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# Pacemaker Heterogeneity in the Suprachiasmatic Nucleus: Origins and Network Implications

Alexis Webb
*Washington University in St. Louis*

WASHINGTON UNIVERSITY

Division of Biology and Biomedical Sciences

Program in Neuroscience

Dissertation Examination Committee:

Erik D. Herzog, Chair

Paul S. G. Stein

Timothy E. Holy

Paul H. Taghert

Kurt A. Thoroughman

Paul A. Gray

PACEMAKER HETEROGENEITY IN THE SUPRACHIASMATIC NUCLEUS:

ORIGINS AND NETWORK IMPLICATIONS

By

Alexis Brooke Webb

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**ABSTRACT OF THE DISSERTATION**

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Professor Erik D. Herzog, Chairperson

In mammals, the suprachiasmatic nuclei (SCN) in the ventral hypothalamus function as a circadian pacemaker, controlling daily rhythms in behavior and physiology. Together the SCN contain approximately 20,000 neurons that maintain rhythms in firing rate and gene expression. Previous studies led to the assumption that single SCN neurons are capable of self-sustained circadian rhythms. Whether and which SCN neurons can maintain cell-autonomous daily oscillations has not been extensively tested. We measured PERIOD2::LUCIFERASE expression in isolated SCN neurons over multiple days to determine if all SCN neurons were circadian. We then examined neuropeptide content of the recorded neurons. We found that when isolated physically or with a blocker of cell-cell communication, SCN neurons expressed a range of circadian periods, amplitudes, and abilities to sustain cycling. Surprisingly, most cells were sloppy oscillators, switching

from rhythmic to arrhythmic or vice versa throughout their lifetime. We also found no evidence for a class of circadian-pacemaker neurons in the SCN based on neuropeptide expression. We conclude that while all SCN neurons are capable of cell-autonomous rhythms, they are intrinsically sloppy with network interactions dramatically increasing the number of circadian neurons.

We next used a mathematical model of the mammalian circadian clock to determine whether rates of gene transcription, protein translation, degradation or phosphorylation might explain the ability of SCN neurons to switch between circadian and arrhythmic behaviors. We found that rhythmicity was more sensitive to the rates of protein translation and degradation. We next tested what effect having neurons with different intrinsic circadian behaviors would have on population synchrony. We simulated cells of known circadian phenotypes (e.g. arrhythmic, damped, or self-sustained) in a pattern defined by small-world network properties and varied the positions and proportions of each oscillator type. We found that increasing the number of damped oscillators or placing them in highly connected locations within the network both augmented the rate at which the network synchronized. We conclude that the SCN likely benefit from a heterogeneous population of oscillators, especially when recovering from an environmental perturbation that causes desynchrony.

Finally, we generated and characterized two independent lines of transgenic mice to test the role of vasoactive intestinal polypeptide (VIP) neurons in circadian rhythmicity.

These mice express Yellow Fluorescent Protein (YFP) under the control of a fragment of the VIP promoter in VIP neurons of the SCN, neocortex, olfactory bulbs, and enteric nervous system.  We crossed these mice to generate a line in which VIP neurons are targeted for deletion using Cre-mediated recombination upon addition of tamoxifen. We observed successful deletion of VIP neurons in cultured SCN explants, but have no evidence to date for deletion of SCN neurons *in vivo* using a variety of protocols. We conclude that our construct is faithfully expressed in VIP neurons and that *in vitro* experiments show promising results for further study.

# **Acknowledgements**

I interviewed at Washington University in January 2004, arriving from Baltimore the day after my birthday. It was my first graduate school interview and I wasn't sure what to expect. I had spent the last 1.5 years teaching high school chemistry to inner city kids and was a little worried how my scientific chops would hold up during the interview process. Thankfully, everyone I met in the Neuroscience program at Wash U over that weekend showed me what a welcoming research community there was to be a part of in Saint Louis. It made my decision to attend graduate school here an easy one. Now, 6 years later, I am so happy to have made Saint Louis my academic and personal home. So many individuals have aided in my professional development and so many have been integral colleagues and friends in this process. I feel very lucky to have had such an opportunity.

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## Chapter 2.

# **Abbreviations**

| | |
|---|---|
| AVP | arginine-vasopressin |
| BAC | bacterial artificial chromosome |
| BRN | basal retinal neuron |
| CBDR | clutter based dimensional reordering |
| CCD | charge-couple device |
| CWT | continuous wavelet transform |
| CreERT2 | Cre-recombinase fused with estrogen receptor T2 |
| *Cry* | *Cryptochrome* gene |
| DD | constant darkness |
| DTA | diptheria toxin A fragment |
| FFT-NLLS | fast Fourier transform – nonlinear least squares fit |
| FLPe | Flipase driver |
| GABA | γ-aminobutyric acid |
| GRP | gastrin releasing peptide |
| IP | intraperitoneal |
| IPSC | inhibitory postsynaptic current |
| LD | light:dark |
| LNv | ventral lateral neuron |
| *luc* | *luciferase* gene |
| MEA | multielectrode array |

| | |
|---|---|
| PACAP | pituitary adenylate cyclase-activing peptide |
| PCR | polymerase chain reaction |
| PDF | pigment dispersing factor |
| *Per* | *Period* gene |
| PER2 | PERIOD2 protein |
| PER2::LUC | PERIOD2::Luciferase fusion protein |
| ROC | receiver operating characteristic |
| SI | synchronization index |
| SCN | suprachiasmatic nucleus |
| TTX | tetrodotoxin |
| VIP | vasoactive intestinal polypeptide |
| *Vipr2* | gene encoding VPAC2 |
| VPAC2 | VIP/PACAP receptor 2 |
| YFP | Yellow Fluorescent Protein |

*"Sleep delays my life. (get up get up)*

*Where does the time go? (get up get up get up)*

*I don't know."*

- Berry/Buck/Mills/Stipe

*"Life moves pretty fast. If you don't stop and look around once in a while,*

*you could miss it."*

- John Hughes

*"A little nonsense now and then is relished by the wisest men."*

- Roald Dahl

# Chapter 1.

# <u>Introduction</u>

**Properties of circadian rhythms**

Every 24 hours the Earth completes one rotation on its axis, creating periods of light and dark, day and night for all organisms that inhabit it. This daily rotation provides a familiar external time reference that dictates behavior – when to sleep and wake, when to feed, and when to avoid predators. Almost all organisms have also evolved to keep a similar internal time without any signals from the environment. Circadian clocks, from the Latin *circa* (about) and *dies* (day), drive rhythms in nearly every aspect of physiology and continue to oscillate even in constant conditions with a period of close to 24 hours. These rhythms can be set or entrained by external cues, such as light, and are stable in the face of temperature changes (Pittendrigh and Daan 1976). In this way, organisms can adapt their internal state to the environment, for example, changing the phase of biological processes, as days get longer or shorter. Proper timing of such events throughout the day and relation to each other greatly improves the fitness of the organism (DeCoursey, Krulas et al. 1997). It is known that circadian dysfunction in humans, such as chronic jet lag and shift work, has adverse health affects, with links to metabolic disorders and cancer (reviewed in (Sahar and Sassone-Corsi 2009). This thesis will address questions regarding the intrinsic cellular characteristics and network organization of the circadian system that lead to these and other properties.

**Single cell circadian oscillators across phyla**

Circadian clocks have evolved multiple times, likely independently, across the tree of life and are found in prokaryotic cyano- and proto-bacteria, as well as the "crown eukaryotic"

kingdoms of fungi, plants, and animals (Dunlap 1999). The existence of clocks amongst

diverse species of life is further evidence that the coordination and timing of daily events

is beneficial to an organism (Paranjpe and Sharma 2005). Appropriate interaction

between biological timing systems and the environment is crucial to survival, whether it

is the proper temporal niche of foraging behavior for rodents to avoid predation or the

yearly migration of monarch butterflies based on the position of the Sun in the sky, which

changes based on both latitude and season (Froy, Gotter et al. 2003). It is now evident

that niche switching between diurnal and nocturnal behavior is dictated by retinal input to

the circadian clock (Doyle, Yoshikawa et al. 2008). Recent work in *Danaus plexippus* has

revealed that the circadian clock lies in the butterfly's antennae, coordinating rhythms

with Sun-compass migration (Merlin, Gegear et al. 2009).

While daily rhythms are most easily recognized in the behavior of an entire animal, even

single cells can be competent circadian oscillators. Among prokaryotes, unicellular

cyanobacteria *Synechoccocus elongatus* show circadian controlled expression of most of

their genome. Real-time bioluminescence recordings show that cyanobacterium can

exhibit stable gene expression rhythms (Mihalcescu, Hsing et al. 2004; Amdaoud,

Vallade et al. 2007). Using a bacterial luciferase reporter and a cooled CCD camera with

high quantum efficiency, Mihalcescu and colleagues were able to detect light from single

cyanobacterium on the order of 10-20 photons per minute, per cell. Results indicate that

the clocks in cyanobacteria are quite stable, impervious to perturbations including cell

division or the activity of neighboring cells.  Remarkably, the period and cycle-to-cycle

precision of the cyanobacterial oscillator can be reconstituted in a test tube by incubating

three proteins (KaiA, KaiB, and KaiC) with adenosine triphosphate. Under these

conditions the phosphorylation state of KaiC exhibits persistent near-24 hour rhythms

(Nakajima, Imai et al. 2005).

Within multicellular organisms, the strongest evidence for single-cell circadian

pacemakers lies in recordings made from the retina of a marine snail, *Bulla gouldiana*

(Michel, Geusz et al. 1993). Basal retinal neurons (BRNs) cultured alone in a microtiter

well transition to a lower membrane conductance around dawn.  Because single BRNs do

not live for many hours during this procedure, Michel and colleagues revealed a daily

rhythm in conductance by sampling different cells around the clock. BRNs provide

critical timing information to photoreceptors and to the entire animal to coordinate daily

locomotor activity.

Potential pacemakers have also been identified in more complex organisms. In

vertebrates, rhythms in melatonin release persist in small pieces of the avian pineal

(Takahashi, Hamm et al. 1980). Perfusate was collected every 90 minutes from isolated

chicken pineal glands in a sterile flow-through culture apparatus. Melatonin levels in

perfusate samples were measured using radioimmunoassay with the concentration of

melatonin peaking during the subjective night. Melatonin rhythms continued for at least 5

days in constant conditions, suggesting that cells within the pineal are autonomous

oscillators. It is not clear which, or if all, cells are responsible for pacemaking within the

pineal. Data from mammalian cells also suggest, but do not prove, their circadian

pacemaking ability.

**The suprachiasmatic nucleus is the master circadian pacemaker in mammals**

In mammals, the master circadian pacemaker is located in the suprachiasmatic nuclei

(SCN) of the ventral hypothalamus, driving rhythms in behavior and physiology, such as

feeding, excretion, sleep-wake activity, locomotor activity, body temperature and blood

pressure regulation, and hormone release (Klein, Moore et al. 1991). The SCN are

bilateral, each nucleus located dorsal to the optic chiasm, separated by the third ventricle,

in total containing about 20,000 neurons. The SCN receive synaptic input and thus light

information from the retina via the retinal hypothalamic tract and send efferent

projections throughout the rest of the hypothalamus and thalamus (Abrahamson and

Moore 2001). The primary neurotransmitters at the site of retinal input are glutamate and

PACAP (Hannibal, Moller et al. 2000), while all SCN neurons appear release GABA and

express its receptors (Gao, Fritschy et al. 1995; Moore, Speh et al. 2002).

By anatomical convention, the SCN is subdivided into two sub-regions, the "core" and

the "shell" (Miller, Morin et al. 1996; Moore 1996). In the mouse, cells within the core

are located ventro-medially, are retinorecipient and co-express gastrin-releasing peptide

(GRP) and vasoactive intestinal polypeptide (VIP) (Abrahamson and Moore 2001). These

cells send dense projections into the shell region. The shell surrounds the core, dorsally

and laterally, and contains primarily vasopressin (AVP) containing neurons (Abrahamson

and Moore 2001). Other neuropeptides found in the SCN include calbindin, calretinin, angiotensin and neurotensin, and met-enkephalin (Abrahamson and Moore 2001).

Lesions of the SCN (Moore and Eichler 1972; Stephan and Zucker 1972; Ibuka, Inouye et al. 1977; Eastman, Mistlberger et al. 1984; Schwartz and Zimmerman 1991) or blocking its electrical activity (Schwartz, Gross et al. 1987; Schwartz 1991) result in behavioral arrhythmicity and loss of physiological cycling. Allografts of SCN tissue to lesioned animals restore rhythmicity with the period of the donor animal (Ralph, Foster et al. 1990). It is likely that these grafts contribute both neural and diffusible signals to the transplanted animal, as the donor SCN can still restore rhythms, though without the same precision and in some, but not all physiological processes, even when enclosed in a dialysis membrane that prevents connections forming with the graft (Lehman, Silver et al. 1987; Silver, Lehman et al. 1990; Ralph and Lehman 1991). These experiments show that the SCN is both necessary and sufficient to drive circadian rhythms. The nature of output signals that coordinate and regulate this behavior to the whole animal is not well understood, but several investigations have pointed to candidate molecules and mechanisms (Kalsbeek and Buijs 1992; Kramer, Yang et al. 2001; Cheng, Bullock et al. 2002; Kraves and Weitz 2006).

**A genetic clock mechanism**

The ability for genes, singly or in combination, to drive behavior is a fundamental observation in biology. The circadian system lends itself to an elegant example.

Mutagenesis screens in the fruit fly, *Drosophila melanogaster*, led to the discovery of

multiple alleles of the same gene leading to three unique behaviors (Konopka and Benzer

1971). Flies that showed long, short, or arrhythmic periods in locomotor activity each

contained a different mutation in the same gene on the X chromosome, *period*. The

population eclosion rhythm was also affected. Homologues have been found in many

organisms, including mice and humans (Shearman, Zylka et al. 1997; Sun, Albrecht et al.

1997; Tei, Okamura et al. 1997). Extensive screens have found mutations in these and

other "clock" genes that lead to changes in circadian behavior (reviewed in (Siepka, Yoo

et al. 2007).

The circadian clock has a molecular basis consisting of interlocking positive and negative

regulatory feedback loops (Reppert and Weaver 2002; Emery and Reppert 2004). Two

transcription factors, CLOCK and BMAL1 (or Mop3) heterodimerize and bind to

regulatory elements on *Period* (*Per1, 2,* and *3*) and *Cryptochrome* (*Cry1* and *2*) genes

called E-boxes, activating transcription. Subsequent translation and accumulation of PER

and CRY proteins leads to the repression of their transcription by inhibiting CLOCK and

BMAL1 (Gekakis, Staknis et al. 1998). After the degradation of the proteins, the process

begins again, taking approximately 24 hours to complete one cycle. The speed of this

molecular clock is tuned by several other elements, including kinases such as casein

kinase I epsilon, which has been shown to control phosphorylation of PER and results in

such circadian mutations as the shortened period of the tau mutant (Lowrey, Shimomura

et al. 2000). An additional negative feedback loop controlled by the REV-ERB proteins

regulates *Bmal1* transcription (Ueda, Chen et al. 2002). A positive feedback loop also

activates *Bmal1* transcription through CLOCK/BMAL1 activation of *Ror* genes and ROR

proteins binding to *Bmal1* (Sato, Panda et al. 2004).

At the organismal level, spontaneous and engineered genetic mutants have been used to

investigate the necessity for, and the redundancy amongst, clock genes. *Bmal1* is required

for circadian rhythmicity, while CLOCK is not (Bunger, Wilsbacher et al. 2000;

Debruyne, Noton et al. 2006; Debruyne, Weaver et al. 2007). Animals lacking both *Per 1*

and *2* or *Cry 1* and *2* are arrhythmic in constant conditions, while mutants lacking only 1

of these genes show some compensatory effects that help to maintain rhythms (Kume,

Zylka et al. 1999; Bae, Jin et al. 2001). A recently discovered mutation in F-box protein

FBXL3, named both Afterhours and Overtime by two independent research groups,

respectively, leads to a longer behavioral period through the stabilization of CRY

proteins (Godinho, Maywood et al. 2007; Siepka, Yoo et al. 2007).

Single SCN neurons contain these intracellular components, making them capable

individual clocks. Gene reporter technology, specifically bioluminescence, allows for

real-time recording of the molecular clockwork in a dish. Transgenic animals containing

*Per1* or *Per2*-driven bioluminescence provide a crucial tool for the study of the clock not

only in the SCN, but also fibroblasts and peripheral tissues throughout the body (Abe,

Herzog et al. 2002; Yamaguchi, Isejima et al. 2003; Welsh, Yoo et al. 2004; Yoo,

Yamazaki et al. 2004). Improvements in low-light imaging have led to single cell

resolution of clock gene expression (Welsh, Imaizumi et al. 2005). A recent study records

single SCN neurons and fibroblasts cultured from some of the mutants described above, and shows sloppy, but circadian rhythms in these cells (Liu, Welsh et al. 2007). Specifically, weak, stuttering and occasional rhythms are seen in single cells lacking *Cry1* and *Per1* or *2*, while cells lacking *Cry2* or *Per3* are predominantly rhythmic. These results support the role of intercellular coupling throughout the animal in the stabilization of intrinsically sloppy cell-autonomous rhythms, a likely reason why a single mutation does not result in behavioral arrhythmicity.


**Evidence that SCN neurons are cell autonomous clocks**

The best evidence that SCN neurons function as pacemakers comes from work monitoring electrical activity from neonatal rat SCN cells dissociated on multielectrode arrays (Welsh, Logothetis et al. 1995). Firing rate rhythms recorded from individual neurons plated at low density ($\sim 3,000$ mm$^2$) continue in constant conditions with a range of phases and periods, seen in approximately 50% of recorded cells, similar to other studies at this density (Liu, Weaver et al. 1997; Herzog, Takahashi et al. 1998). Furthermore, addition of the sodium-channel blocker tetrodotoxin (TTX) for several days blocked firing and presumably cell-cell communication, but did not appear to stop the timekeeping of individual neurons. However, it is likely that even low-density cultures were not free of cell-cell communication, as neurons exhibited inhibitory synaptic currents (Welsh, Logothetis et al. 1995).

Yamaguchi and colleagues were the first to record gene expression, specifically *Period1*-mediated bioluminescence, with single cell resolution in a slice preparation, showing that rhythms continue in the absence of coupling and further strengthening the hypothesis that single SCN neurons are cell autonomous oscillators (Yamaguchi, Isejima et al. 2003). However, the question of whether single SCN neurons are in fact, sustained, autonomous circadian pacemakers remains. A goal of this thesis is to test whether or which SCN neurons would be rhythmic when completely isolated from all other cells. **Chapter 2** investigates whether an identifiable class of pacemakers exists in the SCN using long-term bioluminescence imaging, firing rate recording, and immunocytochemistry. Surprisingly, we find that there is no distinct class of SCN pacemakers and when isolated neurons are unstable oscillators that are likely to lose rhythmicity (Webb, Angelo et al. 2009). We conclude that while all SCN neurons have the capability to sustain cell autonomous rhythms, only a small percentage can successfully do so in the absence of cell-cell communication.

**Potential markers for pacemakers in the SCN**

In **Chapter 2** we test the hypothesis that a specialized sub-class of SCN neurons contains pacemakers by staining cells with neuropeptidergic markers following bioluminescence recording. Here we describe two potential candidates for labeling pacemakers in the SCN.

*AVP*. Vasopressin is expressed in approximately 20% of SCN neurons (Abrahamson and Moore 2001). AVP is found to be rhythmically released from SCN explants following electrical stimulation with a different period than other neuropeptides, suggesting that AVP is found in a distinct population of neurons (Shinohara, Honma et al. 1995). Brattleboro rats lack AVP, but retain circadian rhythms in light-dark and constant light (Groblewski, Nunez et al. 1981). Data suggest that vasopressin is not required for circadian rhythms in sleep-wake or firing rate, but lack of the neuropeptide does affect rhythm amplitude (Brown and Nunez 1989; Ingram, Snowball et al. 1996). Recent bioluminescence recordings of clock gene expression in mice that lack the receptor for VIP and carrying the *mPer1::luc* transgene indicate that cells in the AVP-rich SCN shell maintain molecular rhythmicity in the absence of VIP signaling (Maywood, Reddy et al. 2006).

*VIP*. Vasoactive intestinal polypeptide has the hallmarks to be a key synchronizing agent in the SCN. Approximately 15% of the 20,000 SCN neurons contain VIP and send dense projections throughout the nucleus, as almost 60% of cells, including nearly all the AVPergic neurons, posses its receptor, VPAC2 (Abrahamson and Moore 2001; Kallo, Kalamatianos et al. 2004). Both VIP and VPAC2 mRNA are rhythmically expressed under light-dark conditions in SCN neurons (Shinohara, Funabashi et al. 1999). VIP release is also rhythmic *in vitro* with a different period than AVP release (Shinohara, Honma et al. 1995) and VIP may be important in coding photic information (Shinohara and Inouye 1995). VIP application can also shift behavioral or firing rate rhythms,

suggesting it conveys timing information to SCN neurons (Piggins, Antle et al. 1995;

Reed, Meyer-Spasche et al. 2001).

Animals lacking VIP ($VIP^{-/-}$) or its receptor ($Vipr2^{-/-}$) lose rhythmicity, with only a third

retaining weak behavioral rhythms with multiple circadian periods (Harmar, Marston et

al. 2002; Colwell, Michel et al. 2003; Hughes, Fahey et al. 2004; Aton, Colwell et al.

2005). Therefore, signaling through VIP appears to be important for circadian function at

the level of the intact organism, though its exact mechanism has yet to be fully clarified

(Aton and Herzog 2005). At the cellular level, most SCN neurons from these animals

lack rhythms in firing rate, though approximately 30% show desynchronized but

rhythmic firing with a range of circadian periods (Aton, Colwell et al. 2005). Another

goal of this thesis is to test whether the subclass of VIP neurons in the SCN is essential

for rhythmicity and synchrony. In **Chapter 4** we approach this question by generating

and characterizing a mouse model to specifically delete VIP neurons.

**Cellular pacemakers – are SCN neurons specialized cells?**

The circadian clock in *Drosophila* typifies the idea that a set of neurons can function as

pacemakers. A *Drosophila* brain contains on the order of 100,000 neurons, with circadian

rhythms under the control of a group of about ~150 neurons or clock cells (Nitabach and

Taghert 2008). The neuropeptide pigment-dispersing factor (PDF) is found in a subset of

these clock cells and it appears they are required for proper circadian function, as animals

lacking PDF neurons show impaired behavioral rhythms (Renn, Park et al. 1999). Eight

lateral neurons or LNvs are directly light sensitive and contain PDF, which can drive rhythmicity in other clock cells (Lin, Stormo et al. 2004). LNvs receive input, function as an oscillator, and drive output, thus containing the entire circadian system in this small group of cells.

Are there rules that define a cellular pacemaker? Ideally, a cell must maintain a rhythm autonomously to ensure it can drive rhythms in other cells. A pacemaker must also have a means of output or signaling of that rhythm. The PDF neurons in the *Drosophila* clock described above meet both of these requirements. SCN neurons then have the potential to act as pacemakers by receiving inputs that influence autonomous oscillation and relaying time to other cells, but as we describe in **Chapter 2** do not always behave as such. The apparent lack of a pacemaker "class" of intrinsically rhythmic SCN neurons (Webb, Angelo et al. 2009) suggests that these cells are not specialized relative to other cells in the SCN. All SCN neurons can at one time keep and make pace. However, the network is an essential structure that drives rhythmicity in all cells and for the animal. We evaluate behaviors that arise from building networks from non-specialized SCN pacemakers and other cells, in other words, a network containing a continuum of oscillator types, in **Chapter 3**. We conclude that a network of heterogeneous cells, rather than a specialized class of pacemakers, benefits the system by aiding in flexibility and speed of recovery to perturbations.

**Strategies for neuron-specific deletion**

To understand whether specialized populations of neurons are required for a given output, we must examine how a system functions in their absence. In **Chapter 4** we hypothesize that VIPergic neurons are pacemakers and deleting these neurons will result in behavioral rhythmicity. Existing mouse models lack either the gene for the neuropeptide itself or the gene for its receptor. It is likely that in the absence of either of these molecules, compensatory signaling takes place. Data suggest that other neuropeptides, such as GRP, can signal through the VPAC2 receptor (Maywood, Reddy et al. 2006). Because a fraction of VIP- or VPAC2-deficient animals and SCN neurons from them retain rhythmicity (Aton, Colwell et al. 2005), this or other additional signaling pathways through these cells or both may be sufficient to sustain rhythms in mutant animals. We sought to develop a model where VIP neurons would be specifically deleted, thus removing these potential pacemaker nodes from the network entirely.

Genetic manipulation leading to neuron-specific deletion is a widely used tool in the *Drosophila* community. With the UAS-GAL4 system and cell death genes like *rpr* or *hid*, removing sub-populations of fly neurons is routine (Elliott and Brand 2008). Deletion of PDF neurons in the fly clock leads to a similar behavioral phenotype to the loss of *pdf* itself with the majority of animals showing arrhythmicity in constant darkness (Renn, Park et al. 1999). In the mammalian system, several strategies for conditional gene or cell deletion have emerged. The Cre/LoxP and Flpe recombination systems allow for temporally and spatially specific modification of a gene in a cell population of interest (reviewed in Heldt and Ressler 2009). Mouse lines that in combination can remove populations of cells use Cre drivers to target LoxP sites, turning on genes like diptheria

toxin A (DTA), which results in apoptotic cell death (Ivanova, Signore et al. 2005). We utilize this technology in **Chapter 4** to delete VIPergic neurons using a VIP-driven Cre in combination with a floxed-DTA gene.

**Robustness and precision of the SCN**

Circadian clocks are robust in the face of perturbations. First, clocks are temperature compensated, meaning that changes in temperature do not alter the period of the rhythm at both the animal and cellular level (Bruce and Pittendrigh 1956; Pittendrigh and Caldarola 1973; Izumo, Johnson et al. 2003). Another example of robustness is seen in the ability of circadian clocks to entrain to a range of photoperiods (Gorman, Freeman et al. 1997; Vanderleest, Rohling et al. 2009). Even in the face of stochastic noise due to low numbers of molecules inside cells, intracellular feedback loops in the SCN can continue reliably (Barkai and Leibler 2000; Gonze, Halloy et al. 2002).

A mouse in a cage with a running wheel in constant darkness is a precise machine, exhibiting rhythms in locomotor activity with a period of ~23.7 h that deviates less than 10 minutes every day (Herzog, Aton et al. 2004). *Period1*-mediated bioluminescence rhythms in SCN explants show similar variance in period when compared to behavioral rhythms, but firing rate of single neurons *in vitro* show a significantly broader distribution (Herzog, Aton et al. 2004). This suggests that while single neurons can oscillate in a circadian fashion, they are less precise than the SCN as a tissue and the physiological outputs of the whole animal. Single cells must then synchronize to each

other to drive this robust and precise behavior in the animal. How SCN neurons and the SCN network may contribute to the precision of behavior are discussed in **Chapter 3**. We use a mathematical model the molecular circadian clock and couple single cells together in networks to investigate what properties of oscillators, including heterogeneity and location, may aid in their synchronization to generate a precise output.

**Heterogeneity, plasticity, and inducible clocks**

One aim of this thesis is to examine the properties of individual cellular oscillators in the SCN. First, we identify characteristics of SCN neurons in **Chapter 2** and find that, when isolated, cells exhibit a continuum of oscillator phenotypes (Webb, Angelo et al. 2009). What are the functional consequences of the SCN being comprised of a heterogeneous and often sloppy collection of oscillators? Stochastic behavior often benefits cellular function by adding variability (Raj and van Oudenaarden 2008). While the SCN continues to function following a host of physical and genetic insults discussed above, it must also be flexible in the face of a changing environment. One can imagine that a population of unstable oscillators is more apt to alter behavior than a group of fixed ones. **Chapter 3** formally examines how networks built from heterogeneous oscillators benefit when recovering from a changing state, such as desynchrony.

Photoperiodism is an example of an environmental challenge that the SCN must cope with and recent studies have revealed plasticity in heterogeneous groups of neurons that help in adapting to changing day length (Inagaki, Honma et al. 2007; Vanderleest,

Houben et al. 2007). Neurons must be able to encode both long versus short days to provide the animal with appropriate information regarding the external environment. Using both multi-unit electrical activity and bioluminescence recordings data show that both changes in the phase of rhythms, not the waveform, and different subpopulations of neurons contribute to the changing of behavioral rhythms based on season.

Evidence of inducible circadian clocks also support the idea that SCN oscillators are not static and benefit from behaving in non-identical fashion.  Reindeer in the Arctic Circle must cope with constant light for most of the year. These animals are arrhythmic in during the long summer and winter months, yet can exhibit circadian rhythms in light-dark cycles that exist during the shorter fall and spring seasons (van Oort, Tyler et al. 2005). Voles, a rodent that displays ultradian rhythms in feeding, emerging from their burrows every two to three hours for a snack and thus confusing predators, can be entrained to feed on a circadian cycle when provided with a running wheel or placed on a restricted feeding schedule (van der Veen, Minh et al. 2006). When clock gene expression in the liver was profiled, animals that feed throughout the day showed arrhythmic patterns, while the running wheel or restricted feeding animals had weak circadian rhythms. These data suggest that in the real world animals cope with environmental challenges by having adaptable clocks.

**Mathematical models of SCN function**

Many aspects of the molecular and physiological properties of the SCN are low hanging fruit for mathematical modeling. Circadian stalwarts like Colin Pittendrigh and Jurgen Aschoff relied on models to describe circadian phenomena, before the age of molecular biology, low light imaging, and computers with super processors (Roenneberg, Chua et al. 2008). Early efforts modeled simplified negative feedback loops from organisms such as *Drosophila* and *Neurospera* (Leloup and Goldbeter 1998; Smolen, Baxter et al. 2001) and have since grown to highly detailed complex systems with dozens of equations (Forger and Peskin 2003). Predictions from models like these have led to biological discoveries that were counter to prevailing thought, such as the tau mutation being caused by a gain of function in casein kinase 1 epsilon leading to hyperphosphorylation of PER protein (Gallego, Eide et al. 2006). Models can be deterministic, producing identical, unchanging oscillations over time (Leloup and Goldbeter 2003) or stochastic, which add noise to the system (Forger and Peskin 2004). Stochastic models are better equipped to handle noisy and unstable biological data, and can replicate and predict stuttering behaviors of single neurons from circadian mutants (Liu, Welsh et al. 2007; Mirsky, Liu et al. 2009).

The study of synchronization of these individual oscillators is a second effort in the modeling community. Synchrony has fascinated modelers since Arthur Winfree and Kuramoto, whose models utilize weak coupling to join limit cycle oscillators with a common phase and period (Winfree 1980). Newer models have examined signaling pathways involved in coupling, including VIP (Gonze, Bernard et al. 2005; To, Henson et al. 2007), the heterogeneity of oscillators that are coupled, including damped and non-

rhythmic cells (Antle, Foley et al. 2003; Gonze, Bernard et al. 2005; Antle, Foley et al. 2007; Bernard, Gonze et al. 2007; To, Henson et al. 2007), and the structure of the connections between oscillators that may aid in synchrony (Vasalou, Herzog et al. 2009). Emergent behavior of the system, such as whether a coupled model can replicate seasonal changes, i.e. function as a clock in the real world, is also of interest (Beersma, van Bunnik et al. 2008); Taylor, Webb et al., in press). In **Chapter 3** we first examine the origins of oscillator heterogeneity by probing multi-dimensional parameter space of a deterministic model of the molecular clock. We then ask whether this heterogeneity has implications on the dynamics of synchronization in networks of cells, specifically on the rate of resynchronization following uncoupling.

**SCN as model for study of network architecture and plasticity**

A network is a fundamental organizing principle of the nervous system, one that is placed between neuronal and higher systems level approaches (Churchland and Sejnowski 1988). Networks lend themselves to questions at both levels: by examining the nodes of a network we address questions of cellular and sometimes molecular identity. The connections that build the network are also of interest, as are the properties that emerge from the system as a whole. How do changing the nodes in the network lead to changes in the network output? What about changing the connections? All of these questions are readily addressed using the SCN as a model.

Homeostasis within a network is critical, especially as a system changes over time, either through an alteration in input or characteristics of the network, i.e. properties of nodes or the structure of connections between nodes change but the output remains similar (Marder and Goaillard 2006; Herzog 2007). How a network achieves this balance has been widely studied in a group of oscillators that drives feeding behavior of the lobster, the stomatogastric ganglion (Prinz, Bucher et al. 2004). Experimental and modeling efforts have uncovered how widely a set of properties, in this case conductances of ions across neuronal membranes, can vary and still produce a stereotypical output from the network (Taylor, Hickey et al. 2006).

Homeostasis in network output and variability across nodes is also applicable to the SCN system. The study of the intrinsic properties of nodes and the emergent properties of networks is an overarching theme of this thesis. First, we describe in **Chapter 2** how an SCN neuron could behave with many different circadian phenotypes, even switching between rhythmic and arrhythmic states (Webb, Angelo et al. 2009). Cellular plasticity within a network creates a many potential configurations that could lead to the same output; in **Chapter 3** we observe over a small range of combinations of parameters for clock genes and proteins the extent of possible circadian behavior. When we couple a heterogeneous system of oscillators a coherent output is reached more readily than with an all sustained system. We conclude that having dynamic, changeable nodes benefits a network by providing flexibility and large number of solutions. We develop a model system to probe a subset of SCN cells in **Chapter 4**. By specifically removing VIPergic

neurons, we now have a tool to examine the necessity of a population of nodes on network output.

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# Chapter 2.

## <u>Single SCN neurons are unstable circadian oscillators</u>

This chapter contains the manuscript:

Webb A.B., Angelo N., Huettner J.E., and Herzog E.D. (2009) Intrinsic, non-deterministic circadian rhythm generation in identified mammalian neurons. Proc. Natl. Acad. Sci. U.S.A. *106*, 16493-8

**Abstract**

Circadian rhythms are modeled as reliable and self-sustained oscillations generated by single cells. The mammalian suprachiasmatic nucleus (SCN) keeps near 24-h time *in vivo* and *in vitro*, but the identity of the individual cellular pacemakers is unknown. We tested the hypothesis that circadian cycling is intrinsic to a unique class of SCN neurons by measuring firing rate or *Period2* gene expression in single neurons. We found that fully isolated SCN neurons can sustain circadian cycling for at least one week. Plating SCN neurons at less than 100 cells/mm$^2$ eliminated synaptic inputs and revealed circadian neurons, which contained arginine vasopressin (AVP) or vasoactive intestinal polypeptide (VIP) or neither. Surprisingly, arrhythmic neurons (nearly 80% of recorded neurons) also expressed these neuropeptides. Furthermore, neurons were observed to lose or gain circadian rhythmicity in these dispersed cell cultures, both spontaneously and in response to forskolin stimulation. In SCN explants treated with tetrodotoxin (TTX) to block spike-dependent signaling, neurons gained or lost circadian cycling over many days. The rate of PERIOD2 protein accumulation on the previous cycle reliably predicted the spontaneous onset of arrhythmicity. We conclude that individual SCN neurons can generate circadian oscillations; however, there is no evidence for a specialized or anatomically-localized class of cell-autonomous pacemakers. Instead, these results indicate that AVP, VIP and other SCN neurons are intrinsic, but unstable circadian oscillators which rely on network interactions to stabilize their otherwise noisy cycling.

**Introduction**

Circadian pacemakers are schematized as intracellular transcription-translation negative feedback loops (Reppert and Weaver 2002). In mammals, transcription factors including CLOCK and BMAL1 promote the expression of clock genes including *Period 1 (Per1)* and *2 (Per2)*. The protein products of these genes return to the nucleus following a delay of many hours to repress their own transcription. Genetic deletion of these repressors abolishes circadian rhythms in behavior and physiology (Bae, Jin et al. 2001). The strongest evidence for cell-autonomous, circadian rhythm generation in mammals comes from transcriptional rhythms measured from primary and immortalized fibroblasts (Nagoshi, Saini et al. 2004; Welsh, Yoo et al. 2004).

The mammalian suprachiasmatic nucleus (SCN) of the anterior hypothalamus coordinates daily rhythms including sleep-wake and hormone release (Ralph, Foster et al. 1990). Multielectrode array (MEA) recordings of neuronal firing and luciferase-based reporters of *Per1* and *Per2* expression showed dissociated SCN neurons in the same culture with different circadian periods (Welsh, Logothetis et al. 1995; Liu, Welsh et al. 2007). Furthermore, $Na^+$-dependent action potentials, VIP and its receptor, VPAC2, are required for cellular synchrony and maintaining daily oscillations across the SCN (Yamaguchi, Isejima et al. 2003; Maywood, Reddy et al. 2006). Taken together, these results suggest that single SCN neurons are competent circadian oscillators. However, which, if any, SCN neurons are capable of autonomous rhythmicity in the absence of input from other cells is unknown.

Several classes of neurons within the SCN have been proposed to be intrinsically circadian (Antle and Silver 2005). Two neuropeptides, AVP and VIP, are strong candidates for labeling SCN pacemaking neurons. About 10% of the approximately 10,000 neurons in the unilateral SCN are VIPergic and found primarily in the ventral SCN; AVPergic neurons make up a separate 20% of the population found mostly in the dorsomedial SCN (Abrahamson and Moore 2001). Because AVP and VIP release can have different circadian periods in the same organotypic SCN slice, it was hypothesized that these two groups of neurons are separate oscillators (Shinohara, Honma et al. 1995). Subsequent *in vivo* (De la Iglesia, Cambras et al. 2004) and *in vitro* (Yamaguchi, Isejima et al. 2003; Noguchi, Watanabe et al. 2004) results continue to support the idea that neurons from the dorsal and ventral SCN maintain daily rhythms. We sought to identify intrinsically circadian neurons within the SCN as AVP- or VIPergic.


Here we demonstrate for the first time circadian rhythms in gene expression and firing rate from isolated SCN neurons and their subsequent identification by immunocytochemistry. We find intrinsically rhythmic AVP and VIP neurons. However, not all AVP or VIP SCN neurons are circadian. Furthermore, SCN neurons isolated from their network either physically or by TTX can lose or gain rhythmicity suggesting that SCN neurons are a heterogeneous population of intrinsic, but unstable circadian oscillators.

**Materials and Methods**

CELL CULTURE. SCN were obtained from 1-7 day old homozygous PER2::LUC mice (founders generously provided by J. Takahashi, Northwestern Univ.) housed under a 12h:12h light:dark schedule. For slice cultures, bilateral SCN from 300 μm coronal sections of hypothalamus were cultured on Millicell-CM membranes (Millipore) for a minimum of 3 days (methods modified from (Aton, Huettner et al. 2006). SCN explants were then inverted onto poly-lysine/laminin-coated glass coverslips and maintained at 37°C in 200 μL $CO_2$-buffered medium supplemented with 10% newborn calf serum (Invitrogen) for at least 2 days prior to recording. Dispersed neuron cultures were generated as published (Herzog, Takahashi et al. 1998). Viable cells were plated onto poly-lysine/laminin-coated coverslips attached to 35-mm culture dishes and maintained in 1 mL of $CO_2$-buffered medium that had been conditioned by primary cultures of cortical astrocytes (Banker and Goslin 1991) supplemented with 10% newborn calf serum (Invitrogen) at 37°C. All bioluminescence recordings from dispersed single cells began on *in vitro* day 4 and continued for a minimum of 6 days.



WHOLE-CELL PATCH CLAMP RECORDING. We perfused cultures at 1-2 ml/min with Tyrode's solution containing 150 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES (pH adjusted to 7.4 with NaOH). Whole-cell electrodes were filled with 140 mM cesium glucuronate, 5 mM CsCl, 5 mM $MgCl_2$, 10 mM EGTA, 5 mM ATP, 1 mM GTP, and 10 mM Hepes (pH adjusted to 7.4 with CsOH). The reference electrode was in a well with internal solution connected to the bath by an

agar bridge equilibrated with 4 M KCl. Once whole-cell recordings were established, we perfused with external solution: 120 mM NaCl, 3 mM KCl, 1 mM $NaH_2PO_4$, 4 mM $NaHCO_3$, 10 mM glucose, 5 mM HEPES (pH adjusted to 7.4 with NaOH). Spontaneous IPSCs were recorded at 0 mV (Axopatch 200A amplifier; Molecular Devices, Sunnyvale, CA; filtered at 1 kHz,10 kHz sample rate). After recording IPSCs for 30-60 s in control external solution the superfusion was switched to external solution containing 10 mM KCl. IPSC frequency was quantified using p-Clamp software (Molecular Devices).

SINGLE-CELL BIOLUMINESCENCE RECORDING. We imaged (Versarray 1024 cooled-CCD camera; Princeton Instruments) low density dispersals in 1 mL of air-buffered medium supplemented with 0.1 mM beetle luciferin (Xenogen) and SCN slices in 500 µL at 37°C in sealed Petri dishes. Photon counts were integrated over 1 h with 4 x 4 binning using WinView and quantified using Image J software. To confirm cell health at the end of recording, we added forskolin (10 µM, Sigma) to the medium and recorded for at least 3 more days.  In some SCN slice recordings, we replaced 500 µL of the recording medium with medium containing 0.5 µM TTX (Sigma) and 0.1 mM beetle luciferin. After 6 days, TTX-containing medium was removed and washed with 1-2 full exchanges of recording medium.

MULTIELECTRODE ARRAY (MEA) RECORDING. Long-term firing rate patterns were recorded from cells dispersed from the SCN of C57Bl/6 mice (Harlan; Indianapolis, IN). The SCN from pups (postnatal age 3-5 days) were pooled, dispersed and plated at

approximately 300-700 cells/mm$^2$ or 10,000 cells/mm$^2$ onto multielectrode arrays (Multichannel Systems) according to published methods (Herzog, Takahashi et al. 1998; Aton, Colwell et al. 2005). Every 10 min, the number of spikes with identical amplitude and duration was totaled and stored. Using off-line analysis similar to published methods (Meister 1994; Meister, Pine et al. 1994), we counted action potentials of similar amplitude and duration from individual neurons. The shape of the spike and presence of a clear refractory period were used as criteria for single-unit activity.

ISOLATION OF SINGLE CELLS. Individual neurons in very low-density cultures were identified for isolation following 2 to 3 days of initial bioluminescence recording. All other cells were removed by scraping them from the surface of the culture using a glass micropipette and micromanipulator. Cellular debris was rinsed away using medium in the culture.

IMMUNOCYTOCHEMISTRY. Following 6 days of bioluminescence recording, we treated low density dispersals with colchicine (50 μg/mL, Sigma) for 8-12 h to maximize neuropeptide content. Cultures were then fixed overnight in 4% paraformaldehyde and double-labeled for AVP and VIP using published methods (Aton, Colwell et al. 2005). We stained cultures with anti-AVP (mouse monoclonal, 1:50, gift from Dr. H. Gainer) and anti-VIP (rabbit, 1:2000, Diasorin) followed by donkey anti-mouse-IgG conjugated to Texas Red (1:100, Jackson ImmunoResearch) and donkey anti-rabbit-IgG conjugated to Cy2 (1:50, Jackson ImmunoResearch). Nuclei were visualized using DAPI staining.

Immunopositive cells were counted by two independent observers. An observer blind to the PER2 expression in each cell scored its neuropeptide content from superimposed immunofluorescence and bioluminescence images.

Very low density and control SCN cultures were stained with anatomical markers of neuronal processes and synaptic densities, respectively, anti-beta-3-tubulin (rabbit, 1:500, Covance) and anti-SV2 (mouse monoclonal, 1:50, gift from Dr. K. Buckley) followed by donkey anti-mouse IgG conjugated to Texas Red (1:100, Jackson ImmunoResearch) and donkey anti-rabbit conjugated to Cy2 (1:50, Jackson ImmunoResearch). For all antibodies tested, fluorescence was imaged across cultures with identical exposure time and gain.

ANALYSIS OF RHYTHMICITY. We measured the period of single-neuron PER2::LUC expression and firing rate by FFT-NLLS (Plautz, Straume et al. 1997) and Chi-squared periodogram (Sokolove and Bushell 1978). Cells with a statistically significant period between 14-36 h by both methods were scored as circadian. Confidence intervals were set to 95% and 99% for FFT-NLLS and Chi-squared periodogram, respectively.

ROC ANALYSIS. We used signal detection theory to determine the probability that an ideal observer could predict the cycling behavior of a neuron based on the slope of PER2 accumulation in the previous cycle. For a given slope criterion, we plotted the proportion

of circadian and arrhythmic neurons with greater slopes, generating a receiver operating characteristic (ROC). The area under a ROC is equivalent to the odds of correct detection by an ideal observer (Green and Swets 1966). To calculate each point along the ROC, the proportion of rhythmic neurons that exceeded a given slope criterion were plotted against the proportion of neurons that lost cycling whose slope exceeded the same criterion.

**Results**

**Circadian PER2 expression is rare in isolated SCN neurons.**

We tested the hypothesis that there is a class of specialized, intrinsically circadian neurons within the SCN. Plating SCN neurons at 10,000 cells/mm$^2$ yields a population containing nearly 80% circadian neurons with a similar period (Aton, Colwell et al. 2005) and plating at 3,000 cells/mm$^2$ produces a population of SCN neurons where approximately 50% are circadian with a broad range of periods in the same culture (Welsh, Logothetis et al. 1995; Herzog, Takahashi et al. 1998; Honma, Shirakawa et al. 1998). Because inhibitory synaptic currents were still present even in the low density cultures (Welsh, Logothetis et al. 1995), we tested whether plating at 100 cells/mm$^2$, a density less than 3% of previous methods, would allow simultaneous recordings from cells in which intercellular communication is eliminated. Culture conditions were optimized by growing cells in glial-conditioned medium (Materials and Methods) so that recordings from viable neurons could begin after three days *in vitro* and continue for at least six days (Fig. 1A). Using whole-cell patch, we found that these neurons had normal sodium currents, but showed few or no postsynaptic currents compared to cultures plated
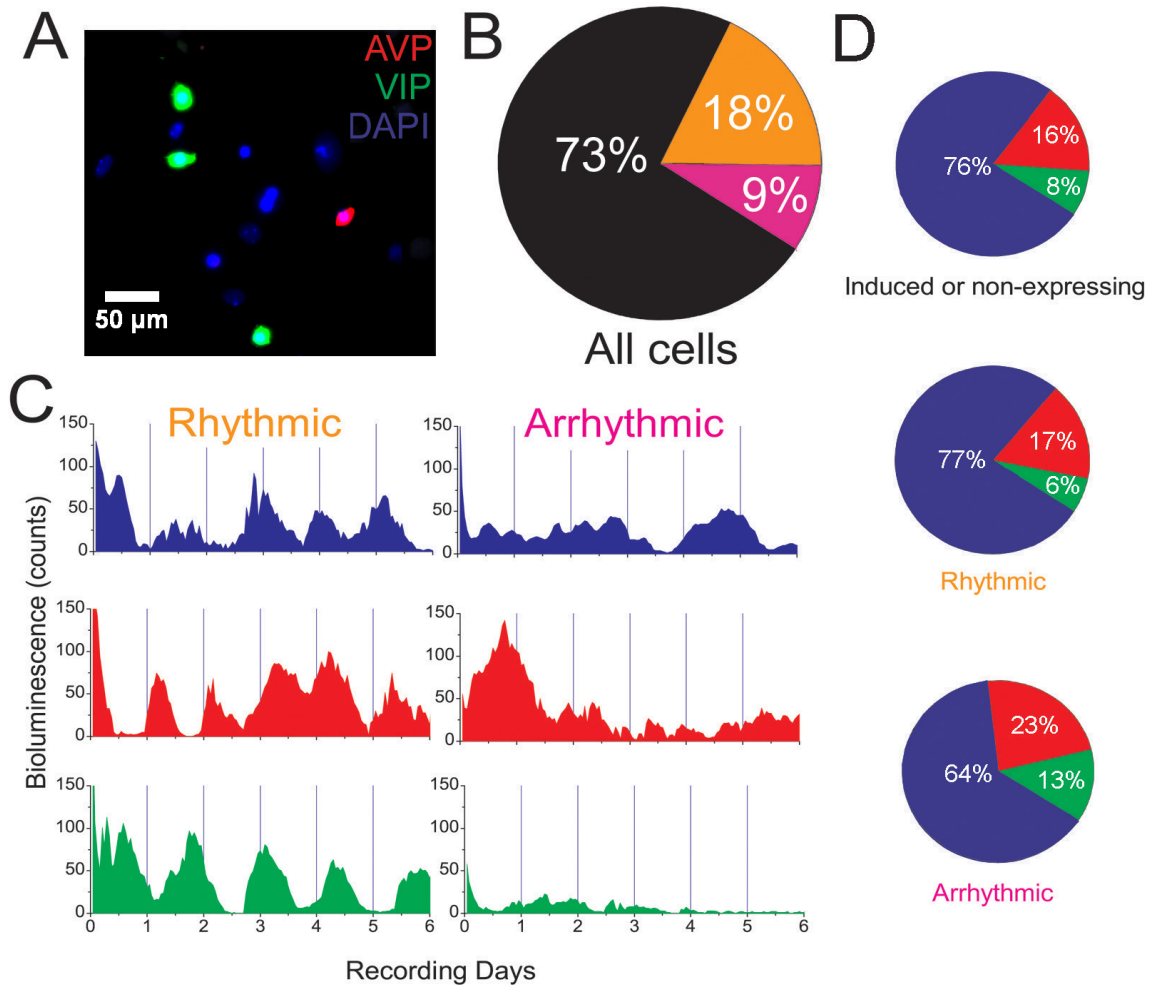
**Figure 1. Intrinsic circadian rhythmicity is rare and found in diverse classes of SCN neurons.** (**A**) A representative region of a recorded and subsequently immunolabeled very low density culture shows SCN neurons containing AVP (red), VIP (green), or neither neuropeptide (blue). (**B**) Of the neurons recorded over 6 days, 73% (n=1027 of 1413 from 14 cultures) expressed PER2-mediated bioluminescence for less than three days (black), 18% were circadian (orange) and 9% were arrhythmic (pink). Most neurons with sustained PER2 expression (n=252 of 386 in 14 cultures) were circadian (orange). (**C**) Representative bioluminescence traces from single identified SCN neurons show circadian or arrhythmic PER2 expression. (**D**) Neurons which did not express (top), or showed circadian (middle) or arrhythmic (bottom) PER2 profiles were similar in their expression of AVP (red), VIP (green) or neither (blue). Thus, multiple cell types were intrinsically circadian and neuropeptide expression did not distinguish between rhythmic and arrhythmic neurons.

at 10,000 cells/mm$^2$, even in the presence of elevated potassium (Fig. 2). We found anatomical markers suggesting some neurons synapsed on themselves in very low-density cultures, but no evidence for intercellular communication (Fig. 3D). These data suggest that, in contrast to previous recordings of dispersed SCN neurons which exhibited synaptic currents (Welsh, Logothetis et al. 1995), these very low density cultures lack intercellular communication.

We recorded bioluminescence for at least six days from individual SCN neurons cultured from PERIOD2::Luciferase (PER2::LUC) homozygous knock-in mice (Yoo, Yamazaki et al. 2004). Surprisingly, we found that most cells luminesced for less than 72 h (73%, n=1027 of 1413 neurons, Fig. 1B). This was highly reproducible between cultures so that the average culture had 71% ± 4% cells (mean ± SD, n=14 cultures), which expressed PER2 for less than 3 days. These cells were viable at the end of the recording based on phase contrast imaging, neuropeptide immunolabeling and nuclear staining. In addition, 10 μM forskolin, a cAMP analog, increased the percentage of PER2 expressing neurons after 6 days of recording to the 70% observed on the first day of recording (Fig. 3B).

Of PER2-expressing neurons, the majority were circadian (65%, n=252 of 386; Fig. 1C and 1D). Rhythmic neurons had periods from 15 to 35h with no correlation between neighbors (closest neurons were more than 50mm apart; Fig. 4) and randomly distributed times of peak bioluminescence (Rayleigh test, $r = 0.21 ± 0.09$, mean ± SD from 14 cultures, $p > 0.1$). Higher mean bioluminescence, however, did not necessarily equate

**A** High Density

10 pA
10 sec

10 pA
0.5 sec

**B**

IPSC amplitude (pA)

10 mM KCl

time (sec)

**C** Low Density

10 pA
10 sec

**D**

IPSC amplitude (pA)

10 pA
50 msec

10 mM KCl

time (sec)

**E**

n = 8    n = 12

IPSC frequency (sec⁻¹)

control
KCl

High Density    Low Density

**F**

High Density    Low Density

IPSC frequency (sec⁻¹)

-80    0

0.5 nA    1 nA

10 msec    10 msec

**G**

High    Low

peak inward current (nA)
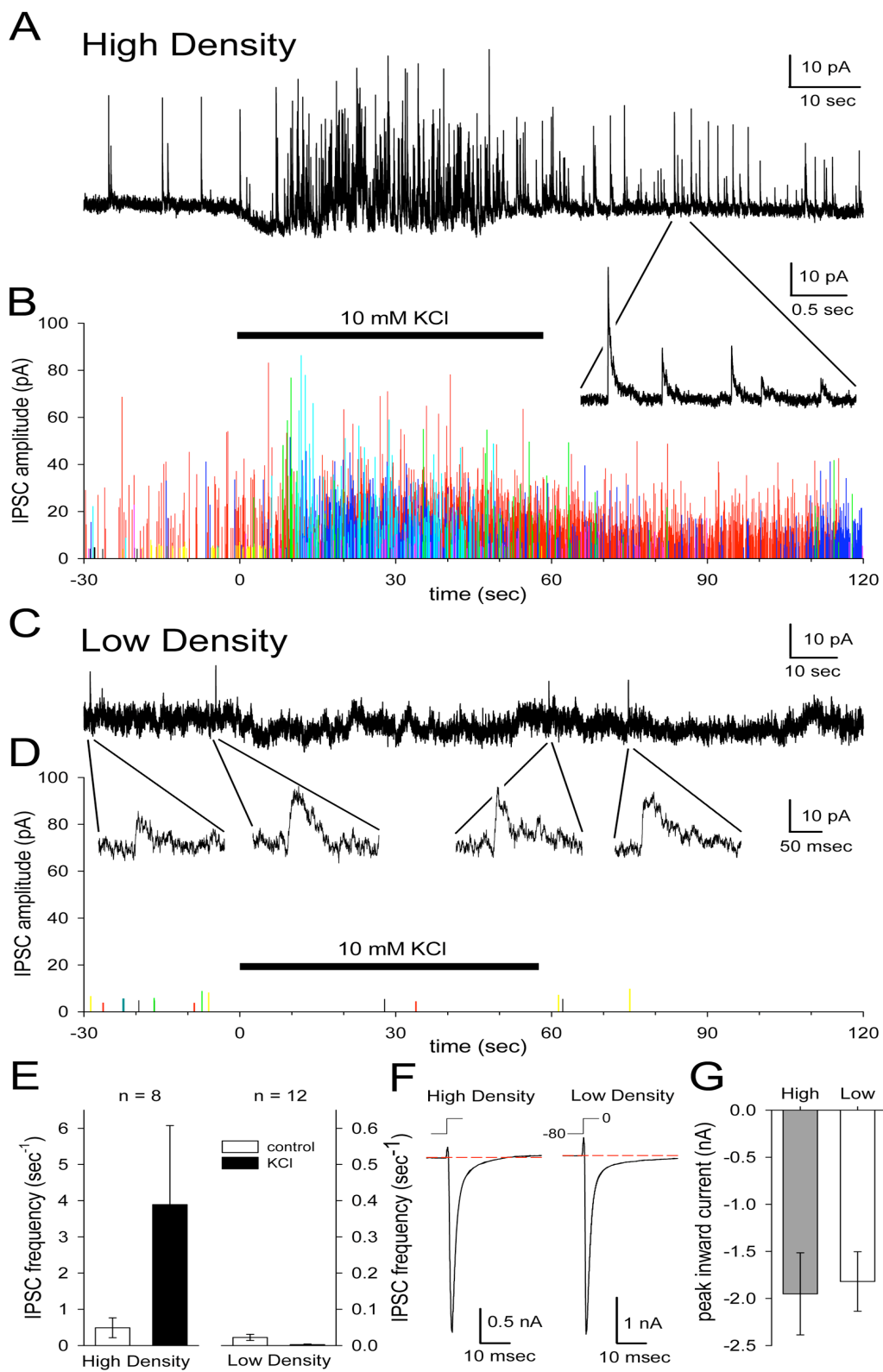
**Figure 2. SCN neurons grown at very low density are healthy and functionally isolated.** (**A**) Representative whole-cell recording of spontaneous IPSCs at 0 mV from a neuron 7d after plating at about 10,000 cells/mm$^2$. Inset shows IPSCs at higher temporal resolution. (**B**) IPSC amplitudes and frequencies were high over 2 min in 8 neurons from 1 culture (data from each neuron in a different color) grown at high density. Bar indicates the time of 10 mM KCl exposure in (**A**) and (**B**). Note the increase in evoked IPSCs. (**C**) Representative whole-cell recording of spontaneous IPSCs at 0 mV from a neuron 7d after plating at about 100 cells/mm$^2$. Insets show small current events, which may reflect rare, spontaneous IPSCs. (**D**) Plots of IPSC amplitude in 12 neurons combined from 2 cultures grown at low density. (**E**) KCl elevation boosted IPSC frequency in high density but not low-density cultures. (**F**) Inward sodium currents evoked by voltage steps from -80 mV to 0 mV in neurons grown at high and low density. (**G**) Peak inward sodium current amplitude ± SEM in neurons grown at high-density (n=8) and low-density (n=12).

A



B



C



D



Very low density

beta-3-tubulin

SV2

Control
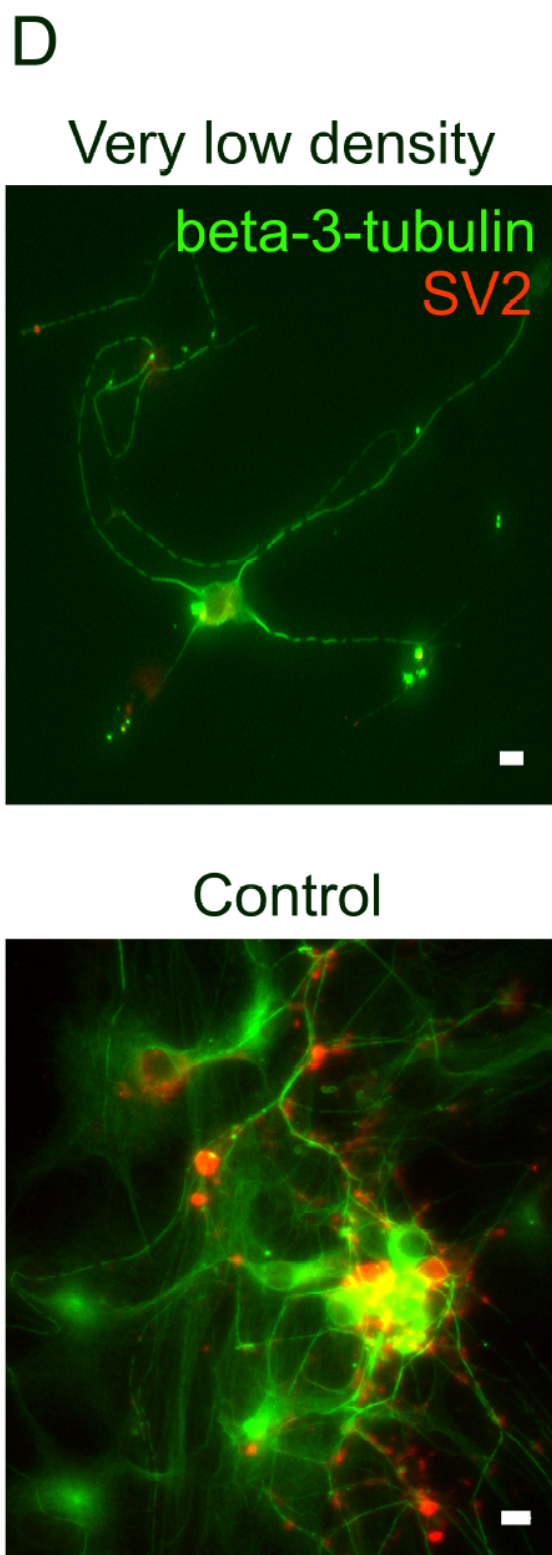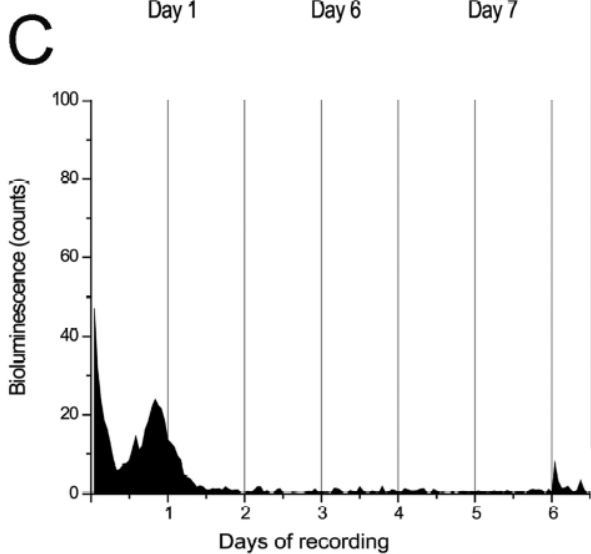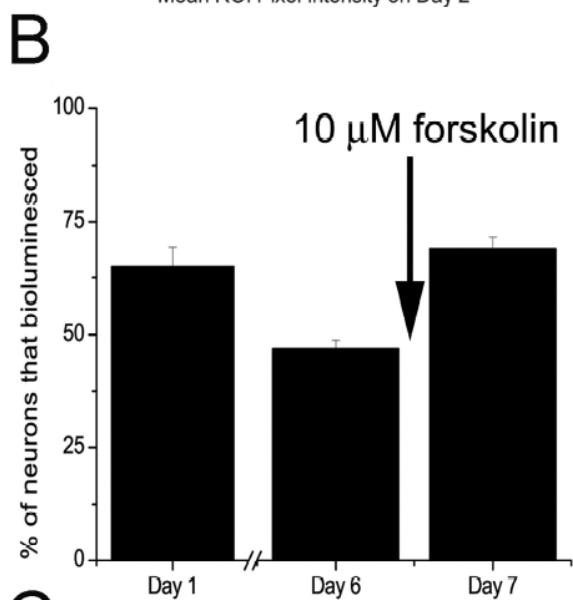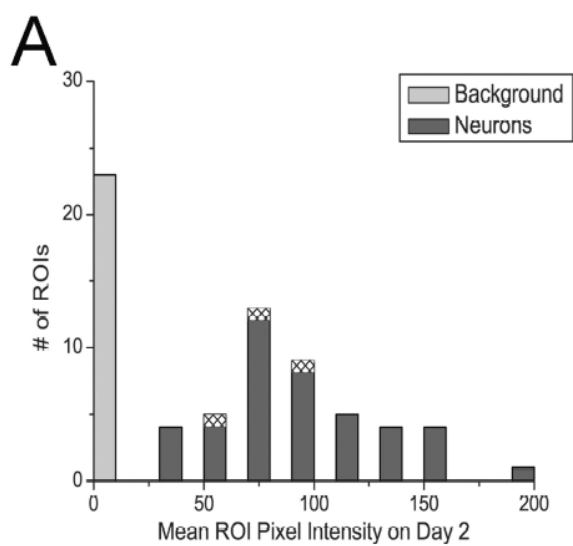
**Figure 3. Neurons in very low-density dispersals are healthy, but most do not express markers for intercellular communication.** In addition to assessments by whole-cell patch recording, multielectrode array recording of action potentials, and bright field and DAPI imaging (Figs. 1, 2 and 6), we tested the viability of low density cultures after 6 days of recording using their response to stimulation and their neuropeptide expression. (**A**) To determine a threshold bioluminescence level for non-expression, we compared maximum pixel intensity during the second 24 h of recording from rhythmic (dark gray) and arrhythmic (white crosshatch) bioluminescent neurons (n=47) or background areas with no cells (n=23, light gray). In these experiments, we found 134 cells, which luminesced well-above background levels, but were clearly not circadian. Subsequently, we defined cells as non-expressing when their maximum bioluminescence was below 5 counts per 24 h. (**B**) Forskolin restored the percentage of bioluminescent neurons to the level seen on the first day of the recording (65% vs. 69% of 241 neurons recorded in 2 cultures). (**C**) Induced and non-expressing neurons also stain for neuropeptides. A representative VIPergic neuron within the field shown in Figure 1A briefly expressed PER2 as can been seen in its bioluminescence trace. We treated all cultures with colchicine prior to fixation to diminish concern for circadian variations in the detectability of neuropeptide content. We found that the percentage of VIP- and AVP- positive neurons was similar in high density and very low-density cultures and in low-density cultures fixed between 10 and 15 days after plating. We conclude that the proportion of each class of peptidergic neuron was relatively stable over the course of the experiment. (**D**) Immunolabeling for beta-3-tubulin, a marker for axons and dendrites, and for the presynaptic protein, SV2, revealed no evidence for intercellular communication in very low-density cultures (top; 10 µm scale bar) and many sites of apposition in control cultures (bottom; 10 µm scale bar). A few neurons in very low-density cultures formed apparent synapses on themselves (autapses), consistent with the rare inhibitory-like currents recorded in these cultures. Cultures of primary cortical astrocytes showed no SV2 staining, similar to neurons plated at very low densities.
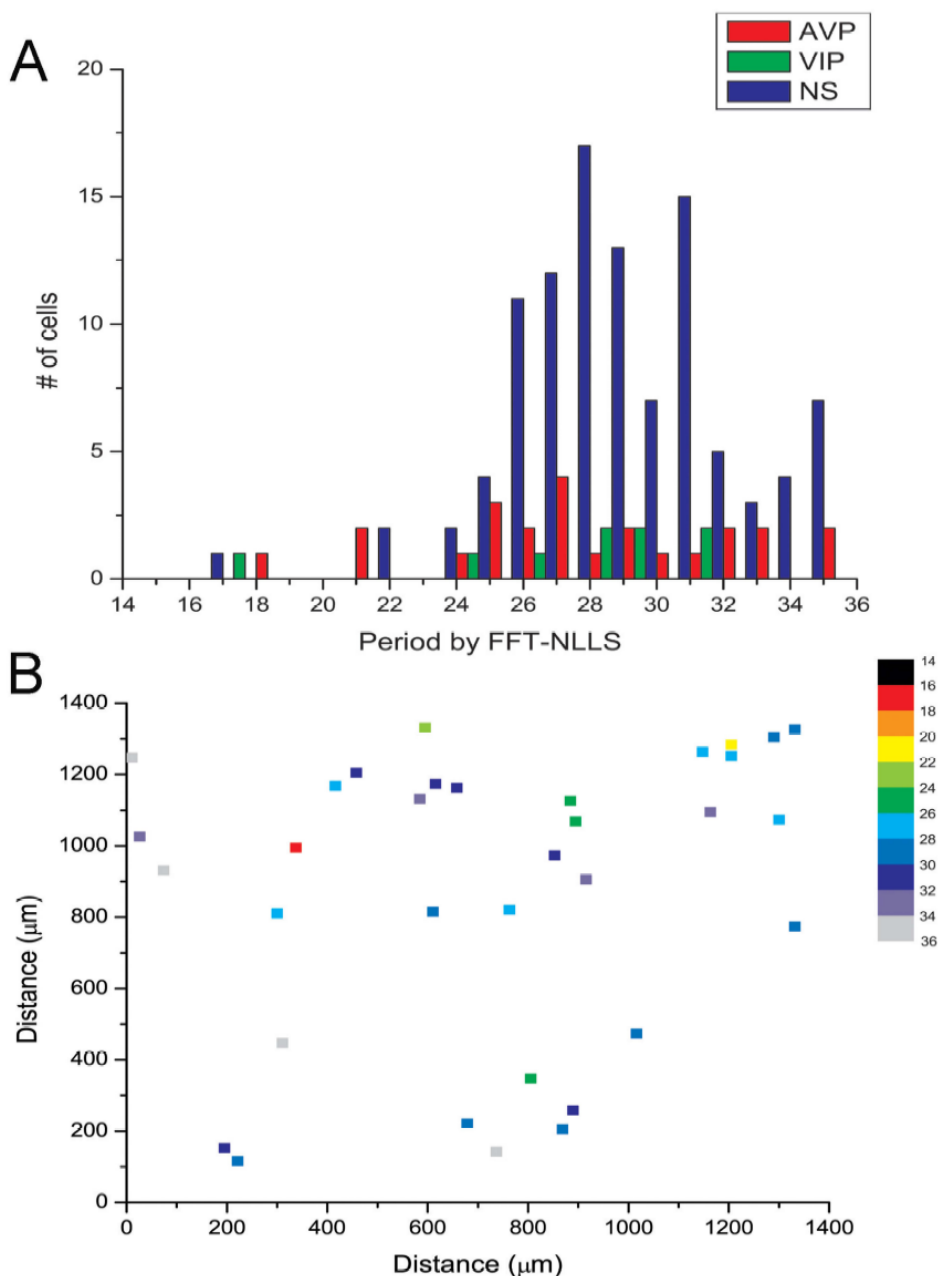
**Figure 4. Very low-density dispersals show no evidence for circadian coupling.** (**A**) Periods as measured by FFT-NLLS from AVPergic, VIPergic, or non-stained neurons (n=139 of 555 recorded and stained cells in five cultures) were broadly distributed and did not differ between neurochemical classes (Forsythe and Brown-Levine's tests, p>0.05). (**B**) Neurons (n=34) in a representative very low-density culture show uncorrelated periods ranging from 14 to 36 h. The period of each circadian neuron is plotted at the location of its soma in hours according to the pseudo-colored scale bar. Neighboring cells tended to have dissimilar periods (regression analysis comparing location with period value, p > 0.05), suggesting a lack of circadian signaling between cells within these cultures.

with rhythmicity (Fig. 5B). Thus, we found that, in contrast to the synchronous rhythms seen in densely-packed SCN neurons (Aton, Colwell et al. 2005), plating SCN neurons at very low density reveals intrinsic PER2 expression is lower in all cells and circadian in approximately 18% of neurons (252 of 1413; 16% ± 2%, mean ± SD from 14 cultures).

We next physically isolated single, bioluminescent neurons by removing all other cells in the dish. We attempted to isolate and record for 5 days from 12 neurons. We selected for cycling neurons and found that 3 of 5 remained circadian for as long as we recorded (6 days; Fig. 6). An additional two neurons were circadian, but at least one other cell was discovered in the dish at the end of the recording. The remaining 5 recorded neurons did not survive the isolation procedure for at least 5 days. Thus, approximately 60% of PER2-expressing cells were circadian in low-density cultures and when isolated. This suggests that rhythms recorded from SCN neurons plated at very low densities are equivalent to recordings from physically isolated neurons. Together, these results provide the best evidence to date that circadian rhythm generation is intrinsic to only a fraction of SCN neurons at a given time.

**Firing rate rhythms in functionally isolated SCN neurons.**

Because we can not exclude the possibility that PER2 or other cellular processes oscillate under conditions when PER2::LUC fails to report rhythms, we investigated an additional, independent reporter of activity in SCN neurons. Extracellular action potentials were
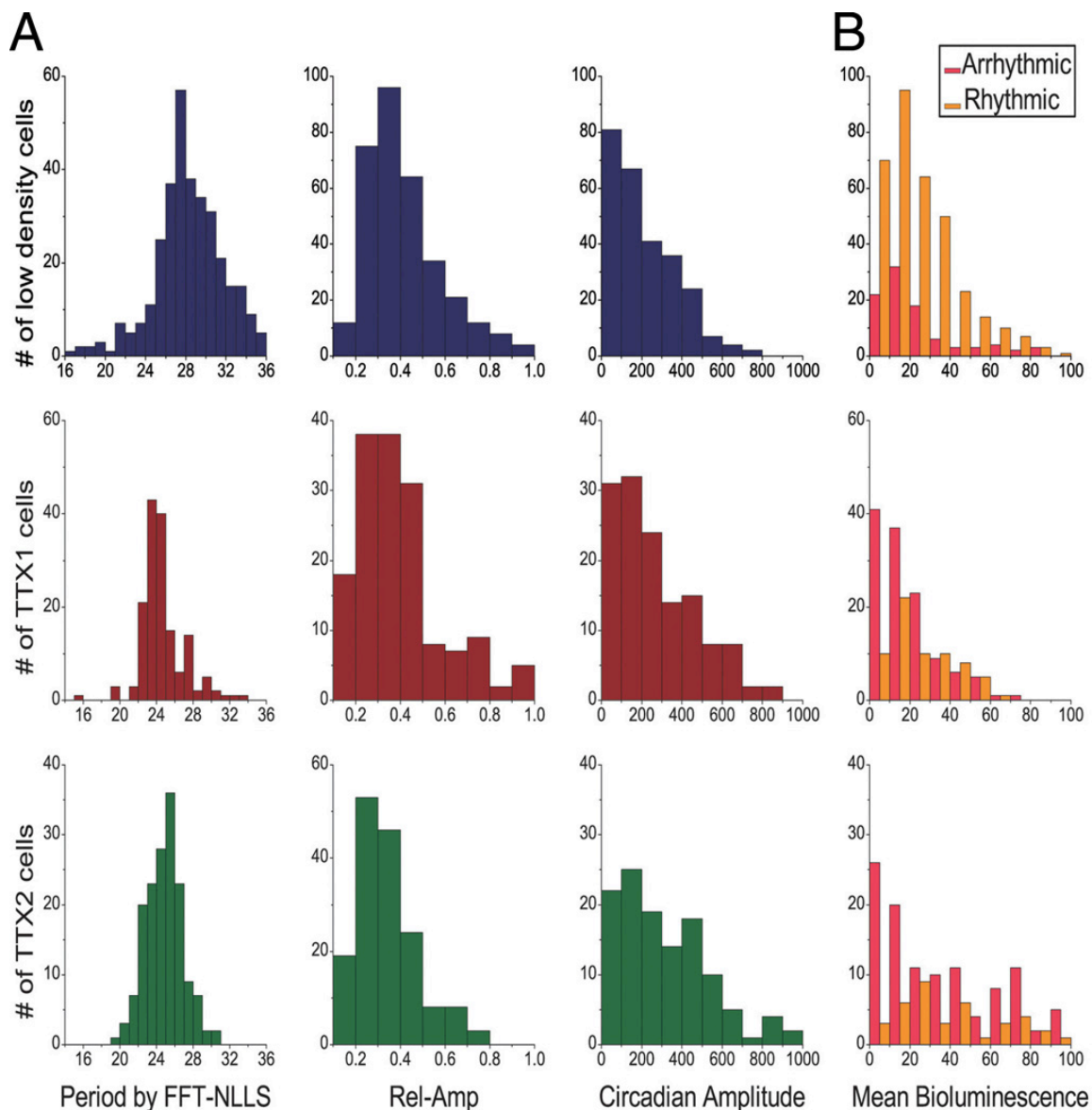
**Figure 5. Characteristics of PER2::LUC expression in single cells provide evidence for a continuum of oscillator phenotypes among SCN neurons, rather than distinct pacemaker classes.** We compared circadian properties and mean PER2 expression of SCN neurons in very low-density dispersals (n=252 neurons of 1413 recorded in 14 cultures) and in SCN explants during the first (TTX1) and second (TTX2) treatments with tetrodotoxin (TTX1, n=158 of 190 recorded in two explants; TTX2, n = 161 of 190 recorded in two explants). (**A**) Circadian neurons recorded in each of the three conditions had broadly distributed circadian properties including period, rel-amp (the ratio of the amplitude error to the most probable amplitude magnitude from a fast-fourier transform-non-linear least squares fit; (Plautz, Straume et al. 1997), and circadian amplitude (the power above the 95% confidence interval at the dominant period in a chi-squared periodogram; (Sokolove and Bushell 1978). Thus, we found no evidence for clustering of properties among circadian neurons. (**B**) The distributions of mean bioluminescence also failed to reveal any differences between rhythmic and arrhythmic neurons in these three culture conditions.
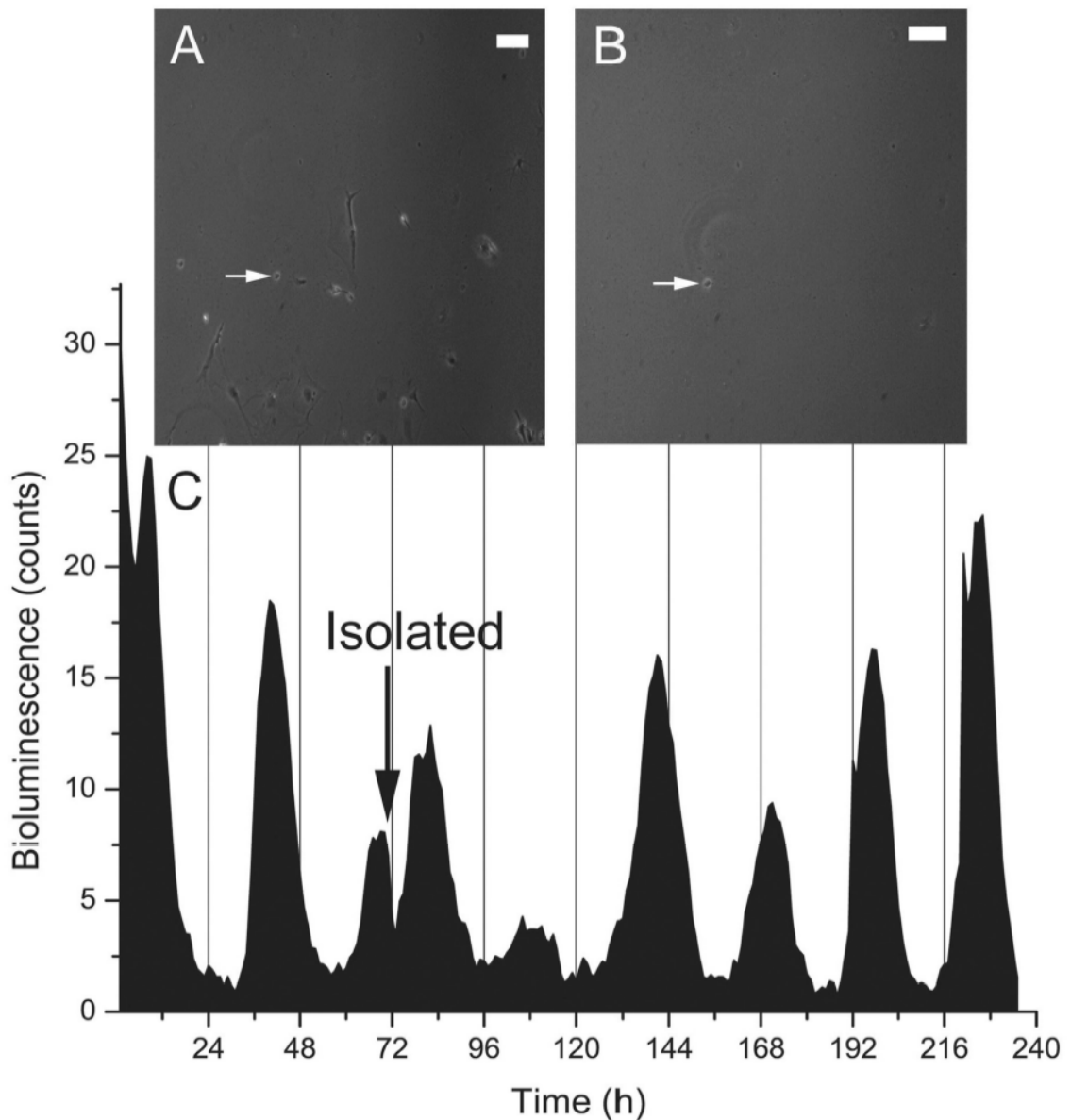
**Figure 6. Circadian rhythms in a fully isolated SCN neuron.** A single, representative SCN neuron (white arrow) recorded prior to (**A**) and following (**B**) removal of all other cells. (**C**) PER2-driven bioluminescence from this neuron had an average period of approximately 27.7 h for the last 5 days following isolation (black arrow). This SCN neuron expressed neither AVP nor VIP.

recorded from neurons one week after plating at 300-700 cells/mm$^2$ on MEAs. We found 33% of SCN neurons which discharged action potentials for at least four days showed circadian rhythms in firing rate (n=6 of 18 neurons recorded from 5 cultures; Fig. 7), consistent with the small percentage of SCN neurons that showed intrinsic PER2 rhythms. Our findings that 18-33% of neurons grown in near isolation remain circadian are consistent with the reported 23-45% of circadian SCN neurons in explants and high-density dispersals lacking VIP, VPAC2, or G$_i$ signaling (Aton, Colwell et al. 2005; Aton, Huettner et al. 2006). Although the chance remains that circadian rhythms persisted in neurons with no or arrhythmic PER2 or firing, the results indicate that the majority of SCN neurons lose circadian cycling when removed from network interactions. We conclude that single neurons are competent circadian pacemakers, which can drive rhythms in firing rate and that intercellular communication dramatically augments the proportion of rhythmic SCN cells.

**Both circadian and non-circadian SCN neurons express AVP or VIP.**

To determine the identity of intrinsically rhythmic neurons, we examined the expression of AVP and VIP following PER2::LUC profiling. The percentages of AVP- or VIPergic neurons were similar in very low density (100 cells/mm$^2$) and control (3000-5000 cells/mm$^2$) SCN cultures (20% and 17% of neurons were AVP-positive, respectively; 8% and 8% were VIP-positive, respectively; 2608 neurons counted in 5 control cultures and 555 neurons counted in 5 very low density cultures). Because these are similar to the
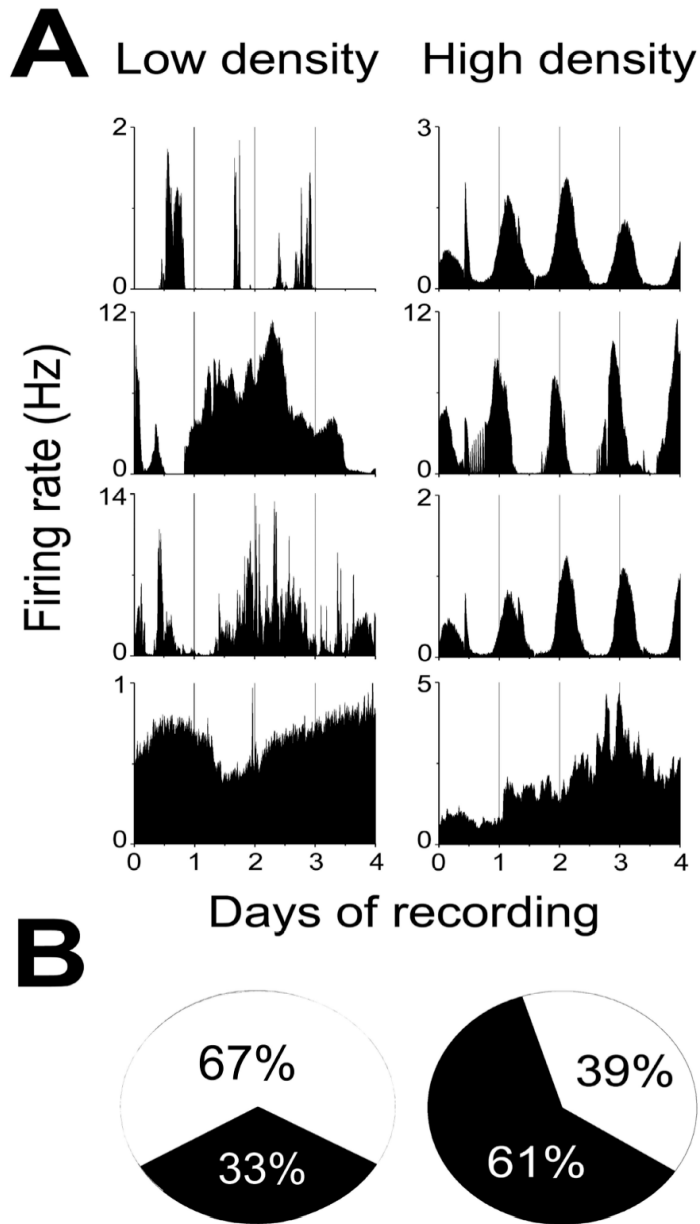
**Figure 7. Compared to SCN neurons plated at high density, only a subset of nearly-isolated SCN neurons maintain circadian firing rate rhythms.** (**A**) Firing rate traces over four days from representative neurons plated and recorded on multielectrode arrays at extremely low density show unstable circadian rhythms (top two traces, left) compared to the more precise circadian patterns in high density cultures (top three traces, right). Arrhythmic firing patterns were more common in low-density cultures (bottom two traces, left) than in high-density cultures (bottom trace, right). (**B**) The proportion of circadian (black) and arrhythmic (white) SCN neurons from low-density cultures compared to high-density cultures. Note that firing rate, like PER2, rhythms were intrinsic to a small fraction of neurons in low-density cultures.

proportions reported in the mouse SCN *in vivo* (Abrahamson and Moore 2001), we

conclude that these classes of neuropeptidergic neurons survive in SCN cultures.

We found that 23% of circadian cells (n=25 of 109 in 5 cultures examined) expressed

either AVP or VIP, while the majority (n=84 of 109) expressed neither AVP nor VIP

(Fig. 1D). Some of the cells, which did not express VIP or AVP in the low-density

cultures, may not have been neurons.  For example, astrocytes can express circadian

rhythms in PER2 (Prolo, Takahashi et al. 2005), but were rare in these cultures based on

phase contrast imaging.  Importantly, the proportion of circadian cells was similar when

comparing PER2::LUC and firing rate data suggesting that the data come from neurons.

The periods of rhythmic AVP and VIP neurons did not differ (AVP: 28.2 ± 0.9 h, mean ±

SD; VIP: 28.5 ± 0.3 h; p > 0.05, Student's t-test; p > 0.05, Brown-Forsythe's and

Levene's tests; Fig. 4A) and were similar to published results from dispersed SCN

cultures (Liu, Welsh et al. 2007). In addition, rhythmic AVP and VIP neurons did not

differ in measures of their rhythm amplitude or expression levels when compared to each

other or to rhythmic neurons that were not stained (one-way ANOVA, p>0.05). Finally,

we compared the predicted and measured likelihoods that an isolated SCN neuron was

rhythmic and expressed AVP, VIP or neither (Table 1). The calculated joint probabilities

were nearly identical to the observed patterns of neuropeptide and circadian PER2

expression suggesting that circadian rhythmicity and expression of AVP or VIP are

independent in isolated SCN neurons. This is the first demonstration that single AVP- or

| Of 555 total neurons | *Circadian Behavior* | | *Neuropeptide expression* | *Probability* | *Observed* |
|---|---|---|---|---|---|
| **Rhythmic** | 109 | | | 0.033 | 0.032 |
| | | **AVP** | 93 | 0.016 | 0.012 |
| | | **VIP** | 46 | 0.15 | 0.15 |
| **Arrhythmic** | 446 | **Other** | 416 | 0.13 | 0.14 |
| | | | | 0.07 | 0.07 |
| | | | | 0.60 | 0.60 |

**Table 1. Comparison of the predicted and observed probabilities of rhythmicity and neuropeptide expression.** To determine the predicted joint probability assuming that circadian cycling is independent of AVP or VIP expression, we calculated the chance that a neuron was rhythmic out of 555 recorded-and-stained neurons, and then multiplied that fraction by the chance a neuron expressed a given neuropeptide. We found these probabilities were similar to the observed likelihoods that a neuron was circadian and expressed AVP, VIP or neither.  For example, 3.2% of the recorded neurons were circadian and expressed AVP.  This is consistent with the prediction that, of SCN AVP-ergic neurons (93 of 555 or 17% of SCN neurons), 20% (109 of 555) will show intrinsic daily rhythms at any given time (0.17*0.20=0.033 or 3.3%).

VIP-containing neurons exhibit intrinsic circadian rhythms in clock gene expression. We conclude that intrinsic circadian cycling is not restricted to a single class of neuropeptidergic neurons in the SCN.

We also tested whether arrhythmic neurons comprised a unique cell class. Neurons which failed to express PER2::LUC rhythmically were AVP- or VIPergic with similar percentages to circadian neurons (Fig. 1D). Arrhythmic AVP and VIP neurons included those that luminesced for less than 72 h (n=92 of 382 neurons in 5 cultures) and those whose PER2 expression was non-circadian (n=23 of 64 neurons). Because AVP and VIP neurons comprise only a fraction of the circadian SCN cells, we asked whether their presence is associated with a circadian phenotype. We found that neither rhythmicity nor arrhythmicity in single SCN neurons is predicted by AVP or VIP expression ($p > 0.05$, Fisher's exact test). We conclude that both circadian and non-circadian SCN neurons form heterogeneous populations (Fig. 5).

**SCN neurons can switch their circadian phenotype.**

When we tested the viability of neurons in low-density dispersals (Figure 2B), we found that application of 10 μM forskolin initiated or ended circadian behavior in 20% of SCN neurons (n=48 of 241 neurons in two cultures). Interestingly, most neurons that changed phenotype became arrhythmic (n=39). These results, combined with the lack of evidence for a neuropeptide class of pacemaker neurons, led us to hypothesize that a cell's ability

to oscillate intrinsically is not deterministic and that circadian cycling becomes random in isolated SCN neurons.

We recorded single-cell bioluminescence under conditions where cell-cell interactions could be reversibly and repeatedly blocked.  Because SCN neurons had been shown to drift out of phase from one another in the presence of TTX and then re-establish their phase relationships when TTX was removed (Yamaguchi, Isejima et al. 2003), we treated organotypic PER2::LUC SCN explants twice with TTX for 6 days at 6 day intervals. We postulated that if circadian pacemaking is neither unique to, nor a determined property of, a class of SCN neurons, repeated TTX treatments would always reveal circadian cells, but not always the same cells.  Under baseline conditions, the periods of bioluminescence rhythms in single SCN neurons were tightly clustered ($25.7 \pm 0.3$ h, mean $\pm$ SD) with synchronized peak bioluminescence values (Rayleigh test, $r = 0.95$, $p < 0.001$). Wash treatment restored rhythmicity to the majority of recorded neurons (n=162 of 190 neurons in two explants). The restored synchrony was significant (Rayleigh test, $r = 0.57$, $p < 0.001$), but less than before TTX, suggesting that complete resynchronization requires more than 6 days.   We found that 50% of neurons lost and 13% sustained circadian rhythmicity in both TTX treatments (n=94 arrhythmic and 24 rhythmic of 190 neurons; Fig. 8). In addition, nearly 38% of neurons (n=72 of 190) gained or lost rhythmicity in the second TTX treatment when compared to the first treatment. Interestingly, the locations of neurons that maintained or increased rhythm amplitude in both TTX applications were scattered throughout the SCN. The locations of arrhythmic cells were also broadly distributed. We then treated a third SCN slice with TTX for 8 days and
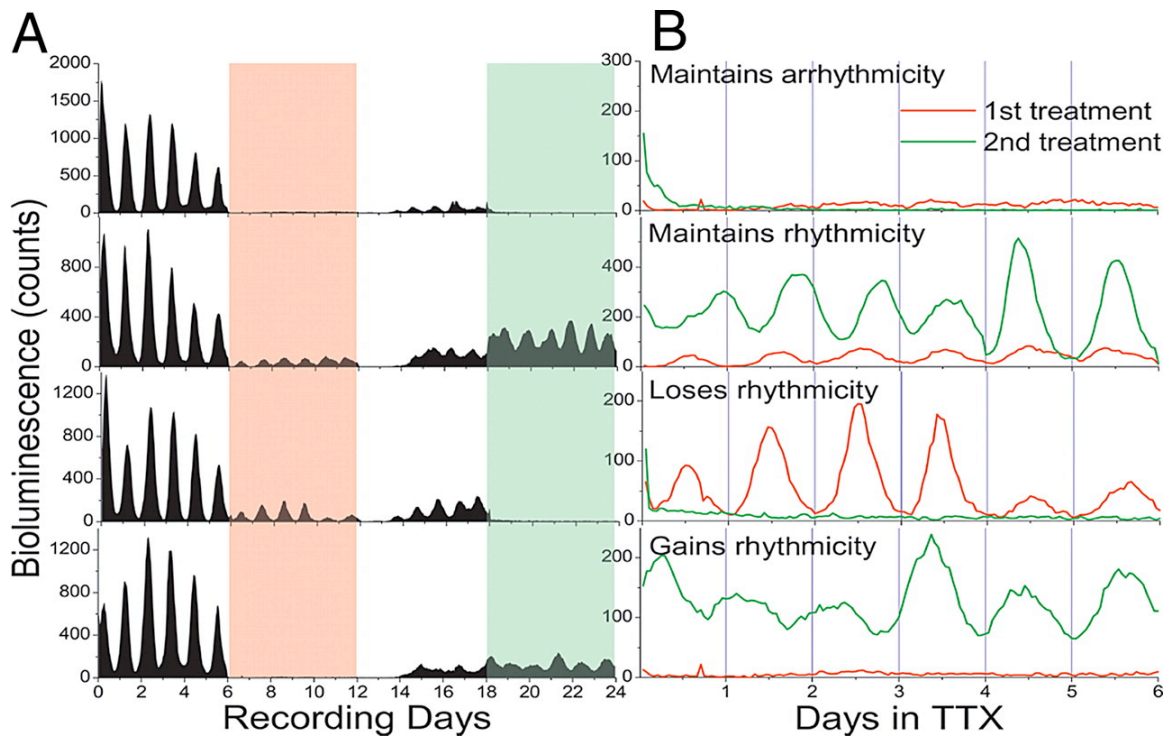
**Figure 8. Repeated TTX treatment revealed switching in circadian behavior of SCN neurons.** (**A**) Long-term recordings of PER2::LUC expression from four representative SCN cells in an explant before, during and between two 6-day TTX treatments (red and green bars). All cells showed drastically reduced PER2::LUC expression and few cells were circadian in either the first or second TTX administration. (**B**) Approximately 13% (n=24 of 190 recorded cells in two cultures) remained rhythmic during both treatments, while 38% became rhythmic or lost rhythmicity in the second TTX treatment.

found that over 70% of neurons lost rhythmicity (n=71 of 98 neurons) and that some

rhythmic neurons skipped a circadian peak (n=18 of 27 rhythmic neurons). When we

treated a control SCN explant with fresh medium every 6 days over a 24-day recording,

we found the number of circadian neurons was similar during the first (n=76 of 100

neurons) and last treatments (n=62 of 100) and no evidence for switching between

rhythmic and arrhythmic states. These observations further support the conclusion that

neurons throughout the SCN are capable of cell-autonomous circadian rhythm generation

and suggest that network interactions stabilize their intrinsically noisy oscillations.

**PER2 accumulation predicts oscillatory ability.**

We examined PER2 expression for patterns that predict the loss of circadian rhythmicity.

When the peak phase or amplitude at the time of TTX application did not correlate with

whether a cell remained rhythmic (Fig. 9), we compared 48-h of bioluminescence from

neurons, which lost (n=25) or sustained (n=25) cycling during TTX treatment. We

aligned traces to 20% of peak expression in the first 24 h and then averaged the

bioluminescence from all neurons in each group in hourly bins (Fig. 10). On the day prior

to losing rhythmicity, neurons had lower peak PER2 expression (Student's t-test, $p <$

$0.0002$), but similar times to peak compared to rhythmic neurons ($p > 0.05$). According to

ROC analysis, an ideal observer could accurately predict the behavior of an SCN neuron

83% of the time using the slope of PER2 accumulation. Random sampling of the same

slope data established a 95% confidence interval (triangles) of the null hypothesis that the
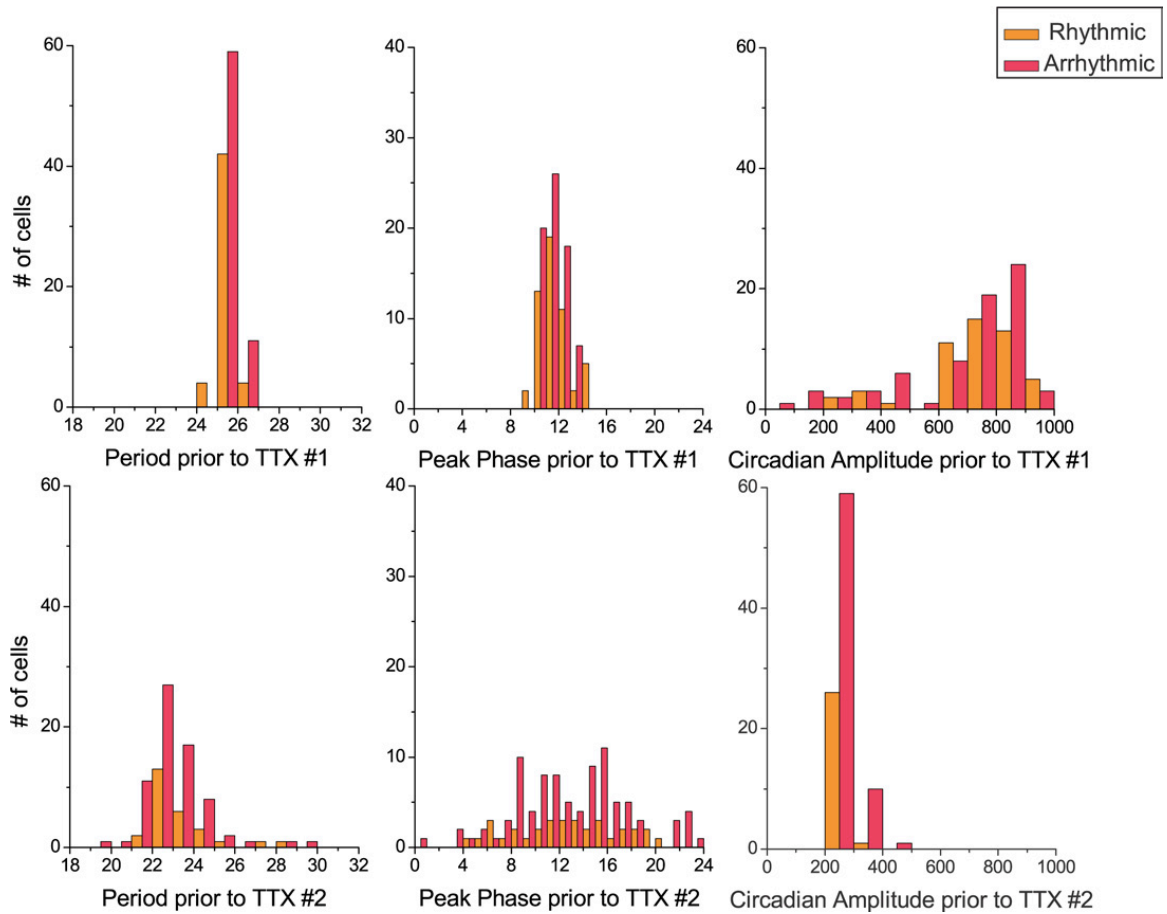
**Figure 9. Period, phase, and circadian amplitude before TTX treatment do not predict whether a neuron will remain circadian in TTX.** Period distribution of PER2::LUC expression for 6 days before the first (top) and second (bottom) TTX treatments for 123 neurons that were either rhythmic (pink) or arrhythmic (orange) shows no correlation of circadian behavior with prior period. Peak phase or circadian amplitude of PER2::LUC expression in the 24-h cycle prior to TTX application also did not correlate with outcome, strongly indicating that each cell's phase or amplitude of oscillation at the time of TTX treatment did not determine whether they would lose rhythmicity.
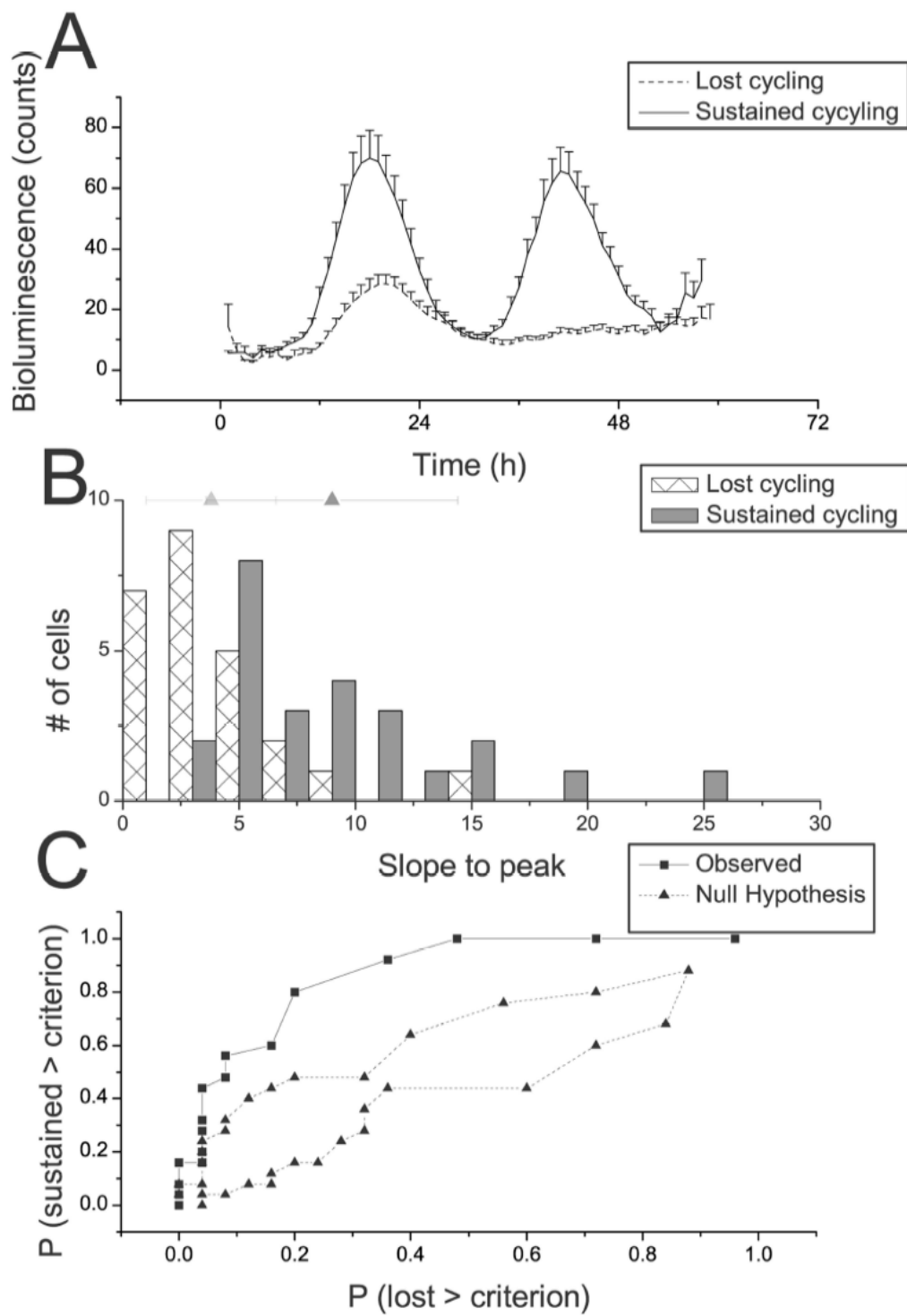
**Figure 10.  Rate of PER2 accumulation is a reliable predictor of changes in oscillatory behavior in single SCN neurons.** (**A**) The average waveforms of neurons which sustained (solid line) or lost (dashed line) circadian expression of PER2 show that the bioluminescence of neurons that failed to continue cycling was lower amplitude and slower to rise on the cycle prior.  Error bars show the SEM of 25 randomly selected neurons in each group. (**B**) The distributions of slope of the rising phase of PER2::LUC expression differed between neurons which lost rhythmicity (white crosshatch) and neurons which continued to cycle (dark gray). Mean and standard deviation are plotted for both groups. (**C**) Receiver operating characteristic (ROC) of 50 neurons whose average waveforms are represented in A (circles). To calculate each point along the ROC, the proportion of rhythmic neurons that exceeded a given slope criterion were plotted against the proportion of neurons that lost cycling whose slope exceeded the same criterion. For example, for a criterion slope of 4 (i.e. bioluminescence increased by at least 4 counts/h from the trough to the peak of the first cycle), we found 23 of 25 neurons sustained rhythmicity (0.92) and 9 of 25 neurons lost cycling (0.36). The ROC curve significantly differed from those generated from randomly sampled data (permutation test, $p < 0.001$).

slope does not predict rhythmicity in the next cycle. The ROC curve significantly differed from these randomly generated data (permutation test, $p < 0.001$). Interestingly, the peak-to-trough amplitude predicted whether a neuron would remain circadian only 71% of the time. Finally, we examined the slope of PER2::LUC expression from rhythmic neurons in untreated SCN explants and found their average slope was 16-times greater than that of TTX-treated neurons which lost rhythmicity. Thus, we found the rate of PER2 accumulation in one cycle was a better predictor than other measures that a neuron would oscillate in the next cycle (Figs. 4, 5, 9).

**Discussion**

The best examples to date of cell-autonomous circadian rhythm generation are unicellular cyanobacteria (Mihalcescu, Hsing et al. 2004) and dinoflagellates (Roenneberg and Morse 1993) and molluscan retinal neurons (Michel, Geusz et al. 1993). Here we have demonstrated that when a single SCN neuron expresses PER2 for multiple days, it is likely to be circadian and can contain AVP, VIP, or another unidentified marker. However, the majority of SCN neurons were arrhythmic and just as likely as rhythmic neurons to express AVP or VIP. In addition, neurons lost or gained daily gene expression rhythms during long recordings where intercellular communication was blocked (i.e. very low density, isolation, and TTX). It is possible that a small, unique class of self-sustained pacemaker cells exists in the SCN (e.g. the 13% of neurons which remained circadian during both week-long TTX treatments). Because these neurons were not found in a reproducible location from explant-to-explant and showed unstable circadian periods and

amplitudes, we conclude that circadian rhythm generation is not localized to a specialized

class of SCN neurons. Instead, it appears that each SCN neuron has about a one in four

chance of expressing PER2 rhythmically in the absence of intercellular communication.

The variable nature of daily rhythms in isolated SCN neurons has been predicted by

models of the transcription-translation circadian feedback loop in which noise

(sometimes associated with low numbers of molecules) was added within a cell (Forger

and Peskin 2004; Gonze and Goldbeter 2006; Liu, Welsh et al. 2007). Under these

conditions, small perturbations (extrinsic or intrinsic to the cell) could push the molecular

oscillator on or off a limit cycle. Other modeling efforts have simulated the SCN as a

network of damped (Bernard, Gonze et al. 2007) or sustained, damped and driven

oscillators (To, Henson et al. 2007). Bernard et al. (Bernard, Gonze et al. 2007) found

that, compared to self-sustained oscillators, damped oscillators more rapidly

synchronized to each other and to environmental cycles, a potential benefit of having

cells which change their rhythm amplitude. A third approach modeled the SCN as

intrinsic oscillators and non-rhythmic gate cells (Antle, Foley et al. 2003; Antle, Foley et

al. 2007). These models showed that non-rhythmic neurons could contribute by gating

input to the SCN depending on feedback from rhythmic cells. Our results support

components of each of these models, by revealing that SCN neurons are heterogeneous,

intrinsically noisy oscillators, which, under some conditions, lose their circadian

fluctuations in gene expression and firing rate. We stress, however, that we find no

evidence for a specialized or anatomically localized class of cell-autonomous oscillators

(or non-oscillatory gate cells). Instead, our results suggest that a single SCN neuron has

the potential to move between these regimes and that the network may contribute to overall rhythmicity.

Randomness in events like transcription has been implicated in generating cell-cell variability and network plasticity (Raj and van Oudenaarden 2008). In addition to the known differences in amplitude and period, we find that SCN neurons also differ in their stability of circadian rhythmicity when isolated from their neighbors. This changeable rhythmicity is reminiscent of the circadian properties of *Drosophila* neurons which vary based on experience (Wulbeck, Grieshaber et al. 2008) and of *Per1-*, *Per2-*, or *Cry1-* mutant SCN neurons which show "stuttering" circadian rhythms (Herzog, Aton et al. 2004; Liu, Welsh et al. 2007). The present results indicate that intercellular interactions acting on noisy gene expression within SCN neurons stabilize cycling which may contribute to both the improved precision (Herzog, Aton et al. 2004; Inagaki, Honma et al. 2007) and adaptable coding of, for example, day length by a population of heterogeneous oscillators (Inagaki, Honma et al. 2007; Ciarleglio, Gamble et al. 2009).

With each neuron having the ability to generate 24-h oscillations or to be driven, the SCN is a multi-potential system. In contrast, fibroblasts have been reported to all oscillate independently without damping *in vitro* (Welsh, Yoo et al. 2004). It is interesting to speculate that SCN neurons rely on signals from other SCN cells for robust daily cycling while allowing for flexibility with changes in environmental conditions. This is consistent with electrophysiological recordings and computational modeling in other neural

networks which have shown neurons which transition between oscillatory regimes depending upon a variety of cellular states (Marder and Goaillard 2006). For example, homeostatic changes in specific ionic conductances can switch neurons in the lobster stomatogastric ganglion from bursting to tonic firing. This kind of tuning provides multiple solutions for individual nodes within a network to produce a system with the desired output. Having a range of solutions lends plasticity, as well as stability, to the system. Individual SCN neurons are variable oscillators when uncoupled, but when connected with each other generate a coherent circadian output. *In vivo* observations of gain or loss of circadian rhythmicity also support our results of instability at the single cell level. For example, locomotor and melatonin patterns lose and regain near 24-h cycling in arctic animals exposed to seasonal changes in day-length (van Oort, Tyler et al. 2005). Food restriction for a few hours each day induces daily oscillations in the dorsomedial hypothalamus of mice (Fuller, Lu et al. 2008). Clock gene expression is non-circadian in the liver of voles until they are provided with a running wheel or daily feeding cycles (van der Veen, Minh et al. 2006).

Previous studies have shown that when intercellular signaling is blocked by one of a variety of mechanisms, the amplitude of PER2 expression diminishes (Colwell, Michel et al. 2003; Yamaguchi, Isejima et al. 2003; Aton, Colwell et al. 2005; Aton, Huettner et al. 2006; Maywood, Reddy et al. 2006). Here we reveal the exciting possibility that network interactions act on the rate of PER2 accumulation in single cells (Fig. 10) to determine their ability to generate and sustain daily rhythms. Slower accumulation of PER2 protein, not the amplitude or time of peak expression, is best correlated with the loss of

rhythmicity in SCN neurons, strongly suggesting that the processes which modulate the

accumulation of PER2 or other clock proteins play a critical role in sustaining circadian

cycling. This is consistent, for example, with the observation that phosphorylation sites

on the PER2 protein play a critical role in both its accumulation and circadian

rhythmicity (Gallego, Eide et al. 2006; Xu, Toh et al. 2007; Meng, Logunova et al. 2008).

Future studies will likely pursue how intercellular signals impact timekeeping processes

including rates of translation and degradation of clock proteins. It remains to be seen how

changes in intracellular state, changes in connectivity within the SCN, or their combined

effects mediate some of the developmental or seasonal adaptations in mammalian

circadian rhythms (Schaap, Albus et al. 2003; Sladek, Sumova et al. 2004; Inagaki,

Honma et al. 2007; Sumova, Kovacikova et al. 2007; Vanderleest, Houben et al. 2007).

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# Chapter 3.

## **<u>Heterogeneous nodes aid in synchrony of the SCN network</u>**

This chapter contains the manuscript:

Webb A.B., Taylor S.R., and Herzog E.D. Heterogeneous nodes aid network synchrony in the mammalian circadian clock. In preparation.

**Abstract**

Several computational models have simulated the transcription-translation feedback loops that drive circadian rhythms using a series of ordinary differential equations. These single-cell models produce oscillations of about 24 hours in the absence of inputs, entrain to light-dark cycles, and when coupled together generate a synchronized rhythm across the population. The implications of variability in the quality of oscillations between cells remain unknown. Recent data from functionally isolated suprachiasmatic nucleus (SCN) neurons show that single cells can exhibit a range of oscillatory phenotypes when removed from the network. We hypothesized that parameters in an existing deterministic model could contribute to different behaviors in SCN neurons. By simulating single cells with a range of parameter values, we found that oscillatory phenotype was more sensitive to changes in rates of translation and degradation of PERIOD than to changes in rates of transcription and phosphorylation. In addition, we wondered what were the potential biological consequences of building a network from heterogeneous oscillators. We observed that the rate of resynchronization in the network depends on the properties and location of damped cells. We found that when coupling is reinstated, network topologies in which damped oscillators are highly connected restore rhythmicity faster than network topologies where rhythmic or mixed cells are highly connected. These results indicate that heterogeneity in oscillatory ability amongst neurons is beneficial and their position within the network can influence the behavior of the system overall.

**Introduction**

The near 24-h oscillations of the circadian clock are modeled as a molecular negative feedback loops within single cells where accumulation of protein represses transcription of its gene (Reppert and Weaver 2002). In mammals, the suprachiasmatic nucleus (SCN), a bilateral structure of 20,000 neurons in the ventral hypothalamus, functions as the master pacemaker with circadian cells driving rhythms in behavior and physiological processes, such as sleep-wake, locomotor activity, temperature, and hormone release (Ralph, Foster et al. 1990). Previously, it had been hypothesized that every SCN neuron acts an autonomous clock, producing daily rhythms in clock gene expression and cellular output in the absence of external signals (Welsh, Logothetis et al. 1995; Liu, Weaver et al. 1997; Herzog, Takahashi et al. 1998; Yamaguchi, Isejima et al. 2003). One alternative suggests that not all SCN neurons are capable of self-sustained rhythmicity, and that perhaps only some can act as pacemakers. Vasoactive intestinal polypeptide (VIP), a neuropeptide found in a subset of SCN neurons, is important in rhythmicity and synchrony (Harmar, Marston et al. 2002; Colwell, Michel et al. 2003; Aton, Colwell et al. 2005) and seemed a likely candidate marker for a class of pacemaker neurons, similar to pigment dispersing factor (PDF) neurons in *Drosophila* (reviewed in Taghert and Shafer 2006). Recent data, however, point to the possibility that, when isolated, SCN neurons exhibit a range of behaviors regardless of neuropeptide expression and are often damped or unstable in their oscillatory ability (Webb, Angelo et al. 2009). Therefore, while all neurons may be capable of autonomous rhythmicity, they are sloppy clocks requiring stabilization from the SCN network to function as robust circadian oscillators. The potential source or sources of this variability is a topic open for investigation.

The advantages of instability in biological systems have been widely documented from emergence of phenotypic diversity through stochastic gene expression in bacteria (Raser and O'Shea 2005) to the root architecture of developing plants (Forde 2009). While the circadian system benefits from its robustness when faced with molecular noise (Gonze, Halloy et al. 2002; Forger and Peskin 2004; Gonze and Goldbeter 2006), observations of plasticity at the organismal level due to environmental perturbation are also common. Behaviors of animals in the "real world" such as predator avoidance, hibernation, migration and mating all require an adaptive clock (DeCoursey, Walker et al. 2000; Davidson and Menaker 2003; Menaker 2006). Computational models of coupling within the SCN have also suggested that heterogeneous, noisy oscillators aid the system. Gonze and colleagues showed that 10,000 Goodwin-type oscillators are readily synchronized using mean-field coupling and that this global coupling results in damped oscillations that speed the synchronization (Gonze, Bernard et al. 2005). This work was later extended to a more complex model and network structure. By building a network of all damped oscillators with SCN-like connectivity, Bernard et al. showed that rhythmicity in single cells and synchrony are co-dependent and that intercellular signaling is key to successfully couple the population together (Bernard, Gonze et al. 2007). A global parameter search for the model by Gonze et al. revealed that coupling drives rhythmicity in damped and even non-oscillatory solutions and that these damped single cell oscillations may be beneficial for efficient synchronization (Locke, Westermark et al. 2008). More recently, a paper applied the insights gained from these models, i.e. that damped oscillators are beneficial for synchrony, to provide a method for precisely

quantifying biological data, allowing for classification of oscillators beyond simply "rhythmic" or "arrhythmic." The authors relate biological data to two models (1) a damped, noise-driven oscillator and (2) a self-sustained oscillator that relaxes to a stable limit cycle; this allows them to assign parameters such as damping rate for a single cell (Westermark, Welsh et al. 2009). While these models have considered the existence and necessity of damped oscillators, to our knowledge no model has examined the consequences of sustained and damped oscillators in combination within a network.

We sought to explain behavior observed in single SCN neurons using a computational model. This model simulates clock gene transcription-translation feedback loops in a single cell with 16 ordinary differential equations and has been shown to oscillate in constant conditions with a near 24-h period and entrain to light-dark cycles (Leloup and Goldbeter 2003). Multiple single cells from this model can generate a coherent synchronized output when coupled together by simulating VIP signaling (To, Henson et al. 2007). Previous sensitivity analysis using different parameter sets revealed that the period of the oscillations was dependent on values related to the synthesis and degradation of *Bmal1* mRNA and its protein (Leloup and Goldbeter 2004). However, the sensitivity of circadian rhythmicity to parameter values has not been explored. By simulating single model cells with parameter ranges that produced a variety of circadian phenotypes, we could identify parameters and parameter combinations that were capable of producing sustained, damped, and arrhythmic oscillations observed in the biological data.

We next assessed the consequences of heterogeneous oscillators on the behavior of the SCN network. Initially, we compared the behavior of single neurons in organotypic SCN explants following the removal of a sodium-channel blocker, tetrodotoxin (TTX). TTX has been used previously to study synchrony in SCN neurons by uncoupling them from each other via action potential mediated synaptic transmission, resulting in arrhythmicity, diminished amplitude and range of circadian periods (Yamaguchi, Isejima et al. 2003; Webb, Angelo et al. 2009). Instead, we proposed to study the dynamics of recovery and resynchronization in SCN neurons following TTX treatment. To gain insight into how the network is organized and potential benefits for this organization, the behavior of cells should be examined not only as they uncouple, but also as they are allowed recouple. Again, we paired biological data with a model to generate SCN-like networks of simulated heterogeneous oscillators that desynchronize and resynchronize. Anatomically, the structure of the SCN is similar to a small world network topology (Watts and Strogatz 1998), with the presence of both local and long-range connections (Abrahamson and Moore 2001). Small world network organization has been shown to improve synchronization in a similar model of the mammalian circadian clock (Vasalou, Herzog et al. 2009). Specifically, we wanted to understand how oscillator type, number of connections, and location within the network may influence how a population of sustained and damped oscillators self-assemble and resynchronize following uncoupling. We therefore simulated resynchronization in a variety of small-world networks of these oscillators to determine factors that may contribute to the behavior we observed in SCN slices.

Here we present data that show small changes in rates of transcription, translation, phosphorylation, and degradation in a model of the mammalian circadian clock can produce a range of circadian behaviors, similar to those observed in isolated SCN neurons. By using a non-biased method of visualizing parameter space, we find that these behaviors are most sensitive to rates of translation and degradation of PERIOD. Using a small world network topology, we built networks of heterogeneous oscillators and found that the percentage and location of damped oscillators in the network influenced the rate of resynchronization. Networks of highly connected damped oscillators resynchronize faster than networks of highly connected sustained oscillators. We conclude that heterogeneous oscillators, particularly damped cells, benefit the network by adding flexibility; this flexibility improves recovery of circadian behavior, such as speeding synchronization following an uncoupling perturbation.

**Materials and Methods**

**<u>SCN CELL CULTURE.</u>** We generated and recorded from low density dispersals and organotypic SCN explants using previously published methods (Webb, Angelo et al. 2009). Briefly, SCN from neonatal PER2::LUC mice were enzymatically digested and cells were dispersed on glass-bottomed dishes coated with polyD-lysine and laminin (Sigma), or were cultured intact on MilliCell membrane (Millipore) pieces before transfer to coated glass-bottom dishes in $CO_2$-buffered medium supplemented with 10% newborn calf serum (Invitrogen).

**BIOLUMINESCENCE RECORDING.** We conducted recordings in air-buffered medium containing 0.1 mM beetle luciferin (BioThema) at 37 °C beginning at day 4 *in vitro* for dispersals or day 2-3 after transfer for explants. We temporally (1 h integration time) and spatially (4x4 pixel resolution) bioluminescence counts using a Versarray 1024 CCD camera (Princeton Instruments).

**TTX TREATMENT AND WASHOUT.** Following 6 days of baseline recording, we treated organotypic SCN explants with 0.5 μM tetrodotoxin (TTX, Sigma) as previously described (Webb, Angelo et al. 2009). TTX remained in the medium for 6 days before the medium was removed and we washed explants with 1 full volume exchange of fresh medium. Recording then continued for at least 6 days to examine rhythms as cells resynchronized after the restoration of cell-cell communication.

**ANALYSIS OF RHYTHMS.** We used NIH ImageJ software to process all images by first subtracting background counts and then measuring pixel intensity over time in a region of interest above each cell. Cells were initially scored as rhythmic or arrhythmic if their gene expression rhythm oscillated with a period between 15 and 35 hours that was statistically significant by both Chi-squared periodogram (Sokolove and Bushell 1978) and FFT-NLLS (Plautz, Straume et al. 1997). When the peak-to-trough amplitude of an oscillating cell's cycle decreased to less than 20% of the peak-to-trough amplitude of the initial cycle, it was considered damped (Prolo, Takahashi et al. 2005).

**MODEL OF MOLECULAR CLOCK IN SINGLE SCN CELLS.** A version of a previously published 16-ordinary differential equation model of the mammalian circadian clock was used to simulate rhythms in single model cells (Leloup and Goldbeter 2003). We altered parameters for rates of transcription, translation, phosphorylation, and degradation of either *Period* or *Bmal1* genes, leaving 50 other parameters set to published basal values (Leloup and Goldbeter 2003), and measured rhythms in gene output. We simulated at least 15 days of gene expression from each cell and analyzed the data after the first 48 h. We then used the crossing of a 3 h and a 24 h running average of the data as phase markers (see Abe, Herzog et al. 2002 for detailed description of these methods) to count the total number of cycles for each cell with the criteria that each cycle had a period of between 15 and 35 h and peak-to-trough amplitude remained above 20% of the initial cycle. Cells were scored as sustained, damped, or arrhythmic based on the number of circadian cycles completed using the same algorithm and criteria applied to the bioluminescence data from SCN neurons.

**VISUALIZATION OF PARAMETER SPACE.** To measure the sensitivity of circadian cycling to clock gene parameters, we organized results from single cells simulations using clutter based dimensional reordering (CBDR), which applies minimization and dimensional stacking algorithms described below. These methods allow visualization of the underlying structure of clock parameter space and gauge the influence of tested parameters relative to output behavior (Peng et al. 2005). We utilized published Matlab

code (Taylor, Hickey et al. 2006) to minimize differences between output scores (sustained, damped, arrhythmic) and cluster behaviors together. The code arranged parameter combinations iteratively until the minimization requirement, i.e. cells with like behavior, were clustered together, was fulfilled. First, the code scans one pair of parameters over a range of values while the remaining parameters are set to basal values. Then we label this grid based on the output for each combination and add it to a larger montage of other parameter pairs. For a useful visualization, the code orders these dimensional stacks to group similar outputs together. Given a unique behavior and parameter combination for each cell, we minimize the stack so that differences between regions of varying outputs are small (in this case, sustained, damped, or arrhythmic patterns in gene expression), and this provides an order ranking of "higher" versus "lower" parameters in the stack. Changes in parameter value that produce larger effects in output phenotype are higher in the stack order.

**MODEL OF SCN COUPLING AND NETWORK TOPOLOGY.** Model cells were coupled together by VIP signaling, simulated as a drive on the rate of *Per* transcription, as previously published (To, Henson et al. 2007). In our model, 20 percent of the 400 neurons were capable of sending a VIP signal and all neurons could respond to VIP. Connections between cells were organized with a small world network topology as in (Vasalou, Herzog et al. 2009) where each VIP cell was coupled to its four nearest neighbors and then had a probability of sending unidirectional long-range connections to other cells in the network. We set the connection probability to p=0.05, resulting in a synchronized system with a range of 4 to ~40 outgoing connections in most networks. To

mimic the TTX experiments, we simulated 6 days with VIP-mediated coupling followed by 6 days with coupling eliminated and then reinstated for up 6-15 days. We assessed the intrinsic circadian expression of each cell as well as the rate of resynchronization of each cell and the ensemble. The network connections and parameter values for each cell did not change throughout the simulation.

**CALCULATION OF SYNC INDEX.** The synchronization index (SI) provides a real-time measure of the phase dispersion across a population of oscillators, which ranges from 1 (all cells peak in phase) to 0 (all cells peak at uniformly-distributed times of the day). We defined SI at each time $t$ by the radius $r$ of the complex order parameter (Strogatz 2000) according to

$$re^{i\psi(t)} = \frac{1}{N}\sum_{j=1}^{N} e^{i\phi_j(t)},$$

where $N$ is the number of cells, $\phi_j(t)$ is the phase of the $j^{th}$ cell at time $t$, and $\psi(t)$ is the average phase of all cells. We compute the instantaneous phase of each cell (simulated or real) by applying the continuous wavelet transform using a Morlet wavelet (Harang et al., 2009) to its trace of *Period* mRNA. The phase of the cell over time may be recovered from the ridges of the transform, which are extracted using a straight-forward algorithm

(Harang et al., 2009; WaveletGUI Package). Briefly, the continuous wavelet transform (CWT) produces a complex-valued field over scales (which may be mapped to instantaneous frequency) and translations (which may be mapped to time). The magnitude of the complex number at a given translation and scale may be interpreted as

the strength of oscillation of the signal at the frequency given by the scale and the time given by the translation. The phase of the complex number at a given translation and scale gives the phase of that oscillatory component. By selecting points with contiguous scales across a range of translations that maximize the magnitude of the CWT (the "wavelet ridge"), we may extract the dominant frequency of the oscillator over time, and from those points extract the phase evolution of the oscillator from the angles of the CWT coefficients.

**Results**

**Small changes can create heterogeneous oscillators**

Recent data from functionally isolated SCN neurons indicated that individual cells are sloppy circadian oscillators with the majority showing weak or arrhythmic patterns in gene expression and firing rate when removed from the network (Webb, Angelo et al. 2009). We utilized a model of the mammalian molecular clock to simulate SCN neurons with a range of circadian and non-circadian behaviors and discover potential sources for such differences. We found that small changes in individual model parameters representing the rate of transcription of the *Period* gene or translation, phosphorylation, or degradation of the PERIOD protein caused cells to transition from circadian to damped to arrhythmic, similar to what was observed in PER2::LUC recording from nearly isolated SCN neurons (Figure 1A). We chose these parameters and ranges in values because they represented points of control for the output we record (PER2::LUC signal) and the chosen values produced all types of behavior we observed in real cells.
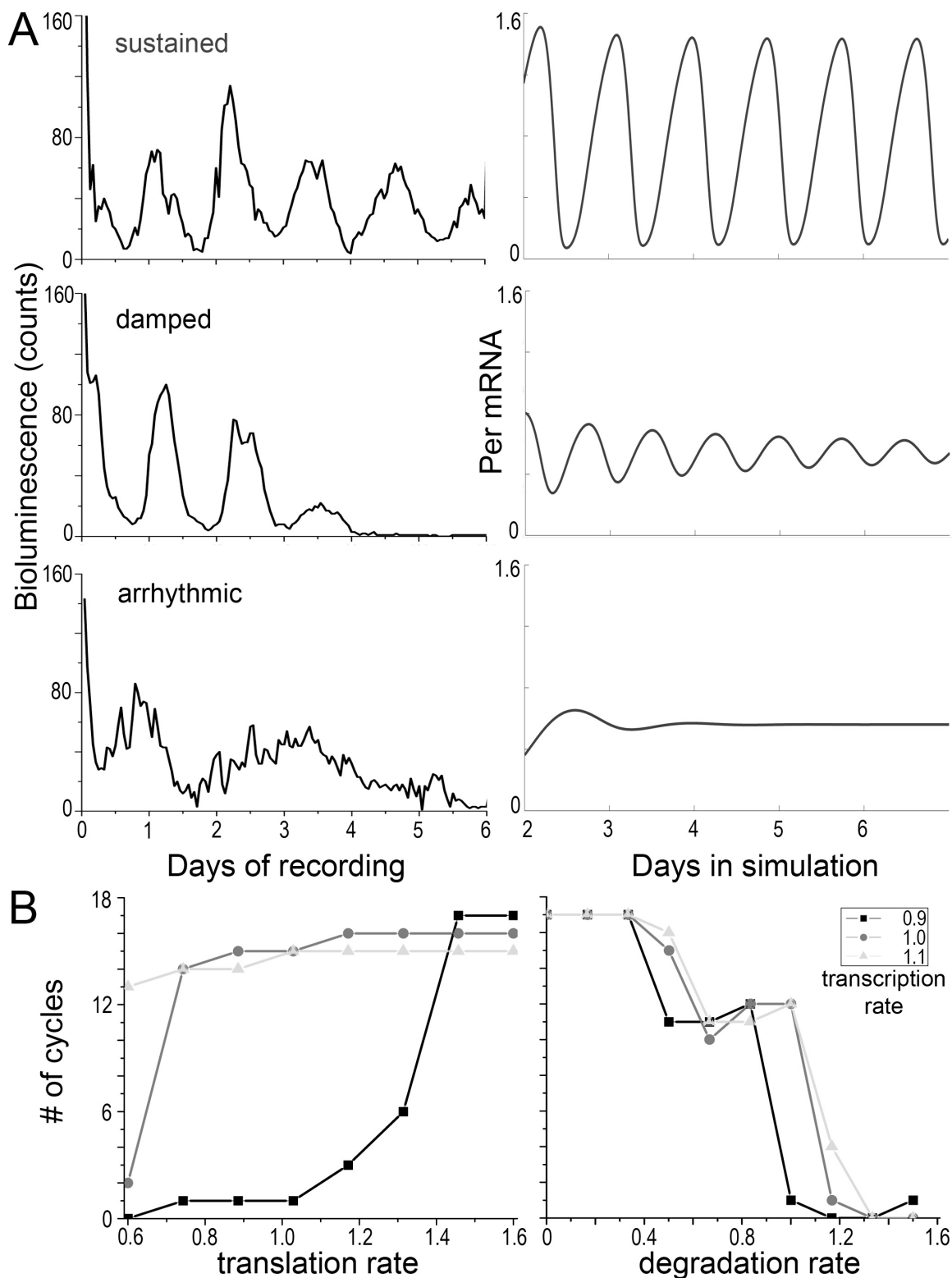
**Figure 1. Circadian phenotypes are readily changeable.** (**A**) Representative *Period* expression from physically isolated SCN neurons recorded *in vitro* (left column) or simulated (right column).  Cells were classified as sustained, damped, or arrhythmic in their circadian expression. To simulate this range of behavior in model cells, the ordinary differential equation model required only small changes in four parameters controlling the rates of Period gene transcription, translation, phosphorylation, and degradation. (**B**) Values for two example parameters corresponding to the translation (left, range) and degradation (right, range) of PERIOD produce all three circadian expression patterns shown above. Each plot shows the number of cycles generated for a given parameter combination. For example, as translation rate increases simulated cells can transition from arrhythmic to sustained regimes over a small range. As the value of transcription rate increases (lines with square (0.9), triangle (1.0), circle (1.1), respectively), the slope of this transition shifts. Comparatively, change in degradation rate also produces all three circadian phenotypes with a more gradual transition that appears less affected by transcription rate.

Simulating single SCN cells with parameter ranges produced not only circadian

oscillations with different periods, but also damped and arrhythmic patterns (Figure 1B).

We then simulated 1296 cells each representing a unique combination of these four

parameters varied over six equal steps. From its *Period* mRNA profile, we categorized

each cell as sustained, damped, or arrhythmic if it oscillated without losing amplitude for

at least 90%, 30-90%, or less than 30% of the simulation time (minimum of 6 days, the

length of time recorded from real cells), respectively.

**Translation and degradation of PERIOD are important for circadian phenotype**

To evaluate our chosen region of parameter space in contributing to sustained, damped,

or arrhythmic output, we looked for an independent method to determine how circadian

behavior varies as function intrinsic properties of the model. We arranged our data using

methods previously applied to the multi-dimensional parameter space of an

electrophysiological model of firing patterns in lobster stomatogastric ganglion neurons

(Taylor, Hickey et al. 2006). By nesting parameter combinations into stacks

(descriptively, 2 parameters vary across the x- and y-axis of a grid; smaller grids then

build into larger ones as more parameters are added) we can view a data set with a large

number of parameters in two dimensions. We chose four parameters that could alter the

amount of *Period* mRNA and protein produced in the model: transcription (vsP),

translation (ksP), phosphorylation (V3PC), and degradation (vmP). The mRNA output

observed after varying each of these four parameters over six equal steps in single cells

included clusters of sustained, damped, and arrhythmic behavior. After applying

dimensional stacking and minimization algorithms to these patterns in gene expression we visualized the underlying structure of parameter space, showing a large region of sustained cells (Figure 2, blue) transitioning to a smaller region of arrhythmic cells (Figure 2, black) with areas of damped cells (Figure 2, cyan) interspersed between these regions. The placement of parameters on this visualization indicated that circadian behavior was more sensitive values for translation and degradation compared to transcription and phosphorylation (Figure 2). Thus, changes in rates of translation and degradation produce larger effects than transcription and phosphorylation, making them higher order parameters.

To determine whether another clock gene influenced the amount of *Period* mRNA in a similar fashion, we compared an additional set of the equivalent parameters for *Bmal1* (Figure 3). In addition, to test for differences across outputs, we compared the levels of both *Period* and *Bmal1* mRNA for each parameter set (Figure 3). Interestingly, we found one of the higher order parameters for the *Bmal1* set was different than what we observed for *Period*. When compared to the visualization of Period parameter space, circadian behavior was more sensitive to the rate of transcription, not translation of *Bmal1*. Both *Period* and *Bmal1* sets included degradation as a higher order parameter. We note that the visualization results these two parameter sets were similar regardless of whether we measured *Period* or *Bmal1* mRNA as an output. *Bmal1* and mRNA instead of *Period*, we found that the higher order parameters did not change.
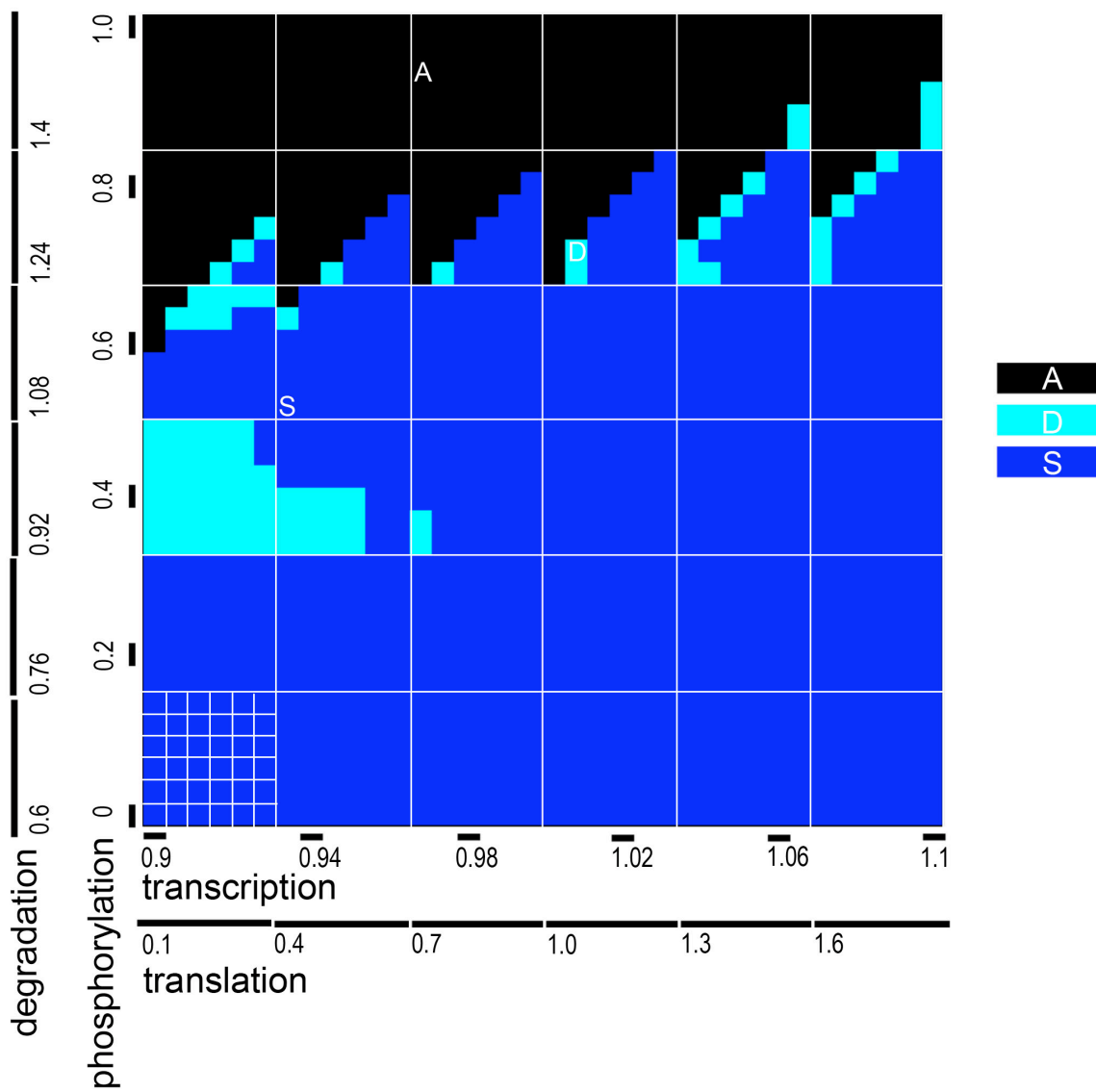
**Figure 2. Two-dimensional visualization of a four-dimensional region of parameter space within the model indicates that circadian phenotype is sensitive to translation and degradation of Period**. Using a minimization algorithm we arrange each small box, representing one of 1296 potential combinations. This non-biased algorithm allows for of grouping cells into distinct areas of rhythmic (blue), damped (cyan) and arrhythmic (black) outputs and therefore suggests the contribution of each of these four parameters to rhythm generation (Taylor et al., 2006). Four parameters representing the transcription, translation, phoshorylation, and degradation of Period are varied at 6 equal steps over a range producing all oscillatory behaviors as seen in Figure 1 and the remaining parameters are set to the published basal values. Axes for small boxes are determined by the lower order or less sensitive parameters, and these are then assembled to form larger, 6x6 squares which vary with the higher order parameters. Rates for translation and degradation appear on the larger axes, indicating that these are higher order parameters whose values produce a more dramatic shift in phenotype when changed. Positions of the sustained (S), damped (D), and arrhythmic (A) simulated cells from Figure 1 are indicated on the visualization of parameter space.
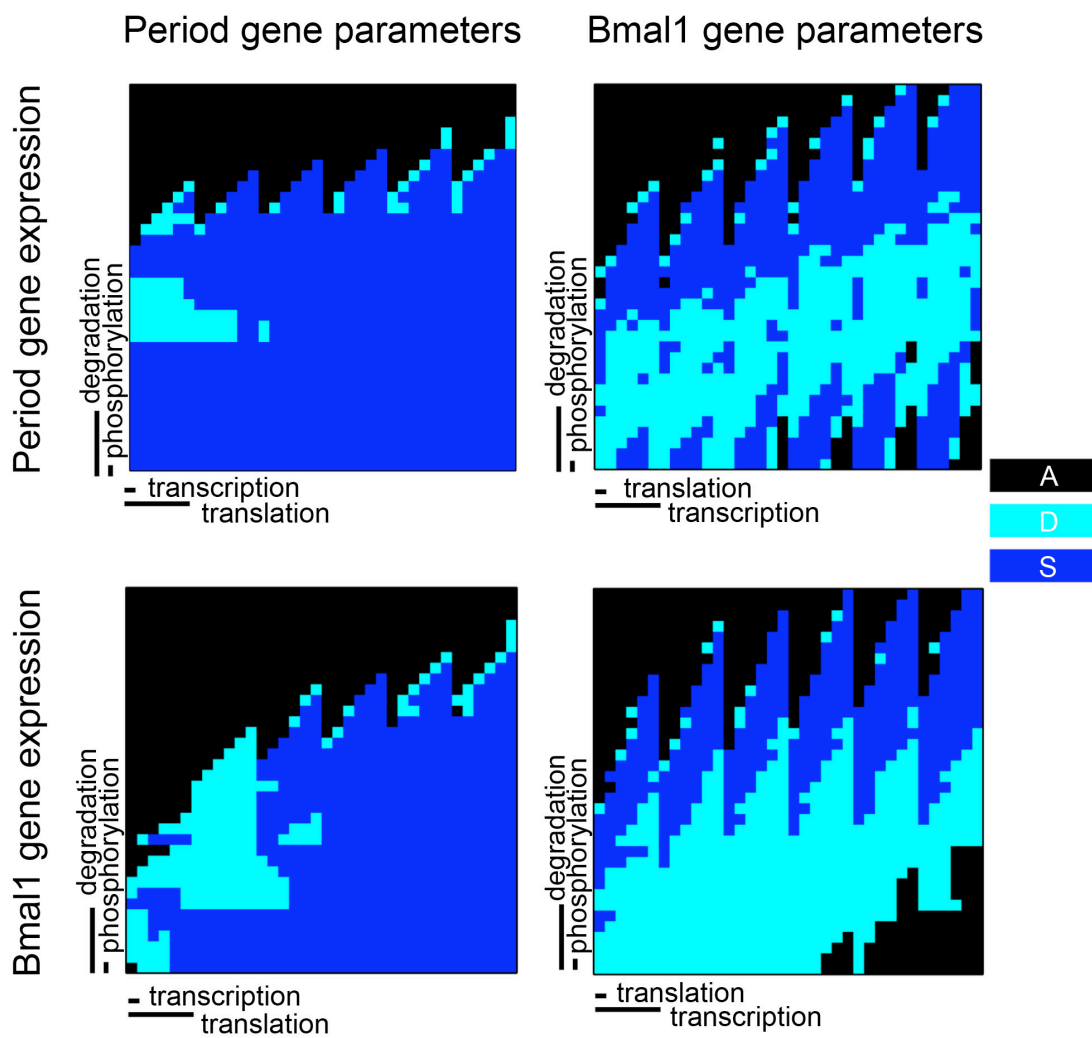
**Figure 3. Comparison of parameter sets and outputs measured on rhythm phenotype.** We examined a parameter set that varied rates of transcription, translation, phosphorylation and degradation of Bmal1, as well as using Bmal1 expression as the output measured, to compare with results shown in Figure 2. Here we replot the visualization from Figure 2 together with three additional plots. The columns show areas of parameter space found by varying rates of transcription, translation, phosphorylation, and degradation of Period (Figure 2) and Bmal1, respectively. The rows indicate the outputs measured (Period, Bmal1) for both of these parameter sets. We found in the area of parameter space chosen for Bmal1 set that changing transcription rates has a larger effect than translation rates on rhythm phenotype, suggesting that for Bmal1 the rate of mRNA production influences whether a rhythm will remain sustained or damp out. Comparing across outputs we note that parameter space effects are consistent regardless of the gene measured. Both Period and Bmal1 outputs show similar, but not identical, parameter space visualizations.

**Examining the kinetics of resynchronization in heterogeneous networks**

Are there benefits to building a network out of heterogeneous and sloppy oscillators? We hypothesized that individual damped oscillators may be beneficial to the SCN network by aiding in its flexibility, especially in the face of changing conditions. To test this, we wanted to examine synchronization of oscillators in the absence and presence of connections. Previously, we observed intrinsic behaviors in single neurons when we treated organotypic SCN slices with tetrodotoxin (TTX) to block spike-dependent signaling and recorded PER2-driven bioluminescence; this treatment reveals cell autonomous properties without physically altering the network (Figure 4). Under these conditions, individual cells displayed a variety of circadian phenotypes, including the sustained, damped, and arrhythmic patterns in gene expression that we observed in physically isolated single SCN neurons (Figure 1A). We also noted that once TTX was removed, cells did not immediately recover rhythmicity and synchrony. When we measured the synchronization index (SI; Materials and Methods) of the population, we found a slow recovery that took the system several days to return to a synchronized state (Figure 4B; SI $\approx$ 0.9). Interestingly, preliminary analysis suggests the neurons that were the first to oscillate after TTX washout were tightly clustered in phase, though not necessarily rhythmic when TTX was present. As more cells regained rhythms, they did so with a broader distribution of phases, with the last cells to be recruited to oscillate significantly broadening the average phase of the population.
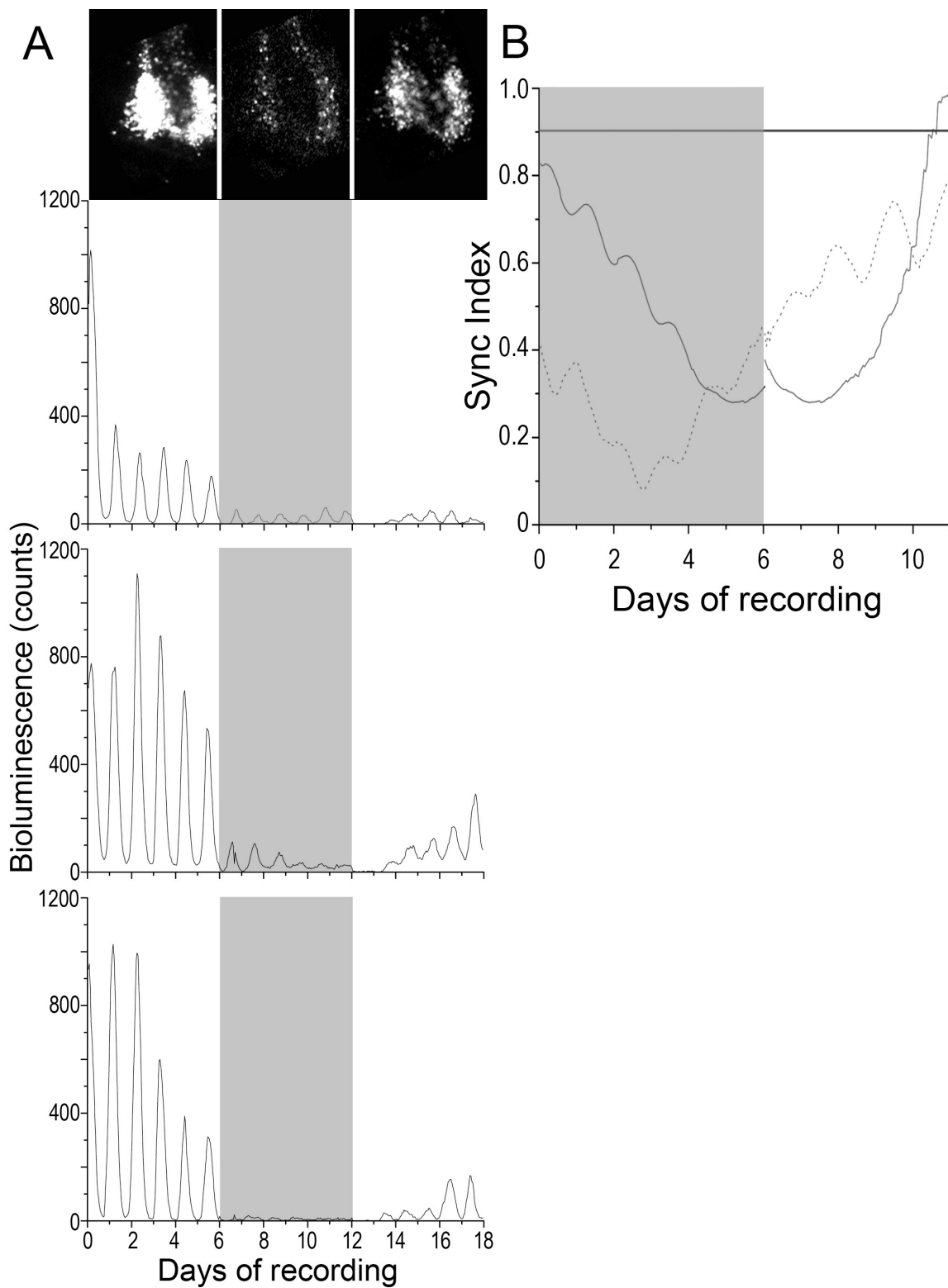
**Figure 4. Coupling restores rhythmicity, but not immediately, to cells in an SCN network.** (**A**) Bioluminescence traces from single neurons in an organotypic SCN explant before, during (gray bar), and following treatment with tetrodotoxin (TTX). TTX minimizes cell-cell communication and reveals intrinsic circadian properties similar to what was seen in isolated SCN neurons in Figure 1A. Here we plot traces from neurons that were sustained, damped, and arrhythmic in TTX, all of which are rhythmic when coupling is in place before treatment. Following washout, rhythms return to all cells, but at different rates. (**B**) SI values from TTX washout in slices. Preliminary data suggests a small population of neurons resynchronizes first and then a slow recruitment of the remaining population is observed following TTX washout. Here we plot SI values calculated from 2 representative SCN slices during TTX (gray bar) and following washout.

This could explain the slower recovery to a fully synchronized system we observed. A previous report noted that phase order was restored to the 48 cells studied within 3 days after TTX is removed by looking at time of peak *Per* expression and that after 7 days original peak to trough amplitude returned (Yamaguchi, Isejima et al. 2003). This study, however, did not calculate SI or use circular statistics to determine the significance of phase clustering.

**Simulated networks of heterogeneous oscillators synchronize faster**

For an investigation of how network structure, including the number and location of damped and arrhythmic oscillators, shapes the kinetics of resynchrony of the population, we built networks containing characterized model cells. Of the 1296 cells from probed parameter space, we organized networks of 400 cells that included both local and global coupling using VIP signaling, similar to work published by Vasalou and colleagues (Vasalou, Herzog et al. 2009). Briefly, 20% of cells in the network release VIP and all cells can respond to VIP signaling. Each VIP cell is connected to its four nearest neighbors and has a probability to send long-range connections to other cells in the network (Figure 5). We first simulated networks where either all or none of the 400 cells were within damped regions of parameter space and found that these networks resynchronized faster than networks of oscillators from sustained regions of parameter space (Figure 6A). Damped networks reached a SI of $\approx 0.9$ within 3 days after coupling was restored, while sustained networks never resynchronized. We should note that all
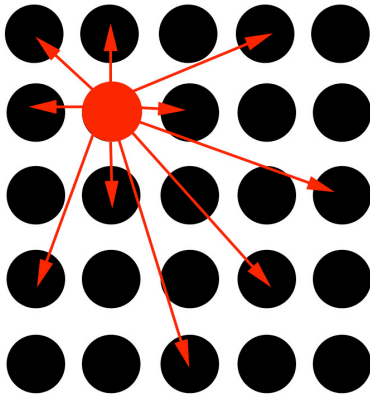
**Figure 5. Small world network topology can create a range in number of connections between nodes.** Small world networks were organized by connecting 20% of cells in the network to four nearest neighbors and then adding the probability for these cells to send a long-range connection (beyond nearest-neighborhood) to any other cell in the network. Here we show an example network highlighting a damped cell (red) placed in a more connected location (on average, 10 connections; range 4-39). This cell sends uni-directional connections (red arrows) to 9 other cells in the network, providing phase and period information that are important in synchronization of the population of oscillators.
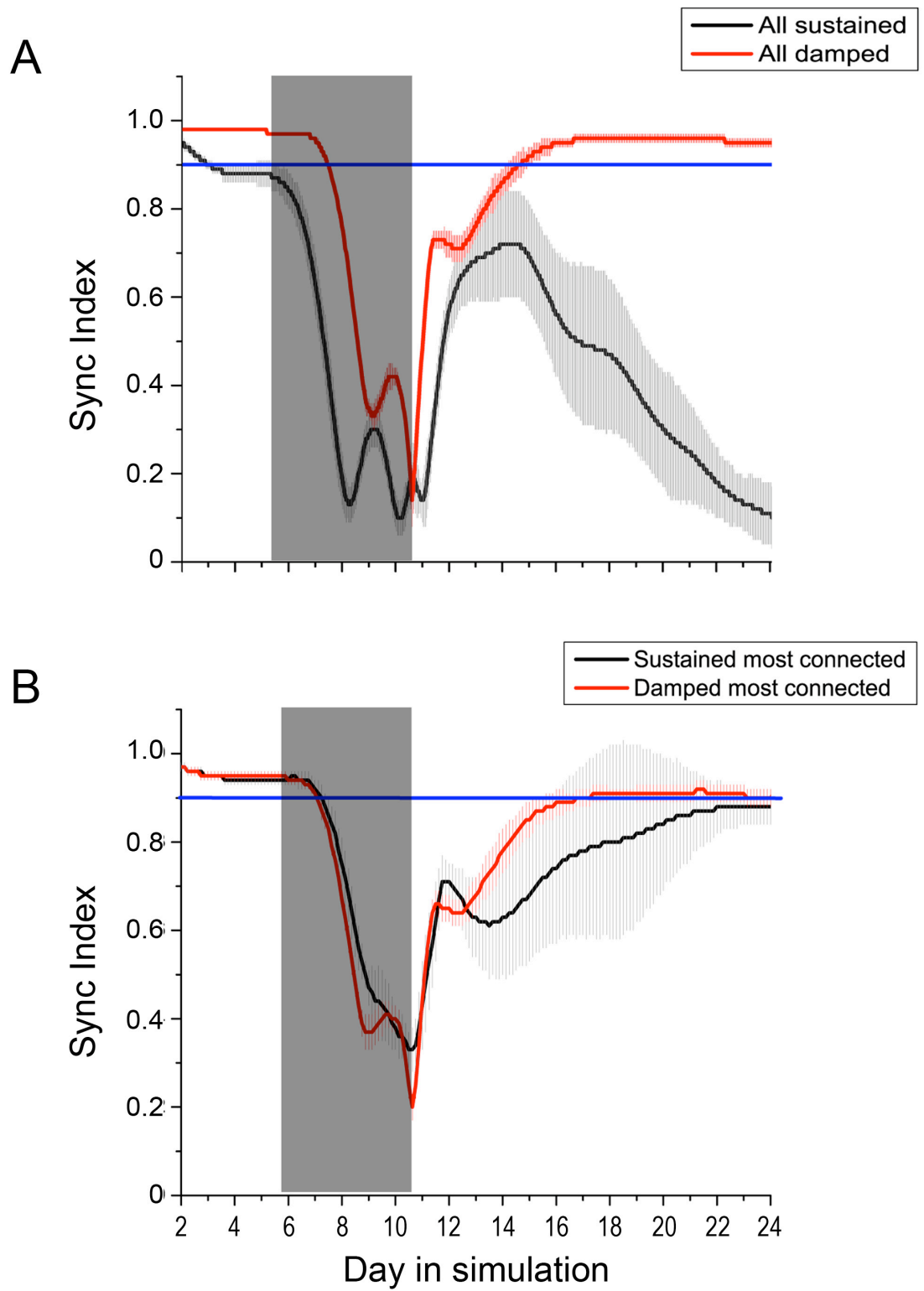
**Figure 6. Networks with damped oscillators synchronize faster.** We measure the synchronization index (SI) of the population in the coupled (days 2-6), TTX-uncoupled (days 6 - 11), and recovery (days 12-24) conditions. (**A**) Average traces of SI values over 10 networks are shown for both all sustained (black) and all damped (red) oscillators. All damped networks resynchronize within 3-4 days after coupling is restored. All sustained networks fail to resynchronize. (**B**) In networks of 80% damped-20% sustained oscillators, we find that networks synchronize faster when damped oscillators are placed at more connected nodes (red) compared to when sustained oscillators (black) are placed there. On average, networks with sustained oscillators at highly connected nodes require around 11 days to resynchronize, compared to highly connected damped networks which take about 5 days.

cells regain rhythms when coupling is restored, but in the all sustained case these cells oscillate with a variety of phases that take longer to synchronize than a network of all damped cells. We then constructed networks with 80% damped cells and 20% sustained cells, proportions similar to what has been observed across individual cells in the SCN in the absence of coupling (Aton, Colwell et al. 2005; Aton, Huettner et al. 2006; Webb, Angelo et al. 2009). In these heterogeneous networks, we changed whether damped cells were placed in the more connected nodes (for one network on average, 10 outputs; range, 4-39 outputs) or if sustained cells were placed in these nodes. We found that when damped cells were placed in the more connected nodes of the network, the population synchronizes faster. The average SI values over time (± SD for 10 networks) are shown for both the sustained-most-connected (black) and damped-most-connected (red) configurations (Figure 6B). Initially the population is synchronized (days 1-6) before coupling is removed and the system desynchronizes as cells drift out of phase with each other. We found that as damped and arrhythmic cells stopped oscillating, we could no longer identify a single discernible phase for calculating SI and these cells were removed until their phase could be determined. On average, only 30-50 cells were removed from the calculation and this lasted for a brief period of time. When coupling was restored each network was able to resynchronize. Several novel features of this process are of note. First, we observed an initial rise in the SI, which we hypothesized, is caused by a group of cells that are initially rhythmic and tightly clustered in phase. Then, as more cells are recruited to resume oscillations, they join the population with a wider distribution of phases that slowly must resynchronize. We find that when damped cells are placed in highly connected nodes, they speed the rate of resynchronization, such that these

networks reach an SI value of 0.9 within 5 days compared to networks with sustained cells at highly connected nodes, which take more than twice as long to resynchronize.

**Discussion**

**Another perspective on sensitivity analysis**

We used a mathematical model of the circadian clock to investigate potential origins of the variability in *Period* gene expression we observed in single SCN neurons. Robustness of a system to perturbations is essential in living cells that must function in the face of both environmental and intrinsic noise. The structure of a system likely contains points or parameters that are more sensitive, and as such, output is readily changed by variation at these points. To examine the robustness of oscillatory behavior in our model we simulated single cells with a range of parameter values in transcription, translation, phosphorylation, and degradation, which in combination produced sustained, damped, and arrhythmic outputs. We classified oscillations based on the number of complete cycles with a circadian period and amplitude that was at least 20% of the amplitude on the second cycle. This classification allowed us to score and include cells that would be discarded as non-oscillatory in other analyses. Our measure of sensitivity therefore is the number of circadian cycles for a given parameter combination, which provides a scale beyond simply scoring between rhythmic and arrhythmic outputs. Another recent model argues the benefits of classification of SCN neurons beyond the dichotomy of rhythmic and arrhythmic class by generating parameters for continuous properties, such as damping rate (Westermark, Welsh et al. 2009).

Using a multi-dimensional parameter space visualization we determined the sensitivity ranking of each parameter individually and found that changes in translation and degradation of *Period* more readily lead to changes in output behavior than transcription and phosphorylation. This is an interesting result, given that we have seen that the rising slope of PER2 protein is a strong predictor whether a cell will fail to complete its subsequent cycle (Webb, Angelo et al. 2009) and other work has shown the changing amount of available PER2 is critical for circadian oscillations (Chen, Schirmer et al. 2009). Mathematically, we can argue that the slope of PER2 accumulation is equivalent to the rate of translation. While the slope's numerical value is not equivalent to the parameter value used in the ordinary differential equations of our model, they are conceptually similar. Degradation, the other higher order parameter in our analysis, also can affect the amount of PER2 in a cell. We hypothesize that these two processes balance each other to regulate the amount of PERIOD protein and proper tuning is essential for circadian oscillations in single cells.

The sensitivity analysis of parameters in combination and of all possible output behaviors adds perspective to previously published systematic evaluations. Robustness analysis in models of the *Drosophila* circadian clock ranked parameters associated with various control processes (transcription and translation, degradation, transport, phosphorylation, and dephosphorylation) that was based on period, amplitude, and later, phase response, of oscillations found that feedback architecture contributed to the stability of the system

overall (Stelling, Gilles et al. 2004; Gunawan and Doyle 2007). Like our multi-dimensional visualization, these studies examined behavior in a more global sense, in contrast to other sensitivity analyses, which examine a single point in parameter space (Leloup and Goldbeter 2004). A global parameter search of a simple Goodwin-type oscillator discovered parameter sets that were unable to produce sustained oscillations in single cells, but when coupled can oscillate and synchronize with other model cells (Locke, Westermark et al. 2008). We also investigated the sensitivity of a range of possible output behaviors to parameter values, including damped and arrhythmic oscillations. We find that small changes in parameter values can produce all circadian phenotypes we observed in the biological data and it is likely that output from single cells is tuned by changing the rates of these molecular control processes similar to what has been observed in other systems (Marder and Goaillard 2006).

**Damped oscillators are necessary for quick resynchronization**

How does a system synchronize when a range of oscillator types are used to populate a network? We sought to expand our sensitivity characterization of individual model cells to understand how a network built from many different circadian phenotypes might synchronize. In addition, we argue that to understand synchrony, both the kinetics of desynchronization (how the system uncouples) and resynchronization (how the system recouples from an uncoupled state) should be examined. Previous work has investigated emergence of synchrony in networks of all damped oscillators (Gonze, Bernard et al. 2005; Bernard, Gonze et al. 2007). We extend this by building small world networks of

previously characterized sustained or damped and arrhythmic model cells to investigate the effects of the number and placement of damped oscillators, i.e. highly connected nodes compared to nodes with few connections, on rates of resynchronization from an uncoupled state.

We compared biological data from SCN slices following washout of TTX and restoration of cell-cell signaling (Figure 5B) to our models and find that networks with 80% damped cells that are placed highly connected nodes (Figure 6B, red line) resynchronize at a similar rate, taking about 5 days to return to a SI value of 0.9. Published data show that the number of damped oscillators and the connectivity of those oscillators contribute to the robustness of synchronization (Bernard, Gonze et al. 2007). Our findings are novel in that they suggest that given the choice between sustained and damped oscillators in a network, placing damped cells at more connected nodes speeds the population's rate of resynchronization from an uncoupled state, not just improving its overall robustness. We demonstrate that having damped oscillators improve a system, specifically its recovery to a synchronized state, compared to sustained oscillators in the same network.

**A model of oscillator self-organization: Resync and recruitment**

How a population of oscillators coordinates to produce a coherent output is a question of interest to biologists, physicists, engineers, and mathematicians (Strogatz 2003). In the circadian field, much investigation has been on synchronizing molecules like PDF or VIP (Aton and Herzog 2005; Stoleru, Peng et al. 2005; Taghert and Shafer 2006), the range in

types of oscillators that can synchronize (Enright 1980; Antle and Silver 2005), and how

the system remains synchronized when faced with changing environmental signals

(Inagaki, Honma et al. 2007; Vanderleest, Houben et al. 2007; Beersma, van Bunnik et al.

2008). We have chosen to focus on fundamental properties of oscillators, including

parameters that create a range of oscillatory phenotypes, and how the choice and location

of oscillators can influence synchronization processes, particularly from an uncoupled

state.


We propose a model where most cells function as damped oscillators in absence of cell-

cell communication and this aids in synchronization of the coupled system. Single

oscillators must couple and together reach a coherent period and phase. We speculate

based on both biological data from SCN slices and a model of networked heterogeneous

oscillators, that when coupling is restored to a dispersed population a small group of cells

is initially rhythmic and synchronized, and then other cells are recruited to the

population. As cells are recruited, they enter with a broader phase distribution than the

initially rhythmic group and over time the system reaches a consensus period and phase

through intracellular signaling. When damped cells signal more cells in the network than

sustained cells, this consensus occurs faster. We believe in both the biological experiment

and our model that coupling is immediate (for example, as soon as TTX is removed, cell-

cell communication via action potentials is possible) and hypothesize that the oscillators

types and their locations in the network determine the rate of resynchrony from an

uncoupled state. Intracellular communication has been shown to support and amplify

circadian rhythms in otherwise sloppy single cells (Aton, Colwell et al. 2005; Aton,

Huettner et al. 2006). We note a potential role for intracellular communication between damped oscillators is to speed synchronization of a population, particularly from a perturbed or uncoupled state.

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# Chapter 4.

## A model to test necessity of a subset of circadian pacemakers

**Abstract**

In mammals, the generation and entrainment of circadian rhythms has been localized to heterogeneous groups of cells. The suprachiasmatic nucleus (SCN) entrains to photic cues and coordinates rhythms in many physiological outputs. Vasoactive intestinal polypeptide (VIP) is rhythmically released in the SCN and is necessary for rhythmicity and synchrony between neurons. We hypothesize that VIP neurons in the SCN are necessary to generate and entrain circadian rhythms. To test this, we developed a transgenic mouse model designed to delete VIP neurons using diphtheria toxin A (DTA). Our BAC transgenic construct (pVIP-YFPstop-Cre) uses the VIP promoter to drive yellow fluorescent protein (YFP) in living VIP neurons. We find faithful expression of our BAC transgenic construct in VIPergic neurons in neural tissues by immunohistochemistry and live imaging. We can excise the YFPstop cassette at FRT sites using the flipase driver (FLPe), allowing the expression of the tamoxifen-sensitive Cre-recombinase (Cre-ERT2) in VIP neurons. We have crossed pVIP-Cre mice with Rosa26-eGFPstop-DTA mice where eGFPstop is flanked by loxP sites. *In vitro* tamoxifen treatment of SCN explants from the pVIP-Cre; Rosa26-eGFPstop-DTA animals significantly reduced the number of VIP neurons relative to controls. We also report initial, unsuccessful attempts to delete VIP neurons *in vivo*. We conclude that our construct provides a novel mouse model that both labels and deletes VIP neurons.

**Introduction**

The surpachiasmatic nucleus (SCN) of the mammalian hypothalamus is the master circadian pacemaker controlling circadian rhythms in behavior and physiology, such as sleep-wake activity, visual sensitivity, body temperature, and hormone release (Klein, Moore et al. 1991). The SCN contains a heterogeneous group of about 20,000 neurons that differ in their inputs, neuropeptide content, and patterns of clock gene expression (Antle and Silver 2005). The role of specific types of SCN neurons in generating circadian rhythms and synchronizing these rhythms to daily light cycles is not completely understood. Recent data show that when isolated, SCN neurons exhibit a range of oscillatory abilities independent of their neuropeptide content and it is likely that all SCN neurons have the ability to function as cell-autonomous pacemakers, but do not always behave as such (Webb, Angelo et al. 2009). Whether a single subpopulation of SCN neurons is necessary for circadian rhythmicity has not been tested.

Vasoactive intestinal polypeptide (VIP) is expressed in approximately 10% of the neurons in the SCN, while nearly 60% of cells within the nucleus contain its receptor (Abrahamson and Moore 2001; Kallo, Kalamatianos et al. 2004). VIP signaling is important for circadian rhythmicity and synchrony in mammals, but its exact mechanism remains to be fully clarified (Aton and Herzog 2005). We have shown that isolated VIP neurons can function as pacemakers, but that they are not always, and not the only neurons in the SCN that can do so. Animals lacking VIP or its receptor, VPAC2, show impaired behavioral rhythms, with only about one third of animals exhibiting circadian

wheel running activity, often with multiple, weak, short free running periods (Colwell, Michel et al. 2003; Harmar 2003; Aton, Colwell et al. 2005). The majority of SCN neurons from these animals are also arrhythmic with the remaining 30% showing sloppy rhythms in firing rate and gene expression with a broad range of periods (Aton, Colwell et al. 2005). Other signaling molecules, like gastrin releasing peptide (GRP), have been shown to synchronize SCN rhythms in the absence of VIP, suggesting that additional pathways may compensate in animals that lack VIP or its receptor (Maywood, Reddy et al. 2006). It is hypothesized that other molecules like GRP, other receptors, or both could function in these cells even in the absence of VIP or VPAC2 protein. Therefore, it is important to assess rhythmicity in the absence of VIP neurons themselves.

The role of VIP signaling in the SCN has strong similarities to signaling through pigment-dispersing factor (PDF) and its receptor amongst clock cells in *Drosophila* (Vosko, Schroeder et al. 2007). The class of PDF-containing neurons, also known as the ventral cluster of lateral neurons (LNvs), is important for circadian behavior (Helfrich-Forster 1998). Animals lacking the gene for PDF or that have PDF+ LNvs genetically ablated show similar circadian deficits, with the majority showing a loss of behavioral rhythms in prolonged constant darkness (Renn, Park et al. 1999). This result suggested that PDF and PDF neurons are required for sustaining circadian rhythmicity. PDF has also been implicated in synchronizing separate populations of oscillators associated with either the morning or evening bouts of activity (Stoleru, Peng et al. 2004). Using a similar approach, we will test whether the subclass of VIP neurons is required in the SCN for rhythmcity and synchrony by deleting them *in vitro* and *in vivo*. We hope to clarify

whether VIP, other signaling pathways, or another unknown cellular state is required

circadian behavior in mammals. If by deleting VIP neurons, we find that all rhythmicity

is lost, we will conclude that VIP neurons are required cellular pacemakers. If we are

unable to delete all VIP neurons, we will quantify the quality of rhythmicity based on the

number of remaining cells. If deleting VIP neurons results in a similar phenotype to the

VIP$^{-/-}$ or *Vipr2*$^{-/-}$ mutations, we will conclude that VIP neurons are not the sole

pacemakers in the SCN and additional signaling is required for circadian rhythms.

Here we present data characterizing a novel mouse model in which VIP neurons

selectively express diptheria toxin A (DTA) under the control of tamoxifen-sensitive Cre-

recombinase, leading to apoptosis in these cells. We first confirm that our construct is

both properly expressed and efficient at deleting VIP neurons *in vitro*. We find a

significant loss of VIP neurons compared to other cell types in SCN explants using our

transgenic model. We plan to test the functional outcomes of this loss of VIP neurons by

measuring gene expression rhythms in SCN explants before and after tamoxifen

treatment. We have also worked to remove VIP neurons in living animals by both global

and SCN-specific tamoxifen delivery. We have found IP tamoxifen injection to be

unsuccessful at initiating recombination *in vivo*; we observed no differences in wheel

running behavior or number of VIP neurons in VIP-deleting animals compared to

controls. Delivery by oral gavage also did not initiate recombination. We have now

delivered tamoxifen directly to the SCN via cannula and will monitor behavioral rhythms

in these animals.

**Materials and Methods**

GENERATION OF pVIP-YFPStop-Cre MOUSE. A bacterial artificial chromosome

(BAC), RP24-131I18, spanning approximately 42 kb upstream and 125kb downstream of

the VIP gene was manipulated using BAC recombineering with galK positive/negative

selection technique (http://recombineering.ncifcrf.gov/). To avoid possible complications

during the expression of Cre recombinase from VIP locus, endogenous loxP and lox511

sites of BAC vector backbone were removed. An insert with enhanced yellow fluorescent

protein (eYFP) sequence followed by two polyA transcriptional stop cassettes was

flanked with two FRT sites which was placed preceding tamoxifen inducible chimeric

Cre-recombinase (Cre-ER(T2)) in pCDNA3 vector. Proper eYFP expression before and

after Flpe recombination was tested in HEK293 cells. This construct was used to generate

an insert by PCR to replace 1951 bp of VIP genomic sequence in BAC RP24-131I18

beginning with the translation initiation site of the gene. Restriction enzyme mapping,

PCR and sequencing was used to confirm proper recombination and insertion. The

purified construct (Appendix A) was injected into mouse (C57Bl/6/CBA F1) blastocysts

by the transgenic core in Ophthalmology at Washington University producing two

founder animals. We have maintained these two lines and have back-crossed founders

onto C57Bl/6 for at least six generations. Genotype for these and all other animals used

have been confirmed with tail-snip PCR.


GENOTYPING. We confirmed the presence of the pVIP-YFPstop-Cre transgene in the

VIP-deleting mouse model using standard tail-snip PCR. Briefly, genomic DNA was

isolated and samples were assayed by PCR with the use of the following primers: VIP/B5 (GCT CTT TCC TTT GTA ACT GTT CCC) and Ysc/F8 (AAA GTA TTA CAT CAC GGG GG). The reaction also contained 0.2 mM dNTPs, 4 M Betaine, and 0.2 µl of KlentagLA in 2 µl of KLA buffer. After samples were heated to 94°C for 2 min, they were subject to PCR for a total of 35 cycles as follows: denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s. The reaction was finished with an extra 5 min elongation period at 72°C, followed by holding at 4°C. Presence of ACTB-FLPe and Rosa-DTA transgenes were detected by PCR using methods previously published (Dymecki 1996; Ivanova, Signore et al. 2005). We confirmed the loss of the YFPstop cassette in pVIP-Cre mice using the following primers for YFP: forward (GTGTCCGGCGAGGGCGAGGGC) and reverse (AAGAAGGCGGGTACCTTACTT). PCR methods were identical to those described above.

IMMUNOSTAINING. We confirmed the expression of our construct in VIP expressing tissues using standard immunohistochemistry methods. Tissue was fixed in 4% PFA and cryoprotected. Sections were double labeled using chicken anti-GFP (1:1000, Aves Labs) and rabbit anti-VIP (1:2000, Diasorin). Fluorescent secondary antibodies, donkey anti-rabbit-IgG conjugated to Cy2 (1:50, Jackson ImmunoResearch) and guinea pig anti-chicken-IgG conjugated to Cy3 (1:200, Jackson ImmunoResearch), were used for visualization.

Following tamoxifen treatment, we confirmed any loss of VIP neurons by labeling for VIP and arginine vasopression (AVP; 1:50, gift from Dr. H. Gainer). Fluorescent secondary antibodies, donkey anti-rabbit-IgG Cy2 (1:50) and donkey anti-mouse-IgG conjugated to Texas Red (1:100, Jackson ImmunoResearch).

LIVE YFP IMAGING. YFP signal in living cells from acute and organotypic SCN slices were visualized using either epi-fluorescence (Nikon) or confocal microscopy (Deltavision). Excitation filters were set between 490 and 510 nm. Emission filters were set between 520 and 550 nm.

COLCHICINE TREATMENTS. To optimize visualization of neuropeptide expression in the brain, colchicine (10mg/mL in 0.9% saline; Sigma) was delivered by intracerebroventricular injection (Karatsoreos, Yan et al. 2004). Briefly, animals were anaesthetized and placed in a stereotaxic apparatus. After the skull was exposed and leveled, a small hole was made above the site of the lateral ventricle (coordinates relative to Bregma: AP +1.0 mm; ML +0.7 mm; DV -3.0 mm from the surface of the brain). 2 microliters of colchicine was injected using a sterile 10 microliter Hamilton syringe. Animals were allowed to recover in their home cage overnight before perfusion.

TAMOXIFEN TREATMENTS. For in vitro tamoxifen treatments, 4-hydroxy-tamoxifen (Sigma) was dissolved in ethanol and then diluted in cell culture medium to a final

concentration of 1 uM. Tamoxifen remained in the medium for 3 days before a full volume medium exchange. Cultures were given a minimum of 4 days to recover before being treated with colchicine and stained for VIP and AVP.

For in vivo tamoxifen treatments, animals were given tamoxifen (Sigma) dissolved in 1:9 ethanol to corn oil by IP injection. Final dosage was either 2 mg/mL per animal once daily for 5 days (Cohort 1), 3 mg/mL per animal twice daily for 5 days (Cohort 2), or 180 mg/kg by weight per animal once daily for 5 days (Cohort 3). Two additional cohorts (Cohorts 4 and 5) were given tamoxifen dissolved in 1:9 ethanol to corn oil by oral gavage, once daily for 5 days. These animals received 200 to 250 µL of 20 mg/mL tamoxifen, for a final dosage of 4 to 5 mg per animal per day. The total number of animals tested across cohorts 1-5 was 35, divided into the following groups: VIP-deleting animals treated with tamoxifen (n=21), VIP-deleting animals treated with vehicle (n=5), littermate controls treated with tamoxifen (n=7), and littermate controls treatment with vehicle (n=2).

A final group of animals (Cohort 6) underwent stereotaxic surgery to target a bilateral cannula (Plastics One) at the SCN for tamoxifen delivery. Briefly, animals were anesthetized and placed in a stereotaxic apparatus. The skull was exposed, leveled, and the cannula guide was positioned above the SCN at -0.5 mm AP from Bregma. Two holes were drilled 0.2 mm on either side of midline. The cannula guide was lowered to -5.0 mm from the surface of the brain and secured with dental cement. Animals were

allowed to recover in their home cages before transfer to cages with running wheels for locomotor behavior monitoring. 4-hydroxy-tamoxifen was dissolved in ethanol and then diluted in 0.9% saline to a final concentration of 1 μM. 0.5 microliters of 4-OH-tamoxifen (Raineteau, Hugel et al. 2006) was delivered via double-barreled cannula under anesthesia once daily for 5 days.

LOCOMOTOR BEHAVIOR ANALYSIS. All animals were housed individually in cages with running wheels with access to food and water. Running wheel activity was recorded in 6-minute bins using Clocklab software (Actimetrics) for at least 10 days in 12:12 LD conditions followed by constant darkness. Animals in Cohort 1 returned to LD during tamoxifen administration, while Cohorts 2-5 received tamoxifen prior to activity monitoring. Cohort 6 was monitored in LD and DD to ensure rhythmic wheel running behavior after surgery. While Cohort 6 was housed in LD, tamoxifen was delivered to each animal under anesthesia via cannula. We analyzed circadian period and amplitude for each condition (LD, DD, pretamoxifen, post-tamoxifen).
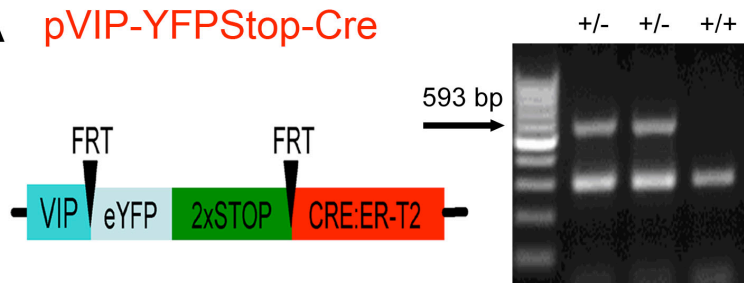
**Results**

**Generation of a mouse model for VIP cell deletion**

Approximately one-third of mice lacking VIP or its receptor, VPAC2, remain behaviorally rhythmic with multiple, weak free running periods (Colwell, Michel et al. 2003; Harmar 2003; Hughes, Fahey et al. 2004; Aton, Colwell et al. 2005). *In vitro*, a
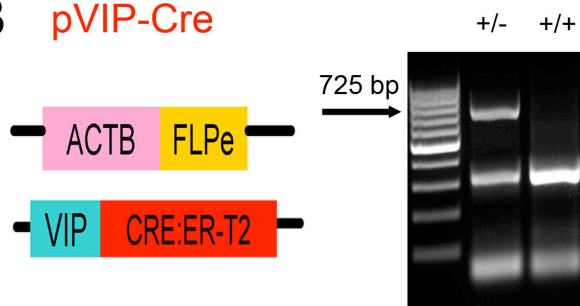
similar proportion of SCN neurons retain rhythms in firing rate, but these neurons are desynchronized with a wide range of periods and phases (Aton, Colwell et al. 2005). In the absence of VIP, another signaling molecule, like GRP, can bind and activate the VPAC2 receptor, restoring synchrony (Maywood, Reddy et al. 2006). Likewise, when VPAC2 is absent, it is possible that VIP signals through other receptors. We hypothesized that by removing the VIP neurons themselves, we could test whether other properties of these cells may be important in driving rhythmicity and synchrony. To this end, we sought to selectively delete VIP neurons in an inducible fashion using tamoxifen-sensitive Cre-recombinase.

We generated a construct containing a BAC spanning 42 kb upstream and 125 kb downstream of the VIP gene (Materials and Methods, Figure 1). For visualization purposes, a YFP cassette followed by two poly-A stop sequences was inserted into the BAC preceding the tamoxifen inducible chimeric Cre-recombinase (Cre-ER(T2)). Flanking this cassette are two FRT sites to allow for its removal in the presence of Flipase (Flpe). We crossed these pVIP-YFP-Stop-Cre mice with a commercially available ACTB-Flpe (Dymecki 1996) line and confirmed the removal of the YFP cassette by immunohistochemistry (Figure 3) and live imaging (Figure 4). When crossed with Rosa-eGFPstop-DTA mice (gift of Dr. S. Hattar) we generate a line of animals (VIP-deleting) that in the presence of tamoxifen should undergo recombination at the loxP sites flanking the eGFPstop cassette and lead to expression of DTA in VIP neurons.

**A** pVIP-YFPStop-Cre

FRT          FRT

VIP | eYFP | 2xSTOP | CRE:ER-T2

+/-  +/-  +/+

593 bp

**B** pVIP-Cre

ACTB | FLPe

VIP | CRE:ER-T2

725 bp

+/-  +/+

**C** pVIP-Cre germline

VIP | CRE:ER-T2

pVIP-YFPStop   pVIP-Cre

~700 bp

593 bp

VIP-Cre

**D** pVIP-Cre; Rosa-DTA

VIP | CRE:ER-T2

loxP          loxP

Rosa26 | 3xSTOP | DTA

300 bp

+/-  +/+
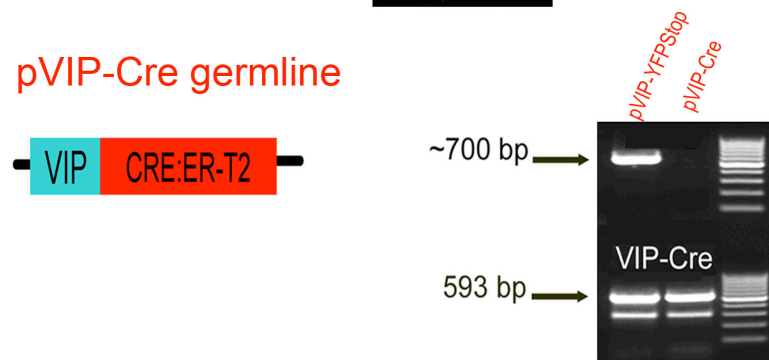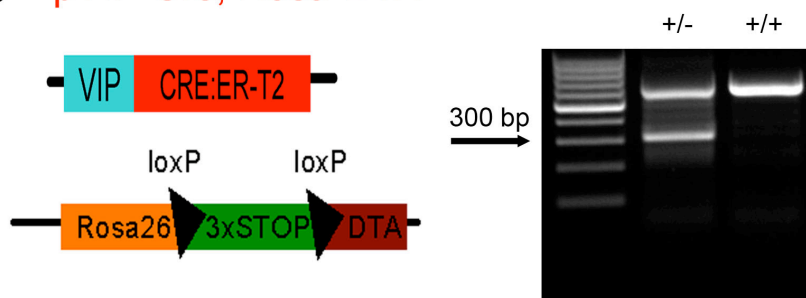
**Figure 1. Strategy for VIP cell deletion using a transgenic BAC.** To specifically target VIP neurons for deletion we employed the following strategy. (**A**) A BAC containing the VIP promoter was used to drive tamoxifen-sensitive Cre recombinase in VIP neurons. (**B**) To remove the eYFP insert surrounded by FRT sites we used the ACTB-FLPe line (Dymecki 1996); purchased from Jackson Labs) and generated a second mouse, pVIP-Cre. (**C**) The loss of the YFP cassette was permanent in the offspring of these animals as confirmed by PCR. We detected a YFP band only in pVIP-YFPStop-Cre animals, but the pVIP-Cre construct was present in both the original and germline animals. (**D**) To target VIP neurons for cell death, we crossed the pVIP-Cre animals with animals expressing Rosa26-eGFPStop-DTA (Ivanova, Signore et al. 2005); gift of Dr. S. Hattar). In the presence of tamoxifen, Cre recombinase will remove the Stop cassette at the loxP sites and allow diptheria toxin to be expressed in VIP cells, leading to apoptosis.

To determine whether our transgenic construct, alone or in combination with other constructs used to generate the VIP-deleting model, did not affect circadian behavior prior to deletion, we measured wheel-running activity in animals not treated with tamoxifen (Figure 2). We found no differences in entrained (12:12 LD cycle) or free running (DD cycle) period, amplitude, or total daily activity in animals containing pVIP-YFPstop-Cre (n=3), pVIP-YFPstop-Cre; ACTB-Flpe (n=2), pVIP-YFPstop-Cre; Rosa-eGFPstop-DTA (n=2), pVIP-YFPstop-Cre; ACTB-Flpe; Rosa-eGFPstop-DTA (n=5), or wild type controls (n=3). Based on these results we believe that there is no leaky expression of Cre-recombinase in the absence of tamoxifen causing alteration of normal behavioral rhythms.

**Faithful expression of pVIP-YFP-Stop-Cre construct**

To confirm the appropriate expression of our construct in VIP-positive neurons, we stained brain and peripheral neural tissues for the presence of YFP using standard immunohistochemical methods. In addition to the approximately 10% of the neurons in the SCN (Abrahamson and Moore 2001), VIP is also expressed in approximately 8% of inhibitory cortical interneurons (Xu, Roby et al.), neurons in the mitral and granule cell layers of the olfactory bulb (Gall, Seroogy et al. 1986), a small percentage of amacrine cells in the retina (Casini, Molnar et al. 1994; Lee, Park et al. 2002), and enteric neurons in the gut from which VIP derives its name (Reinecke, Schluter et al. 1981). A recent study discovered VIPergic neurons in the guinea pig heart (Parsons, Locknar et al. 2006).
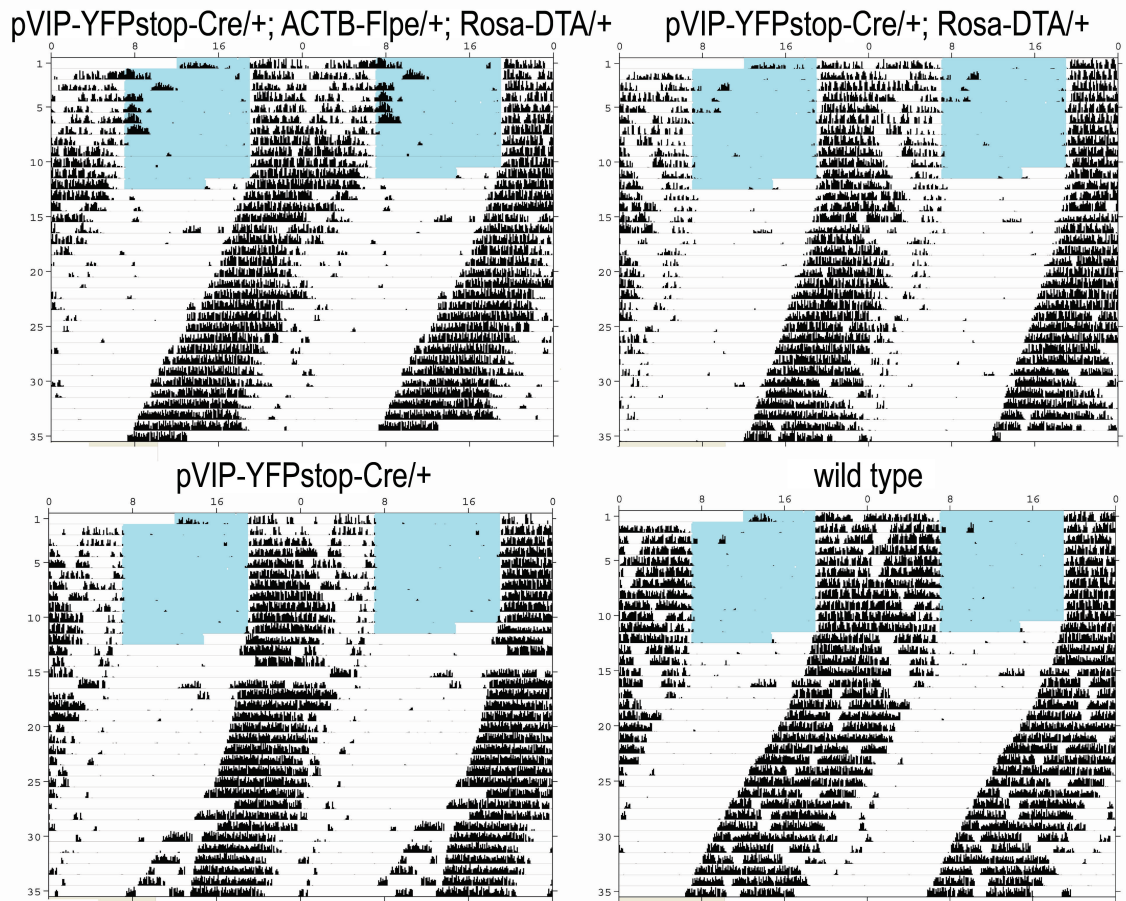
**Figure 2. Mice expressing transgenic alleles have normal wheel running behavior in the absence of tamoxifen.** Representative locomotor activity records from single, double, triple transgenics and a wild-type animal in the absence of tamoxifen. Animals heterozygous for all three alleles in the VIP-deleting model (n=5) showed no significant differences in their circadian period, amplitude, or total daily activity compared to animals with one or two alleles, or non-transgenic littermate controls (n=10). This indicates that Cre expression is not leaky or causing measurable effects in the absence of tamoxifen. Therefore, any behavioral effects seen after the addition of tamoxifen to these animals are likely to be specific to the deletion of VIP neurons.

We also note a transgenic animal using a different VIP BAC promoter (RP23-25A8) driving eGFP shows expression in other brain regions including, the superior colliculus, cerebellum, and brainstem (http://www.gensat.org). We found in the small number of sampled cortical and olfactory bulb sections that 80-100% of VIP-immunopositive neurons were also YFP-positive (n = 20 out of 24 VIP cells in 2 cortical sections from 1 mouse, n = 4 YFP+ cells out 4 VIP cells in 1 olfactory bulb section from 1 mouse; Figure 3). We also confirmed the presence of YFP-positive in neurons of the duodenum, ilium, and colon in the enteric nervous system.

In the SCN, overlap between endogenous VIP and signal from our YFP construct was much less, on average nearly 25% (n = 518 YFP+ out of 2146 VIP cells from 10 animals; Table 1). Some VIP-positive neurons did not contain YFP, suggesting that our construct may not target all VIP cells in the SCN. While about a third of neurons with our construct also contained VIP, we found some YFP-positive neurons that did not express VIP (n = 518 VIP+ out of 1664 YFP cells from 10 animals; Table 1). We suspected that because VIP is expressed rhythmically in the SCN (Shinohara, Funabashi et al. 1999), we may miss a population of VIP neurons depending on circadian time. To alleviate this concern, future experiments should deliver colchicine to the lateral ventricle of animals to minimize vesicle release and concentrate neuropeptide expression in the cell bodies.
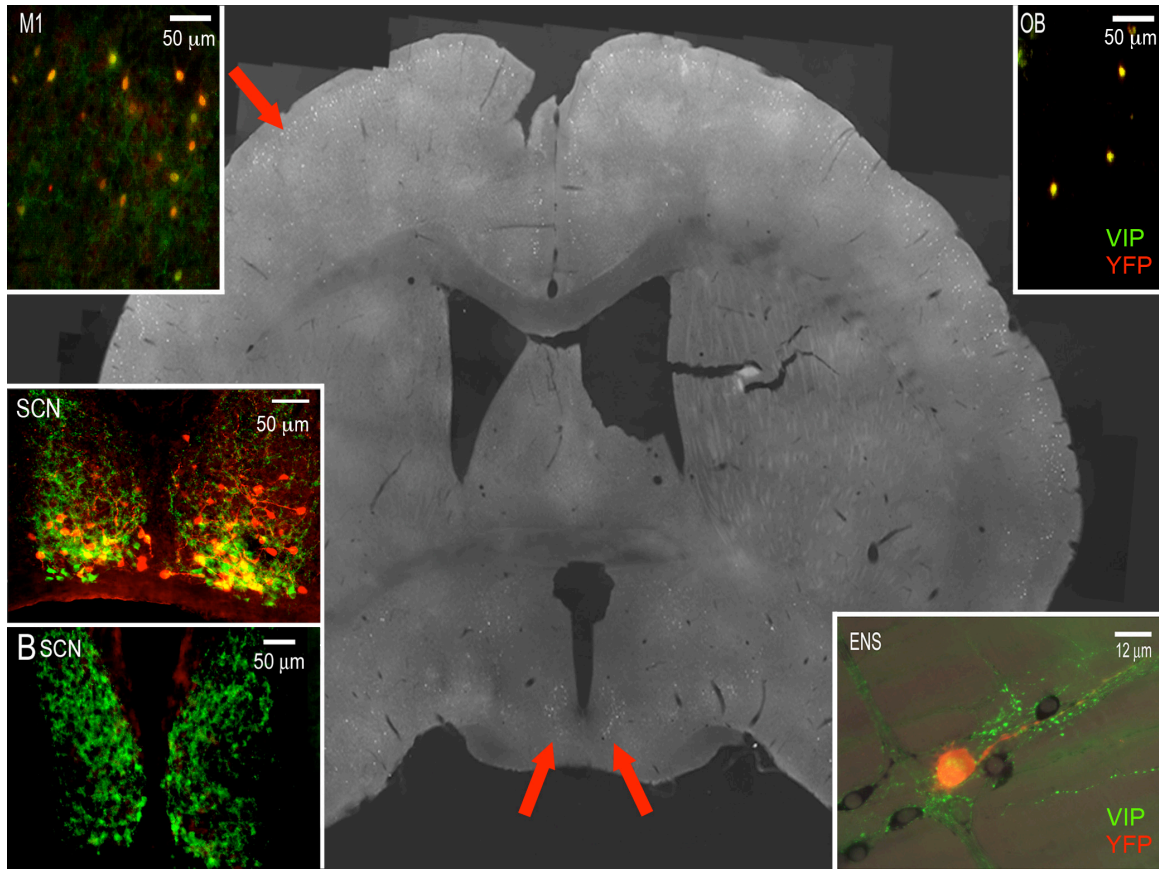
**Figure 3. Faithful expression of pVIP-YFPStop-Cre construct in central and peripheral neural tissues.** Composite image of YFP expression in a coronal section including the SCN and motor cortex (red arrows). Insets show expression from two founder lines of pVIP-YFPstop-Cre. SCN and ENS from line A and motor cortex and OB from line B. Sections are double labeled for YFP (red) and VIP (green). Co-expression (yellow) of endogenous VIP and our construct reporter is seen in all expected tissues. (**B**) We show loss of YFP expression (red) in VIP neurons in the SCN of animals heterozygous for both the pVIP-YFPstop-Cre and ACTB-Flpe alleles.

| | Total VIP+ | VIP+/YFP- | Total YFP+ | YFP+/VIP- | VIP+/YFP+ |
|---|---|---|---|---|---|
| F2 (n=4) | 563 | 520 | 92 | 49 | 43 |
| F3 (n=3) | 648 | 406 | 774 | 532 | 242 |
| F4 (n=3) | 935 | 702 | 798 | 565 | 233 |
| | | | | | |
| Total | 2146 | 1628 | 1664 | 1146 | 518 |
| Average | 215 | 163 | 166 | 115 | 52 |

**Table 1. Summary of double labeling counts of VIP and YFP in the SCN from pVIP-YFPstop-Cre animals.** We compare the total numbers of VIP+, YFP+, and co-labeled neurons from SCN taken from F2 (n=4 animals, 6 sections per animal), F3 (n=3 animals, 8 sections per animal), and F4 (n=3 animals, 7 sections per animal) generations of the pVIP-YFPstop-Cre line.

**YFP in living neurons**

Our construct design includes eYFP to allow for identification and visualization of living VIPergic neurons. This is beneficial for physiologists who would like to record and characterize known cell types. We confirmed live YFP expression in neurons from the SCN and OB in acute and organotypic tissue explants (Figure 4). YFP expression was lost in animals containing both our construct and ACTB-Flpe, showing that in the presence of flipase the eYFPstop cassette was successfully removed at the FRT sites (Figure 4). We confirmed that the loss of the eYFPstop cassette in our construct was passed on via germline transmission in the offspring of animals containing our construct and ACTB-Flpe. Using tail snip PCR we tested these animals for the presence of YFP and found that while they still contained the VIP-Cre band, no YFP band was seen (Figure 1), therefore establishing a second line of pVIP-Cre animals that lack YFP.

*In vitro* **tamoxifen treatment deletes VIP neurons**

To test whether we could successfully drive recombination and delete VIP neurons in the presence of tamoxifen, we cultured SCN explants from pVIP-Cre; Rosa-DTA (VIP-deleting) animals. After one day *in vitro* 4-hydroxy-tamoxifen, the active metabolite of tamoxifen, was added to the cell culture medium at a final concentration of 1 uM. Explants were maintained in tamoxifen-containing medium for 3 days before removal and one full volume exchange of fresh medium. Explants remained in culture for 4 days to recover before treatment with colchicine, fixation and staining for VIP and vasopressin (AVP), as a control for non-specific cell death. Explants from non VIP-deleting littermate
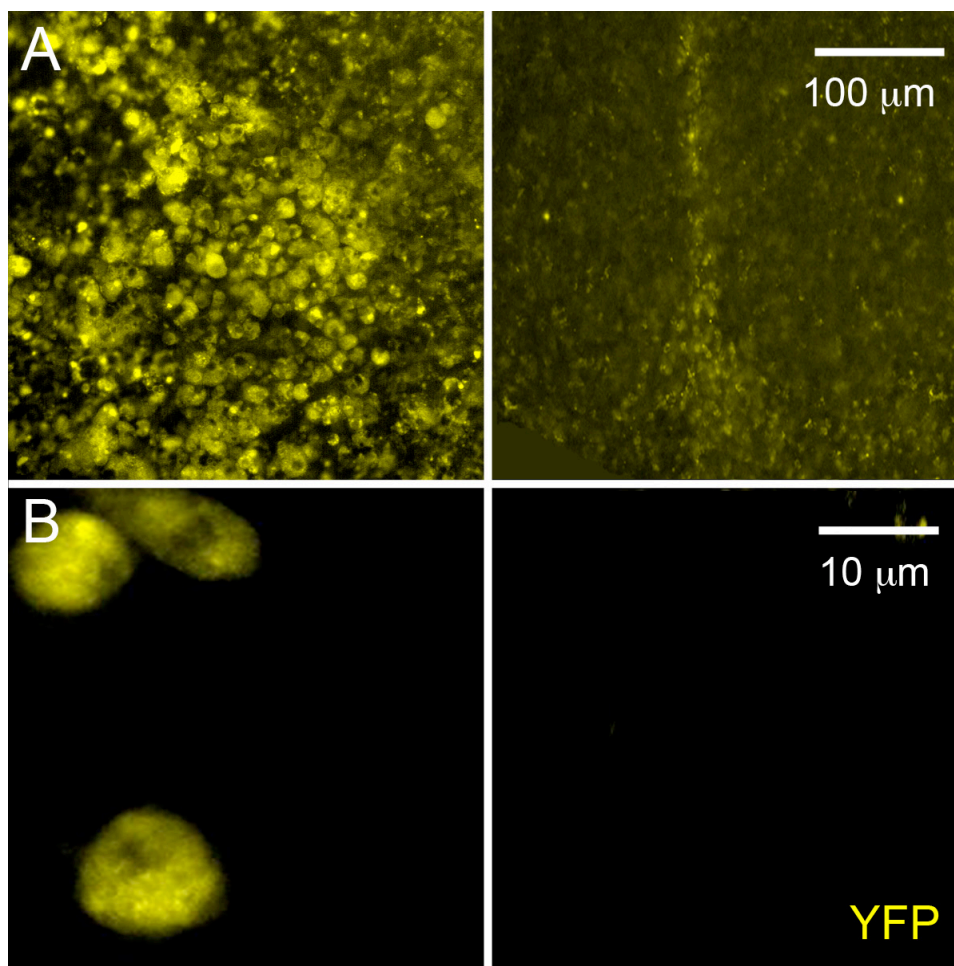
**Figure 4. YFP is visible in living neurons.** Due to the eYFP insert, the presence of our transgenic construct can be confirmed in cultured SCN neurons using fluorescence microscopy. We visualized YFP+ neurons in organotypic SCN explants (**A**) from either pVIP-YFPstop-Cre (left) or pVIP-Cre (right) animals at 20x using an epifluorescence microscope. We also identified YFP+ cell bodies in acute SCN slices (**B**) at 40x using a deconvolution confocal microscope. Tissue from animals that had the YFPstop cassette removed by flipase lacked YFP signal.

controls received identical 3-day tamoxifen treatment and were stained along with the experimental set. Two independent observers, blind to animal group and treatment, counted the number of VIP+ and AVP+ neurons in each SCN. These counts differed by less than 10%. In SCN explants from VIP-deleting animals treated with tamoxifen, we found the number of VIP+ neurons decreased significantly compared to tamoxifen treated controls (Figure 5; n = 25 ± 8 VIP neurons from 3 VIP-deleting explants, n = 74 ± 8 VIP neurons from 3 control explants, $p < 0.05$). We did not observe a significant loss of AVP neurons in VIP-deleting explants compared to controls (Figure 5; n = 137 ± 25 AVP neurons from 3 VIP-deleting explants, n = 163 ± 12 AVP neurons from 3 control explants, $p < 0.5$). Another replicate of this experiment showed a similar trend for both VIP+ and AVP+ neurons in VIP-deleting explants compared to controls (n = 2 explants per group, data not shown). We conclude that tamoxifen treatment leads to significant loss of VIP neurons while sparing other cell types. Any remaining VIPergic neurons in tamoxifen treated VIP-deleting explants appear unhealthy, often with shrunken or misshapen cell bodies, suggesting that these cells were dying at the time of fixation. *In vitro* results suggested that our model works to specifically delete VIP neurons and we proceeded to test VIP-deletion *in vivo*.
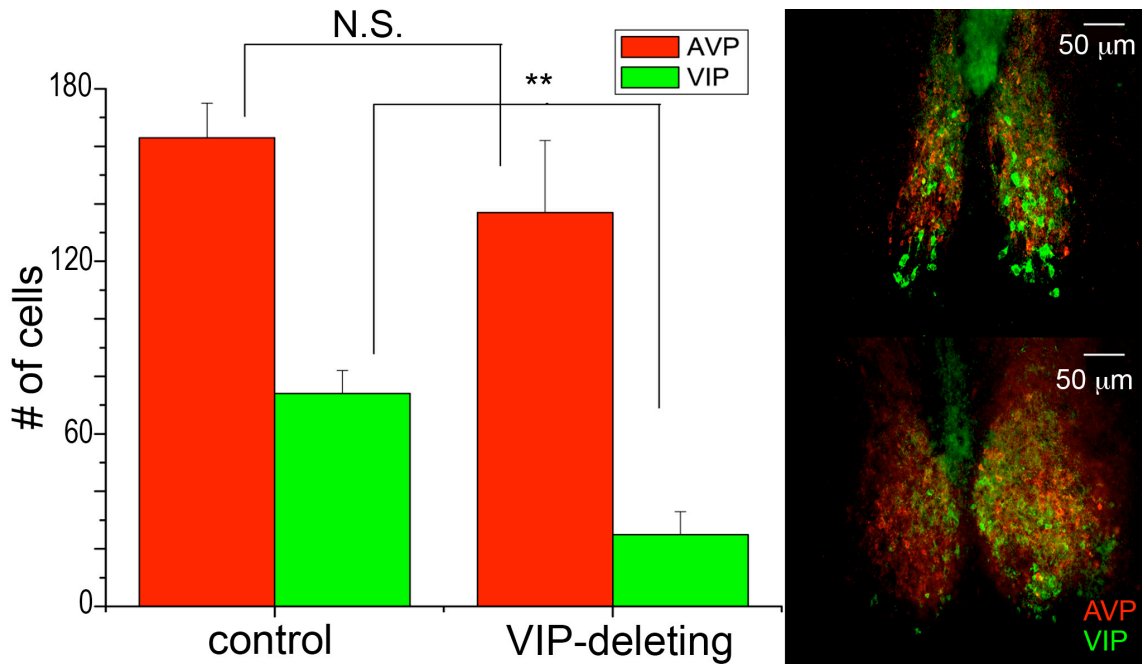
**Figure 5. *In vitro* tamoxifen treatment reduces the number of VIP neurons.** *In vitro* application of 4-OH-tamoxifen reduced the number of VIP neurons compared to AVP neurons. Two observers blind to genotype and treatment counted fluorescent cells; their counts differed by less than 10%. The number of AVP neurons did not appear to differ in SCN slices from control (top, n = 3) and from VIP-deleting animals (bottom, n = 3) while the number of VIP neurons was significantly decreased in the VIP-deleting slices (p<0.05).

**IP tamoxifen injection does not change circadian behavior or VIP neurons**

To test whether we could delete VIP neurons *in vivo* we delivered tamoxifen via intraperitoneal injection to both VIP-deleting animals and littermate controls. We tested three different tamoxifen doses in separate cohorts. Cohort 1 received 0.4 mg once daily for 5 days at two separate times during activity monitoring. Cohort 2 received a higher dose of 0.6 mg twice daily for 5 days. Cohort 3 received an intermediate does based on weight (180 mg/kg) once daily for 5 days. During or following injections all animals were housed individually in cages with running wheels. We recorded behavioral activity in both LD and DD conditions. Compared to controls, VIP-deleting animals that received tamoxifen showed no difference in entrained or free running periods (Figure 6; Table 2). We perfused these animals and examined the number of VIP neurons that remained. Consistent with the lack of a behavioral phenotype, we found no loss of VIP neurons in the VIP-deleting model compared to control animals (Figure 6). We did observe a subtle effect on the LD activity profile of tamoxifen treated VIP-deleting animals compared to controls (Figure 7). While overall activity counts were lower in the VIP-deleting animals, their rising phase of activity was phase advanced compared to controls, showing a trend for increased activity compared to controls in anticipation of lights off. The time of peak activity during the dark phase is similar for both groups, though VIP-deleting animals peak slightly earlier (19.7 h for VIP-deleting compared to 20.2 h for controls).

|  | VIP-deleting | + tamoxifen | control | + tamoxifen |
|---|---|---|---|---|
| **LD** | 24.0 ± 0.1 | 24.0 ± 0.2 | 24.2 ± 0.3 | 23.9 ± 0.1 |
| **DD** | 23.7 ± 0.1 | 24.0 ± 0.4 | 24.5 ± 0.6 | 24.1 ± 0.2 |
| **LD post treatment** | 24.3 ± 0.4 | 24.3 ± 0.2 | 23.8 ± 0.1 | 24.0 ± 0.1 |
| **DD post treatment** | 23.5 ± 0.3 | 23.7 ± 0.1 | 24.0 ± 0.1 | 23.7 ± 0.1 |

**Table 2. Tamoxifen treatment does not affect entrained or free running activity in control and VIP-deleting animals.** We compare wheel-running activity in entrained and free running conditions from vehicle and tamoxifen treated animals before and after 5 days of IP tamoxifen injections and find no significant differences in period.

A  VIP-deleting + IP tamoxifen    wild type + IP tamoxifen

B

AVP
VIP

C

# of cells

Rostral to caudal distance (μm)

AVP
VIP
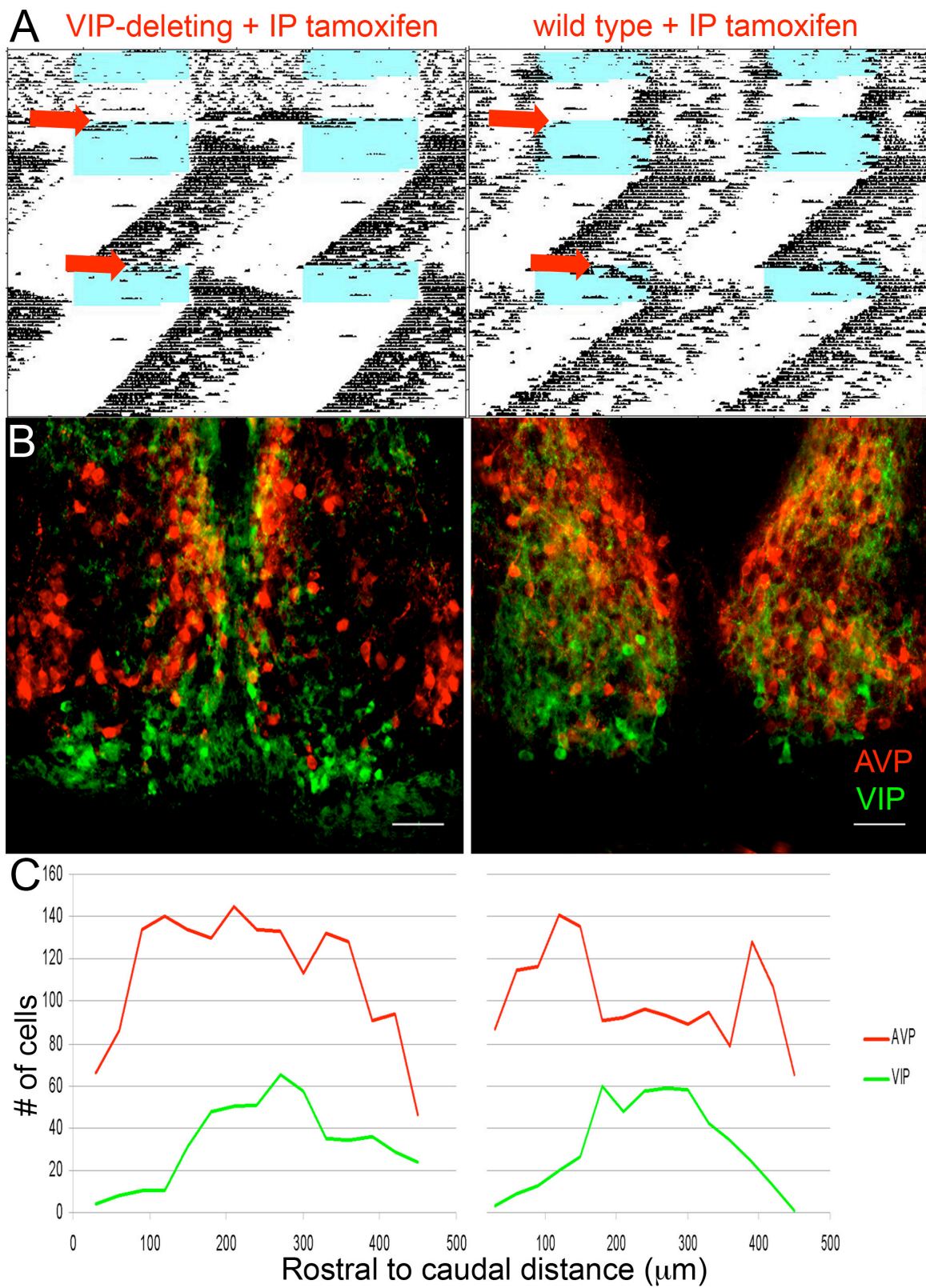
**Figure 6. *In vivo* tamoxifen treatment did not change behavior or number of VIP neurons.** (**A**) Representative actograms from cohort 1. VIP-deleting (n=6) and control animals (n=6) received IP tamoxifen or vehicle for 5 days on two separate occasions (red arrows). The groups did not differ in circadian period, amplitude or daily activity pre- or post-tamoxifen (Table 2). (**B**) Representative SCN sections (scale bar, 50 μm) from each group show similar numbers of VIP+ (green) and AVP+ (red) neurons, as quantified for the rostral to caudal extent of the nucleus in (C). Cohorts 2 and 3 showed similar results in wheel running activity and VIP/AVP staining.
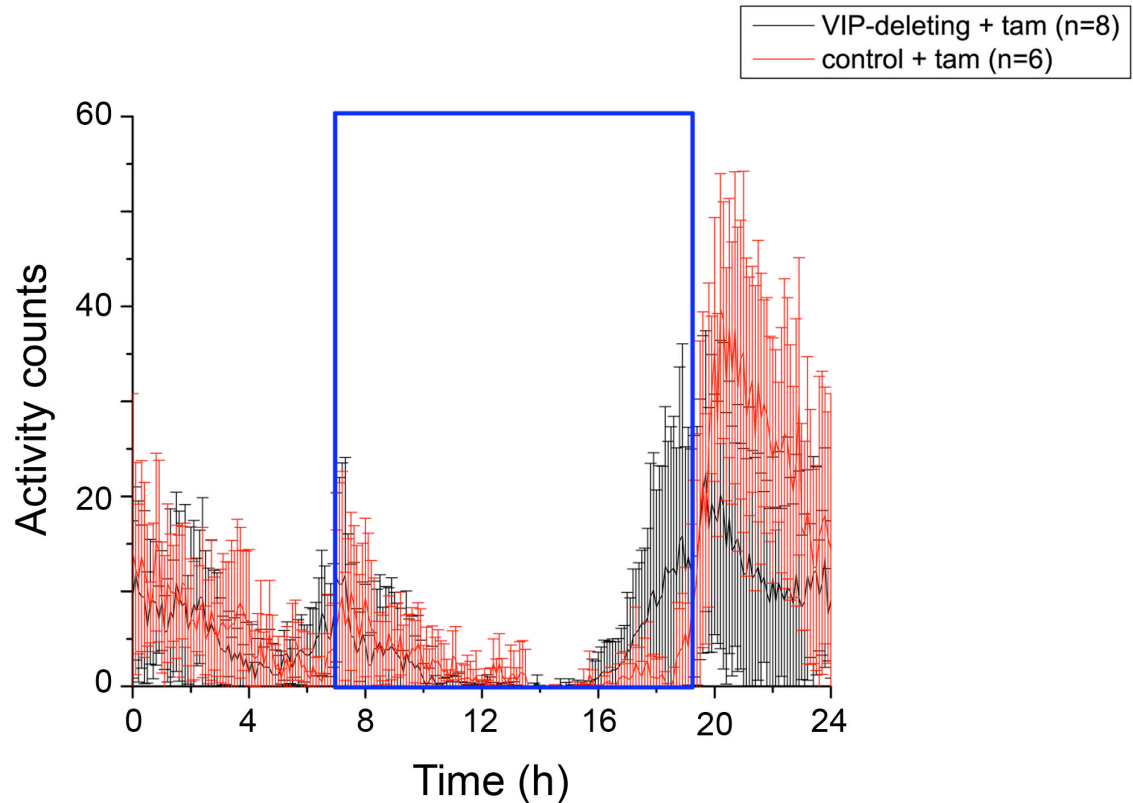
**Figure 7. Average LD activity profile of tamoxifen treated VIP-deleting animals is phase advanced compared to controls.** The 12:12 LD (lights on represented by blue box)activity profiles of VIP-deleting mice (black trace, n=8) show a trend towards running earlier compared to controls (red trace, n=6), increasing activity before lights off at ZT 19. The time of peak activity is similar, but also slightly phase advanced for VIP-deleting animals (19.7 h compared to 20.2 h for VIP-deleting and control groups, respectively). VIP-deleting animals also have lover activity levels overall.

Oral gavage delivery of tamoxifen has been shown to increase efficiency of recombination while minimizing toxicity (Park, Sun et al. 2008). We delivered 4 mg of tamoxifen by oral gavage once daily for 5 days to Cohort 4 (n = 8, VIP-deleting; n = 3, control) and Cohort 5 (n = 2, VIP-deleting; n = 1, control), which included animals that removed *Bmal1* in VIP neurons (n = 2, pVIP-Cre; floxed-*Bmal1*) in addition to the VIP-deleting group. All of these animals also exhibited normal circadian rhythms with no differences in activity in 12:12 LD and DD conditions (free running periods in control, 23.6 h; VIP-deleting, 23.6 h; *Bmal1*-deleting, 23.4 h, respectively) and histological sections from Cohort 4 showed no significant loss of VIP neurons in VIP-deleting animals compared to controls.

After global tamoxifen treatment was unsuccessful in initiating Cre-mediated recombination and because we ultimately sought SCN-specific VIP neuron deletion, we finally delivered tamoxifen directly to the SCN via cannula. A double-barreled cannula guide was implanted in VIP-deleting (n = 2), *Bmal1*-deleting (n = 2) and control animals (n = 2) directly above the SCN and secured with dental cement. Following recovery, these animals were housed in cages with running wheels and their behavioral activity was monitored. We will deliver 4-hydroxy-tamoxifen in 0.9% saline via cannula to the SCN of these animals once daily for 5 days and resume activity monitoring. We expect to observe a change in behavior following SCN-direct tamoxifen delivery.

**Discussion**

**Characterization of a novel VIP cell deletion model**

We have developed and characterized a novel mouse model that marks VIP neurons with

YFP, a benefit for those seeking to measure electrophysiological or other properties of

VIP cells. We believe that our construct is faithfully expressed in VIPergic neurons

throughout central and peripheral neural tissues. By immunohistochemical methods,

nearly 100% VIP neurons in the cortex and olfactory bulb express our construct. The

lower levels of co-expression observed in the SCN should be resolved by treating animals

with colchicine prior to fixation. Colchicine treatment has been shown to increase

neuropeptide content in cell bodies and thus enhance detection (Karatsoreos, Yan et al.

2004). Because endogenous VIP is expressed rhythmically, it is possible that at time of

perfusion some cells did not contain enough VIP to be targeted by our antibody. We also

noticed some YFP+ cells had nearby VIP+ fibers (Figure 3), suggesting that VIP cells

may be surrounding these neurons, but lack a high enough signal in the cell body to show

co-labeling. We expect that by concentrating VIP in the cell bodies we will see better

overlap between our construct and the endogenous population.

Another piece of evidence that increases our confidence that we are driving the construct

in VIP neurons is the significant loss of VIP neurons in the SCN following *in vitro*

tamoxifen treatment. We have observed a nearly complete loss of VIPergic neurons in

SCN slices from VIP-deleting animals treated with tamoxifen (Figure 5). We observe

non-significant loss of other subtypes; specifically, we measured number of AVPergic

cells in the same slice and found similar results in tamoxifen treated VIP-deleting and control animals. Tamoxifen does not appear to have non-specific effects on control animals.

**Limitations and possible solutions for tamoxifen delivery *in vivo***

Based on the literature, inducible Cre activation relies on the molecular mechanisms of nuclear hormone receptors (Garcia and Mills 2002). Cre is fused with an estrogen receptor (Cre-ER) that remains sequestered in the cytoplasm by heat-shock proteins until it is bound by its ligand. For Cre-mediated recombination to take place, a single molecule of tamoxifen must bind to the estrogen receptor and allow Cre to move into the nucleus (Feil, Brocard et al. 1996). Recent studies using a series of IP injections of tamoxifen similar to our method have a range of recombination efficiencies in embryonic (50-66%) and neonatal pups (72%) when delivered to pregnant dams, and as high as 97% when administered in adults (Imayoshi, Ohtsuka et al. 2006; Lagace, Whitman et al. 2007; Slezak, Goritz et al. 2007; Weber, Bohm et al. 2009). Tamoxifen or its active metabolite is believed to pass the blood-brain barrier, as CNS-specific expression patterns are seen following global injection. In some models, oral gavage efficiencies are slightly higher than with IP delivery (Park, Sun et al. 2008).

Our difficulties with *in vivo* VIP cell deletion could be due to a number of technical issues. The dose of tamoxifen that reaches the SCN may not be high enough to cause recombination. We hope by delivering tamoxifen directly to the SCN via cannula we can

circumvent this problem. Our VIP BAC promotor may not be strong enough to drive sufficient Cre expression. We could double the copy number of the pVIP-Cre construct by breeding animals to homozygousity. With animals homozygous for our pVIP-Cre construct, we expect higher levels of Cre expression, and thus higher potential for recombination. It is also possible that removal of the YFPstop cassette by Flipase may have damaged the remaining construct and impaired the level of Cre-recombinase expression. We think this is unlikely, as we do see successful tamoxifen-induced recombination *in vitro*. Alternatively, the problem may not be in our pVIP-Cre construct, but instead in the Rosa-DTA expression. *In vitro* VIP neuron loss in SCN explants from animals as young as P10 suggest that Rosa-DTA is active at early post-natal ages. We speculate that Rosa-DTA expression could be down regulated in older animals and the lack of VIP cell loss is due to no or low amounts of DTA.

**Future experiments to probe the role of VIP neurons**

We have generated a novel mouse model to specifically delete VIP neurons. We have shown that by delivering tamoxifen *in vitro* we can delete VIP neurons at 50-66% efficiency in SCN explants without significantly loss of other cell types. Future experiments will pursue the role of VIP neurons within the SCN network by deleting these cells and measuring changes in rhythmicity and synchrony in the remaining neurons. We hypothesize that loss of VIP neurons will affect the circadian behavior of remaining neurons within an explant, either altering single cell rhythmcity or ability of cells to synchronize to each other. These experiments will require a means to assess gene

expression patterns in single neurons from VIP-deleting animals. Transfection of SCN explants from VIP-deleting animals with viruses containing gene reporter technology, such as PER2::LUC or BMAL1::LUC, will allow us to monitor rhythms prior to and after VIP neuron loss *in vitro*. We will record bioluminescence for several days prior to tamoxifen treatment, continue recording during treatment and after tamoxifen is removed from the medium. We will measure gene expression rhythms in single cells under these conditions and look for differences after VIP neurons have been killed. We can confirm cell loss after recording by staining for neuropeptides. Alternatively, we can culture SCN neurons from these animals on multi-electrode arrays and record firing rate from single cells before and after tamoxifen treatment. These experiments will be the first to test the effect of deleting a specific subpopulation of cells on circadian rhythmicity and synchrony in single cells.

In conclusion, we have begun significant progress towards characterizing a novel mouse model that will specifically delete the population of VIP neurons in the SCN. We have established protocols to test our model *in vitro* and we will further study the effects of VIP neuron loss on rhythmicity and synchrony of single cells. We are working to improve *in vivo* protocols. We hope to delete VIP neurons in the animal to test the effects of cell loss on behavioral rhythmicity. We anticipate that SCN targeted tamoxifen delivery and breeding animals homozygous for our construct will improve chances of Cre-mediated recombination and thus observing VIP cell deletion and behavioral phenotype.

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# Chapter 5.

# <u>Conclusions and future directions</u>

The original aim of this thesis was to test the hypothesis that individual SCN neurons are competent circadian oscillators. Previous *in vitro* studies of firing rate in dispersed SCN neurons led to the assumption that all SCN neurons function as cell autonomous oscillators (Welsh, Logothetis et al. 1995). These cultures showed evidence of intercellular communication, however, an indication that signaling from other cells was capable of influencing the observed behavior. Whether and which single SCN neurons can maintain self-sustained rhythms remained unresolved. Later studies highlighted the crucial roles of cell-cell signaling, specifically through VIP and $G_i$ receptors, in aiding circadian rhythmicity, amplitude, precision, and synchrony (Aton, Colwell et al. 2005; Aton, Huettner et al. 2006). These data also left open questions: if some cells retain circadian rhythms in the absence of intercellular signaling, do they make up a subset of pacemakers? To address these questions, our approach characterized circadian properties across single cells. We sought to extensively test whether isolated SCN neurons, including a single neuron in a dish, are self-sustained oscillators and then determine their cellular identity.

**Seeking SCN pacemakers**

Using very low-density neuronal dispersals, optimized to maintain cell health for the duration of the recording, or SCN explants treated with blockers of cell-cell communication, we recorded SCN neurons that were functionally isolated from all other cells (**Chapter 2**). Our work was the first to show that a single SCN neuron is a capable circadian pacemaker with self-sustained rhythms in gene expression for as long as we

recorded. Unexpectedly, we also found that when uncoupled from the SCN network, most single neurons are rarely pacemakers and that no single subgroup comprises an oscillator class. Even more surprising was the observation that SCN neurons can switch their circadian properties over time. We concluded that individual SCN neurons are plastic, each having the ability to function as cell-autonomous oscillators, but not always behaving as such, and that intercellular signaling dramatically increases the number of circadian cells. This led to further examination, first, of what factors could contribute to a range of circadian phenotypes, and then potential consequences of SCN heterogeneity on the function of the network (**Chapter 3**), as well as the necessity of a single subtype of SCN neurons in that network (**Chapter 4**). The role of heterogeneity in single neurons of the SCN and its contribution to plasticity of the network are recurring themes each discussed briefly below.

**A range of circadian phenotypes**

The three prevailing hypotheses for cell-autonomous circadian rhythm generation in SCN neurons are: 1. All cells are capable of self-sustained rhythms. 2. No cells are capable and network interactions are required to support rhythmicity. 3. A subset of SCN neurons are capable of self-sustained rhythms and comprise a pacemaker class that drives oscillations in the remaining cells. In **Chapter 2** we demonstrated a surprising alternative, that while all SCN neurons appear capable of generating self-sustained rhythms, only a small percentage does so when uncoupled from the network. Instead, we observe a range of behaviors in single cells including sustained, damped, and non-circadian changes in gene

expression and firing rate. SCN neurons have the ability to move along this continuum throughout their lifetime, sometimes switching from self-sustained oscillators to damped, driven cells. We found that the rising slope of PER2 accumulation was a strong predictor of whether a cell would continue to oscillate. We hypothesize that this or other molecular factors contribute to the circadian fate of single SCN neurons, and that future experiments could probe how changes in number of gene transcripts or protein products, or the rate at which they are made, correlate with circadian behavior.

We further examined potential sources for the range of circadian in phenotypes using a mathematical model of the circadian clock (**Chapter 3**). By probing different parameter values that alone or in combination generated sustained, damped, or arrhythmic patterns in gene expression, we determined that output phenotype was more sensitive to rates of translation and degradation. **Chapter 3** also highlights that small changes in molecular events could change the output of single cells and the likelihood that cell-to-cell differences contribute to generating a range of oscillatory phenotypes in the SCN. This result is reminiscent of the stereotyped firing patterns in neurons of the stomatogastric ganglion supporting a range of values in ionic conductances (Prinz, Bucher et al. 2004; Schulz, Goaillard et al. 2006; Schulz, Goaillard et al. 2007). These cells can also transition from one state to another, from tonic firing to bursting for example, simply by small changes in a conductance to maintain homeostasis in network behavior (Marder and Goaillard 2006). In light of these observations, we propose that having a population of neurons with a range of circadian phenotypes might also help the SCN network preserve homeostasis in the face of changing condtions.

**Benefits of sloppy, plastic clocks**

We hypothesize that the major benefit of flexible circadian behavior in single cells is it makes the SCN network more robust to internal or environmental perturbations. As we show in **Chapter** 2, a given SCN neuron is not likely to sustain circadian rhythms if uncoupled from the network, but it does have the ability to do so, and may at a later point. Many studies have extensively described or hypothesized about the differences seen across the SCN in innervation, gene expression, and neuropeptide content (Moore 1996; Hamada, LeSauter et al. 2001; Moore, Speh et al. 2002; Hamada, Antle et al. 2004). Here we add another difference, the ability to sustain oscillations, which seems instead to be dictated by chance (**Chapter 2**). Stochastic noise and instability can be beneficial to systems, increasing the diversity of components and sometimes providing a necessary and sustaining perturbation (Raj and van Oudenaarden 2008). Because SCN neurons are intrinsically unstable it is interesting to speculate that this sloppiness is a fundamental design principle of the network.

It seems intuitive that a system of fixed oscillators is less flexible than a system of plastic ones. If all cells behave identically, how does a system adapt to a new input like a change in day-length? Each cell must receive that input and respond in the same way, or if only a few cells sense the signal and shift their behavior, there must be a period of time before the system resumes a coherent output and all cells are behaving identically again. We show in **Chapter 3** that a network of all sustained cells has difficulty resynchronizing

from an uncoupled state. In the simulation, cells start from initial conditions where they are rhythmic and synchronized, all cells remain oscillatory when uncoupled (unlike the observation in isolated SCN neurons), and continue to oscillate, but not in phase with each other even when coupling is restored. While this observation is surprising, it suggests that a network of all sustained cells with a wide range of periods cannot easily phase lock to one another unless given the proper initial conditions. It seems likely then, that cells with range of oscillatory behaviors, including damped and arrhythmic ones are a critical part of the SCN network.

Likewise, if only a small number of neurons can act as pacemakers, what happens to the system when some or all of these cells are lost? We have begun taking steps to tackle this question, which we describe in **Chapter 4**. Future experiments will examine the necessity of VIP neurons in rhythmicity of the SCN network. The work presented in this thesis is novel in that it claims the SCN functions better because it is comprised of changeable oscillators, and that plasticity in oscillatory ability, even arrhythmicity at the single cell level, improves the system much more so than having a population of identical, all-sustained oscillators.

**Building a better SCN network**

If all SCN neurons were not created equal, we need a set of rules for how this assortment of neurons will assemble into a functional network. Models have proposed defined roles for groups of cells, such as light-sensitive gates that signal a separate population of

oscillators (Antle, Foley et al. 2003; Antle, Foley et al. 2007). Our results also highlight this heterogeneity of SCN neurons, but make a new assertion that the different labels describing circadian behavior are not fixed. We have hypothesized that a network of nodes, which may change their properties over time benefits the system. In the second part of **Chapter 3** we argue that networks of damped oscillators, showing a range of behaviors, resynchronize faster than networks of sustained oscillators. A group of sustained cells, each oscillating at their own pace, seem to march out of step even when they can signal each other, unless they begin in phase. In mixed populations, the first simulations we know of that examine networks including sustained, damped, and arrhythmic oscillators, we find that placing damped oscillators at highly connected nodes speeds resynchronization. We speculate that stiff oscillators cannot adapt to reach a consensus behavior, especially from a desynchronized state, and that signaling from damped and flexible oscillators is required for a robust output at the network level. We have extensively tested the properties of individual nodes of the SCN network and have made predictions about the advantages of heterogeneity. Future work will extend the characterization of the SCN to its network, specifically, to further develop a list of rules describing how these nodes assemble.

We (and others) have proposed the SCN as a good candidate for the study of network function (Strogatz 2001). The SCN is a system that accommodates a range of behaviors at the single cell level and continues to maintain a robust oscillator at the tissue level (Liu, Welsh et al. 2007; Webb, Angelo et al. 2009). The next direction for this research is to increase understanding of how the wiring of the SCN supports synchrony and

rhythmicity and define the organizing principles of the network. Experimental data will

provide metrics of network organization, such as connectivity maps, and simulations will

use agent-based strategies to examine the effects of placement of specific cell types at

different points in the network on rhythmicity and synchrony. In conclusion, our work

highlights the range of circadian behaviors in single SCN neurons and makes predictions

of how this benefits the SCN network. Future studies will begin to characterize properties

of SCN networks and further understanding of fundamental principles underlying the

organization of the mammalian circadian system.

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# Appendix A.

## <u>Sequence of pVIP-YFPstop-CreERT2 construct</u>

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<mark style="background-color: cyan">FRT<u>YFP</u>2xSTOPFRT<mark style="background-color: lime">CREERT2</mark> Insert</mark>

<mark style="background-color: red">End Insert</mark>

<mark style="background-color: magenta">VIP stop codon</mark>

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GACAGCTGCCACGAAGCCGGAAAGGCAGCCCTGCCTGAAGGAAACAGCCA

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