

November 2015

## Behavioral, Neurobiological, and Genetic Analysis of the Circadian Mutant Duper

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**Behavioral, Neurobiological, and Genetic Analysis of the Circadian Mutant Duper**

A Dissertation Presented

By

EMILY NICOLE CORBETT MANOOGIAN

Submitted to the Graduate School of the  
University of Massachusetts Amherst in partial fulfillment  
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

September 2015

Neuroscience and Behavior

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## DEDICATION

To my parents, Jane Marie Corbett and Charles Arthur Manoogian, and my fiancée, Ajay Dhanendra Patel, for their unconditional love, support, and faith in me.

## ACKNOWLEDGEMENTS

I would like to thank my advisor, Eric Bittman, for his superior mentorship. He challenged me so I could learn as much as possible and respected me as an equal. Creating hypothesis, designing experiments, and bouncing ideas off each other was my favorite part of graduate school. Thank you to my amazing committee: Tanya Leise, Bill Schwartz, and Courtney Babbitt. I could not have performed the mathematical analysis without Tanya. Bill provided crucial guidance for both the behavioral and neurobiological work. Courtney offered much needed advice on all genetic studies.

I would also like to thank Jeff Blaustein and Mary Holder for assistance with tissue collection, David Weaver and Hal Gainer for supplying antibodies, Joe Bergan for his assistance with automated image analysis, and John Hogenesch for being a wonderful collaborator and hosting me in his lab for a week to teach me bioinformatics. A special thank you to Vickie Rupp and Animal care, especially Amanda Hageman, for going above and beyond. Thank you to Carrie Mahoney who helped train me, and all of the undergraduate researchers in the lab, especially Julia Napolitano and Michael Thomas. I also want to thank the hamsters that were part of this work.

I would also like to thank Lance Kriegsfeld for making me fall in love with research and taking a chance on me as an undergraduate researcher, my cat Mo, and all of my friends in the NSB program for their continued support.

Finally, I want to thank my parents, Jane and Charles, and my fiancé, Ajay, for making me believe I was capable of anything. My mother has been my inspiration and friend, providing unconditional love and support. Ajay has been loving, patient, encouraging, and supportive. I could not have done it without them. Thank you all.

## ABSTRACT

### BEHAVIORAL, NEUROBIOLOGICAL, AND GENETIC ANALYSIS OF THE CIRCADIAN MUTANT DUPER

SEPTEMBER 2015

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The recently discovered circadian mutant hamster *duper* has a short period of ~23 hours and exhibits exaggerated phase shifts in response to a 15-min light pulse. To increase the understanding of the *duper* mutation, I performed behavioral, neurobiological, and genetic experiments. Behavioral studies using photic and non-photoc stimuli found that large phase shifts exhibited by *duper* hamsters are specific to photic cues, but not to phase. Additionally, 2/3 of *duper* hamsters, but no WT's, displayed transient ultradian wheel-running patterns when transferred from light to dark at CT 18. This suggests that the mutation may weaken coupling among components of the circadian pacemaker. Anatomical and immunocytochemical analysis of the SCN was used to examine the neurobiological mechanisms of large light-induced phase shifts in *duper*s. Brains were collected from *duper* and WT hamsters at CT 12 and 15 as well as 1, 2, 3, 6 and 9 hours following a light pulse, or control handling, at CT 15. Surprisingly, the only difference in PER1 (a core clock protein) expression in the SCN between *duper*s and WT's was seen 2-hours after a light pulse; *duper* hamsters displayed a significantly greater percentage of retinorecipient VIP cells co-labeled with PER1 compared to WT's.

Additional differences between genotypes occurred 9 hours after CT15 (controls). In the SCN, the number of PER1-ir cells was significantly greater in WT than duper hamsters, however this finding was reversed in the PVN. This anatomical mismatch suggests the mutation may affect signaling between the SCN and extra-SCN oscillators. Finally, to identify the genetic basis of the duper phenotype, I crossed dupers with a novel ecotype in order to perform fast homozygosity mapping. Duper transmitted onto the novel ecotype with the predicted Mendelian inheritance of phenotype. I collected DNA from F2 duper hamsters, and expect fast homozygosity mapping will identify candidate genetic regions of the duper mutation. Additional behavioral experiments in F2 dupers demonstrated that duper hamsters are resistant to jet lag. As duper is a unique circadian mutation, understanding of the behavioral phenotype, neurobiological mechanism, and genetic basis of the duper mutation will greatly increase our knowledge of the circadian system.

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# CHAPTER 1

## INTRODUCTION

### Circadian Rhythms

Circadian rhythms are endogenous daily oscillations in physiology and behavior that allow organisms to coordinate their behavior with their environment and maintain internal temporal organization. Circadian rhythms originated over 2 billion years ago at the time of the Great Oxidation Event and have been identified in almost all life forms ranging from archaea to humans (Bell-Pedersen et al., 2005; Whitehead et al., 2009; Edgar et al., 2012; Loudon, 2012). Almost all physiological and behavioral mechanisms are under circadian control, including temperature regulation (Aschoff, 1983; Brown et al., 2002), hormone release (Moore and Eichler, 1972; Legan and Karsch, 1975; Williams III et al., 2010), consolidation of sleep (Edgar et al., 1993; Dijk and Czeisler, 1994), and metabolism (Kalsbeek et al., 2007; Bass and Takahashi, 2010). As such, circadian rhythms play an essential role in the physiological success of an organism. Rhythms are important for survival as they dictate activity patterns that are necessary for predator avoidance and foraging (Bartness and Albers, 2000). In mammals, circadian rhythms regulate reproduction by controlling the timing of ovulation, behavioral receptivity/mate seeking, seasons of fertility, and the success of pregnancies (Eskes, 1984; Miller et al., 2004; Boden and Kennaway, 2006; Alvarez et al., 2008; Kovanen et al., 2010; Pita et al., 2011). Cyanobacterial strains whose endogenous period allow them to adopt an optimal phase relationship with their environment gain an adaptive advantage over competitors that do not (Woelfle et al., 2004). Proper development and survival of new offspring is

also clock-controlled in rodents (Hoshino et al., 2006) and insects (Clayton and Palletta, 1972; Saunders et al., 1976).

Due to the wide reaching role and importance of the circadian clock, disruption of these rhythms can lead to a variety of physiological consequences (Hastings et al., 2003; Takahashi et al., 2008). In mammals, circadian disruption can induce metabolic disorders such as diabetes and obesity (Turek et al., 2005; Marcheva et al., 2010). Reinstating feeding and fasting rhythms using time-restricted feeding (TRF; access to food 8-12 hours/day) in mice, as well as flies, decreases obesity and incidence of diabetes and increases overall health (Chaix et al., 2014; Hatori et al., 2014; Mattson et al., 2014; Zarrinpar et al., 2014; Gill et al., 2015). Modern lifestyle is increasing the number of humans that are prone to circadian disruption due to jet lag (Waterhouse et al., 2007), shift work (CDC, 2011), and delayed sleep onset (Jean-Louis et al., 2000). Rodents that are chronically jet lagged show a decrease in hippocampal neurogenesis (Kott et al., 2012), increased susceptibility to and fatality from bacterial infections, and shorter life span (Davidson et al., 2006). Mice genetically deficient in *Bmal1* (a core clock gene) are unable to maintain endogenous rhythms and display early aging and age-related pathologies (Kondratov et al., 2006). Additionally, many affective disorders including schizophrenia, bipolar disorder, and major depressive disorder are associated with circadian disruption (Mansour et al., 2006; Soria et al., 2010).

Most peripheral organs display circadian rhythms (Balsalobre et al., 1998; Nagoshi et al., 2004; Welsh et al., 2004; Yoo et al., 2004; Evans et al., 2015), and are coordinated by the suprachiasmatic nucleus (SCN) in the hypothalamus that acts as master pacemaker for the organism (Evans et al., 2015). These oscillations can be

observed in cell culture by fusing the LUCIFERASE to a clock protein such as PER2. This is done by inserting the cDNA for *Luciferase* to the 3' end of the genomic sequence of *Per2*. This puts *Luciferase* under the control of the *Per2* promoter so *Luciferase* expression directly represents expression of *Per2* (Yoo et al., 2004). Peripheral oscillators have persistent rhythms *in vitro*, but individual cells will fall out of phase with each other, dampening the rhythm, if synchronizing cues are absent (Yagita et al., 2001; Yoo et al., 2004; Mohawk and Takahashi, 2011). In comparison, individual oscillators within the SCN are able to maintain coordination and sustain robust rhythms *in vitro* for weeks (Welsh et al., 1995; Liu and Reppert, 2000; Yamazaki et al., 2000; Webb et al., 2009). Peripheral organs (except for the retina; Besharse and Iuvone, 1983; Tosini and Menaker, 1998; Green and Besharse, 2004) are unable to maintain rhythms because the individual oscillators do not communicate with each other and will therefore fall out of phase without synchronizing input.

SCN transplant studies that utilized wild type (WT) and mutant hamsters proved that the SCN determines the period of the organism (Ralph et al., 1990; Guo et al., 2006). Hamsters that are homozygous for the circadian mutation *tau* have a free running period of 20 hours in contrast to WT hamsters, which have a period of ~24h. Endogenous rhythms were abolished in WT and *tau* hamsters with SCN lesions and then restored with an SCN transplant from the opposite phenotype. Regardless of phenotype, the host animal adopted period of the SCN donor (*ie* a WT hamster with a *tau* SCN now had a 20h period and vice versa), even though all extra-SCN oscillators must have a different period (Ralph et al., 1990). SCN transplants are also sufficient to restore circadian rhythms in peripheral tissues of mice that are arrhythmic due to genetic mutations (*Clock*

<sup>-/-</sup> or *mCry1<sup>-/-</sup>/Cry2<sup>-/-</sup>*; Sujino et al., 2003). These studies proved that the SCN was not only a part of the control system, but that it alone determined the period of an animal.

### The Molecular Clock

Animal, fungal, and plant molecular clocks are produced from transcriptional-translational feedback loops (Reppert and Weaver, 2000; Harmon et al; 2006; Dunlap, 1999; Fig 1-1). Several core clock proteins are substrate for kinases, and the relatively obscure family of casein kinases, are the most evolutionarily conserved components of clock mechanisms (Reischl and Kramer, 2011; Johnson, et al., 2008). In fact, it is debatable if transcription is necessary for molecular clocks as the cyanobacteria clock is produced solely by oscillating phosphorylation states of clock components: KaiA, KaiB, and KaiC (Johnson, et al., 2008; Nakajima et al., 2005; Nakajima et al., 2010; Reischl

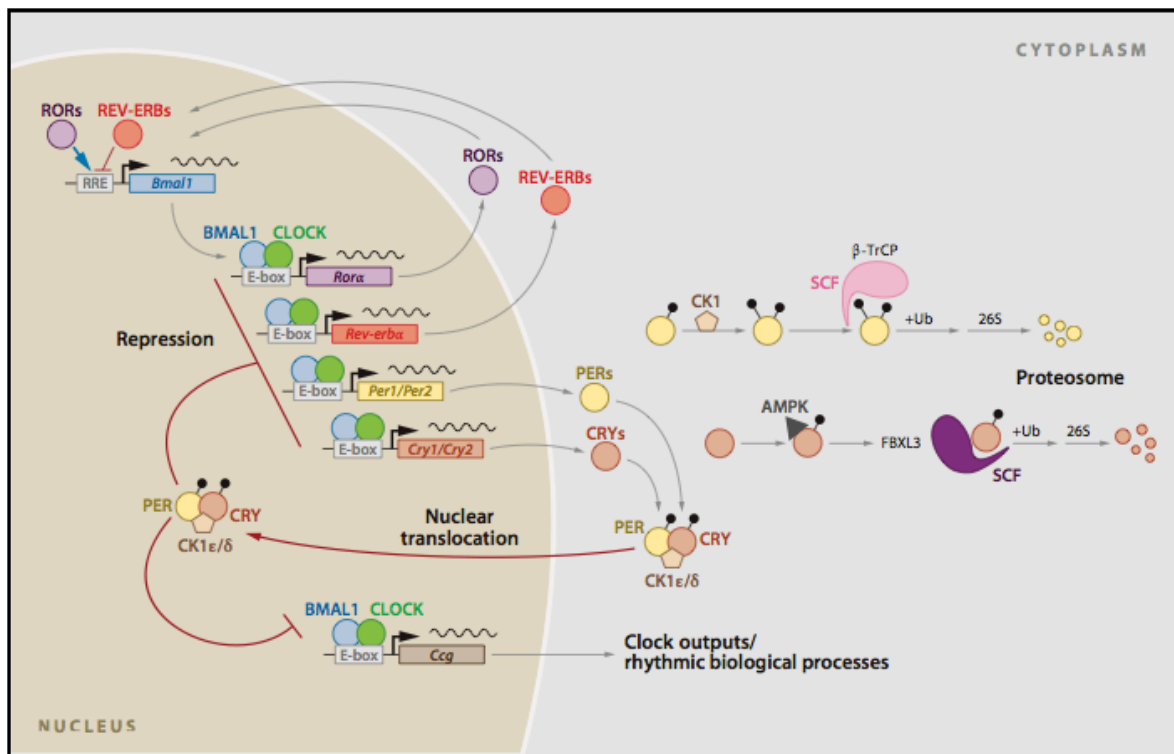


Figure 1-1. Cellular mammalian circadian oscillator. (CCG- clock-controlled gene, P, phosphate; U, ubiquitin; Mohawk et al., 2012)

and Kramer, 2011) and entrain to temperature cues (Yoshida et al., 2009). In mammals, *Period (Per)* 1 and 2, *Cryptochrome Cry* 1 and 2, *Bmal1*, and *Clock* and *Npas2*, are considered core clock genes: if they are compromised (due to a knockout or mutation) animals become arrhythmic (Vitaterna et al., 1994; King et al., 1997; Kume et al., 1999; Van Der Horst et al., 1999; Bunger et al., 2000; Albrecht et al., 2001; Bae et al., 2001; Zheng et al., 2001). However, mice in which a single isoform of *Per* or *Cry* is deleted are able to maintain behavioral rhythms in constant darkness with an altered period and achieve phase shifts (van der Horst et al., 1999; Bae and Weaver, 2003; Zheng et al., 2001). Isoforms of clock genes serve different functions, some of which may be compensatory (Vitaterna et al., 1999; Bae et al., 2001; DeBruyne et al., 2007a/b). For instance, mPER1 interacts with CRY1/2 to influence the phase of transcription, whereas mPER2 modifies gene expression and regulates phosphorylation and transcription by modifying casein kinase 1 $\epsilon$  (CK1 $\epsilon$ ) activity (Bae et al., 2001; Qin et al., 2015; Zheng et al., 1999). *Cry1*<sup>-/-</sup> mice have a ~25 h period and *Cry2*<sup>-/-</sup> have reduced sensitivity to light (Griffin et al., 1999; Vitaterna et al., 1999). NPAS2 can serve as a substitute for CLOCK in the SCN (DeBruyne et al., 2007a) but is insufficient to maintain rhythmicity in peripheral oscillators (DeBruyne et al., 2007b). BMAL1, also known as MOP3, was thought to be the only single isoform of a clock gene necessary for clock function as *Bmal1*<sup>-/-</sup> mice are arrhythmic (Bunger et al., 2000; Boden and Kannaway, 2005). However, further studies have shown that BMAL2 can compensate for BMAL1 (Shi et al., 2010). The original *Bmal1*<sup>-/-</sup> mice may have only been arrhythmic because they also were deficient in BMAL2 (Shi et al., 2010).



In the mammalian clock (Fig 1-1), CLOCK and BMAL1 comprise the positive arm of the TTFL by forming a heterodimer, which binds to an E-box motif (CACGTG) to activate the transcription of *Per* and *Cry*. Upon translation, PER and CRY are both phosphorylated (by CK1 $\epsilon/\delta$  or GSK3 $\beta$  and AMPK respectively) and deactivated, or form a heterodimer (Reischl and Kramer, 2011, Harada et al., 2005; Lamia et al, 2009). The PER/CRY heterodimer is phosphorylated by CK1 $\epsilon/\delta$  for nuclear transport (in a larger complex other proteins; Hirayama et al., 2005) and acts on the CLOCK/BMAL complex to decrease transcription of *Per* and *Cry* (Sato et al., 2006). This is considered the negative arm of the loop. The time to complete this feedback loop determines the period of the clock.

In addition to core loop, other clock components can dramatically alter the period of an organism (Ko and Takahashi, 2006). ROR $\alpha$  and REV-ERB $\alpha$  (retinoic acid-related orphan nuclear receptors) are two such modifiers, which activate or repress the transcription of *Bmal1* respectively, by acting on the RORE (or RRE, retinoic acid-related orphan receptor response element) in the *Bmal1* promoter (Akashi et al., 2005; Guillaumond et al., 2005). In turn, CLOCK/BMAL1 heterodimers also bind to the E-box of *Rora* and *Rev-erba* to regulate their transcription (Fig 1). CLOCK/BMAL1 heterodimers also activate the transcription of the PAR-bZip family members including: DBP, HLF and TEF; the bZip protein, E4BP4 (NFIL3); as well as the basic helix loop helix (bHLH) proteins: DEC1 and DEC2 (BHLHB2, BHLHB3; Gachon 2007; Lowrey and Takahashi, 2004; Ripperger and Schibler, 2006; Takahashi et al., 2008; Mohawk et al., 2012). DBP positively regulates *mPer1* transcription upon binding to D-box elements

in the promoter region (Yamaguchi et al., 2000) and *Dbp*<sup>-/-</sup> mice have a shorter period than WT (Lopez-Molina et al., 1997).

*Tau* mutant hamsters have a period of 20h (homozygous) or 22h (heterozygous) due to a gain of function of CK1ε. When introduced into mice, the *tau* mutation produced a similar effect, whereas *Cklε*<sup>-/-</sup> mice have a longer period than WT (Meng et al., 2008a). CK1δ plays an even larger role than its homolog, CK1ε, in determining period of locomotor rhythms and *Per* expression in peripheral tissues (Etchegaray et al., 2009; Etchegaray et al., 2010; Meng et al., 2010). However, the significance of CK1δ for maintaining rhythms is poorly understood as the *Cklδ*<sup>-/-</sup> is embryonic lethal (Etchegaray et al., 2010). This likely reflects the role of casein kinases in signal transduction pathways and processes other than those that participate in circadian rhythms. These include the Wnt (Davidson et al., 2005) and hedgehog (Jia et al., 2004) signaling pathways. In addition to phosphorylating PER and PER/CRY heterodimers, CK1ε also phosphorylates BMAL1 to positively regulate its activity as a transcription factor for *Per* and *Cry* (Eide et al., 2002). Glycogen synthase kinase-3 (GSK3) α and β isoforms also influence period length by decreasing BMAL1 stability (Sahar et al., 2010; Besing et al., 2015). GSK3's influence on clock mechanisms was originally identified in *Drosophila* as its homolog, *Shaggy*, which phosphorylated TIMELESS (the CRY homolog) and regulates nuclear translocation of the PER/TIMELESS heterodimer (Iitaka et al., 2005). GSK3 shows daily oscillations, yet if SCN slices are chronically dosed with GSK3, BMAL1 rhythmicity is impaired (Besing et al., 2015). Conversely, pharmacological inhibition of GSK3 enhanced the amplitude and shortened period (Besing et al., 2015).

Protein phosphatases (PP) de-phosphorylate proteins creating, further dynamic control of the clock proteins. The role of PP in the circadian clock is still poorly understood, yet research has shown important mechanisms for PP1, PP2A, PP4, and PP5 to regulate rhythms (Reischl and Kramer, 2011). PP1 lengthens period in *Drosophila* (Fang et al., 2007) and in mammals has been shown to increase stability of PER2 by counteracting CK1 $\epsilon$  phosphorylation (Gallego et al., 2006). PP2 plays a large role in the *Drosophila* clock by stabilizing PER, but its role in the mammalian clock is still unknown. *Pp2* deletion or overexpression leads to a long period or arrhythmicity (Sathyanarayanan et al., 2004). PP4 has been shown to regulate the clock only in *Neurospora*, in which *Pp4*<sup>-/-</sup> have a short period and low amplitude rhythms (Cha et al., 2008). In mammals, CK1 $\epsilon$  is the substrate for PP5, which removes autoinhibitory phosphorylations. This action can be counteracted by CRY2. Down-regulation of PP5 disrupts circadian rhythms (Partch et al., 2006).

Ubiquitin ligases regulate the activity of clock proteins. SCF $\beta$ -TRCP (Skp1-Cul1-F-box protein) degrades PER1 and PER2 that have been phosphorylated by CK1 $\epsilon$  (Shirogane et al., 2005; Reischl et al., 2007) and Fbx13 (F box E3 ubiquitin ligase) targets phosphorylated CRY (Busino et al., 2007; Godinho et al., 2007; Siepka et al., 2007; Virshup et al., 2007). Mutations in the coding region of Fbx13 are responsible for both the *Afterhours* and *Overtime* phenotypes, which have a longer period than WT (~26h; Busino et al., 2007; Godinho et al., 2007; Siepka et al., 2007; Virshup et al., 2007).

Post-transcriptional modifications also play a large role in rhythmic protein expression. Approximately 20% of liver proteins display circadian oscillations, yet half of them do not have corresponding rhythmic transcripts (Reddy et al., 2006). BMAL1

acts as a transcription factor for many clock genes, yet there is a wide range in the phase relationship between BMAL1 binding and mRNA accumulation suggesting post-transcriptional regulation. *Rev-erba*, *Rev-erbβ*, *Dbp*, *Tef*, and *Dec2* pre-mRNA closely follows BMAL1 binding, whereas expression of *Per1*, *Per2*, and *Cry2* have ~4hr delay, and *Cry1*, *Rorγ*, and *E4bp4* have ~12hour delay (Rey et al., 2011). Recent studies show that BMAL1 is also an important translational regulator. Phosphorylation of BMAL1 by the mTOR-effector kinase, ribosomal S6 protein kinase 1 (S6K1), allows it to associate with translational machinery for protein synthesis (Lipton et al., 2015).

Additional control of the clock is achieved through histone modifications. SIRT1 controls PER2 deacetylation (Asher et al., 2008) and CLOCK acts on BMAL as a histone acetyltransferase (Doi et al., 2006; Hirayama et al., 2007) to modify gene expression. Although we already know a great deal about the molecular clock, Quantitative Trait Locus (QTL) and RNAi studies indicate some components remain to be identified (Lowrey and Takahashi, 2004; Maier et al., 2009).

### **Circadian Mutations**

Circadian mutants have provided great insight into the molecular clock. The first mammalian circadian mutant, *tau*, had a 20h period when homozygous and 22h period in heterozygous hamsters (Ralph and Menaker, 1988). The mutation was later found to be a gain of function mutation in CK1ε, which destabilized PER to shorten its active period to shorten the period (Meng et al., 2008a). Since then there have been many genetically induced mutations in mice that have further clarified our understanding of the clock mechanism (Table 1-1; Lowrey and Takahashi, 2011). Changes in period are caused by mutations in core clock genes, auxiliary clock genes, coupling factors, and post-

translational events (kinases and ubiquitinases), which alter the stability of clock proteins. Mutations that induce arrhythmicity include deletion of core clock genes (multiple isoforms) and coupling factors.

**Table 1-1. Behavioral phenotypes of mutations in mouse clock and clock-related genes. (Modified Lowrey and Takahashi, 2000).**

Gene(s)	Protein product(s)	Mutant allele(s)	Mutant phenotype(s)	References
<i>Clock</i>	bHLH-PAS transcription factor	<i>Clock</i> <sup><i>Δ19/Δ19</i></sup>	4 hr longer period/arrhythmic	(Vitaterna et al., 1994)
			Rapid resetting	(Vitaterna et al., 2006)
		<i>Clock</i> <sup>-/-</sup>	0.4 hr shorter period	(Debruyne et al., 2006)
			Rapid resetting	(Dallman et al., 2011)
<i>Npas2 (Mop4)</i>	bHLH-PAS transcription factor	<i>Npas2</i> <sup>-/-</sup>	0.2 hr shorter period	(Dudley et al., 2003)
<i>Clock/Npas2</i>	bHLH-PAS transcription factors	<i>Clock</i> <sup>-/-</sup> / <i>Npas2</i> <sup>-/-</sup>	Arrhythmic	(DeBruyne et al., 2007a)
<i>Bmal1 (Arntl, Mop3)</i>	bHLH-PAS transcription factor	<i>Bmal1</i> <sup>-/-</sup>	Arrhythmic	(Bunger et al., 2000)
		<i>Bmal1</i> <sup>-/-</sup> in <i>AVP</i>	1.8 hr Longer period/weakened coupling/rapid resetting	Mieda et al., 2015
<i>Cry1</i>	flavoprotein	<i>Cry1</i> <sup>-/-</sup>	1 hr shorter period	(van der Horst et al., 1999; Vitaterna et al., 1999)
<i>Cry2</i>	flavoprotein	<i>Cry2</i> <sup>-/-</sup>	1 hr longer period	(Thresher et al., 1998;
				van der Horst et al., 1999)

<i>Cry1/Cry2</i>	flavoproteins	<i>Cry1<sup>-/-</sup>/<i>Cry2<sup>-/-</sup></i></i>	Arrhythmic	(van der Horst et al., 1999; Vitaterna et al., 1999)
<i>Per1</i>	PAS protein	<i>Per1<sup>-/-</sup></i>	0.7 hr shorter period	(Cermakian et al., 2001)
		<i>Per1<sup>brdml</sup></i>	1 hr shorter period	(Zheng et al., 2001)
		<i>Per1<sup>ldc</sup></i>	0.5 hr shorter period/arrhythmic	(Bae et al., 2001)
<i>Per2</i>	PAS protein	<i>Per2<sup>brdml</sup></i>	1.5 hr shorter period/arrhythmic	(Zheng et al., 1999)
		<i>Per2<sup>ldc</sup></i>	Arrhythmic	(Bae et al., 2001)
<i>Per3</i>	PAS protein	<i>Per3<sup>-/-</sup></i>	0–0.5 hr shorter period	(Shearman et al., 2000)
<i>Per1/Per2</i>	PAS proteins	<i>Per1<sup>brdml</sup>/<i>Per2<sup>brdml</sup></i></i>	Arrhythmic	(Zheng et al., 2001; Bae et al., 2001)
<i>Per1/Cry1</i>	PAS protein/flavoprotein	<i>Per1<sup>brdml</sup>/<i>Cry1<sup>-/-</sup></i></i>	Normal behavior	(Oster et al., 2003)
<i>Per1/Cry2</i>	PAS protein/flavoprotein	<i>Per1<sup>brdml</sup>/<i>Cry2<sup>-/-</sup></i></i>	<6 months, 1.5 hr longer period; >6 months, arrhythmic	(Oster et al., 2003)
<i>Per2/Cry1</i>	PAS protein/flavoprotein	<i>Per2<sup>brdml</sup>/<i>Cry1<sup>-/-</sup></i></i>	Arrhythmic	(Oster et al., 2002)
<i>Per2/Cry2</i>	PAS protein/flavoprotein	<i>Per2<sup>brdml</sup>/<i>Cry2<sup>-/-</sup></i></i>	0–0.4 hr shorter period	(Oster et al., 2002)
<i>Rev-erba (Nr1d1)</i>	nuclear receptor	<i>Rev-erba<sup>-/-</sup></i>	0.5 hr shorter period; disrupted entrainment	(Preitner et al., 2002)
<i>Rev-erbb (Nr1d2)</i>	nuclear receptor	—	—	—
<i>Rora (Rora)</i>	nuclear receptor	<i>Rora<sup>-/-</sup> (staggerer)</i>	0.5 hr shorter period; disrupted entrainment	(Sato et al., 2004)
<i>Rorb (Rorb)</i>	nuclear receptor	<i>Rorb<sup>-/-</sup></i>	0.5 hr longer period	(Masana et al., 2007)
<i>Rorc (Rorc)</i>	nuclear receptor	<i>Rorc<sup>-/-</sup></i>	Normal behavior	(Liu et al., 2008)

<i>Dec1</i> ( <i>Bhlhe40</i> , <i>Stra13</i> , <i>Sharp-2</i> )	bHLH transcription factor	<i>Stra13</i> <sup>-/-</sup>	0.15 hr longer period	( <a href="#">Nakashima et al., 2008</a> )
<i>Dec2</i> ( <i>Bhlhe41</i> , <i>Sharp-1</i> )	bHLH transcription factor	<i>Sharp-1</i> <sup>-/-</sup>	Delayed resetting	( <a href="#">Rossner et al., 2008</a> )
<i>CK1δ</i> ( <i>Csnk1δ</i> )	casein kinase 1	<i>CK1δ</i> <sup>+/-</sup>	0–0.5 hr longer period	( <a href="#">Xu et al., 2005</a> ; <a href="#">Etchegaray et al., 2009</a> )
<i>CK1ε</i> ( <i>Csnk1ε</i> )	casein kinase 1	<i>CK1ε</i> <sup>-/-</sup>	0.2–0.4 hr longer period	( <a href="#">Meng et al., 2008a</a> ; <a href="#">Etchegaray et al., 2009</a> )
			Rapid resetting	( <a href="#">Pilorz et al., 2014</a> )
		CK1εtau	4 hr shorter period	( <a href="#">Lowrey et al., 2000</a> ; <a href="#">Meng et al., 2008a</a> )
<i>CK1α</i> ( <i>Csnk1α1</i> )	casein kinase 1	—	—	—
<i>Fbxl3</i>	F-box protein	<i>Fbxl3</i> <sup>Ov<sup>tm</sup></sup>	2 hr longer period	( <a href="#">Siepka et al., 2007</a> )
		<i>Fbxl3</i> <sup>A<sup>th</sup></sup>	3 hr longer period	( <a href="#">Godinho et al., 2007</a> )
<i>Bmal2</i> ( <i>Arntl2</i> , <i>Mop9</i> , <i>Clif</i> )	bHLH-PAS transcription factor	—	—	—
<i>Pgc1α</i> ( <i>Ppargc1α</i> )	transcriptional coactivator	<i>Pgc1α</i> <sup>-/-</sup>	0.3 hr longer period	( <a href="#">Liu et al., 2007</a> )
<i>Mtnr1α</i> ( <i>Mell1α</i> )	G protein-coupled receptor	<i>Mtnr1α</i> <sup>-/-</sup>	Normal behavior	( <a href="#">Liu et al., 1997b</a> )
<i>Mtnr1β</i> ( <i>Mell1β</i> )	G protein-coupled receptor	<i>Mtnr1β</i> <sup>-/-</sup>	Normal behavior	( <a href="#">Jin et al., 2003</a> )
<i>Opn4</i>	melanopsin; opsin 4	<i>Opn4</i> <sup>-/-</sup>	Attenuated photic responses	( <a href="#">Panda et al., 2002</a> ; <a href="#">Ruby et al., 2002</a> )
<i>Dbp</i>	PAR bZIP transcription factor	<i>Dbp</i> <sup>-/-</sup>	0.5 hr shorter period	( <a href="#">Lopez-Molina et al., 1997</a> )
<i>Vipr2</i>	G protein-	<i>Vipr2</i> <sup>-/-</sup>	Disrupted	( <a href="#">Harmar et al.,</a>

	coupled receptor		locomotor rhythm	2002; Cutler et al., 2003)
<i>Vip</i>	peptide hormone	<i>Vip</i> <sup>-/-</sup>	1 hr shorter period/arrhythmic	(Colwell et al., 2003)
<i>Prok2</i> (PK2)	secreted protein	<i>Prok2</i> <sup>-/-</sup>	Reduced locomotor activity	(Li et al., 2006)
<i>Nocturnin</i> ( <i>Ccrn4l</i> )	deadenylase	<i>Noc</i> <sup>-/-</sup>	Normal behavior	(Green et al., 2007)
<i>Neuromedin s</i> ( <i>Nms</i> )	Neuropeptide	<i>Nms</i> <sup>-/-</sup>	Normal behavior	(Lee et al., 2015)
		<i>Nms-Clock</i> <sup>A19/Δ19</sup>	1.2 hr longer period	
<i>VPAC2</i> receptor	VIP and PACAP receptor	<i>VPAC2</i> overexpression	0.6 shorter period/ Rapid resetting	(Shen et al., 2000)
<i>V1a</i> and <i>V1b</i> receptors	AVP receptors	<i>V1a</i> <sup>-/-</sup> and <i>V1b</i> <sup>-/-</sup>	Rapid resetting	(Yamaguchi et al., 2013)
<i>Rev-erba/Per1</i>	Clock genes	<i>Rev-erba</i> <sup>-/-</sup> and <i>Per1</i> <sup>-/-</sup>	Rapid resetting	(Jud and Albrecht, 2010)

Other mutations in mice that lead to rapid phase resetting include: overexpression of the VPAC2 receptor (Shen et al, 2000), AVP receptors *V1a*<sup>-/-</sup>/*V1b*<sup>-/-</sup> (Yamaguchi et al., 2013), *Clock*<sup>A19/Δ19</sup> (Vitaterna et al., 2006), *Clock*<sup>-/-</sup> (Dallman et al., 2011), *Rev-erba*<sup>-/-</sup>/*Per1*<sup>-/-</sup> mutants (Jud and Albrecht, 2010), and *Cklε*<sup>-/-</sup> (Pilorz et al., 2014). These studies demonstrate that multiple mechanisms can result in similar phenotypes.

### **Communication Between Oscillators**

Cell autonomous circadian cellular clocks oscillate with periods ranging from 22-30 hours (Welsh et al., 1995; Liu et al., 1997; Ko et al., 2010). Intercellular coupling within the SCN determines the period of an organism by averaging the period of independent oscillators (Liu et al., 1997). The magnitude of phase shifts, and therefore the ability to entrain to the external environment is also regulated by coupling within the



SCN (Meijer et al., 2010; Bordyugov et al., 2011; Ramkisoensing et al., 2014).

Organisms entrain to the photoperiod by phase shifting, and photoperiod also influences intracellular coupling in the SCN (Schaap et al., 2003; vanderLeest et al., 2009). A long photoperiod (longer day than night) leads to decreased coupling between oscillators (Evans et al., 2015), whereas a short photoperiod increases coupling (Schaap et al., 2003). In the SCN, high-amplitude and highly synchronized rhythms exhibit larger phase delays than low-amplitude desynchronized oscillations, in response to 30 min light pulses in early subjective night (vanderLeest et al., 2009). However, behavioral experiments have shown the opposite: animals in short photoperiods, with higher amplitude oscillators, display smaller phase shifts than that of animals in a long photoperiod with lower amplitude rhythms (Ramkisoensing et al., 2014). Coupled SCN networks are also more robust to genetic perturbations than single cell oscillators (Liu et al., 2007).

The main coupling factor in the SCN is vasoactive intestinal peptide (VIP; Colwell et al., 2003; Aton et al., 2005; Aton and Herzog, 2005). VIP cells are mainly located in the ventrolateral SCN, whereas its receptor, VPAC2, is found throughout the SCN (Vertongen et al., 1997; Cagampang et al., 1998). Enzyme immunoassay studies of rats in a LD cycle display oscillations of VIP, with lower levels during subjective day as it is suppressed by light, but do not show rhythmic expression in constant darkness (DD; Shinohara et al., 1993). *In situ* experiments in rats show similar results, but rather than a change in amplitude in LD vs. DD conditions, show a phase shift, with *mVip* peaking earlier in DD (Dardente et al., 2004). Northern blots of *mVip* from rats that were raised in DD demonstrate endogenous rhythmic expression (Glazer and Gozes, 2004). In mice, *in situ* assays displayed high amplitude VIP rhythms in DD and dampened rhythms in LD

(Dardente et al., 2004). Mice deficient in either VIP or VPAC2 have altered circadian rhythms (Shen et al., 2000; Harmar et al, 2002; Colwell et al 2003; Maywood et al., 2006; Brown et al., 2007; Vosko et al., 2007). *Vip*<sup>-/-</sup> mice have low-amplitude rhythms and a short period of cellular oscillations *in vitro* and *in vivo* or are behaviorally arrhythmic (Colwell et al, 2003; Brown et al., 2007). Supplying VPAC2 antagonist further decreases the number of oscillating neurons, indicating that VPAC2 receptors are still being activated by other neuropeptide (Brown et al, 2007). Further evidence shows that VIP is also necessary for intracellular oscillations of cells that express VPAC2 (Maywood et al., 2006). *Vpac2*<sup>-/-</sup> mice are able to entrain to a LD cycle, but become in arrhythmic in DD (Harmar et al., 2002). High levels of either VIP or VPAC2 have provided surprising results. Providing high levels of VIP during subjective night decreases synchrony and increases the ability to phase shift (An et al., 2013). Similarly, VPAC2 overexpressing mice have a shorter period than WT's and show rapid re-entrainment to both phase advances and delays (Shen et al., 2000). These findings suggest that the circadian system entrains through phase and dose dependent responses to VIP (An et al., 2013). Together these findings show that VIP and VPAC2 play a crucial role in synchronizing and maintaining rhythms in the SCN and individual cells.

Most cells within the SCN, including VIP cells, are GABA ( $\gamma$ -aminobutyric acid)-ergic (Van den Pol, 1986; Moore and Speh, 1993; O'Hara et al., 1995; Castel and Morris, 2000). GABA<sub>A</sub> (postsynaptic) and GABA<sub>B</sub> (presynaptic) receptors are located throughout the SCN (Francois-Bellan et al., 1989; Liou et al., 1990; Gao et al., 1995; Chen and van den Pol, 1998; Obrietan and van den Pol, 1998). GABA synchronizes oscillating cells *in vitro* (Colwell, 2000; Liu and Reppert, 2000; Shirakawa et al, 2000)

and acts as a coupling factor between the ventral and dorsal regions of the SCN (Albus et al., 2005). Efferent GABA-ergic projections from the SCN provide synchronizing cues to the ventrolateral preoptic region, which regulate sleep/wake cycles (Sun et al., 2000). Additionally, GABA is thought to regulate afferent input to the SCN, specifically from photic cues (Mintz et al., 2002). In hamsters, microinjections of GABA<sub>A</sub> or GABA<sub>B</sub> agonists to the SCN reduce light-induced phase shifts (Gillespie et al., 1996; Gillespie et al., 1997). However, applying GABA to individual oscillating cells *in vitro* induces phase shifts (Liu and Reppert, 2000) and GABA<sub>B</sub> agonist is sufficient to phase shift cultured SCN slices (Biggs et al., 1998). More recent studies have found GABA destabilizes genetic oscillations in the SCN to regulate phase shifting (Freeman et al., 2013). Taken together these studies display a diverse and crucial role of GABA in the circadian system.

AVP, mainly found in the dorsal medial shell of the SCN, also plays a critical role in interneuronal coupling (Mieda et al., 2015). AVP-specific BMAL1 deletions eliminated circadian oscillations of AVP cells, which resulted in an increase in period, weakened rhythms in SCN slices and enhanced re-entrainment to and 8-hr phase advance (Mieda et al., 2015). Mice deficient in both AVP receptors (*V1a*<sup>-/-</sup> and *V1b*<sup>-/-</sup>) also display rapid resetting to 8-hour phase advances (Yamaguchi et al., 2013). This implies that the daily oscillations of AVP are also necessary to maintain robust rhythms in the SCN.

In addition to having multiple coupling factors, there are also multiple forms of signal transmission within the SCN including synaptic, paracrine, and electrical signaling. Paracrine signaling was illustrated by using PER2::LUCIFERASE SCN slices from mice that were arrhythmic due to deficiencies in either coupling neuropeptides or core clock genes. Rhythms in such slices were restored when they were co-cultured with

WT slices, with the two slices separated by a physical barrier that blocked neural connections but allowed molecular diffusion (Maywood et al., 2011). In addition, gap junctions may play a role in synchronizing the SCN for both astrocytes (Welsh and Reppert, 1996) as well as neurons by regulating spike frequency (Aton and Herzog, 2005; Colwell et al., 2005; Wang et al., 2014). Gap junctions are especially important during neuronal development and are modulated by VIP (Wang et al., 2014). This wide range of coupling factors and mechanisms elucidates the complex network of the circadian system and the many ways it can be regulated.

### **Anatomy of the SCN**

The SCN is roughly subdivided into a “core” and “shell,” and has many cell types (Antle and Silver, 2005; Morin et al., 2007; Yan et al., 2007). Similar anatomical distinctions have been made in mice, rats, hamsters, and humans (Card et al., 1984; Mai et al., 1991; Abrahamson et al., 2001; Moore et al., 2002; Morin et al., 2006). However, aspects of SCN anatomy are species specific. Hamsters have a central nucleus that is defined by immunoreactive (ir) cell bodies of substance P (SP), gastrin-releasing peptide (GRP), calbindin (CalB) and calretinin (CalR; Morin et al., 1992; Miller et al., 1996; Silver et al., 1996b; LeSauter et al., 2002). An equivalent nucleus has not been identified in rats, yet a similar region is defined by enkephalin-IR (ENK) in ground squirrels (Smale et al., 1991). Mice also have CalB, CalR, GRP cells in the SCN, but they are dispersed throughout the SCN (Morin et al., 1996). It was originally thought that the core receives light information from the retina and entrains the shell (Buijs et al., 1996; Tanaka et al., 1997; Bryant et al., 2000; Karatsoreos et al., 2004; Antle and Silver, 2005). However, more recent studies have shown retinal input is distributed throughout the SCN as well as

the subparaventricular nucleus (sPVN; Hattar et al., 2006). In rats these are mainly contralateral projections, whereas mice display an equal number of ipsilateral and contralateral retinal input (Johnson et al., 1988; Abrahamson and Moore, 2001; Hattar et al., 2006; Morin et al., 2006). Hamsters have a complex organization of both ipsilateral and contralateral input (Muscat et al., 2003). The functional significance of anatomical differences between species is still poorly understood, but may influence signaling within the SCN (Morin et al., 2006).

The light receptive cell types within the ventrolateral core include: VIP (Shinohara et al., 1998; Aïoun et al., 1998), CalB (Bryant et al., 2000), and GRP (Ibata et al., 1989; Tanaka et al., 1993; Takata et al., 1997; Aïoun et al., 1998; Shinohara et al., 1998). VIP and GRP both play an important role in synchronizing cells within the SCN and convey retinal information to dorsomedial SCN shell (Piggins et al., 1995; Colwell et al., 2003; Hughes et al., 2004; Aton and Herzog, 2005; Brown et al., 2005). In contrast, the shell is thought to contain a network of cells that remain rhythmic in the absence of light input (Welsh et al., 1995). In rats and hamsters, *in situ* hybridization displays *Per1* expression peaks earlier in the dorsomedial shell and then laterally spreads to the ventrolateral core (Yan and Okamura, 2002; Hamada et al., 2004). This dorsal to ventral pattern of *Per* expression has been shown in SCN slices in *Per1::Luciferase* and *Per1::dsGFP* transgenic mice (Maywood et al., 2006). Long photoperiod (20L:4D) dissociates the phase of the core and shell in mice (Evans et al., 2015). Cells within the shell of the SCN express arginine vasopressin (AVP; Abrahamson et al., 2001; Moore et al., 2002; Card et al., 1984; Mai et al., 1991; Morin et al., 2007; Evans et al., 2015). Dendrites from vasopressinergic cells send efferent projections to nearby regions such as

the subparaventricular nucleus (sPVN) and paraventricular nucleus (PVN), which control locomotor activity and hormone outputs respectively (Vrang et al., 1995; Buijjs et al., 1996; Kalsbeek and Buijjs, 2002; Li et al., 2009; Vujovic et al., 2015). AVP is also involved in coupling to the ventrolateral core of the SCN (Mieda et al., 2015). Despite the phenotypic differences between the shell and the core, all cells within the SCN, as well as all cells throughout the body, most likely have a molecular clock.

### **SCN Output to Peripheral Oscillators**

In mammals, 8-10% of all mRNAs are rhythmically expressed in the liver, heart, kidney, lungs, and SCN (Akhtar et al., 2002; Panda et al., 2002; Storch et al., 2002; Hughes et al., 2009). The circadian clock controls RNA oscillations through modulation of RNA polymerase binding and chromatin remodeling (Koike et al., 2012). Additional regulation of RNA transcriptional oscillations is controlled by the intracellular molecular clock (Yoo et al., 2004) as well as external signals from the SCN (Kornmann et al., 2007). *In vivo*, hepatocyte specific *Bmal1*<sup>-/-</sup> mice were able to maintain circadian rhythmicity of 31 genes, including *mPer2*, but when hepatocytes were oscillated in cell culture was completely arrhythmic (Kornmann et al., 2007). More recent studies show that output from SCN shell neurons specifically determines the phase of peripheral organs (Evans et al., 2015). This illustrates that SCN communication to peripheral organs is sufficient to maintain oscillations of a subset of genes.

The SCN communicates to the periphery through both neural and humoral cues (Lehman et al., 1987; Ralph et al., 1990; Silver et al., 1996a; Guo et al., 2005; Guo et al., 2006; Mohawk et al., 2012). Humoral signals within the brain include AVP (Schwartz and Reppert, 1985; Li et al., 2009; Kalsbeek et al., 2010), prokineticin 2 (PK2; Cheng et

al., 2002), transforming growth factor  $\alpha$  (TGF $\alpha$ ; Kramer et al., 2003), cardiotropin-like cytokine (CLC; Kraves and Weitz, 2006), Glutamate (GLUT; Cui et al., 2001), and GABA (Cui et al., 2001; Kalsbeek et al., 2004). SCN grafts into SCN lesioned hosts hamsters were sufficient to restore locomotor rhythms with the period of the donor (Lehman et al., 1987; Ralph et al., 1990; Meyer-Bernstein et al., 1999), but did not restore entrainment to light or endocrine rhythms (Lehman et al., 1987; Meyer-Bernstein et al., 1999). Transplants of WT SCN, in a semipermeable capsule to allow humoral but not neural communication, into *tau* SCN lesioned animals restored locomotor rhythms (Silver et al., 1996a). Further transplant studies elucidated the differential control of peripheral organs by the SCN (Guo et al., 2006). SCN transplants that were effective to restore behavioral rhythms failed to reinstate rhythms in the heart, spleen, and adrenal medulla (Guo et al., 2006). Parabiosis studies demonstrated that the SCN communicates with peripheral organs via non-neural mechanisms (Guo et al., 2005). Non-neural signals (activity, feeding, temperature, and humoral) are sufficient to entrain the liver and kidney, but not heart, spleen, and skeletal muscle (Guo et al., 2005). Autonomic signaling by the SCN was revealed using viral tract tracers showing SCN neurons communicate to peripheral organs through both parasympathetic (thyroid, liver, pancreas, and submandibular gland) and sympathetic (pineal gland, white and brown adipose tissue, thyroid gland, kidney, bladder, spleen, and adrenal medulla) innervation (Bartness et al., 2001). Real time reporting of PER2::LUC oscillations in liver demonstrated that feeding cues reset the phase of the liver more rapidly in SCN-lesioned animals, suggesting SCN signals increase the robustness of peripheral organs to perturbation (Saini et al., 2013). These studies demonstrate the complex and the differential control of peripheral

oscillators through neural and humoral signals for the SCN, temperature, and intracellular oscillations.

### **Entrainment**

In addition to producing and maintaining endogenous rhythms in the absence of periodic signals, the SCN also receives time cues, aka *zeitgebers* (German for “time giver”) from the environment. The SCN integrates external and internal timing cues to coordinate endogenous rhythms with the environment by adopting its period (Piggins and Guilding, 2011). Entrainment occurs when a self-sustaining oscillator adopts the period of a driving oscillator and maintains a steady phase angle. In mammals, the SCN entrains to the light/dark (LD) cycle, and in turn entrains the peripheral organs. Peripheral clocks entrain to the SCN through multiple mechanisms including neural, behavioral, and humoral outputs. SCN-controlled temperature oscillations (Aschoff et al., 1983; Eastman et al., 1984; Brown et al., 2002), can rapidly phase shift peripheral oscillators, however the SCN is temperature compensated (Ruby et al., 1999; Brown et al; 2002; Kornmann et al., 2007; Abraham et al., 2010; Buhr et al., 2010). Mouse lung fibroblast and slices transfected with *Bmal1::Luc* and *Per2::Luc* reset their phase in response to a REV-ERB $\alpha$  ligand (Meng et al., 2008b). Entrainment is important for an organism because, as previously mentioned, many physiological events need to be anticipated. If an organism were unable to coordinate with its environment, many events would be out of phase, which would presumably decrease fitness. Entrainment is distinct from masking in which the internal clock does not entrain to the environment, but rather independent effector systems respond to environmental cues and are not dictated by an oscillator. Negative masking is evident when a nocturnal animal decreases activity in the presence of light.



Masking may be sufficient for some actions such as predator avoidance, but because the internal clocks are not synchronized, there is a mis-match of external cues and the phase of internal oscillators. It is important for organisms to entrain to the period of their environment as the period of most endogenous clocks varies from 24 hours. Entrainment to a *zeitgeber* whose period that differs from that of the endogenous oscillator is achieved through daily phase shifts.

Phase shifts are achieved by resetting the molecular clock. The phase at which the *zeitgeber* is received determines the direction (advance or delay) and amplitude of the phase shift (Daan and Pittendrigh, 1976d). The strongest *zeitgeber* is light, which acts through direct retinal inputs to the SCN (Daan and Pittendrigh, 1976d; Rusak et al., 1990; Emery et al., 1998), which then resets peripheral clocks through neural and hormonal signals (Guo et al., 2005; Guo et al., 2006; Mohawk et al., 2012). Other stimuli, such as the feeding/fasting cycle, more directly entrain peripheral clocks (Asher and Sassone-Corsi, 2015).

Non-photic stimuli (activity) and photic stimuli affect circadian rhythms at opposite circadian times, (Fig 1-2). Activity pulses of approximately 3-

hours during subjective day have been observed in Syrian hamster to induce phase shifts of 1-3 hours, similar to a light pulse during subjective night (Mrosovsky et al., 1992). To do this the hamster is either given a new wheel, or confined to a new wheel, during subjective day. Although they are not forced to run, the new wheel is sufficient to

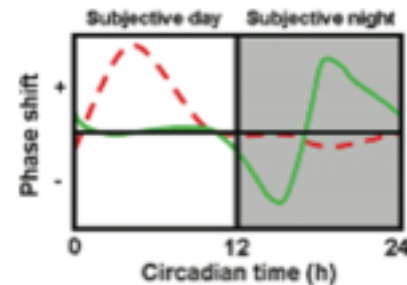


Figure 1-2. PRC in response to a light pulse and a non-photic pulse. Photic PRC (green), non-photic (red; from Piggins and Guilding, 2011).

stimulate activity in when the hamsters would be resting. *Tau* mutants exhibited larger phase shifts in response to novel-wheels during subjective day (Mrosovsky et al., 1992). During this 3-hour period of novel wheel exposure, the hamster must have a minimal amount of activity to achieve a phase shift (Bobrzynska and Mrosovsky, 1998). Sleep deprivation, absent of increased activity, is also sufficient to cause a phase shift (Mistlberger et al., 2002).

### **Phase Shifts**

Two different models have characterized the effect of light on the circadian clock. A parametric model, in which light changes the angular velocity (speeds up or slows down) of the clock, and a non-parametric model, in which light has a discrete effect. If we apply these models to phase shifts, the parametric model suggests that the rate of state variable (clock genes) production and clearance is increased during a manipulation such as a light pulse. The non-parametric model implies that phase shifts are induced when a manipulation causes a rapid change in the values of the state variables. This implies that the phase shift does not occur over time, but rapidly, if not instantaneously.

Phase shifts can be explained with the limit cycle model of circadian rhythms. A limit cycle is a closed trajectory in which the state variables of a dynamical system exhibit oscillating values. The core clock gene products that compose the positive (*Bmal* and *Clock*) and negative arms (*Period* and *Cry*) of the TTFL can be thought of as the state variables whose interaction forms a limit cycle. State variables in these models can include levels of mRNA and proteins. The speed at which proteins are transcribed, translated, and modified (e.g, phosphorylated, ubiquitinated) determines the velocity around the limit cycle. Phase is shown on a limit cycle diagram by isochrons (Fig 1-3).

An isochron is a set of points (values of each state variable) in which trajectories starting at any point on a given isochron will spiral into the limit cycle and end up circling around the limit cycle together, that is, will share a common phase. If a manipulation moves the value of state variable along an isochron, but not to a new isochron, it will not result in a phase shift. However, if a manipulation pushes a state variable to a new isochron, it will result in a phase shift. For example, if PER1 is a state variable (Y), light exposure during late subjective night (CT 21) could increase *Per1* transcription, which would push it to a new isochron to induce a phase advance (Fig 1-3).

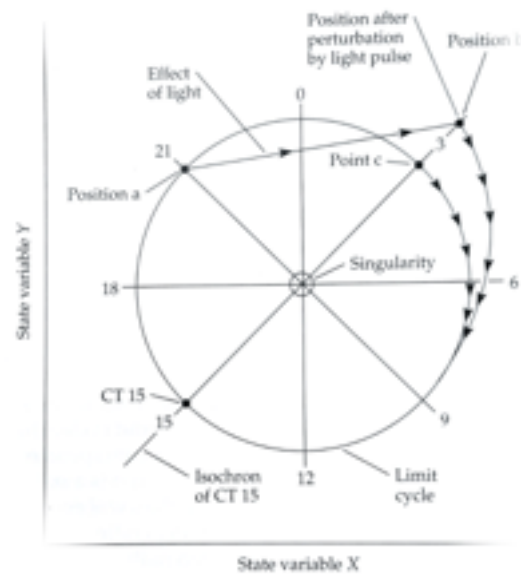


Fig 1-3: Model of effect of light on the limit cycle to induce a phase shift. The light perturbs the state variables from position a, off of the limit cycle to position b, then rejoins the limit cycle at a new phase (from Dunlap, et al. 2004, after Winfree, 1974).

If a limit cycle is modified (change in amplitude or shape), due to changes in expression or function of clock genes or stability of protein products, the period and phase shift amplitude will be greatly affected. This could be caused by a mutation of core clock genes, clock modifiers, promoter sequences, or even non-coding regions. Any of these mutations would modify levels of clock gene expression and/or function, changing the magnitude of the state variables over the course of the cycle. Amplitude can also change due to state variable response to the environment, such as constant light (LL) compared to constant dark (DD).

If the amplitude of the cycle is decreased, a given stimulus can have a much greater effect on the phase of the rhythm (Fig 1-4C). Alternatively, type 0 PRCs (high amplitude resetting) can be achieved by maintaining the amplitude of the limit cycle while increasing the amplitude of the response to a stimulus (Fig 4B).

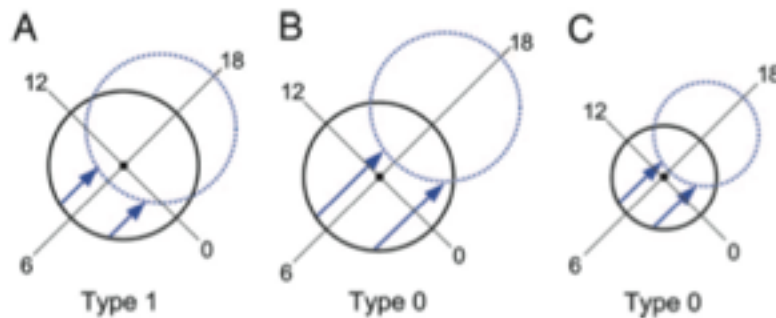


Figure 1-4. Two-dimensional limit cycle model of the circadian system. Black circles are steady state rhythm. Y axis is state variable  $y$ ; X axis is state variable  $x$ . For this example  $y$  and  $x$  could be the positive and negative arm of the TTFL. The blue dotted circle indicates the projected effect of a perturbation to the system (arrows) at all possible phases. A. Type 1 (low-amplitude resetting) phase shift, typical of a WT hamster. B and C. Type 0 (high-amplitude resetting) which can be explained by the stimulus pushing the cycle to a new isochron past the singularity. B Type 0 phase shift due to increased effect of a stimulus with a WT amplitude. C. Type 0 phase shift caused by a decrease in amplitude (due to the mutation) and equivalent to WT response to a stimulus. (from Vitaterna et al., 2006)

Vitaterna et al. (2006) demonstrated that a dominant negative *Clock* <sup>$\Delta 19/\Delta 19$</sup>  mutation in mice was able to affect the resetting of the circadian clock in response to light by decreasing the amplitude of the oscillation, rather than modifying the effect of the perturbation of light on the SCN (Vitaterna et al., 2006). This resulted in a type 0 PRC (Fig 1-4C) rather than type-1 (low-amplitude resetting) PRC in WTs (Fig 1-4A).

*Zeitgebers*, including feeding patterns (Stephan, 2002), activity (Reebs and Mrosovsky, 1989a), and photic input, the strongest cue, influence the SCN (Rusak and Zucker, 1975). There are three major sources of input to the SCN: the intergeniculate leaflet (IGL), the median raphe, and the retina (Cermakian and Sassone-Corsi, 2000; Piggins and Guilding, 2011). Activity feeds back on the SCN through the geniculohypothalamic tract (GHT) from the IGL secreting neuropeptide-y (NPY; Mrosovsky,

2008), GABA (Moore, 1997), and enkephalin (Morin et al., 1992). Wheel running during subjective day can induce phase shifts through suppression of *mPer1* and *mPer2* (Maywood and Mrosovsky, 2001; Brewer et al., 2002). Lesions to the IGL block activity-induced phase shifts (Janik and Mrosovsky, 1994; Wickland and Turek, 1994; Marchant et al., 1997) and microinjections of NPY to the SCN can induce phase advances similar to wheel running (Albers and Ferris, 1984; Biello et al., 1994; Biello and Mrosovsky, 1996). Sleep deprivation, without an increase in activity, can activate the same mechanisms and induce similar shifts (Antle and Mistlberger, 2000). Serotonin (5-HT), also affects non-photoc influence through input on the IGL from the dorsal raphe (Janik and Mrosovsky, 1994; Morin, 1999; Glass et al., 2003). The SCN receives direct serotonin input from the median raphe, which modulates the SCNs sensitivity to light (Glass et al., 1994). Microinjections of a 5-HT agonist, quipazine (semi-specific, binds to 5-HT<sub>2A</sub> and 5-HT<sub>3</sub>), in the SCN are sufficient to induce phase shifts (Prosser et al., 1990). 5-HT<sub>2A</sub> is an excitatory post-synaptic G<sub>q</sub>/G<sub>11</sub> receptor that is modulated by 5-HT<sub>1a</sub> (Eison et al., 1995). 5-HT<sub>3</sub> receptors are ionotropic receptor located both pre- and post-synaptically (Barnes et al., 2009). Quipazine also inhibits light-induced phase shifts (Glass et al., 1994). However, the SCN mainly expresses 5-HT<sub>1a</sub> and 5-HT<sub>7</sub> receptors, which are important for both photic and non-photoc input (Glass et al., 1994; Duncan et al., 1999; Horikawa et al., 2000). Further studies have clarified that 5-HT in the dorsal and medial raphe nuclei are behaviorally regulated by 5-HT<sub>7</sub> and GABA-ergic receptors (Glass et al., 2003).

Light acts on the retina and influences the SCN through the retinal-hypothalamic tract (RHT; Abrahamson et al., 2001; Muscat et al., 2003; Morin et al., 2006; Canteras et

al., 2011). Within the retina, specialized non-visual intrinsically photoreceptive ganglion cells (ipRGCs), which use melanopsin (Opn4) as a photo-pigment, directly project to the SCN and are sufficient for light-induced entrainment and phase shifting (Berson et al., 2002; Hattar et al., 2002; Panda et al., 2002; Lucas, 2011). Although retinal input from rods and cones play a role in light entrainment, *Opn4*<sup>-/-</sup> mice show weakened phase resetting in response to light pulses (Panda et al., 2002). The RHT secretes glutamate and PACAP (Pituitary adenylyl cyclase-activating peptide) on the SCN (Chen et al., 1999; Hannibal et al., 2000). PACAP acts as a modulator of glutamate signaling by acting on VPAC2 receptors (Chen et al., 1999; Vaudry et al., 2000). Glutamate acts on NMDA receptors in the SCN to induce an influx of calcium (Ca<sup>++</sup>), which activates protein kinase A (PKA) to phosphorylate cAMP response element binding protein (CREB). Phosphorylated CREB binds to the cAMP response element (CRE) of the *Per1* and *Per2* promoter to induce transcription (Ginty et al., 1993; Ding et al., 1997; von Gall et al., 1998; Travnickova-Bendova et al., 2002; Quintero et al., 2003; Sakamoto et al., 2013; Golombek and Rosenstein, 2010; O'Neill and Reddy, 2012; Hastings et al., 2014). During subjective night, when PER levels are low, the induction of *Per1* and *Per2* transcription induces phase shifts (Meijer et al., 1988; Shigeyoshi et al., 1997; Mintz et al., 1999; Field et al., 2000; Miyake et al., 2000; Wakamatsu et al., 2001; Yamamoto et al., 2001; O'Neill et al., 2008; Schwartz et al., 2011). PER1 seems to play a larger role as inhibition of *Per1* with antisense oligonucleotides *in vivo* an hour before exposure to light prevents a phase shift (Akiyama et al., 1999). *In situ* labeling of mRNA in hamster SCN following a light pulse indicates that *mPer1* and *mPer2* are transcribed sequentially (Shearman et al., 1997; Shigeyoshi et al., 1997; Yamamoto et al., 2001; Masubuchi et al.,

2005; Krug et al., 2011). Glutamate stimulation in the SCN also induces transcription of immediate early genes, such as *c-Fos* and *JunB* (Rusak et al., 1990; Abe et al., 1991; Abe et al., 1992; Rusak et al., 1992; Schwartz et al., 1994; Wollnik et al., 1995; Vosko et al., 2015). However, the role of immediate early gene transcription is unclear as *c-Fos*<sup>-/-</sup> mice display normal circadian rhythms and phase shifts (Honrado et al., 1996).

The retina innervates the core of the SCN including VIP, GRP, and calbindin cell populations in rats, mice, and hamsters (Ibata et al. 1989; Tanaka et al. 1997; Bryant et al. 2000; Kuhlman et al., 2003; Morin, 2007; Yan et al., 2007). VIP immunoreactive cell bodies receive information from the RHT, and project to the dorsal SCN (Ibata et al., 1989; Kriegsfeld et al., 2004). In *Vip*<sup>-/-</sup> mice, *Per1* and *c-Fos* induction remain normal in VIP cells, but are decreased in the dorsal SCN. These findings indicate that VIP is necessary to relay light input to the dorsal, AVP-ergic, SCN (Vosko et al., 2015).

Although the SCN can achieve rapid phase shifts, it can take multiple cycles for an organism to complete a phase shift in response to a light pulse or a new LD cycle. Double pulse experiments provide an initial 15-min light pulse to induce a phase shift and then 1 or 2 hours later provide a second light pulse. If the animal has completed the phase shift before the second pulse, the second pulse will have an additive effect. If not, the second pulse will have little or no effect. These experiments demonstrated that phase shifts are completed between 1 and 2 hours, which supports the non-parametric model (Pittendrigh, 1967; Elliott and Pittendrigh, 1996; Best et al., 1999). Peripheral organs re-entrain at different rates than the SCN (Yamazaki et al., 2000; Davidson et al., 2008). In order to entrain to a new phase, oscillators are temporarily between two steady states. This is known as a transient interval. Each day of the transient interval, a small phase

shift occurs until the organism is shifted to the new phase and reaches stable entrainment. The differences in rate of phase shifting between the SCN and peripheral oscillators results in mis-alignment of oscillators that compromises physiological fitness (Yamazaki et al., 2000). Chronic mis-alignment due to jet lag or shift work has a wide range of consequences including increase in losses for Baseball teams (Recht et al., 1995), to more severe effects such as increase rates of prostate cancer (Conlon et al., 2007) and metabolic and cardiovascular disease (Sheer et al., 2009).

### The Duper Mutation

The Bittman lab has recently discovered duper, a mutation that has striking effects on circadian function. In 2007, the super duper mutant

hamster was identified, which displayed a short free-running period in constant dark ( $\tau_{DD}$ ) of  $18.09 \pm 0.05$  hours. This mutation arose on the homozygous *tau* (aka super short) mutant background, which had a period of  $20.66 \pm 0.07$  hours. The duper mutation was isolated through backcrosses and on a WT background the period of such single mutants was  $23.11 \pm 0.04$  hours (Monecke et al., 2011). This indicates duper as an epistatic effect to decrease the period of *tau* hamsters by 2 hours. The period of homozygous duper

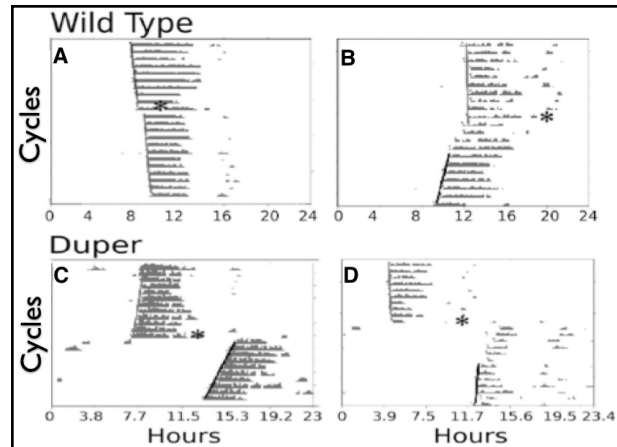


Figure 1-5. Actograms of WT and duper Syrian hamsters that depict phase shift in response to a 15-min light pulse denoted with \* after 10-11 days in DD. Note that activity is plotted modulo  $\tau_{DD}$ ; the endogenous period of the mutant is about 1 hour less than that of the WT. (A, B) Light pulses given to WT hamsters at early and late subjective night respectively. Note that the phase shifts are less than 3 hours and there is a transient period before the phase advance is completed. (C, D) Light pulse given to duper hamsters between early and mid subjective night. Note that the large phase shifts without transient period (Krug et al., 2011).



mutants is similar to that of heterozygous *tau* mutants, yet the coding region of *Cklε* was not mutated in the duper hamster as in *tau* mutants. The coding region of *Cklδ* in duper mutants is also identical to WT hamsters.

Duper is only the second spontaneous circadian mutation that has been identified and is the only recessive circadian mutation. Duper mutants are also unique in that they show a striking exaggeration of phase shifts (type 0 PRC): their clock can be shifted 6-12 hours in response to a 15 minute light pulse 3 or 6 circadian hours after onset of activity (early and mid subjective night, respectively; Krug et al., 2011; Figure 1-5). Dupers that were born and raised in DD also display type 0 PRC (Krug et al., 2011). This proves that large phase shifts are not the result of aftereffects of entrainment to a photoperiod that requires entrainment by phase delays.

Duper hamsters do not show changes in expression of *Per1*, but *Dbp* expression was elevated when compared to WT hamsters both at baseline and 30 mins and 2 hours after a 15-min light pulse at CT15 (Krug et al., 2011). In collaboration with John Hogenesch (University of Pennsylvania), our lab has performed RNA-sequencing (RNA-seq) in liver of duper and WT hamsters. Results have shown that duper is not a mutation in the coding region of any of the following genes that are important components of the clock: *Bmal1*, *Bmal2*, *Clock*, *Npas2*, *Cry1*, *Cry2*, *Per1*, *Per2*, *Per3*, *Rev-erb-α (Nr1d1)*, *Rev-erb-β (Nr1d2)*, *Rora*, *Rorβ*, *Rory*, *Fbxl3*, *Fbxl21*, *Csnk1ε*, *Csnk1δ*, or *Csnk1α* (Bittman and Hogenesch, unpublished). However, with a limited read depth of 20 and a 90% cutoff, it is possible the analysis was too conservative and may be missed sequence changes. Thus, identification of duper will almost certainly reveal novel clock components or ways in which expression of known components is regulated.

Further behavioral analysis of the duper mutants showed that their rhythms are just as precise as WT hamster (Bittman et al., 2012). Furthermore, the light intensity threshold for induction of phase shifts is similar in duper and WT, suggesting that the mutation does not increase PRC amplitude through an action on the input to the pacemaker (Bittman et al., 2014). Aschoff's rule states that nocturnal animals will increase period in LL due to the effect of light on pacemaker velocity (Aschoff, 1960; Daan and Pittendrigh, 1976III; Taylor et al., 2010). However, when kept in LL for 30 days, the duper mutants showed a decrease of period, whereas WT hamsters displayed an increase of period (Bittman et al., 2014). This indicates that for dupers, parametric effects of light play a larger role to accelerate the phase of the clock, compared to delays, effectively shortening the period in LL. Rapid (non-parametric) phase resetting was tested with a double-pulse experiment in which WT and duper hamsters were given two 15 –min light pulses 2 hours apart. The first pulse was given between circadian time 14-16 (CT 14-16; 2-4 hours after lights off, early subjective night) or CT 17-19. As predicted, duper hamsters displayed stronger resetting and exhibited an additive effect of the first pulse. WT hamsters displayed weak resetting and did not show an additive effect of the first pulse (Bittman et al., 2014). This indicates that larger phase shift take longer to complete. These findings suggest that the duper mutation affects the phase response curve, parametric responses to light, and properties of the central pacemaker (Bittman et al., 2014)

Although some circadian mutations resemble aspects of the duper phenotype, no known mutation that replicates the change in period in DD and the photic response. *Per1*<sup>-/-</sup> and *Rev-erba*<sup>-/-</sup> double KO's have a type 0 PRC, and shortened free running period in

constant darkness, but display a small increase in free running period in constant light (Jud et al., 2010), whereas dupers have a decrease in period (Krug et al., 2011). *Clock*<sup>-/-</sup> knockout and *Clock*<sup>Δ19/Δ19</sup> (a dominant negative mutation which alters the C-terminal activation domain of CLOCK protein) mice show exaggerated phase shifts in response to a 6-hour pulse of light, rather than a 15-min pulse as seen in dupers, but do not show a short free running period and large phase shifts are restrict to mid to late subjective night (Dallmann et al., 2011; Vitaterna et al., 2006). Most known clock genes and modifiers have been genetically modified to assess their role in the molecular clock, yet no mutation shares the full phenotype of duper mutants. Therefore, determining the genetic basis of the duper mutation will allow better understanding the circadian system.

There are still many important questions that need to be answered to understand the duper mutation. First, is the duper mutation specific to photic stimuli and/or to phase? Photic and non-photic stimuli use distinct mechanisms to phase shift the molecular clock. Examining the influence of non-photic shifts in duper hamsters may provide insight into the mechanisms of phase shifts. Novel-wheel exposure during subjective day was used to examine if non-glutamatergic signals could also induce exaggerated phase shifts in duper hamsters. Molecular clock components oscillate throughout the circadian day. If the duper mutation alters a specific phase of the clock, molecular clock processes occurring during that time are likely affected by the mutation. Exploration of non-photic and multiple photic stimuli during subjective day will illuminate how the duper mutation alters mechanisms of phase shifting. To do this we performed a variety of non-photic and photic manipulations during subjective day and night. We describe these experiments and their results in Chapter 2.

Second, how is the molecular clock in the SCN affected by light pulses to achieve large phase shifts? The same circadian phenotype can be produced through many different mechanisms. Light pulses during early subjective night induce phase delays by inducing transcription of *Per1* in retinorecipient cells in the SCN. This signal is then conveyed to the dorsal shell of the SCN as well as extra-SCN oscillators (sPVN and PVN), which regulate locomotor and some endocrine rhythms. In response to a 15-min light pulse at CT15, duper hamsters exhibit exaggerated (4-10 hour) phase delays compared to WT's (1-3 hours). As no known circadian mutation can account for the duper phenotype, understanding how the molecular clock is able to achieve large phase shifts in response to light will provide a better understanding of the circadian system. To do this we collected brains from duper and WT hamsters at CT 12 and 15 as well as 1, 2, 3, 6 and 9 hours following a light pulse, or control handling, at CT 15. We used immunocytochemistry to identify light induced proteins, PER1 and c-FOS, and co-labeled them with VIP (retinorecipient cells) and/or AVP-associated Neurophysin (SCN shell, sPVN, and PVN) which have provide anatomical and cell phenotypic information. We describe these experiments and their results in Chapter 3.

Finally, what is the genetic basis of the duper mutation? Although behavioral and neurobiological analysis of the duper mutation is necessary, we will not fully understand the mutation until we know the gene responsible. Fast homozygosity mapping will identify candidate regions of DNA and produce candidate SNPs for the duper mutation. In order to use fast homozygosity mapping, we bred the duper hamsters onto a novel genetic background. Additional behavioral experiments were performed using F2 duper

and WT hamsters to examine how dupers respond to jet lag. These experiments and their results are described in Chapter 4.

As the circadian clock is a fundamental component of regulation of behavior and physiology, identification of the locus of this novel mutation will be an important contribution. Sleep-wake patterns, the timing and success of reproduction and internal physiology, development, and susceptibility to predation are all controlled by circadian mechanisms. In order to understand these behaviors, we need to have a better understanding of the molecular mechanisms that control them. Discovering the genetic cause of the duper mutation could provide a paradigmatic example of how understanding the mechanism by which a single nucleotide change can lead to a profound modification of behavior.

## CHAPTER 2

### PHASE RESETTING IN DUPER HAMSTERS: SPECIFICITY TO PHOTIC ZEITGEBERS AND CIRCADIAN PHASE

The work in this chapter was published in the Journal of Biological Rhythms in April 2015. For full citation see Manoogian et al. 2015.

#### **Abstract**

The duper mutation in Syrian hamsters shortens the free-running period of locomotor activity ( $\tau_{DD}$ ) to about 23 hours and results in a type 0 phase-response curve (PRC) to 15-min light pulses. To determine whether exaggerated phase shifts are specific to photic cues and/or restricted to subjective night, we subjected hamsters to novel wheel confinements and dark pulses during subjective day. Small phase shifts elicited by the nonphotic cue were comparable in mutant and wild-type (WT) hamsters, but dark pulses triggered larger shifts in dupers. To assess further the effects of the duper mutation on light-dark transitions, we transferred hamsters between constant light (LL) and constant dark (DD) or between DD and LL at various circadian phases. Duper hamsters displayed significantly larger phase shifts than WT hamsters when transferred from LL to DD during subjective day and from DD to LL during subjective night. The variability of phase shifts in response to all light/dark transitions was significantly greater in duper hamsters at all time points. In addition, most duper hamsters, but none of the WTs, displayed transient ultradian wheel-running patterns for 5 to 12 days when transferred from light to dark at CT 18. The  $\chi^2$  periodogram and autocorrelation analyses indicate that these ultradian patterns differ from the disruption of rhythmicity by SCN lesions or

exposure to constant bright light. We conclude that the duper mutation specifically amplifies phase shifts to photic cues and may destabilize coupling of circadian organization upon photic challenge due to weakened coupling among components of the circadian pacemaker. Mathematical modeling of the circadian pacemaker supports this hypothesis.

## **Introduction**

Endogenous daily oscillations (circadian rhythms) not only coordinate behavior with the environment but also maintain internal temporal organization. In mammals, circadian rhythms are produced by transcriptional-translational feedback loops involving a set of core clock components that includes the *Period*, *Cryptochrome*, *Clock*, and *Bmal1* genes and their protein products. Posttranslational processes, such as phosphorylation of clock proteins, also determine the period and entrained phase (Meng et al., 2008; Reischl and Kramer, 2011). The duper mutation in Syrian hamsters shortens the period of the free-running locomotor rhythm to approximately 23 h. Duper is the first recessive circadian mutation discovered in mammals, but its genetic basis is not yet known. It differs from the well-studied *tau* mutation in that it is not a sequence change in the coding region of *Csnk1e* or *Csnk1d*, which encode casein kinase 1 $\epsilon$  or 1 $\delta$ , respectively (Lowrey et al., 2000; Monecke et al., 2011).

Although the duper mutation has no effect on the precision or stability of activity rhythms in constant darkness (DD; Bittman, 2012), it markedly amplifies phase-shifting responses to 15-min light pulses (Krug et al., 2011). The effect of duper on responses to non- photic *zeitgebers* is not yet known. Activity pulses of approximately 3-hours during subjective day have been reported to induce phase advances of 1- to 3-hours in Syrian

hamsters, similar in magnitude to the effect of a light pulse during early subjective night (Mrosovsky et al., 1992; Antle and Mistlberger, 2000; Mistlberger et al., 2002; Mistlberger et al., 2003; Webb et al., 2014). Exploration of the influence of duper on nonphotic shifts may provide insight into its effects on circadian organization and reveal fundamental mechanisms of phase shifting. Light-induced phase shifts result from induction of *Per1* and *Per2* transcription upon glutamate release from retinohypothalamic terminals during the subjective night (Yamamoto et al., 2001; Yan and Silver 2002). In contrast, activity-induced phase shifts associated with novel running wheels are caused by suppression of *Period* gene expression in the SCN of wild-type (WT) hamsters during subjective day (Maywood et al., 1999). Furthermore, the magnitude of phase shifts in response to photic stimuli is dependent on photoperiod, whereas that of nonphotic phase shifts is not (Evans et al., 2004). The *tau* mutation has been reported to increase the amplitude of nonphotic responses (Mrosovsky, et al., 1992; Biello and Mrosovsky, 1996). To determine whether exaggerated phase shifts in duper hamsters occur only in response to photic cues or if the mutation also amplifies responses to nonphotic (activity-induced) cues, we examined the effect of 3-hour novel wheel confinements.

The exaggerated effects of light pulses in duper hamsters are confined to the active zone of the PRC, raising the question of whether the effects of the duper mutation on phase lability are confined to subjective night. Dark pulses may be used to explore this question, as these stimuli can induce phase advances and small phase delays when given during subjective day (Boulos and Rusak, 1982; Canal and Piggins, 2006). We subjected hamsters to dark pulses to determine whether the effects of the duper mutation are phase specific as well as *zeitgeber* specific.



Photic pulses (light or dark) are compound stimuli of entry and exit of the new lighting condition. Duper may specifically alter the response to light onset or to light offset. Albers (1986) argued that responses to single transitions summate in order to produce the effect of pulses of light or dark. Whereas DD to LL transitions induce delays during subjective day and advances during subjective night, LL to DD transitions induce phase advances during subjective day and delays during subjective night (Albers, 1986). Thus we examined responses to LL to DD and DD to LL transitions in WT and duper mutant hamsters to determine whether the effects of the dark pulses depend on the phase of entry into dark, the reentry into light, or a combination of the two. Unexpected responses of duper hamsters to transitions from DD to LL led us to compare effects of the mutation to other treatments that compromise the stability of circadian rhythms and to explore the ability of current mathematical models to explain the duper phenotype on the basis of alterations in oscillator coupling.

## **Materials and Methods**

### **Animal Maintenance**

Syrian hamsters (*Mesocricetus auratus*) were group housed in 14:10 light/dark cycle (LD) with ad libitum access to food and water until they reached adulthood (3-8 months). A total of 106 hamsters, including 36 WT (18 male, 18 female) and 70 dupers (44 males, 26 females), were used in these experiments. All hamsters were derived from stock obtained from Lakeview (LAK:LVG) and have been bred in our laboratory for multiple generations as previously described (Monecke et al., 2011). As adults, animals were housed individually with continuous access to a running wheel (17.5 cm diameter) as previously described (Krug et al., 2011). To minimize artifactual changes in phase and

period, cages and water bottles were changed every 30 days during experiment 1 and every 10 to 20 days in experiments 2 and 3. Highly absorbent bedding (Bed-o-cob, Maumee, OH) was used throughout. Hamsters had the same running wheel throughout the experiment except during 3-hour confinement periods in experiment 1, in which they were placed in a novel confinement wheel of the same size. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Amherst and conform to all U.S. federal animal welfare requirements.

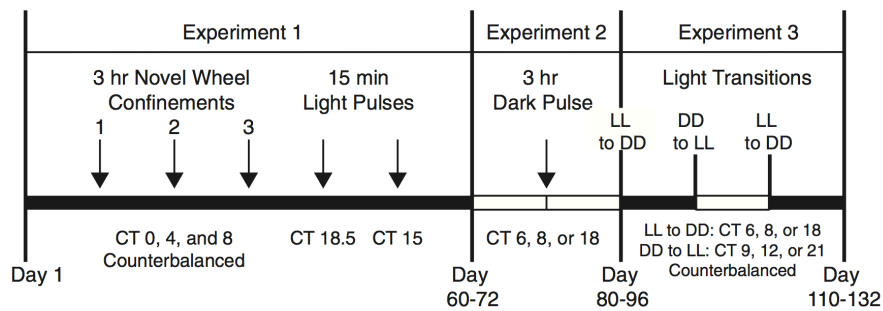


Figure 2-1. Timeline of experiments. Dark bars indicate constant darkness; open bars indicate constant light. The order of CT for novel wheel confinements and light transitions was counterbalanced. Animals were kept in a constant condition (light or dark) for 10 to 12 days between manipulations in all experiments. The 3 experiments generally lasted for a combined length of 110 to 132 days. If an animal repeated a novel wheel confinement due to a lack of activity, the total length of the experiment was extended to as much as 175 days. Novel wheel confinements using a modified Aschoff type II protocol were performed after this experimental timeline with different animals (Fig 2-3).

### Experiment 1: 3-hour Novel Wheel Confinements and Light Pulses

Animals were placed in DD, and running wheel activity patterns were recorded for 10 to 12 days. Actimetrics (Wilmette, IL) software was used to collect activity data in 10-min bins and to assess the quantity and phase of wheel revolutions as previously described (Krug et al., 2011). Hamsters were then placed in a novel confinement wheel for 3 h. A dim red light (<0.1 lux) was used to aid in transfers in and out of confinement wheels. All animals received at least 3 confinements beginning at CT 0, 4, and 8 in counterbalanced order (Fig 2-1). If an animal ran fewer than 3000 revolutions over the course of the 3-hour interval, a confinement was repeated. Although some animals did

not run more than 3000 revolutions after a second confinement at a given phase, no confinement was repeated more than once. Controls were performed at CT 4 by handling animals at the same times as the entry and exit of the confinement to mimic disruption, with- out placement in a novel wheel. To compare the effects of the nonphotic manipulation with the response to light pulses, each hamster received a 15-min light pulse (220 lux, white fluorescent light; Philips [Eindhoven, the Netherlands] 25 watt Hg, F32T8/ADV841/XLL) at CT 18.5 after the effects of the final confinement were assessed. This was followed by 10 to 12 days in DD and a final light pulse at CT 15. Upon finding that novel wheel confinement in DD produced small and inconsistent phase shifts, we per- formed additional experiments using modified Aschoff type II protocols that have been reported to produce larger effects (Webb et al., 2014). Fifteen additional animals (9 WT and 6 duper; 3 and 2 females, respectively) that had not been used in the other experiments were kept in a 14:10 LD cycle (120-150 lux, white fluorescent light) for 10 to 12 days while wheel-running patterns were recorded. At ZT 6 (6 hours before lights off), they were transferred to dark- ness with or without (control) a novel confinement wheel. After 3 h, the hamsters that had been confined to a novel wheel were released and their previous wheel was returned to them. After approximately 10 days of DD, the animals were returned to 14:10 LD for an additional 10 to 12 days. A second novel wheel confinement was performed with the modification that hamsters were exposed to 2 days of constant light (LL) before transfer to DD at ZT 6. All animals (excluding 2 females that were not part of the third manipulation) were subjected to all 3 novel wheel confinements in counterbalanced order. Cages were changed between manipulations at the time of return to the 14:10 LD cycle.

### **Experiment 2: 3-hour Dark Pulses**

After animals completed experiment 1, they were moved either to a 14:10 LD cycle for approximately 10 days or to dim LL. Additional duper females that had been housed in 14:10 LD but had not been included in experiment 1 were added to the study. After 10 to 12 days in dim LL (120-150 lux, white fluorescent light), a 3-hour dark pulse was administered beginning in subjective day (CT 6 and 8) or subjective night (CT 18). At the end of the 3-hour dark pulse, the animals were returned to LL for another 10 to 12 days so that phase shifts could be assessed.

### **Experiment 3: Light-Dark Transitions**

Hamsters were moved from LL (of the same intensity as in experiment 2) to DD at CT 6, 8, or 18 and were maintained in darkness for approximately 10 days. Hamsters were then returned to dim LL at CT 9, 11, or 21 (to match the phase of the return to LL after a 3-hour dark pulse in experiment 2). After 10 to 12 days in dim LL, they were returned to DD at CT 6, 8, or 18 for 10 to 12 days.

### **Statistical Analyses**

Onset of activity was used as the phase marker to assess free-running period (assessed by linear regression; ClockLab software, Actimetrics). Phase shifts were calculated from the least squares fits plotted for 8 to 10 circadian cycles of steady-state wheel running before and after the manipulation (Bittman, 2012). Phase shifts of animals that displayed transients or ultradian behavior after LL to DD transitions at CT 18 were based on the steady state after they spontaneously regained consistent circadian rhythmicity.

All phase shifts are given in circadian hours (hours multiplied by 24 and divided by period of activity onsets preceding the manipulation). Variability of phase shifts was measured using tests for the concentration parameter (described below). The  $\chi^2$  periodogram and autocorrelation were used to determine the period and strength of ultradian rhythms and were based on a minimum of 5 days of behavioral data. Novel wheel confinements and controls with prior exposure to LL or LD were analyzed using a repeated-measures analysis of variance (ANOVA) to compare manipulations within and between genotypes. When a significant difference was found, paired  $t$  tests were used to test for pairwise differences in means.

### Circular Statistics

The resultant vector for a set of phases  $\varphi_k$  given in radians is  $\bar{r} = \frac{1}{N} \sum_{k=1}^N e^{i\varphi_k}$ , the circular mean is  $\varphi = \arg(\bar{r})$ , and the circular standard deviation is  $\sqrt{2(1 - \|\bar{r}\|)}$ . This value was multiplied by  $12/\pi$  to convert phases from radians to circadian hours. To test whether the circular means of 2 or more groups are the same, we applied the Watson-Williams test (circular analog of the 1-factor ANOVA). The concentration parameter  $\kappa$  of a von Mises distribution (circular analog of the normal distribution) describes the spread of values: a higher value of  $\kappa$  corresponds to a narrower concentration about the circular mean (Fisher, 1993). To test whether 2 samples have the same concentration parameters, we applied a 2-sample test of equal concentration parameter with test statistic

$$F = \frac{(n_2 - 1)(n_1 - \|\bar{r}_1\|)}{(n_1 - 1)(n_2 - \|\bar{r}_2\|)},$$

where  $n_1$  and  $n_2$  are the sample sizes of the 2 groups. Circular statistics

were computed using MATLAB 2013a (MathWorks, Natick, MA) and CircStats Version 2012a (Berens, 2009).

## Results

### Experiment 1: 3-hour Novel Wheel Confinement and Light Pulses

Confinement to a novel wheel in DD produced inconsistent phase shifts in both WT and duper hamsters at CT0, 4, and 8 (all  $p > 0.3$ ; Table 2-1 and Fig 2-2). At none of these phases was there a significant effect of genotype on the shift of phase of activity onset after confinement to a novel running wheel. At CT 4, phase shifts of duper but not WT hamsters were significantly larger than those of control animals of the same genotype that were handled but not placed in a novel wheel ( $p = 0.02$  and  $p = 0.08$ , respectively; Table 1). All hamsters were of comparable age when tested, and there were no significant differences between males and females in phase-shift amplitude or the number of wheel revolutions (all  $p > 0.1$ ). The number of wheel revolutions during the

**Table 2-1. Phase shifts in response to novel wheel confinements and light pulses.**

Manipulation	Measurement	All Trials	
		WT	Duper
Novel confinement wheels in DD			
CT 0	Phase shift ( <i>n</i> )	0.2 ± 0.4 (15)	0.04 ± 0.6 (21)
CT 4	Phase shift ( <i>n</i> )	0.1 ± 0.4 (18)	0.3 ± 0.4 (19)*
CT 8	Phase shift ( <i>n</i> )	0.03 ± 0.3 (15)	0.3 ± 0.6 (24)
Revolutions (all confinements)		2551 ± 211(48)*	2583 ± 209 (64)*
Control (DD)			
CT 4	Phase shift ( <i>n</i> )	-0.03 ± 0.08 (5)	-0.003 ± 0.10 (8)
	Revolutions	5 ± 2	10 ± 5
Light pulses			
CT 18.5	Phase shift ( <i>n</i> )	2.6 ± 0.9 (15)*	-11.6 ± 2.6 (16)*†
CT 15	Phase shift ( <i>n</i> )	-1.3 ± 1.1 (15)	-5.6 ± 1.1 (16)†
Modified Aschoff type II novel wheel confinements at ZT 6			
LD to DD	Phase shift ( <i>n</i> )	0.54 ± 0.25 (9)	1.87 ± 0.98 (6)†
	Revolutions	522 ± 123	1047 ± 364
LD to DD Control	Phase shift ( <i>n</i> )	0.58 ± 0.40 (9)	2.07 ± 0.83 (6)†
	Revolutions	3 ± 2	183 ± 76*
LL to DD	Phase shift ( <i>n</i> )	1.95 ± 0.78 (9)*	See text.
	Revolutions	733 ± 165	See text.

All phase shifts are presented as circular mean ± circular SD given in circadian hours (number of animals). Number of revolutions for all experiments is provided as mean ± SEM. Significant differences ( $p < 0.05$ ) between experimental and control groups within genotype and between genotypes are indicated (\* and †, respectively). Where values are not provided for dupers in the modified Aschoff type II novel wheel confinement experiment, the sample size is too small for statistical analysis; see text for details.

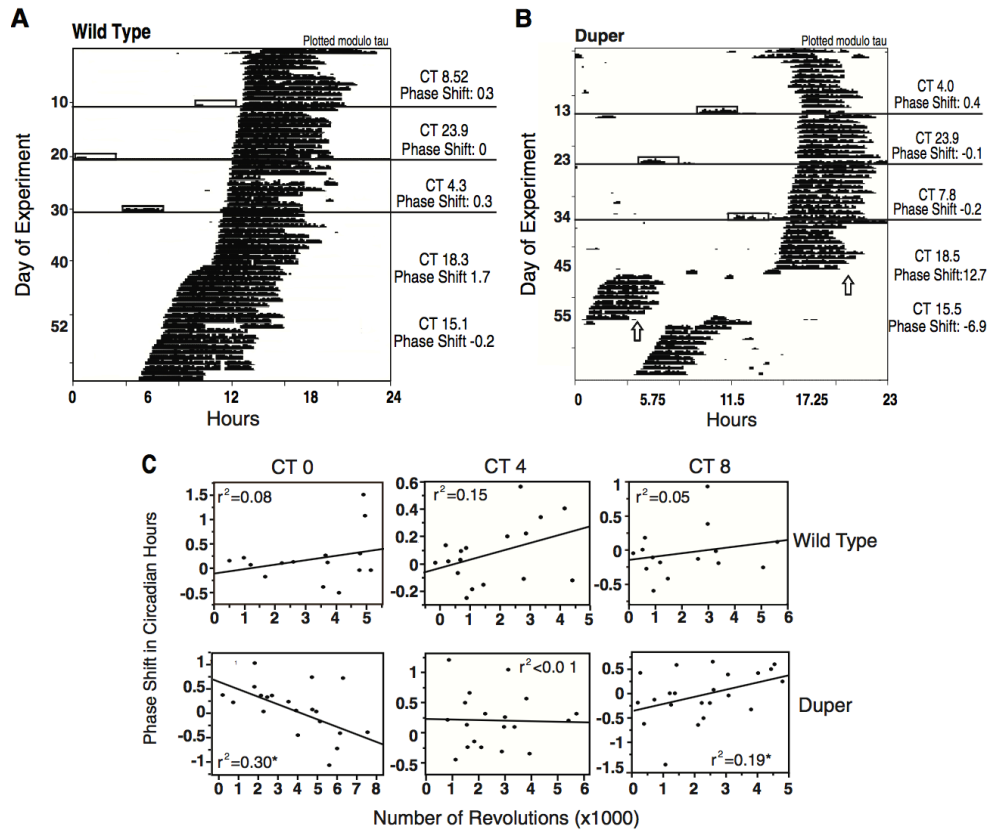


Figure 2-2. Duper and wild-type (WT) hamsters show small and comparable phase shifts in response to novel wheel confinements. Representative actograms of responses of (A) WT and (B) duper hamsters to novel wheel confinement at CT 0, 4, and 8 (open boxes) and to light pulses (vertical arrows) at CT 15 and CT 18.5 are shown. Actograms are plotted modulo  $\tau_{DD}$  for each genotype. (C) Linear regression of phase shifts in circadian time on the number of revolutions during the novel wheel confinement at CT 0, 4, and 8. Significant correlations ( $*p < 0.03$ ) were found at CT 0 and CT 8 in duper hamsters. The number of revolutions during confinement was similar in WT and duper hamsters.

3-hour interval of confinement at all time points was similar between WT and duper hamsters for all trials as well as for trials in which animals ran more than 3000 revolutions. In neither genotype were phase shifts of hamsters that ran more than 3000 revolutions during the 3-hour confinement significantly greater than the average of the entire group, and there was no genotype effect in these more active hamsters upon phase shifts elicited by confinement at CT 0, 4, or 8 (all  $p > 0.2$ ; Table 2-1). Duper hamsters

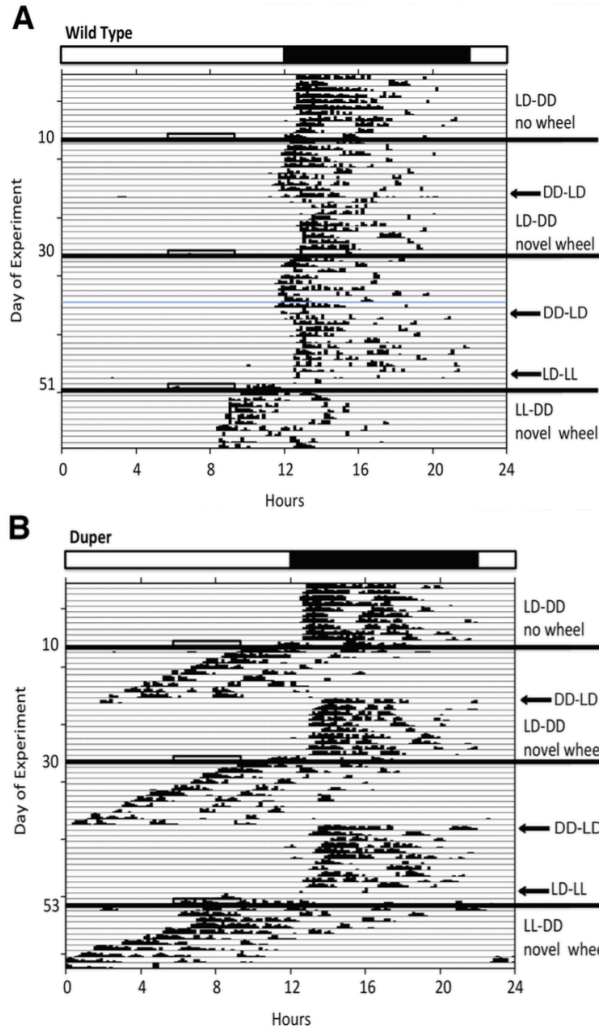


Figure 2-3. Duper and WT hamsters show small and comparable phase shifts in response to novel wheel confinements using a modified Aschoff Type II protocol. Representative actograms of responses of (A) WT and (B) duper hamsters to 3h novel wheel confinements starting at ZT 6 on day 10 including a control transfer from LD to DD (no wheel), LD to DD with a novel confinement wheel, and LD to 2 days of LL preceding a novel wheel in DD. The first 3h in DD (with or without wheel) are indicated by the box. Duper hamsters showed larger shifts upon LD- DD transfer whether or not they were confined for the first 3h to a novel running wheel. (C) Bar graph of phase shifts (mean and standard error) for each manipulation for duper (black) and WT (gray). Significant differences ( $p < 0.05$ ) between manipulations within genotype are indicated with \*, and between genotypes for a given manipulation are indicated with †.

showed a small but statistically significant negative correlation between the number of revolutions and magnitude of phase advances at CT 0 ( $r = 0.30$ , slope =  $-0.001716$ ,  $p = 0.01$ ) and positive correlation at CT 8 ( $r^2 = 0.19$ , slope =  $0.00024$ ,  $p = 0.04$ ; Figure 2-2C). There were no other significant correlations between number of revolutions during the 3-hour wheel confinement and phase shifts.

Further experiments used modified Aschoff type II protocols that have been reported to elicit larger and more consistent nonphotic shifts (Webb et al., 2014). The 3-hour confinement to a novel wheel coincident with transfer from 14:10 LD to DD at ZT 6 induced phase shifts similar to those of nonconfined controls in both WT and duper



hamsters ( $p = 0.84$  and  $p = 0.51$ , respectively; Table 2-1). Dupers showed significantly larger phase shifts than WT hamsters in both conditions (repeated-measures ANOVA:  $p = 0.0007$ ; paired  $t$  test: novel wheel  $p < 0.014$ ; control  $p < 0.006$ ; Table 2-1 and Fig 2-3).

The number of wheel revolutions during the 3-hour interval of confinement was similar between WT and duper hamsters (Table 2-1). In control trials, dupers showed more activity than WT hamsters ( $p < 0.03$ ; Table 2-1). There was no correlation between phase-shift amplitude and number of wheel revolutions within either genotype.

Exposure to 2 days of LL prior to confinement and transfer to DD at ZT 6 amplified phase shifts of WTs compared with those observed after wheel confinement in LD cycles (repeated-measures ANOVA between all manipulations:  $p < 0.001$ ; paired  $t$  test: LL to DD,  $1.95 \pm 0.27$ h vs. LD to DD,  $0.54 \pm 0.09$ h,  $p < 0.001$ ). Wheel confinement of WT hamsters in LL also increased the size of the shift compared with nonconfined controls ( $0.46 \pm 0.19$  h,  $p = 0.002$ ). WT hamsters showed no correlation between phase-shift amplitude and the amount of activity ( $p > 0.1$ ). The 4-duper hamsters subjected to such transfers showed highly variable shifts (0.78, 0.88, 2.29, and  $-9.5$  hour). The duper hamster exhibiting a 9.5-hour phase delay ran more than other hamsters (5557 revolutions). However, other dupers that ran more than 3000 revolutions during confinement had phase shifts comparable to animals that were not active. When given 15-min light pulses after the completion of assessment of the effects of novel wheel confinements in DD, duper hamsters showed larger phase shifts at CT 18.5 and CT 15 than did WT animals (Fig 2-2 A,B and Table 2-1). At both phases, the circular mean phase shifts of the duper hamsters were significantly greater than those of the WT (Watson-Williams test: CT 15,  $F = 98.7$ ,  $p < 0.001$ ; CT 18.5,  $F = 152$ ,  $p < 0.001$ ).

## Experiment 2: 3-hour Dark Pulses

The 3-hour dark pulses at CT 6, 8, and 18 elicited variable phase shifts. Duper hamsters tended to show larger phase shifts than did WT during subjective day (CT 6 and 8; Fig 2-4A,B), but genotype had no significant effect on circular mean (Watson-Williams test,  $F = 1.35$ ,  $p = 0.26$ ; Fig 2-5). Phase shifts in response to dark pulses at CT 6 and 8 were significantly more variable in duper than in WT hamsters (2-sample test of equal concentration parameter,  $F = 10.9$ ,  $p < 0.001$ ). The number of revolution during dark pulse was variable, did not differ significantly between WT and duper hamsters ( $983 \pm 475$  vs.  $186 \pm 156$

revolutions, respectively,  $p > 0.14$ ), and showed no correlation with the amplitude of the phase shift at any time point (CT 6: WT  $r^2 = 0.32$ , duper  $r^2 = 0.09$ ; CT 8:  $r^2 = 0.18$ ,  $r^2 = 0.03$ ; CT 18:  $r^2 = 0.07$ ,  $r^2 = 0.14$ , all  $p > 0.18$ ).

Splitting or arrhythmicity occurred after 3-hour dark pulses in 8 of 21 duper hamsters and 0 of 16 WT hamsters. Two duper mutants split immediately after the dark pulse and 3

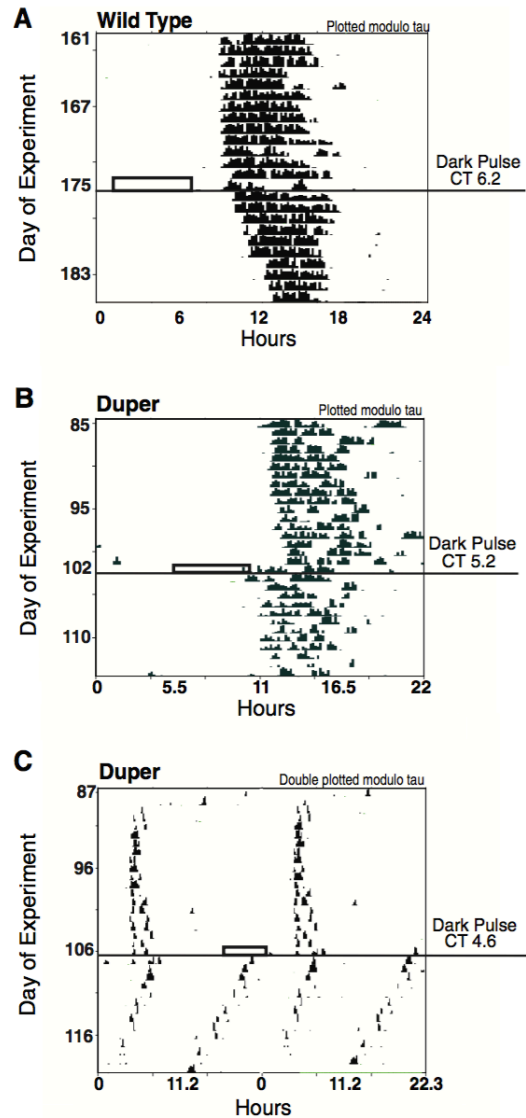


Figure 2-4 The 3-hour dark pulses during LL led to variable phase shifts and splitting in duper but not wild-type (WT) hamsters. Representative examples of responses of (A) WT and (B) duper hamsters to a 3-hour dark pulse at approximately CT 6 are shown. Boxes indicate time of dark pulse, and circadian time of onset is indicated to the right of the actogram. Note that actograms are plotted modulo  $\tau_{LL}$ . (C) Actogram of a duper hamster that split immediately after the dark pulse.

within 12 days after the dark pulse (Fig 2-4C), while 3 became arrhythmic immediately following a dark pulse.

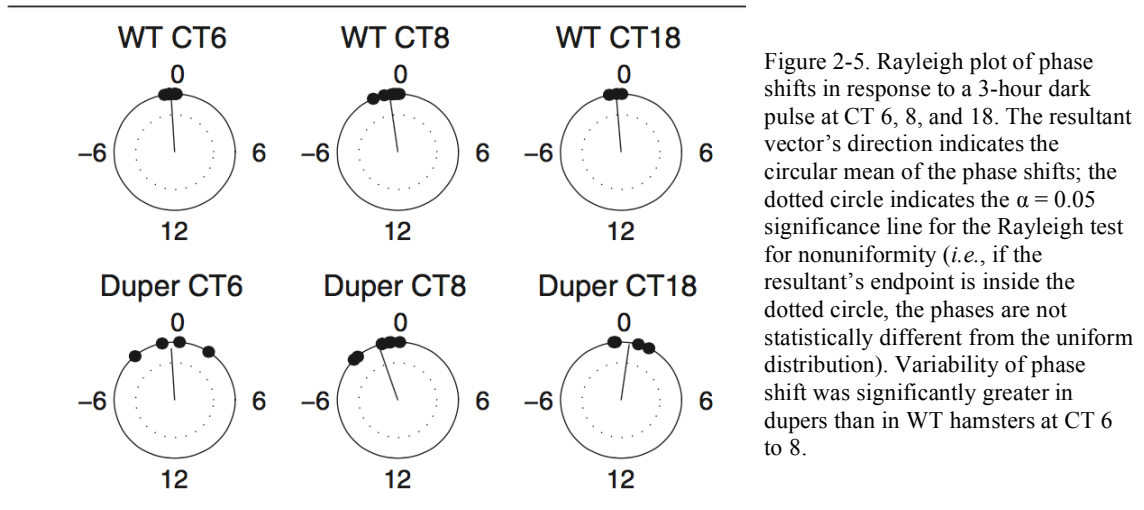


Figure 2-5. Rayleigh plot of phase shifts in response to a 3-hour dark pulse at CT 6, 8, and 18. The resultant vector's direction indicates the circular mean of the phase shifts; the dotted circle indicates the  $\alpha = 0.05$  significance line for the Rayleigh test for nonuniformity (*i.e.*, if the resultant's endpoint is inside the dotted circle, the phases are not statistically different from the uniform distribution). Variability of phase shift was significantly greater in dupers than in WT hamsters at CT 6 to 8.

### Experiment 3: Light-Dark Transitions

Both LL to DD and DD to LL transitions resulted in significantly larger phase shifts in duper than in WT hamsters at particular circadian phases. Both types of light-dark transitions induced phase shifts that were significantly more variable in dupers than in WT hamsters (Fig 2-6).

DD to LL transitions during late subjective day (CT 9) and early subjective night (CT 12) induced significantly larger phase shifts in duper mutants than in WT hamsters (Watson-Williams test:  $F = 99, p < 0.001$ , at CT 9;  $F = 10.3, p = 0.009$  at CT 12; Fig 2-6C,D). Duper hamsters were also more variable (2-sample test of equal concentration parameter:  $F = 6.5, p = 0.02$  at CT 9;  $F = 204, p < 0.001$  at CT 12). WT hamsters showed phase delays of  $1.3 \pm 0.8$  and  $1.2 \pm 0.3$  circadian hours at CT 9 and 12, respectively (circular mean  $\pm$  circular SD). In contrast, duper hamsters exhibited phase advances ranging from 5- to 11-hours at CT 9. At CT 12, both large advances (ranging from 3-7 hours) and delays (8-12 hours) occurred in the mutants (Fig 2-6 C,D).

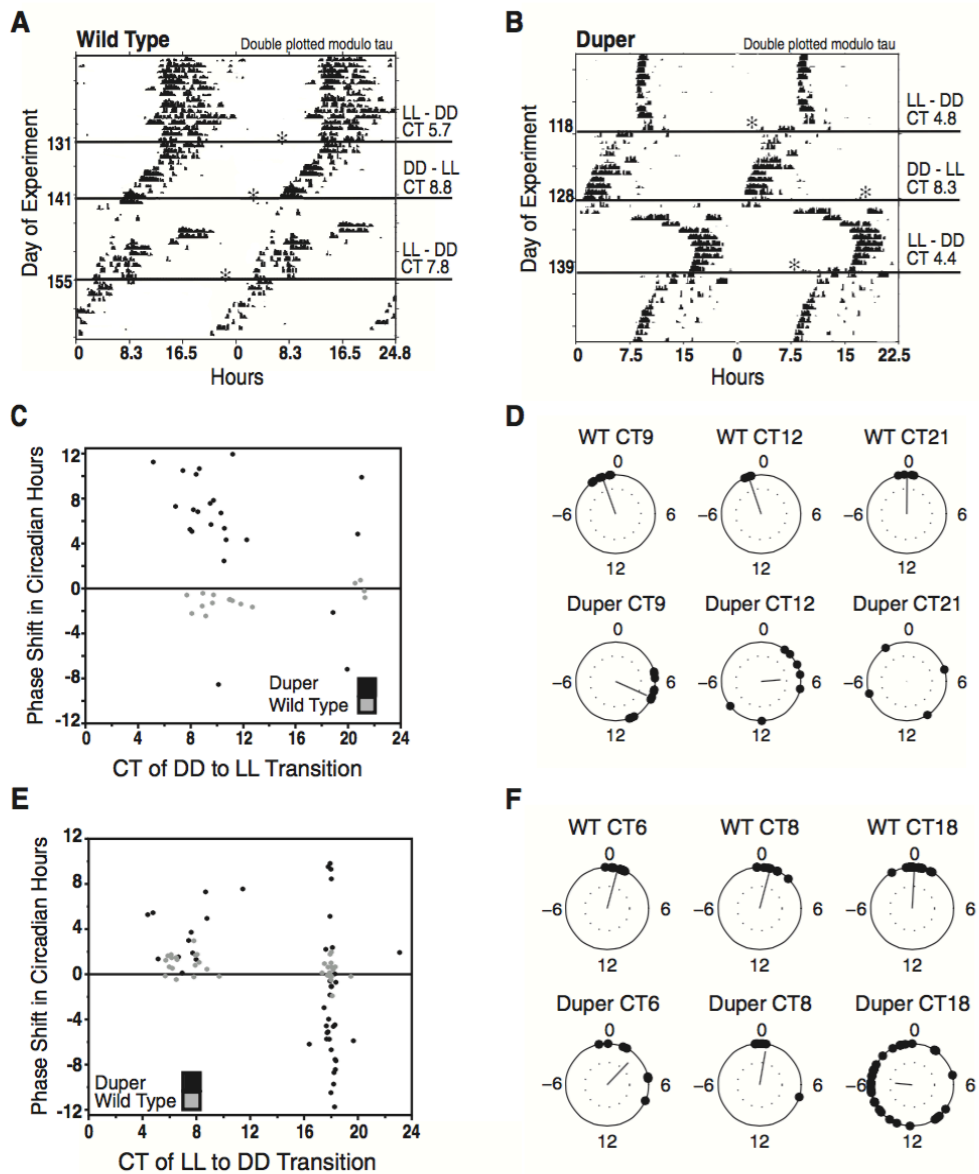


Figure 2-6. Duper hamsters show larger phase shifts than wild-type (WT) hamsters in response to light and dark transitions. Representative responses of (A) WT and (B) duper hamsters to LL to DD and DD to LL transitions. The asterisk (\*) marks time of transition. Acto-grams are double plotted modulo  $\tau$ . (C) DD to LL transitions induced significantly greater phase shifts in duper than in WT hamsters at CT 9 and 12. Seven of 34 duper and 2 of 20 WT hamsters were excluded from analysis because they split in response to the DD to LL transition. (D) Variability of phase shifts was significantly greater in duper hamsters at all time points. (E) LL to DD transitions at CT 18, but not during subjective day, induced significantly larger phase shifts in duper (black points) than in WT hamsters (gray symbols;  $p < 0.001$ ). (F) Phase shifts in subjective night (CT 18) were significantly greater than in subjective day (CT 6 and 8) in both duper and WT hamsters. Variability of phase shifts was significantly greater in duper hamsters at all time points ( $p < 0.001$ ). Dotted circles in E and F are as in Fig 2-5.

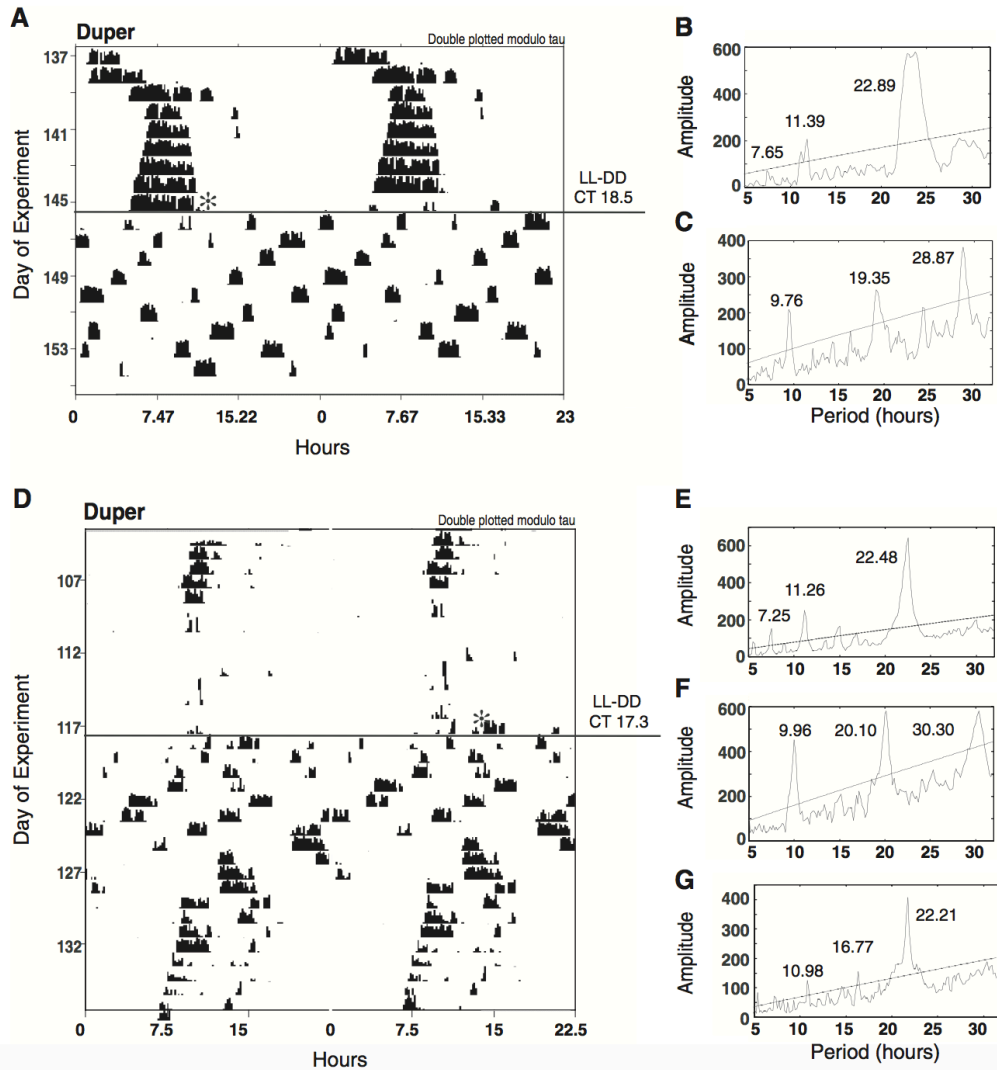


Figure 2-7. LL to DD transition at CT 18 leads to ultradian locomotor rhythms in some duper hamsters. Actograms are plotted modulo  $\tau_{LL}$ . (A, D) Two examples of duper hamsters in which circadian locomotor patterns were transiently disrupted upon transfer from LL to DD during mid-subjective night. Twenty-nine of 43 duper and 0 of 21 WT hamsters displayed ultradian locomotor behavior for at least 5 days following the shift to DD. This did not occur when dupers were shifted from LL to DD at other phases. (B, E) The  $\chi^2$  periodogram analysis of locomotor patterns in LL before the transition; (C, F) analysis of ultradian behavior during its duration after the LL to DD transition; (G) analysis of locomotor patterns after the spontaneous resumption of circadian rhythmicity in DD.

Phase shifts of WT hamsters transferred from DD to LL in late subjective night (CT 21) were uniformly less than 1 hour (mean  $\pm$  SEM,  $0.03 \pm 0.6$ ). Dupers showed great variability in resetting ( $-10.6 \pm 5.4$  circadian hours), with both phase advances and delays occurring and a significantly different concentration parameter than WT (2-sample test

of equal concentration parameter,  $F = 79.0$ ,  $p < 0.001$ ; Figure 2-6C,D). Change in  $\tau$  after DD to LL transitions did not differ between WT and duper hamsters at any phase (WT vs. duper at CT 9:  $0.21 \pm 0.05$  hours vs.  $-0.04 \pm 0.23$  hours; at CT 12:  $0.32 \pm 0.12$  hours vs.  $0.02 \pm 0.23$  hours; at CT 21:  $0.15 \pm 0.05$  hours vs.  $-0.37 \pm 0.44$  hours, all  $p > 0.14$ ) or between phases within phenotype (WT, all  $p > 0.2$ ; duper, all  $p > 0.4$ ).

LL to DD transitions induced similar phase shifts at CT 6 and 8 in duper and WT hamsters (circular mean  $F = 0.98$ ,  $p = 0.33$ , Watson-Williams test), but the variability was much greater in duper hamsters (2-sample test of equal concentration parameter,  $F = 11.6$ ,  $p < 0.001$ ; Fig 2-6E,F). Phase shifts in response to LL to DD transitions at CT 18 were significantly larger in duper than in WT hamsters (Watson- Williams test,  $F = 26.9$ ,

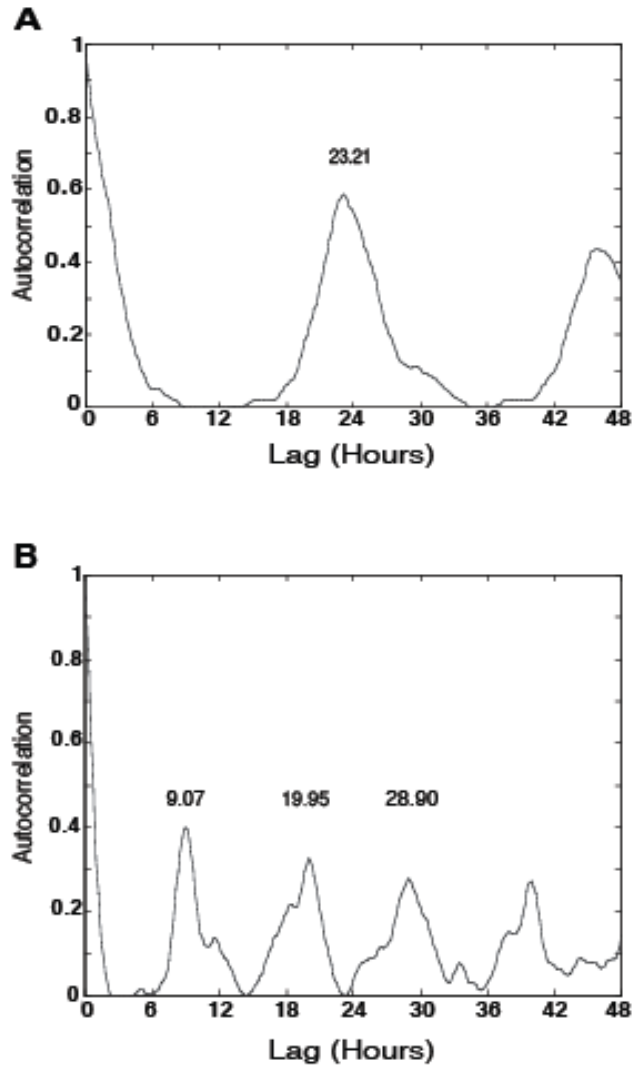


Figure 2-8. Autocorrelation of ultradian behavior in a duper hamster after LL to DD transition at CT 18, for the record shown in Figure 2-7A. (A) Autocorrelation analysis of locomotor patterns for 8 days in LL before the transition. (B) Autocorrelation showing ultradian behavior over 9 days immediately following the transition to DD. Values shown over each peak indicate significant autocorrelation ( $r=0.37$ , first peak); note that period estimates from autocorrelation align closely with periodogram estimates in Fig. 2-7.

$p < 0.001$ ), and duper hamsters again exhibited greater variability (2-sample test of equal concentration parameter,  $F = 23.5$ ,  $p < 0.001$ ; Fig 2-6E,F). Duper and WT hamsters did not differ in change of  $\tau$  after LL to DD transitions at any phase (CT 6: WT  $-0.25 \pm 0.09$  hours vs. duper:  $-0.14 \pm 0.11$  hours; CT8:  $-0.20 \pm 0.07$  hours vs.  $-0.35 \pm 0.29$  hours; CT18:  $-0.20 \pm 0.05$  hours vs.  $0.10 \pm 0.16$  hours, all  $p > 0.3$ ), nor did the phase of transition have an effect on the change of  $\tau$  within either genotype (WT, all  $p > 0.6$ ; duper, all  $p > 0.13$ ).

LL to DD transitions at CT 18 induced ultradian patterns of wheel-running behavior in 29 of 43 duper and 0 of 21 WT hamsters. This did not occur when the dupers were shifted to DD at other phases. Ultradian locomotor behavior lasted for approximately 6 to 12 days following the transition to DD (Fig 2-7). The period of the ultradian rhythms averaged  $7.47 \pm 1.67$  hours (range, 5-10 hours). The period of the ultradian rhythm was not correlated with the number of days of ultradian activity ( $r^2 = 0.04$ ), the amplitude of the phase shift ( $r^2 =$

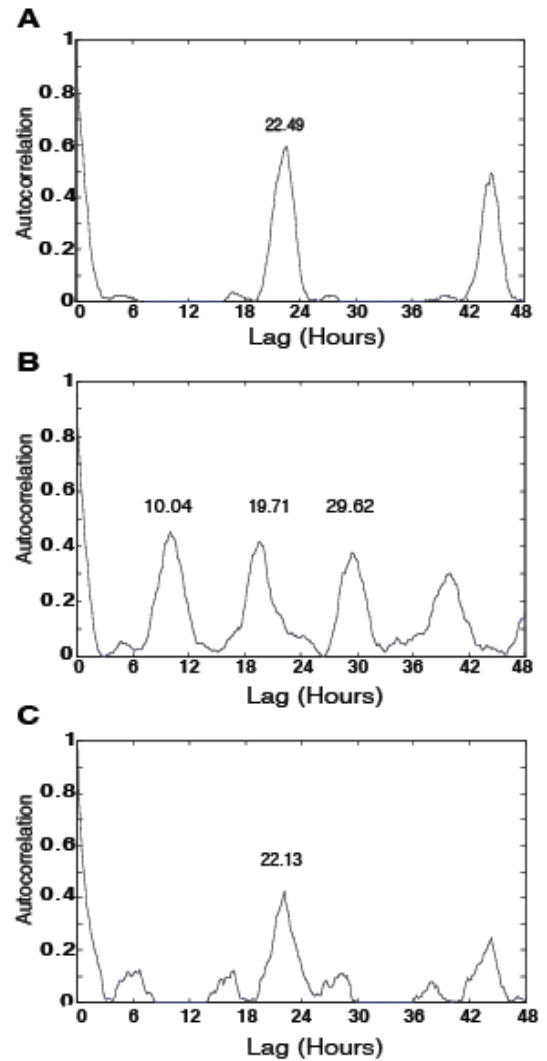


Figure 2-9. Autocorrelation of ultradian patterns and spontaneous recovery of circadian rhythms in a duper hamster after LL to DD transition at CT 18, for the record shown in Figure 6D. (A) Autocorrelation analysis of locomotor patterns in LL (11 cycles) before the transition. (B) Indication of ultradian behavior for the first 7 cycles after the transition ( $r=0.44$ , first peak). (C) Spontaneous reinstatement of circadian rhythms for days 8 after the LL-DD transition.

0.02), or the change in period after rhythmicity was regained ( $r^2 = 0.03$ ; all  $p > 0.4$ ). When kept in DD, all duper hamsters spontaneously regained stable free-running patterns (Fig 2-7D). There was no significant change in the free-running period before ( $\tau_{LL}$ ) versus after ( $\tau_{DD}$ ) the interval of ultradian activity, regardless of whether the  $\chi^2$  periodogram or autocorrelation was used to analyze these effects (Fig 2-8, 2-9).

### **Mathematical Modeling**

To examine whether reduced coupling can account for the altered resetting and other duper properties, we developed a mathematical model to simulate the duper mutation. We sought to determine whether a reduction of coupling strength could reproduce the essential features of the duper behavioral phenotype: shortened period, reduced amplitude (suggested by the gene expression analysis in Krug et al., 2011), type 0 photic PRC, increased range of entrainment (Bittman, 2014), and large, variable shifts following LL to DD transitions at CT 18, sometimes with transient loss of circadian rhythms. To this end, we employed a relatively simple model (Fig 2-10) consisting of coupled Goodwin oscillators adapted from Gonze et al. (2005).

Our model of the circadian pacemaker consists of 6 regional oscillators, mimicking the simple multi oscillator model network postulated by Yamaguchi et al. (2013), except that we include both left and right lobes. Coupling between these oscillators is indicated in Figure 2-10:  $k_A$  denotes the autofeedback of each region to itself (representing communication among neurons within each regional cluster),  $k_C$  denotes coupling between regions in the same lobe, and  $k_I$  denotes interlobe



communication. The autofeedback increases both the amplitude and period of each oscillator and is required for generating self-sustained oscillations.

The WT parameters were chosen to reproduce a typical WT period, responses to light pulses, and ability to entrain to 24-hour LD cycles. In particular, the model with the WT parameter values yields a free-running period of 23.8 hours in DD, a lower range of entrainment of T22, and exhibits a weak resetting curve with all phase shifts less than 2 hours in response to a bright 1-hour photic pulse. Phase shifts in response to LL to DD transitions at all phases are modest (0-2 hours), and oscillators always remain well synchronized. In the absence of coupling, oscillators are self-sustained for the WT parameters.

To simulate mutant behavior, we reduced the parameters controlling strength of coupling, among the components of the model to 63% of its WT value. All other parameter values, including light parameters, are the same as in the WT parameter set. The reduction in the coupling strength parameter results in a free-running period of 22.9 h, significantly reduced amplitude in all components, an extended range of entrainment that goes down to T18.5, and a strong resetting curve with 12-hour phase shifts near CT 18. The response to LL to DD transitions at CT 18 is more dramatic, with severe disruptions of the circadian rhythm sometimes occurring. Slight changes to the period of a light-responsive oscillator result in large changes to the response to LL to DD transitions at CT 18, possibly explaining the large variability observed experimentally in Figure 2-6. See Figure 2-11 for examples of simulated actograms showing DD to LL and LL to DD transitions.

Although the model does not capture all details of hamster activity, it shows that the major features of the duper phenotype can be reproduced qualitatively as a result of reduced coupling within the circadian pacemaker.

### Details of the Mathematical Model

We developed a simple model of the circadian pacemaker to test whether reducing coupling strength within the SCN can qualitatively reproduce essential features of the duper behavioral phenotype: shortened period, reduced amplitude, strong photic resetting, increased range of entrainment, and the highly variable responses to LL-DD transitions at CT18. Our model of the circadian pacemaker consists of six regional oscillators, mimicking the simple multi-oscillator model network of Yamaguchi et al. (2013), which used 4 coupled phase-only oscillators (one core, 2 shell, 1 output (Fig 2-11)). We extend this model by including both left and right lobes and tracking both phase and amplitude. Each regional oscillator in our model is represented by a Goodwin oscillator (Gonze et al., 2005), in which clock gene mRNA  $X$  produces clock protein  $Y$ , in turn activating a transcriptional inhibitor  $Z$ . Light is assumed to increase transcription of  $X$ , as does a neurotransmitter  $V$  that acts to couple the oscillators. The differential equations governing the  $i$ th oscillator are:

$$\begin{aligned}\frac{dX_i}{dt} &= v_1 \frac{K_1^n}{K_1^n + Z_i^n} - v_2 \frac{X_i}{K_2 + X_i} + v_c \frac{KF_i}{K_c + KF_i} + L, \\ \frac{dY_i}{dt} &= k_3 X_i - v_4 \frac{Y_i}{K_4 + Y_i}, \\ \frac{dZ_i}{dt} &= k_5 Y_i - v_6 \frac{Z_i}{K_6 + Z_i}, \\ \frac{dV_i}{dt} &= k_7 X_i - v_8 \frac{V_i}{K_8 + V_i},\end{aligned}$$

where  $F_i$  is the amount of neurotransmitter received by the  $i$ th oscillator, a weighted sum of incoming signals, including from each oscillator to itself:

$$F_1 = \frac{k_A V_1 + k_I V_4}{1 + k_I}, \quad F_2 = \frac{k_A V_2 + k_C V_1 + k_C V_3}{1 + 2k_C}, \quad F_3 = \frac{k_A V_3 + k_C V_2 + k_I V_6}{1 + k_C + k_I}.$$

Expressions for the right lobe are similar. Here  $k_A$  denotes the autofeedback of each region to itself (representing communication among neurons within each regional cluster),  $k_C$  denotes coupling

between regions in the same lobe,

and  $k_I$  denotes inter-lobe

communication. The autofeedback

increases both the amplitude and

period of each oscillator and is

required for generating self-sustained

oscillations; in the absence of

coupling ( $F_i = k_A V_i$ ) the system yields

a self-sustained oscillator for the

WT parameters. The base set of

parameter values is  $k_A = 1.3$ ;  $k_C =$

$0.2$ ;  $k_I = 0.04$ ;  $v_1 = 6.8355$ ;  $K_1 =$

$2.7266$ ;  $n = 5.6645$ ;  $v_2 = 8.4297$ ;  $K_2 = 0.2910$ ;  $k_3 = 0.1177$ ;  $v_4 = 1.0841$ ;  $K_4 = 8.1343$ ;  $k_5$

$= 0.3352$ ;  $v_6 = 4.6645$ ;  $K_6 = 9.9849$ ;  $k_7 = 0.2282$ ;  $v_8 = 3.5216$ ;  $K_8 = 7.4519$ ;  $v_C = 6.7924$ ;

$K_C = 4.8283$ ;  $K=1$ . We induce heterogeneity by multiplying the rate constants of the  $i$ th

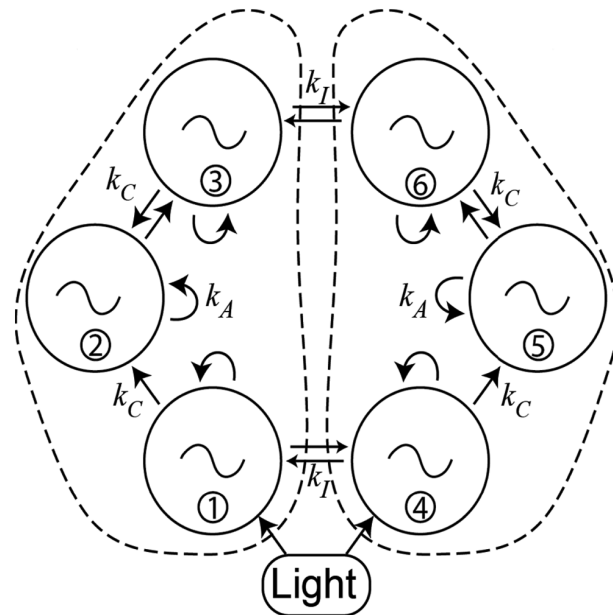


Figure 2-10. Diagram of a simple model of the SCN using 6 Goodwin oscillators. Each oscillator represents a regional cluster of SCN neurons (2 light-responsive core clusters and 4 shell clusters), coupled as indicated by the arrows and organized into left and right lobes. The model is adapted from Gonze et al. (2005).

oscillator by scaling factor  $s_i$ , where  $s_1=1.047$ ,  $s_2=1.005$ ,  $s_3=1.007$ ,  $s_4=1.055$ ,  $s_5=1.003$ , and  $s_6=1.002$ . Note that a higher scaling factor increases the derivative values and so effectively shortens the period.

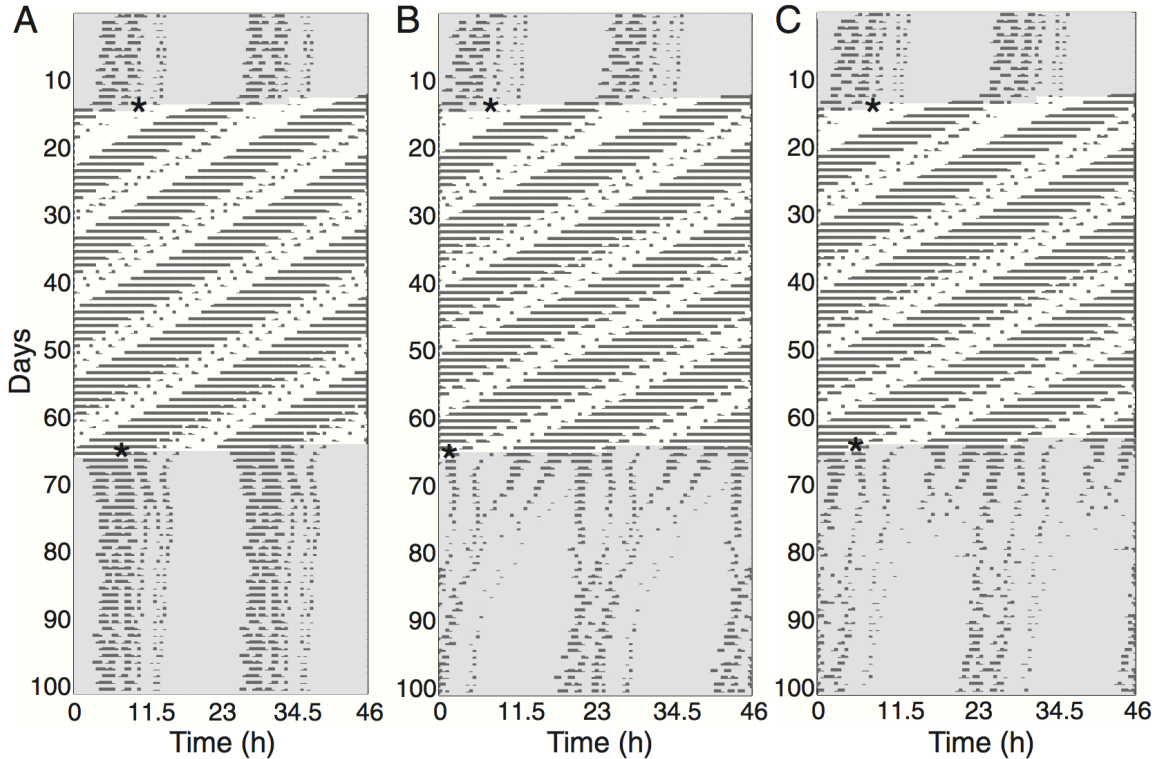


Figure 2-11. Actograms of simulations of the duper mutant with DD to LL and LL to DD transitions at CT 18 (indicated by \*). (A) Simulation using original parameter set with  $s_4 = 1.055$  (which controls the period of oscillator 4), in which oscillators remain synchronized. The system advances by 5 hours in response to the DD to LL transition and delays by 4.5 hours in response to the LL to DD transition. (B) Simulation of system with  $s_4 = 1.058$  (resulting in slightly shortened period), in which oscillators become disassociated in response to the LL to DD transition but resynchronize after several weeks. The system experiences a large, nearly anti-phase shift following the LL to DD transition. (C) System with  $s_4 = 1.059$ , in which left and right sets of oscillators split for a week following the LL to DD transition, then exhibit weak rhythms for a week before spontaneously regaining a coherent free-running circadian rhythm. Actograms are constructed from the simulations by adding Gaussian noise at 10% of the maximum amplitude for each oscillator, then smoothing with a discrete wavelet transform to isolate the circadian component for each oscillator in the system. Activity occurs when any oscillator is near its peak value. Circadian time CT 12 corresponds to activity onset in the simulated actograms.

The WT parameter set yields a free-running period of 23.8h in DD ( $L=0$ ), has lower range of entrainment T22 ( $L=0.25$  during light half of T-cycle,  $L=0$  during dark half), and exhibits a weak resetting curve with all phase shifts less than 2h in response to a bright 1h photic pulse ( $L=0.8$  during pulse;  $L=0$  otherwise). Phase shifts in response to

LL-DD transitions at all phases are modest, 0-2h, and oscillators always remain well synchronized. To simulate LL, we set  $L=0.3$ .

The duper mutation is simulated by decreasing the coupling parameter  $K$  from 1 to 0.63 (leaving all other parameters identical to WT values), resulting in a free-running period of 22.9h, significantly reduced amplitude in all oscillators, an extended range of entrainment that goes down to T18.5, and a strong resetting curve with jump near CT18. Values of  $L$  are same as in WT simulations. The amplitude of oscillation in all state variables is greatly reduced, and response to LL-DD transitions at CT18 is more dramatic, with severe disruptions of the circadian rhythm sometimes occurring, as shown in Figure 2-11. Slight changes to the period of one of the oscillators results in large changes to the response to LL to DD transitions at CT 18, explaining the large variability observed in Figure 2-6. Increasing the light intensity parameter  $L$  in the LL simulations also shortens the period, as observed in the duper hamsters (Bittman, 2014).

Actograms are constructed from the simulations by adding Gaussian noise at 10% of the max amplitude for each oscillator, then smoothing with a discrete wavelet transform to isolate the circadian component and identify peaks of  $Z_i$ . Activity occurs when any oscillator is near its peak  $Z$  value. Circadian time CT12 corresponds to activity onset in the simulated actograms.

## **Discussion**

As part of an ongoing effort to analyze the duper mutation, we have sought a deeper understanding of its behavioral phenotype. We used novel wheel confinements, dark pulses, and light/dark transitions to determine whether large phase shifts of duper mutants are specific to certain *zeitgebers* or restricted to particular circadian phases. Our

results provide no evidence that the *duper* mutation amplifies circadian responses to a non-photoc cue. This suggests that the mutation selectively alters responses to photic *zeitgebers* and/or its effects are restricted to subjective night. Unlike WT hamsters, *duper* mutants that were confined to novel wheels in DD showed significantly greater phase shifts at CT 4 than did handled controls. Nevertheless, the effect was small even in the *duper* hamsters. Genotype did not influence levels of activity over the 3 hours spent in the novel wheel. Furthermore, restriction of our analysis to animals that ran more than 3000 revolutions over the course of the confinement revealed no effect of genotype. Despite the fact that our animals were comparatively active, the phase shifts that we observed were much smaller than those elicited by light in WT, let alone *duper* mutant hamsters. Given that the *tau* mutation can shift the phase of peak sensitivity to nonphotoc *zeitgebers* (Mrosovsky et al., 1992; Biello and Mrosovsky, 1996) it is possible that we might find a greater response of *duper* hamsters at other circadian times, but the variability and small amplitude of the effects of the novel wheel in both genotypes at 3 different phases during the subjective day make this doubtful.

Although the phase shifts we observed in WT hamsters placed in novel wheels in DD were smaller than some that have been reported previously, the effects of this procedure have been highly variable in other laboratories (Reebs and Mrosovsky, 1989b; Mrosovsky et al., 1992; Bobrzynska and Mrosovsky, 1998; Duncan et al., 2014). Mrosovsky et al. (1992) reported that novel wheel confinement elicited only small phase shifts at CT 0 and 8. At CT 4, they obtained variable results: although shifts as great as 2 to 4 hours occurred in many of the hamsters, some animals exhibited little or no phase shift even when they ran a substantial amount (Mrosovsky et al., 1992). Previous studies

reported even more varied results with a 2-hour pulse at CT 6, with most animals displaying phase shifts of less than an hour, similar to controls (Reebs and Mrosovsky, 1989a). Due to the variable results, Reebs and Mrosovsky (1989a) repeated the experiment with varying durations of novel wheel confinement (1-, 3-, and 5-hours). They found that 3- and 5-hour confinements showed significantly larger phase shifts (2-3 hours) than the 1-hour confinements (0-1 hours) but still noted that about 25% of the animals in longer confinements did not shift.

Other investigations of nonphotic *zeitgebers* have used modified Aschoff type II protocols, and some laboratories have found larger effects using such procedures (Bobrzynska and Mrosovsky, 1998; for review, see Webb et al., 2014). Thus, we followed our initial studies comparing novel wheel confinement in DD with additional experiments using Aschoff type II designs. Our findings were comparable to those of others who have found highly variable results and lack of an effect of the novel wheel using comparable procedures (Evans et al., 2004; Duncan et al., 2014). Although duper hamsters did show larger phase shifts when exposed to a light/dark cycle prior to novel wheel confinement at ZT 6 (compared to DD before confinement), this is likely a confound of the light stimulus given that there was no difference between control and experimental conditions. Although we found that WT hamsters had significantly larger phase shifts when exposed to LL for 2 days prior to confinement than when the novel wheel was introduced at ZT 6 of a 14:10 schedule or at CT 4 or 8 after 10 days in DD, this procedure also confounded the light with the nonphotic stimulus, and hamsters to whom a wheel was provided at the time of LD to DD transfer at ZT 6 showed no greater phase shifts than did controls. The profound differences between duper and WT hamsters

in responses to transfer between LL and DD (experiments 2 and 3) greatly complicate interpretation of Aschoff type II designs in experiments on nonphotic phase shifting. Furthermore, the short period of duper hamsters results in a more positive phase angle and greater masking in LD cycles (Krug et al., 2011). This complicates assessment of the circadian phase at which the nonphotic stimulus is provided and makes comparison of duper and WT responses still more difficult.

Dark pulses provide another tool to explore the effects of the duper mutation during subjective day. In previous studies using WT Syrian hamsters, a 2- to 3-hour dark pulse initiated at CT 6 to 8 resulted in an average phase advance of 2.5 hours, with a range of 0 to 9 hours (Boulos and Rusak, 1982; Canal and Piggins, 2006). Although we found the response of duper hamsters to be variable, none of our hamsters showed shifts larger than 4 h. Previous studies have shown that activity during the dark pulses contributes to the amplitude of the phase shift (Reebs et al., 1989). However, we found no significant difference between duper and WT hamsters in running activity during the 3 hours of darkness. Furthermore, the number of revolutions during the 3-hour dark pulse was not correlated with the amplitude of phase shifts. These observations further support the hypothesis that the effect of the duper mutation is specific to photic stimuli.

The light pulses typically used to provide insight into entrainment are complex stimuli. They include not only the interval of exposure to light or dark at a specific phase of a free run in otherwise constant lighting conditions but also acute transfers into or out of light and darkness. We used light to dark (LL to DD) and dark to light (DD to LL) transitions to isolate these events and to investigate further the effects of exposure to light and darkness at various circadian phases. The lability of phase in response to both DD to LL



and LL to DD transitions was uniformly greater in duper than in WT hamsters. Consistent with previous studies (Albers, 1986; Aschoff, 1994), WT animals displayed phase shifts of 2-hours or less at all phases tested, regardless of whether the transition was from light to darkness or vice versa. In contrast, duper hamsters experienced very large phase shifts, and within groups of mutants experiencing transitions at the same phase, we observed some delays and some advances. It is possible that shifts in response to a DD to LL transition during subjective day (during the dead zone of the PRC for light pulses) are not immediate but are triggered by an effect of light after the start of subjective night. However, dupers experienced larger phase shifts in response to a DD to LL transition at CT 9 or 12 than they did to a 15-min light pulse at CT 12 (Krug et al., 2011).

Although the duper mutation does not affect the stability or precision of free-running circadian rhythms in DD (Bittman, 2012, 2014), several of our observations of responses to photic manipulations provide evidence of lability and can be used to gain insight into circadian organization. First, we found that more than half of the duper hamsters transferred from LL to DD at CT 18 experienced a striking loss of circadian rhythmicity. In Siberian hamsters, a 5-hour delay of an LD cycle with lights off at CT 17 can induce arrhythmicity or lead to an inability to re-entrain (Ruby et al., 1996). This state seems to reflect a compromise of pacemaker function (Grone et al., 2011). Thus, it seems possible that the duper mutation may disrupt the SCN function or block its output or that the integration of cellular rhythms may be altered in a way similar to that seen in constant bright light. To evaluate these possibilities, we used autocorrelation analysis to compare these records of duper hamsters showing ultradian rhythms after this transition

with those of SCN-lesioned *tau* mutant hamsters in DD (Bittman and Monecke, unpublished data) or of dupers in constant bright light (~300 lux; Bittman, 2014). SCN-lesioned *tau* hamsters displayed weak circadian rhythms or were arrhythmic but did not show ultradian wheel-running activity. Duper hamsters split or became arrhythmic when maintained in constant light but did not exhibit ultradian rhythms of locomotor behavior. We conclude that ultradian wheel-running patterns of duper hamsters when transferred from light to dark at CT 18 are a novel transition state rather than a suppression of SCN function.

A second indication of an effect of the duper mutation on the lability of the circadian system is our observation that a quarter of dupers split either immediately or within 10 days after a 3-hour dark pulse (Fig 2-4C). Splitting implies decoupling of circadian components, such as the morning and evening oscillators (Pittendrigh and Daan, 1976; Daan and Berde, 1978). Boulos and Rusak (1982) observed occasional splitting several days after administration of dark pulses to WT hamsters, but immediate splits have not been observed previously. The immediacy and frequency of splitting in the dupers, which we did not observe in any of the WTs, suggests that the duper mutation may weaken coupling of pacemaker components.

A third indication that duper destabilizes the circadian system is the high variability of phase shifts in response to dark pulses at CT 6 and 8 and at all LL to DD and DD to LL transitions. An effect of duper to reduce oscillator amplitude (Krug et al., 2011) may also explain increased variability in phase shifts. Exaggerated responses of core clock components downstream from the perturbation of *Per1* may contribute to or account for variability and lability of resetting.

Taken together, our results provide insight into the coupling strength and stability of the duper circadian system. We used simulations based on a mathematical model to test whether the behavior of duper mutants can be mimicked by manipulation of a coupling parameter. We found that reduction of coupling strength qualitatively captures the essential features of the duper behavioral phenotype: shortened period, reduced amplitude, type 0 photic PRC, increased range of entrainment, and large, variable shifts following LL to DD transitions at CT 18, sometimes with transient loss of circadian rhythms. While a simplified model cannot capture all details of hamster activity, the modeling does reproduce the features of interest in the duper phenotype through an overall decrease in coupling strength. Destabilization of the circadian pacemaker in dupers may be a consequence of reduced communication among the constituent components.

Although the genetic basis of the duper mutation is as yet unknown, our observations provide a basis on which to generate testable hypotheses. We cannot rule out the possibility that the duper mutation affects cell-autonomous properties, perhaps by altering expression of core clock genes. Nevertheless, our modeling suggests that a reduction of coupling between SCN neurons can parsimoniously explain the wide range of altered circadian dynamics observed in the duper mutant.

The retinorecipient core of the SCN is most directly affected by photic cues and thus may be the locus of changes that alter circadian function in duper mutant hamsters. Manipulation of VIP/ VPAC2r (Aton et al., 2005; Hughes and Piggins, 2008), GABA (Freeman et al., 2013), vasopressin receptors (Yamaguchi et al., 2013), BMAL1 (Ko et al., 2010), or CLOCK (Vitaterna et al., 2006; Shimomura et al., 2013) reduces oscillator

amplitude or cellular coupling. The decreased amplitude that is often a consequence of weakened coupling can drive some of the changes in dynamics, including type 0 PRCs. In various clock mutants, reduced amplitude of oscillations of gene expression has been observed to result in enhanced phase resetting. This may be explained theoretically using simple amplitude models (Lakin-Thomas et al., 1991; Vitaterna et al., 2006). Furthermore, coupling can directly affect circadian period. For instance, increased VIP activity in the SCN lengthens period (Aton et al., 2005; Pantazopoulos et al., 2010; Lucassen et al., 2012), and this finding can be reproduced by manipulating a VIP-like coupling mechanism in a model (Gonze et al., 2005).

We propose that the *duper* mutation affects a signaling pathway critical to normal coupling among neurons in the SCN. Further genetic studies to identify the location of the *duper* mutation, and thus elucidate the molecular clock mechanism, will test this prediction.

## CHAPTER 3

### ANATOMICAL AND IMMUNOCYTOCHEMICAL ANALYSIS OF EFFECTS OF THE DUPER MUTATION ON CLOCK PROTEINS IN THE SCN AND PVN

#### **Introduction**

Endogenous circadian rhythms are necessary for organisms to coordinate their physiology with the environment. Because the period of circadian rhythms deviates from 24 hours, organisms must phase shift (advance or delay) daily to entrain to the period of the environment. In mammals, phase shifts are initiated as light regulates transcription of *Per1/2* in order to reset the molecular clock.

The molecular clock is composed of transcription/translational feedback loops (TTFL) of core clock genes: *Clock*, *Bmal1*, *Per1/2*, and *Cry1/2* (Ko and Takahashi, 2006). CLOCK/BMAL1 form a heterodimer that binds to E-boxes in the promoters of *Per1/2* and *Cry1/2* to induce transcription. Once translated, PER and CRY form a heterodimer that, when phosphorylated, translocates to the nucleus and binds to the CLOCK/BMAL1 complex to inhibit its transcriptional activity (Reischl and Kramer, 2011, Harada et al., 2005; Lamia et al, 2009). In mice, there are 6 E-boxes in the *Per1* promoter, whereas hamsters have 8. A difference in non-canonical E-Boxes (CANNTG) between *Per1* and *Per2* leads to differential timing of expression in mice; *Per2* transcription lags that of *Per1* (Yamajuku et al., 2010). The duration to complete these loops is the period of the clock. Based on *in situ* hybridization experiments in rodents (including hamsters) and *in silico* models based on WT and mutant mice and U2-OS cells, transcription of *mPer1* is low during the night and peaks during mid-subjective day

(Yamamoto et al., 2001; Relógio et al., 2011; Hirota et al., 2012). In hamsters and *in silico* models, PER protein lags mRNA by approximately 4-6 hours and peaks in early subjective night (Maywood et al., 1999; Relógio et al., 2011; Hirota et al., 2012; John et al., 2014; Figure 3-1).

### The SCN's Response to Light

In mammals, light can phase shift the circadian clock by way of direct projections of the retinal-hypothalamic tract (RHT)

to the suprachiasmatic nucleus (SCN),

which is located in the hypothalamus

just above the optic chiasm. SCN

transplants have illustrated it as the

master pacemaker that determines the

period of the organism (Ralph et al.,

1990; Guo et al., 2006). The SCN

coordinates peripheral oscillators

through neural and humoral cues

(Lehman et al., 1987; Ralph et al., 1990;

Silver et al., 1996a; Guo et al., 2005;

Guo et al., 2006; Mohawk et al., 2012).

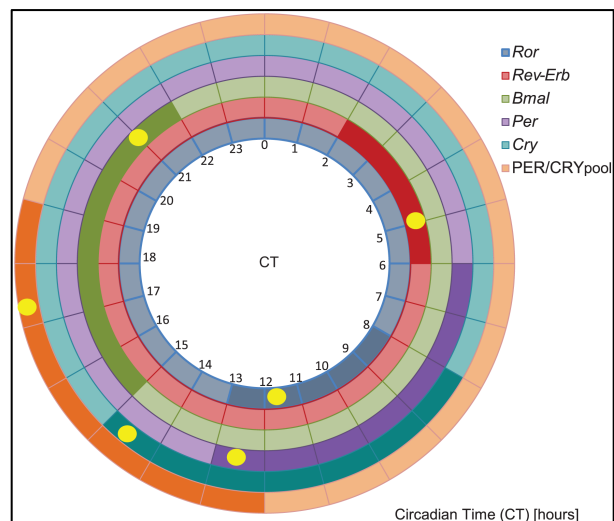


Figure 3-1. Diagram depicts clock gene RNA and protein expression patterns. The phase of expression, based on circadian time (CT) in hours, for 5 RNAs (*Ror*, blue; *Rev-Erb*, red; *Bmal*, green; *Per*, purple; *Cry*, teal) and nuclear pool of the PER/CRY protein heterodimer (orange). Dark tones indicate the phase of high expression based on published data. Yellow circles represent peak expression based on *in silico* experiments from Relógio et al. 2011. Figure taken from Relógio et al. 2011.

Retinal projections to the SCN co-release glutamate and pituitary adenylylate cyclase activating polypeptide (PACAP; Hannibal et al., 2000). PACAP modulates the SCN's response to light by enhancing glutamate induced phase shifts in early subjective night and suppressing phase shifts in late subjective night (Chen et al., 1999; Bergström

et al., 2003). PACAP activates receptors PACAP-R1 and -R2 to induce phase shifts through adenylate cyclase activation of cAMP and cAMP-independent  $\text{Ca}^{++}$  influx (Hannibal et al., 1997; Harrington et al., 1999; Kopp et al., 1999). Glutamate binds to multiple ionotropic receptors in the SCN including N-methyl-d-aspartate (NMDA; NMDA1), amino-methyl propionic acid (AMPA; GluR1, GluR2, and GluR4), and Kainate (KA) receptors (Gannon and Rea, 1994; Von Den Pol et al., 1994). Ionotropic receptor binding induces an influx of  $\text{Ca}^{++}$  that activates CaM Kinase II/IV (Schurov et al., 1999) and MAPK (Obrietan et al., 1998; Antoun et al., 2012). These kinases phosphorylate cAMP response element binding protein (CREB). Phosphorylated CREB acts as a transcription factor by binding to the cAMP response element (CRE) in the promoters of *Per1* and *Per2* (Ginty et al., 1993; Ding et al., 1997; von Gall et al., 1998; Tischkau et al., 2002; Travnickova-Bendova et al., 2002; Quintero et al., 2003; Sakamoto et al., 2013; Golombek and Rosenstein, 2010; O'Neill and Reddy, 2012; Hastings et al., 2014).

In hamsters, light exposure at CT15 or CT16 (early to mid subjective night) stimulates significant increases in *mPer1* within 30 mins (Yamamoto et al., 2001; Krug et al., 2011). Rapid induction of *Per1* in response to light has also been demonstrated in mice and rats (Shigeyoshi et al., 1997; Yan et al., 1999; Yan and Silver, 2002). *In vivo* inhibition of *Per1* induction in mice prevents light -induced phase shifts (Akiyama et al., 1999). Therefore, exposure to light during subjective night (when *Per1/2* levels are low) most likely resets the molecular clock by shifting the phase of *Per1/2* expression.

Although PER1 inhibition of CLOCK/BMAL transcriptional activity is mainly CRY dependent, *Cry1/2* do not have a CRE in the promoter region, and do not display an

immediate increase in mRNA in response to light. However, mCRY1 displays light induced increases several hours after a light pulse (Field et al., 2000). This suggests that PER-dependent nuclear accumulation of CRY may play a large role in light induced phase resetting (Lee et al., 2001; Reppert and Weaver, 2002). However, PER1/2 can also form homodimers, and biochemical experiments demonstrate that PER2 can directly bind to CLOCK/BMAL1 independent of CRY (Chen et al., 2009). In *Drosophila*, PER alone is sufficient to suppress CLK/CYC (homologs of CLOCK/BMAL; Nawathean and Rosbash, 2004). These studies suggest that light induced PER1/2 may have a small role as a transcriptional repressor independent of CRY.

Light also induces transcription of immediate early genes (IEGs) including *c-Fos* and *JunB*, in the SCN (Earnest et al., 1990; Rusak et al., 1990; Abe et al., 1991; Abe et al., 1992; Rusak et al., 1992; Schwartz et al., 1994; Wollnik et al., 1995; Vosko et al., 2015). Like *Per1/2*, the *c-Fos* promoter contains CREs (Sheng and Greenberg, 1990; Boutillier et al., 1992). C-FOS and JunB form heterodimers (activator proteins-1; AP-1) that bind to AP-1 sites (TGACTCA) and act as transcriptional regulators (Sheng and Greenberg, 1990). In humans, AP-1 sites are located in promoter regions for cytokines and oncogenes. Yet because AP-1 sites are so widely distributed throughout the genome, it is difficult to determine if they specifically regulate a certain types of genes (Zhou et al., 2005). FOS has also acts on enhancer elements to alter histone acetylation (Malik et al., 2014). This control of activity-dependent gene expression is critical for nervous system function (Malik et al., 2014) and may influence the timecourse and nuclear residence time of c-FOS. In the SCN, there are conflicting reports concerning the role of c-FOS in phase shifting. *c-Fos*<sup>-/-</sup> mice are still able to achieve phase shifts, indicating that



c-FOS is unnecessary (Honrado et al., 1996). However, in rats, blocking both *c-Fos* and *JunB* expression with antisense oligonucleotides inhibited light-induced phase shifts (Wollnik et al., 1995). This suggests that although c-FOS alone is not necessary for phase shifts, activation of AP-1 sites may play a significant role.

Immediate early genes are used as a marker for general neuronal activity because their transcription is induced by a wide variety of external stimuli, and they have a short half-life of 10-15 min (Bullitt, 1990; Sheng and Greenberg, 1990; Hoffman et al., 1993; Best et al., 1999). *Per1* expression in the SCN core peaks about 1 hour after light exposure and approximately 0.5 hours later in the shell (Hamada et al., 2004; Vosko et al., 2015). PER protein expression is delayed by approximately 4-6 hours (Maywood et al., 1999; Relógio et al., 2011). However, c-FOS protein expression peaks approximately 1 hour after exposure to light (Vosko et al., 2015). The short half-life and rapid expression of c-FOS allow for examination of the SCN's temporal response to light.

Post-translational events also play a large role in determining the period of an organism and the magnitude and speed of phase shifts. CK1 $\epsilon/\delta$  phosphorylate PER1/2 monomers to tag them for ubiquitination, as well as PER/CRY dimers to aid in nuclear translocation. The *tau* mutant hamster has a 20-hour period in homozygotes or a 22-hour period in heterozygotes in DD due to a gain of function of CK1 $\epsilon$  (Ralph and Menaker, 1988; Gallego et al., 2006; Meng et al., 2008). CK1 $\epsilon$  sets the period of the clock by regulating PER1/2 (Maywood et al., 2014). Despite the role of CK1 $\epsilon$  to set the period of the clock, *Ckl1 $\epsilon$ <sup>-/-</sup>* mice do not have a change of period (Pilorz et al., 2014). Mutations in *Drosophila* have illustrated that protein phosphatases (PP) act to lengthen (PP1) or shorten (PP2) period (Sathyanarayanan et al., 2004; Fang et al., 2007). In mammals, PP1

increases stability of PER2 by counteracting CK1 $\epsilon$  phosphorylation (Gallego et al., 2006), but the effect on the clock is less well understood. CK1 $\epsilon$  is also a substrate for PP5. Down-regulation of PP5 disrupts circadian rhythms (Partch et al., 2006). The *duper* mutation may affect post-translational regulation as it can modify both period and speed of re-entrainment.

### **Communication within the SCN and Peripheral Oscillators**

The hamster SCN is regionally divided into the ventrolateral “core,” which receives retinal input that controls oscillations of *Per1/2* and the dorsomedial “shell,” which maintains rhythmic oscillations independent of light and conveys phase to peripheral clocks (Hamada et al., 2004; Morin et al., 2007; Evans et al., 2015). Peptide distribution in the mouse SCN is not as clearly defined (Morin et al., 2007). Cell types of the SCN core include: gastrin-releasing peptide (GRP), calbindin (CalB), and vasoactive intestinal peptide (VIP; Iбата et al. 1989; Tanaka et al. 1997; Bryant et al. 2000; Kuhlman et al., 2003; Hamada et al., 2004; Morin, 2007; Yan et al., 2007). VIP acts as a coupling factor between neurons within the SCN and communicates retinal input to the shell, which only has sparse retinal input (Colwell et al, 2003; Piggins and Cutler, 2003; Hamada et al., 2004; Kriegsfeld et al., 2004; Aton et al., 2005; Aton and Herzog, 2005; Morin et al., 2007). The VIP receptor, VPAC2, is expressed throughout the SCN (Vertongen et al., 1997; Cagampang et al., 1998). *Vip*<sup>-/-</sup> mice either exhibit a decrease in the period of their locomotor rhythm (~23 hours) or are arrhythmic in DD (Colwell et al., 2003). Multi- and single- unit electrode recording of SCN slices from *Vip*<sup>-/-</sup> mice demonstrate that the SCN is arrhythmic (Brown et al. 2007). The arrhythmicity of the SCN is due to arrhythmicity of individual neurons (Brown et al., 2007), as well as a lack

of synchronization between oscillating neurons (Brown et al., 2007; Maywood et al., 2011). If a VPAC2 receptor antagonist is applied, the number of arrhythmic neurons is increased (Brown et al, 2007). This suggests that rhythmic locomotor activity in *Vip*<sup>-/-</sup> mice is due to VIP independent activation of VPAC2. *Vpac2*<sup>-/-</sup> mice are unable to entrain to a LD cycle and become arrhythmic in DD (Harmar et al, 2002; Colwell et al 2003; Maywood et al., 2006; Brown et al., 2007; Vosko et al., 2007). Overexpression of VPAC2 results in a shorter period in DD (~23.4 hours) and fewer transients in jet lag paradigms (Shen et al 2000). Additionally, the SCN shell projects to the SCN core, in which AVP may act as coupling factor (Morin et al., 2007; Maywood et al., 2011; Yamaguchi et al., 2013; Mieda et al., 2015).

The subparaventricular nucleus (sPVN) receives a dense efferent SCN projection (Saper et al., 2005). Retrograde and anterograde tracing in rats demonstrated that AVP and VIP neurons in the SCN project to the sPVN and PVN (Watts and Swanson, 1987; Watts et al., 1987). In mice and rats, the sPVN and PVN control the phase of locomotor and a portion of endocrine output respectively (Vrang et al., 1995; Buijs et al., 1996; Kalsbeek and Buijs, 2002; Vujovic et al., 2015). Additional projections from the SCN to the habenula regulate the temporal pattern of activity within a rest-activity cycle (Paul et al., 2011), and projections to the dorsal medial hypothalamus and the locus coeruleus likely affect wakefulness (Mahoney et al., 2013). Eliminating the SCN efferent outputs to the sPVN with knife cuts in rats abolished circadian oscillations in plasma levels prolactin and LH and locomotor behavior (Watts et al., 1989). In addition to neural projections, the SCN also communicates with other oscillators through humoral cues. Encapsulated SCN transplants in hamsters demonstrated that humoral signals from the

SCN are sufficient to restore locomotor rhythms but are insufficient to restore rhythms of reproductive responsiveness, melatonin, and endocrine systems (Lehman et al., 1987; Silver et al., 1996a; Meyer-Bernstein et al., 1999).

### **The Duper Mutation**

The recently discovered duper mutant hamster has a free running period of ~23 hours in DD and high-amplitude phase resetting (type 0 phase response curve): in response to a 15-min light pulse during early or late subjective day, duper mutants exhibit phase shifts of 4-12 hours, compared to WT, which shift 1-3 hours (Krug et al., 2011; Manoogian et al., 2015). However, novel wheel access did not induce larger phase shifts in duper hamsters than in WT (Manoogian et al., 2015). Previous studies analyzing the “super duper” mutant hamster (duper mutation on a *tau* mutant background) found that there were no differences in *Per1* induction 30 mins and 2 hours after a light pulse in WT and super duper hamsters (Krug et al., 2011). However, albumin site D-binding protein (*Dbp*), a PAR bZIP transcription factor that regulates many clock controlled genes and acts as a transcription factor for *Per1* (Ripperger et al., 2000), had higher basal levels in super duper mutants and increased expression at both time points after a light pulse compared to WT hamsters (Krug et al., 2011). I set out to examine PER protein levels, which are critical given that post-transcriptional and post-translational regulators modify circadian period (Xu et al., 2005; Godinho et al., 2007; Siepka et al., 2007; Meng et al., 2008; Etchegaray et al., 2009).

The object of these experiments is to test the hypothesis that exaggerated phase shifts in duper hamsters are due to increased induction or prolonged expression of PER1 in retinorecipient (VIP positive) cells. Alternatively, the duper mutation may affect

signaling between the retinorecipient cells in the ventrolateral SCN and the dorsal SCN or between the dorsal SCN and extra-SCN oscillators. The duper mutation may also affect molecular clock processes that occur after the initial PER1 induction by light. Given the behavioral effects of the mutation, I set out to explore whether duper influences light induction of early molecular steps in the SCN that lead to phase shifts.

Co-expression analysis of PER1 with VIP and AVP allows us to investigate regional and cell phenotype specific differences in PER1 expression. If the mutation alters SCN and extra-SCN communication, rather than photic input, I predict, in hamsters that receive a light pulse in early subjective night, PER1 in VIP positive cells will resemble expression in WTs and regions distal to the SCN core (SCN shell, sPVN, and PVN) will have delayed expression of PER1. Combining temporal and spatial analysis of PER1 allowed me to investigate how the duper mutation alters the molecular clock to achieve large phase shifts in response to light.

## **Materials and Methods**

### **Animal maintenance**

Male and female Syrian hamsters (*Mesocricetus auratus*) were group housed in a 14:10 LD cycle (~150 lux) until they reached adulthood (3-6 months). Ad libitum food and water was provided and highly absorbent bedding (Bed-o-cob, Maumee, OH) was used at all times. A total of 124 hamsters were used.

### **Experimental Design**

As adults, hamsters were individually housed and provided a running wheel (17.5 cm), as previously described (Krug et al., 2011). Hamsters were then moved to DD to be phenotyped by assessing free running period and response to a 15-min light pulse at

CT15. Animals remained in DD for 10-12 days before and after the pulse in order to measure the phase shift before being transferred back to LD for 10-12 days. After duper mutants were identified, they returned to DD and on the 10<sup>th</sup>-12<sup>th</sup> day, duper and WT hamsters were sacrificed at CT12 or CT15 or received either a light pulse or control cage handling at CT 15 and tissue was collected 1, 2, 3, 6, and 9 hours later (5-6 hamsters per group).

### **Tissue collection**

Hamsters were anesthetized with 0.3 ml of sodium pentobarbital (80mg/kg) and perfused with 100ml of 0.1M PB followed by 300ml of 4% paraformaldehyde. Post-perfusion, the brain was extracted. Brains were soaked in 4% paraformaldehyde overnight and then infiltrated in 20% sucrose in 0.1M PB at 4° C (~2 days). They were then sliced 40um thick on a freezing microtome. Tissue was collected in a 1 in 4 series and stored in cryoprotectant at -20° until stained.

### **Antibodies**

Double label immunocytochemistry (ICC) was used to detect VIP and PER1 protein and triple label to detect PER1, c-FOS, and AVP associated Neurophysin (AVP-NP). Primary antibodies included: VIP made in guinea pig (1:5000; Peninsula Labs, San Carlos, CA, T-5030); PER1 R43 made in rabbit (1:5000; from David R. Weaver; LeSauter et al., 2012); c-FOS made in goat (1:1000; Santa Cruz Biotech, Dallas, TX, sc-52-G); AVP associated Neurophysin P45 made in mouse (1:50; Gainer lab, ATC CRL 1798). Secondary antibodies were Alexa Fluor 488-conjugated AffiniPure Donkey anti Guinea Pig (DAGP; 1:300; Jackson Immuno Research, West Grove, PA, 706-545-148); Cy3 Donkey anti-Rabbit (DAR; 1:500; Jackson Immuno Research, 711-165-152); Alexa

Fluor 488 Donkey anti-Goat (DAG; 1:300; Jackson Immuno Research, 705-545-003); Cy5 Donkey anti-mouse (DAM; 1:300; Jackson Immuno Research, 715-175-150). NP is used as a marker for AVP because it is co-produced and NP as the carrier protein for AVP (Schmale et al., 1983). Additional ICC studies in rats have verified that Neurophysin and AVP are co-expressed in the SCN (Vandesande et al., 1975). Chemicon and ICN AVP antibodies were not used because they did not clearly stain cells in the SCN. Additionally, because both Chemicon and ICN antibodies were made in rabbit, we could not use them in combination with the PER1 R43 antibody.

### **Immunocytochemistry (ICC)**

4-6 sections containing mid-SCN, caudal-SCN, and PVN were chosen for each hamster. Only mid-SCN was analyzed for the PER1-VIP double label, as VIP cells were not located in caudal SCN or PVN. Due to the large number of animals, each ICC experiment was split into two runs, each with half of the animals from each group. On day one, sections were rinsed 4x (5 min each) in 0.1M Phosphate Buffer Solution (PBS), blocked for 1-hour in PBS+ comprised of 0.4% Triton X-100 (Electrophoresis grade; Fisher Scientific, Pittsburg, PA) and 0.1% Bovine Serum Albumin, Factor V (Sigma, St. Louis, MO) in 0.1M PBS, and placed in the first primary antibody to incubate overnight (17-18 hours at room temperature on a rocker). All antibodies (ab) and fluorophores were diluted in PBS+. On day two, sections were rinsed in 0.1M PBS 4x (5 min each) and then incubated in the first secondary ab for 2-hours at room temperature on a rocker. Sections were rinsed again and incubated in the second primary ab overnight. This process was repeated until the double or triple label ICC was complete. After the incubation in the last secondary ab, tissue was rinsed 4x in 0.1M PB and mounted onto subbed slides. When

dry, slides were cover-slipped using Aqua-Poly (Polysciences, Warrington, PA, 18606-20).

### **Image analysis**

Images (snap shots and z-stacks) were taken at 10 and 20X using a Zeiss 700 confocal microscope. FIJI (Fiji Is Just ImageJ; <http://fiji.sc/Fiji>; Schindelin et al., 2012) NIH imaging software was used for all image analysis. A researcher who was blind to the genotype and manipulation manually identified co-labeled PER1-VIP and PER1 cells counts. PER1 cell counts were replicated with FIJI 3D automated cell. c-FOS cells counts were also automated. For manually identified co-labeled PER1-VIP cells, the intensity of PER1 was determined by automated identification. Automated processing of PER1 and c-FOS was performed as follows: (1) “Gaussian blur 3D” to smooth cells, (2) “subtract background” (rolling ball, 50 pixels): local background is determine for every pixel by averaging the 50 pixels around that pixel, which is then subtracted, (3) “threshold” more than 2 standard deviation above background (threshold=99.54%), (4) “watershed” to separate touching objects, (5) “erode” to removes pixels from the edges of objects (helps separate cells), and (6) “dilate” to add pixels to the edges of objects. Steps 4-6 were used to help differentiated individual cells that were near each other. The density of VIP and AVP-NP fibers precluded use of an automated cell counter. VIP networks were manually identified and intensity of VIP and PER1 staining were measured using FIJI. Sections were categorized as mid-SCN, caudal SCN, or PVN based on expression of VIP and AVP fibers distribution (Fig 3-2A,B). Ventral and dorsal SCN were determined by separating the SCN halfway between the rostral and caudal boarders as defined by VIP or



AVP fibers (Fig 3-2D). Rostral-SCN sections were not used, as they did not contain VIP or AVP cell bodies.

### **Statistical Analysis**

Due to the small n value (4-7 animals/group) and variation within groups, it is difficult to have enough power for a variety of statistical analyses. Small n-values are typical in neuroimaging studies in mammals (Maywood et al., 1996; Yamamoto et al., 2001; Kriegsfeld et al., 2003; Krug et al., 2011; Lokshin et al., 2015). Although analysis of variance (ANOVA), is often performed in such studies, the assumption of normal distribution of data, is not satisfied. Accordingly, I used the Kruskal-Wallis test, a non-parametric one-way analysis of variance that does not assume a normal distribution, to analyze differences between genotype for each manipulation and effects of light at each time point. The Bonferroni correction was used for multiple comparisons (within individual analyses). The Bonferroni correction for comparison of the effects of light within genotype set the threshold  $\alpha=0.0125$ , and for effects of genotype at each time point at  $\alpha=0.0045$ . As most findings were not significant after these corrections, results are reported as significant if  $p<0.05$  and corrected  $\alpha$ -values are noted.

### **Results**

As expected, PER1-ir was distributed throughout the SCN and PVN. In hamsters that received a light pulse, c-FOS-ir cells were found throughout the SCN and PVN, but were concentrated in the ventral core of the SCN (Fig 3-2A,B). The precise distribution of PER1-ir and c-FOS-ir cells depends upon the manipulation and phase in which the animals was sacrificed. VIP cell bodies were only identified in ventral mid-SCN. However VIP fibers were densely distributed throughout mid-SCN (Fig 3-2A). In caudal-

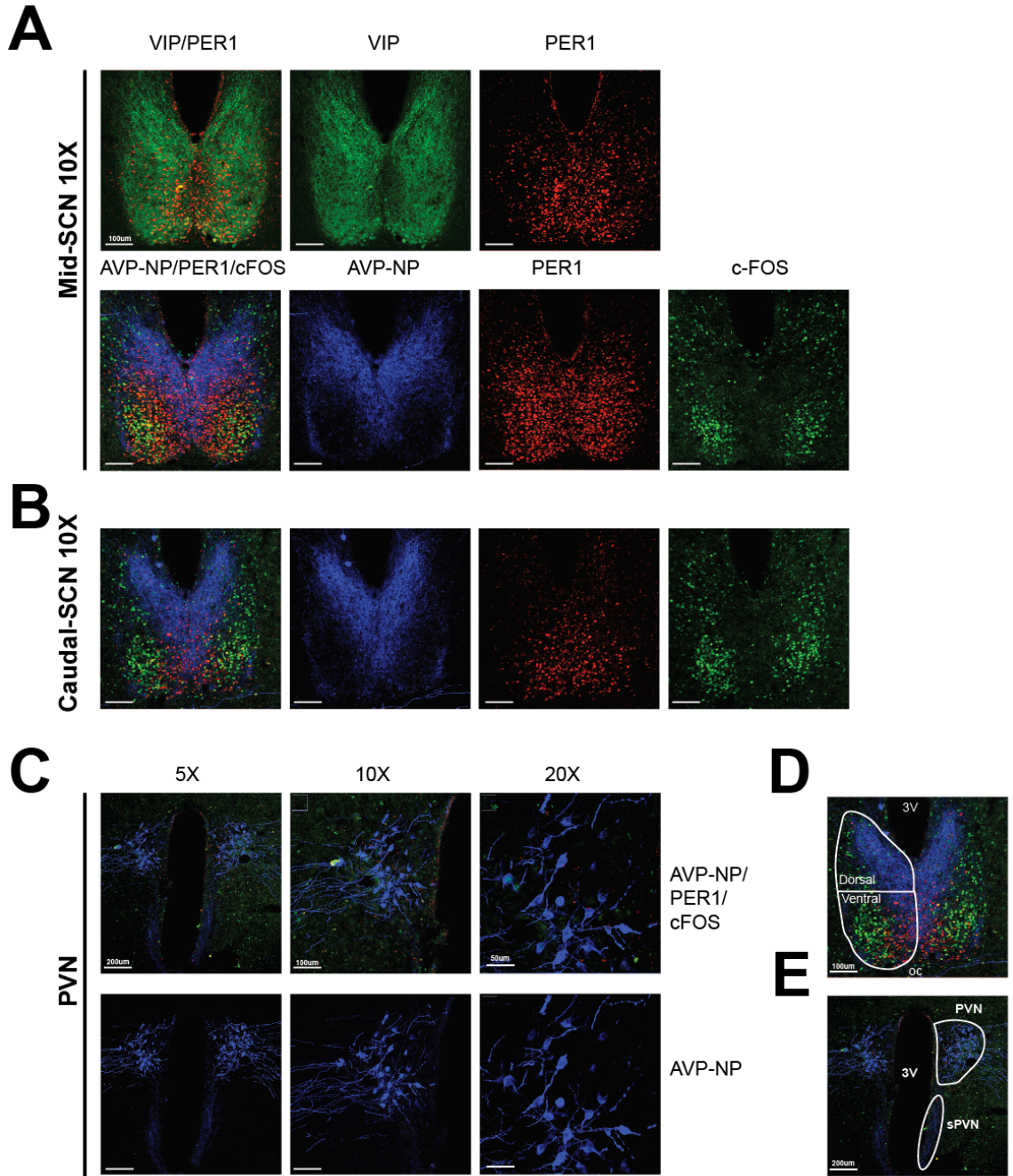


Figure 3-2. Representative images of mid-SCN, caudal-SCN, and PVN sections from a WT hamster 1 hour after a light pulse at CT15. (A) Mid-SCN: 10X images of double label VIP (green, Alexafluor 488) and PER1 (red, Cy3; top) and triple label for AVP-NP (blue, Cy5), PER1 (red, Cy3), and c-FOS (green, Alexafluor 488). (B) 10 X images of caudal-SCN triple labeled for AVP-NP, PER1, and c-FOS. (A,B) Composite (left) and individual labels are shown (right). (C) PVN triple labeled for AVP-NP, PER1, and c-FOS. Composite images (top), AVP-NP only (bottom); (left) 10X zoomed out PVN, (middle) 10X right PVN, (right) 20X PVN. (D) 10X caudal-SCN defining regions of ventral and dorsal SCN. (E) 10X zoomed out representative image depicting definition of PVN and sPVN.

SCN, VIP fibers were identified in dorsal, but not ventral SCN, and few or no VIP-ir cells were present. AVP-NP-ir cells were located in the dorsal SCN shell and PVN (Fig

3-2). There was a dense AVP-NP fiber network in the SCN shell with projections to the ventral SCN, and though the sPVN and PVN. Fewer AVP-NP fibers were present in the PVN (Fig 3-2).

### **Mid-SCN: Effects of Light**

c-FOS-immunoreactive (ir) and PER1-ir cell counts were increased in response to light in both duper and WT hamsters (Fig 3-3). The number of c-FOS-ir cells was significantly higher 1 and 3 hours after a light pulse compared to controls, in dupers (CT15 +1,  $p < 0.02$ ; CT15+3,  $p < 0.03$ ; corrected  $\alpha = 0.0125$ ) and WTs (CT15 +1,  $p < 0.01$ ; CT15+3,  $p < 0.01$ ; corrected  $\alpha = 0.0125$ ; Fig 3-3A). At 6 hours after a light pulse, there was a significant increase in the number of PER1-ir cells compared to controls in WT hamsters ( $p < 0.02$ ; corrected  $\alpha = 0.0125$ ), but it was not significant in duper hamsters ( $p < 0.13$ ; corrected  $\alpha = 0.0125$ ; Fig 3-3C). Duper and WT hamsters displayed increases in PER1-ir cell counts in response to light 9 hours after light exposure (duper,  $p < 0.02$ ; WT,  $p < 0.04$ ; corrected  $\alpha = 0.0125$ ; Fig 3-3C).

The number of VIP-ir cells was not affected by light (Fig 3-4C). The total number and percentage of VIP cells co-labeled with PER1 was significantly increased 6 hours after a light pulse compared to controls in dupers and WTs ( $p < 0.02$  and  $p < 0.03$ , respectively; corrected  $\alpha = 0.0125$ ; Fig 3-4D). 9 hours after a light pulse, duper hamsters also displayed a greater percentage of VIP cells co-labeled with PER1 compared to controls ( $p < 0.01$ ; corrected  $\alpha = 0.0125$ ), but WT hamsters did not ( $p < 0.09$ ; corrected  $\alpha = 0.0125$ ; Fig 3-4D). There was no effect of light on mean intensity of PER1-ir cells in the SCN, or in PER1-ir cells co-labeled with VIP (Fig 3-5A,B).

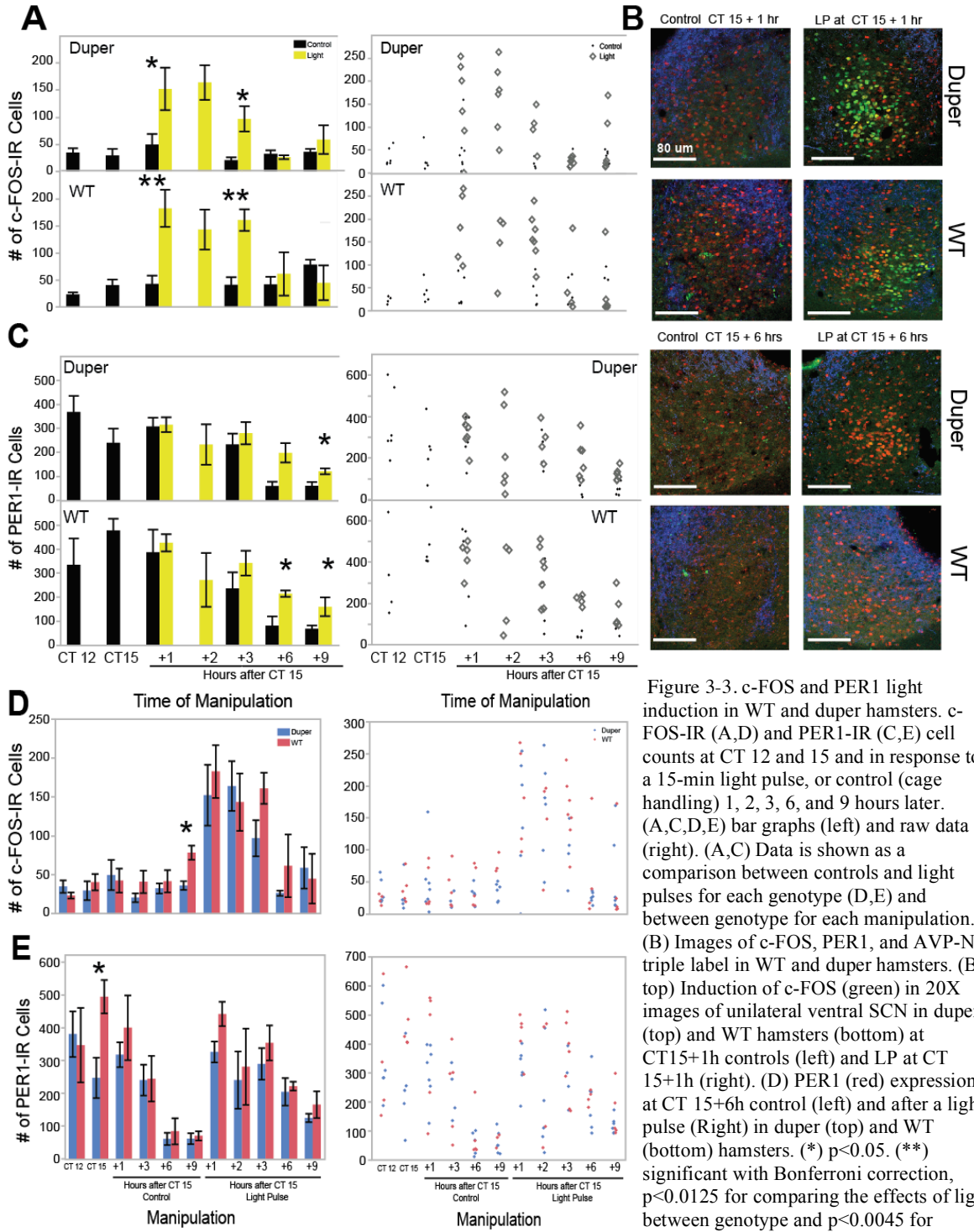


Figure 3-3. c-FOS and PER1 light induction in WT and duper hamsters. c-FOS-IR (A,D) and PER1-IR (C,E) cell counts at CT 12 and 15 and in response to a 15-min light pulse, or control (cage handling) 1, 2, 3, 6, and 9 hours later. (A,C,D,E) bar graphs (left) and raw data (right). (A,C) Data is shown as a comparison between controls and light pulses for each genotype (D,E) and between genotype for each manipulation. (B) Images of c-FOS, PER1, and AVP-NP triple label in WT and duper hamsters. (B, top) Induction of c-FOS (green) in 20X images of unilateral ventral SCN in duper (top) and WT hamsters (bottom) at CT15+1h controls (left) and LP at CT 15+1h (right). (D) PER1 (red) expression at CT 15+6h control (left) and after a light pulse (Right) in duper (top) and WT (bottom) hamsters. (\*)  $p < 0.05$ . (\*\*) significant with Bonferroni correction,  $p < 0.0125$  for comparing the effects of light between genotype and  $p < 0.0045$  for comparing genotype at each time point.

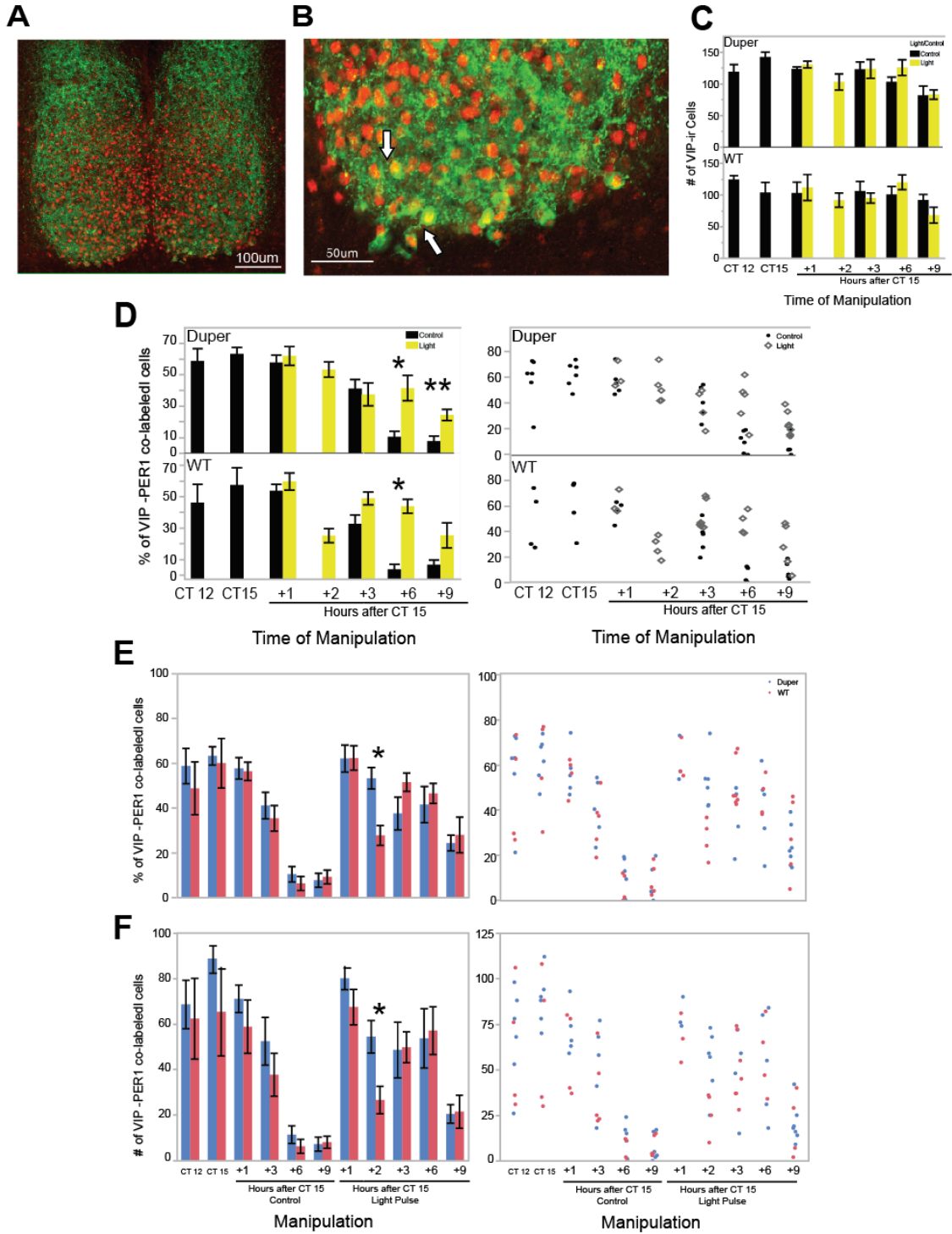


Figure 3-4. PER1-ir expression in VIP-ir cells increases 6 hours after a light pulse in early subjective night. (A) 10X image of mid-SCN labeled for VIP (green) and PER1 (red) from a WT hamster at CT 15. (B) 20X image of SCN from (A-left), arrows point to two examples of VIP cells co-labeled with PER1. (C) Bar graph of number of VIP-ir cells. (D and E) Bar graph (left) and raw data (right) representing the % of VIP cells co-labeled with PER1. Data are compared between light exposed and control groups within genotype (D) and between genotype at for each manipulation (E). (F) Bar graph (left) and raw data (right) of number of VIP cells co-labeled with PER1. (\*)  $p < 0.05$ . (\*\*) significant with Bonferroni correction,  $p < 0.0125$  for comparing the effects of light between genotype and  $p < 0.0045$  for comparing genotype at each time point.

### **Mid-SCN: Effects of Genotype**

The number of c-FOS-ir and PER-ir cells did not differ between dupers and WTs in response to light. However, WT hamsters displayed more c-FOS-ir cells 9 hours after CT15 in control animals ( $p < 0.03$ ; corrected  $\alpha = 0.0045$ ; Fig 3-3E,F). WTs also exhibited more PER1-ir cells at CT15 ( $p < 0.04$ ; corrected  $\alpha = 0.0045$ ). The percent of VIP cells co-labeled with PER1 was significantly higher in duper than in WT hamsters 2 hours after a light pulse ( $p < 0.01$ ; corrected  $\alpha = 0.0045$ ; Fig 3-4E,F). There was no effect of genotype on mean intensity of PER1-ir cells within the SCN or co-labeled with VIP (Fig 3-5C).

### **Caudal-SCN**

Analysis of caudal-SCN was subdivided to ventral and dorsal regions (Fig 3-2) because PER1-ir and c-FOS-ir expression differed between these regions at specific time points. Mid-SCN analysis was not subdivided as ventral and dorsal because no differences were observed between regions.

### **Caudal-SCN: Effects of Light**

c-FOS-ir and PER1-ir cell counts in the ventral region of caudal SCN sections were increased in response to light in both duper and WT hamsters in the same manner as mid-SCN. The number of c-FOS-IR cells was significantly greater in animals that received a light pulse than in controls 1 and 3 hours after the pulse in dupers (CT15 +1,  $p < 0.01$ ; CT15+3,  $p < 0.01$ ) and WTs (CT15 +1,  $p < 0.02$ ; CT15+3,  $p < 0.04$ ; corrected  $\alpha = 0.0125$ ; Fig 3-6B). PER1-ir cell counts were increased in response to light in both duper and WT hamsters 6 (duper,  $p < 0.01$ ; WT,  $p < 0.01$ ) and 9 (duper,  $p < 0.002$ ; WT,  $p < 0.03$ ; corrected  $\alpha = 0.0125$ ) hours after a pulse (Fig 3-6D). WT hamsters, but not dupers,

also exhibited higher PER1-ir counts 3 hours after the light pulse at CT15 ( $p < 0.04$ ; corrected  $\alpha = 0.0125$  Fig 3-6D).

In the dorsal region, dupers showed a significant increase in c-FOS-ir cells 1 hour after a light pulse compared to controls ( $p < 0.03$ ), but differences were not significant 3

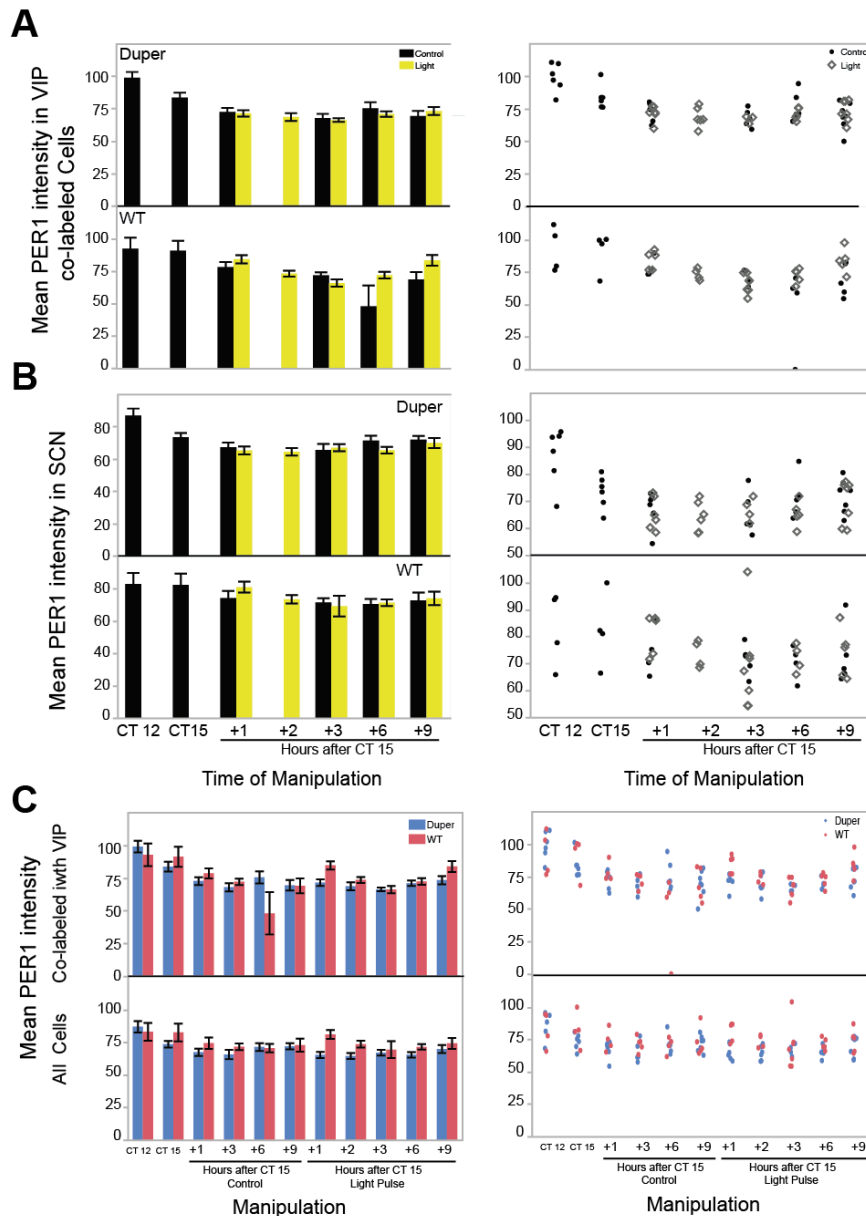


Figure 3-5. Intensity of PER1-ir cells is not affected by genotype or light. (A and B) Bar graph (left) and raw data (right) of mean PER1-ir intensity (arbitrary units, au) in cells co-labeled with VIP (A) and all cells in the SCN (B) compared between light exposed and control groups within genotype. (C) Bar graph (left) and raw data (right) of mean intensity of PER1-ir cells co-labeled with VIP (top) and all PER1 cells in the SCN (bottom) between genotype at for each manipulation.



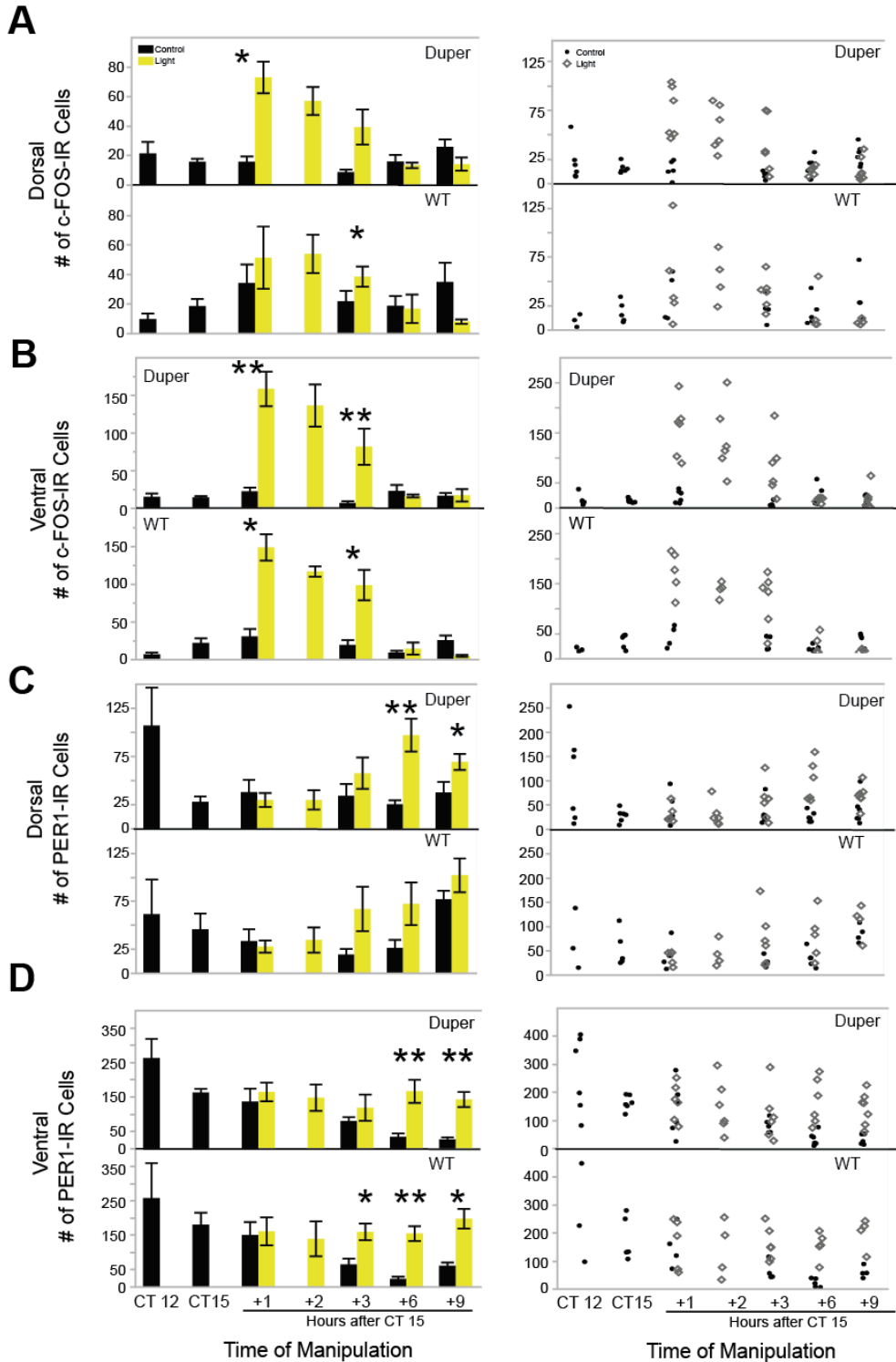


Figure 3-6. PER1-ir expression lags expression of c-FOS-ir in caudal SCN. Results from light exposed and control groups are plotted within genotype. Number of c-FOS-ir (A,B) and PER1-ir (C,D) cells at CT12, 15, and 1,2,3,6, and 9 hours after a light pulse or control at CT 15 in ventral (A,C) and dorsal (B,D) SCN. Data are represented in bar graphs (left) and raw data points (right). (\*)  $p < 0.05$ . (\*\*) significant with Bonferroni correction,  $p < 0.0125$  for comparing the effects of light between genotype and  $p < 0.0045$  for comparing genotype at each time point.



hours after a light pulse ( $p < 0.06$ ; corrected  $\alpha = 0.0125$ ; Fig 3-6A). In WT hamsters the number of c-FOS-ir cells did 1 hour after a light pulse did not differ from controls ( $p < 0.09$ ; corrected  $\alpha = 0.0125$ ) but there was a significant increase 3 hours after a light pulse at CT15 ( $p < 0.02$ ; corrected  $\alpha = 0.0125$ ; Fig 3-6A). PER1-ir cell counts were significantly greater in duper hamsters 6 and 9 hours after the light pulse than in controls ( $p < 0.01$  and  $p < 0.04$  respectively; corrected  $\alpha = 0.0125$ ; Fig 3-6D). WT hamsters did not display a significant change in PER1-ir cell counts in dorsal region of caudal SCN in response to light (Fig 3-6D).

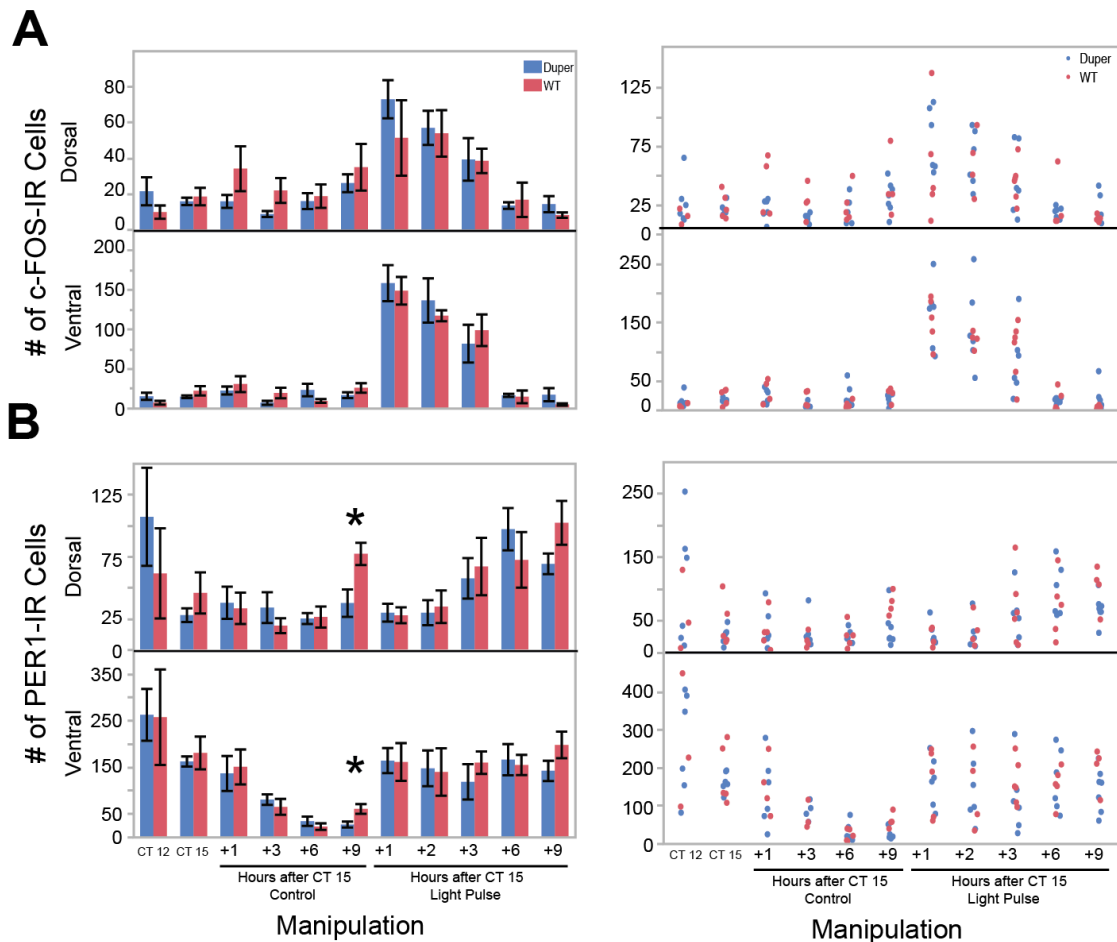


Figure 3-7. c-FOS-ir and PER1-ir expression show no difference between genotype in response to light in caudal SCN. Data is compared between genotype for each manipulation. Number of c-FOS-ir (A) and PER1-ir (B) cells at CT12, 15, and 1,2,3,6, and 9 hours after a light pulse or control at CT 15 in ventral (bottom) and dorsal (top) SCN. Data is represented in bar graphs (left) and raw data points (right). WT hamsters show a greater number of PER1-ir cells than dupers 9-hours after CT15 (controls, B). (\*)  $p < 0.05$ .

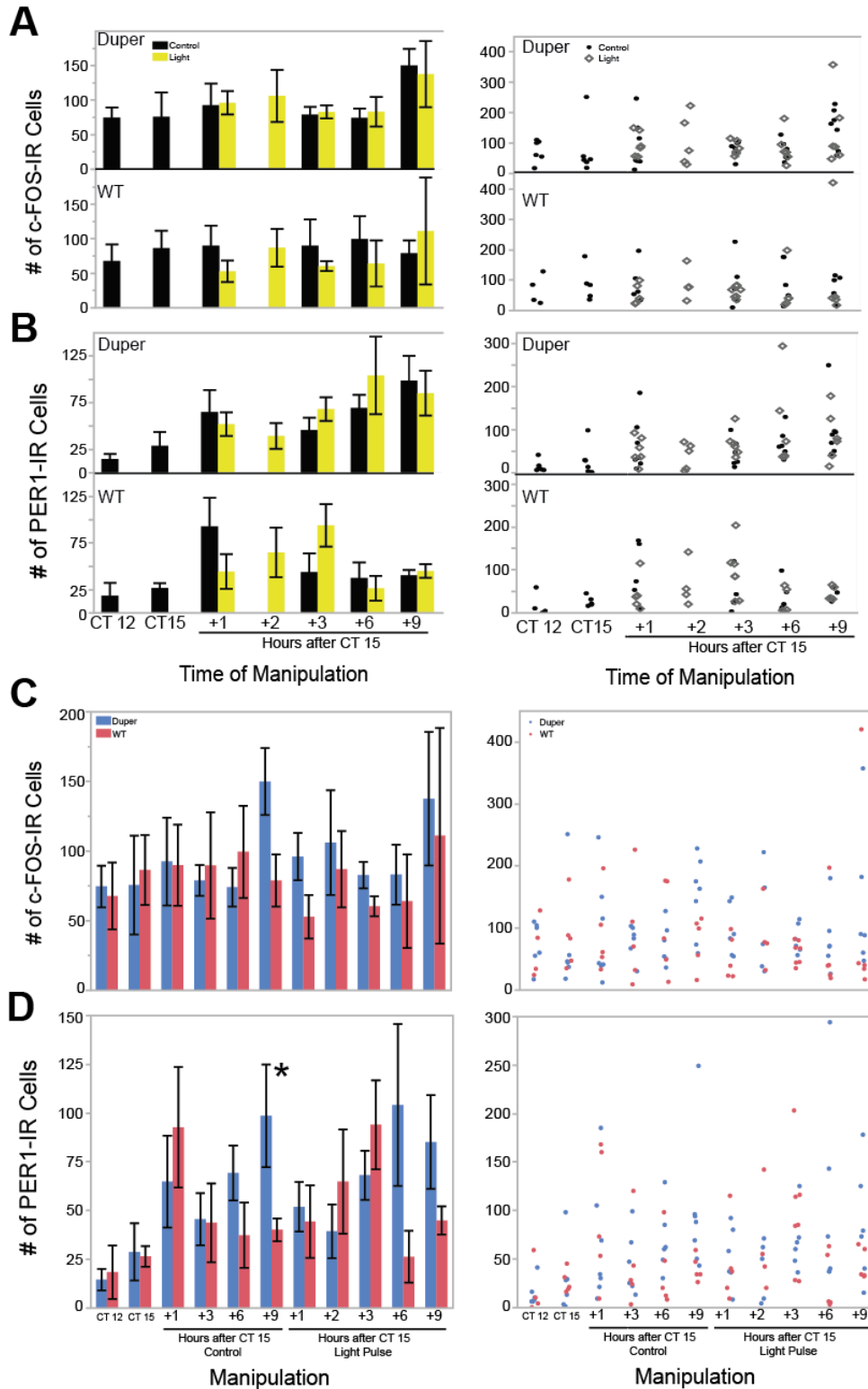


Figure 3-8. c-FOS-ir and PER1-ir expression show no response to light in the PVN. Data are compared between light exposed and control groups within genotype (A,B) and between genotype at for each manipulation (C,D). Number of c-FOS-ir (A,C) and PER1-ir (B,D) cells at CT12, 15, and 1,2,3,6, and 9 hours after a light pulse or control at CT 15 in PVN. Data are represented in bar graphs (left) and raw data points (right). (\*)  $p < 0.05$ .

### **Caudal-SCN: Effects of Genotype**

There were no differences between duper and WT in the number c-FOS-ir cells in ventral or dorsal regions of caudal SCN at any time point (Fig 3-7A). PER1-ir cell counts differed between duper and WT controls only 9 hours after CT15, in both ventral ( $p < 0.03$ ) and dorsal ( $p < 0.05$ ) regions of caudal SCN (corrected  $\alpha = 0.0045$ ; Fig 3-7B).

### **PVN: Effects of Light**

There were no differences in c-FOS-ir or PER1-ir cell counts between control and light pulsed animals for dupers or WTs in the PVN (Fig 3-8A,B).

### **PVN: Effects of Genotype**

The number of c-FOS-ir cells was not significantly different between duper and WT hamsters at any time point. However, at 9 hours after CT 15 (control) duper hamsters displayed a trend towards a greater number of c-FOS-ir cells than WTs ( $p < 0.07$ ; corrected  $\alpha = 0.0045$ ; Fig 3-8C). PER1-ir cell counts were significantly greater in duper than in WT hamsters 9 hours after CT15 (control;  $p < 0.02$ ; corrected  $\alpha = 0.0045$ ).

### **Discussion**

In response to a light pulse at CT15, PER1 expression in mid- and caudal-SCN lags c-FOS by approximately 3-5 hours in duper and WT hamsters. To our knowledge, this has not previously been reported. Previous studies have illustrated that *Per1* mRNA peaks 1 hour after a light pulse in early subjective night and begins to decrease 2 hours following the pulse (Yamamoto et al., 2001; Krug et al., 2011). I observed increases in c-FOS 1 and 3 hours after a light pulse, but no increase in the number of PER1-ir cells until 6 and 9 hours after a light pulse for either duper or WT hamsters in the SCN Fig 3-(3-7)). This difference in mRNA and protein levels seen for PER1 but not c-FOS, supports

previous findings that there are important post-transcriptional and post-translational regulators for *Per1* (Cao et al., 2015). It is possible that c-FOS protein is more rapidly expressed than PER1 due to a difference in size and complexity of post-transcriptional processing. In mice, c-FOS is a 380 amino acid (aa) protein, whose gene has 4 exons. In contrast, PER1 is 1291aa in length, and comprised of 25 exons. Because PER1 is approximately 4x the size of c-FOS, and comprised of 6x the exons, the temporal difference in protein expression may be due to additional time needed for transcriptional and translational processing of PER1. In mice, a 30-min light pulse at CT16 induced an increase in PER1 4 hours after the pulse. PER1 levels remained significantly greater in light pulsed animals compared to controls at CT6 and 8, yet these levels were much lower than PER1 levels at CT4 (Yan and Silver, 2004). As PER1 expression in response to light has not been previously described in hamsters, more frequent time points are needed to increase precision of analysis.

In the mid-SCN, there were no differences between WT and duper hamsters in the number c-FOS-ir or PER1-ir cells in response to light. As light induces transcription of *c-Fos* and *Per1* through CRE binding, similar numbers of c-FOS-ir cells between genotypes indicates that the duper mutation does not directly affect retinal input to the SCN and/or the early phases of the SCN's processing of the light stimulus. There were also no differences in PER1-ir cell count between dupers and WTs. This is consistent with *in situ* hybridization studies in super duper and WT hamsters that reported similar induction of *Per1* mRNA by a light pulse at CT 15 (Krug et al., 2011).

This study is the first to characterize the pattern of PER1 protein in VIP cells. PER1-ir expression in VIP cells showed similar results to overall PER1-ir cell counts.

This indicates that light had the same effect on the retinorecipient VIP cells in the SCN in both duper and WT hamsters. However, at 2 hours after the light pulse, the percentage of VIP cells co-labeled with PER1 was significantly higher in duper compared to WT hamsters. Interestingly, there were no differences in the number of PER1-ir cells between duper and WT hamsters at 1- or 3 hours after the light pulse, or in total number of PER1-ir cells (Fig 3-4). The difference in VIP and PER1 co-labeled cells 2 hours after the pulses seems to be caused by a decrease in WTs opposed to an increase in duper hamsters. This finding is difficult to interpret and is further complicated by the lack of control group at that time point. It is possible that possible that PER1 in VIP cells is expressed in 2-waves in WTs, whereas dupers display a sustained response. Sustained PER1 in duper hamsters could be due to changes in retinal response in VIP cells through promoter or enhancer binding. For example, phosphorylated CREB and/or IEGs may have an increased binding affinity for CRE and/or AP-1 sites, respectively, in the *Per1* promoter. Post-translational events could also explain increased stability of PER1. It is also important to consider that neither duper or WT hamsters display a significant increase in the number of PER1-ir cells (specifically in VIP cells, or the SCN) in response to light compared to controls until 6 hours after the light pulse. Therefore, it is possible that the difference between WT and duper hamsters in VIP cells co-labeled with PER1 2 hours after a light pulse may be independent of light. Further experiments assessing PER1 in VIP cells in control animals 2 hours after CT15 are necessary to determine if this effect is light dependent.

In the dorsal region of caudal-SCN, WT hamsters had significantly more PER1-ir cells than duper hamsters 9 hours after CT 15 in control groups (Fig 3-6, 3-7). However,

in the PVN this was reversed: duper hamsters exhibited a greater number of PER1-ir cells than WT (Fig 3-8D). These results suggest that the duper mutation may affect extra-SCN signaling. Both AVP and VIP project to the sPVN and the PVN. Additionally, overexpression of VPAC2 can lead to rapid phase resetting (Shen et al., 2000), which is also observed in *V1a/V1b*<sup>-/-</sup> mice (Yamaguchi et al., 2013), and duper hamsters (chapter 4). VPAC2 overexpression mutant mice also exhibit a decrease in period (~23.6 hours; Shen et al., 2000). Taken together, this suggests that the duper mutation may enhance signaling to extra-SCN regions that control locomotor output (sPVN) to decrease the period of locomotor rhythms and rapid and large phase shifts. However, due to the low number of samples and high variability, replicating these results, as well as collecting additional time points during subjective day could help clarify these findings.

c-FOS-ir and PER1-ir cell counts in the PVN were significantly affected by light in duper or WT hamsters. However, 2 of 6 of duper hamsters also displayed more PER1-ir cells at 6 and 9 hours after a light pulse at CT 15 compared to WT in the PVN. As the PVN does not receive as many projections from the retina as the SCN (Youngstrom et al., 1991), it is possible that changes in c-FOS and PER1 expression in response to light are delayed in the PVN. Additional time points (10-15 hours) after a light pulse would be needed to answer this question.,

It is also important to note that because duper hamsters have an ~23 hour period, each circadian hour is about 57.5 min in length. Thus 9 hours after CT 15, duper and WT hamsters are in a slightly different phase (dupers ~ CT 24.5, WT ~CT 24). This is not a large difference in phase, but it may affect the elevated number of PER1-ir cells in dupers 9 hours after CT 15 in controls. It is more likely that there is difference in phase angle

between WTs and dupers (between onset of activity and peak of PER1, or in the relative timing of different molecular events such as peak vs trough of PER1) that the current time sampling rate is too coarse to detect. Although using 60-minute hours does create minor differences in phase, this approach facilitates a direct comparison of the time course of protein accumulation and decay.

Although this study did not identify expected differences between duper and WT hamsters in response to light, dupers displayed a significantly greater percentage of VIP cells co-labeled with PER1 compared to WTs 2 hours a light pulse at CT 15. This increase of PER1 in duper hamsters supports the second alternative hypothesis, that the duper mutation affects molecular clock mechanisms that occur after light has induced the synthesis of PER1. We cannot completely rule out the first hypothesis, as we need additional time points to accurately assess the duration of PER1 induction by light. However, we can rule out the idea that light induces a greater magnitude of PER1 in response to light, as the peak number of PER1-ir cell count and mean intensity did not differ between duper and WT hamsters. There were also no differences between WT and duper in the number of c-FOS-ir cells in response to light. Additionally, the difference in PER1 expression in caudal-SCN and PVN between genotypes at 9 hours after CT 15 (controls) supports the first alternative hypothesis that the duper mutation affects signaling between the dorsal SCN and extra-SCN oscillators. Further analysis of c-FOS and PER1 expression in AVP cells of the dorsal SCN and the sPVN will help to answer this question. Additional discussion of results is presented in Chapter 5.

## Future Directions

In order to understand the mechanism of exaggerated phase shifts in the duper hamster, we need to understand the mechanisms distal of retinal input. To do this, we can distinguish which cells in the SCN are co-labeled with AVP. As AVP is located in shell neurons, which are rhythmic in the absence of light, it is possible that PER1 expression is altered by the duper mutation in that subset of cells. This is supported by data showing differences in PER1 expression in the dorsal region of caudal SCN, which contains a majority of shell neurons. Although the differences were subtle, further anatomical identification may elucidate a more significant finding.

Duper may also affect other factors of the molecular clock aside from PER1. For instance, PER2 can bind to the CLOCK/BMAL complex in place of CRY (Chen et al., 2009). The mutation could increase the ability of PER2 to suppress CLOCK/BMAL transcriptional activity, which could explain both a decrease in period in DD by shortening the phase of *Per1/2* transcription. Additionally, because *Cry1/2* do not have a CRE element in their promoter, increased activity of PER2 could also explain exaggerated phase shifts in response to light. As there are many molecular events that could be affected by duper, it is necessary to identify the gene in order to fully understand the mechanisms of the duper mutation.



## CHAPTER 4

### GENETIC ANALYSIS OF THE DUPER MUTANT HAMSTER

#### Introduction

In order to understand the mechanism responsible for the duper phenotype, the genetic mutation must be identified. Our laboratory supplied the Broad Institute for Biomedical Research with genomic DNA from WT Syrian hamsters, and assemblies have been deposited in the National Institute for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov/genome/> 11998). RNA-seq was used to analyze the transcriptomes of liver of duper and wild type hamsters sacrificed at mid subjective day (CT6) and mid subjective night (CT15; Table 2; Hogenesch and Bittman, unpublished). Analysis was done with a sequencing depth of 20 and 90% sequence similarity, meaning that 18 of 20 reads need to demonstrate the same SNP to be

Gene	# Reads in WT	# Reads in Duper	WT_freq	Duper_freq	Fold change
Bmal1	2884	3048	1.75E-05	1.57E-05	-1.11
Bmal2	0	0			
Clock	1412	1424	8.54E-06	7.33E-06	-1.17
Npas2	176	188	1.07E-06	9.68E-07	-1.10
Cry1	2742	810	1.66E-05	4.17E-06	-3.98
Cry2	2258	3060	1.37E-05	1.57E-05	1.15
Per1	2434	7538	1.47E-05	3.88E-05	2.63
Per2	7048	4346	4.26E-05	2.24E-05	-1.91
Per3	4740	3118	2.87E-05	1.60E-05	-1.79
Fbxl3	2076	2836	1.26E-05	1.46E-05	1.16
Fbxl21	310	582	1.88E-06	3.00E-06	1.60
Rora	2836	2662	1.72E-05	1.37E-05	-1.25
Rorb	0	0			
Rorc	11306	13980	6.84E-05	7.20E-05	1.05
Rev-alpha	234	400	1.42E-06	2.06E-06	1.45
Rev-beta	10820	10344	6.55E-05	5.32E-05	-1.23
Csnk1e	1404	1520	8.50E-06	7.82E-06	-1.09
Csnk1d	5854	7592	3.54E-05	3.91E-05	1.10
Total reads	165254568	194299900	1	1	1.00

Table 4-1. De novo RNA sequencing of Syrian hamster liver reveals substantial clock gene expression. Duper and control livers from 3 to 5 unrelated animals were obtained at CT15 and total RNA was prepared. Ribosomal RNA was depleted by RiboZero gold, and the remaining RNA was used in total RNA sequencing using the TruSeq library preparation kit. Approximately 300,000,000 reads were obtained on an Illumina HiSeq 2000 instrument for both samples. As there was no genome sequence at the time, and all clock genes have not yet been cloned from Syrian hamsters, de novo transcript assembly was performed using the Trinity algorithm. Scaffolds were converted to a searchable database, and clock genes were identified using protein sequences from mouse clock genes as input in a TBLASTN search. Bowtie was also used to align RNA-seq reads to the transcript scaffolds created by Trinity. Clock genes are indicated by their official mouse symbols, and the number of RNA seq reads aligned to each gene is listed for both the control (WT) and duper hamsters. All clock genes save *Arntl2* and *Rorb* were detected. Down regulated genes are indicated in red, while upregulated genes are in green. No coding region mutation was found in any of the clock genes, but due to depth of sequencing, and the difficulty in getting complete cDNA sequences from de novo RNA sequence assembly, one or more may have escaped detection.

considered a candidate. It is possible that the cutoff was too conservative and may have excluded the genetic mutation responsible for duper. As none of the known clock genes had a coding mutation, however, identification of duper will likely reveal novel clock components and/or ways in which expression of known components is regulated.

Fast homozygosity linkage mapping will be used to generate novel candidates for the mutation. As duper is a recessive mutation, all phenotypic mutants will be homozygous for the mutation. By crossing dupers with a novel genetic background, we will be able to isolate a small number of loci as candidates for the duper mutation (Fig 4-2, 4-3). Fast homozygosity mapping does not require full chromosomes for

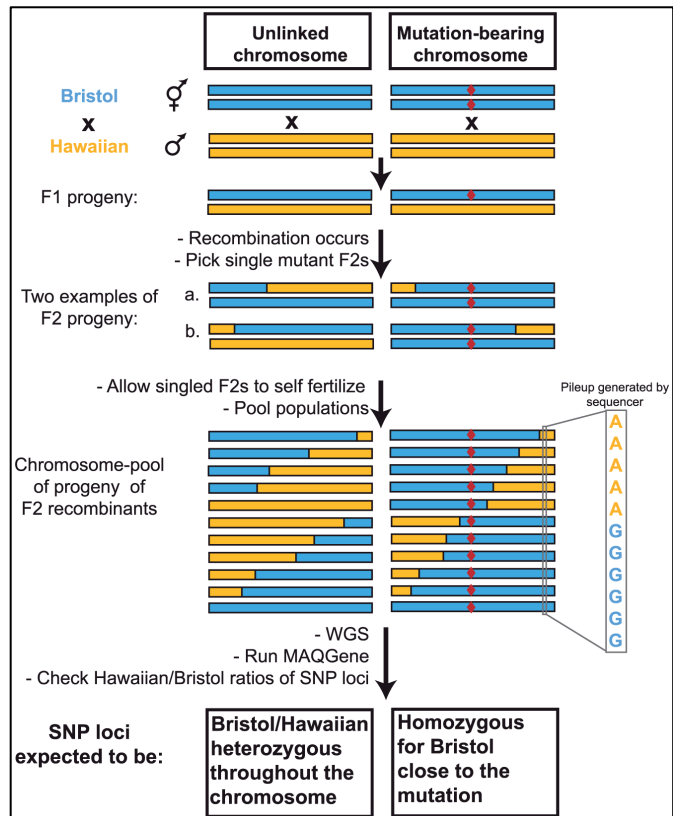


Figure 4-2. A diagram illustrating how fast homozygosity mapping is able to identify a loci specific to a recessive mutation. (Taken from Doitsidou et al 2010).

genetic sequencing. This is important, as the Syrian DNA is still in scaffolds (*i.e.*, without chromosomal assignment). Fast homozygosity mapping will identify candidates single nucleotide polymorphisms (SNPs) associated with the duper mutation. Results will be improved with high numbers of F2 dupers on Bio 14.6 background and high levels of recombination around the mutation. Based on fast homozygosity mapping in zebrafish, we predict that 25-50 F2 dupers will be needed to identify a small number of candidate

regions (Voz et al., 2012). In order to pursue next generation sequencing and thus identify candidate loci to identify the specific mutation, it is first necessary to assess transmission of duper in crosses with this distant ecotype.

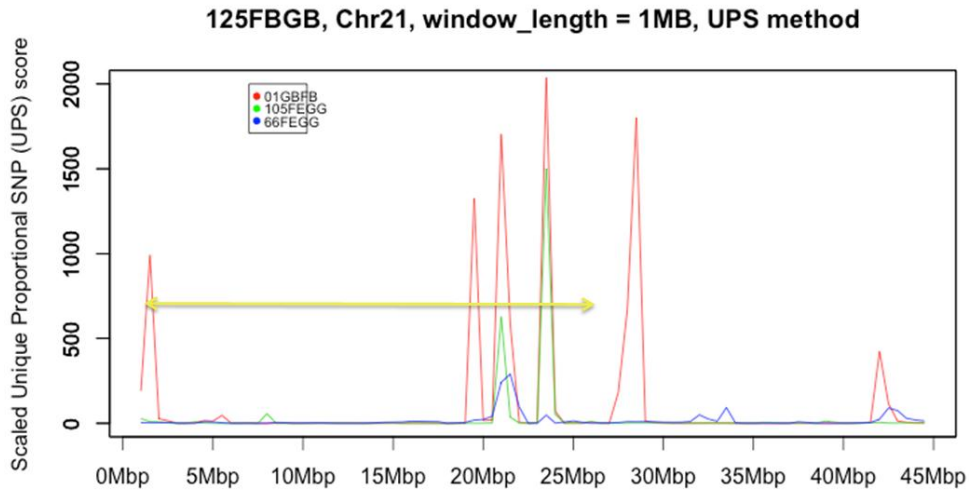


Figure 4-3. Fast homozygosity mapping in zebrafish. Bowen et al., 2011 method was used to map developmental mutant zebrafish. Using a well-characterized mapping panel of SNPs, the location was determined to be in a 25 megabase interval (indicated by yellow arrow). Implementation of Unique Proportional SNP (UPS) method shortened the interval to four regions of < 0.5 Mbps (indicated as red peaks). The UPS method is similar to the Bowen method, but disregards SNPs shared between mutant and mapping populations and assess SNP frequency. Regions of SNPs unique to mutants are noted by peaks. Unrelated mutants are indicated in green and blue. Known and candidate genes were mapped to these intervals and deleterious SNPs were determined for these genes. (Figure made by John B. Hogenesch, unpublished).

Total RNA was taken from Bio 14.6 and Weinert hamsters (wild caught; Weinert et al., 2001) to determine if they were genetically distinct from Lakeview hamsters. Weinert hamsters exhibited 20,441 SNPs, and Bio 14.6 hamsters have 24,297 SNPs that are unique from duper in cDNA, most of which are silent (Bittman and Hogenesch, unpublished). As the number of SNPs in genomic DNA will be much greater than that of cDNA, this approach hold great promise to identify the region containing the duper allele with high resolution. Both strains were sufficiently distinct from duper, but Bio 14.6 hamsters were chosen, as they are easier to obtain and crosses between WT and Bio 14.6

hamsters have already been reported to produce viable and fertile offspring (Mitsuhashi et al., 2003).

Bio 14.6 hamsters have been reproductively isolated since the 1960's (Bajusz, et al., 1996; Forman et al., 1972), have white fur with black ears, and are used in multiple cardiovascular studies, as they carry a recessive deletion of *δ-sarcoglycan*, which affects skeletal, smooth and cardiac muscle (Sakamoto et al., 1997; Rotundo et al., 2011). *δ-sarcoglycan* is one of four components of the sarcoglycan complex, which is a sub-complex of the dystrophin-glycoprotein complex (DGC). DGC mediates interactions between the cytoskeleton, membranes, and extracellular matrix (Lapidos et al., 2004). Bio 14.6 hamsters develop congestive heart failure and edema by 200 days of age (Chimoskey et al., 1984). Further studies show a decrease in lifespan starting at 250 days (Penev et al., 1998). Parabiosis studies with Bio14.6 and WT hamsters can triple their lifespan (Chimoskey et al., 1984). Research has shown that Bio14.6 hamsters are also deficient in natriuretic factor, and that congenital heart failure and edema are not a direct result of the cardiomyopathy, but rather the inability to excrete salt and water due to a lack of humoral mediators (Chimoskey et al., 1984). When challenged with severe jet lag (12-hour phase shifts every 7 days), the lifespan of Bio 14.6 hamsters decreased by 11% (Penev et al., 1998). Although shorter lifespan is challenging, the mutation allows us to assess the impact of the duper mutation on the effects of jet lag in hamsters pre-disposed to cardiac disorders. If the duper mutation helps the organism rapidly re-entrain to a new phase, then we predict that F2 duper hamsters with a *δ-sarcoglycan* deletion would be resistant to the negative effects of jet lag.

Duper was found on a *tau* mutant background of the LVG strain, which descended from hamsters of the Lakeview strain. They have been inbred since 1987 (Ralph and Menaker, 1988). Duper was isolated on a WT background by backcrossing with WTs (LVG strain) that were more recently obtained from the Lakeview hamstery. Therefore the duper mutation will only be expressed in Lakeview DNA.

To differentiate the SNP responsible for the duper mutation from random non-functional SNPs in LVG hamsters, 3 duper mutants were bred with a WT Bio 14.6 hamster male (a genetically distinct ecotype) obtained from BioBreeders (Fitchburg, MA). Crossing duper with a novel ecotype was necessary for two reasons. First it is important to compare littermates, as they will share a majority of random non-functional SNPs. This decreases the amount of variation between WT, heterozygous duper, and duper hamsters in order to ease identification of SNPs that are specific to duper. Second, our hamsters were inbred to isolate duper so candidate SNPs found using RNA-seq may be irrelevant to the duper phenotype, but have been inherited in the duper line. The genetically distinct hamster will not have inherited such random mutations. Thus, in F2 littermates for the Bio 14.6 WT and duper cross, only SNPs linked to (in the chromosomal neighborhood of) the duper locus will assort with the recessive phenotype.

It is unknown whether the duper mutation will transmit onto a novel background. I set out to determine whether the mutation is expressed on a novel ecotype, and then to examine whether the period and PRC in response to a 15-min light pulse is inherited in a Mendelian pattern or is sex-linked. In order to begin to assess linkage of recessive traits, I examined the independent assortment of the cardiomyopathy (and thus the  $\delta$  – *sarcoglycan* deletion, although this was not directly assessed), white coat color and the

duper phenotype in Bio14.6 hamsters. I predicted that all F1s would have a WT phenotype (because the mutant allele is recessive) and F2s would be 25% WT, 50% heterozygous, and 25% homozygous duper for the duper mutation. If the mutation transmits, phenotypically 25% will be duper and 75% WT.

If we are able to produce F2s with a duper phenotype, then we will be able to analyze the consequences of circadian phenotype on jet lag. Organisms entrain to the environment by phase shifting. We hypothesize that duper hamsters are resistant to jet lag due to their ability to achieve large phase shifts in response to light. We predict F2 duper hamsters will rapidly (1-3 days) re-entrain to a LD cycle after an 8-hr phase advance or delay. F2 hamsters that are phenotypically WT and LVG WT hamsters will take 7-9 days to achieve an 8-hr phase advance and 3-8 days to achieve an 8-hour phase delay.

## **Materials and Methods**

### **Animal Maintenance**

Syrian hamsters were raised and kept in 14:10 LD with *ad libitum* food and water as described in chapters 2 and 3. At approximately 3 months of age, hamsters were individually housed for phenotyping. Running wheels were only provided during phenotyping. Six additional LVG WT hamsters (3 male) were purchased to compare phenotype with F2s. These animals were approximately 2 months old when we received them.

### **Breeding**

A Bio 14.6 male was phenotyped and mated with a female LVG-duper hamster. Phenotype was determined by measuring free running period in DD and phase shift amplitude in response to a 15 min light pulse at CT 15 (early subjective night) and

CT18.5 (late subjective night). Additional pairings with 2 Bio 14.6 females were attempted but were unsuccessful. The F1 litter consisted of 10 animals (5 female, 5 male). Once F1s reached adulthood, they were phenotyped and were mated with an F1. This produced 5 litters of F2 hamsters. Once the F1 mothers' pups were weaned and estrous cycles were regular, they were mated again, with a different F1 male. This was repeated, for a total of 3 litters from each F1 female (15 F2 litters in total) producing a total of 154 F2 hamsters. 2 hamsters died before they could be phenotyped.

### **Jet Lag**

14 F2 WT, 9 F2 duper, and 5 LVG WT hamsters were subjected to an 8-hr phase advance as well as an 8-hour phase delay. LVG WT hamsters did not receive phase delay. Hamsters kept in a 14:10 LD cycle for 10 days of stable entrainment. On the 10<sup>th</sup> day, the LD cycle was phase advanced by 8 hours by shortening the dark phase to 2 hours. Phase delays were achieved by lengthening the dark phase to 18 hours. All animals remained in the new LD cycle until they all had stably entrained for 7-10 days. The number of days it took for animals to entrain to the new phase was assessed. Re-entrainment was considered complete when the onset of activity was stable (within 30 minutes) with the 24 period of the LD cycle for at least 3 days.

### **Statistical Analysis**

$\chi^2$  analysis was used to compare the frequency of large phase advances (>2 hours) and phase delays (>4 hours) between LVG duper (previously characterized), F2 duper, and F2 WT hamsters. Two-sample t-tests were used to compare the number of days it took to complete an 8-hour phase advance or delay between dupers and WT hamsters. As

all animals were subjected to both advances and delays, paired-tests were run to compare the number of days needed to complete the phase shift within genotype.

### DNA collection and isolation

Ear snips were taken from F0 Bio 14.6 and duper parents, and all F2 dupers.

Genomic DNA was isolated and purified for sequencing as in Krug et al, 2011.

### Results

As predicted, the F0 Bio 14.6 hamsters and the F1s (heterozygotes) demonstrated the WT phenotype. In the second generation, the duper mutation transmitted onto the Bio14.6 with the predicted Mendelian pattern of inheritance for a recessive trait: 39/152

hamsters (22 males, 17 females; 25.7%) displayed the duper phenotype (Fig 4-

4). F2 dupers (based on  $\tau_{DD}$ ) also displayed the expected phase delays (>4 hours, based on phase shifts of LVG-duper hamsters) in response to a light pulse at CT 15 ( $\chi^2=3.42$ ,  $p=0.064$ ) and phase advances (>2 hours;  $\chi^2=1.4$ ,  $p=0.24$ ; Fig 4-4, 4-5). White pelage was

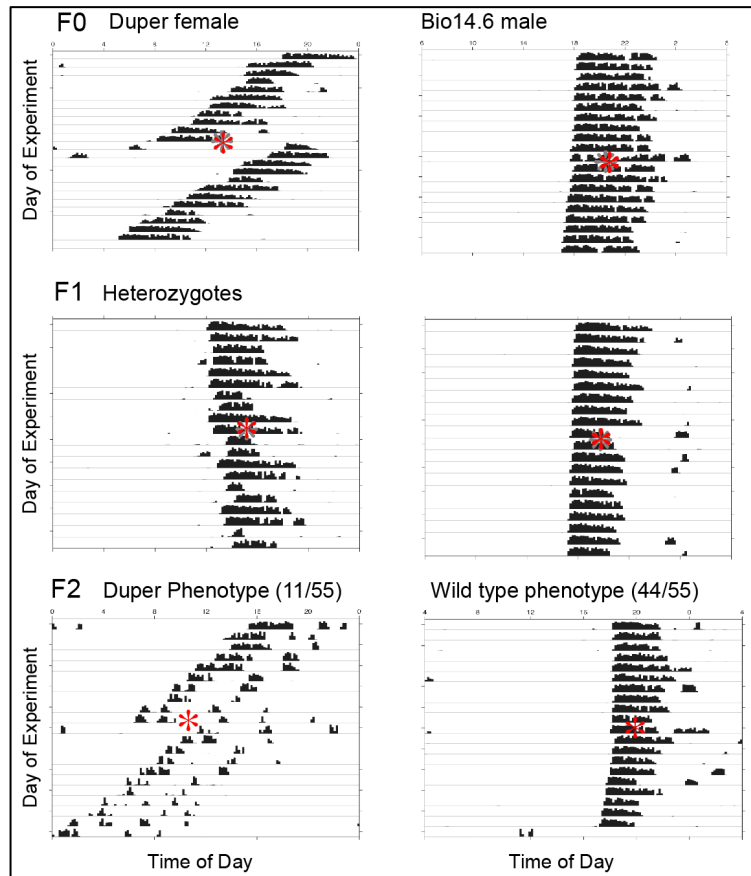


Figure 4-4. The duper mutation transmits to the Bio 14.6 background. Actograms of F0, F1 (hets), and F2 WT, and duper hamster free running in DD with a light pulses at CT15. F2 dupers on a Bio 14.6 background display the expected duper phenotype.



independently assorted from duper as expected, with 8/39 (20.5%) dupers co-expressing the recessive phenotypes ( $\chi^2=0.491$ ,  $p=0.48$ ). Similarly, only 4 of 39 (10.3%) duper experienced heart failure typical of the recessive  *$\delta$ -sarcoglycan* mutation. A total of 18/152 F2 hamsters (4 F2 duper, 2 died before phenotyped) died due to cardiomyopathies at ~130 days old; hamsters without the mutation are expected to have a lifespan of 16-22 months. This also suggests that the recessive traits assort independently. However, because the cardiomyopathic phenotype is exhibited later in life, it is possible that F2 dupers, that are currently adolescents or young adults, also carry the  *$\delta$ -sarcoglycan* mutation but have not yet expressed the phenotype. Therefore we cannot quantify how many F2 duper hamsters also exhibit the cardiomyopathic phenotype.

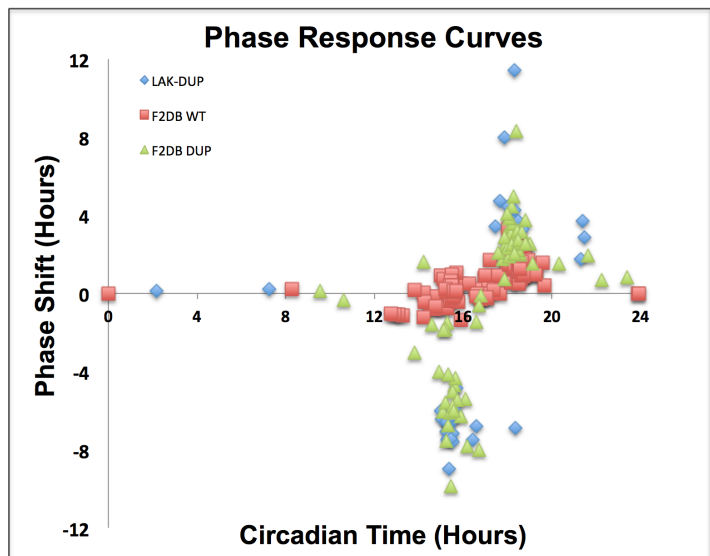


Fig 4-5. Phase response curve of F2 WT, F2 dupers, and F0 Lakeview dupers in response to a 15 min light pulse. Phase shifts are provided in hours.

In jet lag experiments, F2 dupers displayed rapid re-entrainment to 8-hour phase advances, completing phase shifts in 3.2 +/- 0.5 days compared to 14.6 +/- 0.9 days for F2 WT (p<0.0001), and 11.2 +/- 3.1 days for LVG WT. In response to 8-hr phase delays, F2 dupers re-entrained more rapidly than F2 WT (2.7 +/- 0.3 days vs 10.2 +/- 2.7 days; p<0.0001; Fig 4-6). WT hamsters took significantly longer to entrain to phase advances

than delays ( $p < 0.009$ ). Duper hamsters did not exhibit a significant difference in the number of days needed to complete an 8-hour advance or delay ( $p < 0.77$ ).

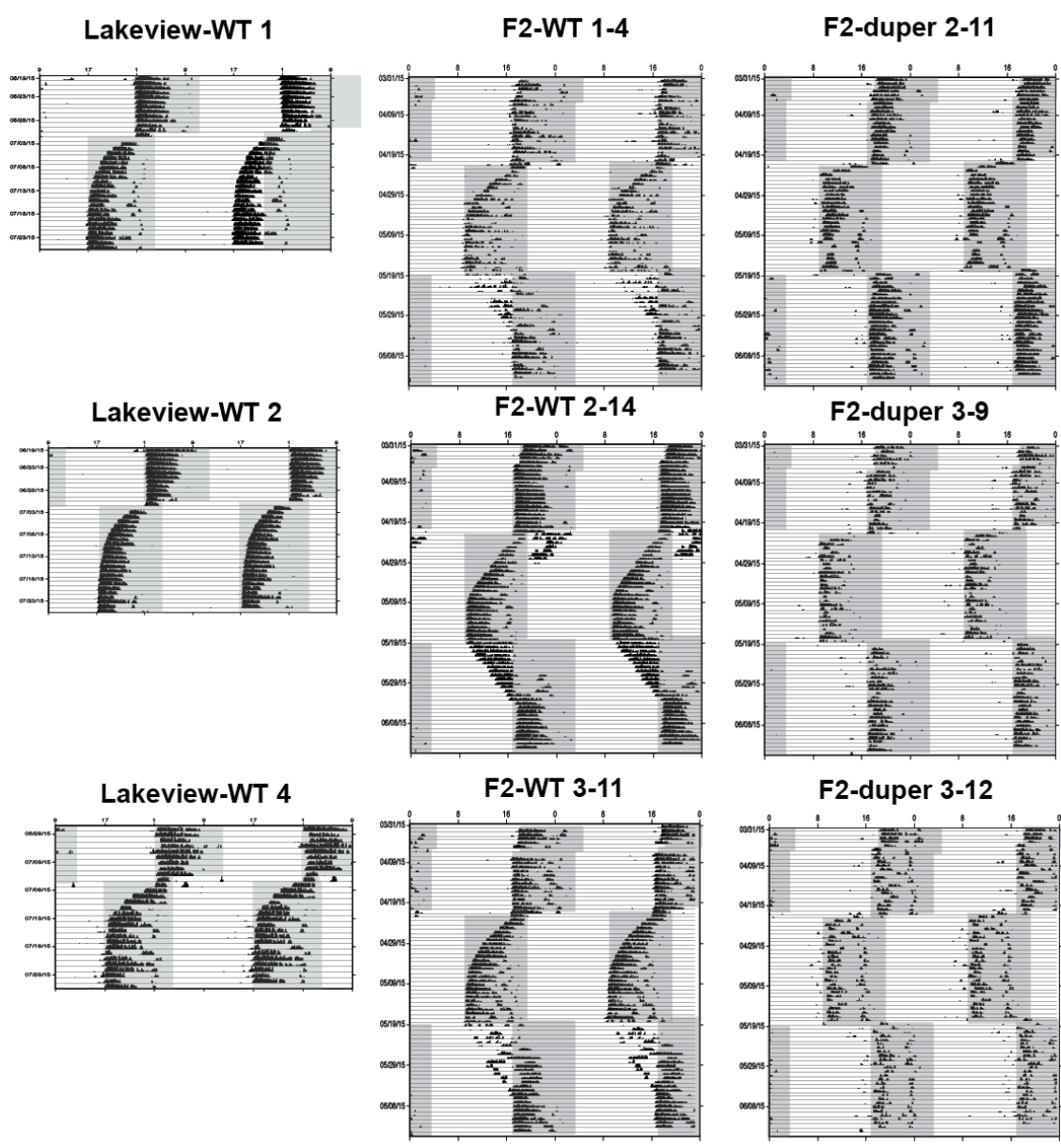


Figure 4-6. Wild type hamsters re-entrain to 8h advances or delays of the 14L:10D cycle, but duper hamsters rapidly re-entrain to 8-hour phase shifts. Actograms of 3 F2 WTs (middle), F2 dupers (right) subjected to and 8-hour phase advance followed by and 8-hour phase delay. 3 Lakeview WTs (Left) only received an 8-hour phase advance. Gray bars indicate lights are off.

## **Discussion**

As expected, the duper phenotype transmitted onto the Bio14.6 background in a Mendelian inheritance pattern. This new line of duper hamsters will make possible the identification of the region containing the genetic mutation. The hamster genome is ~2.50 Gb, which is similar in size to the mouse (~2.57 Gb). Based on preliminary data using cDNA, the coding regions of duper on a LVG background and Bio14.6 hamsters are expected to have one SNP, on the average, every 3500 base pairs. Preliminary data also indicate that Bio 14.6 hamsters have ~24,000 SNPs relative to duper hamsters in cDNA. As we will be using genomic DNA for fast homozygosity mapping, we expect a much greater number of SNPs. There is currently no information on the recombination frequency in hamsters. Generally, there is greater recombination frequency towards the tip of the chromosome. However, recombination frequency varies between species, and within species there are variations within chromosomes and between alleles (Jensen-Seaman et al., 2004). This makes it difficult to predict the recombination frequency in hamsters, and thus the number of F2 dupers that will be needed to identify the chromosomal region containing the allele with good resolution.

Based on previous studies using fast homozygosity mapping in zebrafish, which have a ~1.39 Gb genome, we predict that we will need 25-50 F2 dupers. We currently have samples from 39 F2 dupers). A greater number of samples will decrease the number and size of candidate regions as well as the size of regions identified.

We are unable to accurately predict the size of the regions identified by fast homozygosity mapping, as it will depend upon the density of markers around the gene, the recombination frequency, and the genome assembly. However, previous experiments

using fast homozygosity mapping in zebrafish, using 20-25 pooled samples with 1 SNP per 130 base pairs, have produced candidate regions ranging in size from <0.5-3.2Mb (Voz et al., 2012; Hogenesch et al., unpublished, Figure 4-3).

The rapid re-entrainment to both 8-hour advances and delays exhibited by F2 dupers demonstrates that the duper mutants are behaviorally resistant to jet lag. F2 WT hamsters exhibited a slow rate of re-entrainment that was similar to genetically WT hamsters recently purchased from Lakeview. It is possible that phase shifts in duper hamsters are due to negative masking to light. To examine this, we would need to transfer hamsters to DD after the phase shift was achieved to ensure that their endogenous rhythms had shifted. Re-entrainment to 8-hour phase delays is complete in 2-6 days in mice (Shen et al., 2000; Yamaguchi et al., 2013). In response to 12-hour phase-shifts every 7 days, Bio 14.6 hamsters were not able to re-entrain within 7 days in response to the first shift, but seemed to re-entrain more rapidly to some (but not all) of subsequent shifts (Penev et al., 1998). However, because these animals were not released into DD following the shift, it is unclear if they re-entrained. It is possible that the shift in activity was a result of negative masking in response to light. Syrian hamsters were not able to entrain to 6-hour phase advances every three days (Gibson et al., 2010). The slower re-entrainment rate to phase delays seen in F2 WT hamsters (~10days) may be due to a difference in methods. In our experiment phase delays were achieved by lengthening the dark phase to 18 hours, whereas 8-hour phase delays in mouse studies were instituted by lengthening the light phase to 22 hours. This difference may induce more rapid re-entrainment by inducing a larger phase shift on the day of the delay.

The length of time to achieve a phase shift can be predicted by the PRC. As duper hamsters display phase shifts of 4-12 hours in response to a 15 min light pulse, it is not surprising that they can re-entrain to an 8-hour phase shift in a matter of days. Of course this is influenced by the phase at which they are exposed to light, as light during subjective day falls in the dead zone (does not elicit a phase shift) for dupers and WTs. As WTs display 0.5-3 hour phase shifts, it is expected that achieving an 8-hour phase shift would take many days.

Preliminary studies in our lab assessed the mechanism of re-entrainment in WT hamsters. We tested the hypothesis that re-entrainment to 8-hour phase shifts was achieved after the first 2 days of the new LD, but locomotor rhythms were delayed. Alternatively, 8-hour phase shifts might be achieved by shifting the clock a small amount each day until light no longer is present in the active zone of the PRC. In order to examine this question, we subjected 4 F2 WT hamsters to either an 8-hour phase advance or delay of the 14L:10D cycle. 2 days later, animals were moved to DD. If the SCN had achieved the full phase shift, then while in DD, the animals should have continued to shift their activity onsets until the new phase was achieved. If re-entrainment is truly gradual, with light information inducing incremental phase shifts throughout the interval over which transients occur, hamsters transferred to DD on the second day after the shift should run with their endogenous period of ~24 hours, with onset of activity determined by their last day in LD. We found that the alternative hypothesis was correct; hamsters maintained the phase of activity at the second day of the shifted cycle.

Mice with overexpressing VPAC2, *V1a/1b*<sup>-/-</sup>, and *Cklε*<sup>-/-</sup> mice all exhibit rapid re-entrainment to large phase shifts (Shen et al., 2000; Yamaguchi et al., 2013; Piorz et al.,

2014). However, unlike *duper*, the period of VPAC2 overexpression mice is only decreased by ~0.6 hours (~23.4 hours; Shen et al., 2000), and *V1a/V1b<sup>-/-</sup>* and *Ckl1ε<sup>-/-</sup>* mutants exhibit a WT period of ~24 hours (Yamaguchi et al., 2013; Pilonis et al., 2014) compared to ~23 hour period in *duper*s. However it is still possible that the *duper* mutation affects PER stability or CK1ε enzymatic activity. The information from the RNA-seq data is also limited in that it is based on liver samples, not SCN, and only assessed during one point in early subjective night. It is possible that the amplitude or phase of *CK1ε* expression is altered at another phase, or that the mutation has an effect on an enzyme that modifies clock proteins which is specific to the SCN.

Although *duper* mutants are behaviorally resistant to jet lag, it is still unknown if peripheral clocks shift at the same pace. Jet lag has been shown to decrease lifespan (Pinev et al., 1998; Davidson et al., 2006) and inhibit neurogenesis (Gibson et al., 2010). It is widely assumed that these adverse effects are the result of the internal desynchrony that occurs when animals are subjected to such shifts. Genetically induced circadian disruption (*Bmal1<sup>-/-</sup>*) exhibit early aging and age related pathologies (Kondratov et al., 2006). Furthermore, circadian disruption is associated with many affective disorders including schizophrenia, bipolar disorder, and major depressive disorders (Mansour et al., 2006; Soria et al., 2010). If the *duper* mutation shifts both neural and peripheral clocks at the same speed, it may illuminate a way for humans to achieve large phase shifts but escape the negative consequences of circadian desynchrony. If not, jet lag may induce greater internal phase mis-alignment in *duper* than in WT hamsters. Further assessment of how peripheral clocks respond to phase shifts is needed to understand the consequences of the *duper* mutation.

Now that transmission of the duper mutation on a novel ecotype and phenotype has been assessed, we can pool the isolated DNA from F2 dupers and use fast homozygosity mapping to identify the genetic basis of the duper mutation.

## CHAPTER 5

### DISCUSSION: WHAT WE KNOW ABOUT THE DUPER MUTANT HAMSTER

#### Behavior

The Syrian hamster mutant, *duper*, has a period of ~23 hours, compared to ~24 hours in WT hamsters. Also in contrast to WTs, *duper*s show a type 0 PRC in response to light pulses (Krug et al., 2011). Despite having a short period, *duper* hamsters' biological clock is just as precise as WTs (Bittman, 2012). In contrast to WTs, which lengthen their period when kept in constant light, *duper* hamsters further decrease their period thus breaking Aschoff's rule. However, *duper* hamsters do not display an altered threshold for light-induced resetting of circadian phase (Bittman, 2014). In order to better understand the *duper* mutation, we performed a series of behavioral experiments to determine whether the effects of this mutation are specific to photic stimuli and/or phase.

We hypothesized that the *duper* mutation is specific to photic stimuli and not to the phase of the manipulation. Novel wheel running is a non-photic stimulus that induces phase shifts during subjective day (Mrosovsky et al., 1992). We found that novel wheel running was insufficient to induce large phase shifts in *duper* hamsters. This suggests that the mutation is specific to photic stimuli; however, other non-photic stimuli, such as food availability or temperature, have not been tested. As light pulses only induce phase shifts during subjective night, we used 3-hour dark pulses as a photic stimulus during subjective day to examine if the mutation is phase specific. Dark pulses induced exaggerated phase shifts in some *duper*s, but in no WTs. We also observed that *duper* phase shifts were significantly more variable than WTs. Additionally, 8/21 *duper*s and 0/16 WTs split their



behavioral rhythms following a 3- dark pulse. The splitting of locomotor rhythms indicates a de-coupling of oscillators, presumably in the SCN pacemaker. Variability may be due to the complex nature of dark pulses. Dark pulses affect the clock in three ways: first, a LL to DD transition; second, exposure to dark for 3-hours; and third, DD to LL transition. Each of these manipulations can induce a phase shift. Therefore, the phase shift resulting from a dark pulse may result from a combination of these events. To further analyze the effect of light during subjective day, we used LL to DD and DD to LL transitions. Duper hamsters exhibited exaggerated phase shifts to both transitions during both subjective day and night. Again, duper hamsters displayed more variability than WT hamsters. Additionally, LL to DD transitions during subjective night induced ultradian locomotor activity in 29/43 duper hamsters and 0/21 WTs. Mathematical modeling can reproduce this ultradian behavior due to LL to DD transitions as well as a short period in DD by weakening coupling between oscillators within the pacemaker. Taken together, these findings support the hypothesis that the mutation is specific to photic stimuli, but not specific to phase.

Although we performed multiple behavioral manipulations, there are still limitations. Although duper hamsters exhibited large phase shifts in response to LL to DD and DD to LL transitions during subjective day, it is possible that the transitions did not affect the clock until subjective night. Dupers also displayed greater variability of phase shifts in response to both light transitions and dark pulses than WT hamsters. These issues make it difficult to assess accurately the affects of light during subjective day.

Wheel running activity has been shown to have an inverse relationship to the period of locomotor activity in hamsters (Weisgerber et al. 1997; Mrosovsky, 1999). High levels of activity decrease the period of hamsters by  $\sim 0.2$  hours (Mrosovsky et al., 1999). However, this finding is not always reproducible even in the same lab (Aschoff et al., 1973; Weisgerber et al., 1997; Mrosovsky, 1999). As the duper phenotype has only been assessed with wheel running, further analysis is necessary to prove that the duper phenotype is independent of locomotor feedback. Locomotor rhythms can be measured using laser beam crosses or a temperature monitor in place of a wheel. Based on observations that dupers that exhibit low levels of activity still have an  $\sim 23$  hour period, I predict that dupers will exhibit the same phenotype without a wheel.

### **Duper on a Novel Ecotype and Jet Lag**

In order to use fast homozygosity mapping to identify the duper mutation, it is necessary to express the duper phenotype on a WT background genetically distinct from the LVG (Lakeview) hamster line. As expected, the duper mutant phenotype transmitted to a genetically distinct line of hamsters (Bio 14.6) in the same recessive Mendelian pattern as the LVG hamster strain on which it was discovered. In addition to phenotyping the F2 litters (measured  $\tau_{DD}$  and response to light pulses at CT 15 and 18.5), we performed behavioral experiments to test if duper hamsters are resistant to jet lag.

I hypothesized that duper hamsters would be resistant to jet lag due to their ability to rapidly achieve large phase shifts in response to a 15-min light pulse. I predicted that following an 8-hour phase advance or delay, duper hamsters would rapidly entrain to the new phase ( $\sim 1-3$  days), whereas WT hamsters will take  $\sim 7-9$  days (shift approximately 1 hour per day) to complete the phase shift. My results supported the hypothesis that duper

hamsters re-entrained rapidly compared to WT hamsters. In response to an 8-hour phase advance, F2 duper hamsters entrained to the new phase of the LD cycle in 2-3 days whereas F2 WT hamsters took ~14 days to achieve the shift. LVG WT took ~11 days to achieve an 8-hour phase advance. F2 duper hamsters also achieved an 8-hour phase delay in 2-3 days whereas F2 WTs took ~10 days.

These results demonstrate that duper hamster's locomotor rhythms are resistant to jet lag; yet we do not know if peripheral oscillators are also able to achieve rapid phase shifts. If so, there should be a decrease in internal desynchrony and the duper mutation could provide valuable information in determining whether rapid re-entrainment reduces the negative consequences of jet lag or shift work. However, if the peripheral oscillators do not rapidly shift, the duper mutation may exacerbate the negative effects of jet lag by increasing the duration and magnitude of phase mis-alignment of peripheral oscillators.

Previous studies have shown that chronic jet lag or shift work schedules increase the risk of both metabolic and cardiovascular disease and can decrease lifespan (Penev et al., 1998; Yamazaki et al., 2000; Davidson et al., 2006; Conlon et al., 2007; Sheer et al., 2009). The Bio14.6 hamsters have a cardiomyopathy due to a deletion of *δ-sarcoglycan*, which affects skeletal, smooth and cardiac muscle (Sakamoto et al., 1997; Rotundo et al., 2011). A sub-line of this mutation (TO-2) is exacerbated by circadian disruption, leading to a decrease in lifespan, upon repeated 12-hour phase shifts every 7 days (Penev et al., 1998). In humans, shift work, which disrupts the clock with frequent phase shifts similar to chronic jet lag, can perturb circadian control of sympathetic and vagal autonomic control (Furlan et al., 2000) and daily blood pressure oscillations (Chau et al., 1989; Martino and Young, 2015). As duper hamsters are resistant to jet lag, duper expressed on

a Bio14.6 background provides a unique opportunity to analyze the cardiovascular benefits of the duper phenotype when challenged with jet lag. If the duper mutation decreases internal desynchrony induced by jet lag, duper hamsters with a cardiomyopathy will have a longer lifespan than WT cardiomyopathic hamsters.

Although portions of the duper phenotype resemble effects of other circadian mutations in mice (VPAC2 overexpression, *Clock*<sup>-/-</sup>, *Clock*<sup>Δ19/Δ19</sup>, *V1a/1b*<sup>-/-</sup>, *Cklε*<sup>-/-</sup>) none of them fully replicate the phenotype (Shen et al., 2000; Vitaterna et al., 2006; Dallman et al., 2011; Yamaguchi et al., 2013; Piorz et al., 2014). *Clock*<sup>Δ19/+</sup>, *Cklε*<sup>-/-</sup>, *V1a/1b*<sup>-/-</sup>, and VPAC2 overexpression mutant mice display rapid resetting in response to either a 6- or 8-hour phase advance or delay (Shen et al., 2000; Vitaterna et al., 2006; Yamaguchi et al., 2013; Piorz et al., 2014). Unlike duper, in response to a 1-hour light pulse *Cklε*<sup>-/-</sup> mice increase phase shifts by only 1 hour compared to WTs, and do not have a type 0 PRC (Piorz et al., 2014). *Clock*<sup>Δ19/+</sup> and *Clock*<sup>-/-</sup> mice exhibit type 0 PRCs to long light pulses of 6- or 4-hours respectively (Vitaterna et al., 2006; Dallman et al., 2011). It is unclear how this compares to duper hamsters, as 15-min light pulses have not been performed. In contrast to dupers, *Clock*<sup>Δ19/+</sup> mutants increase  $\tau_{DD}$  by 1-hour and by 4-hours in homozygotes (Vitaterna et al., 2006). Although *V1a/1b*<sup>-/-</sup> mice show rapid resetting in a phase shift paradigm, they do not display a change in  $\tau_{DD}$  compared to WTs (Yamaguchi et al., 2013). VPAC2 overexpression ( $\tau_{DD}$  = 23.26; Shen et al., 2000) and *Clock*<sup>-/-</sup> ( $\tau_{DD}$  = 23.6 or 23.3; DeBruyne et al., 2006; Dallman et al., 2011, respectively) in mice result in a decrease in  $\tau_{DD}$ . *Clock*<sup>-/-</sup> mice also defy Aschoff's rule, and displayed a decrease  $\tau_{LL}$ , similar to duper mutants (Dallman et al., 2011). Furthermore, RNA-seq indicates that duper is not due to a mutation in the coding regions in any of the core clock

genes, or in *CK1ε/δ* (Bittman and Hogenesch, unpublished). RNA sequencing was performed with a read depth of 20 and a cutoff of 90%, which means 18 of every 20 reads would need to exhibit the duper mutation to be identified. It is possible that these measures were too strict, so that a mutation in the coding sequence could have been missed. Taken together, these findings indicate that the duper phenotype is caused by a novel mutation, which will aid in our understanding of molecular clock mechanism.

### **Neurobiological Analysis**

I hypothesized that exaggerated phase shifts seen in duper hamsters are due to increased amplitude or prolonged expression of *Per1* in retinorecipient (VIP positive) cells, compared to WT. My first alternative hypothesis is that the duper mutation affects signaling between the retinorecipient cells in the ventrolateral SCN and the dorsal SCN or between the SCN and extra-SCN oscillators. My second alternative hypothesis is that the duper mutation affects the molecular clock in distal to initial PER1 induction by light.

Based on previous literature, I predict that a 15-min light pulse at CT15 will induce *c-Fos* and *Per1* expression in the ventrolateral SCN (VIP cells) for both genotypes. If the null hypothesis is correct and duper affects the photic signaling pathway upstream of, or including, activation of the CRE, I anticipate changes in both *c-Fos* and *Per1* expression in duper hamsters compared to WT. If alternative hypothesis 1 is correct, and the mutation alters communication between the SCN and extra-SCN, rather than photic input, I predict PER1 co-labeled with VIP positive cells in dupers will resemble expression in WTs and regions distal to the SCN core (SCN shell, sPVN, and PVN) will have delayed expression of *Per1*. Finally, if the second alternative hypothesis is correct and the mutation affects the post-transcriptional or post-translational modification of

PER1, I predict only temporal expression of *Per1* will be modified. Combining temporal and spatial analysis of PER1-ir allowed me to investigate how the *duper* mutation alters the molecular clock to achieve large phase shifts in response to light.

As expected, the number of c-FOS-ir cells was significantly increased 1 and 3 hours after a light pulse in *duper* and WT hamsters in mid- and caudal-SCN. In both WT and *duper* hamsters, PER1 protein expression lagged reported *Per1* mRNA expression by 4-6 hours in mid- and caudal- (ventral and dorsal) SCN peaking ~6 hours after a light pulse. The temporal difference between c-FOS and PER1 protein expression was unexpected and may be due a difference in size as well as post-transcriptional and post-translational complexity. Furthermore, this finding may be related to the additional role of FOS, but not PER, as an enhancer (Malik et al., 2014).

There was also no significant difference in the intensity of PER1-ir cells between genotype at any time point. The only significant effect of genotype in response to light was at 2-hours after a light pulse. *Duper* hamsters exhibited significantly more VIP cells co-labeled with PER1 than WT hamsters (Fig 3-4). This difference is due to a decrease in co-labeled cells in WT hamsters 2-hours after the pulse, which is surprising given that that the total number and percentage of co-labeled cells does not differ between genotype at 1- or 3-hours after a light pulse. This may reflect a sustained response to light in retinorecipient cells of the SCN in *duper* hamsters compared to WTs. However, because this pattern of a decrease 2-hours after light exposure followed by a second increase 3-hours after has not before been documented, it will be important to replicate these findings.

There were no significant effects of light on the number of PER1-ir or c-FOS-ir cells in the PVN. It is possible that I did not see a difference in the PVN because I did not wait a sufficient amount of time following light pulse to take samples. Additional time points at 12- and 15-hours after the pulse may have revealed a change in PER1-ir in the PVN.

An effect of genotype was also seen 9-hours after CT15 (control). WT hamsters displayed a greater number of PER1-ir cells in caudal-SCN than dupers. However, in the PVN at the same phase this was reversed: duper hamsters exhibited more PER1-ir cells than WTs. This may indicate that the duper mutation affects signaling between the SCN and extra-SCN oscillators. To understand how the duper mutation alters the phase of endogenous PER1 expression in DD, additional samples need to be taken during subjective day so we can compare levels at phases throughout a full circadian day.

*In situ* hybridization analysis of *Per1* expression in the SCN in response to a 15-min light pulses shows no differences between WT and duper hamsters (Krug et al., 2011). Taken together, light induced *Per1* mRNA and protein suggest that the mutation alters mechanisms downstream from PER1 and is independent of the photic signaling pathway. Specifically, because photic and non-photoc (activity) cues both act of the CRE of the PER1 promoter region, it is likely that changes in clock mechanism in dupers is distinct from to P-CREB induced *Per1* transcription. Additionally, there was no difference in the intensity of PER1-ir cells in the SCN, which implies that the amount of PER1 produced does not differ between duper and WT hamsters. However, it is possible that the mutation only affects CREB signaling to stimulate transcription, and does not alter the decrease in signal from non-photoc cues. Activity induced shifts signal the SCN

through 5-HT, which influences *Per1* transcription at the CRE, and NPY, which acts through PKC to regulate E-box induced transcription by modulating CLOCK and BMAL1 phosphorylation (Shim et al., 2007; Robles et al., 2010). PKC also acts as a regulator of light input during subjective night by interacting with PER2 (Jakubcakova et al., 2007). Chemical inhibition of PKC enhances light-induced phase delays in mice (Lee et al., 2007), yet *Pkca*<sup>-/-</sup> mice exhibit impairments to light entrainment (Jakubcakova et al., 2007). As novel-wheel running was insufficient to induce large phase shifts in duper hamsters, it is possible that E-box controlled *Per1* transcription is also not affected by the mutation. This could be examined using ChIP (chromatin immunoprecipitation) to identify complexes, and the phase at which they bind, to E-boxes in the *Per1* promoter in the SCN. Samples would need to be taken at least every 4 hours from animals kept in DD.

It is still possible that the mutation affects coupling between oscillators within and/or distal to the SCN. Interestingly, there were a greater number of of PER1-ir cells 9-hours after CT 15 (control) in WTs compared to dupers in the dorsal region of caudal SCN, which contains shell neuron that receive input from ventral SCN, but at the same phase in the PVN, duper hamsters displayed more PER1-ir cells. This finding supports the first alternative hypothesis - that the duper mutation alters signaling within the SCN or with extra-SCN oscillators. Mathematical modeling of the duper mutation based on behavioral data also suggests that the mutation is due to a weakening of regional oscillator coupling within the SCN (Manoogian et al., 2015). Furthermore, two mutations in mice that demonstrate rapid re-entrainment to 8-hour phase advances and delays



similar to *duper* (VPAC2 overexpression and *V1a/1b*<sup>-/-</sup>) are due to changes in coupling (Shen et al., 2000; Yamaguchi et al., 2013).

We could further examine coupling between the ventral and dorsal SCN by shifting the LD cycle (Albus et al., 2005; Meijer et al., 2010). Mice that are subjected to a 6-hour phase delay of the LD cycle demonstrate rapid resetting of the ventrolateral retinorecipient cells of the SCN core, but resetting in the dorsomedial shell is delayed for several days (Albus et al., 2005; Meijer et al., 2010). As the shell controls locomotor rhythms by communicating with the sPVN (Buijs et al., 1996; Vujovic et al., 2015), the delay in shifting may explain why it takes multiple days for WT hamsters to achieve a phase shift. To assess if the *duper* mutation is due to a change in coupling between the ventral and dorsal SCN, we could subject *duper* and WT hamsters to a 8-hour phase advance and then collect the brain 1, 2, 3, 5, and 10 days after the phase shift. Triple label immunocytochemistry for AVP, VIP, and PER1 will allow us to analyze the cell phenotype and anatomy of PER1. Based on mathematical modeling, which suggests the *duper* mutation is due to weakened coupling between oscillators, I predict that the dorsal SCN (AVP-ir cells) will take longer to complete the phase shift in *duper* than in WT hamsters. However, because *duper* hamsters are behaviorally resistant to jet lag, and locomotor rhythms are controlled by the dorsal SCN, an alternative prediction is that the dorsal SCN will achieve a phase shift more rapidly in *duper* compared to WT hamsters.

We can also examine coupling between the rostral and caudal SCN by modifying the photoperiod. AVP, but not VIP, is present in rostral SCN. In contrast to dorsomedial AVP expression in mid- and caudal-SCN, AVP is distributed evenly throughout rostral SCN (Morin et al., 2007; Yan et al., 2007). In a long photoperiod, the rostral and caudal

SCN desynchronize and the peak phase of electrical activity of subpopulations of the SCN is widely distributed in mice (Inagaki et al., 2007; Meijer et al., 2010) and hamsters (Jagota et al., 2000). In short days, the range in phase of peak electrical activity is much more narrow (Meijer et al., 2010). To test coupling between the rostral and caudal SCN in duper hamsters, we can subject duper hamsters to a long photoperiod and then label AVP using ICC to observe the phase of the subpopulations of the SCN on multiple days after exposure to the new LD cycle. If the duper mutation weakens coupling as expected, I predict that the rostral and caudal SCN will fall out of phase faster once exposed to a new photoperiod compared to WTs.

It is also possible that the mutation alters the TTFL distal to PER1 translation. This is supported by an increase in co-labeled VIP and PER1-ir cells, but not c-FOS-ir cells, 2-hours after a light pulse in duper hamsters compared to WTs. Mutations in post-translational regulators have demonstrated similar aspects of the duper phenotype. The *Ckl1 $\epsilon$ <sup>-/-</sup>* mutant mouse exhibits large phase shifts similar to duper, but unlike duper, exhibit a long period in DD (Meng et al., 2008a; Pilorz et al., 2014). We know that duper hamsters do not have the same mutation because both genomic DNA sequencing and RNA-seq data shows that there is no mutation in CK1 $\epsilon$  or 1 $\delta$  mRNA (Monecke et al., 2011; Bittman and Hogenesch, unpublished). However, it is possible that duper affects CK1 $\epsilon$  or 1 $\delta$  substrates. The *tau* mutant hamster, in which the duper mutation was originally expressed, has a period of 20 hours due to a gain of function of CK1 $\epsilon$  (Meng et al., 2008). When duper and *tau* are co-expressed (super duper), duper has an epistatic effect and decreases the period by 2 hours to ~18 hours, compared to decreasing the period by 1 hour on a WT (LVG or Bio14.6) background (Krug et al., 2011). CK1 $\epsilon$

shortens the nuclear half-life of PER1/2 by increasing the speed of ubiquitination through phosphorylation, and also aids the nuclear translocation of the PER/CRY heterodimer. In addition to acting as a transcriptional repressor, PER2 promotes CK1 $\epsilon$  activity by inhibiting autoinactivation, but inhibits its ability to phosphorylate some substrates and stimulate CRY2 phosphorylation (Qin et al., 2015). Additionally, CK2 can either stabilize PER2 (Maier et al., 2009), or cooperate with CK1 $\epsilon$  to promote PER2 degradation (Tsuchiya et al., 2009). BMAL1 is also a substrate for CK2 $\alpha$ , which aids its nuclear entry (Tamaru et al., 2009). O-GlcNAc transferase (OGT) is another post-translation modifier that reciprocally regulates GSK3 $\beta$  to modulate the speed of the clock (Kaasik et al., 2013). As post-translational modification can alter both period and phase re-setting of the molecular clock, it is plausible that the duper mutation affects post-translational regulation.

Taken together, my results suggest that the mutation may alter the post-translational or –transcriptional regulation of Per1 in the SCN and may also modify signaling between the ventral and dorsal SCN or the SCN and extra-SCN oscillators, such as the PVN. Because PER1-ir cells in the SCN had similar mean intensity in duper and WTs, I conclude that phase shifts are not achieved by increasing PER1 in response to light. However, it is unclear whether light increases the duration of elevated PER1-ir cells in the SCN as both WT and duper hamsters both displayed increased PER1 expression 9 hours after the light pulse. Additional samples taken 10-12 hours after the pulse would be needed to answer this question.

### **Future Directions**

Additional anatomical (sPVN and PVN) and phenotypic (AVP co-labeled) analysis of PER1-ir and c-FOS-ir cells will further clarify changes in extra-SCN oscillators. Although we have learned a great deal from the duper mutation, we need to identify the gene in order to understand the mechanism. We have produced a line of dupers on a novel ecotype, which enables us to use fast homozygosity mapping to rapidly identify the affected locus. Once the gene is identified, further studies will be necessary to confirm the predicted mechanism.

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