5-2018

Copper in the Suprachiasmatic Nucleus: Copper Signaling, Homeostasis, and Circadian Rhythms and Trace Metals in the Master Clock

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To the Graduate Council:

I am submitting herewith a dissertation written by Yukihiro Yamada entitled "Copper in the Suprachiasmatic Nucleus: Copper Signaling, Homeostasis, and Circadian Rhythms and Trace Metals in the Master Clock." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biochemistry and Cellular and Molecular Biology.

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A Dissertation Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Yukihiro Yamada
May 2018

ACKNOWLEDGEMENTS

There are countless reasons for which I am grateful to a great number of people who have helped me reach this finish line, and many to whom I am indebted in completing this work. First and most important to my time in graduate school is my mentor and PhD advisor, Dr. Rebecca Prosser. Starting a new project with many unforeseen paths, her knowledge and wisdom guided me through the ups and downs of research and kept me focused on the goals ahead. Her virtuous patience, understanding, and professionalism are ideals of mentorship that truly lead by example and showed me what it means to be a leader in academia and in the broader research community. But it is for Rebecca's kindness to believe in and support me with both courage and strength of faith in me that I will never be able to thank her enough, to repay the debt of gratitude for what that meant to me. Indeed, words cannot describe how humbled I am that Rebecca, together with my committee, remained committed to my growth and progress throughout the many struggles, internal and external, before and after my leave of absence. Thank you Drs. Jim Hall, Matt Cooper, Albrecht von Arnim, and Brad Binder for an unforgettable experience in my PhD defense, and great memories in the journey along the way; to Dr. McKee who gave me my first opportunity and the members of his lab, especially Rihui Yan, who gave me and my family so much hope that is priceless to us; and to the BCMB staff, current department head Dr. Daniel Roberts and former committee member and head, Dr. Cynthia Peterson for giving me the opportunity and support to begin my career and complete my goals. A special thank you to LaShel Brown and Lori Daniels for helping me in my time of trouble, and to Cheryl Hodges for help with a once-in-a-lifetime trip to Montana. And to Dr. Binder for your advice and encouragement through a difficult time in my studies. I would like to thank the many other members of the BCMB department, current and former, and the dedicated staff of the University of Tennessee Graduate School, who have helped me big and small. All of you in your dedication and support of students have made students' wishes come true, thank you.

There are many BCMB and other students to whom I owe much gratitude for teaching me many things, especially my classmates and many former graduate students. A special thanks to Dr. Nathan Stebbins who spent countless hours teaching me different aspects of science and continues to inspire me further. And of course, thank you to the many past and present Prosser

Lab members. Thank you Dr. Xiang Mou for inspiring me and for the many memories before you left the lab. Thank you Dr. Beth Cantwell for mentoring and training me. A special thanks to former undergraduates who helped me through different stages of my project and graduate school: Dr. Alicia McClintock, Katherine Hatcher, Melissa Lee, Stephanie Zale, Eric Olberding, and Veronica Go. To Dr. Jonathan Lindsay, Joanna Cooper, and Kathryn Abrahamsson. To all of you and the times we shared that made the lab feel like home, and to Rebecca who made us all feel like we belong, thank you. This dissertation comes from all of your all's support and work.

Lastly, I could not have started or finished graduate school without the support and love of friends and family. Friends who I made through graduate school and in science, friends outside of science, those who are like family to me, and my family and wife. I would like to express again, my gratitude for my lab family –Jonathan, Joanna, Kate, and Rebecca– and extended, Cody Burleson, Katherine Hatcher, Melissa Lee, Andrew Stafford, Drs. Jim Hall and Matt Cooper, as well as former Cooper lab members. I will miss the times we spent in Knoxville, at conferences and elsewhere as our bonds were defined beyond science and its benchtops. To my father and mother, brothers and sisters. To Mary Jane, her parents and their families. Your patience, support, and love means everything to me. I am blessed and forever grateful, for where I have come from, gone through, and gotten to, I could not have without you all, and may this dissertation be one of many, many ways in which your support helped to make happen. Thank you to everyone who keeps me going, who helped me along the way, and to those who continue to believe in me. Thank you Mary Jane, this is to you.

ABSTRACT

Herein, we present data supporting a role for copper (Cu) and Cu homeostasis in the suprachiasmatic nucleus (SCN), the location of the mammalian circadian pacemaker. Although many studies have investigated the function and effects of Cu in synaptic function and receptor signaling in the brain and other tissues, these results are the first to directly link Cu with the SCN master clock and circadian neuronal activity rhythms. Previous work using extracellular recordings of SCN neurons in *ex vivo* hypothalamic slices has demonstrated that resetting the circadian clock, e.g. by glutamate (Glu) treatment during the night, induces shifts in the phase of SCN neuronal activity rhythms that correspond to phase shifts in circadian behavioral activity (Albers et al., 2017; Golombek and Rosenstein, 2010; Herzog et al., 2017; Lindsay et al., 2014; Prosser, 1998). Here we first have demonstrated that both Cu chelation and Cu application are able to induce night-time phase shifts in neuronal activity rhythms *in vitro*. Second, we have shown that these two treatments affect *N*-methyl-D-aspartate receptor (NMDAR) and Glu neurotransmission differently. Since Glu phase-shifts the SCN clock through NMDAR-mediated, calcium-dependent signaling pathways and activation of other pathways, we pharmacologically tested several of these pathways to investigate how application of Cu or the Cu chelator, tetrathiomolybdate (TTM), induces NMDAR-independent and dependent phase shifts, respectively. Our results demonstrate that Cu induces mitogen-activated protein kinase (MAPK)-dependent phase shifts in the absence of NMDAR-mediated calcium influx. On the other hand, the specific extra- and intracellular mechanisms by which Cu removal induces phase shifts remain unclear. Lastly, we have preliminary results indicating that concentrations of Cu in the SCN are comparable to other brain regions, and show day-night expression of two Cu transporters, copper transporter 1 (CTR1) and ATPase-7A (ATP7A) in the SCN. We discuss these findings in light of the existing literature and current models of SCN circadian oscillator mechanisms. Our results together with published findings suggest that Cu homeostasis is tightly regulated in the SCN, and that changes in Cu levels serves as a time cue for the circadian clock. Future research can elucidate how Cu (dys)regulation interacts with oscillations in SCN neuronal firing and signaling activity and whether Cu or other trace elements influence SCN metabolic and redox activity.

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ABBREVIATIONS

Aβ	amyloid β
AP5	(2R)-amino-5-phosphonovaleric acid
APP	amyloid precursor protein
ATOX1	antioxidant protein 1
ATP7A	ATPase-7A
AVP	arginine vasopressin
BCS	bathocuproine disulfonate
BDNF	brain-derived neurotropic factor
$[Ca^{2+}]_I$	intracellular calcium concentration
COX	cytochrome c oxidase
CREB	cAMP-/Ca^{2+}-response element binding protein
CTR1	copper transporter 1
Cu	copper
EBSS	Earles' balanced salt solution
ERK	extracellular-signal related kinase
GABA	γ-aminobutyric acid
GABAa-R	GABA receptor A
Glu	glutamate
GRX	glutaredoxin
GSH/GSSG	Reduced glutathione/oxidized form
LD	light-dark
LRP1	low-density lipoprotein-related receptor 1
LTP	long-term potentiation
MAPK(K)	mitogen-activated protein kinase (kinase)
MEK	MAPK/ERK kinase
MT	metallothionein
NAD(H)	nicotinamide adenine dinucleotide (reduced)

NADP(H)	NAD phosphate (reduced)
NMDAR	NMDA receptor
NOS	nitric oxide synthase
PKG	cGMP-dependent protein kinase
ppb	parts per billion
PrPc	Cellular prion protein
ROS	reactive oxygen species
RyR	Ryanodine receptor
SCN	suprachiasmatic nucleus
sGC	soluble guanylyl cyclase
SOD1	Cu/Zn-superoxide dismutase 1
TNFα	tumor necrosis factor-alpha
TrkB	tropomyosin receptor/tyrosine receptor kinase B
TrkB-I	TrkB receptor inhibitor
TTFL	transcription/translation feedback loop
TTM	tetrathiomolybdate
VGCC	voltage-gated calcium channel
VIP	vasoactive intestinal polypeptide
ZT	zeitgeber time

CHAPTER ONE

INTRODUCTION

1.1 Circadian Rhythms

1.1a Three Interlinked Clocks in Circadian Biology

Organisms have an internal timekeeping system to synchronize their physiology and behavior and coordinate these activities to increase benefits and reduce harms present in the environment. With light being the dominant environmental stimuli, ~24-h ("circadian") clocks evolved to respond to changes in the light-dark cycle (LD) to maintain synchrony with the daily rotation of the Earth. Many important physiological processes and behaviors are controlled by such clocks and exhibit a circadian rhythm with coordinated peaks and troughs to minimize energy and resource loss, maximize energy/resource utility, and facilitate homeostatic processes (reviewed in Asher and Sassone-Corsi, 2015; Asher and Schibler, 2011; Bailey et al., 2014; Bass and Lazar, 2016; Bass and Takahashi, 2010; Cribbet et al., 2016; Farajnia et al., 2014; Gamble et al., 2014; Man et al., 2016; Nakagawa and Okumura, 2010; O'Neill et al., 2013; Panda, 2016; Reddy and Rey, 2014; Riede et al., 2017; Silver and Kriegsfeld, 2014; Wilking et al., 2013).

With the evolution of photosynthetic organisms such as cyanobacteria, circadian timekeeping allowed for timely regulation of proteins and molecules in light harvesting as well as upregulation of processes in response to UV exposure and DNA damage (Cockell and Rothschild, 1999; Gehring and Rosbash, 2003; Hitomi et al., 2000; Lucas-Lledo and Lynch, 2009; Mei and Dvornyk, 2015; Milev and Reddy, 2015). Analogously, mitochondrial processes such as oxidative phosphorylation require coordinated activity of enzymes and abundance of molecules involved in ATP production (Cela et al., 2016; Chiang et al., 2014; Isobe et al., 2011a, b; Masri et al., 2013; Neufeld-Cohen et al., 2016; Peek et al., 2013). Because food/energy availability and mitochondrial processes were evolutionarily linked to the circadian oscillators, organisms also

coordinate anabolic processes including protein synthesis in a circadian fashion (Chaix et al., 2016; Huang et al., 2013; Panda, 2016; Wang et al., 2015a). Thus, energy storage and metabolism are central to the evolution of circadian biology, while the "gears" for circadian timekeeping are internal to the cell and autonomous.

In order to coordinate processes with the environment and anticipate daily rhythms in the availability of light or food sources, organisms possess an autonomous and temperature compensated (largely unaffected by fluctuations in environmental temperature) circadian timekeeping system in the form of molecular feedback loops (rev. in Chaix et al., 2016; Herzog et al., 2017; Hurley et al., 2016; Ukai and Ueda, 2010). Constituting the gears and mechanics of such molecular feedback loops are the circadian transcription and translation of transcriptional activators and repressors, which are thusly called central, or core, clock genes/proteins. In mammals, the transcriptional activators CLOCK and BMAL1 working as a dimer form the positive loop to transcribe mRNA for transcriptional repressors, in particular PER and CRY proteins of the negative loop, which feedback and block CLOCK and BMAL1 transcription until the repressor complexes of PER and CRY are removed and degraded (Herzog et al., 2017; Partch et al., 2014; Takahashi, 2017). The transcription/translation feedback loop (TTFL) is well established as the primary basis by which organisms maintain endogenous ~24-h rhythms indefinitely even in the absence of cyclic photic signals (e.g., during continuous dark or light). However, in constant light or constant dark conditions most organisms will exhibit rhythms with periods slightly greater or less than 24-h, demonstrating the requirement for external cues to synchronize their clocks with the Earth's 24-h LD cycle. Circadian time cues or "zeitgebers" ("timegivers") affect endogenous molecular clocks through activation of signaling pathways and/or changes in the cellular context that directly affect the kinetics or activity levels of clock genes/proteins. Depending on time-of-cue, such external input can advance or delay the molecular clock and its output in order to synchronize the organism's activities to the environment.

As mentioned, coordination of metabolic activity is fundamental to the evolution of circadian clocks, and research has demonstrated how clock proteins regulate various metabolic activities via transcription/translation (rev. in Asher and Sassone-Corsi, 2015; Bass and Lazar,

2016; Panda, 2016). Conversely, availability of energy-providing molecules (glucose, ATP, NADH, etc.) or other molecules directly related to photosynthesis or cellular respiration also affect the core molecular clock (Asher and Sassone-Corsi, 2015; Bailey et al., 2014; Choudhary et al., 2016; Feeney et al., 2016; Feng et al., 2017; Panda, 2016; Reddy and Rey, 2014). Generally, lack of ATP and other energy-associated substrates reduces other cellular functions including synthesis, transport, or degradation of molecules involved in faithful circadian timekeeping. Because the clock regulates protein and lipid metabolism, circadian input to the mitochondria allows for coordinated generation of ATP and cofactors such as NADH/FADH required for protein/lipid metabolism and function (Aviram et al., 2016; Bellet et al., 2016; Chiang et al., 2014; Hirano et al., 2017; Kumar Jha et al., 2015; Loizides-Mangold et al., 2017; Masri et al., 2014; Milev and Reddy, 2015; Neufeld-Cohen et al., 2016; Panda, 2016; Poggiogalle et al., 2017; Reddy and Rey, 2014; Robles et al., 2014; Robles et al., 2017; Wang et al., 2017).

Cellular activities produce free radicals and reactive oxygen species (ROS) that can affect the redox state of the cell and lead to oxidative stress. The redox state of the cell in turn can directly affect the function of some clock proteins (e.g. Rev-Erbα) (Bailey et al., 2014; Carter et al., 2017; Hirano et al., 2017; Hirayama et al., 2007; Ivleva et al., 2005; Milev and Reddy, 2015; Qian et al., 2012a; Rey et al., 2016; Rutter et al., 2001; Shang et al., 2012; Sundar et al., 2017; Wende et al., 2016; Wood et al., 2010; Yang et al., 2014; Yoshida et al., 2011). In addition, oxidative damage of clock proteins may enhance their degradation or hinder their function. It is still unclear whether such effects on the core molecular clock are important for adjusting other processes to the metabolic state of the cell(s) or are just epiphenomenal to high cellular activity. Nonetheless, one important clock function may be to coordinate enzymatic removal of ROS to when peak oxidative stress is anticipated.

Interestingly, the removal of ROS and cellular redox reactions involve redox recycling of antioxidant enzymes and molecules, (Chakravarty and Rizvi, 2012; Cho et al., 2014; Edgar et al., 2012; Hardeland et al., 2003; Hirayama et al., 2007; Hoyle and O'Neill, 2015; Kil et al., 2015; Milev and Reddy, 2015; O'Neill and Feeney, 2014; O'Neill et al., 2011; Patel et al., 2014; Putker and O'Neill, 2016; Reddy and Rey, 2014; Toledano and Delaunay-Moisan, 2015), and studies using

mature red blood cells has demonstrated circadian oscillations in the redox states of antioxidant enzymes in the absence of transcription/translation feedback loops (Cho et al., 2014; Homma et al., 2015; O'Neill and Reddy, 2011). In addition, genomics and other experiments provide emerging evidence for redox oscillations as a primary cellular rhythm intimately tied to cellular energy production (Mendez et al., 2016; Putker et al., 2017; Rey et al., 2016; Rhee and Kil, 2016; Toledano and Delaunay-Moisan, 2015; Wende et al., 2016), and redox molecules are more broadly conserved among biology than specific clock genes/proteins (Causton et al., 2015; Edgar et al., 2012; Fanjul-Moles, 2013; Hoyle and O'Neill, 2015; O'Neill et al., 2011; Olmedo et al., 2012). However, it is not known how ubiquitous circadian redox oscillators are, as well as how cells coordinate molecular, metabolic, and redox rhythms to generate output adjusted to input from the environment.

1.1b The suprachiasmatic nucleus and mammalian circadian rhythms

In the mammalian brain, a central pacemaker or "master clock" in an area of the hypothalamus called the suprachiasmatic nucleus (SCN) governs circadian processes through outputs to other brain regions and various tissues/organs which themselves contain circadian clocks. Input from the SCN synchronizes the clocks within each organ system and coordinates the activity of different organ systems across the day (Bass and Lazar, 2016; Cribbet et al., 2016; Gamble et al., 2014; Kumar Jha et al., 2015; Riede et al., 2017; Silver and Kriegsfeld, 2014). Without daily SCN output, organs lose temporal coordination with each other, and within an organ the amplitude of circadian rhythms dampens within days as the cells become desynchronized (Brown and Azzi, 2013; Evans, 2016; O'Neill et al., 2013). In contrast, dispersed SCN cells *in vitro* can maintain circadian clock activity for weeks at the single cell level in the absence of daily synchronizing input (Honma et al., 1998; Noguchi et al., 2017; Welsh et al., 1995; Welsh et al., 2010). In addition, cultured SCN tissue slices maintain high amplitude circadian rhythms as a result of endogenously produced synchronizing agents such as vasoactive intestinal polypeptide (VIP).

In vivo, light stimulates melanopsin containing retinal ganglion cells, which project to the SCN through the retinohypothalamic tract, and induces glutamate release from axon terminals (Baver et al., 2008; Berson et al., 2002; de Vries et al., 1993; Ding et al., 1994; Doyle et al., 2008; Drouyer et al., 2007; Ebling et al., 1991; Hattar et al., 2002; Mikkelsen et al., 1995; Mintz and Albers, 1997; Mintz et al., 1999; Tsai et al., 2009; Vindlacheruvu et al., 1992; Wong et al., 2007). During the subjective night, i.e. night according to the animal's pacemaker, glutamate release onto SCN neurons resets their clocks by activating calcium-dependent signaling pathways (Albers et al., 2017; Colwell, 2000; Fukushima et al., 1997; Golombek and Rosenstein, 2010; Hamada et al., 1999; Tominaga et al., 1994). Resetting of the SCN clock results in either an advance or delay shift in the phase of circadian activity ("phase shift"). However, during the subjective day, photic cues (i.e. light/glutamate) are not able to reset the SCN clock and induce phase shifts. Physiologically, this time-dependent regulation and sensitivity to photic input underlies the ability to synchronize to the external LD cycle, which is called photic entrainment. Several non-photic cues can reset the SCN clock also, such as behavioral activity, cocaine, and serotonin (Antle and Mistlberger, 2000; Edgar et al., 1993; Glass et al., 2012; Prosser et al., 1993; Prosser et al., 1992; Prosser et al., 1990; Prosser et al., 2014; Stowie et al., 2015; Webb et al., 2014; Yamakawa et al., 2016). The phase-shifting effect of these cues are seen when they occur in the subjective day.

A unique feature of the SCN is the diurnal increase in neuronal activity and firing rate that peaks around mid-subjective day (rev. in Albers et al., 2017; Colwell, 2011; Hastings et al., 2014; Herzog et al., 2017; Nakagawa and Okumura, 2010; Riede et al., 2017). This diurnal peak in SCN neuronal activity occurs in both diurnal and nocturnal animals. The increased daytime activity of SCN neurons, seen at the level of increased cellular respiration and an increased ratio of action potential generation, involves an increase in excitatory drive arising from circadian changes in expression and activity of ion channels and pumps. Exactly how the TTFL clock regulates these ionic currents and ultimately the rhythm in neuronal excitability is not well understood. On the other hand, emerging evidence suggests that the enhanced daytime SCN neuronal activity functions as an input for pacemaker activity and not just an output of the TTFL clock (Granados-Fuentes et al., 2015; Hermanstyne et al., 2017; Jones et al., 2015; Kononenko et al., 2013). In a

subset of SCN neurons, conditional blocking of synaptic transmission disrupts the TTFL clock (Lee et al., 2015). Furthermore, optogenetic excitation of SCN neurons is sufficient to induce phase shifts in molecular (*ex vivo*) and behavioral rhythms (Jones et al., 2015). One simple explanation is that these manipulations alter VIP release, which affects the TTFL by activating cyclic AMP and Gq-dependent calcium signaling pathways (Enoki et al., 2017; Han et al., 2012; Jones et al., 2015). Although most SCN neurons release GABA, due to the multifaceted, circadian physiology of GABA signaling in the SCN (Alamilla et al., 2014; Albers et al., 2017; Albus et al., 2005; Belenky et al., 2003; Choi et al., 2008; De Jeu and Pennartz, 2002; DeWoskin et al., 2015; Evans et al., 2013; Fan et al., 2015; Freeman et al., 2013; Gribkoff et al., 2003; Hamada and Shibata, 2010; Hummer et al., 2015; Moldavan et al., 2017; Walton et al., 2017), the role of GABA neurotransmission remains unclear and/or has not been addressed in these studies.

The SCN also exhibits a daily rhythm in cellular redox state driven by TTFL output (Wang et al., 2012). The ratio of oxidized forms of redox molecules to reduced redox molecules (FAD(ox) to NADPH; DHA-dehydroascorbic acid to ascorbic acid) and global protein glutathiolation are higher in SCN tissue during the early subjective night, reflective of a relatively oxidized state during the night and reduced state during the day (Wang et al., 2012). The ~24-h rhythm in redox state is absent in SCN tissue from arrhythmic mice lacking the core clock protein BMAL. Furthermore, pharmacological manipulation using oxidizing or reducing agents demonstrate that the cellular redox state is linked to neuronal excitability rhythms in the SCN (Wang et al., 2012). A reduced state allows for increased K^+ influx and decreased K^+ efflux, most likely through redox modulation of leaky K^+ channels and voltage-gated K^+ channels respectively, and is involved in the relatively depolarized state of SCN neurons during the day. The importance of redox in circadian neuronal excitability and K^+ channels has also been demonstrated in *Drosophila* circadian clock neurons and behavior (Fogle et al., 2015). In addition to reports of redox effects on K^+ channels, an increased oxidative environment can change the function of other channels, and proteins in general, indirectly via redox cofactors and directly through modifications including glutathiolation and nitrosylation. Although the physiological consequences of the aforementioned rhythm in global protein glutathiolation are yet unknown, nitrosylation may be important in SCN rhythmicity (Riccio et al., 2006).

While questions regarding the connections between redox state on neuronal excitability remain largely unanswered, studies have shown that a variety of circadian clocks are sensitive to oxidative stress and changes in redox state (Mendez et al., 2016; Milev and Reddy, 2015; Putker and O'Neill, 2016; Wende et al., 2016). These include non-mammalian model organisms, such as zebrafish, *Arabidopsis*, *Neurospora*, and cyanobacteria (Hirayama et al., 2007; Ivleva et al., 2005; Ivleva et al., 2006; Kim et al., 2012; Lai et al., 2012; Qian et al., 2012a; Wood et al., 2010; Yoshida et al., 2011). In mammalian cells, such as mouse hepatocytes and embryonic fibroblasts, oxidative stress affects clock proteins and their functions (Gupta and Ragsdale, 2011; Rutter et al., 2001; Shang et al., 2012; Tamaru et al., 2013; Yang et al., 2014). Furthermore, TTFL-driven circadian oscillations in nicotinamide phosphoribosyltransferase (NAMPT), the rate-limiting enzyme in NAD^+ biosynthesis, produce oscillations in NAD^+ (Bellet et al., 2013; Nakahata et al., 2009; Ramsey et al., 2009). NAD^+ is a cofactor for the transcriptional suppressor SIRT1, and increased NAD^+ abundance suppresses CLOCK:BMAL complex-dependent transcriptional activation, inhibiting the core positive loop of the TTFL clock. Pharmacologically or genetically changing NAD^+ abundance and dynamics disrupts TTFL output (Nakahata et al., 2009; Ramsey et al., 2009). On a separate note, *in vitro* DNA binding by the transcriptional activators and core clock proteins, CLOCK and BMAL, increases in the presence of NADH or NADPH, the reduced forms of NAD^+ or $NADP^+$, respectively (Rutter et al., 2001; Yoshii et al., 2013). Notably, NADH is required for oxidative phosphorylation, and mitochondrial energy production is affected by the ratio of these redox pairs (Jokinen et al., 2017; Mendez et al., 2016; Scialo et al., 2017; Verdin, 2015). The effects of redox agents on NAD^+/NADH ratio and rhythmic output has not been reported in the SCN. However, the redox oscillation in the SCN mentioned previously would favor a nighttime increase in the oxidized NAD^+, which is consistent with increased *nampt* mRNA and SIRT1 levels in the SCN during the night (Chang and Guarente, 2013). Similarly, the role of diurnally fluctuating NADPH in circadian biology has recently been reported, and manipulation of NADPH rhythms and redox have been shown to affect clock gene expression, albeit not in the SCN (Putker et al., 2017; Rey et al., 2016). In summary, circadian oscillations in the redox state of SCN neurons is involved in neuronal excitability and presumably TTFL output of the master clock,

hence changes in the redox state caused by excess ROS/oxidative stress or external input will likely shift SCN circadian output.

1.2 Copper is an Essential Trace Element

1.2a Copper in biology

Copper is an essential trace element involved in energy production and cellular redox activities. Depending on cellular location and protein coordination, copper (Cu) switches oxidation state ($Cu^{2+}/^{+}$) making it uniquely important as an essential cofactor of several enzymes such as cytochrome c oxidase in the mitochondrial electron transport chain (Gaier et al., 2013a; Scheiber et al., 2014). Total intracellular Cu concentration is thought to range from nanomolar to micromolar (Balamurugan and Schaffner, 2006). However, the intracellular concentration of unbound Cu is virtually zero in most cells under homeostatic conditions, due to an abundance of proteins that bind Cu to protect against Cu-induced ROS generation and oxidative stress (Hung et al., 2010). Metallothioneins, ATOX1 (antioxidant protein-1), and GSH (glutathione) play a primary role in Cu homeostasis by binding and buffering against the toxic effects of Cu (Baker et al., 2017a; Bhattacharjee et al., 2017; Calvo et al., 2017; Hatori et al., 2017; Hatori and Lutsenko, 2016; Ohrvik et al., 2017; Scheiber et al., 2014).

The necessity for Cu in mammalian development is evidenced by embryonic lethality of homozygous knockout of the Cu import protein CTR1 (copper transporter 1) in mice (Kuo et al., 2001). Furthermore, systemic Cu deficiency, as occurs in Menkes disease, has moderate to severe effects on development and neurological function, and disruption of Cu delivery due to mutations, dietary deficiency, or chelation can lead to improper mitochondrial function, iron metabolism, and neuronal function (Gaier et al., 2013a; Greenough et al., 2016; Kawahara et al., 2017; Medeiros, 2017; Opazo et al., 2014; Scheiber et al., 2014). In addition, mutations in mitochondrial proteins involved in Cu transport and incorporation in cytochrome c oxidase lead to defects in mitochondrial oxidative phosphorylation and cardiac hypertrophy (Baertling et al.,

2015; Baker et al., 2017b; Boulet et al., 2017; Dodani et al., 2011b; Freisinger et al., 2004; Ghosh et al., 2014; Hlynialuk et al., 2015; Jaksch et al., 2001a; Jaksch et al., 2001b; Leary et al., 2007; Leary et al., 2013; Leary et al., 2004; Pacheu-Grau et al., 2015; Punter et al., 2000; Stiburek et al., 2009; Stroud et al., 2015; Vesela et al., 2004; Yang et al., 2010). In some animal and cellular models of these mutations, Cu supplementation rescues the mitochondrial and cardiac pathologies, illustrating the intimate connection between intracellular Cu and mitochondrial activity (Baertling et al., 2015; Elsherif et al., 2004a; Elsherif et al., 2004b; Ghosh et al., 2014; Jaksch et al., 2001b; Jiang et al., 2007; Johnson and Newman, 2007; Leary et al., 2013; Li et al., 2015a; Medeiros, 2017; Zhou et al., 2008; Zhou et al., 2009).

Because Cu can switch oxidation states, excessive or dysregulated Cu can generate reactive oxygen species and cause oxidative damage intracellularly or extracellularly. Thus, there are several mechanisms for buffering, storing, and removing Cu. When Cu levels exceed the capacity of Cu-storage into vesicles or buffering proteins (e.g. metallothioneins), insufficiently buffered Cu can generate ROS via Fenton chemistry (Baker et al., 2017a; Bhattacharjee et al., 2017; Hatori and Lutsenko, 2016; Hordyjewska et al., 2014; Ohrvik et al., 2017). In mammals, two P-type ATPases, ATP7A and ATP7B, have a prominent role in intracellular Cu homeostasis and transport (Lutsenko, 2016; Migocka, 2015; Yu et al., 2017). Mutations in ATP7A disrupt Cu absorption in the gut and intracellular delivery to Cu-dependent enzymes, resulting in Menkes disease (Kaler, 2014; Lenartowicz et al., 2015; Zlatic et al., 2015). Conversely, mutations in ATP7B transporters, which relocate to the plasma membrane in response to excess intracellular Cu and function in Cu efflux, underlie Wilson's disease. Toxic Cu accumulation in Wilson's disease is the result of dysfunctional ATP7B and inability to pump excess Cu out of cells (Bandmann et al., 2015; Burkhead et al., 2011; Lutsenko, 2014). Neurological manifestations can occur in both diseases, but studies on the neurobiological basis of these symptoms are lacking. Nevertheless, by perturbing Cu regulatory mechanisms or Cu levels directly, studies have demonstrated a physiological role of Cu in modulating the activity of various proteins inside and outside the cell, including signaling pathways.

Exogenous Cu is reported to activate multiple signaling pathways depending on cell or tissue type (Barthel et al., 2007; Chen et al., 2009; Eckers et al., 2009; Mattie et al., 2008b). Many studies have looked into the role of Cu in cancer because of its role in MAPK and hypoxia inducible factor-1α (HIF-1α) signaling, and their relevance in growth factor receptor (EGFR/VEGFR) signaling, angiogenesis and tumor proliferation (Acevedo et al., 2017; Brady et al., 2017; Brady et al., 2014; Calderon-Aparicio et al., 2015; Chen et al., 2006; Chen et al., 2009; Chesi et al., 2016; Denoyer et al., 2015; Gaitanaki et al., 2007; Hassouneh et al., 2007; Henry et al., 2006; Ishida et al., 2013; Kadowaki et al., 2009; Kim et al., 2015; Kumar et al., 2010; Li et al., 2015b; Liu et al., 2016a; Mattie et al., 2008a; Pan et al., 2002; Redman et al., 2003; Rigiracciolo et al., 2015; Tsai et al., 2012; Yee et al., 2017; Yip et al., 2011; Yoo et al., 2012). Importantly, MAPK signaling is not only under circadian control but plays an important role in entrainment of the clock (Antoun et al., 2012; Butcher et al., 2002; Butcher et al., 2005; Butcher et al., 2003; Cao et al., 2015; Dziema et al., 2003; Hainich et al., 2006; Pizzio et al., 2003; Pizzio et al., 2005; Sanada et al., 2000; Sato et al., 2014). On the other hand, several clock proteins interact/modulate HIF-1α activity, including in cancer cells (Choudhry and Harris, 2017; Eckle et al., 2012; Ghorbel et al., 2003; Kobayashi et al., 2017; Peek et al., 2017; Suyama et al., 2016), but the interactions between the clock and HIF-1α are still unclear (Bozek et al., 2007; Bozek et al., 2009; Chilov et al., 2001; Okabe et al., 2014; Yu et al., 2015). Nonetheless, Cu-induced changes in MAPK and HIF-1α activity, as well as other signaling molecules, could affect the clock through changes in clock gene/protein expression and activity. In summary, Cu is involved in metabolic, redox, and signaling aspects of various cells and tissues and tight homeostatic regulation is critical for cellular function.

1.2b Copper in the Brain

Brain Cu concentrations range from 1 to 4 ug/g wet tissue in mice and 5-15 ug/g wet tissue in humans, but there is considerable variability depending on brain region (Genoud et al., 2017; Hare et al., 2012; James et al., 2017; Keen and Hurley, 1979; Lovell et al., 1998; Lutsenko et al., 2010; Magaki et al., 2007; Prohaska, 1987; Pushie et al., 2011; Rajan et al., 1997; Xu et al., 2017; Zatta et al., 2009). In the brain, perturbation of Cu levels leads to complex changes in synaptic

function that are often biphasic (D'Ambrosi and Rossi, 2015; Peters et al., 2011). The effects on synaptic function result from Cu's role in both excitatory and inhibitory transmission, and may differ across brain regions depending on the distribution of neurotransmitter receptors. In hippocampal brain slices, bath-application of Cu blocks long-term potentiation (LTP) (Doreulee et al., 1997); inhibition of hippocampal LTP was further demonstrated by dietary supplementation or chronic injection of Cu *in vivo* (Goldschmith et al., 2005; Leiva et al., 2009). Cu inhibition of excitatory transmission is due to blocking calcium influx either through NMDA receptors (NMDAR) or postsynaptic voltage-gated calcium channels (e.g. L-type) (Doreulee et al., 1997; Gaier et al., 2013a; Leiva et al., 2009; Morera et al., 2003; Schlief et al., 2006; Stys et al., 2012b; Vlachova et al., 1996; You et al., 2012). Many groups have demonstrated inhibition of NMDAR-mediated calcium influx by application of exogenous Cu, while studies using Cu-specific chelators to remove endogenous Cu have provided evidence for a physiological role of Cu in modulating NMDAR activity (Doreulee et al., 1997; Gaier et al., 2013a; Horning and Trombley, 2001; Marchetti, 2014; Marchetti et al., 2013; Schlief et al., 2005; Schlief and Gitlin, 2006; Schlief et al., 2006; Stys et al., 2012b; Trombley and Shepherd, 1996; Vlachova et al., 1996; Weiser and Wienrich, 1996; You et al., 2012).

While total extracellular Cu concentrations (bound + unbound) can range from high nanomolar to low micromolar (rev. Mathie et al., 2006), studies have found a releasable pool of Cu stored in secretory vesicles (Dodani et al., 2011a; Hartter and Barnea, 1988; Kardos et al., 1989). Cu can reach micromolar concentrations (~15µM) within the synaptic cleft and can modulate neurotransmitter signaling and synaptic activity in an ATP7A-dependent manner (Schlief et al., 2005; Schlief et al., 2006). Electrophysiology studies demonstrated that applying low micromolar Cu can inhibit NMDA, AMPA, and GABAa receptor activity (Kumamoto and Murata, 1995; Ma and Narahashi, 1993; Sharonova et al., 1998; Trombley and Shepherd, 1996; Vlachova et al., 1996; Weiser and Wienrich, 1996). A number of Na^+, Ca^{2+} and K^+ channels are also inhibited by micromolar amounts of Cu (Castelli et al., 2003; Horning and Trombley, 2001; Mathie et al., 2006; Morera et al., 2003; Niu et al., 2006), some of which are known to play important roles in SCN neuronal activity rhythms (Itri et al., 2005; Kent and Meredith, 2008; Kim et al., 2005; Meredith et al., 2006). Interestingly, one study has shown a biphasic effect of Cu on

cell firing, where low nanomolar concentrations of Cu increase the firing rates of olfactory epithelial neurons, while ≥1μM Cu decreases firing rates (Aedo et al., 2007). The mechanisms through which Cu inhibits many of the different receptors and ion channels have not been elucidated. However, in some cases, direct oxidization of thiol groups by Cu seems to be involved in the inhibitory effects seen at higher concentrations, as treatment with the reducing agent dithiotreitol blocks or reverses the Cu effects (Aedo et al., 2007; Morera et al., 2003; Weiser and Wienrich, 1996). Cu may also modulate neuronal activity indirectly by S-nitrosylation of NMDAR and other proteins (Schlief and Gitlin, 2006; Schlief et al., 2006).

1.2c Copper and Circadian Clocks

The central goal of this research is to determine whether Cu in the SCN participates in circadian clock functioning. Because Cu's role in biology is pleiotropic, even in the brain there are many possible links between circadian output and Cu homeostasis, some of which are reviewed below. Importantly, this project is the first to explore Cu's role in the SCN.

Increased SCN neuronal activity during the day creates a higher metabolic demand as reflected in upregulation of mitochondrial enzymes and increased mitochondrial activity (Gellerich et al., 2013; Isobe et al., 2011b). Rhythmic activity of cytochrome c oxidase peaks during the day in SCN mitochondrial extracts, which also exhibit increased membrane potential (Isobe et al., 2011b). Cu is essential for normal cytochrome c oxidase function, and hence oxidative phosphorylation, and the TTFL clock may coordinate Cu homeostasis to ensure timely cellular Cu import and/or delivery to mitochondrial proteins. Interestingly, in *Arabidopsis thaliana*, clock proteins have been shown to regulate the expression of Cu import and export proteins in such a way that cytosolic Cu may oscillate also (Penarrubia et al., 2010; Perea-Garcia et al., 2016a; Perea-Garcia et al., 2016b). On the other hand, unanticipated changes in cytosolic and mitochondrial Cu levels may induce responses that feedback on TTFL clock function. In *Arabidopsis*, a Cu-metabolism relationship has been shown to affect circadian output (Andres-

Colas et al., 2010; Perea-Garcia et al., 2016a; Perea-Garcia et al., 2016b). Similarly, exposure to Cu has been shown to affect circadian gene expression in zebrafish (Vicario-Pares et al., 2017).

In a study of Cu transporter expression across different brain regions, ATP7A and ATP7B mRNA are highest in the hypothalamus (Platonova et al., 2005b). Hypothalamic cells also express mRNA for CTR1 (Platonova et al., 2005b). Additionally, in the hypothalamus, ATP7A protein is expressed specifically in neurons and partially present in the plasma membrane, whereas ATP7B is mostly found associated with ependymal cells lining the third ventricle (Platonova et al., 2005a). Interestingly, there is an ATP7B variant selectively expressed in the pineal gland and retina that exhibits circadian expression, with highest mRNA levels during the night (Borjigin et al., 1999). Rhythmic expression in the pineal gland requires SCN circadian clock signaling, and light-dark cycles are needed for both retinal and pineal rhythms to be maintained. In addition, the roles of ATOX1, ATP7A, and ATP7B in Cu homeostasis have been shown to be affected by redox cycling of glutathione (GSH/GSSG) by glutaredoxin-1 (Baker et al., 2017a; Bhattacharjee et al., 2017; Brose et al., 2014; Hatori et al., 2012; Hatori et al., 2017; Hatori and Lutsenko, 2016; Hatori et al., 2016; Lutsenko, 2016; Singleton et al., 2010). Since glutaredoxin utilizes NADPH in GSH redox (Ivarsson et al., 2005; Johansson et al., 2004; Mailloux and Treberg, 2016; Reinbothe et al., 2009), the circadian control and diurnal changes in NADPH as well as (global) glutathiolation levels (Putker et al., 2017; Rey et al., 2016; Wang et al., 2012) raise the possibility of circadian, posttranslational control of Cu homeostatic proteins. These findings point to the possibility of circadian regulation of copper transporters/homeostasis and a putative role in rhythmic SCN neuronal output.

Increases in Cu abundance promote an oxidative environment through increases in ROS generated through Cu participating in Fenton-type reactions and mitochondrial activity. Although acute changes in Cu abundance may be rare physiologically, circadian systems provide a unique possibility that day/night differences in Cu levels could be tied to physiological changes in ROS levels. Intriguingly, structural and *in vitro* studies suggest that the circadian hormone melatonin physically interacts with Cu and can scavenge and protect against Cu-induced free radical generation (Galano et al., 2015; Ghosh et al., 2017; Parmar et al., 2002; Perez-Gonzalez et al.,

2017; Romero et al., 2014). Several antioxidant molecules are under circadian control, including Cu/Zn-dependent superoxide dismutase and glutathione (Wilking et al., 2013), and antioxidant responses are neuroprotective against Cu-induced oxidative stress. Notably, ATP7A dysfunction and resulting Cu dyshomeostasis induces redox imbalances among different cellular compartments, and particularly affects mitochondrial redox state by increased Cu oxidization of GSH (Bhattacharjee et al., 2016). Along similar lines, GSH affects CTR1-mediated Cu import, and conversely, Cu influx affects GSH levels; Cu import and antioxidant status are thus linked to protect against Cu-induced ROS generation/oxidative stress (Bhattacharjee et al., 2017; Chen et al., 2008a; Jazvinscak Jembrek et al., 2014; Kumar et al., 2016; Maryon et al., 2013; Mercer et al., 2016; Ozcelik and Uzun, 2009; Scheiber and Dringen, 2011). Since redox/oxidative stress has been shown to influence various circadian parameters as mentioned in section 1.1, acute changes in intracellular Cu levels may shift the clock through these and other related mechanisms. In brief, Cu-dependent changes in the activity of redox molecules may affect neuronal excitability and TTFL clocks in the SCN.

In summary, studies support three interconnected oscillatory loops, including redox cycles, controlling circadian activity and output. In the SCN, these oscillators function in coordination to generate diurnal neuronal firing rhythms, which in turn directly or indirectly can affect these oscillators. Copper levels are tightly regulated by various homeostatic mechanisms, and changes in Cu concentration affects various processes, often in an inverse or biphasic manner. Cu's roles include neuromodulation at various receptor-ion channels, intracellular signaling pathways such as MAPK/ERK, and as a redox-active element for various metabolic activities. Several cuproproteins, such as Cu transporters and Cu-dependent enzymes, are expressed in the hypothalamus and may have a role in circadian rhythms. Here we are investigating the role of Cu in the SCN circadian clock.

CHAPTER TWO

COPPER CHELATION AND EXOGENOUS COPPER AFFECT RECEPTORS IN THE SUPRACHIASMATIC NUCLEUS

This chapter is adapted for dissertation formatting from the following publication:
Yamada Y, and Prosser RA (2014) Copper chelation and exogenous copper affect circadian clock phase resetting in the suprachiasmatic nucleus *in vitro*. Neuroscience 256: 252-261

2.1 Introduction

In mammals, the suprachiasmatic nucleus (SCN) in the hypothalamus governs circadian rhythms as the master pacemaker. The SCN is responsible for coordinating various peripheral clocks and synchronizing the entire system to environmental light/dark cycles (entrainment) (Welsh et al., 2010). The SCN clock entrains to light/dark cycles via retinohypothalamic innervation from specialized retinal ganglion cells, which release glutamate (Glu) upon light stimulation (de Vries et al., 1993; Ebling, 1996). Glu activation of postsynaptic *N*-methyl-D-aspartate receptors (NMDAR) is the dominant cue for photic entrainment, where influx of Ca^{2+} through NMDAR leads to activation of Ca^{2+} dependent signaling pathways (Ding et al., 1994). Activation of downstream kinases and transcription factors leads to induction of immediate-early genes and clock-associated genes involved in the molecular feedback loops that underlie cellular circadian rhythmicity (Golombek and Rosenstein, 2010; Welsh et al., 2010). These events reset the phase of the SCN circadian clock, and phase-resetting ultimately keeps physiological and behavioral processes coordinated with the environment.

The process of SCN circadian clock phase resetting involves complex cellular mechanisms. One mechanism involves brain-derived neurotrophic factor (BDNF), which activates the tyrosine kinase receptor TrkB (Liang et al., 2000; Liang et al., 1998; Mou et al., 2009). The exact mechanism by which TrkB activation couples with SCN clock phase-resetting is unknown but may involve modulation of NMDAR signaling as shown in other systems (Carreno et al., 2011; Mizuno et al.,

2003). Previous research has found that copper (Cu) can inhibit NMDAR activation and can interact with proteins upstream and downstream of TrkB signaling pathways (Hwang et al., 2007; Thompson et al., 2011; Turski et al., 2012; Vlachova et al., 1996; Weiser and Wienrich, 1996). We set out to investigate potential modulatory effects of Cu in the SCN. To this end, we chose a potent Cu-specific chelator, tetrathiomolybdate (TTM) to decrease bioavailable Cu. TTM has been used in clinical studies of Wilson's disease to reverse the pathological Cu accumulation caused by mutations in the copper transporter ATP7B (Brewer et al., 2003). TTM at nanomolar and low micromolar concentrations is well tolerated in cell culture and can chelate multiple Cu (up to 6) ions (Juarez et al., 2006; Kumar et al., 2010; Lowndes et al., 2009; Zhang et al., 2009b). Here, we report the results of *in vitro* electrophysiological recordings of neuronal activity from SCN brain slices following treatments designed to increase or decrease Cu availability. The results suggest that Cu functions to regulate glutamate signaling and clock resetting in SCN neurons, demonstrating the need for further research on the role of transition metals in SCN circadian clock phase regulation.

2.2 Methods

2.2a Brain slice preparation

Coronal brain slices (500 u) containing the SCN were prepared during the daytime from adult male C57BL/6Nhsd mice (Harlan Laboratories) housed in a 12:12 light/dark cycle. Slices were maintained at the interface of a Hatton-style brain slice chamber as described (Prosser, 1998). Brain slices were continuously perfused with Earle's balanced salt solution (EBSS) supplemented with glucose, bicarbonate and gentamicin at pH 7.4. Both the slice chamber and media reservoir were oxygenated (95% O2 / 5% CO2) and maintained at 37°C. All experimental protocols were approved by the University of Tennessee Knoxville Institutional Animal Care and Use Committee.

2.2b Drug treatments

All treatments were performed on day 1 in vitro. All drugs were prepared in warm, oxygenated EBSS. At the onset of the drug treatments, perfusion of the standard medium was stopped and the medium in the chamber replaced with fresh medium containing the experimental treatments. After 10 min this medium was removed and perfusion with untreated medium was reinstated. Previous experiments have demonstrated that changing the perfusion medium by itself does not affect the phase of the circadian clock (Biello et al., 1997; Gillette and Prosser, 1988; Prosser et al., 1989; Prosser et al., 2003). CuCl2, CoCl2, Glu, NMDAR antagonist AP5 ((2R)-amino-5-phosphonovaleric acid), and AMPA receptor antagonist NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f] quinoxaline-2,3-dione) were purchased from Sigma-Aldrich (St. Louis, MO). TTM and bathocuproine disulfonate (BCS) salts (Sigma-Aldrich) were used to chelate copper. Trk receptor antagonist K252a (EMD Biosciences (Philadelphia, PA)) and TrkB inhibitor, TrkB-Fc (R&D Biosciences (Minneapolis, MN)) were also used in this study. For experiments involving the TrkB inhibitor (TrkB-I), slices were pre-treated for 5 min, followed by a 10 min treatment of TrkB-I in combination with Glu, TTM, or CuCl2.

2.2c Single-unit recording and data analysis

Single-unit recordings were performed on day 2 in vitro for most experiments, and on day 3 in vitro where indicated. The procedure for neuronal recordings has been described previously (Prosser, 1998). Briefly, the spontaneous activity of single SCN neurons was recorded extracellularly using glass capillary microelectrodes filled with 3M NaCl. Each neuron was recorded for 5 min, and the data stored for later determination of firing rate using a DataWave system (Berthoud, CO). Typically, 4–7 cells were recorded during each hour. These individual firing rates were then used to calculate 2 h running averages, lagged by 1 h (± SEM), to obtain a measure of population neuronal activity. As in previous studies (Mou et al., 2009; Prosser, 1998), the time of peak neuronal activity was assessed visually by estimating, to the nearest quarter hour, the time of symmetrically highest activity. For example, if the two highest 2 h means are

equal, then the time of peak is estimated to be halfway between them. Phase shifts were calculated as the difference in time-of-peak of untreated slices vs. drug treated slices. Using these methods, the consistency of the results obtained for each experimental manipulation is such that differences in phase of as little as one hour are often statistically significant with few replicates (Chen et al., 1999; Prosser, 1998). Statistical analyses were performed using Sigmastat (San Jose, CA, USA). One-way ANOVA was used to test for significant differences between treatment and control experiments, followed by Holm-Sidak test for post hoc comparisons.

2.3 Results

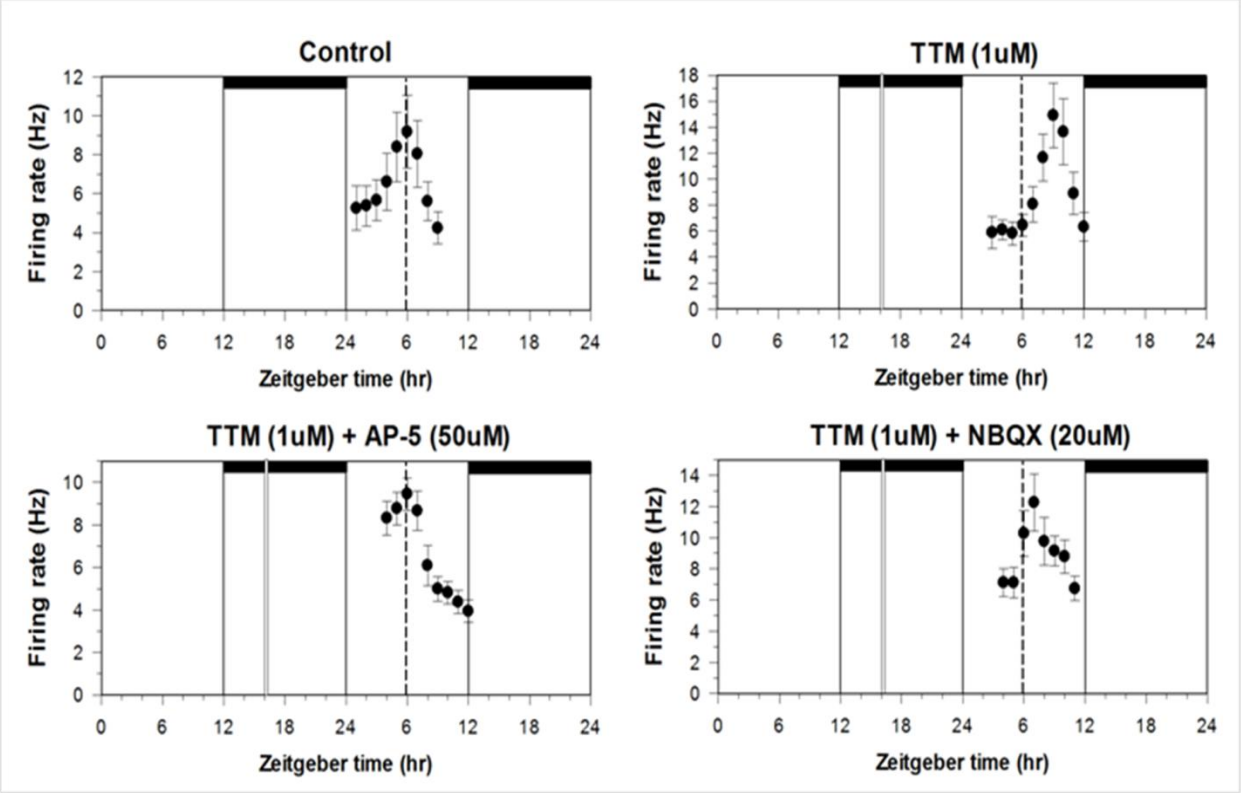
2.3a Copper chelation phase-shifts the SCN circadian clock

In our SCN slice recordings, the population neuronal firing rates of untreated control slices peaked during mid-subjective day on 2 d *in vitro* with a mean (± SEM) time-of-peak of Zeitgeber time (ZT) 6.06 ± 0.07 (n = 4; Fig. 2.1a; ZT 0 = lights-on and ZT 12 = lights-off in the animal colony). These results are consistent with previous studies showing that peak activity occurs around mid-day on days 1-3 *in vitro* (Gillette and Prosser, 1988; Prosser et al., 1989) . To assess the role that Cu plays in the SCN circadian clock, we began by using a high affinity Cu-specific chelator, TTM to reduce available Cu levels in the SCN. Bath application of 1µM TTM at early subjective night (ZT16) induced a significant delay in peak neuronal activity, with a mean time-of-peak occurring at ZT9.0 ± 0.24 (n = 4, $p<0.001$; Fig. 2.1). As expected, the delayed time of peak neuronal activity seen on day 2 *in vitro* reflects a permanent shift of the underlying circadian clock, as we observed a similarly delayed time-of-peak on the third day *in vitro* (mean phase shift: -3.0 h ± 0.14, n = 3; Fig. 2.1b).

The ~3-h phase shifts induced by TTM at ZT16 are similar to those induced by Glu activation of NMDAR (Biello, 2009; Mou et al., 2009). Since Cu is known to inhibit NMDAR activity (Trombley and Shepherd, 1996; Vlachova et al., 1996), we hypothesized that TTM phase-shifts the SCN circadian clock by decreasing Cu inhibition of NMDAR activity. If correct, lower doses of

Figure 2.1. TTM treatment at early-subjective night (ZT16) induces phase-delays

Top: Shown are the 2 h means ± SEM of SCN neuronal activity recorded on day 2 in vitro from control slices and slices treated at ZT16 with the compounds indicated. 10 min treatment with 1µM TTM induces a phase-delay that is blocked by co-application of AP5 and 10µM Cu. NBQX partially blocks the effect of TTM. Horizontal bars: time of lights-off in the animal colony; vertical bars: time of drug treatment; dotted line: mean time-of-peak in control experiments. Bottom: Histogram plot summarizing the mean phase shifts ± SEM resulting from the indicated treatments. Phase delays induced by 1µM TTM occur on day 2 and day 3 in vitro. 10nM TTM does not induce phase delays by itself but induces phase delays in the presence of 1µM Glu. 1µM Glu by itself does not have an effect. One-way ANOVA indicated a significant effect of treatments ($F = 23.23$). Numbers in parentheses indicates number of experiments. *Phase delays significantly different from (untreated) control slices based on post hoc (Holm-Sidak) test ($P < 0.01$). Delays are plotted as negative values. ZT, zeitgeber time.



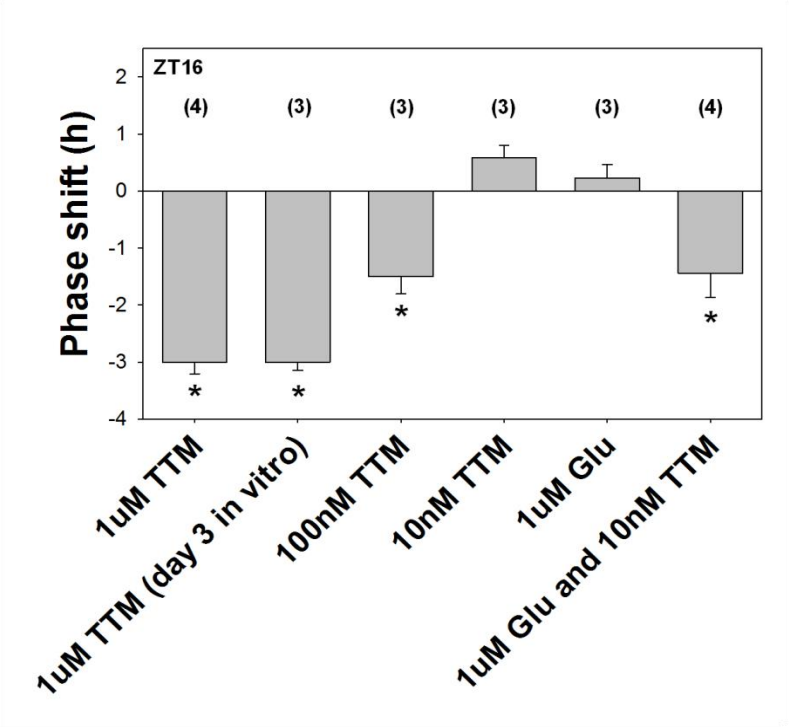


Figure 2.1 continued

TTM would be less effective at removing this inhibition. Consistent with this, a 10-fold lower concentration of TTM (100nM) also induced phase delays but of a smaller magnitude (mean phase shift of -1.50 h ± 0.31, *n* = 4, p<0.01; Fig. 2.1b), while a 100-fold concentration (10nM) did not induce significant changes (mean phase shift: 0.58 h ± 0.22, *n* = 3). This theory also assumes that there is a low concentration of endogenous Glu, below what is needed to activate NMDAR until the inhibition by Cu is removed by TTM. On-going studies in our lab have demonstrated that 10µM Glu and higher concentrations cause ~3-h phase delays when applied at ZT16 (e.g., see Fig. 2.3), but 1µM Glu does not cause phase delays (mean phase shift: 0.23 h ± 0.24, *n* = 3; Fig. 2.1b). However, a combined treatment of 10nM TTM and 1µM Glu causes phase delays (mean phase shift: -1.44 ± 0.43, *n* = 4, p<0.01; Fig. 2.1b), suggesting a synergistic effect in activating NMDARs.

2.3b TTM-induced phase shifts depend on NMDAR and TrkB receptor activity

To further investigate whether TTM-induced phase delays involve NMDAR and other relevant receptors, we used various antagonists in combination with TTM. In support of our hypothesis, co-application of the NMDAR antagonist AP5 (50µM) blocked TTM-induced phase delays (mean phase shift: -0.17 h ± 0.44, *n* = 3; Fig. 2.2). Previous reports indicate that AMPA receptors participate in photic phase shifts upstream of NMDAR, likely by removing the magnesium block of NMDAR channels (Mintz et al., 1999; Mizoro et al., 2010). Consistent with this, the AMPA receptor antagonist NBQX (20µM) attenuated TTM-induced phase delays (mean phase shift: 0.83 h ± 0.44, *n* = 3; Fig. 2.2). Neither AP5 nor NBQX affected the phase of SCN neuronal activity rhythms when applied alone to the brain slices (mean phase shifts, respectively: -0.25 h ± 0.38, *n* = 3; -0.17 h ± 0.08, *n* = 3; data not shown).

Glu-induced phase shifts also require activation of TrkB receptors by its ligand BDNF (Allen et al., 2005; Liang et al., 2000; Mou et al., 2009). We therefore used the non-selective Trk receptor inhibitor K252a to test whether the TrkB receptor is similarly involved in TTM-induced phase delays. Co-treatment with K252a (1µM) blocked TTM-induced phase delays (mean phase shift: 0.33 h ± 0.72, *n* = 3; Fig. 2.2). To further test TrkB receptor involvement, we used a TrkB

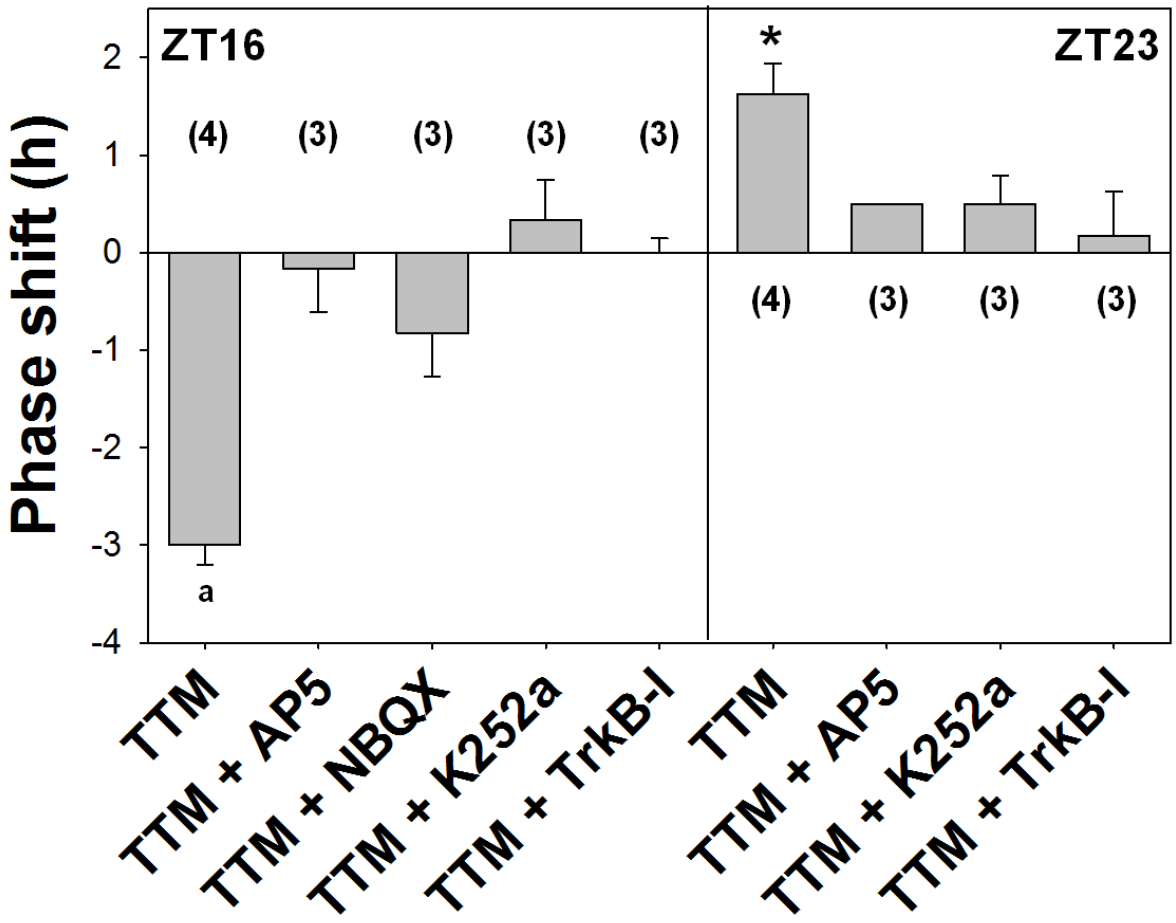


Figure 2.2. TTM-induced phase shifts require NMDA and TrkB signaling

Histogram plot summarizing the mean phase shifts ± SEM resulting from the indicated treatments, where TTM concentration is 1µM. ZT16: TTM-induced phase delays are blocked by AP5, NBQX, K252a, and TrkB-I (inhibitor). ZT23: TTM induces phase advances that are blocked by AP5, K252a, and TrkB-I. One-way ANOVA indicated a significant effect of treatments ($F = 18.31$). *Phase delays significantly different from control ($p<0.05$). [a]Data repeated from Fig. 2.1 for clarity.

fusion protein (TrkB-I) to bind BDNF and inhibit activation of TrkB signaling selectively. We found that TTM-induced phase delays were also blocked by TrkB-I (1µg/mL) co-treatment (mean phase shift: 0.0 h ± 0.14, *n* = 3; Fig. 2.2), Analogous to previous results from our lab with K252a (Mou et al., 2009), TrkB-I did not cause phase delays when applied alone (mean phase shift: -0.08 h ± 0.55, *n* = 3; data not shown). We also confirmed that TrkB-I treatment inhibits Glu-induced phase delays (mean phase shift: 0.25 h ± 0.25, *n* = 3; data not shown).

Glu activation of NMDAR during late subjective night (ZT23) causes ~2-3 h phase advances through somewhat different intracellular mechanisms from ZT16 (Mou et al., 2009). Therefore, we investigated whether TTM applied to SCN brain slices at ZT23 mimics the phase-advancing effects of late-night Glu applications. We determined that 1µM TTM induced phase advances when applied to SCN brain slices at ZT 23 (mean phase shift: 1.63 h ± 0.31, *n* = 4, $p<0.05$; Fig. 2.2). Together, these results indicate that Cu chelation can induce photic-like phase delays and phase advances when applied to the SCN *in vitro*.

Furthermore, we found that the phase advances induced by TTM application at ZT 23 were blocked by co-treatment with AP5 (mean phase shift: 0.5 h ± 0.0, *n* = 3), K252a (mean phase shift: 0.58 h ± 0.22, *n* = 3), or TrkB-I (mean phase shift: 0.17 h ± 0.46, *n* = 3; Fig. 2.2), analogous to the results of our ZT16 experiments. Together, the results suggest that TTM-induced phase shifts at both ZT16 and ZT23 are dependent on NMDAR and TrkB receptor activity in a manner similar to Glu-induced phase shifts.

2.3c Cu application phase-shifts the SCN circadian clock

To confirm whether TTM-induced phase delays result from Cu depletion, we used a different Cu chelator, bathocuproine sulfonate (BCS). Bath-application of 10µM BCS caused phase delays similar to TTM (mean phase shift: -2.67 h ± 0.44, *n* = 3, $p<0.001$; Fig. 2.3). Furthermore, co-application of 10µM Cu with TTM prevented TTM-induced phase delays (mean phase shift: -0.25 h ± 0.20, *n* = 3; Fig. 2.3), whereas 10µM cobalt was unable to block the effect

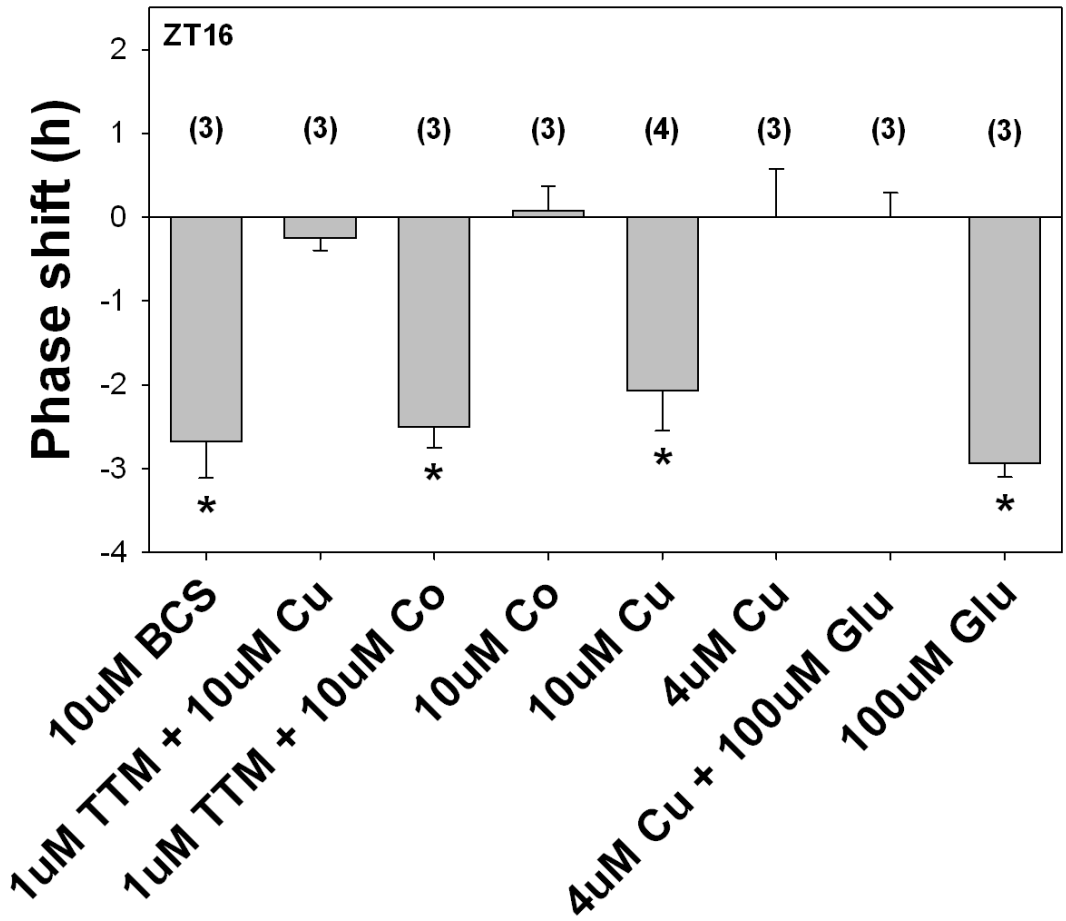


Figure 2.3. 10µM Cu induces phase delays at ZT16

Histogram plot summarizing the mean phase shifts ± SEM resulting from the indicated treatments. 10µM of the Cu chelator, bathocuproine sulfonate (BCS), induces phase delays similar to TTM. Co-application of 1µM TTM with 10µM Cu results in no phase delays, but the combination of TTM with 10µM Co2+ causes phase delays.10 min treatment with 10µM Cu induces phase delays, but 4µM Cu does not. However, 4µM Cu inhibits Glu (100µM)-induced phase delays, resulting in no shift. One-way ANOVA indicated a significant effect of treatments ($F = 14.97$); *$p<0.05$ (compared to control).

of TTM (mean phase shift: -2.25 h ± 0.25, *n* = 3, p<0.001; Fig. 2.3). Application of cobalt alone did not have an effect (mean phase shift: 0.08 h ± 0.22, *n* = 3; Fig. 2.3). Thus, two Cu chelators induce phase delays, while saturating TTM with excess Cu inhibits TTM-induced phase delays.

Next, we investigated the effects of Cu treatment on the SCN circadian clock phase. First, we applied the same concentration of Cu (10µM) as that which inhibited TTM-induced phase delays at ZT16. Interestingly, treatment with 10µM Cu alone at ZT16 resulted in phase delays with a mean shift of -2.06 h ± 0.07 (*n* = 4, p<0.01; Fig. 2.3). Given that TTM and Cu applied individually both induced phase delays, the lack of phase shift induced by the combined treatment reported above led us to explore Cu effects in more detail. Based on the maximum estimated 6:1 binding ratio of Cu to TTM, a combined treatment of 10µM Cu and 1µM TTM could result in a net increase in Cu levels of 4µM. Therefore, we applied 4µM Cu to investigate the dose-dependency of Cu-induced phase delays. As shown in Fig. 2.3, this concentration of Cu had no effect on the time of peak neuronal activity in the SCN (mean phase shift: 0.0 h ± 0.58, *n* = 3). These results suggest that with the combined TTM/Cu treatment, a portion of the Cu binds to and saturates the chelation capacity of TTM, while the remaining Cu is insufficient to induce a phase shift on its own.

Based on previous reports suggesting that Cu can inhibit Glu signaling (Vlachova et al., 1996; Weiser and Wienrich, 1996), next we tested whether Cu could inhibit Glu-induced phase delays at ZT 16. Since 10µM Cu alone causes phase delays, for these experiments we needed to use the lower (4µM) concentration of Cu. We also used a lower concentration of Glu (100µM) that still induces a robust phase shift (mean phase shift: -2.9 $\pm$ 0.17 h, *n* = 3, p<0.001; Fig 2.3), to increase the potential of seeing an inhibition. Co-application of 4 µM Cu and 100µM Glu induced no phase shift (mean phase shift: -0.0 h ± 0.29, *n* = 3; Fig. 2.3), demonstrating that, while 4µM Cu applied alone has no phase-shifting effect, it nonetheless is able to inhibit Glu-induced phase delays**.** These results demonstrate that the effects of exogenous Cu on SCN clock phase regulation are highly dependent on its concentration, with lower levels capable of inhibiting Glu-induced phase shifts and higher levels capable of mimicking Glu-induced phase shifts.

Importantly these results are also consistent with our hypothesis that TTM-induced phase shifts involve chelation of endogenous Cu.

2.3d Cu-induced phase shifts are not NMDAR dependent

To begin exploring the cellular mechanisms underlying 10µM Cu-induced phase shifts, we first tested whether Cu-induced phase delays involve either NMDA or TrkB receptors. We found that Cu-induced phase delays were not inhibited by either AP5 (mean phase shift: -1.75 h ± 0.18, n = 3, $p<0.05$) or NBQX (mean phase shift: -2.5 h ± 0.38, n = 3, $p<0.001$; Fig 2.4). In contrast, Cu-induced phase delays were inhibited by K252a (mean phase shift: -0.38 h ± 0.28, n = 3) and TrkB-I (mean phase shift: 0.33 ± 0.22, n = 3; Fig 2.4).

Similar to our results at ZT 16, treatment with 10µM Cu at ZT23 induced phase advances (mean phase shift: 1.56 h ± 0.36, n = 4, $p<0.05$; Fig. 2.4) that were not sensitive to AP5 (mean phase shift: 1.92 h ± 0.55, n = 3, $p<0.05$; Fig. 2.4). However, unlike our results at ZT 16, neither K252a co-application nor the more specific TrkB-I inhibitor blocked Cu-induced phase advances (mean phase shift, respectively: 2.17 h ± 0.60, n = 3, $p<0.01$ and 1.69 ± 0.45, n = 4, $p<0.05$; Fig. 2.4). The above data suggest that the mechanisms involved in Cu-induced phase shifts are different from TTM-induced phase shifts. Moreover, the mechanisms associated with Cu-induced phase delays and phase advances also appear to be different.

2.4 Discussion

These experiments are the first to demonstrate that SCN circadian clock phase is affected by *in vitro* treatments that either increase or decrease Cu levels. These data implicate endogenous Cu in the regulation of mammalian circadian rhythms. The concentrations of physiologically relevant transition metals (Cu, Zn, Fe) have been shown to vary between brain regions, and these metals have diverse tissue-specific functions. Little is known about their

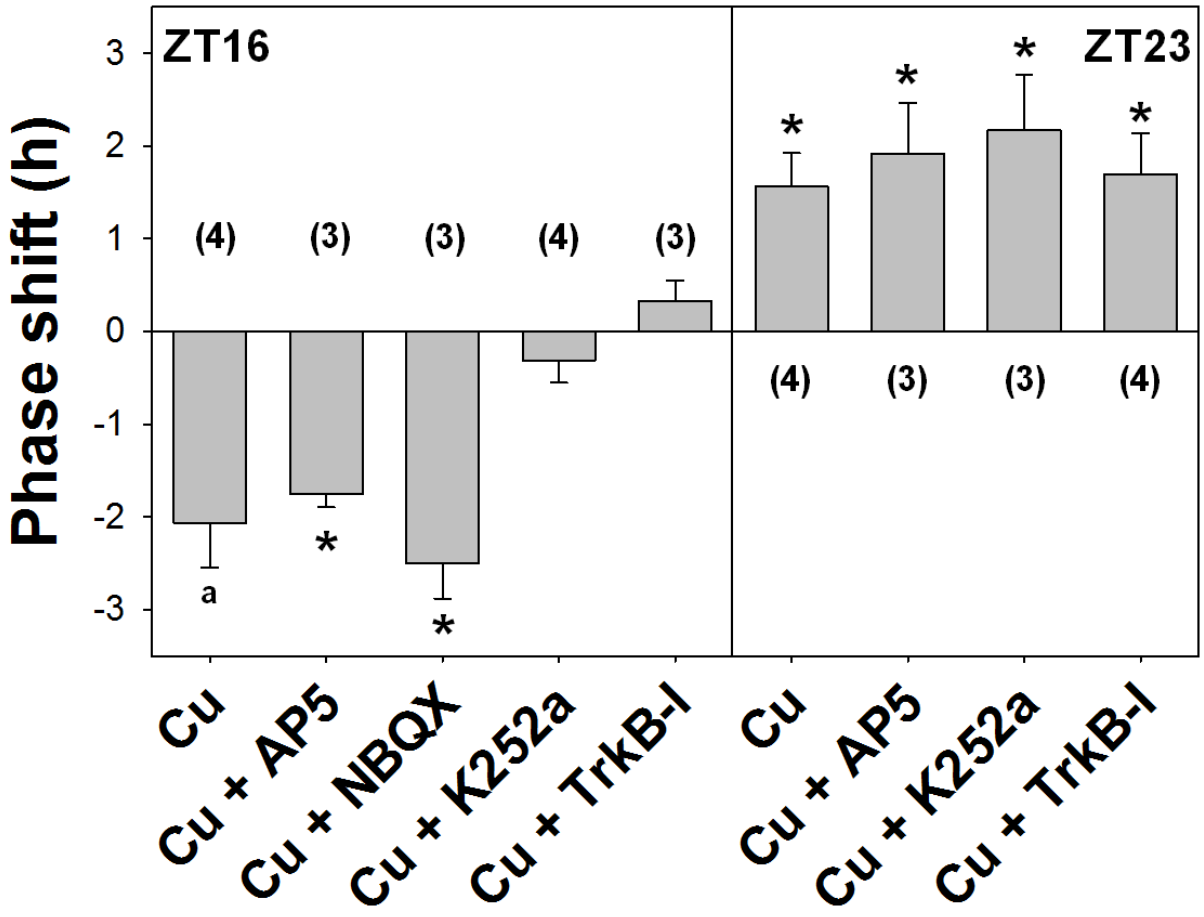


Figure 2.4. Cu-induced phase shifts do not depend on NMDAR signaling

Histogram plot summarizing the mean phase shifts ± SEM resulting from the indicated treatments. ZT16: Neither AP5 nor NBQX affects Cu-induced phase delays, whereas K252a and TrkB-I inhibited the effect of Cu. ZT23: 10µM Cu causes phase advances, but AP5, K252a, nor TrkB-I blocked these phase advances. One-way ANOVA indicated a significant effect of treatments ($F = 19.93$). *Phase advances significantly different from (untreated) control slices ($P < 0.05$). Advances are plotted as positive values. [a]Data repeated from Fig. 2.3 for clarity.

functions in circadian biology. By investigating the phase-shifting effects of TTM and Cu, this study provides the groundwork for future research regarding the role of Cu in modulating Glu signaling in the SCN and in turn, whether the SCN clock regulates Cu availability in SCN cells and other brain areas.

Brain Cu concentrations range from 1 to 4 ug/g wet tissue in mice and 5-15 ug/g wet tissue in humans, but there is considerable variability depending on brain region (Keen and Hurley, 1979; Lovell et al., 1998; Lutsenko et al., 2010; Magaki et al., 2007; Prohaska, 1987; Rajan et al., 1997; Zatta et al., 2009). Because high Cu concentrations can be toxic to cells, most cellular Cu is restricted by binding to storage/buffering proteins, as well as through Cu transport. Total extracellular Cu concentrations (bound + unbound) can range from high nanomolar to low micromolar (rev. Mathie et al., 2006). Total intracellular Cu concentration is thought to range similarly from nanomolar to micromolar (Balamurugan and Schaffner, 2006). However, the intracellular concentration of unbound Cu is virtually zero under homeostatic conditions due to an abundance of proteins that can bind Cu and respond to sudden influxes (Hung et al., 2010).

In hypothalamic and hippocampal neurons, studies have found a releasable pool of Cu stored in vesicles (Dodani et al., 2011a; Hartter and Barnea, 1988; Kardos et al., 1989). Two P-type ATPases, ATP7A and ATP7B, are responsible for intracellular Cu transport. Mutations in ATP7A disrupt Cu absorption and delivery to Cu-dependent enzymes, resulting in symptoms of Cu deficiency (as occurs in Menkes disease). Mutations in ATP7B result in Wilson's disease, marked by excess Cu accumulation in tissues. Neurological manifestations can occur in both diseases, but studies on the neurobiological roles of ATP7A/B in general are lacking. In response to excess intracellular Cu, these transporters relocate to the plasma membrane and function in Cu efflux in different tissues. In neurons, Cu can reach micromolar concentrations (~15µM) within the synaptic cleft and can modulate neurotransmitter signaling and synaptic activity in an ATP7A-dependent manner (Schlief et al., 2005; Schlief et al., 2006). In a study of Cu transporter expression across different brain regions, ATP7A and ATP7B mRNA is highest in the hypothalamus (Platonova et al., 2005b). Hypothalamic cells also express mRNA for the Cu import protein, copper transporter 1, although protein levels were not reported (Platonova et al., 2005b). In a

recent proteomic study of SCN tissue, rhythmic expression of the Cu chaperone and transporter ATOX1 was reported (Lee et al., 2013); ATOX1 interacts with ATP7A and appears to be required for normal Cu efflux (Hamza et al., 2003; Hamza et al., 1999) Additionally, in the hypothalamus, ATP7A protein is expressed specifically in neurons and partially present in the plasma membrane, whereas ATP7B is mostly found associated with ependymal cells lining the third ventricle (Platonova et al., 2005a).

In hippocampal neurons, ATP7A has been found to traffic to the plasma membrane in response to KCl-induced depolarization—as well as in response to NMDA or Glu treatment—an event accompanied by an increase in Cu efflux (Schlief et al., 2005; Schlief et al., 2006). Both KCl- and NMDAR-induced re-localization of ATP7A can be blocked by NMDAR antagonists or Ca^{2+} chelation (Schlief et al., 2005). In a more recent study, the link between Ca^{2+} signaling and Cu efflux in hippocampal neurons was further confirmed using both Cu^{2+}-specific fluorescent sensors and X-ray fluorescence microscopy (Dodani et al., 2011a). Cu release in response to Glu is postsynaptic and is thought to modulate postsynaptic neuronal activation. Importantly, our data are the first to functionally link Cu and NMDA signaling in the hypothalamus. It is tempting to speculate that, as in the hippocampus, vesicular release of Cu by SCN neurons may modulate NMDAR activity. Because Glu is released by retinal ganglion cells when they are stimulated by light, high daytime Glu activation of NMDAR may increase ATP7A localization to the plasma membrane of SCN neurons and enhance synaptic release of Cu, which could dampen NMDA receptor activity. In fact, a similar idea has been proposed for *Arabidopsis thaliana*, where cytosolic Cu levels may oscillate in a circadian fashion as a result of feedback loops regulating the expression and activity of Cu transporters (Penarrubia et al., 2010).

Interestingly, there is an ATP7B variant selectively expressed in the pineal gland and retina that exhibits circadian expression, with highest mRNA levels during the night (Borjigin et al., 1999). Rhythmic expression in the pineal gland requires SCN circadian clock signaling, while light-dark cycles are needed for both retinal and pineal rhythms to be maintained. Using organotypic pineal cell culture, the authors also showed that activation of β-adrenergic receptors or treatment with a cAMP analog can induce transcription of the ATP7B variant (Borjigin et al.,

1999). Together, these studies raise the possibility that cytosolic Cu levels and proper delivery of Cu to cuproproteins may be regulated in a circadian fashion in multiple endogenous oscillators in a manner that reflects day-night variations in metabolic demands. Thus, it will be important to determine whether the Cu transporters described above are found in the SCN, and assess whether cytosolic Cu levels oscillate in SCN cells.

Early electrophysiology studies demonstrated that applying low micromolar Cu can inhibit NMDA, AMPA, and GABAa receptor activity (Kumamoto and Murata, 1995; Ma and Narahashi, 1993; Sharonova et al., 1998; Trombley and Shepherd, 1996; Vlachova et al., 1996; Weiser and Wienrich, 1996). A number of Na^+, Ca^{2+} and K^+ channels are also inhibited by micromolar amounts of Cu (Castelli et al., 2003; Horning and Trombley, 2001; Mathie et al., 2006; Morera et al., 2003; Niu et al., 2006), some of which are known to play important roles in SCN neuronal activity rhythms (Itri et al., 2005; Kent and Meredith, 2008; Kim et al., 2005; Meredith et al., 2006). Interestingly, one study has shown a biphasic effect of Cu on cell firing, where low nanomolar concentrations of Cu increase the firing rates of olfactory epithelial neurons, while $\geq$1μM Cu decrease firing rates (Aedo et al., 2007). As the physiological effects of Cu on postsynaptic receptors are dependent on Cu concentration and localization, speculation on how Cu modulates the activity of the various receptors in the SCN *in vivo* is difficult. Based on the results presented here, however, we hypothesize that there are diurnal changes in the effects of extracellular Cu and the overall function of Cu in the SCN.

The mechanisms through which Cu inhibits many of the different receptors and ion channels have not been elucidated. However, in some cases, direct oxidization of thiol groups by Cu seems to be involved in the inhibitory effects seen at higher concentrations, as treatment with the reducing agent dithiotreitol blocks or reverses the Cu effects (Aedo et al., 2007; Morera et al., 2003; Weiser and Wienrich, 1996). Cu may also modulate activity indirectly by S-nitrosylation of NMDAR and other proteins (Schlief and Gitlin, 2006; Schlief et al., 2006), a mechanism reported to be important in SCN rhythmicity (Riccio et al., 2006). Conversely, a recent study using hippocampal neurons (You et al, 2012) together with earlier reports (Martin et al, 1971; Vlachova et al 1996) suggests that Cu inhibition of NMDA signaling could involve an interaction with

glycine/D-serine. Clearly, the mechanism through which Cu acts in the SCN deserves further investigation.

In the present study, we used TTM to investigate whether endogenous Cu modulates SCN circadian rhythms. TTM is able to compete with high affinity cuproproteins (e.g. metallothionein) for Cu and is suggested to have sub-nanomolar affinity (Lowndes et al., 2009; Ogra et al., 1996). To evaluate whether the effects of TTM are specifically attributable to Cu chelation, we tested a second Cu chelator, BCS. Since BCS also caused phase delays when applied at ZT 16, this strongly indicates that it is the removal of Cu that causes the phase delays. Consistent with this, combining Cu and TTM at a 10:1 ratio (which would prevent chelation of endogenous Cu) resulted in no phase delay. Conversely, cobalt had no effect on TTM-induced phase delays. Since 10nM TTM did not induce phase delays, this suggests that there is enough Cu in the slice to saturate and block the effect of 10nM TTM. However, when combined with a concentration of Glu (1µM) that by itself does not induce phase delays, we observed an additive effect of 10nM TTM and 1µM Glu. These results suggest that TTM does chelate Cu at this low nanomolar concentration and thereby increases the sensitivity of SCN neurons to Glu.

Our results suggest that TTM induces both phase advances and phase delays by relieving Cu antagonism of NMDAR activity, as the NMDAR antagonist AP5 blocked the early- and late-night effects of TTM while low (4µM) concentrations of Cu inhibit Glu-induced phase shifts. The results from NBQX experiments suggest that AMPA receptors also contribute to TTM effects. Since TrkB receptor activation is necessary for Glu-induced phase shifts, the K252a and TrkB-I inhibition of TTM-induced phase shifts is also consistent with Cu modulation of NMDAR activity (Liang et al., 2000; Michel et al., 2006; Mou et al., 2009).

Additionally, our results raise the question whether TTM-induced phase shifts require endogenous Glu in our SCN slices. One study demonstrated that on average, ~200 picomoles (per 15 minutes) of Glu is released at early night in rat SCN slices, and application of 1µM NMDA increased Glu release (Hamada et al., 1998). Furthermore, the authors used enucleated rats to demonstrate that spontaneous Glu release does not require retinal ganglion cells and intact retinohypothalamic input to SCN neurons. If TTM chelation of Cu leads to increased NMDAR

activation, then this may stimulate additional release of Glu in the SCN slice and enhance NMDAR activity even further. Our data showing that 10nM TTM enabled 1µM Glu to induce phase delays support such a model. Moreover, 100nM TTM alone induced small phase delays while 1µM Glu treatment did not. This suggests that there is enough Cu present in the SCN to block NMDAR activation by 1µM Glu treatment in addition to endogenous Glu, but upon sufficient removal of Cu inhibition, endogenous Glu alone is capable of phase-shifting the clock. Whether TTM effects require endogenous Glu and whether Cu chelation increases Glu release in the SCN remains untested. Altogether, our experiments with TTM suggest an important role of Cu in modulating glutamatergic tone in the SCN, and Cu's potential *in vivo* role in modulating retinohypothalamic innervation warrants further investigation.

The data regarding Cu-induced phase shifts is less straight-forward. One study using cultured cortical neurons demonstrated that 10µM Cu significantly increases phosphorylation of Trk receptors in a matrix metalloproteinase dependent manner (Hwang et al., 2007). Because K252a and TrkB-I blocked Cu-induced phase delays at ZT16, it will be important to determine whether Cu increases TrkB phosphorylation in the SCN. However, neither K252a nor TrkB-I blocked Cu-induced phase advances at ZT23. This suggests that the effects of Cu at ZT23 involve distinct cellular mechanisms from those at ZT 16. On first glance, it is surprising that both Cu removal and Cu addition induced photic-like phase shifts. However, Cu has been shown to have biphasic effects at low nanomolar versus low micromolar concentrations (Aedo et al., 2007; Peters et al., 2011). This is similar to the modulatory activities of zinc (II), which have also been shown to have biphasic effects at low and high concentrations (Blakemore and Trombley, 2004; Lorca et al., 2011; Tian et al., 2010). The involvement of glycine and/or glycine receptors in these biphasic effects have been demonstrated for both Cu and zinc (Doi et al., 1999; Miller et al., 2005; You et al., 2012; Zhang and Thio, 2007), and glycine itself has biphasic actions in the SCN (Ito et al., 1991; Mordel et al., 2011). Hence, the differences in effects of Cu chelation versus Cu application are likely to be pleiotropic and complex.

Evolutionarily, the effects of copper removal and addition might relate to the need for SCN cells to tightly regulate copper levels. Cu is required for normal metabolic activity of cells,

and both copper excess and copper deficiency are toxic or fatal to most cells, a fact brought especially to bear in Menkes' and Wilson's disease patients. Further, there is precedence for a Cu-metabolism relationship affecting some circadian parameters, as seen in *Arabidopsis* (Andres-Colas et al., 2010). In any case, our experiments demonstrate that the mechanisms involved in TTM-induced phase shifts differ to some extent from Cu-induced phase shifts: TTM-induced phase shifts involve activation of NMDA signaling, while Cu affects circadian rhythms independent of Glu receptor signaling. In summary, our data demonstrating that both increasing and decreasing Cu levels can affect the phase of the SCN circadian clock lays the foundation for future research on the function of Cu in the SCN circadian clock.

CHAPTER THREE

COPPER CHELATION AND EXOGENOUS COPPER AFFECT INTRACELLULAR SIGNALING IN THE SCN

3.1 Introduction

Copper is an essential trace element involved in energy production and cellular redox activities. Depending on cellular location and protein coordination, copper (Cu) switches oxidation state (Cu2+/+) making it uniquely important as an essential cofactor of several enzymes, such as cytochrome c oxidase in the mitochondrial electron transport chain (Gaier, Eipper et al. 2013, Scheiber, Mercer et al. 2014). Because of the redox activity of Cu, however, excessive and/or dysregulated Cu can generate reactive oxygen species and cause oxidative damage intracellularly or extracellularly. On the other hand, systemic Cu deficiency has moderate to severe effects on development and neurological function, as seen in Menkes disease, while disruption of Cu delivery due to mutations, dietary deficiency, or chelation can lead to improper mitochondrial function, iron metabolism, and neuronal function (Gaier, Eipper et al. 2013, Scheiber, Mercer et al. 2014). Thus, Cu homeostasis requires highly regulated, intricate mechanisms, many of which are evolutionarily conserved from yeast to humans and yet poorly understood in the brain.

By perturbing Cu regulatory mechanisms or Cu levels directly, studies have demonstrated a physiological role of Cu in modulating the activity of various proteins inside and outside the cell. By knocking out copper transporter 1 (CTR1) or by chelation of extracellular Cu, diminishing Cu influx decreases phosphorylation and activation of the MAPKs, extracellular signal-regulated kinase1/2 (ERK1/2), possibly through a direct mechanism (Chen, Lan et al. 2009, Tsai, Finley et al. 2012, Turski, Brady et al. 2012, Brady, Crowe et al. 2014). The activator of ERK1/2, MAPK/ERK kinase1 (MEK1) has been shown to bind Cu with fentomolar or tighter affinity, and MEK1 phosphorylation of ERK increases in the presence of Cu (Turski, Brady et al. 2012, Brady, Crowe

et al. 2014). In addition, exogenous Cu is reported to activate other signaling pathways depending on cell or tissue type probably as a result of oxidative stress due to excess or dysregulation of free Cu (Barthel, Ostrakhovitch et al. 2007, Mattie, McElwee et al. 2008, Chen, Lan et al. 2009, Eckers, Reimann et al. 2009). Thus Cu modulation of signal transduction may occur through different mechanisms and have different roles depending on tissue.

In the brain, perturbation of Cu levels leads to complex changes in synaptic function that are often biphasic. The complex effects of Cu illustrate its role in excitatory and inhibitory transmission, and they depend on the neurotransmitter receptors and their distribution as well as the cellular context or brain region. In hippocampal neurons, bath-application of Cu in hippocampal slices blocks LTP (Doreulee, Yanovsky et al. 1997), and inhibition of hippocampal LTP was further demonstrated by diet or chronic injection of Cu *in vivo* (Goldschmith, Infante et al. 2005, Leiva, Palestini et al. 2009). Cu inhibition of excitatory transmission is due to blocking either calcium influx through NMDA receptors (NMDAR) or postsynaptic voltage-gated calcium channels (e.g. L-type) (Vlachova, Zemkova et al. 1996, Doreulee, Yanovsky et al. 1997, Morera, Wolff et al. 2003, Schlief, West et al. 2006, Leiva, Palestini et al. 2009, Stys, You et al. 2012, You, Tsutsui et al. 2012, Gaier, Eipper et al. 2013). Many groups have demonstrated inhibition of NMDAR-mediated calcium influx by exogenous Cu, and moreover, use of Cu-specific chelators to remove endogenous Cu has provided evidence for a physiological role of Cu in modulating NMDAR activity (Trombley and Shepherd 1996, Vlachova, Zemkova et al. 1996, Weiser and Wienrich 1996, Doreulee, Yanovsky et al. 1997, Horning and Trombley 2001, Schlief, Craig et al. 2005, Schlief and Gitlin 2006, Schlief, West et al. 2006, Stys, You et al. 2012, You, Tsutsui et al. 2012, Gaier, Eipper et al. 2013, Marchetti, Baranowska-Bosiacka et al. 2013, Marchetti 2014). Furthermore, our studies of the suprachiasmatic nucleus (SCN) using hypothalamic slices suggest a role for endogenous Cu in modulating NMDAR-mediated effects (Yamada and Prosser 2014).

The SCN in the hypothalamus is the master clock governing mammalian circadian rhythms in coordination with external signals, where the light/dark cycle is the dominant environmental cue or zeitgeber (“time-giver”) (Albers et al., 2017; Evans, 2016; Golombek and Rosenstein, 2010; Herzog et al., 2017; Welsh et al., 2010). Light-induced NMDAR signaling to the SCN occurs via

axons from retinal ganglion cells, which release glutamate (Glu) and other neuromodulatory factors onto SCN neurons (Baver et al., 2008; Berson et al., 2002; de Vries et al., 1993; Ding et al., 1994; Doyle et al., 2008; Drouyer et al., 2007; Ebling et al., 1991; Hattar et al., 2002; Mikkelsen et al., 1995; Mintz and Albers, 1997; Mintz et al., 1999; Tsai et al., 2009; Vindlacheruvu et al., 1992; Wong et al., 2007). During the night, Glu activation of NMDAR shifts the phase of SCN rhythmic activity by resetting the clocks of SCN neurons ("phase shifts"), which requires calcium-dependent signal transduction to activate transcription and translation of various molecules (Aguilar-Roblero et al., 2007; Albers et al., 2017; Asai et al., 2001; Colwell, 2000, 2001; de Vries et al., 1994; Ebling et al., 1991; Fukushima et al., 1997; Golombek and Rosenstein, 2010; Hamada et al., 1999; Mintz and Albers, 1997; Mintz et al., 1999; Tischkau et al., 2003a; Tominaga et al., 1994; Vindlacheruvu et al., 1992; Watanabe et al., 1994). Resetting the SCN clock phase during the early night results in phase delays, while phase resetting during the late night results in phase advances. This is because different signaling molecules are involved in early or late night effects downstream of calcium influx and calcium-mediated nitric oxide synthase (NOS) activation (Agostino et al., 2004; Ding et al., 1994; Ding et al., 1997; Ferreyra and Golombek, 2001; Fukushima et al., 1997; Golombek et al., 2004; Golombek and Rosenstein, 2010; Harrington et al., 1999; McNulty et al., 1998; Plano et al., 2012; Watanabe et al., 1994). Phase delays require ryanodine receptor activation and release of intracellular calcium from the ER, whereas phase advances require activity of soluble guanylyl cyclase and cGMP-dependent protein kinase (PKG) (Aguilar-Roblero et al., 2007; Aguilar-Roblero et al., 2016; Ding et al., 1998; Ferreyra et al., 1998; Ferreyra and Golombek, 2001; Golombek et al., 2004; Mathur et al., 1996; Mercado et al., 2009; Oster et al., 2003; Plano et al., 2012; Prosser et al., 1989; Tischkau et al., 2004; Tischkau et al., 2003b; Weber et al., 1995). However, both night-time phase shifts involve MEK activity and phosphorylation of ERK1/2 (Antoun et al., 2012; Butcher et al., 2002; Butcher et al., 2005; Cao et al., 2015; Cao et al., 2008; Coogan and Piggins, 2003; Dziema et al., 2003; Hainich et al., 2006). Since Cu may be required for MEK activity and ERK signaling, here we investigate the role of various signaling pathways in the SCN mediating the effects of exogenous Cu and chelation of endogenous Cu in phase-shifting the SCN clock.

Previously we have shown that bath-application of 10µM Cu alone, but not 4µM Cu, phase-shifts SCN neuronal activity rhythms. Interestingly, 4µM Cu inhibits co-applied Glu from inducing NMDAR-mediated phase shifts, and furthermore, chelation of endogenous Cu induces NMDAR-mediated phase shifts without Glu co-treatment. These results suggest biphasic effects, where too little or too much Cu activates different signaling pathways. We hypothesize that: excess Cu induces MAPK-dependent phase shifts as it relates to oxidative stress signaling; however Cu under homeostatic control regulates synaptic signaling and functions to maintain normal SCN activity rhythms. Consistent with this hypothesis, our results presented here build on our understanding of phase-shifting mechanisms and the effects of Cu dysregulation on SCN rhythmic activity.

3.2 Methods

3.2a Brain slice preparation

Coronal brain slices (500 u) containing the SCN were prepared during the daytime from adult male C57BL/6Nhsd mice (Harlan Laboratories) housed in a 12:12 light/dark cycle. Slices were maintained at the interface of a Hatton-style brain slice chamber as described (Prosser, 1998). Brain slices were continuously perfused with Earle's balanced salt solution (EBSS) supplemented with glucose, bicarbonate and gentamicin at pH 7.4. Both the slice chamber and media reservoir were oxygenated (95% O2 / 5% CO2) and maintained at 37°C. All experimental protocols were approved by the University of Tennessee Knoxville Institutional Animal Care and Use Committee.

3.2b Drug treatments

All treatments were performed on day 1 *in vitro*. All drugs were prepared in warm, oxygenated EBSS. At the onset of the drug treatments, perfusion of the standard medium was

stopped and the medium in the chamber replaced with fresh medium containing the experimental treatments. After 10 min this medium was removed and perfusion with untreated medium was reinstated. Previous experiments have demonstrated that changing the perfusion medium by itself does not affect the phase of the circadian clock (Biello et al., 1997; Gillette and Prosser, 1988; Prosser et al., 1989; Prosser et al., 2003). $CuCl_2$, $CoCl_2$, Glu, NMDAR antagonist AP5 ((2*R*)-amino-5-phosphonovaleric acid), and AMPA receptor antagonist NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f] quinoxaline-2,3-dione) were purchased from Sigma-Aldrich (St. Louis, MO). TTM and bathocuproine disulfonate (BCS) salts (Sigma-Aldrich) were used to chelate copper. Trk receptor antagonist K252a (EMD Biosciences (Philadelphia, PA)) and TrkB inhibitor, TrkB-Fc (R&D Biosciences (Minneapolis, MN)) were also used in this study. For experiments involving the TrkB-Fc inhibitor (TrkB-I), slices were pre-treated for 5 min, followed by a 10 min treatment of TrkB-I in combination with Glu, TTM, or $CuCl_2$. Intracellular kinases were inhibited similarly using the PI3K inhibitor LY294002 (Sigma) or ryanodine receptor inhibitor dantrolene (Sigma). In the case of the MEK1/2 inhibitor U0126 (Cell Signaling) or PKG inhibitor KT5823 (Cayman Chemicals), slices were pre-treated for 10 min and post-treated for an additional 5 min. After drug or vehicle wash-out, slices remained in perfused media conditions until collection for immunoblotting or through the duration of single-unit recording experiments.

3.2c Immunoblotting

Hypothalamic slices from 2 mice were trimmed and reduced to isolate SCN tissue and incubated in the brain slice chamber. Incubated slices were collected at midday or early night (ZT6, ZT16) in microcentrifuge tubes set in dry-ice and stored immediately at -80°C. Frozen SCN slices were lysed with modified RIPA buffer, sonicated briefly, rotated for 25 min, and centrifuged at full-speed (17,000g) for 10 min to remove insoluble cell remnants. For each sample, protein concentration was measured using Bradford assay (Thermoscientific) and adjusted for volume before addition of 5X running buffer (reducing). Samples were boiled at 70°C and run on 4-12% SDS-PAGE gradient gels (Novex), transferred onto immobilon-fl PVDF membranes (EMD Millipore), and incubated in 5% non-fat milk for 1 hr for blocking. Antibodies for ATP7A (chicken)

and CTR1 (rabbit) were purchased from Abcam and applied overnight at 1:1000 and 1:500 concentrations in 2% milk after blocking. Corresponding Li-COR secondary antibodies were applied at 1:20,000 for 1-1.5 hr and processed for imaging following standard protocols *per* Li-COR instructions.

3.2d Single-unit recording and data analysis

Single-unit recordings were performed on day 2 *in vitro* for most experiments, and on day 3 *in vitro* where indicated. The procedure for neuronal recordings has been described previously (Prosser, 1998). Briefly, the spontaneous activity of single SCN neurons was recorded extracellularly using glass capillary microelectrodes filled with 3M NaCl. Each neuron was recorded for 5 min, and the data stored for later determination of firing rate using a DataWave system (Berthoud, CO). Typically, 4–7 cells were recorded during each hour. These individual firing rates were then used to calculate 2 h running averages, lagged by 1 h (± SEM), to obtain a measure of population neuronal activity. As in previous studies (Mou et al., 2009; Prosser, 1998), the time of peak neuronal activity was assessed visually by estimating, to the nearest quarter hour, the time of symmetrically highest activity. For example, if the two highest 2 h means are equal, then the time of peak is estimated to be halfway between them. Phase shifts were calculated as the difference in time-of-peak of untreated slices vs. drug treated slices. Using these methods, the consistency of the results obtained for each experimental manipulation is such that differences in phase of as little as one hour are often statistically significant with few replicates (Chen et al., 1999; Prosser, 1998). Statistical analyses were performed using Sigmastat (San Jose, CA, USA). One-way ANOVA was used to test for significant differences between treatment and control experiments, followed by Holm-Sidak test for *post hoc* comparisons.

3.2e Analysis of Cu levels by ICP-MS

During the day (ZT4-12), SCN tissue samples were dissected similarly to immunoblot samples. Samples were partially dried on filter paper to wick excess media were collected in Fisherbrand polypropylene PREMIUM microcentrifuge tubes and weighed before storing at -80°C. 500 µL of ultrapure 40% nitric acid was added to each sample vortexed and agitated before low-speed spin down. In a heat-bloc set at 60°C, samples were uncapped and dissolved in nitric acid, allowing for safe evaporation of volatile substances within a contained, clean vial-and-beaker covered system to avoid contamination of samples (fume hood). Completely digested samples were diluted with 500 µL, ultrapure 2% nitric acid and filtered using sterile syringe filters (Fisher), washed with 2% nitric acid into pre-cleaned scintillation vials until a final volume of 5 mL was reached. Samples were analyzed using a modified Perkin Elmer ICP-MS (inductively coupled plasma mass spectrometer) calibrated by a multi-element (calibration standard 3, Perkin Elmer) method.

3.3 Results

3.3a Cu- and TTM-induced phase delays during the early night

Our previous research found that treating SCN-containing brain slices either with exogenous Cu or with the Cu chelator TTM induces phase delays when applied during the early night and phase advances when applied during the late night (Yamada and Prosser, 2014). These effects mimic the phase shifts induced by *in vivo* light pulses and *in vitro* Glu application. We also determined that TTM-induced phase shifts require NMDA receptor activation while Cu-induced phase shifts do not. To further elucidate the cellular mechanisms underlying Cu- and TTM-induced phase shifts, here we focused on downstream mechanisms previously shown to be involved in Glu-induced phase shifts, first investigating early night phase delays, and then investigating light night phase advances.

Glu is known to activate MAPK signaling in the SCN, and inhibiting MAPK activation blocks Glu-dependent phase delays *in vivo* and *in vitro*. Previous studies have shown that changes in Cu levels affect MAPK signaling: increasing Cu increases MEK1 activation while decreasing or chelating copper, by TTM for example, dampens or blocks MAPK/ERK signaling ((Brady et al., 2017; Brady et al., 2014)). Therefore, we started by investigating whether Cu- or TTM-induced phase delays involve MAPK signaling.

As reported previously (Yamada and Prosser, 2014), the mean (± SEM) time of peak neuronal activity in the SCN in untreated (control) brain slices is ZT 6.06 ± 0.07 (n = 4), and ZT16 application of either 10µM Cu or 1µM TTM induces significant phase delays (relative to time-of-peak firing of control slices; Cu, mean phase shift: -2.06 h ± 0.48, n = 4 ($p<0.001$, vs. untreated slices) ; TTM, mean phase shift: -3.0 h ± 0.24, n = 4 ($p<0.001$). Here we found that co-application of the MEK1 inhibitor U0126 did not block TTM-induced phase delays (mean phase shift: -2.83 h ± 0.44, n = 3 ($p<0.001$)). However, Cu-induced phase delays were blocked by co-treatment with U0126 (mean phase shift: 0.5 h ± 0.29, n=3 ($p=0.50$, vs. untreated slices)). These data are summarized in Fig 3.1. This suggests that MAPK signaling is involved in Cu-induced but not TTM-induced phase delays.

Intracellular calcium release from the endoplasmic reticulum through activation of ryanodine receptors has also been shown to be necessary for Glu-induced phase delays: blocking ryanodine receptors with dantrolene inhibits Glu-induced phase delays (Aguilar-Roblero et al., 2007; Aguilar-Roblero et al., 2016; Mercado et al., 2009). Here we assessed the effects of dantrolene on Cu- and TTM-induced phase delays. We found that co-treatment with dantrolene at ZT16 had no effect on Cu-induced phase delays (mean phase shift: -1.92 h ± 0.08, n=3 ($p<0.001$, vs. untreated slices)), suggesting ryanodine receptor activity is not required for Cu-induced phase delays the early night. However, dantrolene did block TTM-induced phase delays (mean phase shift: -0.42 h ± 0.36, n = 3 ($p=0.58$)), similar to its effects on Glu-induced phase delays at ZT16 (mean phase shift: -0.13 h ± 0.32, n=2). These results are summarized in Fig 3.1 and 3.2 (One-way ANOVA for ZT16 data above, $F=24.65$ ($p<0.001$)).

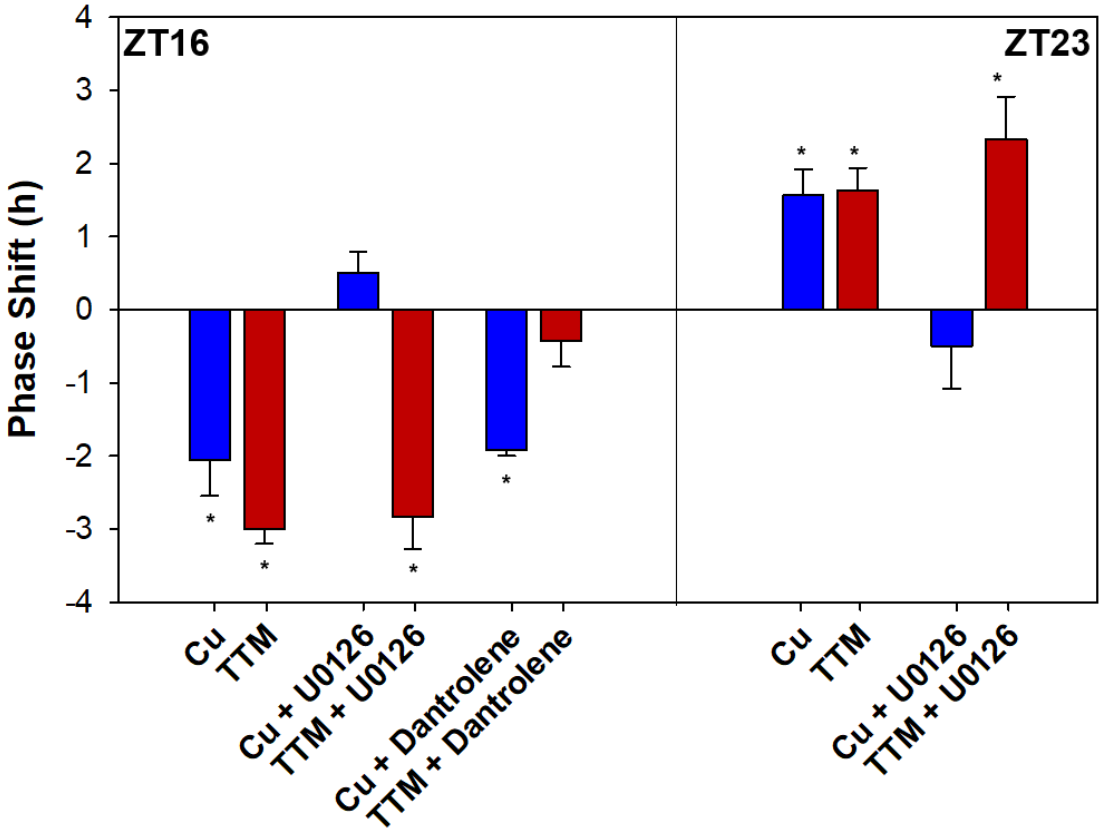


Figure 3.1. Effects of MEK1/2 inhibition and ryanodine receptor inhibition on Cu and TTM-induced phase shifts at ZT 16 and 23

Histogram plot summarizing the mean phase shifts ± SEM resulting from the indicated treatments at two different time points. (Left) 10µM Cu and 1 µM TTM induce phase delays at ZT16 which require different mechanisms as tested by the MEK1/2 inhibitor U0126 and ryanodine receptor inhibitor dantrolene. Dantrolene blocked TTM effects. (Right) At ZT23, Cu and TTM induces phase advances. At either time point, Cu-induced phase shifts are blocked by U0126, which has no effect on phase shifts induced by TTM. *Phase shifts significantly different from (untreated) control slices based on post hoc (Holm-Sidak) test ($P < 0.005$). Delays are plotted as negative values. Advances are plotted as positive values. ZT, zeitgeber time, n=3-4 for all experiments. Cu and TTM alone data repeated from Fig. 2.1 and 2.3 for clarity.

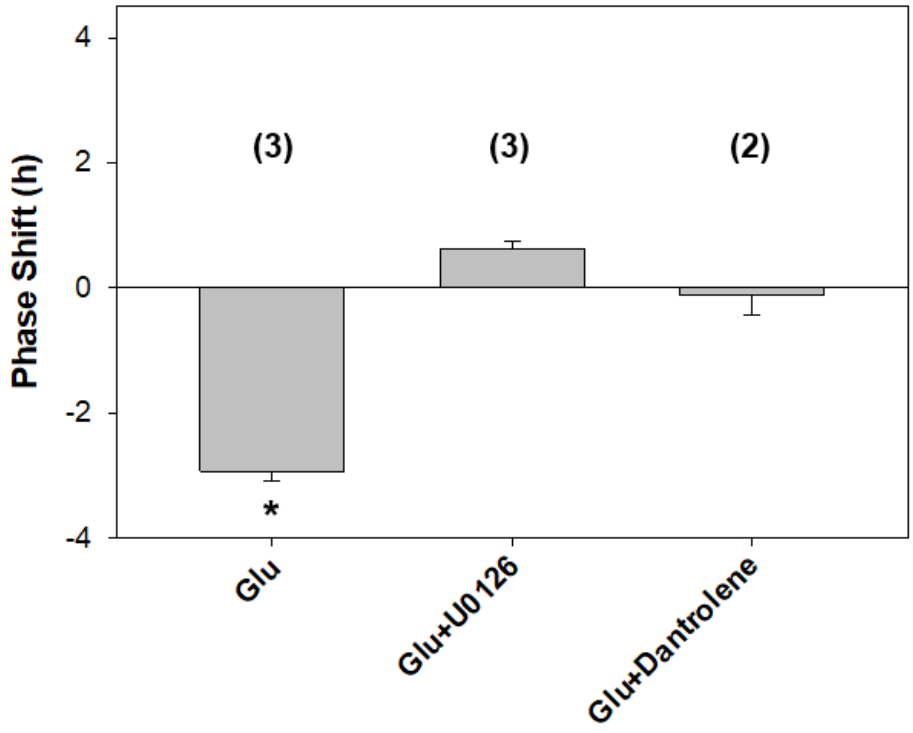


Figure 3.2. Effects of MEK1/2 inhibition and ryanodine receptor inhibition on Glu-induced phase delays at ZT16

Histogram plot summarizing the mean phase shifts ± SEM resulting from the indicated treatments at ZT16. Glu-induced phase delays are blocked by the MEK1/2 inhibitor, U0126, and ryanodine receptor inhibitor, dantrolene. *Phase shifts significantly different from (untreated) control slices based on post hoc (Holm-Sidak) test ($P < 0.001$). Delays are plotted as negative values. ZT, zeitgeber time. Numbers in parentheses indicates number of experiments.

3.3b Cu- and TTM-induced phase advances during the late night

In our previous work, we showed that *in vitro* application of either Cu or TTM at ZT23 induces phase advances: applying 10μM Cu to SCN slices results in a ~1.5-h phase advance (mean phase shift: 1.56 h ± 0.36, n = 4 ($p<0.05$)) while application of 1 μM TTM induces a mean phase shift of 1.63 h ± 0.31, n = 4 ($p<0.05$). Similar to experiments investigating Glu-induced phase delays, the MEK1/2 inhibitor U0126 has also been shown to block Glu-induced phase advances (Butcher et al., 2005; Coogan and Piggins, 2003), so we performed analogous experiments at ZT23 for Cu and TTM. Co-application of U0126 at ZT23 blocked Cu-induced phase advances (mean phase shift = 0.5 h ± 0.58, n = 3 ($p=0.33$, vs. untreated slices)). On the other hand, U0126 did not block TTM-induced phase advances (mean phase shift: 2.33 h ± 0.58, n = 3 ($p<0.01$)). These data are summarized in Fig 3.1 (One-way ANOVA for ZT23 results, $F=8.77$ ($p<0.005$)). Because dantrolene does not inhibit Glu-induced phase advances (Ding et al., 1998), we did not assess the effects of dantrolene on Cu- and TTM-induced phase advances. The results thus far suggest that MAPK signaling plays an important role in Cu-induced phase shifts at ZT16 and ZT23 but is not involved in TTM-induced phase shifts.

3.3c NOS signaling in Cu- and TTM-induced phase shifts

Nitric oxide (NO) production by NO synthase (NOS) is another cellular mechanism involved in Glu-induced phase shifts, and NO has been shown to activate ryanodine receptors in addition to other signaling molecules (Kakizawa, 2013; Kakizawa et al., 2012; Mikami et al., 2016; Vielma et al., 2016; Wang et al., 2010a). Therefore we investigated the role of NO in Cu- and TTM-induced phase shifts by inhibiting NOS activity with L-NAME (Fig. 3.3). Consistent with previous reports, co-treatment of L-NAME at ZT16 blocked Glu-induced phase delays (mean phase shift: -0.13 h ± 0.31, n=4 ($p=0.70$, vs. untreated slices)). Cu-induced phase delays were not affected by co-application of L-NAME at ZT16 (mean phase shift: -2.5 h ± 0.5, n = 2). When co-applied at ZT23, however, L-NAME blocked Cu-induced phase advances (mean phase shift: 0.19 h ± 0.47, n = 4 ($p=0.96$)). Next, we blocked NOS activity with L-NAME to assess whether TTM-induced phase

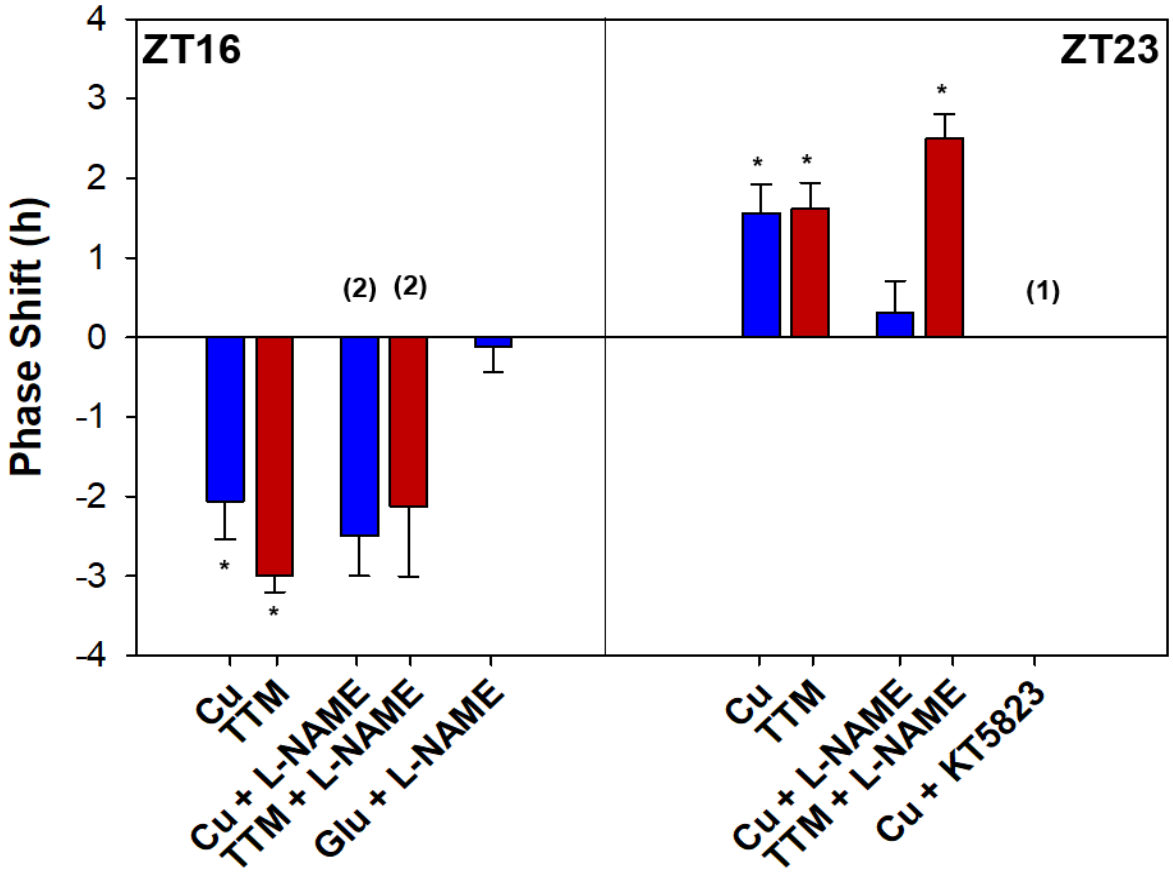


Figure 3.3. NO signaling involvement in Cu and TTM induced phase shifts

Histogram plot summarizing the mean phase shifts ± SEM resulting from the indicated treatments at two different time points. (Left) L-NAME inhibition of nitric oxide synthase does not appear to block Cu- or TTM-induced phase delays at ZT16 (N=2, for both however). In contrast, co-application of L-NAME does block Glu effects at ZT16, consistent with previous reports. (Right) L-NAME blocked Cu-induced phase advances but had no effect on TTM-induces phase advances. Treatment with the PKG inhibitor KT5823 also blocked Cu effects at ZT23, which supports a role for NOS in Cu-induced phase advances. However, as an N=1, this result need to be confirmed. *Phase shifts significantly different from (untreated) control slices based on post hoc (Holm-Sidak) test ($P < 0.05$). Delays are plotted as negative values. Advances are plotted as positive values. ZT, zeitgeber time; n=3-4 for all experiments, except where indicated in parentheses.

shifts require NOS activity. Surprisingly, L-NAME did not affect TTM-induced phase delays (mean phase shift: -2.25 ± 0.75, n = 2) or phase advances (mean phase shift: 2.5 h ± 0.31, n = 4 ($p<0.001$)), suggesting that NOS activity is not involved in TTM-induced phase shifts at either ZT16 or 23.

During the late night, NO activates soluble guanylyl cyclase production of cGMP, which in turn activates cGMP-dependent protein kinase (PKG) (Ding et al., 1998; Ding et al., 1997; Ferreyra and Golombek, 2001; Golombek et al., 2004; Golombek and Rosenstein, 2010; Liu et al., 1997; Mathur et al., 1996; Oster et al., 2003; Plano et al., 2012; Tischkau et al., 2003b; Weber et al., 1995). Inhibition of PKG blocks Glu-induced phase advances ((Ding et al., 1998; Golombek et al., 2004; Mathur et al., 1996; Weber et al., 1995)). If Cu-induced phase shifts of the SCN clock at ZT23 require NOS production of NO, then it is possible that downstream PKG activation is also involved in Cu-induced phase advances. To test this idea, we inhibited PKG with KT5823, which has been shown to block Glu-induced phase advances. Our preliminary data indicate that, similar to L-NAME, KT5823 blocked Cu-induced phase advances when co-applied at ZT23 (phase shift: 0 h, n=1). This is shown in Fig. 3.3. These results suggest that Cu-induced phase delays do not require NOS production of NO during the early night, while NOS-dependent signaling pathways that include PKG activity play a role in Cu-induced phase advances during the late night.

3.3d PI3K/Akt signaling in TTM-induced phase shifts

We have previously shown that TTM-induced phase shifts require activation of TrkB receptors, similar to Glu-induced phase shifts (Mou et al., 2009; Yamada and Prosser, 2014). TrkB is known to activate PI3k/AKT signaling as well as MAPK/ERK signaling (Hua et al., 2016; Marsden, 2013; Van't Veer et al., 2009; Yoshii and Constantine-Paton, 2007, 2014; Zhou et al., 2010). Since TTM-induced phase shifts were not blocked by inhibition of MAPK or NO signaling cascades, we tested whether the PI3K/Akt signaling pathway is involved in TTM-induced phase shifts, using the PI3K/Akt inhibitor, LY294002. Co-treatment with LY294002 did not block TTM-induced phase shifts at ZT16 (mean phase shift: -1.83 h ± 0.51, n = 3 ($p<0.01$)) or at ZT23 (mean phase shift: 3 h

± 0.76, n = 3 ($p<0.001$)). To summarize, TTM-induced phase shifts do not require NOS, MEK or PI3K activity, but TTM-induced phase delays require ryanodine receptor activation and presumably calcium release from the ER.

3.3e Expression of Copper Transporters in the SCN

In addition to the above results, our previous experiments suggest that decreasing Cu levels affects SCN neuronal activity rhythms by strengthening extracellular glutamatergic signaling (i.e., TTM-induced phase shifts are inhibited by the NMDA inhibitor AP5), while increasing Cu modulates circadian phase through NMDA-independent mechanisms. Because Cu homeostasis is highly regulated in various tissues including many regions of the brain, we reasoned that SCN neurons regulate extracellular Cu levels in order to modulate or gate glutamatergic input. To begin investigating this idea, we performed immunoblot experiments on SCN tissue extracts to assess expression of two Cu homeostasis proteins, ATP7A and CTR1. In response to various stimuli including Cu overload, ATP7A is known to translocate to the plasma membrane of neurons and pump Cu out. On the other hand, CTR1 is the primary Cu importer of mammalian cells. We collected SCN samples during midday (ZT6), early night (ZT16), and late night (ZT23) corresponding to when Glu does not induce phase shifts (ZT6) and when it induces large nighttime phase shifts (ZT16/23). We observed no difference in CTR1 expression at these time points (n=6 (One-way ANOVA, $F= 1.92$, $p=0.18$)). However, our results (n=4) suggest higher ATP7A expression during the early night ($P<0.05$). These data are summarized in Fig. 3.4. These data support the potential for a day-night difference in Cu homeostatic regulation and export by ATP7A in the SCN.

3.3f Preliminary Analysis of Cu levels in SCN

Using SCN extracts collected during the late day, we performed preliminary experiments using ICP-MS to measure Cu levels. In our first run, we used 12 mice per sample to obtain 25mg of SCN tissue, as well as 50mg of hypothalamic tissue surrounding the SCN remaining after

Figure 3.4. Expression of copper transporters in the SCN

(Top) Representative western blot showing a ~180kDa band corresponding to ATP7A and a ~30kDa band for CTR1 Cu transporters in SCN tissue lysates collected at ZT6, ZT16, or ZT23 after *in vitro* incubation. Lysates of mouse cortex were included for comparison and positive control. Actin was used as a loading control. Western blots were imaged on a Li-Cor Odyssey CLx imaging system. *Denotes ATP7A band of interest. (Bottom) Histogram plot showing diurnal differences in ATP7A expression (adjusted/relative to actin, n=4), left, and no differences in CTR1 expression (relative to actin, n=6), right. Data normalized to ZT6 to show fold changes in night (ZT16) versus day (ZT6). *Significantly different from ZT6, $p<0.05$.

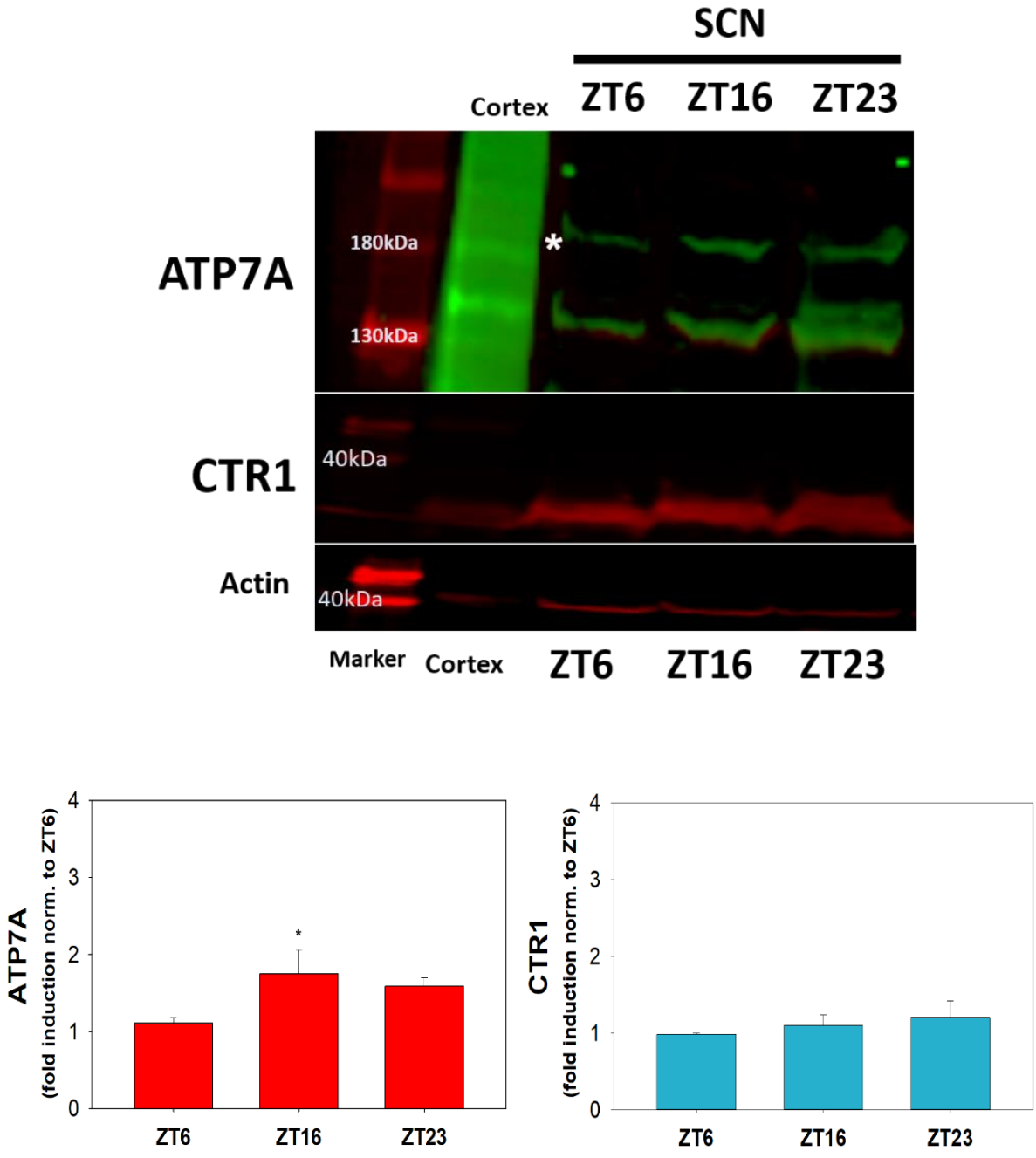


Figure 3.4 continued

removal of both nuclei. From 2 SCN samples, we obtained values of 14.66ppb (parts per billion) and 7.98ppb of Cu, which when adjusted for volume measured and SCN tissue mass, yields an average concentration (± SEM) of 4.53 ± 0.95 ug/g. The extra-SCN hypothalamic tissue concentrations for 3 samples were 1.98 ± 0.35 ug/g. On the second run, we reduced the number of mice per sample to 4 with total tissue collected at 5.6mg and 7.4mg for two samples. Average Cu concentration for this run was: SCN, 8.02 ± 0.83 ug/g (n=2); extra-SCN 4.55 ± 0.01 ug/g (n=2). Combined, the results indicate that we detected higher levels of copper in SCN extracts compared to hypothalamic tissue immediately adjacent to, and surrounding the SCN.

3.4 Discussion

The relationship between trace metals, circadian rhythms, and metabolism is still largely uninvestigated, but several studies in plants demonstrate rhythms in copper and iron homeostasis and how they pertain to circadian activity and metabolism of plants (Andres-Colas, Perea-Garcia et al. 2010, Perea-Garcia, Andres-Colas et al. 2010, Chen, Wang et al. 2013, Hong, Kim et al. 2013, Tissot, Przybyla-Toscano et al. 2014). Since Cu plays a role in iron homeostasis, it is noteworthy that a circadian rhythm in iron levels in the midbrain of mice has been reported (Unger, Earley et al. 2013, Unger, Jones et al. 2014). In addition, iron deficiency has been shown to affect circadian wheel-running activity and is strongly associated with restless leg syndrome, which has a circadian component and manifests as a sleep disorder (Dowling, Klinker et al. 2011, Earley, Connor et al. 2014, Furudate, Komada et al. 2014). On a slightly different note, circadian rhythms in magnesium levels have been demonstrated in mammalian U2OS and mouse fibroblast cells, correlating with rhythmic transcription of Mg^{2+} transporters, and changes in Mg^{2+} availability/transport can affect the period, amplitude, and phase of molecular clocks, as well as metabolic (ATP/protein translation) rhythms (Feeney et al., 2016). Even though Cu is essential to cellular respiration and other metabolic activities in the brain, there are no reports of how SCN neurons regulate Cu homeostasis and its transport to the mitochondria. In this study, we have tested several of the intracellular signaling pathways potentially involved in phase-shifting of

neuronal activity induced by Cu application or deficiency via Cu chelation, and hence pathways responding to changes in Cu availability and homeostasis.

3.4a Intracellular mechanisms of Cu- and TTM-induced phase shifts

We have shown previously that bath-applying 4μM Cu to SCN-containing brain slices for 10min does not result in phase delays, but application of 10μM Cu induces phase shifts (Yamada and Prosser, 2014). In addition, we have previously shown that 4μM Cu blocks Glu-induced phase shifts and that 10μM Cu-induced phase shifts are not blocked by NMDAR inhibition. Thus, consistent with other studies, we believe that at micromolar levels, Cu blocks NMDAR activation by Glu and therefore calcium influx via NMDAR is not involved in Cu-induced phase shifts (Black et al., 2014; Gasperini et al., 2015; Marchetti et al., 2014; Schlief et al., 2005; Schlief et al., 2006; Trombley and Shepherd, 1996; Vlachova et al., 1996). In this study, we show that Cu-induced phase shifts require MEK1 activity, and that during the late night, but not early night, they require NOS activation. For late-night phase advances, activation of NOS and cGMP-dependent PKG is required where nitric oxide activates soluble guanylyl cyclase which produces cGMP (Ding et al., 1998; Ding et al., 1997; Ferreyra and Golombek, 2001; Golombek et al., 2004; Golombek and Rosenstein, 2010; Liu et al., 1997; Mathur et al., 1996; Oster et al., 2003; Plano et al., 2012; Tischkau et al., 2003b; Weber et al., 1995). Consistent with this, inhibition of PKG blocked Cu-induced phase advances. It is unclear how Cu induces NOS-dependent phase advances without prior activation of NMDAR and influx of calcium. This may involve Cu activation of signaling pathways such as MAPK or more indirectly through changes in redox-sensitive proteins or displacing zinc from sites able to bind either Cu or Zn. Displacement of Zn or sufficient changes in redox activity within SCN neurons may relate to the amount of excess Cu necessary to phase shift the clock. Nevertheless, it remains unknown in general, how MAPK and PKG pathways work or interact in SCN neurons to reset their clocks.

On the other hand, we have shown that lowering the amount of endogenous Cu by chelation induces NMDAR-mediated phase shifts. From our prior experiments and studies by

other labs (Dodani et al., 2014; Gasperini et al., 2015; Schlief et al., 2006; You et al., 2012), TTM likely exerts its effect through removal of Cu and Cu's inhibitory effect on NMDAR, which could allow for endogenously released Glu to then activate NMDARs. A recent study by Brancaccio demonstrated a role for NR2C subunit-containing NMDARs in SCN rhythmicity and explored how rhythmic release of Glu from astrocytes at night can be manipulated to activate presynaptic NR2C containing SCN neurons (Brancaccio et al., 2017). Postsynaptic NMDARs are located in the ventral region of the SCN, which receives glutamatergic input from the eyes via the retinohypothalamic tract, and their role in Glu-induced phase shifts has been well established (Baver et al., 2008; Berson et al., 2002; de Vries et al., 1993; Ding et al., 1994; Doyle et al., 2008; Drouyer et al., 2007; Ebling et al., 1991; Hattar et al., 2002; Mikkelsen et al., 1995; Mintz and Albers, 1997; Mintz et al., 1999; Tsai et al., 2009; Vindlacheruvu et al., 1992; Wong et al., 2007). As TTM-induced phase shifts appear to depend on an increase in intracellular calcium, consistent with inhibition of TTM-induced phase delays at ZT16 by dantrolene, our results implicate calcium-dependent signaling pathways. In contrast, MEK1-dependent phase shifts induced by application of Cu are not sensitive to dantrolene inhibition of ryanodine receptors. Since Cu has also been shown to inhibit various types of calcium channels (Castelli et al., 2003; Lu et al., 2009b; Mathie et al., 2006; Shcheglovitov et al., 2012), TTM may have multifaceted effects on calcium dynamics in the SCN, though this remains untested. Thus, the effect of removing Cu's inhibitory effects on NMDAR and other receptor-ion channels may result in multiple presynaptic and/or postsynaptic changes in SCN neurons.

Both Glu- and TTM-induced phase shifts require TrkB signaling in conjunction with NMDAR activation. However, downstream of these cell surface receptors, the differences and similarities between Glu and TTM effects are intriguing. While Glu induces phase shifts during the early or late night by activating NMDAR, NOS, and MAPK signaling, TTM-induced phase shifts do not appear to require MAPK or NOS activity downstream of NMDAR-mediated calcium influx. Several studies have demonstrated that TTM blocks MEK1, putatively by preventing Cu from getting to MEK1's Cu-binding site (Brady et al., 2017; Brady et al., 2014; Turski et al., 2012). Indeed, our results show that TTM-chelation of Cu in SCN slices results in phase shifts that are independent of MEK1 activity. In addition, we used LY294002 to inhibit PI3K/Akt because TrkB

signaling activates PI3K/Akt in addition to MAPK pathways. PI3K/Akt inhibition had no effect on TTM-induced phase shifts, however, similar to another study looking at PI3K/Akt's role in phase-shifting SCN rhythms (Cao et al., 2011),

Interestingly, TTM-induced phase shifts were not blocked by the NOS inhibitor L-NAME, which is activated downstream of Glu/NMDAR signaling in the SCN. Why NOS and nitric oxide signaling are not required in TTM effects and how ryanodine receptors are activated by Cu chelation are important questions needing to be resolved. Decreases in available Cu may directly activate signaling pathways upstream of ryanodine receptors and/or exert its effect indirectly through changes in oxidation state or redox associated proteins. Our L-NAME experiments suggest that nitric oxide modification (nitrosylation) and modulation of ryanodine receptor activity does not play a role in TTM-induced phase shifts. However, other redox-related changes to ryanodine receptors may be induced by Cu removal. We have not yet tested dantrolene's effects on TTM-induced phase advances; however, ryanodine receptors are reportedly not involved in Glu-induced phase advances at ZT23 (Ding et al., 1998). Moving forward, testing calcium-dependent signaling pathways downstream of NMDAR-dependent TTM effects remains an important task, as well as eliminating other potential players in SCN rhythms such as protein kinase A and PKG. Based on the severity and variety of neurological symptoms seen in Cu deficiency, namely Menkes disease, Cu's potential involvement in many neuronal processes, including circadian time-keeping, is not surprising.

3.4b Potential role of endogenous Cu and homeostasis in SCN

Research on Cu chelators, Cu-related pharmaceuticals, and proteins involved in trace element homeostasis and their interactions with Cu and other metals is a critical part of understanding and treating diseases related to Cu, even certain neurodegenerative diseases and cancers. Treatments using TTM or its derivatives have not only been investigated in regard to Wilson's disease, caused by excessive Cu accumulation in various organs including the brain, but also in various cancer studies due to their inhibitory effects on MEK1-dependent ERK1/2 signaling

as well as on extracellular matrix changes as it relates to angiogenesis (Brady et al., 2014; Brewer, 2014; Chisholm et al., 2016; Henry et al., 2006; Pan et al., 2002; Wei et al., 2014; Wei et al., 2012). Because of the short (10min) application of TTM, we do not expect that this treatment significantly affects other intracellular enzymes that depend on Cu, such as the redox scavenger Cu/Zn-superoxide dismutase or cytochrome c oxidase (complex IV of electron transport chain) (also see Chapter 2 data using membrane impermeable, BCS chelator). Since TTM's direct interaction with Cu is most likely extracellular, it appears that there are physiologically relevant amounts of extracellular Cu, and potentially synaptic Cu, in the SCN, whereby extracellular chelation induces inter- or intracellular signaling. Many other studies have implicated an important role for extracellular or synaptic Cu in different brain regions, some of which are discussed below (D'Ambrosi and Rossi, 2015; Gaier et al., 2013a; Scheiber et al., 2014; Zlatic et al., 2015). Although some Cu may be introduced in the incubation media, our media does not include serum, and intra- versus extracellular levels of Cu should be physiologically equilibrated prior to drug treatments, such that our *in vitro* experiments have consistently modeled *in vivo* results in terms of intra-SCN signaling. Future experiments should investigate intra- versus extracellular Cu levels, as well as trace level metal contamination occurring in our media solutions. In any case, the removal of extracellular Cu has been shown to disrupt import of Cu through CTR1, and CTR1 import of Cu has been directly linked to MEK1 activity (Brady et al., 2014; Turski et al., 2012). A model comparing the cellular mechanisms associated with Cu, TTM, and Glu-induced phase shifts is presented in Fig. 3.5.

In this study, we show that CTR1 is present in the SCN and that SCN slices contain Cu. Although we need to perform additional ICP-MS experiments to demonstrate low micrograms of Cu per gram of SCN tissue with confidence, these values are highly consistent with several studies using ICP-MS. The inter-run difference in amounts is likely due to the amount of tissue sampled for each and overall optimization of the SCN tissue digestion methodology to minimize loss of cellular Cu among different samples. In addition, our results indicate a difference in ATP7A regulate changes in extracellular Cu in response to signaling events, some of which could be controlled by the circadian clock, not just in response to when intracellular Cu levels increase. A

Figure 3.5. Phase-shifting circadian neuronal activity rhythms in the SCN by copper, glutamate, and tetrathiomolybdate (TTM)

(Left) Application of Cu induces night-time phase shifts by inducing MEK-dependent MAPK signaling independently of NMDAR activity. During the early night (ZT16), TrkB may play a role upstream of MAPK signaling or through a separate mechanism involving other kinases. During the late night (ZT23), nitric oxide synthase (NOS)-mediated activation of cGMP-dependent, protein kinase G is involved but not TrkB signaling. (Middle) Endogenous Cu blocks endogenous, extracellular Glu accumulation during the night but is not able to inhibit phasic release of Glu at the synapse (via retinohypothalamic input, for example). Glu-induced phase shifts are mediated by various signaling mechanisms downstream of TrkB and calcium signaling pathways, including ryanodine receptor-mediated calcium release from the endoplasmic reticulum during the early night and NO-induced production of cGMP by soluble guanylyl cyclase (sGC) and activation of PKG during the late night. Phosphorylation of CREB is one of the key transcription factors connecting receptor signaling to changes in core clock proteins (of the transcription-translation-feedback-loop). (Right) TTM binds extracellular Cu, increasing NMDAR activity during the late night when extracellular levels of Glu are higher. Increase in intracellular calcium by NMDAR activity, which may or may not be at the synapse, together with TrkB signaling leads to night-time expression during the night versus day. This raises the interesting possibility that ATP7A may phase shifts through yet unknown mechanisms. Ryanodine receptor activity is required for TTM-induced phase delays at ZT16. Pathways in green have been demonstrated and connected to phase shifts for the corresponding times. Grey arrows represent untested connections. Location and function of the Cu-transporter ATP7A have not been investigated in the SCN.

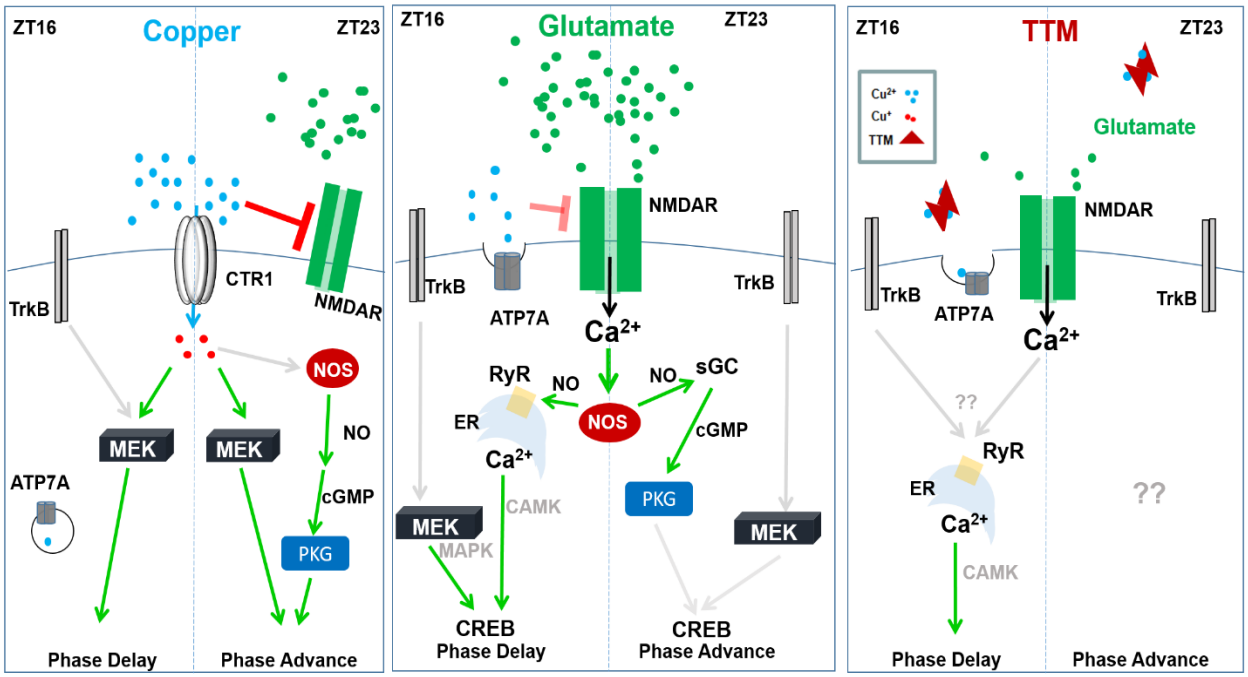


Figure 3.5 continued

related idea is that changes in extracellular SCN Cu could be driven by Glu input, primarily from retinohypothalamic axons, which occurs *in vivo* when light stimulates specialized retinal cells. Glu stimulation of hippocampal neurons has been shown to induce the Cu-transporter ATP7A to move to the membrane and increase Cu export (Schlief et al., 2005; Schlief et al., 2006). This increase in extracellular Cu was shown to protect hippocampal neurons against Glu/NMDAR excitotoxicity, and Cu chelation increases the sensitivity of hippocampal neurons to Glu insult (Schlief et al., 2006). As various loss-of-function mutations in ATP7A cause Menkes disease, seizures due to dysfunction of ATP7A in neurons more than likely relate to increased susceptibility to Glu excitoxicity (Hodgkinson et al., 2015; Prasad et al., 2011). Interestingly, SCN neurons are robustly resistant to Glu excitotoxicity, and ERK1/2 signaling in SCN tissue has been reported to be neuroprotective against excess Glu (Bottum et al., 2010; Karmarkar et al., 2011). Although the protective effect of Cu against excitotoxic Glu insult is redox sensitive and linked to decreased NMDAR-mediated calcium influx, the involvement of ERK1/2 has not been investigated in this context. As Cu increases MAPK signaling via MEK1 and chelation has the opposite effect, activation of this pathway may be involved in a negative feedback loop with ATP7A translocating to the membrane. In addition, a potential mechanism for Cu inhibition of NMDAR currents involves Cu-bound cellular prion protein nitrosylating NMDARs (Black et al., 2014; Gasperini et al., 2015; Schlief et al., 2006; You et al., 2012). Based on our results, we hypothesize that: ATP7A-released, endogenous Cu blocks Glu activation of NMDAR, in particular where rhythmic Glu release occurs at night and potentially during the day when Glu does not cause phase shifts; both Cu modulation of NMDAR and Cu-dependent MEK1 signaling contribute to neuronal resistance to Glu excitotoxicity in the SCN. In general, tight homeostatic control of intra-/extracellular Cu by ATP7A, CTR1 and other Cu regulatory proteins is vital to neuronal survival, in that excess Cu is neurotoxic.

3.4c Cu dyshomeostasis and pathological implications

Dysfunction or loss of circadian rhythms can result from aging and neurodegeneration, and oxidative stress plays a central role in the pathological process of both (Aziz et al., 2009;

Bellanti et al., 2017; Colwell, 2011; Cuesta et al., 2012, 2014; Fahrenkrug et al., 2007; Fifel, 2017; Fifel et al., 2014; Harper et al., 2008; Hu et al., 2013; Hu et al., 2009; Kudo et al., 2011a; Kudo et al., 2011b; Li et al., 2017a; Liu and Chang, 2017; Liu et al., 2000; Meijer et al., 2012; Morton et al., 2005; Stevanovic et al., 2017; Van Erum et al., 2017; Wang et al., 2015b; Wang et al., 2016; Wilking et al., 2013; Wu et al., 2006; Wu and Swaab, 2007; Zhou et al., 2016). Studies indicate that Cu accumulates in specific brain regions of aged subjects, and it is still unknown how this normally occurring phenomena affects neurons and their communication (Braidy et al., 2017; Fu et al., 2015a; Pushkar et al., 2013; Singh et al., 2013b; Wang et al., 2010b; Zatta et al., 2008). In neurodegenerative diseases, long-term Cu dyshomeostasis plays a role in oxidative damage, and redox active Cu accelerates formation of toxic oligomers associated with some neurodegenerative factors (Ahuja et al., 2015; Atwood et al., 2000; Dell'Acqua et al., 2015; Drew and Barnham, 2011; Greenough et al., 2016; Kawahara et al., 2017; Kitazawa et al., 2009; Lan et al., 2016; Lin et al., 2010; Lu et al., 2009a; Mayes et al., 2014; Okita et al., 2017; Singh et al., 2013b; Squitti, 2014; Strozyk et al., 2009; Villar-Pique et al., 2016). In our experiments we bath-applied 10µM Cu to SCN brain slices for 10 min to induce phase shifts. Although we believe the acute increase in Cu levels are well within a tolerable range, some of the effects of bath-applying Cu could be interpreted as less physiological and as such perhaps mimic the initial responses to excess Cu or Cu dysregulation. When Cu levels exceed the capacity of Cu-storage into vesicles or buffering proteins (e.g. metallothioneins), excess free Cu can generate reactive oxygen species via Fenton chemistry (Baker et al., 2017a; Bhattacharjee et al., 2017; Hatori and Lutsenko, 2016; Hordyjewska et al., 2014; Ohrvik et al., 2017). The importance of metallothioneins in Cu homeostasis has been demonstrated in the context of Alzheimer's and Parkinson's disease (Chung et al., 2010; McLeary et al., 2017; Meloni et al., 2007; Meloni et al., 2008; Okita et al., 2017). In addition, Cu's ability to displace intracellular stores of zinc, including from Zn-bound metallothioneins, may play a role in various instances of neurotoxic oxidative stress, such as in experiments looking at paraquat-related Parkinson's disease models for example. On a related note, in a recent genetic selection study using CRISPR, some of the key mediators of paraquat-induced neuronal death were ATP7A, CTR1 and the antioxidant Cu/Zn-superoxide dismutase (Reczek et al., 2017). Furthermore, in Alzheimer's patients and mouse models, Cu is found in high

concentrations extracellularly in association with amyloid plaques, yet low intracellular Cu concentrations have also been reported (Greenough et al., 2016; James et al., 2017; Xu et al., 2016; Xu et al., 2017). Amyloid β has a high affinity for Cu and can sequester Cu extracellularly (Hatcher et al., 2008; James et al., 2017; Jiang et al., 2013; Lu et al., 2015; Sarell et al., 2009; Singh et al., 2013b; Syme et al., 2004). Chelation of Cu by amyloid β in hippocampal neurons has been shown to affect NMDAR function and decrease synaptic efficacy (Stys et al., 2012a; You et al., 2012). Such extracellular sequestration of Cu by amyloids may also decrease influx of Cu and reduce ERK1/2 signaling. Thus, some circadian abnormalities observed in aged subjects or those with Alzheimer's disease, for example, may partially involve Cu dyshomeostasis and warrants further study.

This study expands on our previous work exploring the interaction of NMDAR signaling and Cu in SCN circadian rhythms and further establishes a putative role for endogenous Cu in maintaining normal clock function. We believe that Cu has physiological roles in maintaining clock activity both intracellularly and extracellularly within the SCN, and disruption of Cu homeostasis may underlie some cases of abnormal circadian activity, and vice-versa. As Cu homeostasis is a key component of various neuronal processes, as especially exemplified by Menkes and Wilson's disease, further study of Cu's role in inter-/intraneuronal signaling in the SCN will greatly contribute to our understanding of Cu physiology in excitotoxicity and pathology.

CHAPTER FOUR

COPPER IS A KEY ELEMENT OF THE MASTERCLOCK

4.1 Looking back, thinking ahead: Copper in the SCN

Herein, we have established a starting point for investigating the role of Cu and Cu homeostasis in the SCN, location of the mammalian circadian pacemaker. Although many studies have investigated the roles and effects of Cu in synaptic function and receptor signaling in the brain and other tissues, these results are the first to directly link Cu with the SCN master clock and circadian neuronal activity rhythms. First, we have demonstrated that both Cu chelation and Cu application are able to induce night-time phase shifts in neuronal activity rhythms *in vitro*. Second, we have shown that these treatments affect NMDARs and Glu neurotransmission differently. Since Glu-mediated NMDAR activation phase shifts the SCN clock through calcium-dependent signaling pathways and requires concurrent activation of other pathways, we pharmacologically tested several of these pathways to investigate how the Cu chelator TTM induces NMDAR-dependent phase shifts. However, the specific extra- and intracellular mechanisms by which Cu removal initiates these events remain unclear. Similarly, we do not know fully how addition of Cu activates intracellular signaling pathways leading to night-time phase shifts, although our results demonstrate that Cu induces MAPK-dependent phase shifts in the absence of NMDAR-mediated calcium influx. In addition, Cu-induced phase delays during the early night do not involve ryanodine receptor-mediated calcium release from the endoplasmic reticulum, while phase advances do not require concurrent TrkB signaling. Below, we also discuss results from TTM and Cu treatment at ZT6 (Fig. 4.1), which suggest that the Cu's role is dynamic and changes during the night versus day. Lastly, we have preliminary results indicating that concentrations of Cu in the SCN are comparable to, or possibly higher than, other brain regions, and that there is expression of two Cu transporters, CTR1 and ATP7A in the SCN. These results are incorporated into models (Fig. 4.2, 4.3) and discussed in more detail below. In this final

chapter, I will discuss the main concepts arising from these data and tie these ideas to the findings of others, with the goal of providing a foundation for future investigations.

4.2 Cu in the SCN: regional distribution and homeostasis

One of the outstanding questions concerns Cu distribution and homeostasis in relation to its function in the SCN. The SCN contains functionally heterogeneous groups of neurons across ventral and dorsal regions of the SCN, with ventral neurons receiving retinal and other afferent input, and pacemaking dorsal neurons responsible for high amplitude, rhythmic activity/output and control of extra-SCN clocks (Evans, 2016; Evans and Gorman, 2016; Herzog et al., 2017). Although most SCN neurons are GABAergic, they are functionally differentiated by additional neuropeptides and molecular characteristics, where in general, ventral neurons secrete vasoactive intestinal polypeptide (VIP) to couple with shell neurons, which in turn signal and coordinate activity with other neurons via arginine vasopressin. In addition, astrocytes are now known to play a substantial role in SCN neuronal timekeeping (Barca-Mayo et al., 2017; Brancaccio et al., 2017; Duhart et al., 2013; Tso et al., 2017). Even though our experiments are focused on regulation of baseline SCN neuronal activity, the potential role of astrocytes in Cu homeostasis should not be overlooked, as astrocytes are known to both store and release Cu (Pal and Prasad, 2014; Scheiber and Dringen, 2013; Scheiber et al., 2010a; Scheiber et al., 2012). There is, however, stronger evidence for the role of Cu in neurons, such that astrocytes may primarily function to maintain adequate regional levels of Cu and protect neurons from excess extracellular Cu. Indeed, a recent study demonstrates that neurons have twice the concentration of Cu (per milligrams of total protein content) than astrocytes (Hare et al., 2013). Thus, looking at circadian dynamics of Cu distribution and homeostasis in the SCN could shed light on the importance and function of Cu in both neurons and astrocytes.

4.2a Cu homeostatic mechanisms: transporters and chaperones

Of special interest going forward will be experiments that characterize Cu dynamics across multiple 24-h cycles together with assessing the circadian profiles of homeostatic proteins such as CTR1 and ATP7A. Our results indicate that ATP7A expression may be higher at night (ZT 16) than the day (ZT 6), while CTR1 expression does not differ between these two times. However, the subcellular localization of CTR1 could change across different circadian times as well as in response to various external inputs and environmental changes. As noted previously (see Chp3 discussion), ATP7A's subcellular localization has been shown to change depending on signaling events. *In situ* localization of ATP7A and CTR1 across circadian time as well as the use of live imaging studies could shed light on Cu export and distribution dynamics.

Changes in ATP7A localization are thought to be mediated by ATOX1 in a redox state dependent manner. In the SCN, ATOX1 binding and shuttling of Cu to ATP7A-associated trans-golgi secretory pathways would likely be highest during the day when these neurons are in a more reduced state (Brose et al., 2014; Hatori et al., 2016; Singleton et al., 2010; Wang et al., 2012). Although ATP7A can funnel Cu to secretory vesicles, at the trans-golgi network, ATOX1 and ATP7A are thought to pass Cu onto Cu chaperones and other cuproproteins (Bhattacharjee et al., 2016; Comstra et al., 2017; Gaier et al., 2013b; Hatori et al., 2016; Lutsenko, 2016; Telianidis et al., 2013). These chaperones then transport Cu to other subcellular locations such as mitochondria and/or transfer Cu to cuproproteins. Through ROS or antioxidant signaling and cuproproteins, the mitochondria can feedback on ATOX1/ATP7A activity to decrease mitochondrial Cu uptake and increase vesicular Cu (Baker et al., 2017a; Bhattacharjee et al., 2016; Hatori et al., 2016; Leary et al., 2013; Lutsenko, 2016). Thus, the potential implications of possible diurnal ATOX1 and ATP7A activities most likely depend on factors related to tissue-specific demands on Cu distribution, which still needs to be studied in the SCN. There are several additional Cu homeostatic proteins also worth nothing for future SCN studies, including Cu storage and buffering metallothioneins and the mitochondrial Cu chaperone, SCO1. SCO1 is a critical Cu chaperone for assembly/synthesis of cytochrome c oxidase, without which mitochondria cannot properly produce ATP through oxidative phosphorylation. It is important to

note here that SCO1 is functionally linked to localization of CTR1, hence Cu import, and a SCO1 mutation is embryonically lethal owing to rapid degradation of CTR1 and Cu deficiency (Baker et al., 2017b; Hlynialuk et al., 2015).

4.2b Cu homeostasis, PrPc, and APP/Aβ

There are many other proteins shown to influence Cu levels, availability, and distribution. ATP7B is another ATPase Cu pump, which when mutated results in Cu accumulation and a condition known as Wilson's disease, a condition associated with various neurological symptoms including parkinsonism, ataxia, dystonia, and seizures. ATP7B is localized in the parenchyma and sites of high Cu concentration such as ventricles, where ependymal ATP7B works to efflux Cu into the cerebrospinal fluid (Choi and Zheng, 2009; Davies et al., 2013; Hare et al., 2012; Ohrvik et al., 2017). Indeed, the 3rd ventricle that borders the SCN is reportedly enriched in Cu (Davies et al., 2013; Fu et al., 2015a; Fu et al., 2015b; Hare et al., 2012; Pushie et al., 2011). The cellular prion protein, PrPc has been suggested to act as a sensor and potential docking site for extracellular Cu, which modulates PrPc interactions with other surface or extracellular proteins (e.g. for nitrosylation), and plays a role in metal homeostasis (Brown, 2003; Cheng et al., 2006a; Gasperini et al., 2016; Gasperini et al., 2015; Hodak et al., 2009; Pushie et al., 2011; Rachidi et al., 2003; Stys et al., 2012a; Urso et al., 2012; Vassallo and Herms, 2003; You et al., 2012). Although it is still unclear how PrPc interacts with metal homeostasis machinery, the endocytosis of Cu-bound PrPc is thought to be (indirectly) involved, and this endocytosis is mediated by high affinity interaction with the low-density lipoprotein-related receptor 1 (LRP1) (Hooper et al., 2008; Jen et al., 2010; Parkyn et al., 2008; Pauly and Harris, 1998; Rushworth et al., 2013; Taylor and Hooper, 2007; Urso et al., 2010). Interestingly, our lab has demonstrated that LRP1 is expressed in the SCN, and that inhibiting LRP1 binding to its ligands blocks Glu-induced phase shifts (Cooper and Prosser, 2017). Preliminary evidence suggests increased LRP1 expression during the night when NMDAR activation by Glu resets the clock.

Various proteins involved in Alzheimer's disease also affect Cu homeostasis. Knockout mice lacking amyloid precursor protein (APP) and its paralog APLP2, which have high affinity Cu binding sites, show reductions in brain Cu levels (Barnham et al., 2003; Ciccotosto et al., 2014; Gough et al., 2014; James et al., 2017; Spoerri et al., 2012; Treiber et al., 2004). The β-secretase BACE1, which cleaves APP and promotes amyloid β (Aβ) processing by γ secretase, is a Cu-binding protein that has recently been implicated in Cu compartmentalization, potentially through binding Copper Chaperone of SOD1 (Cu/Zn-superoxide dismutase 1) (Dingwall, 2007; Liebsch et al., 2017). Similarly, the γ secretase presenilin has been shown to play a role in Cu uptake and homeostasis (Greenough et al., 2011; Southon et al., 2013). Although the mechanisms by which these amyloidogenic proteins affect Cu homeostasis is yet to be established, the well-documented involvement of Cu dysregulation in various neuropathologies is consistent with the double-sided role of Cu deficiency and Cu excess in neuronal synaptic signaling, metabolism, and redox regulation. Thus, the following section discusses Cu's role in neurodegeneration and how studying Cu in the SCN can help elucidate emerging links between circadian rhythms and neurodegenerative diseases.

4.3 Cu, neurodegeneration, and oxidative stress

The link between neurodegeneration and circadian rhythms is one of the many emerging areas of interest in neuroscience and chronobiology. Several studies have demonstrated deficits in circadian activity in neurodegenerative diseases, as well as changes in pathological behavioral and neurobiological profiles that have a circadian component, and studies in the SCN have demonstrated direct mechanisms of clock disruption associated with these conditions. Studies show that disrupted circadian/sleep rhythms can precede more overt symptoms of neurodegeneration (Aziz et al., 2009; Bellanti et al., 2017; Colwell, 2011; Cuesta et al., 2012, 2014; Fahrenkrug et al., 2007; Fifel, 2017; Fifel et al., 2014; Harper et al., 2008; Hu et al., 2013; Hu et al., 2009; Kudo et al., 2011a; Kudo et al., 2011b; Li et al., 2017a; Liu and Chang, 2017; Liu et al., 2000; Meijer et al., 2012; Morton et al., 2005; Stevanovic et al., 2017; Van Erum et al., 2017;

Wang et al., 2015b; Wang et al., 2016; Wilking et al., 2013; Wu et al., 2006; Wu and Swaab, 2007; Zhou et al., 2016). Similarly, Cu has been implicated in various neurodegenerative diseases, owing to the variety of processes involving Cu and the role of Cu dyshomeostasis in neurotoxicity. From our data and others, we suggest that endogenous Cu modulates synaptic signaling and plays a role in several features of SCN neuroglial mechanisms, including clock function.

4.3a PrPc, LRP1, Aβ

As discussed in Chapter 3, the SCN exhibits a high resistance to excitotoxicity, and we postulate that this may involve endogenous Cu inhibition of NMDAR and its putative role in MAPK pathway activation. Cu has been shown to reduce NMDAR-mediated Glu excitotoxicity through a process that involves Cu efflux via ATP7A and/or PrPc-bound, Cu-mediated nitrosylation of NMDAR (Gasperini et al., 2015; Hodgkinson et al., 2015; Schlief et al., 2006; Stys et al., 2012a; You et al., 2012). Notably, deficits in circadian/sleep rhythms arising from PrPc knockout have been reported [as reviewed in (Castle and Gill, 2017)]. One possibility is that Cu neuromodulation of NMDAR is disrupted in these mice, resulting in disrupted circadian activity. As mentioned, PrPc may play a role in Cu homeostasis, potentially via LRP1 endocytosis of Cu-bound PrPc, but the physiological relevance of this mechanism in Cu homeostasis is contentious. In addition, it is not known how LRP1 affects Cu/PrPc-induced nitrosylation of NMDAR and Cu reduction of Glu excitotoxicity (Hooper et al., 2008; Jen et al., 2010; Parkyn et al., 2008; Rushworth et al., 2013). However, as discussed below, complex interactions of Cu, PrPc, and LRP1 have been reported in regard to neurotoxicity and a central Alzheimer's disease protein, Aβ.

Cu accumulation in aged mice is correlated with decreased LRP1 in endothelial cells and decreased LRP1-mediated, amyloid beta (Aβ) clearance across the blood-brain barrier in a transgenic amyloidogenic Alzheimer's mouse model (Singh et al., 2013b). Interestingly, down-regulation of LRP1 by Cu involves Cu-induced nitrosylation and degradation of LRP1, possibly mediated by Cu-dependent PrPc-LRP1 interaction and endocytosis (Singh et al., 2013b). The resulting increase in Aβ accumulation further exacerbates Alzheimer's pathology by disrupting

Cu distribution and homeostasis, which in turn increases BACE1 and inflammation. Similar results have been reported by another group (Kitazawa et al., 2009; Kitazawa et al., 2016). Because Aβ binds Cu with nanomolar affinity, the Cu/LRP1-dependent Aβ accumulation likely underlies the observed Cu redistribution and accumulation outside of neurons, as seen in human brain tissue (James et al., 2017). Furthermore, the ability of Aβ to chelate Cu ions has been shown to cause neuronal death due to dysregulation of NMDAR, in connection with PrPc (You et al., 2012). Thus, by removing Cu from synapses and disrupting its physiological role, Aβ-Cu complexes can induce downregulation of LRP1 at the parenchyma, leading to a decrease in Aβ clearance from the brain and even greater accumulation, while simultaneously increasing neuronal death via NMDAR over-activation. To add further insult, interaction of PrPc with Aβ has been shown to induce neurotoxicity through LRP1-depedent endocytosis (Rushworth et al., 2013). Although the actions of Cu in this latter process have not been evaluated, we speculate that Aβ-binding of PrPc is less likely to occur in healthy tissue where Cu-binding to PrPc *and* PrPc endocytosis and cell surface trafficking by LRP1 are all properly regulated. Thus, further studies on Cu and LRP1 in the SCN, in general and in relation to excitotoxicity and Aβ-induced deficits could yield surprising and novel discoveries about SCN clock function.

4.3b GSH/SOD1, oxidative stress, and inflammation

Another feature pertinent to SCN clock function and neurodegeneration is Cu's general role in redox and oxidative stress. First, Cu is known to oxidize various molecules in the cell, and high cellular demands for Cu and inadequate buffering by Cu binding proteins and antioxidants can lead to cellular damage and death. Several studies have demonstrated how a proportional balance of Cu and reduced glutathione/GSH are important for reducing oxidative damage and cell survival, particularly in response to neurotoxic conditions (Du et al., 2008; Hatori et al., 2012; Kumar et al., 2016; Liddell and White, 2017; Mercer et al., 2016; Ozcelik and Uzun, 2009; Samuele et al., 2005; Singleton et al., 2010; White et al., 1999; White and Cappai, 2003). Mitochondrial energetics plays a critical role in determining cellular Cu distribution and metabolism, ROS and GSH levels, and highly metabolically active neurons must coordinate antioxidant processes to

protect against ROS/oxidative stress (Grimm and Eckert, 2017; Requejo-Aguilar and Bolanos, 2016; Stefanatos and Sanz, 2017). Indeed, neuronal differentiation involves concomitant changes in redox status, glutathione antioxidant system and Cu shuttling via the redox-sensitive ATOX1 (Hatori et al., 2016). Similar to ATOX1, ATP7A/B also possess redox-sensitive, cysteinyl thiol groups that when glutathiolated inhibit Cu binding to the pumps until de-glutathiolated by GSH; Cu increases glutaredoxin 1-mediated reduction of oxidized glutathione (GSSG), allowing for ATP7A/B-mediated Cu efflux (Singleton et al., 2010). Furthermore, knockdown of glutaredoxin 1 or chemical depletion of GSH results in intracellular Cu accumulation (Singleton et al., 2010). Conversely, when ATP7A-mediated Cu transport is defective and Cu accumulates in mitochondria, this increases GSSG and sensitizes cells to GSH depletion (Bhattacharjee et al., 2016). Thus, increases in Cu decrease the GSH/GSSG ratio, since GSH is utilized to protect against Cu redox and move Cu to secretory vesicles and out of the cell, and mitochondrial antioxidant systems play a key role in replenishing GSH through mechanisms under circadian control (e.g. NADPH and periredoxin/thioredoxin) (LeVault et al., 2016; Mendez et al., 2016; Milev and Reddy, 2015; Patel et al., 2014; Putker et al., 2017; Rey et al., 2016; Rhee and Kil, 2016).

Another important antioxidant implicated in neurodegeneration is Cu/Zn-superoxide dismutase (SOD1) (e.g., associated with familial Amyotrophic Lateral Sclerosis; ALS). Intriguingly, SOD1 activity exhibits diurnal variation in mouse liver and pineal gland (Cipolla-Neto et al., 1993; Jang et al., 2011). In a recent study using a novel CRISPR genetic screening method in human Jurkat cells, SOD1, ATP7A, and CTR1 were identified as key modifiers of paraquat-induced oxidative stress, where SOD1 and CTR1 are required for protection against paraquat-induced cell death, and the presence of ATP7A decreases resistance to paraquat (Reczek et al., 2017). Increased SOD1 activity due to loss of ATP7A function is a potential mechanism identified in resistance to paraquat, albeit many alternative mechanisms remain untested. Paraquat exposure is a risk factor for developing Parkinson's disease and is used in mice to model loss of dopaminergic neurons. In general, both disruption of Cu distribution and Cu-dependent iron homeostasis have been identified in Parkinson's disease, and so the prominent role of Cu homeostasis proteins in paraquat-induced cell death is a promising discovery. Another intriguing idea is the physiological interaction of SOD1 and the protein DJ-1, also called Park7, because

mutations in DJ-1 are causally related to familial, early-onset Parkinson's (Girotto et al., 2014; Knippenberg et al., 2013; Lev et al., 2015; Lev et al., 2009; Milani et al., 2013; Morimoto et al., 2010; Wang et al., 2011; Yamashita et al., 2010). DJ-1 has been shown to bind Cu and transfer Cu to SOD1 (Bjorkblom et al., 2013; Girotto et al., 2014; Puno et al., 2013), as well as to bind and exert a protective effect against mutant SOD1 models of familial ALS (Knippenberg et al., 2013; Wang et al., 2011; Yamashita et al., 2010). Given the diurnal rhythms in redox and SOD1 seen in different tissues, and the potential circadian function of redox in the SCN, these studies on Cu homeostasis and antioxidants demonstrate a strong basis for a link between Cu physiology and circadian deficits in oxidative stress-related neurodegeneration.

The last feature to discuss here in terms of potential Cu-related mechanisms in SCN and circadian deficits in neurodegeneration is the role of astrocytes and inflammation in Cu metabolism. Astrocytes are important players in Cu homeostasis, especially in protecting neurons against excess Cu. Astrocytes store and release Cu as needed under physiological conditions (Hare et al., 2013; Pal and Prasad, 2014; Qian et al., 2012b; Scheiber and Dringen, 2013; Scheiber et al., 2012). Cu uptake by astrocytes is partially involved in stably maintaining extracellular GSH despite sudden increases in extracellular Cu (Pope et al., 2008). However, antioxidant stabilization of GSH by astrocyte-conditioned media alone is observed also. In line with these observations, astrocytes have been shown to increase export and cellular concentration of reduced glutathione when treated with Cu, surprisingly without cellular increases in its oxidized form (Scheiber and Dringen, 2011). In addition, astrocytic release of pyruvate has been implicated in protecting against oxidative effects of extracellular Cu (Wang and Cynader, 2001). Despite being relatively resilient to the damaging effects of Cu, excess Cu resulting from Cu dysregulation has been shown to be toxic to astrocytes due to unbuffered increases in intracellular Cu (Bulcke et al., 2015; Chen et al., 2008b; Merker et al., 2005; Ramirez-Ortega et al., 2017; Scheiber et al., 2010b). Given the short duration and low concentrations of exogenous Cu used in our experiments (and continued robust neuronal activity rhythms exhibited by the SCN tissue after the experimental treatments), we do not believe astrocytic death to be a pertinent factor to our SCN data. Nonetheless, determining the effects of acute and

long term Cu chelation and Cu application on SCN astrocytes *in vivo* and *in vitro* would be useful in regard to circadian rhythms and Cu-related brain pathology.

Studies to date on the effects of Cu and Cu chelation/deficiency on inflammation have all focused on tissue/cell lines outside the brain (Ansteinsson et al., 2009; Chen et al., 2015; Di Bella et al., 2017; Liu et al., 2016b; Persichini et al., 2006; Wei et al., 2014; Wei et al., 2012); and hence, the physiological relevance of these results are unclear in the context of SCN tissue and our data. However, Cu homeostasis and inflammatory responses appear to feedback on each other under pathological conditions through nitric oxide signaling and various inflammatory factors, in particular tumor necrosis factor-alpha (TNF-α) (Becaria et al., 2006; Hu et al., 2016; Kitazawa et al., 2016; Lu et al., 2009a; Rossi-George and Guo, 2016; Rossi-George et al., 2012; Sun et al., 2017; Terwel et al., 2011). Importantly, TNF-α has been shown to phase shift the SCN molecular clock *in vitro* and *in vivo* via actions mediated by astrocytes (Duhart et al., 2013; Leone et al., 2012). Furthermore, TNF-α has been shown to increase spontaneous firing of SCN neurons by a nitric oxide-dependent mechanism (Nygard et al., 2009). In addition, TNF-α signaling is primarily responsible for lipopolysaccharide-induced phase shifts in behavioral rhythms (Duhart et al., 2013; Leone et al., 2012; Paladino et al., 2014), and several studies have shown that Cu affects inflammatory responses to lipopolysaccharide challenge in relation to neurological function (Kitazawa et al., 2016; Patel et al., 2013; Rossi-George and Guo, 2016; Rossi-George et al., 2012; Wei et al., 2011; Wei et al., 2012). In fact, both ATOX1 and ATP7A may mediate Cu's role in various neuro-immunological responses (Chen et al., 2015; Patel et al., 2013). Thus, these studies highlight a need to investigate Cu homeostasis as it relates to SCN astrocytic function and the interaction between circadian rhythms and inflammation.

In summary, this section reviewed the role of Cu in SCN function as it relates to neurodegenerative diseases, in terms of signaling, redox/oxidative stress, and inflammation. Both Cu chelation and Cu treatment paradigms have been used extensively in these areas, yet our study is the first to perform such experiments in the SCN. Given our experimental results and the various findings described above, it is clear that investigating Cu neurochemistry and

physiology in the SCN could greatly enhance understanding of SCN circadian clock function and diseases of the brain.

4.4 Zinc and iron in rhythm

Cu homeostasis also plays a role in both zinc physiology and iron homeostasis, which have been frequently explored in various brain regions, yet remains relatively uninvestigated in SCN and mammalian circadian rhythms. Zn and Fe rhythms in human blood, milk, and urine have been reported, and there are indications that the clock modulates Zn/Fe metabolism (Araki et al., 1983; Couturier et al., 1988; Guillard et al., 1979; Hongo et al., 1993; Kanabrocki et al., 2007; Kanabrocki et al., 2008; Krebs et al., 1985; Markowitz et al., 1985; Picciano and Guthrie, 1976; Scales et al., 1988; Schumann and Haen, 1988; Taylor and Ghose, 1986). Because Zn and Fe also have various neurobiological functions, dysregulation of these ions has been implicated in neurodevelopmental problems, seizures, and neurodegeneration. Thus, in the next section, I will briefly discuss Zn and Fe physiology in the brain and how they may play a role in the SCN; overall, in light of a Zn-Fe-Cu axis, these ideas reinforce the conclusion that Cu homeostasis and dysregulation require further investigation in the SCN.

4.4a Zn physiology in sleep and circadian rhythms

Constant light has been shown to affect plasma Cu and Zn levels (Morton, 1990). Higher circulating Zn levels have been linked to better sleep quality and duration in different human populations and in mice (Cherasse et al., 2015; Ji and Liu, 2015; Kordas et al., 2009; Luojus et al., 2015; Rondanelli et al., 2011; Saito et al., 2017; Song et al., 2012; Tan et al., 1995; Zhang et al., 2009a). Zn supplementation has been shown to decrease sleep onset latency and to improve sleep quality (Kordas et al., 2009; Rondanelli et al., 2011; Saito et al., 2017). In a study by Song et al., women who had the highest percentage of optimal sleep duration had the highest serum and hair Zn/Cu ratios out of 126, adult Korean women (2012). In another study using mice, Zn

increased non-rapid eye movement (NREM) sleep when given at the onset of dark phase before their normal active period but not when given during the subjective day prior to sleep onset (Cherasse et al., 2015).

Similar to Cu, Zn is involved in many neuronal functions, including modulation of various receptors, intracellular signaling, and apoptosis (Blakemore and Trombley, 2017; Que et al., 2008). As a neurotransmitter, presynaptic Zn is found co-packaged with Glu in vesicles via activity of the transporter ZnT3; in the synapse it can activate a Zn-specific, G-protein coupled receptor, while free, excess Zn is taken back in by reuptake transporters (Ketterman and Li, 2008; Khan et al., 2014). Additionally, there are other transporters for Zn, such as ZIP1, which can form a complex with NMDARs to modulate postsynaptic structure (Mellone et al., 2015). As nearly all SCN neurons are GABAergic, a physiological role of presynaptic Zn/ZnT3 in SCN function is only likely to occur at the glutamatergic inputs to the SCN, such as from retinal ganglion cells or on SCN efferent neurons projecting to the paraventricular thalamus (Alamilla and Aguilar-Roblero, 2010). A neuroprotective and regenerative role for Zn in retinal ganglion cells has been described in relation to ZnT3 and Zn release from retinal cells (Bai et al., 2013; Chappell and Redenti, 2001; Li et al., 2017b). The most likely function of synaptic or extracellular Zn in the SCN would be to modulate NMDA receptors and possibly GABAa receptors, as well as in synaptic plasticity.

NMDAR in the SCN are primarily composed of two NR2B (GluN2B) and two NR1 (GluN1) subunits (Bendova et al., 2012; Clark and Kofuji, 2010; Wang et al., 2008). Because NR2B subunits-containing NMDARs exhibit low-affinity Zn binding, low micromolar applications of Zn have only modest inhibitory effects on most NMDAR-mediated currents in the SCN (Clark and Kofuji, 2010; Wang et al., 2008). However, high-affinity Zn inhibition (~1nM) of NR2A-containing NMDARs has been demonstrated (Bottum et al., 2010; Clark and Kofuji, 2010; Moriya et al., 2000); interestingly, from electrophysiology data, these NR2A-mediated currents appear to be relatively magnesium insensitive, supporting an NR1/NR2A, heterotetrameric complex with either NR2C or NR2D (Clark and Kofuji, 2010). Thus, similarly to Cu, Zn inhibition of presynaptic NR2A/NR2C-containing NMDAR could have important functions in modulating presynaptic neurotransmitter/neuropeptide release. Interestingly, a recent study has shown that NR2C-

containing NMDAR are expressed presynaptically in the SCN, and that they participate in regulating SCN rhythms through astrocytic Glu release rather than stimulus-driven, phasic Glu, (Brancaccio et al., 2017)

Several studies have explored the effects of Zn application on GABAa receptors (-R) in the SCN, especially in regard to diurnal differences and GABAa-R subunits (Belenky et al., 2003; Kawahara et al., 1993; Kretschmannova et al., 2005; Kretschmannova et al., 2003; Strecker et al., 1999). In the SCN, the role of GABAa-R is highly complicated by the effects of day-night changes in chloride equilibrium and synaptic versus extrasynaptic receptor localization. Of note, Zn has been shown to shift the postsynaptic chloride equilibrium via complex effects on the potassium chloride co-transporter 2 that depend on presynaptic input and neuron type (Chorin et al., 2011; Di Angelantonio et al., 2014; Hershfinkel et al., 2009; Saadi et al., 2012). Indeed, the mechanisms and effects of Zn in postsynaptic neurons depend on the activity and function of both presynaptic and postsynaptic neurons (Anderson et al., 2015; Izumi et al., 2006; Kalappa and Tzounopoulos, 2017; Lavoie et al., 2011; Perez-Rosello et al., 2015; Vergnano et al., 2014). In addition, an endogenous role for tonic, non-synaptic extracellular Zn in modulating extrasynaptic NMDAR has been recently found in the dorsal cochlear nucleus (Anderson et al., 2015). In our preliminary ICP-MS studies of SCN tissue, we measured a higher Zn content than Cu (approximately double), but it would be premature to speculate on the implications of these data.

How Cu dyshomeostasis may affect these various processes is unclear due to the lack of studies involving Cu and Zn, but one emerging mechanism is the possibility that accumulation of free Cu can displace Zn from Zn-binding proteins such as metallothioneins or postsynaptic proteins (Baecker et al., 2014; Isaev et al., 2016; Meloni et al., 2007; Meloni et al., 2008; Tanaka and Kawahara, 2017). Intracellularly, Zn has a multitude of effects, ranging from stabilizing protein interactions at the postsynapse (Fernandez et al., 2008; Jan et al., 2002; Lee et al., 2017; Sun et al., 2013; Tao-Cheng et al., 2016); to intracellular modulation of ion channels (Di Angelantonio et al., 2014; Gao et al., 2017; Pitt and Stewart, 2015; Schulien et al., 2016; Tuncay et al., 2013; Wang et al., 2001; Woodier et al., 2015; Xia et al., 2000; Xie et al., 2004; Yi et al., 2013), including calcium release via ryanodine receptors (Pitt and Stewart, 2015; Schulien et al.,

2016; Tuncay et al., 2013; Wang et al., 2001; Woodier et al., 2015; Xia et al., 2000; Xie et al., 2004; Yi et al., 2013); to TrkB transactivation (Huang and McNamara, 2010, 2012; Huang et al., 2008); and ROS generation and apoptosis (Bishop et al., 2007; Chang et al., 2010; Clausen et al., 2013; Liao et al., 2011). Although later studies have cast doubt on TrkB transactivation, Zn affects BDNF levels potentially via upregulation of Zn-dependent matrix metalloproteinase 9 (Helgager et al., 2014; Yoo et al., 2016), and more generally, the role of Zn in synaptic plasticity and modulation of long-term potentiation is well established (Ando et al., 2010; Fujise et al., 2017; Grabrucker et al., 2011; Hagmeyer et al., 2015; Izumi et al., 2006; Kalappa and Tzounopoulos, 2017; Kirsten et al., 2015; Kodirov et al., 2006; Pan et al., 2011; Perez-Rosello et al., 2013; Quinta-Ferreira and Matias, 2005; Sindreu et al., 2011; Sindreu and Storm, 2011; Takeda et al., 2015; Takeda et al., 2017; Vergnano et al., 2014). Of particular relevance is Zn binding and strengthening of CRY1 and PER2 clock protein dimerization in a potentially redox sensitive manner (Schmalen et al., 2014). Although we haven't addressed the many other ways in which Zn may be a key micronutrient in neuronal circadian rhythms, the discussion above highlights the value of studying Zn in different brain regions. And Zn —which is an effective treatment for toxicological Cu in Wilson's disease cases— tends to compete with Cu metabolism.

4.4b Fe in sleep/circadian rhythms, Cu, and heme

Clock control of Fe homeostasis is closely coupled to metabolic demands, and the co-evolution of these systems is inherently driven by cellular handling of oxygen as a byproduct of photosynthesis and as an electron acceptor in oxidative phosphorylation. For organisms that utilize photosynthesis, circadian regulation of Fe is critical for optimally timed chloroplast function, complete with mechanisms to adjust to seasonal variation in LD cycle changes (Botebol et al., 2015; Chen et al., 2013b; Duc et al., 2009; Hong et al., 2013; Salome et al., 2013; Tissot et al., 2014; Wilson and Connolly, 2013). As Fe is also critical to mitochondrial ATP production and the bioenergetics of heterotrophs, food intake influences circadian Fe metabolism, and Fe status in turn influences activity levels and cycles as seen in various mammalian species (Dean et al., 2006; Dowling et al., 2011; Hunt et al., 1994; Kordas et al., 2009; Schumann and Haen, 1988;

Simcox et al., 2015; Unger et al., 2009). Long-term shifts in LD cycle affect Fe concentration in the frontal lobe (Karakoc et al., 2011).

In addition, Fe deficiency negatively impacts sleep quality, and Fe supplementation of Fe can improve sleep duration particularly in groups at risk for deficiency (Dean et al., 2006; Kordas et al., 2009). Indeed, Fe deficiency underlies a subgroup of Willis-Ekbom disease patients, who exhibit periodic limb movements with an apparent circadian/sleep component, and studies and treatment have targeted Fe's role in dopamine synthesis (Allen and Earley, 2007; Baier and Trenkwalder, 2007; Connor et al., 2009; DeAndrade et al., 2012; Dowling et al., 2011; Frauscher et al., 2009; Freeman and Rye, 2013; Furudate et al., 2014; Trenkwalder et al., 2008), As an essential cofactor for tyrosine hydroxylase, Fe dysregulation has also been strongly implicated in Parkinson's pathogenesis. Restless legs symptoms are reportedly common in these patients, which correlate with increased severity of motor and sleep dysfunction and is linked to Fe dysregulation (Piao et al., 2017). Circadian regulation of dopaminergic circuits has been well established both by peripheral clocks and by the master clock (Brooks et al., 2011; Sleipness et al., 2007; Verwey et al., 2016). Notably, tyrosine hydroxylase is regulated by the clock (Bussi et al., 2014; Chung et al., 2014; McClung et al., 2005), and one study has shown that iron deficiency displays a diurnal component in dopamine metabolism (Bianco et al., 2009).

The direct mechanisms by which the circadian clock affects Fe homeostasis in the brain, upstream of changes in expression of Fe-binding proteins, are yet unclear, In mouse colon tumor tissue, CLOCK/BMAL1 were recently identified as transcription factors for iron regulatory protein 2 (IRP2), which is a central component controlling various Fe proteins (Okazaki et al., 2016). Although other clock-controlled components are involved in circadian Fe regulation (Ben-Shlomo et al., 2005; Okazaki et al., 2010), of primary interest to our work are: (diurnally expressed) PrPc and Cu-mediated interactions via Cu-dependent ferroxidases ceruloplasmin and hephaestin. Although the role of PrPc in Fe homeostasis has been reported in various models in the past, these often involved the scrapie form of PrPc involved in prion disease (Fernaeus et al., 2005a; Fernaeus and Land, 2005; Fernaeus et al., 2005b; Singh et al., 2011; Singh et al., 2009a; Singh et al., 2009b; Singh et al., 2009c; Singh et al., 2012). Nonetheless, there are data suggesting a

physical interaction between PrPc and Fe, and more recent studies have investigated a putative ferrireductase domain that mediates interactions with transport machinery to increase Fe import (Asthana et al., 2017; Bhupanapadu Sunkesula et al., 2010; Das et al., 2010; Haldar et al., 2015; Lee et al., 2007; Park et al., 2008; Singh et al., 2013a; Tripathi et al., 2015). In addition, PrPc knockout mice may also have decreased cellular Fe content due to decreased systemic Cu and Cu-dependent ferroxidase activity (Gasperini et al., 2016; Pushie et al., 2011). Systemic decrease or dysregulation of ferroxidase activity results in deficits in Fe storage and delivery to various tissues, and can result in brain Fe deficiency and neurological symptoms as in aceruloplasminemia (Gulec and Collins, 2013; Ha et al., 2016; Kono, 2012; McCarthy and Kosman, 2014). At the cellular level, however, Cu-dependent ferroxidase activity is required for Fe export via ferroportin, and as such, Cu dysregulation also causes Fe accumulation in the brain (McCarthy and Kosman, 2013, 2014; Vashchenko and MacGillivray, 2013; Welch et al., 2007). Furthermore, ferroxidase deficits due to cell- or brain region-specific Cu dysregulation is causally linked to Fe-induced oxidative stress underlying dopaminergic neurodegeneration as seen in Parkinson's disease (Bharucha et al., 2008; Boll et al., 2008; Boll et al., 1999; Dusek et al., 2015; Rubio-Osornio et al., 2013; Song et al., 2017).

From the evidence above, it is not surprising that studies have shown how Fe physiology may conversely feedback on neuronal circadian biology, particularly as a cofactor in heme. In the SCN, where heme metabolism is circadian, exogenous heme or inhibition of its degradation disrupts PER2 transcriptional rhythms (Guenthner et al., 2009; Rubio et al., 2003). In Drosophila neurons, many Fe homeostatic proteins appear to modulate their clock proteins, PER and TIM, (Mandilaras and Missirlis, 2012). Underlying these observations is the fact that PAS-domains found in clock proteins NPAS, CLOCK, and PER function in heme binding (Hayasaka et al., 2011; Kitanishi et al., 2008; Lukat-Rodgers et al., 2010). Perhaps even more intriguing is that binding of heme to Rev-Erb proteins affects Rev-Erb's interaction with nuclear corepressors, and hence, modulates their role in circadian transcription regulation in a putatively, redox-dependent mechanism (Carter et al., 2017; Gupta and Ragsdale, 2011; Matta-Camacho et al., 2014; Yin et al., 2007). There are other heme- or Fe-binding proteins involved in the clock, including sGC, and iron homeostatic proteins under circadian control which may feedback and modulate iron levels

(Girvan and Munro, 2013; Okazaki et al., 2016; Okazaki et al., 2010; Robles et al., 2014; Schaap et al., 2013; Simcox et al., 2015; Zhang et al., 2017). Dexras1 is particularly intriguing as it is: a) under circadian control (Gerstner et al., 2006; Hahnova et al., 2016; Takahashi et al., 2003); b) plays a role in photic and non-photic phase shifting responses via MAPK signaling (Cheng et al., 2006b; Cheng et al., 2004; Hahnova et al., 2016); and c) is activated (putatively via nitrosylation) downstream of NMDAR and NO signaling, resulting in Fe influx and increased excitoxicity (Cheah et al., 2006; Chen et al., 2013a; Choi et al., 2013; White et al., 2016). Thus, the role of Fe in heme redox biology and circadian molecular rhythms demands further inquiry and understanding. Proteins which can bind both Cu and Fe –such as PrPc and alpha-synuclein (amyloidogenic protein found in Lewy bodies and Parkinson's disease), multicopper ferroxidases, and putative interacting partners including amyloid precursor protein, and their degenerative, neurotoxic forms– are implicated in various aspects of neurobiology and psychopathology, including excitotoxicity-induced seizures, depression, and fatal brain diseases. These Cu/Fe-associated proteins are implicated in sleep and circadian rhythms to varying degrees, and circadian deficits together with more conspicuous sleep-wake and monoaminergic disorders can appear when these proteins are dysregulated, such as by abrupt or harmful environmental factors, metabolic/genetic/nutritional deficiencies, or Zn dyshomeostasis. Therefore, in some cases, the early detection of various neurological conditions not yet outwardly apparent may be aided by monitoring circadian outputs; conversely, awareness and avoidance of disruptors of circadian biology, including exposure to toxic metals, may prevent or prolong onset of neuropathology, even cancer.

I end this section by reframing this under-appreciated, and poorly understood area in SCN physiology of metals in rhythms, behavior, and neurodegeneration. Cu, Fe, and Zn accumulate with age and correlate with mitochondrial dysfunction and dysregulation of cellular metabolic activities, both of which tie into excess oxidative stress and eventual neurodegeneration. Ideally, the SCN pacemaker should be able to quickly pick up on various external cues, yet maintain a robust, autonomous function. At the same time, it must optimize metabolic and cellular redox activities for growth or health, maximize antioxidant and tissue repair and protective mechanisms, and minimize environmental hazards (e.g. UV/ozone) and external-internal conflict

(e.g. jetlag) in order to coordinate peripheral clocks in the brain and body for evolutionarily adaptive behaviors, especially protecting and increasing brain functionality. Thus, the SCN is a critical model for metallo-neurobiology and will define new approaches to increase health and well-being in conjunction with nutrition, exercise, and self-awareness of key, internal diurnal processes. By further studying these trace metals in neurophysiology and SCN rhythms, we can begin to explore new solutions to physical and mental health, perhaps even new approaches to cancer and neurodegeneration, where Fe/Cu/Zn chelation therapy trials have already begun.

4.5 Conclusion and future directions: Cu and SCN function

We have only scratched the surface of the myriad questions that come out of this research. Focusing back on our model, there remains many questions regarding the effects of TTM and exogenous Cu. We hypothesize that extracellular Cu modulates NMDAR activity in the SCN, and at night it inhibits the effects of tonic or extrasynaptic Glu that are not related to photic input. This is supported by our Cu chelation data, but as stated, we do not know the specific mechanisms by which Cu removal directly affects receptors, signaling and metalloproteins. We need to assess if and which pools of Cu are directly affected by TTM (and BCS) using fluorescent Cu-specific sensors and/or synchrotron X-ray fluorescence, for example. In-depth characterization of basal level Cu distribution and homeostasis in astrocytes and neurons over circadian time, as discussed earlier, as well as after Glu and other circadian-related manipulations would indirectly support some of our hypotheses.

In addition, Cu has been shown to inhibit or modulate calcium, GABAa-R, and even K^+ channels, but we have not tested these ideas yet, particularly in regard to TTM effects. As we theorize that calcium-dependent signaling is involved in TTM-induced, nighttime phase shifts, investigating calcium dynamics and channel activity, respectively, by imaging or patch-clamp electrophysiology in tandem with inhibitors would be critical in confirming TTM effects. Calcium imaging and electrophysiology would also implicate which neurons are not directly affected by

TTM in regard to ventral input (e.g. GABA, VIP) to dorsal neurons in the SCN shell region versus selective activation of dorsal neurons only.

On a different note, TTM-induced phase shifts during the midday (ZT6) are not mediated by NMDAR, when Glu does not induce phase shifts either (Fig. 4.1). Previous research on possible mechanisms for daytime phase shifts in general have focused on suppression of neuronal firing, such by GABAa-R, serotonin receptors, and K^+ channels. It is possible that endogenous Cu inhibits hyperpolarizing currents and TTM has the opposite effect, resulting in suppression of neuronal activity. In general, however, micromolar Cu is thought to suppress neuronal firing, yet application of Cu at ZT6 did not induce daytime phase shifts. We suggest two possible explanations: First, TTM could suppress firing of VIP-expressing neurons by decreasing Cu in the ventral region, where restoring endogenous Cu inhibition to those specific, extracellular sites is delayed; and the effect of Cu bath-application is both temporary and spread throughout SCN regions. Alternatively, the divergence of daytime effects between Cu chelation versus Cu application on SCN circadian rhythms may functionally relate to ERK1/2 activity rhythms. During the day when phosphorylated ERK1/2 activity is the highest, increasing MAPK signaling by Cu application would not be expected to have an effect on SCN activity rhythms even if this were possible. In fact, non-photic phase shifts induced by dark pulses and sleep deprivation during day have been shown to suppress the high ERK1/2 phosphorylation/activity. Since TTM would likely also suppress MEK1-MAPK signaling, these reports are consistent with our data showing no effect of MEK1/2 inhibition on TTM-induced, daytime phase advances. Thus, daytime effects of TTM are likely multifactorial and require further studies on ion channels, neuronal activity, and intracellular signaling. Additionally, cAMP-dependent Protein Kinase A has been implicated in daytime suppression of neuronal firing and phase shifts and would be an important pathway to investigate.

Of major interest for future studies is to determine whether Cu inhibition of NMDAR activity requires PrPc and/or nitrosylation of NMDAR. Although protein level data have not been reported, circadian expression of PrPc mRNA with a peak in early subjective night has been shown in the SCN (Cagampang et al., 1999). Since the role of Cu-dependent PrPc in modulating NMDAR

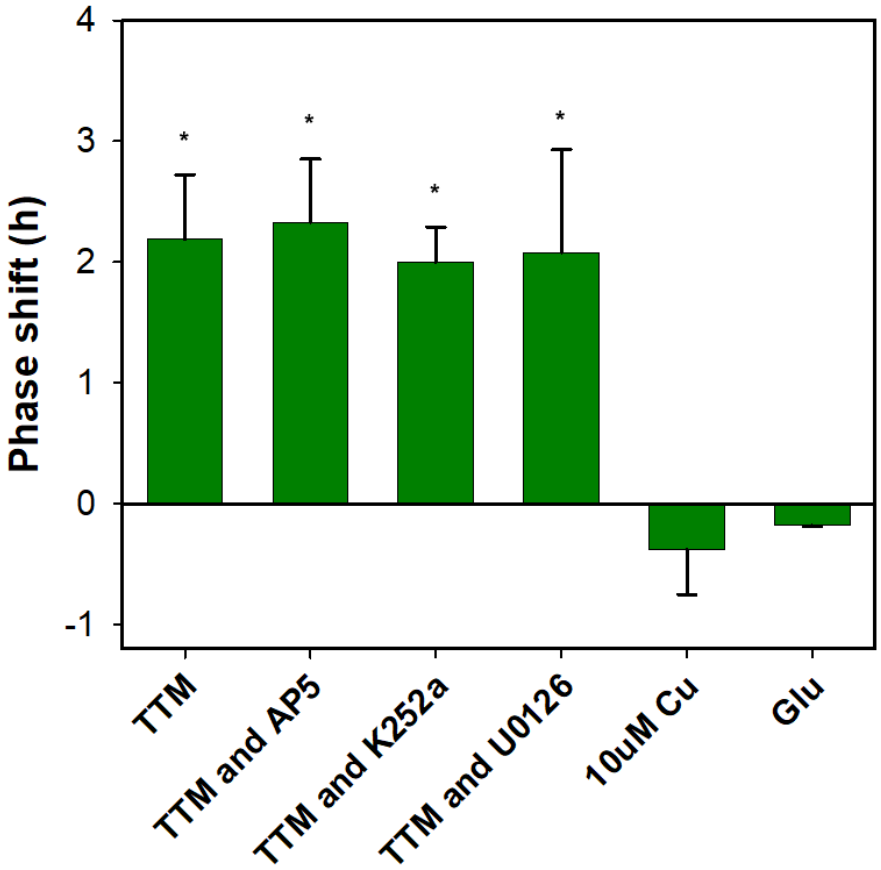


Figure 4.1. TTM, but not Cu, induces daytime phase advances at ZT6

Histogram plot summarizing the mean phase shifts ± SEM resulting from the indicated treatments. 10min TTM (1µM) treatment at midday (ZT6) induces phase advances, which are not blocked by AP5, K252a, and U0126 (inhibitors for NMDAR, TrkB, and MEK1/2, respectively). Neither 10min application of 10µM Cu nor 1mM Glu alone phase-shift SCN neuronal activity rhythms at ZT6. One-way ANOVA indicated a significant effect of treatments ($F = 8.641$, $p<0.001$). Advances are plotted as positive values. *Phase advances significantly different from control ($p<0.05$). N=3-4 for all experiments.

has been linked to LRP1 endocytosis, these studies would additionally intersect with LRP1 research by our group. Whether the endocytosis of NMDAR and the functions of LRP1 in receptor endocytosis are relevant to SCN neurons remains unknown. If neither nitrosylation nor Cu-LRP1 interactions are involved in NMDAR modulation by Cu, a less obvious mechanism may involve Zn displacement from redox-sensitive sites and/or complex, biphasic characteristics of both Cu and Zn which have been shown to activate or decrease neuronal firing and ion channel activity depending on low nanomolar versus micromolar Cu or Zn concentrations (Aedo et al., 2007; Delgado et al., 2006; Peters et al., 2011). Neither Zn imaging nor chelation studies have been performed with SCN tissue/neurons, but these experiments could prove highly insightful and establish a completely new role for Zn in the brain and circadian rhythms. Future experiments could test the synaptic effects of applying both Cu and Zn together, with or without the presence of metalloproteins such as APP or amyloid β, in order to examine circadian disruptions in receptor signaling and neurotoxicity in the context of trace metal dyshomeostasis and SCN pathophysiology in neurodegenerative diseases.

One of our main general questions regarding Cu in the SCN concerns redox and oxidative stress. Again, Cu is known to induce nitrosylation of proteins, and this may relate to our result that Cu-induced phase advances required NO signaling. Neither nitrosylation of NMDAR and other proteins in the SCN nor rhythms in nitrosylation in tissues have been reported, to the best of our knowledge. Indeed, nitrosylation of NMDAR may only occur under certain conditions, such as when it is physiologically important to inhibit and internalize NMDAR following phasic activation. In addition, Cu and Cu homeostasis are intertwined with redox rhythms, and many aspects of redox rhythms are unknown in the SCN. One of the primary players in physiological Cu-redox activities is glutathione and the enzyme glutaredoxin, and the function and importance of the glutathione system is understudied in the SCN and circadian rhythms in general. As depicted in the models below (Figs. 4.2 and 4.3), we hypothesize that: diurnal differences in GSH metabolism influences cellular Cu distribution and metabolism in a circadian manner, as governed by underlying molecular, metabolic, and redox oscillations; and diurnal changes in Cu metabolism feedback on these underlying clocks—inducing phase shifts when signaling pathways, mitochondria, and ROS/antioxidant activity are uncoupled by shifts in Cu homeostasis.

Figure 4.2. Model of SCN Cu physiology during the day.

During the day, Cu is readily imported into SCN neurons by CTR1, which requires the reduction of Cu^{2+} to Cu^{+} (not shown here). This would be potentially facilitated by the relatively reduced state of SCN neurons and by higher levels of reduced glutathione (GSH) during the day. By modifying ATOX1 via glutaredoxin1 (GRX), directly binding Cu immediately upon import, or through indirect mechanisms, GSH has been shown to influence Cu uptake. High affinity Cu binding by metallothioneins (MT) is crucial in protecting against Cu redox activity (i.e. Fenton chemistry) and consequent oxidative stress. Cu chaperones such as ATOX1, CCS (Copper Chaperone for Superoxide Dismutase), and mitochondrial Cu chaperones (e.g. SCO1, COX11, etc.) deliver Cu to ATP7A-associated secretory vesicles, Cu/Zn-dependent SOD, and mitochondrial targets related to cytochrome c oxidase (COX), respectively.

During the day, (light-stimulated) glutamatergic input to SCN neurons does not induce phase shifts, and hence, SCN clock(s) utilize various mechanisms to temporally gate the effects of light/glu signaling to coordinate SCN activity rhythms with external daytime cues. These clock-regulated mechanisms will feedback on the clock(s) overtime, so that entraining cues such as light during the night, can shift the clock in a time-dependent manner. The molecular clock drives/regulates a variety of processes, including high neuronal firing and $[Ca^{2+}]_i$, Ca-dependent and independent signaling pathways, mitochondrial processes, $Fe^{3+/2+}$-/heme-related metabolism, and antioxidant levels. In turn, cellular metabolic activities, particularly within the mitochondria, feedback on to the clock by affecting energy substrate levels (e.g., ATP/AMP), redox-cycling metabolites (NAD(P)H, FADH), and possibly via increased ROS generation/oxidative stress leading to signaling responses and depletion of GSH. Cu metabolism and homeostasis may also play a role in these processes/daytime features. Cu may affect activity of redox-sensitive K^{+} channels through direct interaction or indirectly by modulating ROS/antioxidant mechanisms (H_2O_2, SOD, GSH). Higher Cu import during the day could: activate MEK1 via putative, high affinity (attomolar) binding and underlie higher MAPK activation during the day; influence COX-dependent oxidative phosphorylation and ATP production; and generally promote oxidization once GSH levels decrease (e.g. protein-thiol oxidation, lipid peroxidation, and zinc displacement). Other known intracellular mechanisms relate to metabolism or signal transduction and include,

binding-based inhibition of phosphodiesterases and phosphatases, as well as modulation of transcription factors (SP1, AP1, NRF2) and proteases/protease inhibitors.

The last set of mechanisms may not be as relevant to Cu physiology, and rather be associated with Cu dyshomeostasis and betray cellular pathophysiology, yet the difference between normal Cu function and dysregulation may be functionally relevant in the SCN. The gating of exogenous Cu effects during the day versus night, and the shift in rhythms induced by either Cu chelation or addition –nighttime only for addition– suggests that the status of Cu homeostasis is an important metabolic and physiological cue between the SCN and other tissues and brain regions—relaying information related to food consumption/energy state, liver function, inflammation, and other processes in the context of circadian or sleep-wake rhythms. In a similar vein, Cu levels have been shown to influence neuropeptide Y transcription, neuroplasticity, synchronized neuronal firing, as well as circadian genes, activity and behaviors (in plants and aquatic animals); and on the other hand it is required for iron homeostasis, norepinephrine synthesis, thermoregulation, neuropeptide activation (e.g. AVP amidation), and protection against seizures and aberrant neuronal activity. Thus, tight control of (intra)cellular Cu distribution is essential for a host of processes, and the window between Cu physiology and pathophysiology is narrow.

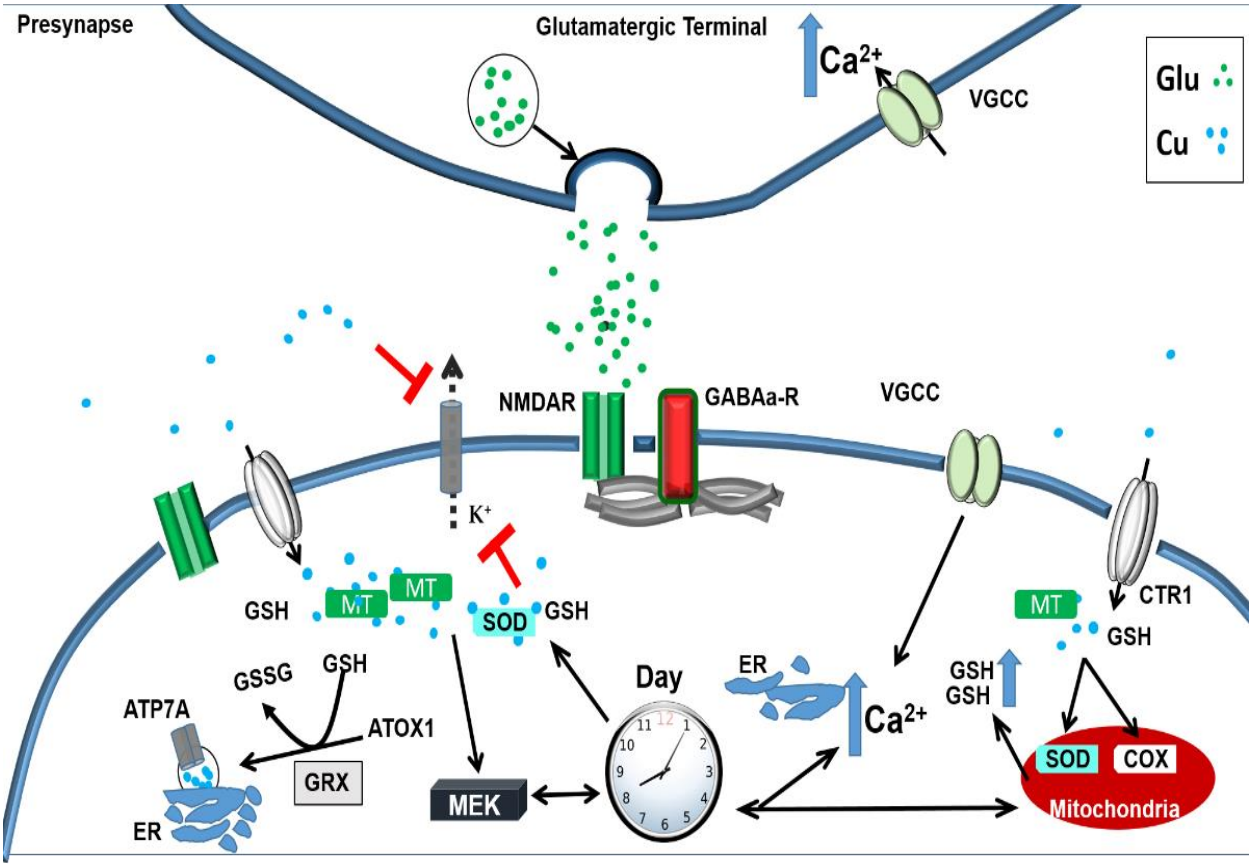


Fig. 4.2 continued

Figure 4.3. Model of SCN Cu physiology during the night.

Cu (blue dots) can inhibit various ion channels, including voltage-gated Ca^{2+} channels, which may partially underlie, decreased postsynaptic $[Ca^{2+}]_I$ and neuronal firing during the night in the SCN. Extracellular Cu inhibits NMDAR-mediated, calcium influx during the night. Thus, we believe that TTM chelation of Cu induces $[Ca^{2+}]_I$ increase during the night, and underscore future experiments looking at TTM-induced changes in $[Ca^{2+}]_I$ and Ca^{2+} channel activity. ATP7A movement to the plasma membrane and efflux of Cu has been shown to suppress NMDAR overactivation and Ca^{2+} excitoxicity. ATP7A translocation can occur in response to excess Cu but also Ca^{2+} influx and K^+-induced neuronal depolarization/firing, which raises the possibility of a negative feedback loop. Thus, high neuronal firing and $[Ca^{2+}]_i$ during the day may be followed by increased Cu efflux at later time points, which requires increased ATP7A activity and is consistent with increased ATP7A expression during the early night (Fig. 3.4). The increased Cu flux and sequestering into secretory vesicles would use up reduced glutathione (GSH) more rapidly in order to de-glutathiolate ATOX1 and ATP7A (via glutaredoxin-1, GRX) for increased Cu binding/transport activities. This redox-cycling process would require reduction of ATOX1/ATP7A by GSH at a high rate, resulting in a higher ratio of oxidized glutathione (GSSH) to GSH during the night. This is consistent with the increased, global protein glutathiolation in SCN tissue seen during the night, when SCN neurons are also more oxidized (Wang et al., 2012).

Another facet of Cu inhibition of NMDAR activity is Cu-induced nitrosylation of the receptor, possibly via cellular prion protein (PrPc). Cu-bound PrPc is able to physically interact with NMDARs, and this Cu-induced interaction is necessary for PrPc-mediated nitrosylation of NMDARs. Research by our lab has shown that low-density lipoprotein receptor-related protein-1 (LRP1) is important in glu/NMDAR-dependent phase shifts during the night when LRP1 expression may be higher than the day. Because LRP1 is involved in endocytosis of PrPc and NMDARs, and Cu promotes PrPc endocytosis, one intriguing possibility is that LRP1 endocytoses PrPc which are bound to NMDARs in the presence of Cu. (The PrPc-associated endocytic actions of LRP1 may or may not occur extrasynaptically). Since PrPc-bound NMDARs are likely nitrosylated, LRP1 may play a role in recycling nitrosylated NMDARs to return them to the cell

surface free of this inhibitory modification. Consistent with this hypothesis, blocking LRP1's extracellular binding sites and hence, its cell-surface activities, blocks glu-induced phase shifts. Together with Cu's neuromodulatory role, it is possible that many NMDARs exhibit increased nitrosylation during the night, especially pertinent given that SCN astrocytes release higher amounts of glu during the night (as depicted by green dots).

Lastly, processes activated by the circadian clock during the day underlie the transition and changing of cellular features in the SCN associated with the night, some of which negatively feedback on daytime processes—including an increased role or distribution of extracellular Cu during the night (hypothetical). Eventually negative-feedback mechanisms wind down, and suppression of daytime processes decreases. However, excess Cu (>4μM) disrupts this timed "wind down" through a variety of mechanisms implicated in our pharmacological experiments (but many of which remains to be tested). An acute excess of intracellular Cu –modeled by exogenous application experiments– during the early night could signal a prolonged daytime or delay the onset of late-night cellular states, whereas during the late night, could signal an advance due to environmentally-linked changes in circadian behavior/physiology. Many disease states influenced by the clock (e.g. Alzheimer's, infection, cancer) exhibit chronic Cu dysregulation and highlight the need for future research in circadian Cu physiology and dyshomeostasis.

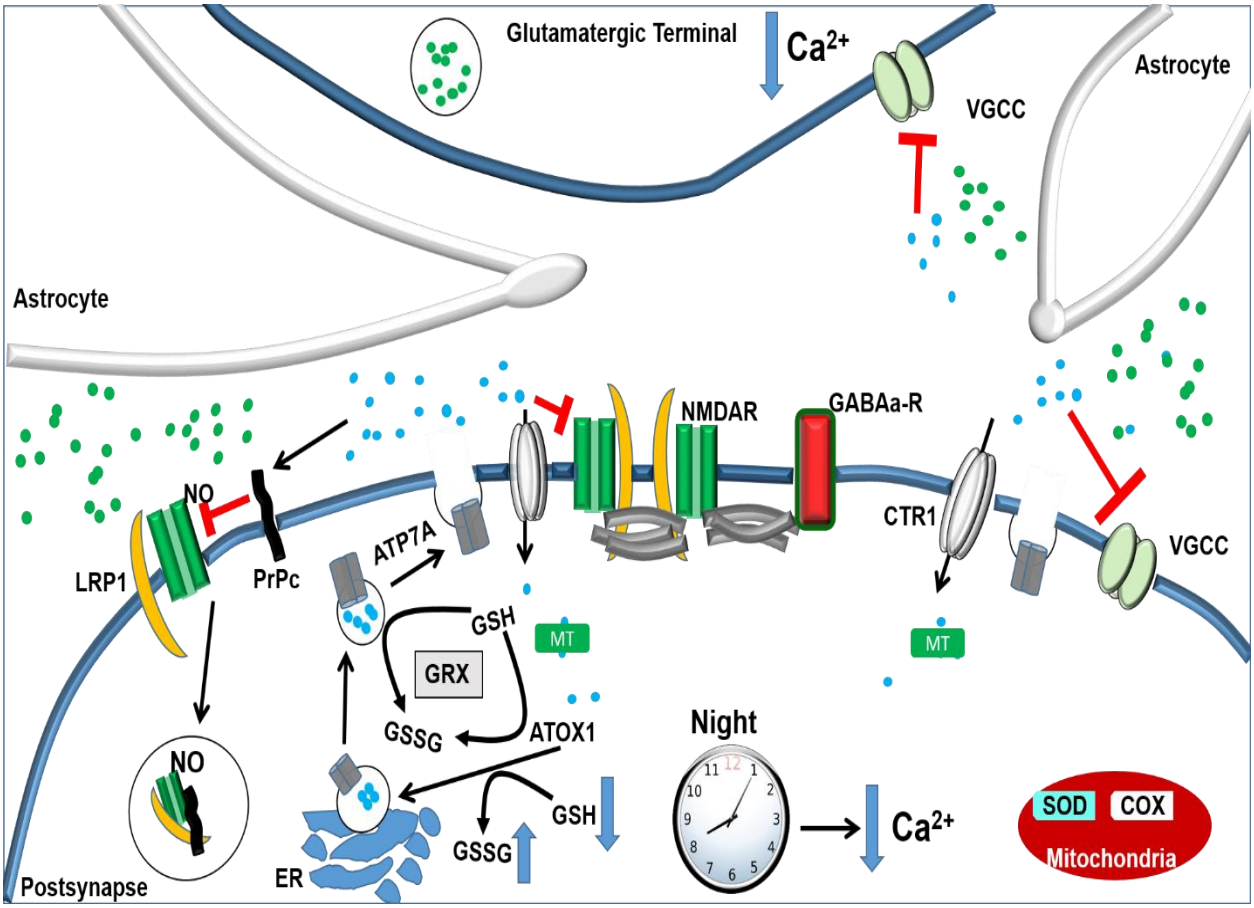


Figure 4.3 continued

In addition, Cu plays a major role in iron homeostasis and are similar in their redox active chemistry. Nonetheless, because Fe and Cu are differently involved in various aspects of cellular redox activities, not just limited to mitochondria, and heme plays a key part in circadian rhythms, studies on Fe/heme-dependent signaling *per se* merit much more involved inquiries. Furthermore, Cu, Zn, and Fe homeostasis all intersect in respect to oxidative stress and mechanisms of cell death. Brain cells are all highly sensitive to excess trace elements to a varying degree depending on brain region-associated metabolic processes, redox physiology, and neuroglial interactions. In various ways, these metals affect inflammatory processes by increasing oxidative stress and participate in programmed cell death, both physiologically and in disease states. The robustness of the SCN clock may be adaptive, to be resilient to changes in oxidative stress and be protected from acute neurotoxic insults under relatively normal conditions, but in cases of neurodegenerative diseases and chronic dysregulation, its output rhythms may be sensitively tuned to environmental inputs and offer insight into synaptic dysregulation. Since Cu, Zn, and Fe have established roles in synaptic function and their dyshomeostasis is strongly implicated in oxidative stress and various neurotoxic/-degenerative states, the SCN and its robust cellular rhythms provide a unique opportunity to study both synaptic dysfunction and oxidative stress in regard to trace metal dyshomeostasis and neurodegeneration.

Finally these data may relate to how the three oscillators found in the SCN intersect. Cu, Zn, and Fe are all involved in various aspects of cellular metabolism, from glucose regulation to mitochondrial oxidative phosphorylation. Cu and Zn interact with extracellular and intracellular signal transduction mechanisms, while Fe primarily affects molecular pathways intracellularly via heme and redox-associated activities. The regulatory mechanisms controlling extracellular Cu and Zn are strongly influenced by redox and hence, redox modulates the effects of Cu and Zn on interneuronal and neuroglial communication.

Together these highly diverse and complexly intertwined activities provide a picture of how these oscillators became interconnected as they evolved, creating a system that is robustly sensitive and adaptive to environmental conditions. From the unicellular organisms evolving in a metal-rich, redox active environment to photosynthetic organisms in oxidant rich conditions and

to brain cells that control sleep/wake and feeding; from the non-transcription based circadian redox rhythms to the signal transduction pathways that help integrate external stimuli; from the extracellular mechanisms regulating intercellular communication that connect cells of different tissue systems to the modulation of synaptic and extrasynaptic neuronal input/output—coordinated by various circadian clocks with different gears. Through their roles in cellular energetics and redox, in extra- and intracellular signal transduction, and in oxidative stress and cell death: Cu, Zn, and Fe have a unique possibility in their interconnected homeostasis to play a fundamental role in circadian timekeeping of the SCN master clock. Together they can serve as metabolically and pathologically pertinent inputs to the SCN from the body, help coordinate and synchronize the complex circuit of the SCN network, and thus, reflect the functional status of various cellular processes and overall brain health. In this study of Cu and SCN neuronal activity rhythms, we have only uncovered one part of one process in this evolutionarily created puzzle. But with more time and hands, we can take this watch apart, piece by piece.

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VITA

Yukihiro Yamada was born in Hiroshima, Japan, to Takashi and Kikue and as the younger brother to Hiro and Mio. He grew up in Cork, Ireland, while his father Takashi earned his PhD in material science there, and attended St. Colomba's Boys School until age 11 (interrupted by ~2 years in South Wimbledon, England). He moved to Knoxville, Tennessee, and attended Webb Middle School. He also formally learned Japanese during these years and attended East Tennessee Japanese School (ETJS). After graduating from Farragut High School and ETJS in 2004, Yuki double-majored in Biochemistry & Cellular and Molecular Biology (BCMB) and philosophy at the University of Tennessee, Knoxville, earning his BS in spring 2008. He became best friends with his future wife Mary Jane during college and started dating in 2007 till they married in 2015. Having taught languages, both formally (Foreign Language Academy, Knoxville) and privately, and Math at ETJS, he was inspired by great teachers at UT who spoke the language of science or philosophy. So he took more classes, undergraduate and graduate, and decided to work in a lab. Under the supervision of Drs. Rihui Yan and Bruce Mckee, Yuki worked with fruit flies to study mutations in meiosis genes. Then, as a BCMB graduate student (from fall 2009 to fall 2017), he continued his education in diverse subjects, including philosophy and statistics, while teaching biology labs and performing experiments. In Dr. Rebecca Prosser's lab, he learned about circadian rhythms and acquired a strong taste for copper. Being passionate about many other things, especially basketball and music, his goal is to never stop learning and growing. He moved to Seattle, WA, with his beautiful and amazing wife to start a new journey.