates from both genotypes are plotted in F.

Table 3. Phenotypic characteristics of NMS-Cre;VGAT^{flox} mice

Parameters	NMS-Cre(+);VGAT^{Flox}	NMS-Cre(-);VGAT^{Flox}	P Value
Free running period ChiSq (hr)	23.90 ± 0.08	23.78 ± 0.05	ns
Period amplitude	1520 ± 175.5	1852 ± 93.1	ns
Activity level (Wheel rev cpm)	13.71 ± 2.59	17.31 ± 0.93	ns
Phase angle of entrainment (hr)	0.618 ± 0.214	0.365 ± 0.179	ns

Values presented as mean ± SEM; n = 4 for NMS-Cre(+);VGAT^{Flox}; n = 8 for NMS-Cre(-);VGAT^{Flox} and all intact SCN VGAT animals. ChiSq, Chi square periodogram. Cpm, counts per minute. ns, not significant.

Table 4- 3 Phenotypic characteristics of NMS-Cre;VGAT^{Flox}

Discussion

The first goal of this project was to construct a SCN-specific Cre driver mouse line for the study of circadian pacemaker functions. To my knowledge, gained from attending multiple rhythm society meetings and in consultation with other major circadian researchers in the field, no SCN-specific Cre mouse line has been reported. SCN specificity is important for insightful studies of circadian function because SCN is currently the only known nucleus in the brain that is necessary and sufficient to drive circadian behavior rhythm (Moore and Eichler 1972; Stephan and Zucker 1972; Ralph, Foster et al. 1990). And since every cell in the body possess the machinery to potentially become clock cells, more importantly, nucleus such as DMH has been shown to oscillate by non-photic stimuli (Gooley, Schomer et al. 2006; Mieda, Williams et al. 2006), a SCN-specific Cre driver can be a useful tool to genetically dissect circadian pacemaker

functions and enable ever expanding behavior studies in fields affected by circadian physiology.

NMS-Cre driver mouse

With that goal in mind, I generated a BAC transgenic mouse line that specifically expresses iCre at the SCN. ROSA-YFP reporter shows that Cre recombinase expression covers the majority of SCN in percentage similar to the most abundant SCN neurotransmitter, GABA. NMS-Cre driven YFP staining completely co-localized with AVP and VIP. While it is known that very few SCN neurons are required to maintain a behavioral circadian rhythm (Harrington, Rahmani et al. 1993), the complete and specific SCN coverage of the SCN-iCre driver mouse was a compelling reason to go ahead with generating a mouse line sensitive to genetic ablation of the SCN. Although this began purely as a positive control experiment to show the sufficient iCre coverage, its success would be another first in the circadian field. Until now, only SCN enriched drivers are available. These so-called "enriched" drivers are not suited for genetic ablation studies because they are widespread in the CNS or even throughout the periphery. Therefore, ablation strategy of delivering toxin to sensitized cells would cause extensive non-specific effects that could make results difficult to interpret.

Genetic ablation of SCN neurons

There are two available mouse lines with abundant reports of CNS ablation experiments reported: ROSA-DTA (Ivanova, Signore et al. 2005) and the iDTR (Buch, Heppner et al. 2005). I decided to breed the NMS-IRES2-iCre line to both because they

had disparate attractiveness in subsequent experimental design for behavior characterization. The ROSA26-DTA line carries a flox-stop-flox DTA cassette in the ROSA26 locus. Neurons expressing Cre recombinase begin to produce toxin as soon as the NMS-Cre is expressed. I had thought that this line would allow simpler experimental design and provide cleaner data for analysis. Unfortunately, the cross produced a very low ratio of pups that carry both the NMS-Cre and ROSA-DTA locus. This suggested that NMS expressing cells might be required for normal physiological development. The very few surviving animals had less robust free-run rhythm and one animal exhibited large phase-shift when pulsed by light. I ultimately abandon this line because of the low yield in obtaining double positive animals and concern that the few surviving animals might be the results of an altered NMS-Cre expression pattern.

The advantage of the iDTR line was that animals can develop normally and I would have temporal control of when the DT-sensitive cells were ablated. For behavior studies, I was able to establish baseline activity records prior to toxin administration and use the same individual as control. The NMS-Cre(+);iDTR line bred well and had a ratio that matches Mandelian inheritance. These mice have the same baseline free-run rhythm in constant darkness as their NMS-Cre(-);iDTR littermates. When received an IP injection of diphtheria toxin (DT), the iCre positive iDTR animals became active in a non-consolidated fashion during the next subjective day and their activity pattern became arrhythmic. DT administered animals in both groups appeared healthy and maintained similar total amount of wheel-running activity. DT disrupts a cell's translational machinery, but affected cells die over a period of a few days (Gropp, Shanabrough et al.

2005). The rapid behavior change suggests that protein synthesis disruption in the SCN neurons immediately causes the animal to lose their timing acuity.

One caveat of the iDTR strategy is the variable in toxin delivery to sensitized neurons. Current data showed that some Cre recombinase positive neurons survived even after two rounds of injections. This result is consistent with others that use the iDTR line (Gropp, Shanabrough et al. 2005; Hatori, Le et al. 2008). These remaining SCN neurons might be sufficient to either partially maintain or reestablish activity rhythm at a different phase and/ or period. A study has been planned to investigate whether the remaining number of Cre positive neurons correlate with the extent of free-run disruption. It is important to point out that DT had no effect on NMS-Cre(-);iDTR animals. After DT administration, NMS-Cre(-);iDTR animals maintained all circadian activity parameters such as phase, period, and robustness that were established prior to DT administration. Current data indicates that NMS-Cre line covers sufficient portion of the SCN that can disrupt normal circadian pacemaker properties and results in arrhythmic behavior in constant darkness.

NMS-Cre(+);Bmal1$^{flox/flox}$ maintains behavioral circadian rhythm

Animals without the Bmal1 gene cannot maintain behavioral circadian rhythm (Bunger, Wilsbacher et al. 2000). Bmal1 dependent core clock genes do not oscillate in SCN of Bmal1 KO animals. Therefore, NMS-Cre(+);Bmal1$^{flox/flox}$ animals that free-run with a normal period was a surprised finding. Although period was normal, NMS-Cre(+);Bmal1$^{flox/flox}$ animals do have two circadian parameters that are subtly different from their control littermates. First, NMS-Cre(+);Bmal1$^{flox/flox}$ have a significantly

advanced phase angle upon released into constant dark. Second, NMS-Cre(+);Bmal1$^{flox/flox}$ animals have a higher amplitude than its control littermates.

The advanced phase angle finding is consistent with the phenotypic characteristic of Bmal1 KO mouse (Bunger, Wilsbacher et al. 2000). In that report, the authors pointed out that Bmal1 KO mouse have altered activity on light-dark cycles and two out of seventeen analyzed KO animals failed to entrain altogether. The milder phenotype is likely resulting from the presence of SCN neurons without Cre expression. Since the NMS-Cre lesion experiments suggest that SCN neuronal networking is important for maintenance of behavioral circadian rhythm and SCN neurons are thought to synchronize with each other, these data suggest that the NMS-Cre(+) neurons, which are supposedly lacking Bmal1 and thus non-oscillatory cell-autonomously, can still serve as a conduit for inter-neuron synchronization within SCN. Current data cannot exclude a possible extra-SCN effects that can rescue behavioral rhythm from a SCN without a clock.

In order to explain how a difference in amplitude can be obtained from animals behave with similar periods, one can compare the NMS-Cre(+);Bmal1$^{flox/flox}$ animals to Clock mutant chimeras. Principal component analysis performed on Clock mutant chimeras shows that measures of circadian period and circadian amplitude vary independently (Low-Zeddies and Takahashi 2001). The NMS-Cre(+);Bmal1$^{flox/flox}$ SCN is likely to beahve similar to one of the intermediate Clock mutant chimera animals where the period of behavioral rhythm is essentially normal, but have highly variable amplitude.

Knocking out GABAergic neurotransmission

With the NMS-Cre line as an enabling tool to specifically manipulate the pacemaker neurons, it is now possible to test pacemaker component functions that affect animal behavior. By collaborating with Drs. Tong and Lowell who have recently made a line of floxed VGAT animals (Tong, Ye et al. 2008), I am able to test the requirement of GABAergic neurotransmission in the SCN.

In situ hybridization data showed that the VGAT expression in the SCN was largely eliminated in the NMS-Cre(+);$VGAT^{flox/flox}$ SCN. These animals maintained free-running activity rhythm similar to their control littermates. NMS-Cre(+);$VGAT^{flox/flox}$ animals have normal phase response curve and have similar activity level compared to their littermates. These data suggest that, in vivo, GABAergic neurotransmission in the NMS-Cre(+) neurons is not required for their behavioral rhythm's entrainment, synchronization, and output.

This finding is consistent with ex vivo slice experiments where rhythmic luciferase expression driven by the Period 2 promoter showed that GABA receptors anatagonism does not affect oscillation of SCN neurons (Aton, Huettner et al. 2006). Although a formal examination of the core clock feedback loop in the NMS-Cre(+);$VGAT^{flox/flox}$ SCN is yet to be done, the largely normal free-running rhythm of the NMS-Cre(+);$VGAT^{flox/flox}$ animal suggests that the NMS-Cre(+);$VGAT^{flox/flox}$ SCN core clock is oscillating with the same period as its wild type littermates. It is known that behavioral free-running rhythm persists even when the core clock oscillation is dampened (Vitaterna, Ko et al. 2006). Recent reports also suggest that a normal behavioral rhythm does not necessarily come from SCN with normal oscillation in gene expression (Pendergast, Friday et al. 2009; Shi, Hida et al. 2010). In order to formally examine the

core clock gene oscillating properties, the NMS-Cre(+);VGAT$^{flox/flox}$ animals are currently being bred to Per2::Luc animals for ex vivo slice experiment.

GABAergic neurotransmission's role in pacemaker neuron synchronization

GABA has been proposed as a possible synchronization factor for SCN neurons because administration of exogenous GABA is sufficient to synchronize dispersed SCN neurons (Liu and Reppert 2000), differentially affect SCN neuronal firing between morning and night time of the day (Wagner, Castel et al. 1997), and cohesive firing in multiple parts of the SCN is disrupted by GABA receptor antagonism (Albus, Vansteensel et al. 2005). However, behavior data of NMS-Cre(+);VGAT$^{flox/flox}$ animals does not support this theory. It is possible that lack of GABA in the SCN can be compensated by other neurotransmitters or the gap junction-mediated electrical coupling. Neither can the current data deny a possible extra-synaptic GABAA receptor pathway (Ehlen and Paul 2009) that might be sufficient in maintaining behavior rhythm. NMS-Cre line would be useful in creating a SCN specific GAD65 and GAD67 deficient animal to genetically test that possibility.

Intercellular coupling had been reported to be a mechanism that confers robustness against clock mutation (Liu, Welsh et al. 2007). In that report, oscillatory properties of either intact or dispersed SCN neurons carrying clock mutations were shown to be distinct. Therefore, suggesting that the intact nature of the SCN explants can compensate for the genetic defects of the molecular clock. This logical deduction is reasonable and can be supported by various preceding reports (Aton and Herzog 2005; Aton, Huettner et al. 2006). However, Liu et al did not present any possible candidate

factors for the proposed coupling mechanism. The current behavior data from NMS-Cre(+);VGAT$^{flox/flox}$ cannot support GABAergic neurotransmission as the sole coupling factor. However, it is possible that a lack of SCN GABAergic neurotransmission could potentiate mild behavioral circadian rhythm phenotypes in the various clock mutants. If so, GABAergic neurotransmission would be the first bona fide factor that enables the pacemaker to resist genetic perturbation of the intracellular clock. The NMS-Cre line could enable future research in the field of the hierarchical SCN driven system biology to delineate pacemaker functions with or without a SCN derived coupling factor.

GABAergic neurotransmission's role as a pacemaker output factor

GABAergic neurotransmission has been postulated as a possible output factor of the circadian pacemaker because nearly all SCN neurons are GABAergic. There are abundant SCN projection to hypothalamic sites involved in control of behavioral and physiological functions (Saper, Lu et al. 2005). GABA receptors have been implicated in the regulation of the pacemaker's response to light (Ralph and Menaker 1989). However, wheel-running behavior of NMS-Cre(+);VGAT$^{flox/flox}$ animals indicate that GABAergic neurotransmission cannot be accounted for as an output factor. It is possible that wheel-running behavior, in particular, is compensated by diffusible or non-synaptic factors. Nevertheless, NMS-Cre(+);VGAT$^{flox/flox}$ animal's ability to phase-shift in response to light pulse suggests, at the very least, GABA receptors in the synapse cannot be responsible for light-induced pacemaker output for wheel-running behavior. GABAergic neurotransmission might still have a role in circadian pacemaker output in that cannot be restored without direct synaptic connection. Various pharmacological experiments

suggest that GABAergic neurotransmission plays a role in circadian pacemaker controlled neuroendocrine functions (Kalsbeek, Cutrera et al. 1999; Kalsbeek, La Fleur et al. 2004; Kalsbeek, Palm et al. 2006; Kalsbeek, Perreau-Lenz et al. 2006). These physiological functions might be where GABA exerts its most potent effect. The NMS-Cre(+);VGAT$^{flox/flox}$ line is the most direct tool for that line of discovery.

In conclusion

Taken together, in vivo data presented in this chapter suggest a model that an intra-SCN neuronal network is required to maintain a consolidated behavioral circadian rhythm (Figure 4- 9). With a properly networked SCN, non-oscillatory neurons can synchronize their neuronal activity to a small population of oscillating SCN neurons and then drive circadian rhythm. Despite the fact that virtually all SCN neurons are GABAergic, GABA is an unlikely transmitter for this intra-SCN networking.

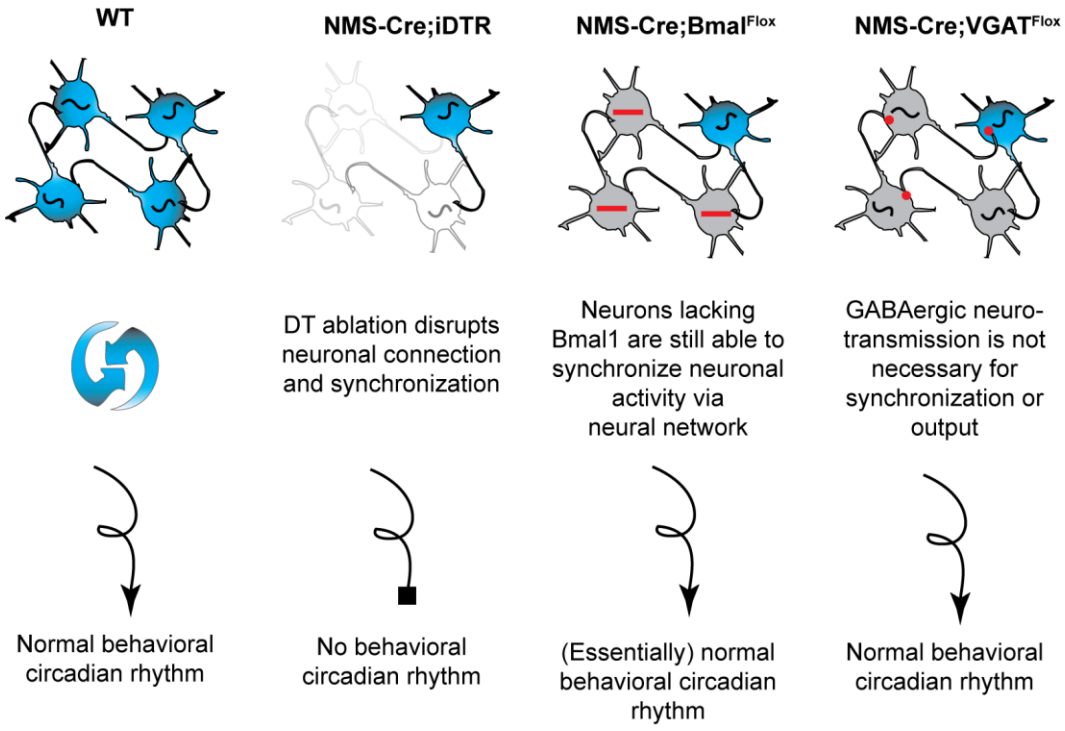

Figure 4- 9 A model of intra-SCN netowrking

Chapter four references

Albus, H., M. J. Vansteensel, et al. (2005). "A GABAergic mechanism is necessary for coupling dissociable ventral and dorsal regional oscillators within the circadian clock." Current Biology **15**(10): 886-893.

Aton, S. J. and E. D. Herzog (2005). "Come together, right...now: synchronization of rhythms in a mammalian circadian clock." Neuron **48**(4): 531-534.

Aton, S. J., J. E. Huettner, et al. (2006). "GABA and G(i/o) differentially control circadian rhythms and synchrony in clock neurons." Proceedings of the National Academy of Sciences of the United States of America **103**(50): 19188-19193.

Belenky, M. A., Y. Yarom, et al. (2008). "Heterogeneous expression of gamma-aminobutyric acid and gamma-aminobutyric acid-associated receptors and transporters in the rat suprachiasmatic nucleus." Journal of Comparative Neurology **506**(4): 708-732.

Buch, T., F. L. Heppner, et al. (2005). "A Cre-inducible diphtheria toxin receptor mediates cell lineage ablation after toxin administration." Nat Methods **2**(6): 419-426.

Bunger, M. K., L. D. Wilsbacher, et al. (2000). "Mop3 is an essential component of the master circadian pacemaker in mammals." Cell **103**(7): 1009-1017.

Cha, J. H., M. Y. Chang, et al. (2003). "Transgenic mice expressing the diphtheria toxin receptor are sensitive to the toxin." Mol Microbiol **49**(1): 235-240.

Ehlen, J. C. and K. N. Paul (2009). "Regulation of light's action in the mammalian circadian clock: role of the extrasynaptic GABAA receptor." Am J Physiol Regul Integr Comp Physiol **296**(5): R1606-1612.

Gillespie, C. F., E. M. Mintz, et al. (1997). "GABA(A) and GABA(B) agonists and antagonists alter the phase-shifting effects of light when microinjected into the suprachiasmatic region." Brain Research **759**(2): 181-189.

Gooley, J. J., A. Schomer, et al. (2006). "The dorsomedial hypothalamic nucleus is critical for the expression of food-entrainable circadian rhythms." Nat Neurosci **9**(3): 398-407.

Gropp, E., M. Shanabrough, et al. (2005). "Agouti-related peptide-expressing neurons are mandatory for feeding." Nat Neurosci **8**(10): 1289-1291.

Harrington, M. E., T. Rahmani, et al. (1993). "Effects of damage to SCN neurons and efferent pathways on circadian activity rhythms of hamsters." Brain Res Bull **30**(5-6): 655-669.

Hatori, M., H. Le, et al. (2008). "Inducible ablation of melanopsin-expressing retinal ganglion cells reveals their central role in non-image forming visual responses." PLoS One **3**(6): e2451.

Honma, S., T. Shirakawa, et al. (1998). "Circadian periods of single suprachiasmatic neurons in rats." Neuroscience Letters **250**(3): 157-160.

Ivanova, A., M. Signore, et al. (2005). "In vivo genetic ablation by Cre-mediated expression of diphtheria toxin fragment A." Genesis **43**(3): 129-135.

Kalsbeek, A., R. A. Cutrera, et al. (1999). "GABA release from suprachiasmatic nucleus terminals is necessary for the light-induced inhibition of nocturnal melatonin release in the rat." Neuroscience **91**(2): 453-461.

Kalsbeek, A., S. La Fleur, et al. (2004). "Suprachiasmatic GABAergic inputs to the paraventricular nucleus control plasma glucose concentrations in the rat via sympathetic innervation of the liver." Journal of Neuroscience **24**(35): 7604-7613.

Kalsbeek, A., I. F. Palm, et al. (2006). "SCN outputs and the hypothalamic balance of life." J Biol Rhythms **21**(6): 458-469.

Kalsbeek, A., S. Perreau-Lenz, et al. (2006). "A network of (autonomic) clock outputs." Chronobiol Int **23**(3): 521-535.

Ko, C. H. and J. S. Takahashi (2006). "Molecular components of the mammalian circadian clock." Hum Mol Genet **15 Spec No 2**: R271-277.

Kondratov, R. V., A. A. Kondratova, et al. (2006). "Early aging and age-related pathologies in mice deficient in BMAL1, the core component of the circadian clock." Genes & Development **20**(14): 1868-1873.

Liu, A. C., D. K. Welsh, et al. (2007). "Intercellular coupling confers robustness against mutations in the SCN circadian clock network." Cell **129**(3): 605-616.

Liu, C. and S. M. Reppert (2000). "GABA synchronizes clock cells within the suprachiasmatic circadian clock." Neuron **25**(1): 123-128.

Low-Zeddies, S. S. and J. S. Takahashi (2001). "Chimera analysis of the Clock mutation in mice shows that complex cellular integration determines circadian behavior." Cell **105**(1): 25-42.

Lowrey, P. L. and J. S. Takahashi (2004). "Mammalian circadian biology: elucidating genome-wide levels of temporal organization." Annu Rev Genomics Hum Genet **5**: 407-441.

McIntire, S. L., R. J. Reimer, et al. (1997). "Identification and characterization of the vesicular GABA transporter." Nature **389**(6653): 870-876.

Mieda, M., S. C. Williams, et al. (2006). "The dorsomedial hypothalamic nucleus as a putative food-entrainable circadian pacemaker." Proc Natl Acad Sci U S A **103**(32): 12150-12155.

Moore, R. Y. and V. B. Eichler (1972). "Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat." Brain Res **42**(1): 201-206.

Moore, R. Y. and J. C. Speh (1993). "GABA is the principal neurotransmitter of the circadian system." Neurosci Lett **150**(1): 112-116.

Pendergast, J. S., R. C. Friday, et al. (2009). "Endogenous Rhythms in Period1 Mutant Suprachiasmatic Nuclei In Vitro Do Not Represent Circadian Behavior." Journal of Neuroscience **29**(46): 14681-14686.

Ralph, M. R., R. G. Foster, et al. (1990). "Transplanted suprachiasmatic nucleus determines circadian period." Science **247**(4945): 975-978.

Ralph, M. R. and M. Menaker (1989). "GABA REGULATION OF CIRCADIAN RESPONSES TO LIGHT .1. INVOLVEMENT OF GABAA-BENZODIAZEPINE AND GABAB RECEPTORS." Journal of Neuroscience **9**(8): 2858-2865.

Rudic, R. D., P. McNamara, et al. (2004). "BMAL1 and CLOCK, two essential components of the circadian clock, are involved in glucose homeostasis." Plos Biology **2**(11): 1893-1899.

Sagne, C., S. ElMestikawy, et al. (1997). "Cloning of a functional vesicular GABA and glycine transporter by screening of genome databases." Febs Letters **417**(2): 177-183.

Saper, C. B., J. Lu, et al. (2005). "The hypothalamic integrator for circadian rhythms." Trends Neurosci **28**(3): 152-157.

Shi, S., A. Hida, et al. (2010). "Circadian Clock Gene Bmal1 Is Not Essential; Functional Replacement with its Paralog, Bmal2." Curr Biol.

Stephan, F. K. and I. Zucker (1972). "Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions." Proc Natl Acad Sci U S A **69**(6): 1583-1586.

Sun, Y. M., Z. H. Yang, et al. (2006). "The mortality of MOP3 deficient mice with a systemic functional failure." Journal of Biomedical Science **13**(6): 845-851.

Tong, Q., C. P. Ye, et al. (2008). "Synaptic release of GABA by AgRP neurons is required for normal regulation of energy balance." Nat Neurosci **11**(9): 998-1000.

Vitaterna, M. H., C. H. Ko, et al. (2006). "The mouse Clock mutation reduces circadian pacemaker amplitude and enhances efficacy of resetting stimuli and phase-response curve amplitude." Proc Natl Acad Sci U S A **103**(24): 9327-9332.

Wagner, S., M. Castel, et al. (1997). "GABA in the mammalian suprachiasmatic nucleus and its role in diurnal rhythmicity." Nature **387**(6633): 598-603.

Welsh, D. K., D. E. Logothetis, et al. (1995). "INDIVIDUAL NEURONS DISSOCIATED FROM RAT SUPRACHIASMATIC NUCLEUS EXPRESS INDEPENDENTLY PHASED CIRCADIAN FIRING RHYTHMS." Neuron **14**(4): 697-706.

Wojcik, S. M., S. Katsurabayashi, et al. (2006). "A shared vesicular carrier allows synaptic corelease of GABA and glycine." Neuron **50**(4): 575-587.

Yamaguchi, S., H. Isejima, et al. (2003). "Synchronization of cellular clocks in the suprachiasmatic nucleus." Science **302**(5649): 1408-1412.

CHAPTER FIVE
Results

THE BEHAVIORAL CIRCADIAN RHYTHM OF NMS KO MOUSE

Summary Abstract

The suprachiasmatic nucleus (SCN) of the hypothalamus contains a self-sustained circadian pacemaker. Its neurons receive photic input via the retinohypothalamic tract, phase-lock the pacemaker with the photic input, and send out a clock signal that influences daily onset of behavioral activity. Neuromedin S (NMS) is a recently identified neuropeptide. A whole-brain expression survey of the mouse NMS transcript by in-situ hybridization (ISH) detected signal exclusively in the SCN. Based on this unique expression pattern, NMS was hypothesized to play a role in circadian physiology.

To determine NMS' function in behavioral circadian rhythms, a NMS-null (KO) mutant mouse line was generated by homologous recombination in ES cells. The NMS KO mice are apparently healthy, and are normal in a battery of metabolic and behavioral screening tests.

NMS KO animals have well-consolidated behavioral rhythm under continuous darkness, suggesting that the oscillatory machinery of the clock genes is intact. However, when subjected to 8-hour photic phase advancement, NMS KO animals shift their activity onset time quicker than their wild type littermates. Consistent with this result, NMS KO animals' internal clock rhythm also adjusts to light cycle advancement in less time than wild type littermates. Expression level of mPer1, a known light-inducible core clock gene, was measured to test whether NMS KO animals have an altered response to

light stimuli. Both ISH and real-time quantitative PCR analysis showed normal induction of mPer1 mRNA in SCN in response to light pulse, suggesting that SCN neurons of NMS KO mice have normal light input connections and respond normally to light.

It is known that an overtly normal behavioral rhythm can be maintained even with reduced oscillation amplitude of the molecular pacemaker. Reduced clock amplitude may also allow large phase shifts in response to light. To test this possibility, the oscillation amplitude of mPer1 and mPer2 expression levels in SCN under continuous darkness was assessed, and found that NMS KO animals have normal amplitudes. To further dissect pacemaker functionality in vivo, a phase-response curve (PRC) was constructed by applying 6-hour light pulses at various times of the day. KO animals' PRC exhibited exaggerated phase shifts near its inflection point, a transition at ~CT17 (the 17th hour of subjective circadian time) where light-induced phase delay changes to phase advancement.

Constant light condition is known to disrupt or lengthen free-running period (tau) to longer than twenty-four per cycle in wild type mouse, but return to shorter than twenty-four hours after returning to constant darkness. NMS KO animals also exhibited lengthening of the tau in constant light; however, their tau did not return to the average tau of the NMS KO animals that were not exposed to constant light.

Taken together, current data suggest that NMS has a unique role in keeping the faithfulness, or "stubbornness," of the circadian pacemaker to an established light cycle and fortifying its resistance to perturbation. To my knowledge, the NMS-null mouse is the first example showing rapid entrainment to new light cycle while maintaining a robust internal rhythm under continuous darkness. The current conclusions implicate the

NMS neuropeptidergic pathway as a possible pharmaceutical target for treatment of circadian-related disorders of sleep/wake often caused by the round-the-clock lifestyle of modern human society.

Results and Discussion

NMS is expressed exclusively in the mouse SCN

A number of genes are found to express in the SCN. However, all are either every sparsely expressed, or it has additional widespread expression pattern throughout the brain. Since NMS was reported in the rat as a gene highly expressed in SCN, a decision was made to screen for the central expression pattern of NMS in the mice. In situ hybridization screening for NMS expression was performed on a male C57Bl6/J brain from the olfactory bulb to the brain stem, and detected signal exclusively in the SCN (Figure 5- 1). The results suggest that NMS neurons could include neurons that function as circadian pacemakers. Pharmacological data from Mori et al also suggested that NMS might have a role in behavioral circadian rhythm (Mori, Miyazato et al. 2005). Two lines of investigation were proposed: (1) Develop a Cre recombinase driver line for genetic manipulation of circadian pacemaker neurons, (2) Create a mouse line with a NMS-null allele to study the function of NMS gene in behavioral circadian rhythm.

In order to accomplish both objectives in the most time and cost efficient manner, a knock-in (NMS-KI) strategy was employed by replacing the NMS start codon with a Cre recombinase expression cassette. The NMS signal peptide sequence was also removed to ensure that a translated alternatively spliced NMS transcript will not end up in the endoplasmic reticulum. Details of targeting strategy, ES cell screening, and

genotyping methodology are listed in the material and method chapter. Development of the NMS-Cre driver is detailed in material and method section of this dissertation. Although NMS-KI and BAC transgenic ATG replacement of a Cre recombinase cassette failed to produce Cre recombinase expression pattern similar to the endogenous NMS, a bicistronic strategy produced a SCN-specific Cre transgenic mouse that is detailed in the previous chapter. The remainder of this chapter will describe the circadian phenotypes characterized in the NMS-KI mouse.

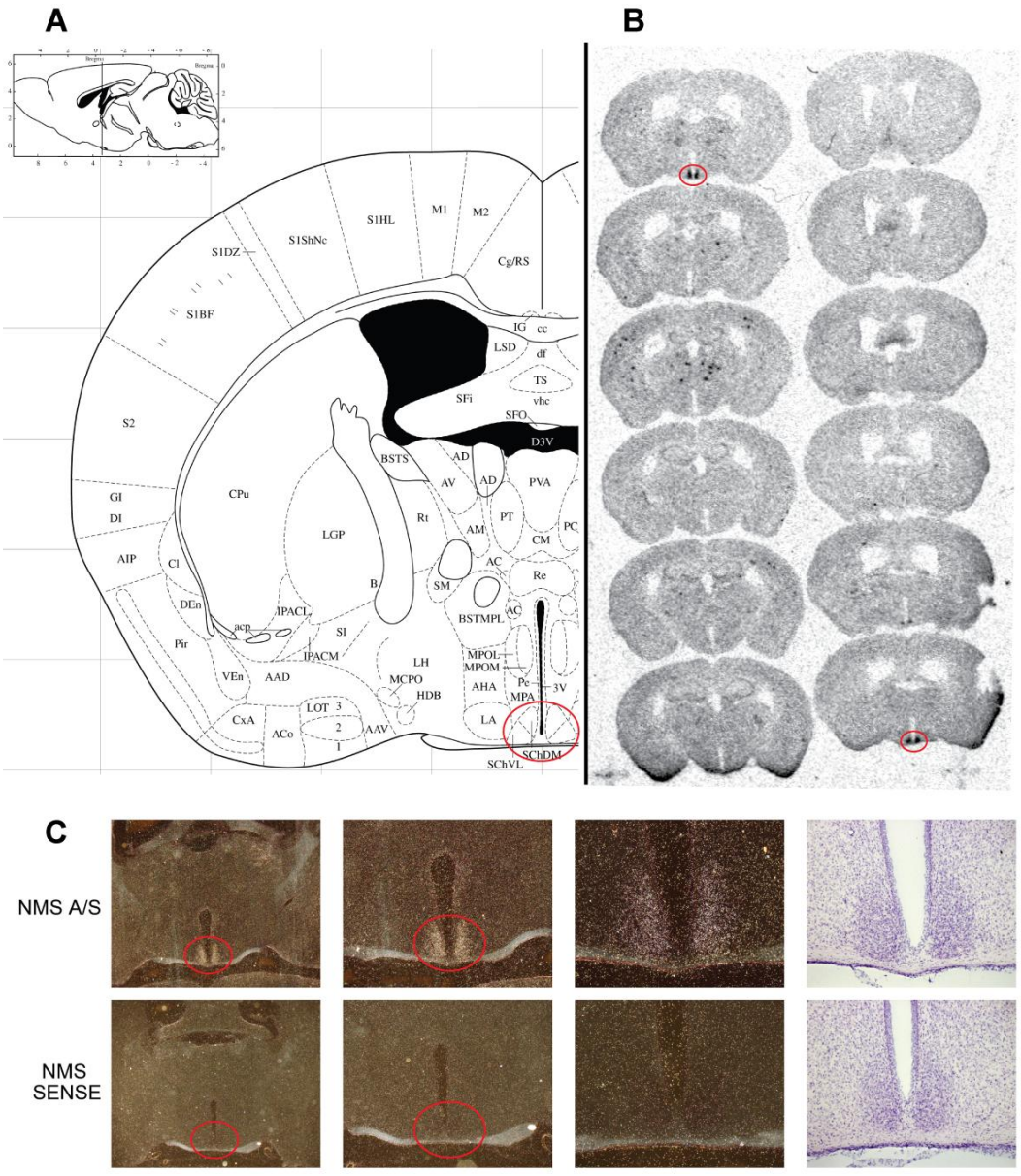

Figure 5- 1 NMS expression is detected exclusively in the SCN. An entire male C57BL6 brain was sectioned, mounted, and hybridized to in vitro transcribed sense and anti-sense 35S-labeled riboprobes in order to assess NMS expression throughout the brain. Red ovals indicate SCN location on the brain atlas (A), autoradiogram (B), and dark-field microscopy (C). No signals were detected using sense probe (C).

NMS-KI is NMS KO

In order to verify that NMS-KI animals are without functional NMS, a novel probe-based multiplex real-time PCR assay was developed to positively identify the altered signal sequence in the NMS-KI hypothalamic transcripts. The multiplex assay identified the three possible allele combinations (Table 5- 1). This data suggests that NMS-KI is a null allele that cannot produce a properly processed NMS peptide.

All commercially available antibodies targeting the NMS peptide are rat-specific. Attempts to use rat specific NMS antibody for immunohistochemistry has failed to produce a positive signal in the mouse. Custom developed antibody using synthetic NMS peptides have not produced a positive IHC, either.

Genotype	KI / KI	KI / WT	WT	NTC
KI-probe	+	+	n/d	n/d
WT-probe	n/d	+	+	n/d

Signal peptide sequence from NMS-KI animal is not detected in mRNA isolated from NMS-KI hypothalamus. A Taqman-probe based real-time PCR reaction detects the presence of cDNA made from reverse transcription of hypothalamic mRNA. +, positive detection between cycle 20-30 of the real-time PCR reaction. n/d, not detected.

Table 5- 1 Wild type signal peptide sequence is not detected by probe-based real-time QPCR in homozygous NMS-KI hypothalamic punch out.

NMS KO mice are normal in metabolic and behavioral screening tests

There is currently no published report of animals lacking the NMS neuropeptide. However, NMU KO animals are reported to show increased body weight, hyperphagia, and decreased locomotor activity and energy expenditure (Hanada, Teranishi et al. 2004). Since both NMS and NMU activate the NMU1R and NMU2R receptors, animals lacking NMS could exhibit similar phenotype. However, metabolic and behavioral screening tests on mixed background NMS KO animals appeared to be normal (Table 5- 2). This is expected because NMS has a different expression pattern than NMU. In the brain, NMU is found to express highly in ARC and affect gene expression in hypothalamic regions involved in control of feeding behavior (Hanada, Teranishi et al. 2004). NMS is detected exclusively in the SCN. NMS KO animals are fertile and show identical appearance as its wild type littermates.

NMS KO mice rapidly adjust to 8-hour phase advancement

Because of NMS' exclusive expression pattern at the SCN, the lack of NMS gene was speculated to have an impact on NMS KO's behavioral circadian rhythm. The jet-lag paradigm was tested on the KO animals because of the clinical relevance to stress imposed on the circadian physiology by trans-meridian travel and shift-work schedule. Individually housed wild-type and KO animals have free access to food, water, and running-wheels. During the first batches of pilot experiments, telemetry transponders were also implanted in the abdomen for voluntary activity and body temperature recording. Drinking water activities were also recorded. The records of those parameters were the same as the wheel running activity, and they are not shown here. Animals were

acclimated to the wheels in 24-hour daily cycles with 12-hours light and 12-hour dark (12L: 12D) lighting schemes. Once the activity onset time were established, the lighting schedule was advanced by eight hours. The activity onset time of NMS KO (N = 9) animals shifted quickly to the new phase within three days, while control animals needed six days or longer (Figure 5- 2A-C). Two KO animals' data were excluded. The first one was eliminated from analysis because its onset time was delayed by two hours on day one; this level of delay was not seen in control group. Another KO animal data was excluded because its onset time shifted nearly six hours later than the mean of the group and slower than even the slowest wild type control. One wild type animal's data was excluded because the animal had low activity level and ceased wheel-running altogether after day four. Repeated measure two-way ANOVA indicate that there is a significant difference (P = 0.009) between the two genotypes in phase-shift. Post hoc analysis indicates a significant difference in activity onset time on days 2, 3, and 4 after phase-shift.

NMS KO mice are normal in metabolic and behavioral screening tests

Test Name	Difference from WT littermates	N of subjects (WT / KO)
Body weight (normal chow)	Slightly lighter	10 / 10
Body weight (32% HF)	Slightly lighter	7 / 4
Metabolic cage (5 wks old)	No difference	2 / 4
Gross neurological function	Normal	9 / 11
General appearance	Normal	9 / 11
Sensorimotor reflexes	Normal	9 / 11
Elevated plus maze	No difference	9 / 11
Open field	No difference	9 / 11
Fear conditioning - Cue	Normal	9 / 11
Fear conditional - Context	Normal	9 / 11
Stimulus response	Normal	9 / 11
Pre-pulse inhibition	Normal	9 / 11
Rotorod	No difference	9 / 11
Hot plate	Normal	9 / 11

Mixed background (C57 and 129) animals were used. Tests were administered from top of the list to the bottom, in the order of their disrutiveness to normal animal activity.

Table 5- 2 NMS KO mice are normal in metabolic and behavioral screening tests

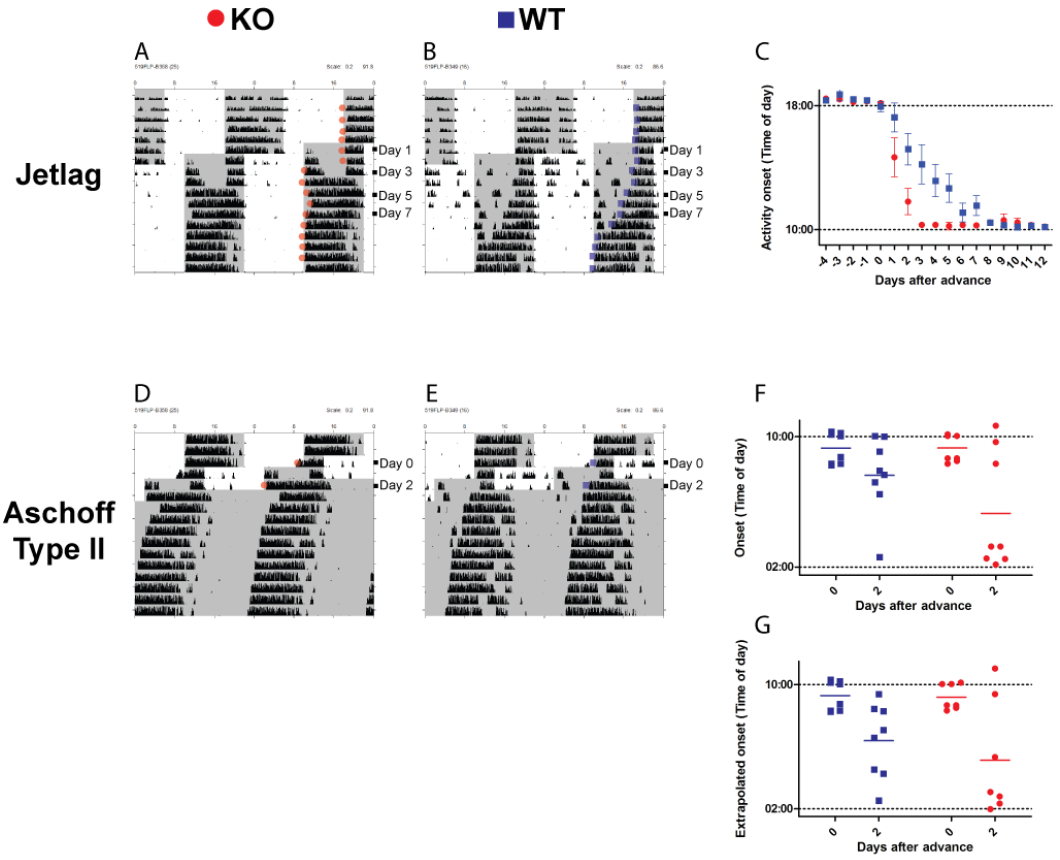

Figure 5- 2 NMS KO animals adjust activity onset time quicker than control wild type animals. Wheel running activity of NMS KO (N = 9) and control wild type (N = 9) animals were recorded every 60 seconds. Activity onset times were determined using Clocklab software analysis program with onset fit parameters set for 6 hour activity band detection. Onset time were individually verified and adjusted for obvious incorrect calls and daylight savings time, when applicable. Jetlag experiments (A-C) were performed by 8-hour phase advancement of dark onset. Actograms were double plotted with following day activity plotted immediately to the right of the current day. Day 1 (A and B) indicate the first day in the completed advanced light cycle. The red dot and blue square (A-C) indicate the determined onset time. Activity onset times of the day were plotted against days before and after phase advancement. The P = 0.009 by repeated measured two-way ANOVA. Post hoc analysis indicates significant difference between genotypes on days 2, 3, and 4 after phase shift.
NMS KO animals (N = 7) adjust their internal pacemaker quicker than control wild type animals (N = 8). Modified Aschoff type II paradigm (D-G), two cycles of 8–hour light phase advancement followed by free-run in constant darkness, were used to determine both the onset time of day 2 (F) and phase of internal clock (G). The

extrapolated day 2 onset hours were visually identified by extended regression line drawn through clocklab determined onset time from day 5 – 13.

It has been demonstrated that an abrupt shift in light dark cycle disrupt synchronous gene expression rhythm in the SCN (Nagano, Adachi et al. 2003). The slow SCN resynchronization to phase advancement was implicated for causing the symptoms experienced during jetlag. In order to determine the internal pacemaker's phase in response to rapid phase shift, NMS KO animals were subjected to a modified version of the Aschoff type II paradigm. Animals subjected to the modified Aschoff type II paradigm received two cycles of the phase advancement then were released into constant darkness. The free-running onset times were determined after five days in constant darkness. Overall, a larger portion of the NMS KO group adjusted its activity onset time after two days of phase advancement cycles. Looking at the extrapolated day 2 onset time, NMS KO's internal clock was also adjusted closer to the new phase (Figure 5- 2D-G).

These series of behavioral experiments suggests that NMS KO animals do not suffer jetlag because their internal pacemaker is able to respond and resynchronize to the new phase quicker than control wild type animals. Animals without an internal pacemaker are known to phase-shift rapidly to environmental time cues such as light through masking. However, this is not the case for NMS KO animals because they clearly demonstrate the ability to free-run under constant condition (Figure 5- 2D). The robust free-running rhythm suggests that NMS' circadian oscillator is functional and that its output pathways for locomotor activity are intact.

NMS KO animals have normal response to light stimuli

The canonical clock gene, mPer1, is known to be induced at the SCN by light stimulation during the night (Shigeyoshi, Taguchi et al. 1997). In order to determine if altered SCN response to light stimuli plays a role in the rapid phase-shift, NMS KO animals were subjected to light pulse at ZT 17 and core clock gene expression analyzed. After a one hour light pulse, SCN mPer1 expression were induced to similar levels between WT and KO animals (Figure 5- 3). This result indicates that animals lacking NMS respond normally to phase-shifting light pulse and that the input pathway from the retina to the SCN pacemaker neurons is intact.

NMS KO has normal clock oscillation amplitude

It is known that, in case for Clock mutant animals, an overt behavioral circadian rhythm can be maintained by an oscillator with reduced amplitude (Vitaterna, Ko et al. 2006). Furthermore, Clock mutants rapidly entrain to light-cycle phase-advancement (Nakamura, Yamazaki et al. 2008). In order to examine in vivo oscillation of core clock gene at the SCN, NMS KO and WT animals were released to constant dark environment and brains were collected every four hours for 24 hours. In situ hybridization of mPer1 (Figure 5- 4) and mPer2 mRNA (Figure 5- 5) levels indicate that NMS KO has normal clock oscillation amplitude. As a peripheral control, the liver clocks were also monitored and they oscillate within normal levels (Figure 5- 5D). Therefore, a dampened SCN oscillator is not likely to be the reason for a rapid resynchronization of NMS KO SCN to phase advancement. The current data also suggest that NMS is not involved in the SCN

core clock gene expression rhythm. Also, NMS is not likely to be an output molecule for synchronization of peripheral clocks.

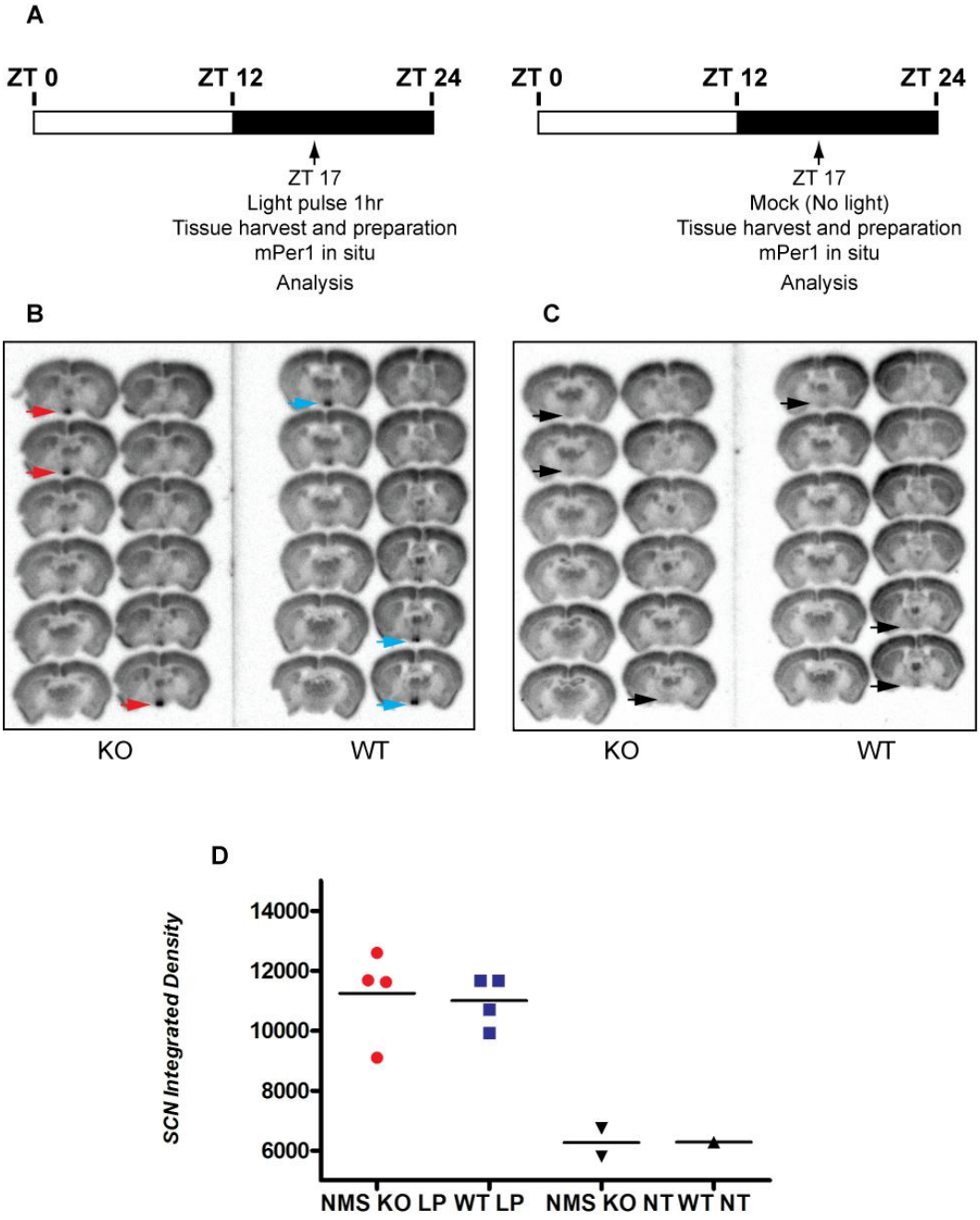

Figure 5- 3 SCN of NMS KO animals respond normally to light pulse. Animals from both genotypes (N = 4 per group) were subjected to light pulse (LP) in their home cage for 60 minutes beginning at ZT 17 (Panel A). After LP, animals were perfused with saline followed by 4% PFA. Mock group brains were collected at ZT 18 without receiving LP. The 35 micron thick frozen coronal sections were mounted on slides for in situ hybridization using 35S-labeled mPer1 riboprobes. Hybridized slides were exposed to storage phosphor screen for 24 hours followed by data collection on a GE Typhoon phosphor image scanner (Panels B and C). SCN specific integrated density were extract from TIFF image files analyzed using Adobe Photoshop CS4 Extended counting module (D).

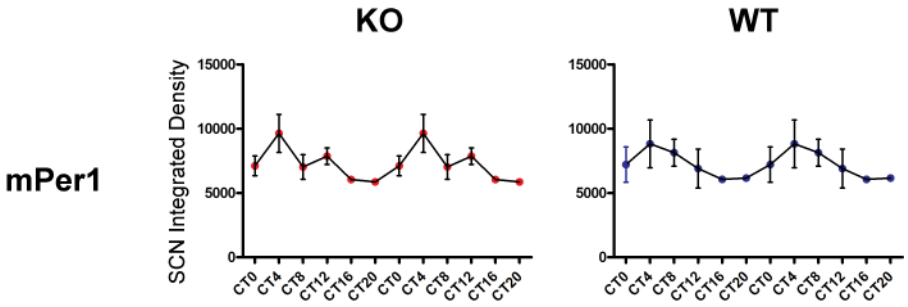

Figure 5- 4 mPer1 oscillate normally in NMS KO animals

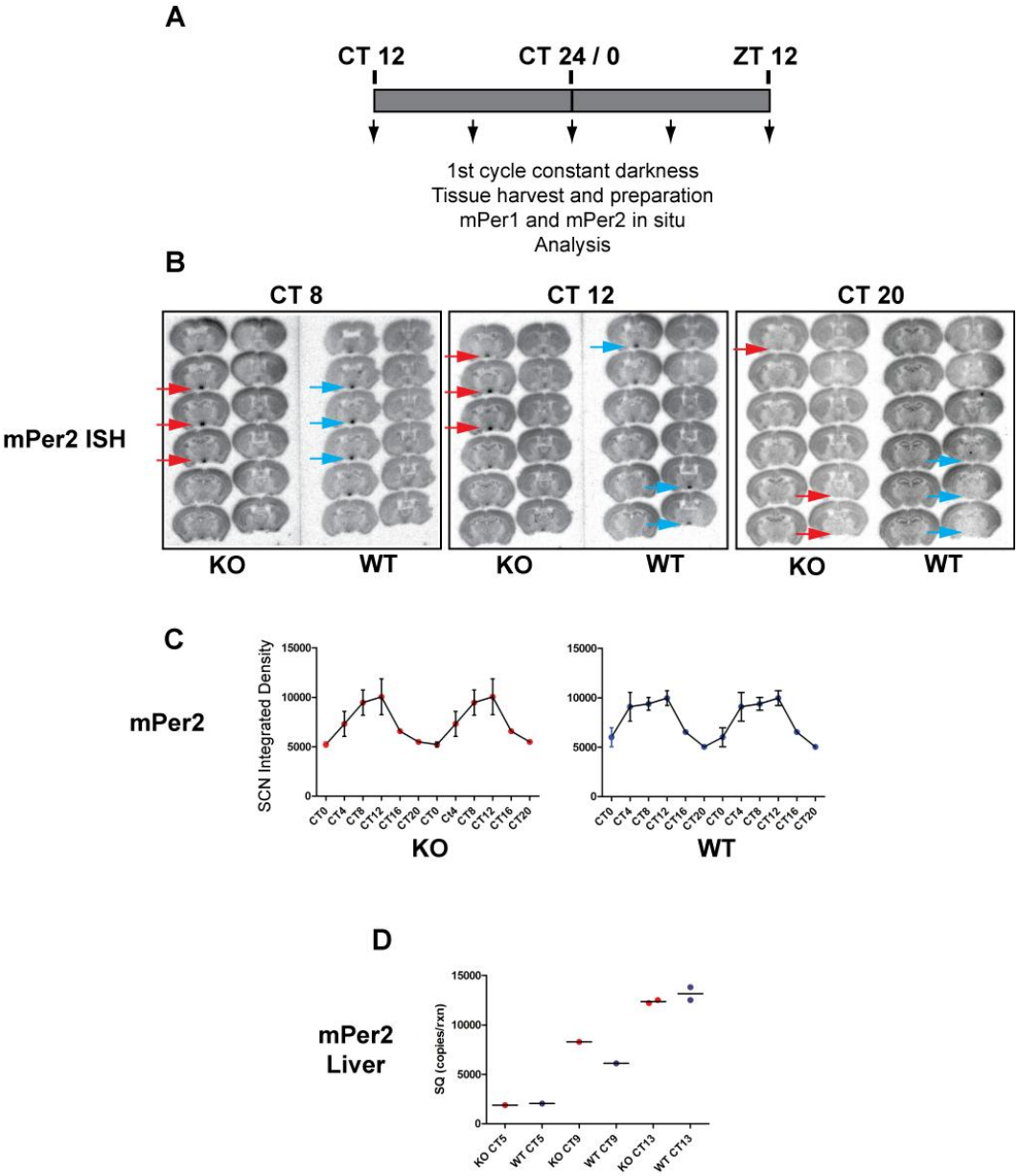

Figure 5- 5 Core clock genes oscillate normally in SCN and liver of NMS KO animals. Animals (N = 3 per group for each time point. N = 1 for CT 14 and CT 20 where signals levels were expected to be at the nadir) of both genotypes were placed in constant darkness and brains were collected at 4-hour intervals for 24 hours (Panel A) then subjected to in situ hybridization. Two time points (Panel B, CT 8 and CT 12) where highest expression levels of mPer2 detected by phosphor screens are shown. CT 20 represents the lowest expression level. Representative signals

used for analysis are indicated by red arrows (NMS KO) and blue arrows (WT). Panel C shows the SCN specific integrated densitometry reading where dots represent the mean value and error bars plot ± SEM. Liver mPer2 expression levels were measured by quantitative real-time PCR analysis, panel D. SQ, cDNA starting quantity estimated by mPer2 plasmid standard curve.

NMS KO has quasi-normal phase response curve to light

The normal functionality of the pacemaker can be represented by the phase-response curve to light (Daan and Pittendrigh 1976). During early subjective night, light pulse delays subsequent phase of activity onset. During late subjective night, light pulse advances subsequence phase of activity onset, while pulses administered during the subjective day has little to no phase effect. The range of the PRC, in terms of phase delay and advancement, is also limited to no more than 6 hours either direction. In case of heterozygous Clock mutants, the PRC appear to show exaggerated shifts that resemble a type 0 curve (Vitaterna, Ko et al. 2006). When PRC to light pulse was initially constructed for NMS KO animals on N1 mixed 129 and C57 background, large phase-shifts were detected near the inflection point (near CT17) of the PRC (Figure 5- 6). Whereas for WT animal, phase-shifts near the inflection point appeared subtle with shifts no more than three hours, some NMS KO animals shifted as much as 11 hours. Because constructing PRC using Aschoff type I protocol is time and resource consuming, Aschoff type II protocol was employed to test the occurrence of exaggerated phase shift specifically at ZT 17. However, N5 NMS KO animals pulsed at ZT 17 with Aschoff type II protocol did not exhibit the exaggerated phase shift. Since results from both Aschoff types I and II protocols are considered equal in the field, both sets of data are combined in (Figure 5- 6). It is possible that strain difference can contribute to the disappearance of

exaggerated phase shift at ZT 17. The possible difference between the two protocols could also be revealed here. However, this can be technically difficult because it will require finding a number of NMS KO animals exhibiting large phase shift by Aschoff type I protocols first.

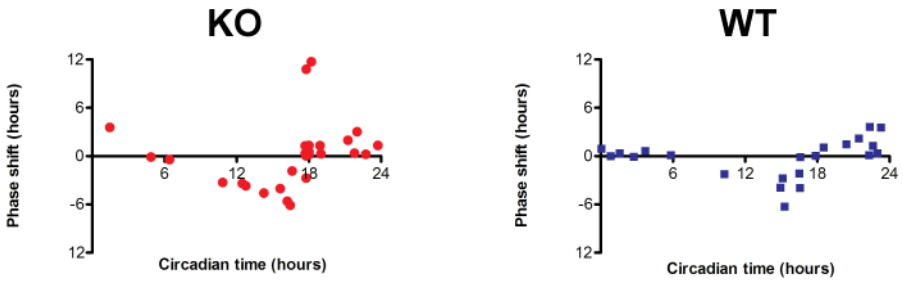

Figure 5- 6 Phase response curve to light is quasi-normal in NMS KO animals. Phase response curve to 6-hour of light pulse to NMS KO (N = 28) and WT control (N = 22) are presented. The x-axis indicate the CT at the beginning of light pulse, and the y-axis indicate the amount of phase shift. Positive phase shift indicate phase advancement after light pulse. Negative phase shift indicate phase delay.

Constant light reveals a period defect in NMS KO animals

Constant light (LL) desynchronizes rhythmic gene expression in SCN neurons (Ohta, Yamazaki et al. 2005). Although the mechanism that propels mice to have longer period than twenty-four hour in LL is unknown, it is generally assumed that behavioral rhythm reflects the status of SCN neurons. Recent report have shown that activity rhythm affected by LL (medium intensity 500 lux) is quickly restored upon releasing to constant darkness in forty-six wild type mice and all of them return to tau shorter than twenty-four hours (Chen, Seo et al. 2008). Wild type mice subjected to LL (green LED)

exhibit period longer than twenty-four hours, but their tau return to less than twenty-four hours upon released back to constant dark condition. In contrast, NMS KO animals that were first exposed to LL then return to constant darkness have smaller change in tau and maintain a longer than twenty-hour tau (Figure 5- 7). All NMS KO animals not exposed to LL exhibited either similar or shorter tau in constant darkness (Figure 5- 7F).

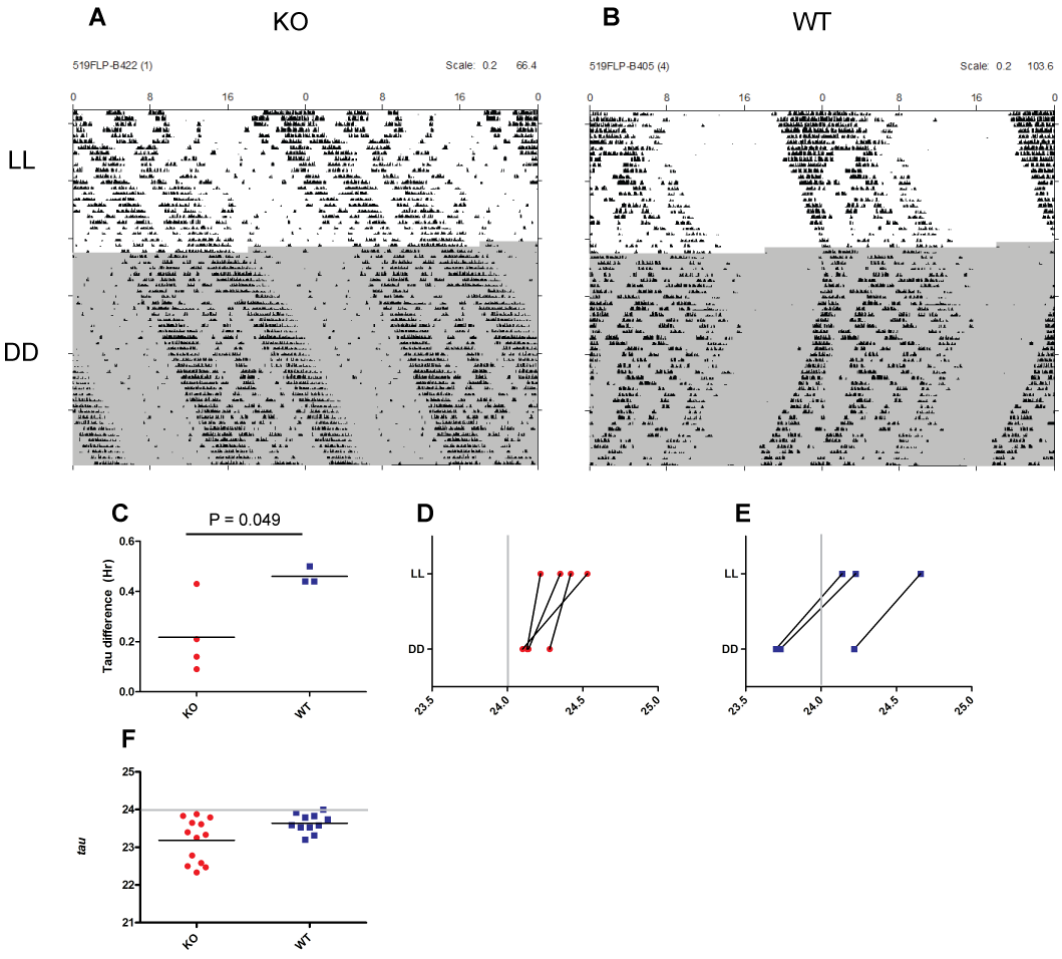

Figure 5- 7 NMS KO animals exposed to constant light maintain a period that is longer than 24 hours. NMS KO (N = 4) and WT control (N = 3) animals were

entrained to 12L:12D cycles, exposed to constant green LED light (LL) for 30 days then returned to constant dark (DD) for 30 days (A and B). The difference of period in LL and DD were plotted in C. Horizontal line is the mean. P = 0.049 were determined by Mann Whitney test. Stabilized Tau determined in LL and DD of NMS KO animal (D) and wild type control (E) were plotted. Grey vertical line indicates Tau of 24 hours. NMS KO animals not exposed to LL do not exhibit free-running period longer than 24 hours (N = 13).

Two possible scenarios can be suggested from the current result: (1) a distinct oscillatory mechanism drive the activity rhythm in LL, and NMS is involved in switching between LL mechanism and the constant dark mechanism; (2) the shorter than twenty-four hour activity rhythm is maintained by both the canonical intracellular core clock and a NMS-mediated coupled SCN neuronal network. When lacking NMS, the intracellular clock, along with other coupling factors, maintains the longer than twenty-four hour tau set forth in LL.

In support of the first scenario, it has been speculated that components of a circadian oscillator network can rearrange to accommodate disparate conditions such as constant dark versus constant light (Roenneberg and Merrow 2003). A rearranged oscillator network explains how mPer2 knockout animals can have activity rhythm in constant light condition, but not in constant dark condition (Steinlechner, Jacobmeier et al. 2002). A direct supporting evidence for the second scenario is lacking. G-protein coupled receptor binding peptidergic neurotransmitter such as Vasoactive Intestinal Polyeptide has been implicated as a SCN neuronal activity synchronization factor (Aton, Colwell et al. 2005). NMS, with its receptors in the SCN, could very well function as an intercellular coupling factor that conditions a stressed pacemaker to return to normal range.

Chapter five reference

Aton, S. J., C. S. Colwell, et al. (2005). "Vasoactive intestinal polypeptide mediates circadian rhythmicity and synchrony in mammalian clock neurons." Nature Neuroscience **8**(4): 476-483.

Chen, R. M., D. O. Seo, et al. (2008). "Strong Resetting of the Mammalian Clock by Constant Light Followed by Constant Darkness." Journal of Neuroscience **28**(46): 11839-11847.

Daan, S. and C. S. Pittendrigh (1976). "Functional-Analysis of Circadian Pacemakers in Nocturnal Rodents .2. Variability of Phase Response Curves." Journal of Comparative Physiology **106**(3): 253-266.

Hanada, R., H. Teranishi, et al. (2004). "Neuromedin U has a novel anorexigenic effect independent of the leptin signaling pathway." Nat Med **10**(10): 1067-1073.

Mori, K., M. Miyazato, et al. (2005). "Identification of neuromedin S and its possible role in the mammalian circadian oscillator system." Embo J **24**(2): 325-335.

Nagano, M., A. Adachi, et al. (2003). "An abrupt shift in the day/night cycle causes desynchrony in the mammalian circadian center." J Neurosci **23**(14): 6141-6151.

Nakamura, W., S. Yamazaki, et al. (2008). "In vivo monitoring of circadian timing in freely moving mice." Curr Biol **18**(5): 381-385.

Ohta, H., S. Yamazaki, et al. (2005). "Constant light desynchronizes mammalian clock neurons." Nature Neuroscience **8**(3): 267-269.

Roenneberg, T. and M. Merrow (2003). "The network of time: Understanding the molecular circadian system." Current Biology **13**(5): R198-R207.

Shigeyoshi, Y., K. Taguchi, et al. (1997). "Light-induced resetting of a mammalian circadian clock is associated with rapid induction of the mPer1 transcript." Cell **91**(7): 1043-1053.

Steinlechner, S., B. Jacobmeier, et al. (2002). "Robust circadian rhythmicity of Per1 and Per2 mutant mice in constant light, and dynamics of Per1 and Per2 gene expression under long and short photoperiods." Journal of Biological Rhythms **17**(3): 202-209.

Vitaterna, M. H., C. H. Ko, et al. (2006). "The mouse Clock mutation reduces circadian pacemaker amplitude and enhances efficacy of resetting stimuli and phase-response curve amplitude." Proc Natl Acad Sci U S A **103**(24): 9327-9332.