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The roles of vasoactive intestinal polypeptide in circadian entrainment of suprachiasmatic nucleus

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THE ROLES OF VASOACTIVE INTESTINAL POLYPEPTIDE IN CIRCADIAN ENTRAINMENT OF SUPRACHIASMATIC NUCLEUS

By

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ABSTRACT OF THE DISSERTATION

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In mammalian hypothalamus, the suprachiasmatic nucleus (SCN) generates daily behavioral and physiological rhythms as a circadian pacemaker. The 20,000 SCN neurons synchronize to each other and to the ambient cues to generate coherent daily rhythms. Vasoactive intestinal polypeptide (VIP), a neuropeptide produced by SCN neurons, plays a major role in synchronizing SCN neurons to each other. Whether VIP mediates synchrony to environmental cues and how synchrony within the SCN is achieved has not been examined extensively. We recorded PERIOD::LUCIFERASE (PER2::LUC) expression from SCN explant cultures over multiple days following VIP application at different circadian time points to generate a phase response curve which reliably predicted the phase relationship between the SCN and daily increases in VIP. VIP shifted PER2::LUC rhythms in time- and dose-dependent manner. VIP rapidly increased intracellular cAMP in most SCN neurons and simultaneous antagonism of adenylate cyclase (AC) and phospholipase C (PLC) was required to block the VIP-

induced phase shifts of SCN PER2 rhythms. We conclude that VIP entrains circadian timing among SCN neurons through rapid and parallel changes in AC and PLC activities.

While performing the experiments mentioned above, we found that a single VIP pulse reliably reduced the PER2::LUC rhythm amplitude in the SCN explants. The amplitude reduction was dose-dependent, but not circadian. We found that the amplitude reduction was primarily explained by reduced synchrony among SCN neurons, with little effect on the amplitude of individual neurons. To test if VIP modulates the amplitude of circadian rhythm *in vivo*, we compared the effects of light on locomotor rhythms in wild-type and VIP-deficient mice. We found that constant light reduced the amplitude of behavioral rhythms in wild type, but not in *Vip-/-*, mice. Because, theoretically, reduced synchrony among oscillators can facilitate their entrainment to periodic signals, we tested if VIP accelerates entrainment of animals to an 8-h advanced light-cycle or SCN explants to a 10-h advanced temperature cycle. We found that VIP doubled the speed of circadian entrainment both *in vivo* and *in vitro*. We conclude that reduced synchrony by VIP accelerates entrainment.

Finally, we characterized the spatiotemporal expression of one of the three major VIP receptors, VPAC2R, in various brain areas and SCN. We characterized the specificity of a new antibody and found moderate to weak levels of VPAC2R in cortex, hippocampus, olfactory bulb, cerebellum, arcuate nucleus in hypothalamus, amygdala and ventrolateral thalamus and high levels in the SCN. VPAC2R expression was observed from rostral to caudal SCN with stronger expression in dorsomedial area. SCN neurons expressing VIP

or vasopressin all expressed VPAC2R. We found intracellular VPAC2 expression mainly along cell bodies and dendrites, but not along their axons. We found that VPAC2R levels in the SCN do not oscillate in light-dark cycles or in constant conditions. We conclude that VPAC2R presents broadly in the SCN throughout the day to mediate circadian synchrony in the SCN.

Taken together, these experiments suggest that VIP signaling mediate entrainment to the daily light cycle and an altered schedule by jet lag or day night shift work through wide expression of VPAC2R in the SCN.

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Abbreviations

AC	adenylate cyclase
AVP	arginine-vasopressin
CCD	charge-coupled device
CMV	cytomegalovirus
DD	constant darkness
Edelfosine	(7R)-4-Hydroxy-7-methoxy-N,N,N-trimethyl-3,5,9-trioxa-4-phosphaheptacosan-1-aminium-4-oxide
ELISA	enzyme-linked immunosorbent assay
GRP	gastrin releasing peptide
FRET	Förster resonance energy transfer
IBMX	3-isobutyl-1-methylxanthine
LD	light:dark
LL	constant light
luc	luciferase gene
MAP2	Microtubule-associated protein 2
MDL	MDL-12,330a
PACAP	pituitary adenylate cyclase-activating peptide
PDF	pigment dispersing factor
PDFR	pigment dispersing factor receptor
PER2	PERIOD2 protein
PER2::LUC	PERIOD2::Luciferase fusion protein
PLC	phospholipase C

PMT	photomultiplier tube
PRC	phase response curve
r	length of a mean vector in the Raleigh plot
SI	synchronizing index
SCN	suprachiasmatic nucleus
THFA	9-(Tetrahydro-2-furyl)-adenine
U73122	1-[6-[[(17*b*)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino] hexyl]-1*H*-pyrrole-2,5-dione
VIP	vasoactive intestinal polyepeptide
Vipr2	gene encoding VPAC2
VPAC2R	Vasoactive intestinal polypeptide receptor 2

Chapter 1.

Introduction

Properties of circadian rhythms

The day night cycle on Earth generates a temporal niche to all of its organisms. They need to decide when to sleep, eat and avoid their predators. Circadian (from the Latin circa – "about", and dies – "day") rhythms function to drive behavioral and physiological oscillations following the 24-hour light dark cycle. These rhythms are innate in most of organisms, persistent even in constant conditions with a period close to 24 hours, and resistant to change their periods over a range of temperatures. Though circadian rhythms persist with a period close to 24 hours even in the absence of timing cues, they synchronize to cyclic timing cues via periodic adjustments (entrainment) (Pittendrigh & Daan 1976b). The proper adjustments are vital to organisms' health and survival (DeCoursey *et al.* 1997). This applies to humans too, as shown in adverse effects of jet lag and day night shift works on physical and mental dysfunctions (Cho 2001; Davidson *et al.* 2006; Sahar & Sassone-Corsi 2007). This thesis addresses mechanisms regarding the entrainment of the circadian system.

Circadian rhythms have been reported and described in multiple specifies ranging from prokaryotes such as cyano- and proto-bacteria to eukaryotes including fungi, plants, and animals (Sweeney & Hastings 1960; Pittendrigh 1967; Pittendrigh & Daan 1976b; Czeisler *et al.* 1981; Kondo *et al.* 1993; Dunlap 1999). The existence of circadian rhythms in diverse phylogenic trees, along with evolution, indicates beneficial aspects of the coordination between biological timing processes and environmental fluctuations. In the animal kingdom, for instance, circadian clocks play a role in optimizing animals' adaptation to an ambient light dark cycle for their survival (Paranjpe *et al.* 2005), or

guiding migrations of birds or butterflies by positioning the Sun's location in the sky (Froy *et al.* 2003). In human society, circadian rhythms affect well being of everyday life. Modern affairs disrupting our circadian rhythms such as trans-meridian travels, day night shift work, and day light savings are shown to cause lower efficiencies at work, cognitive deficits, or even a variety of illnesses (Davidson *et al.* 2006; Czeisler & Gooley 2007). In clinics, doctors are trying to find optimal time points of drug treatment based on our circadian clocks (i.e. chronotherapy) (Yamamoto *et al.* 1997). It is still not clear how circadian systems re-synchronize to advancing or delaying entrainment cycles.

The suprachismatic nucleus (SCN) as a master circadian pacemaker

In mammals, suprachiasmatic nucleus (SCN), one of the nuclei located in the ventral hypothalamus, generates rhythms in physiology and behavior including sleep and waking, feeding, excretion, blood pressure regulation, body temperature, locomotor activity, and hormonal release (Moore *et al.* 1991). The bilateral nuclei are composed of about 20,000 neurons, located just above the optic chiasm and separated by the third ventricle.

Lesions of the SCN (Moore & Eichler 1972; Stephan & Zucker 1972; Ibuka *et al.* 1977; Eastman *et al.* 1984; Schwartz & Zimmerman 1991) or electrical silencing *in vivo* (Schwartz *et al.* 1987; Schwartz 1991) cause loss of rhythmicity in behavior and physiology. SCN allografts to lesioned animals successfully restore rhythmicity in behavioral rhythms with the period of donor SCN (Ralph *et al.* 1990). The restoration of the rhythmicity may depend on direct neuronal connections and diffusible signals (Lehman *et al.* 1987; Silver *et al.* 1990; Ralph & Lehman 1991). Therefore, the SCN is

necessary and sufficient to generate circadian rhythms. The nature of these output signals needs further examination, but several candidate molecules have been suggested (Kalsbeek & Buijs 1992; Kramer *et al.* 2001; Cheng *et al.* 2002; Kraves & Weitz 2006).

The SCN shows daily rhythms in 2-deoxyglucose uptake (Schwartz & Gainer 1977), cAMP and Ca^{2+} content (Murakami & Takahashi 1983; Ikeda *et al.* 2003), CREB-mediated gene expression (Obrietan *et al.* 1999), electrical activity (Inouye & Kawamura 1979), neurotransmitter release (Schwartz & Reppert 1985) *in vivo*, and electrical activity (Groos & Hendriks 1982; Shibata & Moore 1988; Herzog *et al.* 1996), neuropeptide release (Shinohara *et al.* 1995; Watanabe *et al.* 2000), gene expression (Yamazaki *et al.* 2000) *in vitro*. These rhythms in various transcription factors, messenger molecules and firing rate rhythms indicate the diverse, but orchestrated actions of the SCN to output pathways as a master circadian pacemaker.

An intracellular clock mechanism

The advent of 'clock genes' arose in a relatively recent period from various species including cyanobacteria, fungi, fruit flies, and mammals by genetic screens and discovery of spontaneous mutations (Konopka & Benzer 1971; King *et al.* 1997; Ishiura *et al.* 1998; Dunlap 1999; Emery & Reppert 2004). Though different species have different genetic components, homologous mechanisms generate daily oscillations across species. Fundamentally, the circadian clocks are composed of interlocking positive and negative feedback loops, where a lag in transcription and translation generates near 24-hour oscillations (Reppert & Weaver 2002; Emery & Reppert 2004). In mammals, a negative

feedback loop inhibits the expression *Period (Per1, 2 and 3)* and *Cryptochrome (Cry1 and 2)* genes by their own protein products, PER and CRY. *Per* and *Cry* transcriptions are activated by the binding of CLOCK and BMAL1 (Mop3) heterodimer complex on their regulatory element, E-box (Gekakis *et al.* 1998). The accumulated PER and CRY products, in turn, translocate into the nucleus, and repress the activity of CLOCK and BMAL1. Subsequently, the reduction of PER and CRY level disinhibits CLOCK and BMAL1 activity to increase *Per* and *Cry* transcription. Another negative feedback loop inhibits *Bmal1* transcription through the binding of REV-ERB to ROR-element (RORE) of *Bmal1* promoter (Ueda *et al.* 2002). A positive feedback loop activates *Ror* gene through CLOCK and BMAL1, resulting in the increase of *Bmal1* transcription by binding of ROR to its RORE (Sato *et al.* 2004).

Genetic screens and findings of spontaneous mutations revealed the roles of clock genes in the quality and speed of the rhythms. Single knockout of each gene except knocking out *Bmal1* does not abolish the rhythms completely, and animals show arrhythmicity in constant darkness only when they lack *Bmal1* or both *Per1* and *2* or *Cry1* and *2* (Kume *et al.* 1999; Bae *et al.* 2001; Debruyne *et al.* 2006; Debruyne *et al.* 2007). Interestingly, the knockout of Bmal1 leads to the decreased Bmal2 expression, which may mimic the effect of knocking out the both genes (Shi *et al.* 2010). The phosphorylation of PER proteins by a variety of kinases including casein kinase I epsilon facilitates degradation of PER, thus it regulates the speed of the oscillations. Therefore, mutations in the kinase or phosphorylation sites of PER lead to the lengthening or shortening of period in gene expression and behavioral rhythms in flies (Lin *et al.* 2005), rodents (Lowrey *et al.* 2000;

Meng *et al.* 2008), and human (Toh *et al.* 2001; Xu *et al.* 2005). Interestingly, a mutation reducing the activity of an F-box protein, FBXL3 (Afterhours or Overtime) is shown to lengthen the behavioral period through the stabilization of CRY proteins (Godinho *et al.* 2007; Siepka *et al.* 2007). The redundancy of gene components may improve the precision of cellular rhythms or their stability against perturbations (Stelling *et al.* 2004).

Intercellular communication in the SCN

When SCN neurons lose intercellular communication, they fail to reliably generate coherent gene expression or firing rate rhythms (Aton & Herzog 2005). If individual SCN neurons contain the intracellular clock, why do they require the intercellular communication? The research investigating roles of the intercellular communication started recently since the development of two techniques: transgenic animals express LUCIFERASE proteins driven by *Period* and the usage of cooled charged device camera (Yamazaki *et al.* 2000; Yoo *et al.* 2004; Welsh *et al.* 2005). The imaging of the SCN neurons from these transgenic animals using techniques for low-light imaging enabled to visualize the roles of the communication. A chronic blockade of voltage gated sodium channel by tetrodotoxin (TTX) treatment desynchronizes SCN neurons, leading to the amplitude dampening of gene expression rhythms (Yamaguchi *et al.* 2003). Similarly, SCN neurons plated in very low density show sloppy oscillations with a broad period distribution (Webb *et al.* 2009). In a study using different circadian mutant SCN, the coupling of SCN neurons is shown to overcome the genetic defects. The circadian gene expression rhythms are intact in *Per1-/-* or *Cry1-/-* or *Cry2-/-* SCN explants, but their SCN neurons lose rhythmicity (*Per1-/-* or *Cry1-/-*) or synchrony (*Cry2-/-*) when they are

dissociated (Liu *et al.* 2007). Therefore, coupling or synchrony among SCN neurons endows robustness to individual SCN neurons against perturbations. Indeed, the gene expression rhythms in SCN slice cultures failed to entrain to a 6-hour temperature pulse, or 20 hour- or 28 hour cycles initially, but entrained after the chronic treatment of TTX (Abraham *et al.* 2010; Buhr *et al.* 2010). However, these results also suggest that the reduced synchrony among the SCN neurons might be beneficial to entrainment to an ambient cycle. This thesis investigates this possibility. **Chapter 3** examines whether the SCN or animals with reduced synchrony entrains more quickly to a largely advanced light- or temperature cycle than synchronized ones.

Light effects of circadian rhythms

Periodic timing stimuli (i.e. light) entrain circadian rhythms within the range of entrainment capacity (Pittendrigh & Daan 1976b). By entraining to the daily onset of light, circadian pacemakers synchronize their period to the light cycle with a stable phase relationship. Entrainment is achieved when the period of internal clocks (τ) is equal to the period of the entraining stimulus (T; 24 hours in case of light), which requires daily phase adjustments of circadian clock ($\Delta\Phi$: phase shifts) (Pittendrigh & Daan 1976b). Their relation is as follows:

$$\tau - T = \Delta\Phi$$

The direction and size of phase shifts by light depends on when the stimulus hit the circadian clcoks, which is summarized in phase response curve (PRC). Assuming that the light rapidly shifts the circadian rhythms (non-parametric entrainment), the PRC predicts: 1) what time the light stimulus need to be given for entrainment, 2) how many cycles the

circadian clocks take to entrain, 3) a period range of the light stimulus to which a certain period of circadian clocks is able to entrain and 4) phase angle of the entrainment (Johnson *et al.* 2003). Indeed, evidence for non-parametric entrainment was demonstrated in the eclosion rhythm of flies (Zimmerman *et al.* 1968), locomotor rhythm of nocturnal rodents (Pittendrigh & Daan 1976b), *in vitro* oscillation of genes in cyanobacteria (Yoshida *et al.* 2009). Although the SCN rapididly shifts in response to stimuli (Best *et al.* 1999), non-parametric entrainment has not been shown yet. Another goal of this thesis is to demonstrate whether the SCN entrains to a repeated stimulus via rapid adjustments of its phase. In **chapter 2**, we demonstrate the non-parametric entrainment of the PER2 expression rhythms in the SCN to repeated pulses of VIP, a neuropeptide expressed in the SCN (An *et al.* 2011).

On the other hand, constant light or irregular light pulses (i.e. critical light pulse) appear to affect the synchrony among circadian cells. Locomotor activity of hamsters or mice in constant light for more than a month showed rhythms with a longer period or arrhythmicity or two bouts of activity in a cycle (i.e. split) (Pittendrigh & Daan 1976a; Ohta *et al.* 2005). Importantly, the SCN neurons from arrhythmic mice were desynchronized, but the neurons from rhythmic ones remained synchronous (Ohta *et al.* 2005). A critical light pulse generates two desynchronized rhythms of gene expression in rat SCN (Ukai *et al.* 2007), and arrhythmicity or rhythms with no amplitude in body temperature, plasma cortisol in human (Jewett *et al.* 1991). It is not known what mediates the light effects on split or arrhythmic behaviors or desynchrony among the SCN neurons. In **chapter 3**, we find VIP reduces the synchrony among the SCN neurons, resulting in

the amplitude reduction in their output rhythm, the behavioral rhythm (equal to the split or arrhythmic behaviors) in constant light.

VIP signaling and its roles

VIP gene and biochemical properties. The *Vip* gene, encoding a 28-amino acid peptide, is located on human chromosome 6p21-6qter (Gozes *et al.* 1987) and mouse chromosome 10 A1 (Lamperti *et al.* 1991). VIP belongs to the VIP peptide family with other neuropeptides including the peptide with N-terminal histidine and C-terminal isoleucine (PHI), pituitary adenylate cyclase activating polypeptide (PACAP), GH-releasing hormone (GHRH), secretin, gastric inhibitory polypeptide (GIP), glucagon, and glucagon-like polypeptide I (GLP-I) (Usdin *et al.* 1994). VIP and PHI are encoded by two neighboring exons, and share the same precursor protein, but are processed by tissue-specific alternative splicing as shown in bird hypothalamus (Talbot *et al.* 1995). The vicinity of its transcription start site contains cAMP-response element (CRE), raising the possibility of its regulation by CREB (Fink *et al.* 1991). The *Vip* gene expression is regulated by various factors including retinoic acid (Georg *et al.* 1994) and estrogen (Kasper *et al.* 1992). The trigger mechanisms of the VIP release are not well-studied except the NO-mediated release in enteric nervous system (Kim *et al.* 2003), or light- or circadian release in the SCN (Shinohara *et al.* 1993; Shinohara *et al.* 1995).

VIP receptors and their biochemical properties. So far, the known VIP-binding receptors are vasoactive intestinal polypeptide receptor 1 (VPAC1R, ~70 kDa), vasoactive intestinal polypeptide receptor 2 (VPAC2R, ~60 kDa), and pituitary adenylate

cyclase-activating polypeptide receptor 1 (PAC1R, ~50 kDa), which are encoded by *Vipr1* (~22kb), *Vipr2* (30 kb), and *Adcyap1r1* genes, respectively. They belong to secretin family (class B) of Gs-protein coupled receptors (GPCR) with seven-transmembrane helices (Langer & Robberecht 2007). GPCR is known to change its conformation to interact with heterotrimetric G-proteins, and facilitate the exchange of GDP to GTP on the Gα subunit upon its ligand binging. Generally, adenylate cyclase is a known downstream pathway of these receptors through the $G\alpha_s$, but couplings to phospholipase C (PLC) and the calcium/InsP3 pathway through $G\alpha_q$ or $G\alpha_i$ is also reported (Langer & Robberecht 2007). After binding to their ligands, VIP or PACAP, VPAC1R and VPAC2R are rapidly phosphorylated (Langer *et al.* 2005), which promotes the internalization through β-arrestin (Reiter & Lefkowitz 2006). The receptor regulation may control the delivery and amplification of the signal from the ligand. VPAC1R appears to be more sensitive to VIP and PACAP than VPAC2R (Usdin *et al.* 1994). VPAC2R binds equally to VIP and PACAP, while PAC1R poorly interacts with VIP, binding to PACAP preferentially (Christophe 1993; Lutz *et al.* 1993).

Functions and signaling in CNS and PNS. VIP modulates behavioral and physiological functions acting through various brain areas. Its roles in circadian rhythms are covered in detail below. It modulates neuronal firing in various brain areas such as the cortex (Sessler *et al.* 1991), SCN (Reed *et al.* 2002), hippocampus (Yang *et al.* 2009), spinal cord (Phillis *et al.* 1978), midbrain (Haskins *et al.* 1982), and locus coeruleus (Wang & Aghajanian 1990). Also, it increases the communication of glia with neurons by promoting secretion of cytokines and growth factors (Schettini *et al.* 1994). VIP is

involved in neurotrophic actions during the brain development (Gozes & Brenneman 1989) and after an injury in spinal cord, cerebellum, hippocampal and cerebral cortical cultures (Brenneman *et al.* 1995; Cavallaro *et al.* 1996; Kim *et al.* 2005). In behavior, it regulates sleep, and learning and memory. VIP, at least in part, is shown to increase REM sleep by the involvement of prolactin release (el Kafi *et al.* 1995).VIP effects on learning and memory differ (improve or deteriorate) with different locations, concentrations and duration of the injection, thus its role is still in debate (Gozes *et al.* 1993; Yamaguchi & Kobayashi 1994). Recently, humans with a microduplication in the *Vipr2* gene show higher chances of having schizophrenia (Vacic *et al.* 2011), indicating the relation of VIP with psychological disorders.

The downstream components delivering VIP signaling are heterogeneous in different brain areas. To modulate firing rates, VIP requires protein kinase A (PKA)-mediated cAMP signaling in noradrenergic neurons of the locus coeruleus (Wang & Aghajanian 1990), but does not require it to depolarize retinal horizontal neurons (Lasater *et al.* 1983). To promote the survival of neurons, VIP requires cAMP signaling in developing sympathetic neuroblasts (Pincus *et al.* 1994), but requires protein kinase C not PKA in postnatal mouse brains (Rangon *et al.* 2005). In the SCN, phase shift of neuronal firing rate rhythms by VIP required PKA (Meyer-Spasche & Piggins 2004), while induction of *Period* by VIP required PLC (Nielsen *et al.* 2002). Therefore, VIP uses different downstream components at various brain areas depending on its functions, and if a novel VIP action is known in a particular brain area, its downstream components may not be the same with the already known ones. **Chapter 2** identifies the downstream components

of VIP-induced phase shifts in PER2 expression rhythms in the SCN. We found that only simultaneous blockade of adenylate cyclase (AC) and PLC activities suppresses the VIP-mediated phase shifts of gene expression rhythms. We conclude that VIP shifts circadian rhythms in the SCN through parallel changes in AC and PLC activities in the SCN.
The roles of VIP in the PNS were not extensively studied, but some research suggests its involvements in pain sensation (Payan & Goetzl 1988) or in pathogenesis in systemic sclerosis (Matucci-Cerinic *et al.* 2001).

Expression profiles in the SCN. VIP-immunoreactivity is broadly observed in the SCN (Romijn *et al.* 1997; Abrahamson & Moore 2001; Kalamatianos *et al.* 2004). VIP-ergic neurons in ventrolateral SCN project their dense fibers throughout the SCN, mainly to the dorsomedial SCN, where vasopressin-expressing (AVP-ergic) cells are located (Abrahamson & Moore 2001). In the SCN, VIP-ergic neurons and AVP-ergic neurons contribute to about 15 % and 20 % of total population respectively. The SCN shows a strong *Vipr2* mRNA signal, no, little if any, *Vipr1* mRNA signal, and modest *Adcyap1r1* mRNA signal (Usdin *et al.* 1994; Cagampang *et al.* 1998b; Kalamatianos *et al.* 2004). With the slight difference between species, it is expected that approximately 30 % of VIP neurons and about 60 % of AVP-ergic neurons express VPAC2R respectively (Kalamatianos *et al.* 2004; Kallo *et al.* 2004). Since previous research detected the VPAC2R expression with its mRNA probe or β-galactosidase driven by human *Vipr2* promoter, it is not known the VPAC2R expression along neuronal projections, its intracellular localization, and percentage of VPAC2R-positive neurons. These data will identify the role of VPAC2 in synaptic transmission, and the number of neurons directly

responsive to VIP. VIP-immunoreactivity in the SCN is not rhythmic in constant condition, but rhythmic in diurnal condition, showing a decrease in the presence of light (Shinohara *et al.* 1993). Whether and how VPAC2R is rhythmically expressed in the SCN is not clear because of inconsistencies between research on its temporal mRNA profiles (Cagampang *et al.* 1998c; Shinohara *et al.* 1999). **Chapter 4** characterizes the spatiotemporal expression of VPAC2R with a specific antibody against VPAC2R. We found that VPAC2R is present in all SCN neurons throughout the day, overlaps with the VIP and AVP expression, and functions in receiving synaptic information along dendrites and cell bodies. We conclude that VPAC2R exists broadly in the SCN throughout the day to mediate circadian synchrony in the SCN.

Roles in the SCN. VIP mediates circadian synchrony in the SCN. VIP is released from retinorecipient VIP-ergic neurons by a photic stimulus, and conveys the photic information to the SCN (Abrahamson & Moore 2001). Supporting this idea, behavioral rhythms of animals lacking VIP (*Vip-/-*) or VPAC2R (*Vipr2-/-*) have aberrant phase angle, different phase shifts and entrainment rates with light pulses or light dark schedules (Harmar *et al.* 2002; Colwell *et al.* 2003). Animals lacking PAC1R do not have significant deficits in their light responses, indicating that PAC1R is not essential in the SCN (Hannibal *et al.* 2001). As the light transiently induces PER proteins (Field *et al.* 2000), VIP application induces *Period* gene transcription in SCN explant cultures (Nielsen *et al.* 2002). *Vipr2-/-* mice failed to induce the *Period* genes after the photic stimulation (Harmar *et al.* 2002). As an entraining agent like light, VIP application also shifts behavioral (Piggins *et al.* 1995), firing rate (Reed *et al.* 2001), and AVP-release

rhythms (Watanabe *et al.* 2000). However, this research has not identified whether VIP entrains the SCN rhythms. In **chapter 2**, we generate a complete VIP PRC of PER2 expression rhythm, and entrain the PER2 rhythms by applying VIP daily as predicted by our PRC. The resulting PRC is not identical to the light PRC (An *et al.* 2011). Interestingly, the application of pigment dispersing factor, a functional homolog of VIP in insects, generates a similar PRC about behavioral rhythms in cockroach to the VIP PRC of PER2 expression rhythms, but not like light PRC (Petri & Stengl 1997). Since the light regulates the levels of various neuropeptides and second messengers (Gillette & Mitchell 2002), which applications also induce phase shifts of the SCN rhythms, we conclude that VIP modulates the light-mediated phase shifts of PER expression rhythms in an orchestration with diverse agents.

Another role of VIP is to generate and sustain synchrony among the SCN neurons. VIP release is rhythmic even in constant conditions. *Vip*-/- mice were arrhythmic (60 % of the total) or weakly rhythmic with multiple components in constant darkness or with daily light pulses in 24 hour cycle (skeletal photoperiod) (Colwell *et al.* 2003; Aton *et al.* 2005). Vipr2-/- mice showed similar behavioral defects in the same conditions (Harmar *et al.* 2002; Aton *et al.* 2005). In cellular level, the neurons from these mutants are arrhythmic, or desynchronized each other if they are rhythmic (Aton *et al.* 2005; Maywood *et al.* 2006). Importantly, daily VPAC2R agonist treatments restore the rhythms and synchrony among the *Vip*-/- SCN neurons (Aton *et al.* 2005). Therefore, VIP is necessary and sufficient to generate rhythms of the SCN neurons and synchrony among them. Surprisingly, we found that VIP application reduces the synchrony among the SCN, and

investigateed the desynchronizing effect of VIP on the SCN in **chapter 3**. VIP application reduces synchrony among the SCN neurons throughout the day in dose-dependent manner, resulting in a decrease in amplitude of ensemble rhythms. This result and previous research suggest that VIP switch its role from a synchronizing agent to a desynchronizing agent depending on different doses (high dose: desynchronizing, low dose: synchronizing) or situations (applied in coupled system: desynchronizing, applied in uncoupled system: synchronizing). We conclude that VIP acts as a versatile agent, which regulates synchrony in the SCN in different contexts.

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Chapter 2.

VIP entrains circadian oscillators

This chapter contains the manuscript:

An, S., Irwin, R. P., Allen, C. N., Tsai, C., Herzog, E.D. (2011) Vasoactive intestinal polypeptide requires parallel changes in adenylate cyclase and phospholipase C to entrain circadian rhythms to a predictable phase. *J Neurophysiol.*

Abstract

Circadian oscillations in the suprachiasmatic nucleus (SCN) depend on transcriptional repression by the Period (PER) 1 and 2 proteins within single cells and on vasoactive intestinal polypeptide (VIP) signaling between cells. Because VIP is released by SCN neurons in a circadian pattern and following photic stimulation, it has been suggested to play a role in synchronization to environmental light cycles. It is not known, however, if or how VIP entrains circadian gene expression or behavior. Here, we tested candidate signaling pathways required for VIP-mediated entrainment of SCN rhythms. We found that single applications of VIP reset PER2 rhythms in a time- and dose-dependent manner that differed from light. Unlike VIP-mediated signaling in other cell types, simultaneous antagonism of adenylate cyclase (AC) and phospholipase C (PLC) activities was required to block the VIP-induced phase shifts of SCN rhythms. Consistent with this, VIP rapidly increased intracellular cAMP in most SCN neurons. Critically, daily VIP treatment entrained PER2 rhythms to a predicted phase angle within several days, depending on the concentration of VIP and the interval between VIP applications. We conclude that VIP entrains circadian timing among SCN neurons through rapid and parallel changes in AC and PLC activities.

Introduction

Coordinated rhythms across populations of neurons are believed critical to many behavioral and cognitive functions (Buzsáki 2006). The mechanisms that synchronize the periods of neural oscillators can include gap junctions which produce in-phase rhythms(Schneider *et al.* 2006; Mancilla *et al.* 2007), reciprocal inhibition producing either in-phase or anti-phase cycling(Wang & Rinzel 1992) and fast, weighted, excitatory synapses producing a range of phase relationships(Smarandache *et al.* 2009). Daily, or circadian, rhythms in behavior and physiology, however, depend on the neuropeptide, vasoactive intestinal polypeptide (VIP). The mechanisms by which VIP synchronizes circadian rhythms among cells are unknown.

The daily resetting of circadian timing establishes a stable phase relationship (i.e. the phase angle of entrainment) between behavioral and physiological rhythms and environmental cues. VIP is well-positioned to reset circadian oscillators in the brain to each other and to exogenous timing cues. *Vip* and its receptors, *Vipr1* and *Vipr2*, are expressed in the central and peripheral nervous systems (Dietl *et al.* 1990; Mohney & Zigmond 1998; Chaudhury *et al.* 2008) including in the suprachiasmatic nucleus (SCN), a master circadian pacemaker (Cagampang *et al.* 1998c; Shinohara *et al.* 1999). VIP applied to SCN explants in the late subjective night induces the transcription of *Period (Per) 1* and *2*, two genes implicated in rhythm generation and entrainment (Nielsen *et al.* 2002). VIP can shift the daily rhythms in locomotion (Piggins *et al.* 1995) and in electrical discharge (Reed *et al.* 2001) and vasopressin release (Watanabe *et al.* 2000) of SCN explants. These actions of VIP in the SCN have been shown to depend on the

activities of phospholipase C (PLC) (Nielsen *et al.* 2002), adenylate cyclase (AC) or protein kinase A (PKA) (Meyer-Spasche & Piggins 2004), but the signaling underlying entrainment by VIP has not been studied.

A phase response curve (PRC) plots the steady state shift in a rhythm as a function of the time of stimulation. The PRC can be used to predict features of entrainment including the phase angle of entrainment, the range of periods to which the oscillator can entrain, and how long it will take to entrain (Pittendrigh 1960). Importantly, existing PRCs have not been tested for their ability to predict these features of SCN entrainment. This study aimed to generate a PRC to VIP that would predict features of entrainment and could be used to test the underlying molecular mechanisms and kinetics. We combined pharmacology with recordings of bioluminescence from a reporter of PERIOD2 levels and of Förster resonance energy transfer (FRET) from a reporter of cAMP levels. We found that VIP directly entrains the PER2 rhythms of SCN neurons through rapid, parallel changes in AC and PLC signaling.

Materials and Methods

ANIMALS. PERIOD2::LUCIFERASE **(**PER2::LUC) knock-in mice (Yoo *et al.* 2004) (founders generously provided by J. S. Takahashi, Univ. of Texas Southwestern Medical Center, Dallas, TX) were housed in a 12 h/12 h light/dark cycle and bred as homozygous pairs in the Danforth Animal Facility at Washington University. All procedures were approved by the Animal Care and Use Committee of Washington University or Oregon Health Sciences University and followed National Institutes of Health guidelines.

DRUGS. VIP was purchased from Bachem (King of Prussia, PA) or Tocris (Ellisville, MO). MDL-12,330a (MDL), 9-(Tetrahydro-2-furyl)-adenine (THFA), 1-[6-[[(17*b*)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino] hexyl]-1*H*-pyrrole-2,5-dione (U73122), forskolin and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma (Saint Louis, MO). (7R)-4-Hydroxy-7-methoxy-N,N,N-trimethyl-3,5,9-trioxa-4-phosphaheptacosan-1-aminium-4-oxide (Edelfosine) was from Tocris (Ellisville, MO). Drugs were dissolved in DMSO or de-ionized water as stock solutions, stored at -20 °C and diluted with culture medium so that the final DMSO concentration was below 0.4 % of the total volume. Culture media consisted of DMEM (Sigma, Saint Louis, MO) supplemented with 2% B27 (Invitrogen, Carlsbad, CA), 10 mM HEPES (Sigma, Saint Louis, MO) and 2.2 mg/ml $NaHCO_3$ (Invitrogen, Carlsbad, CA). VIP was dissolved in culture medium and vehicle controls consisted of an equal volume of culture medium.

BIOLUMINESCENCE RECORDING. We recorded bioluminescence rhythms from 300 μm, coronal SCN slices from PER2::LUC mice (age 8-20 days) using a photomultiplier tube (PMT) (HC135-11 MOD, Hamamatsu Corp., Shizuoka, Japan) as described (Abe *et al.* 2002). SCN explants were cultured on 0.4 mm membrane inserts (Millipore, Billerica, MA) in sealed 35-mm culture dishes (BD Biosciences, San Jose, CA) with 1 ml pre-warmed air-buffered medium supplemented with 10% newborn calf serum (NCS) (Invitrogen, Carlsbad, CA) and 100 μM beetle luciferin (Promega, Madison, WI) as a final concentration at 34°C. Bioluminescence counts were integrated and stored at 1-min intervals for up to 15 days of recording.

During drug applications, 500 µl of the culture medium was mixed with the VIP, antagonists or vehicle (culture medium, 5-50 µl) and added back to the culture dish. For the phase shift experiments, circadian times were calculated in hours from the peak of the PER2::LUC rhythm (CT 12) (Yoo *et al.* 2004). To minimize artifacts, treatments were added without subsequent removal.

To measure the phase shift, Bioluminescence data were detrended by subtracting a 24-h running average (Abe *et al.* 2002) and the daily peak of expression was determined using an acrophase fitting function with Clocklab software (Actimetrics, Wilmette, IL). Phase shifts were measured as the time difference between linear regressions of the acrophases on the days prior to a treatment and the 4 to 5 days after treatment. In some cases, the shift was measured after one to three cycles of transient shifts. Period of the PER2::LUC rhythm was measured as the average time between acrophases from at least four days of recording. The induction of PER2 expression was measured by averaging raw bioluminescence signal of the cycle with the VIP or vehicle treatment. All statistics were performed with Origin 7.0 software (Origin, Northampton, MA).

ELISA. We measured VIP concentrations to determine the profile of the neuropeptide from 8 mouse SCN explants treated with either 1 µM VIP (n = 4) or vehicle (n = 4). We collected 40 µl of medium from each culture 0, 10, 30, 60, 120 min, and 24 h after treatment, froze at -35°C immediately and stored at -80°C. A competitive enzyme-linked immunosorbent assay (ELISA) was performed according to the manufacturer's protocol (Peninsula Laboratories, San Carlos, CA). Absorbance was read at 450nm with a

microplate spectrophotometer (Molecular devices, Menlo Park, CA). A standard curve was generated with serially diluted standards ranging from 0 to 10 ng / ml and IC_{50} was 0.24 ng / ml.

cAMP MEASUREMENT. SCN cultures were prepared from neonatal Sprague-Dawley rats and transfected with a cAMP reporter using the biolistic method as previously described (Ikeda *et al.* 2003). Briefly, neonatal rat pups (3 - 7 days old) were decapitated, the brains removed, and 200-300 μm thick coronal slices cut with a vibrating-blade microtome (Camden Instr., Lafayette, IN). The slices were placed on Millicell-CM membranes (30-mm diameter, 0.4 μm, Millipore, Billerica, MA) and maintained in an incubator at 37 °C with 5% CO_2. The organotypic cultures were grown in culture media consisting of: DMEM/High without L-glutamine and with sodium pyruvate (Hyclone, Thermo Scientific, Waltham, MA), 2% B27 supplement (GIBCO, Carlsbad, CA), 10 mM HEPES (GIBCO, Carlsbad, CA), and 1X GlutaMax (GIBCO, Carlsbad, CA).

cAMP activity was measured using a fusion protein consisting of cyan fluorescent protein (CFP), a truncated Epac1 expressing a cAMP binding site and yellow fluorescent protein (YFP) (DiPilato *et al.* 2004a; Dunn *et al.* 2006a). The cDNA for ICUE2 was kindly provided by Dr. Jin Zhang and Dr. Marla B. Feller (DiPilato *et al.* 2004a; Dunn *et al.* 2006a; Violin *et al.* 2008). A Helios Gene Gun (Bio-Rad Laboratories) was used according to the manufacturer's instructions to transfect the ICUE2 cDNA driven by a CMV promoter, into 2- to 20-day-old cultures. Individual neurons were imaged between 2 and 7 days after transfection. Slice cultures were transferred to a recording chamber

(35°C) with a laminar flow (6-8 ml/min) of an ACSF solution consisting of (in mM): NaCl, 124; KCl, 2.5; NaH_2PO_4, 1.2; $MgCl_2$, 1.2; $CaCl_2$, 2.4; glucose, 10; $NaHCO_3$, 24; adjusted to 300 mOsm and bubbled with 5% CO_2 and 95% O_2. The recording chamber was located on the stage of an inverted microscope (Nikon TE2000E, Toyko, Japan) and illuminated using a xenon-arc lamp, passed through a 436/20 nm filter (Chroma, Tech. Corp, Bellows Fall, VT) within a Lambda 10-3 filterwheel (Sutter Instruments, Novata, CA) and with light reflected by a 455dcxru dichroic filter (Chroma, Tech. Corp, Bellows Fall, VT). Images were visualized using an ORCA ER CCD camera (Hamamatsu Photonics, Hamamatsu, Japan) after passing through a Dual-View beam splitter at 505dcxr (Optical Insights, Tucson, AZ), with 535/40 and 480/30 nm emission filters. Data acquisition was controlled by Metafluor software (Molecular Devices, Sunnyvale, CA) with binning and light exposure optimized to minimize photobleaching. The FRET ratio, fluorescence at 535nm/480nm after background subtraction at each wavelength, was normalized using the ratio before application of VIP. Neurons were identified by morphological appearance. At the end of each experiment neurons were treated with forskolin (20 μM) and 3-isobutyl-1-methylxanthine (IBMX, 75 μM). Cells that did not respond were excluded.

STATISTICS. Comparison between two different groups with one variable was performed using one-way ANOVA with a Scheffé post hoc test and comparison between two different groups with two variables using two-way ANOVA with a Tukey post hoc test. Values were considered significant if $p < 0.05$. All statistics and curve fits were performed with Origin 7.0 (OriginLab, Northampton, MA).

Results

VIP shifts of PER2::LUC rhythms depended on phase and dose.

We monitored the effects of 10 nM to 100 μM VIP on PER2-driven luciferase activity in SCN explants. The half-life of VIP applied to SCN cultures was about 2 h based on ELISA measurements **(Fig. 1)**. VIP application near the peak of PER2 expression (CT12) reduced the subsequent amplitude and delayed the peak of subsequent cycles compared to vehicle-treated cultures **(Fig. 2A)**. This manuscript focuses on the phase-shifting effects of VIP, leaving cause and relevance of the amplitude effects for a subsequent analysis. The steady-state phase shift after four days **(Fig. 2B)** was measured as a function of VIP concentration **(Fig. 2C)**. When applied at CT12, VIP induced a dose-dependent delay in the peak of PER2 expression with a threshold around 100 nM ($p<0.05$ compared to vehicle treated, One-way ANOVA, Scheffé post hoc, $F_{1,15,} =11.15$), an EC_{50} near 500 nM and saturation around 10 μM. VIP also induced a transient increase in PER2 expression with a similar dose-dependence (**Fig 2E**). The threshold for VIP-mediated PER2 induction was around 100 nM, similar to the threshold for phase shifting ($p<0.05$ compared to vehicle treated, One way ANOVA, Scheffé post hoc, $F_{1,6} =7.85$; EC50 near 100 nM and saturation around 5 μM). Thus, a 10-fold increase in VIP concentration approximately doubled the steady-state phase delay of SCN rhythmicity ($r^2 = 0.98$, $n =$ 30 SCN explants).

To measure whether VIP has phase-dependent effects on SCN rhythms, either 100 nM or 10 μM VIP was applied at various circadian time points. The resulting VIP phase response curves (PRCs) had a large delay zone from early subjective day to early

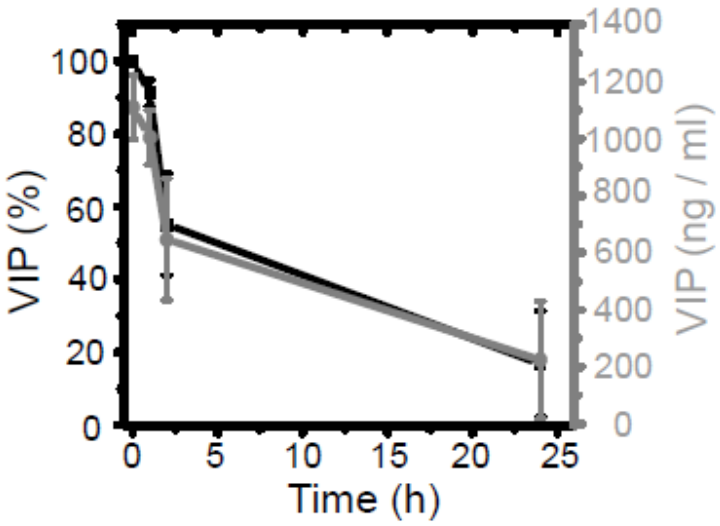
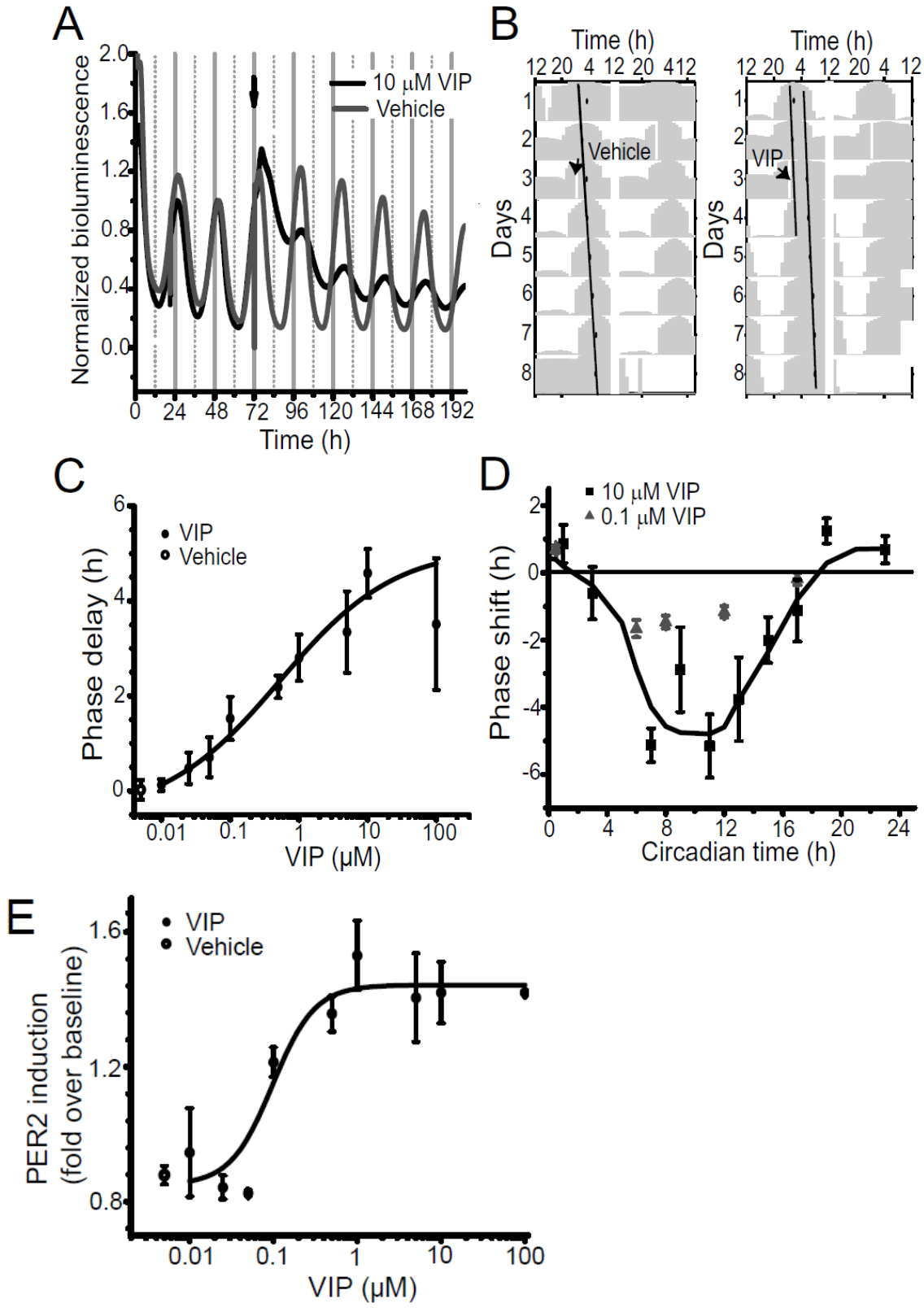


Figure 1. VIP added to the medium halved in concentration after 2 hours. The percentage (black line) and concentration (grey line) of the remaining VIP in the culture medium was measured using ELISA. After 2 hours, about half of the VIP remained and only 20 % of VIP remained after 1 day.


A
Normalized bioluminescence
2.0
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Time (h)
12 20 4 12 20 4 12
VIP
Days
1 2 3 4 5 6 7 8
C
Phase delay (h)
6 4 2 0
VIP
Vehicle
0.01 0.1 1 10 100
VIP (μM)
D
Phase shift (h)
2 0 -2 -4 -6
10 μM VIP
0.1 μM VIP
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E
PER2 induction
(fold over baseline)
1.6 1.2 0.8
VIP
Vehicle
0.01 0.1 1 10 100
VIP (μM)

Figure 2. VIP phase shifted PER2::LUC rhythms in the SCN. (A) Representative PER2::LUC traces with 10 μM VIP (black) or vehicle (gray) treatment at CT12 (arrow). Each PER2::LUC rhythm was normalized to the peak prior to treatment. **(B)** Double-plotted actograms of the bioluminescence traces from Fig. 1A where each line shows 48 h of data beginning with the last 24 h of bioluminescence from the line above. The acrophase (black circle) of PER2::LUC expression was the daily peak of a sine function fit to each cycle's bioluminescence trace (Herzog et al. 2004). Phase shifts were measured as the time difference between linear fits to the acrophases before and after the treatment. **(C)** The dose-dependent phase delays induced by VIP applied at CT 12. Above 100 nM VIP and below 10 μM VIP, the delay of the PER2::LUC rhythm increased linearly with logarithmic increases in VIP concentration. Data were fitted with a logistic function (black line). **(D)** Phase response curves for 10 μM (square) or 100 nM (triangle) VIP (n = 63 and n=33, respectively) as a function of circadian time of VIP application. Phase delays and advances are displayed as negative and positive values respectively. The PRC for 10 μM VIP was fitted with a fast Fourier transform, adjacent-point average (line). Note that the PRC is dominated by delays and unlike the PRC to light. (E) Concentration-response curve for the transient induction of PER2 by VIP. Data were fitted with a logistic function (black line).

subjective night (CT3-18) and a small advance zone from late subjective night to early subjective day (CT19-1) with significant differences between the times of advances and delays (p<0.01, $F_{1,115}$ =7.11 , n =63, Two-way ANOVA, Tukey post hoc; **Fig. 2D**). VIP (10 μM) treatments at CT11-12 induced a phase delay which was similar in magnitude at one, two and three days following treatment (p=0.77, One-way ANOVA, $F_{1,18}$=0.09). In contrast, the same VIP treatment at CT19-23 induced advances which were larger when measured the day after treatment than on subsequent days (p=0.02, One-way ANOVA, $F_{1,16}$=6.17**; Fig. 3**). VIP (10 μM) induced larger delays than 100 nM VIP at most of the time points tested except the early subjective day. Vehicle treatment induced no or little shift (p<0.05, Two-way ANOVA, Tukey post hoc, compared with 10 μM VIP, n= 41). Therefore, adjustment of daily PER2 rhythms depended on the time of administration and concentration of VIP.

Blockade of both AC and PLC activities was required to suppress VIP-induced phase shifts.

Previous findings indicate that in the SCN, VIP may signal through cAMP- or Ca^{2+}-mediated pathways. We examined the effects of two inhibitors of AC and two inhibitors of PLC on VIP-induced phase shifts. At the time of VIP administration , we included MDL-12,330a (MDL), an irreversible, competitive inhibitor of AC (Lippe & Ardizzone 1991), 9-(Tetrahydro-2-furyl)-adenine (THFA), a noncompetitive inhibitor of AC (O'Neill *et al.* 2008), U73122, an inhibitor of PLC (Smith *et al.* 1990), or (7R)-4-Hydroxy-7-methoxy-N,N,N-trimethyl-3,5,9-trioxa-4-phosphaheptacosan-1-aminium-4-oxide (edelfosine), a specific inhibitor of PLC (Powis *et al.* 1992). The VIP-induced

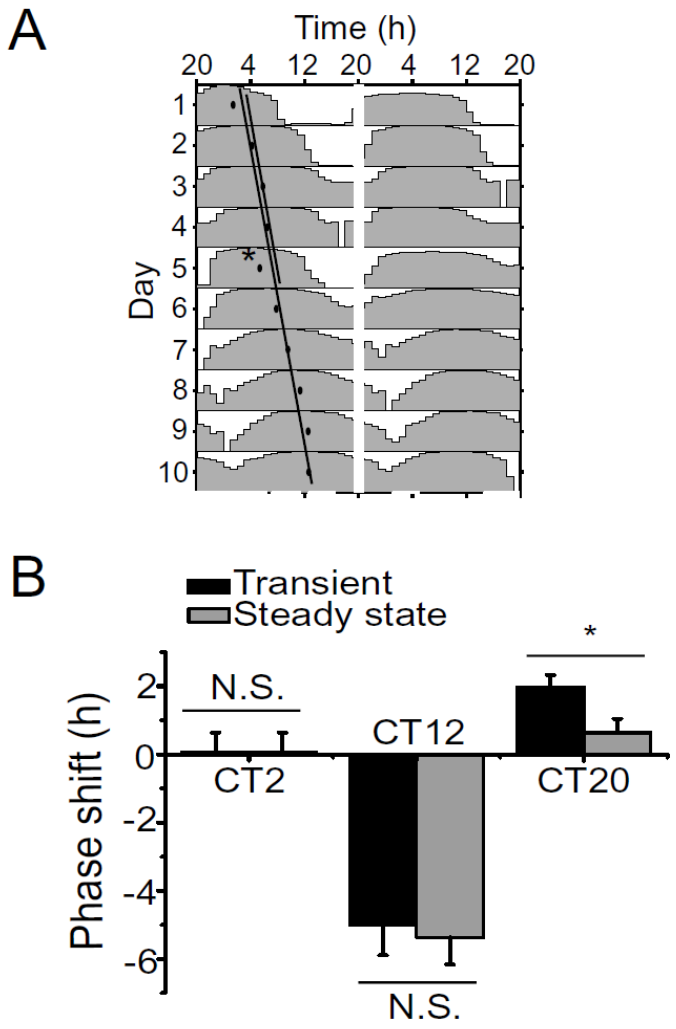

Figure 3. VIP treatment during the late subjective night induced transient shifts of PER2::LUC rhythms. (A) Representative actogram of PER2::LUC expression shows that on the day after VIP application, the SCN rhythm was advanced by several hours (*) and on subsequent days established a smaller steady-state phase shift (black line). **(B)** Transient phase shifts were significantly larger than steady-state shifts following VIP application around CT 20 (mean ± SEM; $p<0.05$, One-way ANOVA, $F_{1,16}=6.17$), but not at other times.

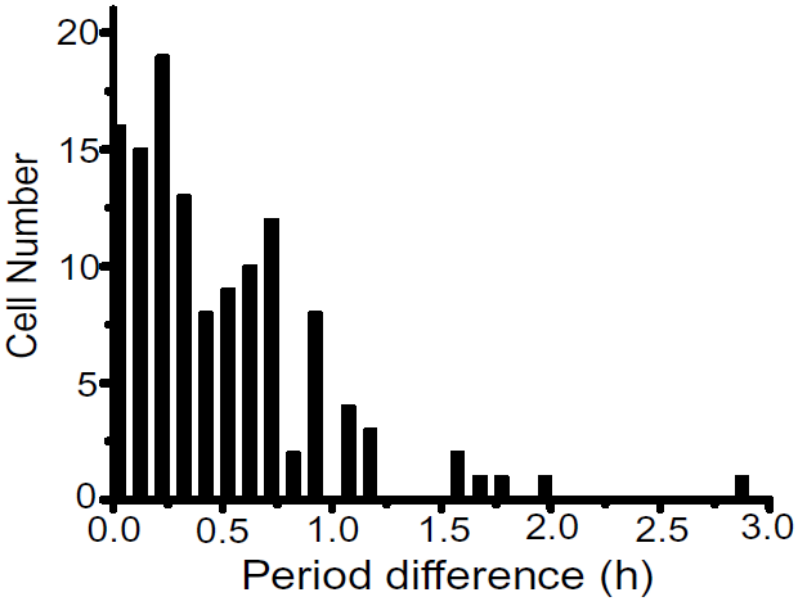

Figure 4. VIP pulses rapidly changed the phase, but not the period of SCN cultures. The period before and after 10 μM VIP treatment differed by less than 1 hour in most SCN cultures (n=116 of 126). This is further evidence that VIP can entrain the SCN through rapid shifts in the circadian oscillator.

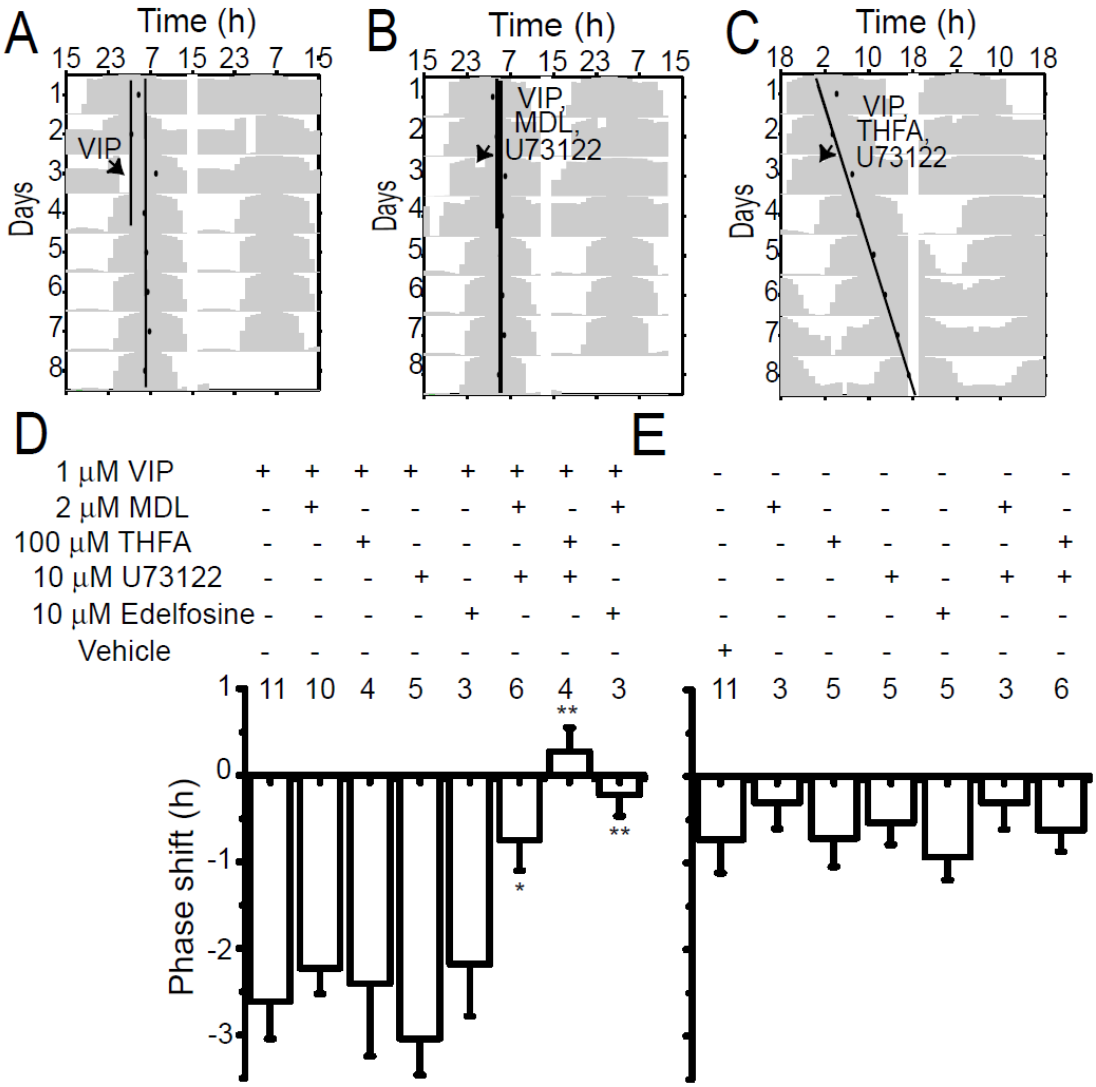

Figure 5. Blockade of cAMP and PLC signaling was required to reduce VIP-induced phase shifts. (A, B and **C**) Representative actograms of PER2::LUC rhythms with treatment of 1 µM VIP alone, VIP with 2 µM MDL (inhibitor of AC) + 10 µM U73122 (inhibitor of PLC), VIP with 100 µM THFA (inhibitor of AC) + 10 µM U73122 respectively. The inhibitor cocktails were applied 1 hour prior to the VIP application at CT12. (**D**) The phase shift (mean ± SEM) of PER2::LUC rhythms by VIP applied at CT12 was significantly attenuated by the combination of the AC and PLC inhibitors (* $p<0.05$ and ** $p<0.01$, One-way ANOVA with Scheffé post hoc test). (**E**) In the absence of VIP, the inhibitors induced little to no phase shifts ($n = 25$, when compared with vehicle-treated cultures, $p> 0.05$). The number of cultures recorded is indicated for each treatment.

phase shifts of the PER2::LUC rhythms at CT12 were significantly reduced by the combined application of MDL (2 μM) or THFA (100 μM) with U73122 (10 μM) or edelfosine (10 μM) 1-h prior to VIP application (* $p<0.05$, $F_{1,15}$ =8.47 for MDL+U73122; ** $p<0.01$, $F_{1,12}$=10.6 for THFA+U73122; ** $p<0.01$, $F_{1,15}$ = 15.89 for MDL+edelfosine, One-way ANOVA, Scheffé post hoc; **Figs. 5A-D**). None of the inhibitors alone reduced the VIP-induced phase shift compared with VIP ($p>0.05$; **Fig. 5D**) or induced a significant shift compared with vehicle ($p>0.05$, One-way ANOVA, Scheffé post hoc; **Fig. 5E**). The inhibitors alone or in combination also did not reduce the amplitude of PER2 rhythms compared with vehicle. These results indicate that VIP signals in parallel through AC and PLC to phase shift SCN rhythms.

VIP elevated cAMP in most SCN neurons.

To measure the responsiveness of individual SCN neurons to VIP, the cAMP reporter, ICUE2, was transfected into individual SCN neurons **(Fig. 6A)**. The ICUE2 and other Epac-based FRET reporters detect increases in cAMP as a decrease in the YFP/CFP ratio reliably in fly brain (Shafer *et al.* 2008), cultured retina (Dunn *et al.* 2006b), hippocampus (Nikolaev *et al.* 2004), dorsal root ganglion (Murray *et al.* 2009), and mammalian cell lines (DiPilato *et al.* 2004b; Nikolaev *et al.* 2004; Ponsioen *et al.* 2004). To confirm the reliability of the ICUE2 in the SCN, we applied a combination of forskolin (20 μM), an activator of most ACs and 3-isobutyl-1-methylxanthine (IBMX, 75 μM), an inhibitor of cAMP phosphodiesterase (PDE) to the ICUE2-expressing SCN neurons at the end of each experiment. This treatment reduced the normalized FRET ratio (**Fig. 6A)** within 4 min by 0.14 ± 0.02 ratio units in 21 neurons from 10 slice cultures.

Application of VIP (1 μM) for 1 min reduced the FRET ratio in 10 SCN neurons in 4 slice cultures, consistent with a second messenger process **(Fig. 6B** and **D)**. The duration of the FRET response varied with some neurons returning to near baseline within 10 min while others persisted longer than 20 min or as long as we recorded. Addition of THFA (100 μM) to the bath 2 min prior to and during VIP application significantly reduced the response of SCN neurons (measured 3 min after VIP, $t_{13} = 3.88$, $p \leq 0.005$ unpaired two-tailed t-test, $n = 11$, 6 slice cultures, **Figs. 6C, D**) suggesting that intracellular VIP signaling acts, at least in part, through the cAMP signaling.

VIP entrained the PER2::LUC rhythms to a predicted phase angle.

We investigated if and how the SCN circadian pacemaker might entrain to daily VIP stimulation. Our VIP PRC predicted that daily VIP treatment should fall around CT2 to entrain the SCN, approximately 10 h before the peak of PER2::LUC bioluminescence (CT12). VIP (10 nM or 25 nM) or vehicle was applied to SCN cultures for 5 consecutive days starting on the fifth day of the bioluminescence recording. The period of SCN cultures was not altered by the vehicle treatment (before: 24.5 ± 0.3 h vs. during: 24.5 ± 0.2 h; $n = 7$ cultures, $p = 0.97$, One-way ANOVA; **Figs. 7A, D**). In contrast, the circadian period was shortened by daily, 25 nM VIP with the bioluminescence peak occurring 10.1 ± 0.4 h after VIP on the last day of application (period before: 24.7 ± 0.2 h vs. days 2-5 of the treatment: 24.4 ± 0.2 h, $n = 7$ cultures; **Figs. 7C, D**). Notably, the phases of cultures treated with 25 nM VIP were more synchronized (Rayleigh test, $p < 0.05$, $r = 0.97$) than those of vehicle-treated cultures ($p > 0.1$, $r = 0.52$; **Fig. 7E**). A lower dose of VIP (10 nM) had a smaller effect on the period (before: 25.0 ± 0.3 h vs. during: 24.7 ± 0.2 h) and a less

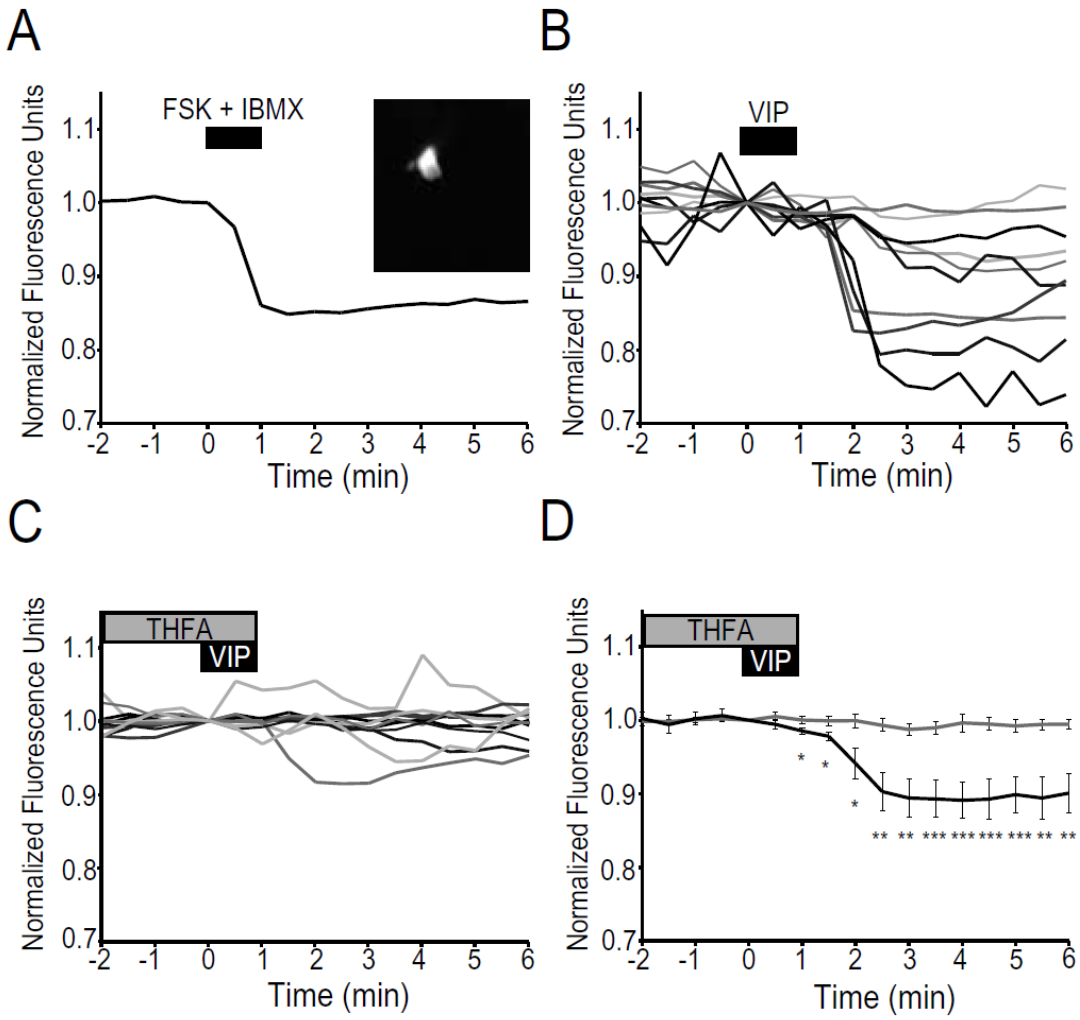

Figure 6. VIP increased cAMP in SCN neurons. (A) A representative SCN neuron expressing the ICUE2 reporter (inset image) showed a reduced FRET ratio (535nm/480nm) after treatment with forskolin (20 µM) and IBMX (75µM), indicative of increased intracellular cAMP. The data were normalized to the ratio at the initiation of treatment. **(B)** Treatment with VIP (1 µM) reduced the FRET ratio in SCN neurons. Each line represents a different neuron. **(C)** Pretreatment (2 min) with THFA (100 µM) attenuated the VIP (1 µM)-induced reduction in the FRET ratio. **(D)** Plot of the mean ± SEM responses to VIP (black, $n = 10$, Fig. 3B) and THFA + VIP (gray, $n = 11$, Fig. 3C) showing that THFA treatment significantly reduced cAMP induction by VIP (unpaired two-tailed *t*-test, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.005$).

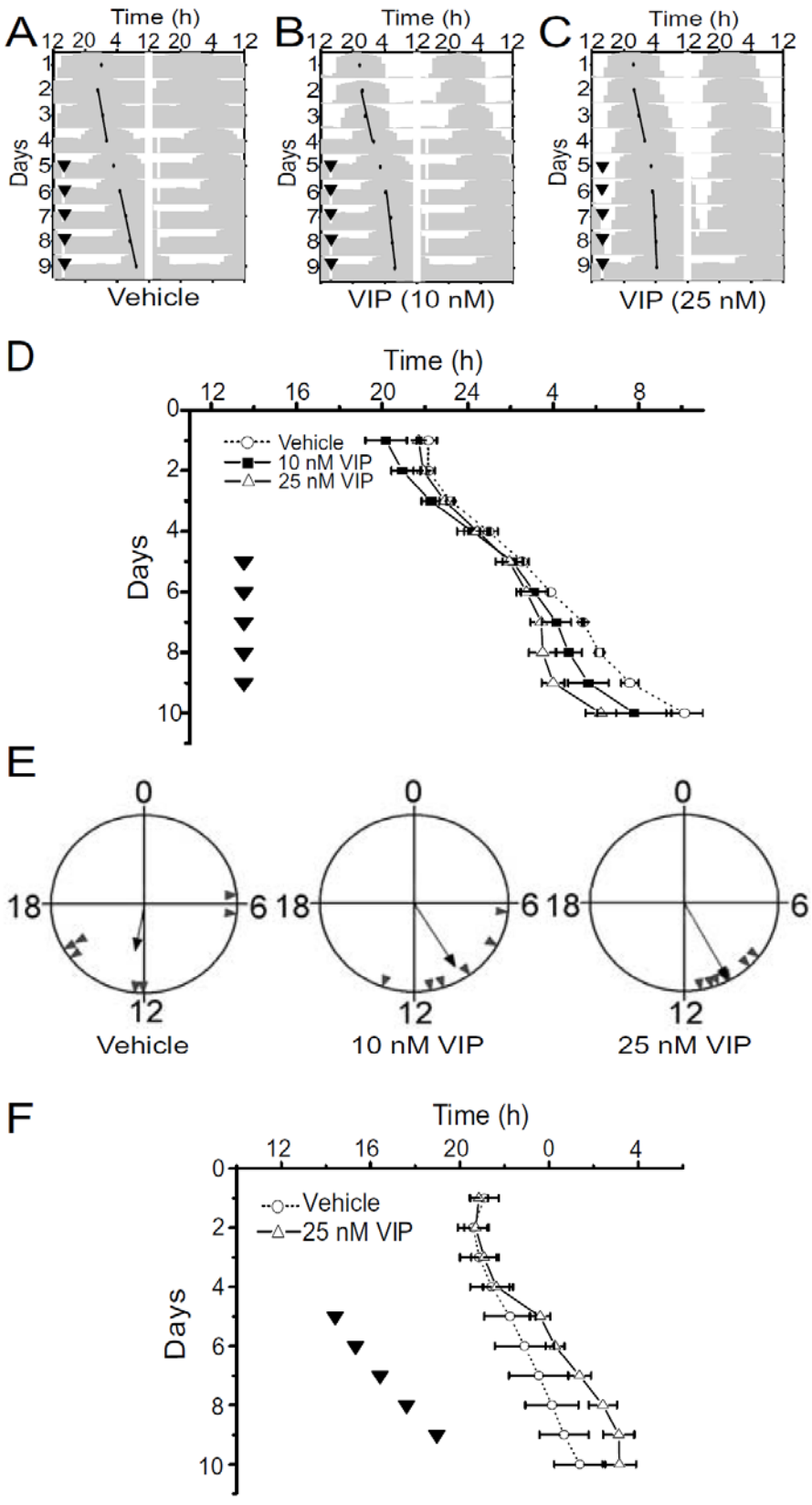
A
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Figure 7. VIP entrained PER2::LUC rhythms. (A, B and **C**) Representative actograms of SCN PER2::LUC rhythms during five consecutive days of treatment (triangles) with either vehicle, 10 nM VIP or 25 nM VIP. Note that the daily peak of PER2::LUC rhythms shifted to follow the daily VIP pulse by approximately 10 h and free-ran from that phase after the last VIP treatment. (**D**) The daily peak of PER2::LUC rhythms (mean ± SEM) of SCN cultures treated with VIP or vehicle (triangles) indicated that VIP entrained circadian rhythms in the cultured SCN (vehicle, n=2; 10 nM VIP, n=3; 25 nM VIP, *n* =3). Similar results were found in two additional replications of this experiment. (**E**) Representative Rayleigh plots showing the phase angle of the PER2::LUC expression (triangles) from cultures treated with vehicle (*n* = 7), 10 nM (*n* = 6) or 25 nM (*n* =7) VIP on the fifth day of treatment. The time between the peak phase of the SCN cultures and the time of treatment is the phase angle of entrainment. Note that cultures treated with VIP had similar times of peak PER2::LUC bioluminescence whereas vehicle treated cultures did not tend to peak at similar times. (**F**) The daily peak of PER2::LUC rhythms (mean ± SEM) over 10 days showed that SCN cultures (open triangles) synchronized their phase and period to 25 nM VIP applied every 25 h (filled triangles) for 5 consecutive days and free-ran (open circles) when treated with vehicle.

reliable effect on the phase angle of entrainment (10.0 ± 1.1 h; n = 6 cultures; **Figs. 7B, D**). Application of 25 nM VIP on a 25-h cycle also entrained the period of SCN cultures (24.4 ± 0.1 h before and 25.0 ± 0.2 h during the application; n = 4 cultures) to the predicted phase so that the VIP application fell 7.6 ± 0.7 h before the peak of bioluminescence **(Fig. 7F)**. In contrast, vehicle treatments every 25-h failed to change the period (24.4 ± 0.2 h before versus 24.6 ± 0.1 h during; *n* = 2 cultures). Therefore, daily VIP applications entrained PER2::LUC rhythms to the predicted phase angles.

Discussion

The mechanisms by which circadian rhythms synchronize to daily timing cues have been formally described as a result of rapid changes in either phase or period of the endogenous oscillator (Comas *et al.* 2006). Rapid phase adjustment, or non-parametric entrainment, has been shown in the eclosion rhythm of flies (Zimmerman *et al.* 1968), locomotor activity of nocturnal rodents (Pittendrigh & Daan 1976b), and *in vitro* oscillation of cyanobacterial genes (Yoshida *et al.* 2009) and predicted for the SCN (Best *et al.* 1999). Our results implicate VIP in rapid phase adjustment of the SCN on a daily basis. Single pulses of VIP shifted the phase, rather than the period, of the SCN **(Fig. 4)** and repeated pulses entrained SCN rhythms. Importantly, VIP doses near the threshold for phase shifts, when applied daily, entrained circadian rhythms of PER2. VIP similarly entrains circadian rhythms in cortical astrocytes (Marpegan *et al.* 2009). Thus, we postulate that VIP shifts the oscillations of SCN cells through rapid changes in their clock gene expression. In the SCN, VIP release is both circadian (Shinohara *et al.* 1995) and increased by light (Shinohara *et al.* 1993; Shinohara *et al.* 1995). Based on the PRC

measured here, we make two predictions: 1) increases in VIP release due to light during the day delay SCN rhythms and, 2) in the absence of light, circadian release of VIP peaking around midday delays free-running SCN rhythms. These predictions are consistent with the advanced phase angle of entrainment in a light cycle and shortened free-running period in constant darkness reported in VIP-deficient mice (Colwell *et al.* 2003).

The PRC to VIP described here is the first based on shifts in PER2 expression in the SCN, differs in shape and amplitude from a PRC to light and also differs in some respects from published responses to VIP. The PRCs for VIP-induced shifts in vasopressin and multiunit firing rate rhythms were previously described as light-like with advances three to eight times larger than the shifts reported here (Watanabe *et al.* 2000; Reed *et al.* 2001). We found that advances were larger when measured on the day after VIP treatment (as was done in previous studies) compared to the steady-state shift **(Fig. 3)**. This illustrates that the isolated SCN can exhibit large, transient adjustments in phase similar to what has been described for behavioral shifts to light during the late night. Importantly, PRCs based on the first day or two after a treatment can differ substantially from the steady state PRC. By measuring the steady-state PRC to VIP applied at many different circadian times from long-term recordings of SCN, we conclude the VIP PRC differs from the effects of light *in vivo* or glutamate *in vitro* on SCN rhythms. The VIP PRC is dominated by a large delay zone. Although this could be unique to PER2, it is consistent with the period lengthening effects of chronic VIP infusion on locomotor activity (Pantazopoulos *et al.* 2010).

Interestingly, the PRC to VIP in the SCN shares a similar shape and amplitude as the PRC to the neuropeptide, pigment dispersing peptide (PDF), in the cockroach (Petri & Stengl 1997). PDF in flies appears to play roles similar to mammalian VIP in entrainment and synchrony among circadian oscillators (Lin *et al.* 2004). Thus, the steady-state PRC with a large delay zone and low amplitude, narrow advance zone may have features which facilitate coordinated rhythmicity in populations of circadian cells.

The rate of entrainment and the phase angle of entrainment both depended on the concentration of VIP and whether VIP was applied on a 24-h or a 25-h cycle. Thus, aging, light intensity and other events which change VIP levels or time of release would be expected to impact circadian behaviors. This is consistent with, for example, the evidence that age-related changes in VIP timing and levels are intimately associated with the menopause in rats (Krajnak *et al.* 1998; Gerhold *et al.* 2005). It is clear, however, that VIP is not the sole entraining agent of the SCN since, for example, mice lacking VIP or its receptor can still entrain to light cycles. Photic entrainment likely depends on neuropeptides and transmitters including VIP (Piggins *et al.* 1995; Watanabe *et al.* 2000; Reed *et al.* 2001), pituitary adenylate cyclase-activating polypeptide (Hannibal *et al.* 1997; Harrington *et al.* 1999), gastrin releasing peptide (Albers *et al.* 1995; Piggins *et al.* 1995; McArthur *et al.* 2000; Kallingal & Mintz 2006), glutamate (Meijer *et al.* 1988; Ding *et al.* 1994; Asai *et al.* 2001) and NO (Ding *et al.* 1994) and intracellular signals including cAMP (Prosser & Gillette 1989) and cGMP (Prosser *et al.* 1989; Liu *et al.* 1997).

Although the exact sites of action for VIP-induced entrainment in the SCN are unknown, we found VIP increased intracellular cAMP in individual SCN neurons **(Fig. 6B)**. The VPAC2 receptor, encoded by the Vipr2 gene, is presumed to be the primary mediator of VIP activity since the loss of the receptor produces a phenotype similar to loss of VIP (Harmar *et al.* 2002; Colwell *et al.* 2003). Consistent with our findings, Vipr2 mRNA (Cagampang *et al.* 1998c; Shinohara *et al.* 1999) and a transgenic reporter using the human Vipr2 promoter (Kallo *et al.* 2004) have been shown to be widely expressed in the SCN.

We found convergence of VIP signaling to both the cAMP and Ca^{2+} signaling pathways in the SCN. Previous reports have shown that prolonged blockade of cAMP production or Ca^{+2} in the rodent SCN or snail retina stop the progression of circadian rhythms (Ralph *et al.* 1992; Khalsa *et al.* 1993; Lundkvist *et al.* 2005; O'Neill *et al.* 2008). Here, low doses of antagonists against cAMP and Ca^{+2} signaling suppressed VIP-induced shifts while blockade of a single pathway had little effect on shifts. This implicates Gs in VIP-mediated responses and also raises the possibility for the involvement of Gq or Gi/o activities (Trimble *et al.* 1987; Van Rampelbergh *et al.* 1997; Gillette & Mitchell 2002; Hains *et al.* 2004; Aton *et al.* 2006; Stewart *et al.* 2007) and suggests that at least two, and possibly interconnecting, pathways are involved. Furthermore, the antagonists had little effect on PER2 rhythms over 5-6 days, suggesting that the mechanisms mediating shifts are more sensitive to cAMP and Ca^{+2} levels than the mechanisms involved in rhythm generation. It is important to note that a subjective day (Prosser & Gillette 1989), unlike our VIP PRC.

Taken together, the findings presented here implicate VIP in the synchronization of SCN neurons to each other and environmental cycles via increases in AC and PLC signaling to rapidly shift clock gene rhythms.

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Chapter 3.

VIP accelerates entrainment of the SCN to an environmental cycle

This chapter includes the manuscript:

An S., Harang R., Meeker K., Granados-Fuentes D., Tsai C., Petzold L.R., Doyle III F.J. Vasoactive intestinal polypeptide speeds circadian entrainment by reducing synchrony. In preparation.

Abstract

Shift work or travel across time zones can result in desynchronization of the body's circadian rhythms from the local light-dark cycle. To date, there is no prevention or cure for jet lag. In mammals, the suprachiasmatic nucleus (SCN) generates and sustains circadian rhythms. In the SCN, about 20,000 neurons are coupled, and oscillate in synchrony, generating high-amplitude rhythms. SCN cells depend on vasoactive intestinal polypeptide (VIP) to synchronize to each other and to generate coordinated circadian rhythms in physiology and behavior. We serendipitously found that VIP reduced the amplitude of the PERIOD2 (PER2) ensemble rhythms in the SCN and synchrony among SCN neurons in a dose-dependent manner. Consistent with our findings *in vitro*, light-suppression of circadian rhythms in locomotor activity was reduced in mice deficient for VIP. Based on properties of weakly coupled oscillators, we hypothesized that reducing synchrony among SCN cells could facilitate synchronization to environmental cues. We found that *in vivo* or *in vitro* delivery of VIP to the SCN approximately halved the time required to entrain to an 8-10-h advance in the light or temperature cycle. We conclude that treatments which reduce synchrony among the SCN neurons can alleviate jet lag.

Introduction

Circadian rhythms synchronize (entrain) to daily environmental cues such as light and dark. In modern life, circadian oscillators in suprachiasmatic nucleus (SCN), a master circadian pacemaker, entrain slowly following trans-meridian time travel or during shift work. The long term misalignment between the internal timing and altered light dark cycles can even induce physiological and psychological abnormalities including depression, the risk of cancer, heart problems and obesity, and increased mortality (Cho 2001; Davidson et al. 2006). .

The neurons in the SCN synchronize to each other and to ambient oscillations of light. To generate reliable oscillations in output rhythms and convey timing information throughout the body, they require vasoactive intestinal polypeptide (VIP) signaling (Harmar *et al.* 2002; Aton *et al.* 2005; Maywood *et al.* 2006). VIP is released in the SCN as a function of circadian time and light intensity (Shinohara *et al.* 1994; Shinohara *et al.* 1995; Honma *et al.* 1998). In the absence of VIP (*Vip-/-*) or its receptor, VPAC2R (*Vipr2-/-*), animals become arrhythmic with fragmented activity, and their SCN neurons are arrhythmic or desynchronized each other (Harmar *et al.* 2002; Colwell *et al.* 2003; Aton *et al.* 2005; Maywood *et al.* 2006). VIP application to the SCN explant cultures induces *Period* (*Per*) 1 and 2 (Nielsen *et al.* 2002), two genes implicated in light-induced resetting (Akiyama *et al.* 1999; Tischkau *et al.* 2000; Tischkau *et al.* 2003), and shifts rhythms in behavior and SCN physiology (Piggins *et al.* 1995; Watanabe *et al.* 2000; Reed *et al.* 2001; Meyer-Spasche & Piggins 2004; An *et al.* 2011). Notably, daily VIP or VPAC2R agonist pulses entrain rhythms in SCN explants or *Vip-/-* SCN neurons (Aton *et*

al. 2005; An *et al.* 2011). VIP has therefore been described as a synchronizer or coupling factor in the mammalian circadian system.

Recent research suggests that desynchrony among or damping of individual oscillators can promote entrainment. In entrainment experiments using temperature cycles or pulses, the desynchronizing agents such as chronic tetrodotoxin treatment, a blocker of voltage-gated sodium channels, or MDL-12,330a, a blocker of adenylate cyclase activity, augment the entrainment of SCN slices (Abraham *et al.* 2010; Buhr *et al.* 2010). Since the SCN is composed of about 20,000 circadian cells, the desynchrony leads to the amplitude reduction of circadian rhythms in the whole SCN (Yamaguchi *et al.* 2003; Abraham *et al.* 2010). This amplitude reduction in the whole SCN is, therefore, different from the amplitude reduction due to the damping of individual neurons. Damped or weak oscillators are predicted to be readily entrainable in a computer simulation and one experimental result using *Clock*/+ heterozygote SCN (Vitaterna *et al.* 2006; Abraham *et al.* 2010; Komin *et al.* 2011). However,it has not been examined, whether the desynchrony or damping facilitates the entrainment to an entraining cue *in vivo*, and whether a transient desynchrony among the SCN cells also promotes the entrainment.

Here, we report that VIP administration reduced the amplitude of ensemble PER2 rhythms and synchrony among SCN cells. The amplitude reduction of behavioral rhythms by constant light depended, in part, on VIP. In addition, we found that VIP administration in the early subjective day sped entrainment of circadian rhythms to an advanced light- or temperatureschedule both *in vivo* and *in vitro*. These data suggest that

treatments which reduce the synchrony among the circadian oscillators will ameliorate the negative consequences of jet lag or day night shift works.

Materials and Methods

ANIMALS. Mice were housed in a 12 h/12 h light/dark cycle in the Danforth Animal Facility at Washington University. PER2::LUC knock-in mice (Yoo et al., 2004) (founders generously provided by J. S. Takahashi, Univ. of Texas Southwestern Medical Center, Dallas, TX) and *Vip-/-* (generous gift from C. S. Colwell, University of California, Los Angeles, Los Angeles, CA) mice were backcrossed with C57BL/6 (purchased from Charles River Laboratories), and bred as homozygous pairs. All procedures were approved by the Animal Care and Use Committee of Washington University and followed National Institutes of Health guidelines.

CULTURES. For recording of population rhythms, 300 μm coronal SCN slices from 8- to 20-day-old PER2::LUC pups were obtained and immediately located under the photomultiplier tube (PMT) (HC135-11 MOD, Hamamatsu Corp., Shizuoka, Japan). Maintenance, recording of and VIP treatment of cultures were performed as described previously (An *et al.* 2011).

For recording of single cell rhythms from slices, the coronal SCN slices were maintained for 2-5 days on the 0.4 mm membrane inserts (Millipore, Billerica, MA) with 1 ml CO2-buffered medium (CO2 DMEM) supplemented with 10 % new born calf serum (NCS) (Invitrogen, Carlsbad, CA). Slices were then inverted onto poly-D-lysine (PDL) and

laminin-coated glass coverslips and maintained for 6-8 days with 250-400 µl of the CO2 DMEM with the 10 % NCS until the recording.

DRUGS. VIP was purchased from Bachem (King of Prussia, PA). Culture media consisted of DMEM (Sigma) supplemented with 2% B27 (Invitrogen), 10 mM HEPES (Sigma) and 2.2 mg/ml NaHCO3 (Invitrogen). For *in vitro* recording, VIP was dissolved in culture medium and vehicle controls consisted of an equal volume of culture medium. For *in vivo* injection, artificial cerebrospinal fluid (aCSF) was used to dissolve VIP.

BIOLUMINESCENCE RECORDING. We recorded bioluminescence rhythms from 300 µm, coronal SCN slices from PER2::LUC mice (age 8-20 days) using a photomultiplier tube (PMT) (HC135-11 MOD, Hamamatsu Corp., Shizuoka, Japan) as described previously (An et al., 2010). SCN explants were cultured on 0.4 mm membrane inserts (Millipore, Billerica, MA) in sealed 35-mm culture dishes (BD Biosciences, San Jose, CA) with 1 ml pre-warmed air-buffered medium supplemented with 10% newborn calf serum (NCS) (Invitrogen) and beetle luciferin (Promega, Madison, WI) at 34°C. Bioluminescence counts were integrated and stored at 1 min intervals for up to 15 days of recording. To analyze the amplitude changes after VIP application, the recordings were detrended as described previously (An *et al.* 2011). Then, the detrended traces were divided by the peak prior to the application and top-to-bottom values of the each half cycle were measured. To reduce the initial culture-to-culture variations, we divided each value with an average of 2-3 cycles before the treatment. The display of bioluminescence

traces and statistics were done using Origin 7.0 software (Origin, Northampton, MA) unless mentioned otherwise.

SINGLE-CELL BIOLUMINESCENCE RECORDING. We recorded the bioluminescence rhythm for single cells from slice cultures with an inverted microscope (TI-S/L100, Nikon) and Andor iXon EMCCD (12 x 512 pixels, 16um pixel size) cooled charged device camera (Andor technology) at -80 °C in 1 ml air-buffered medium with luciferin and 10% NCS. Cultures were maintained in custom-built incubator (In Vivo Scientific) at 34 °C.At the forth or fifth cycle, we applied 150 nM VIP or vehicle into the culture medium and continued the recording for 4-5 days. Photon counts were integrated over 1 minute or 10 minutes with Micro-Manager and processed with ImageJ (both National Institute of Health). Cells clearly distinguishable from surrounding cells were analyzed. To generate a raster plot using ImageJ, normalized data of the bioluminescence traces was made by dividing with the peak prior to the application using detrending macro (kindly provided by Dr. Marpegan). For the better contrast, these processed data were subtracted by their trough value prior to the application so that the peak-to-trough values are 1-0. Rayleigh statistics were done using Oriana (Kovach Computing Services, UK).

MICROINJECTION. For stereotaxic surgery, mice were anesthetized with isofluorane (Butler Animal Health Supply, Dublin, OH) mixed with continuous oxygen flow. Brain was positioned flat along the Bregma to Lambda. A stainless steel guide cannula (4.0 mm, 26 gauge; Plastics One, Roanoke, VA) was inserted perpendicular to the skull, fixed with

mixture of dental cement (CO-ORAL-ITE Dental MFG, Diamond Springs, CA) and methyl methacrylate (Sigma, Saint Louis, MO). The coordinate for the SCN was from Bregma: +0.5 mm posterior, +0.2 mm lateral, and +4.1 mm depth from the brain. 30 or 300 pmole of VIP (1.5 mM) or vehicle (aCSF) was injected with 200 nl volume in a polyethylene tubing connected to 20 µl Hamilton syringe. The VIP or vehicle was delivered using a microinjection pump (Model KDS 310, Analytical West Inc.) with the flow rate of 50 nl / min.

BEHAVIORAL RECORDING. Adult male mice were housed in individual cages established with running wheels and maintained in light-tight chambers with fluorescent bulbs (General Electric). Locomotor activity was measured in 6 min-bin using Clocklab (Actimetrics, Wilmette, IL). For measuring the light-induced VIP effect, the C57BL/6 (n = 18) and *Vip-/-* (n = 15) mice were kept in constant light for about 40 days followed by constant darkness for 10-11 days. For measuring the effect of VIP injection, 26 C57BL/6 mice were kept for 7 d in 12 h/12 h light/dark cycles, cannulated and recovered for another 7 d followed by VIP (n = 14) or vehicle injection (n = 12). After the injection, recording was resumed followed by light off. Recording was maintained for 13 days with a light onset being advanced for 8 h. For better display purpose, a moving average with 4-h time window was done in time series data of locomotor activity.

Results

VIP reduces the amplitude of PER2::LUC rhythm.

As part of an analysis of VIP-induced phase shifts of the SCN (An et al., 2011), we recorded PER2-driven bioluminescence from SCN explants treated with 10 nM to 100 μM VIP. We noted a highly reproducible and sustained reduction in SCN rhythmicity following VIP **(Fig. 1A)**. We measured the peak-to-trough amplitude 48 h after VIP application and found a dose-dependent amplitude reduction in PER2 rhythms with an EC_{50} near 150 nM and saturation above 10 μM **(Fig. 1B)**. Within this range, a 10-fold increase in VIP concentration caused a 50 % decrease in the PER2 amplitude ($r^2 = 0.99$, n = 31 explants). Following 1 μM VIP, the peak-to-trough amplitude was rapidly reduced and then slowly recovered over approximately 9 days **(Fig. 2)**. The amplitude reduction was similar regardless of the time of VIP application ($p > 0.05$, $F_{2,59} = 22.76$, n = 74, Two-way ANOVA; **Fig. 1C**).

VIP reduces the synchrony of SCN populations.

The amplitude reduction recorded from the population of SCN cells could reflect reduced synchrony among SCN cells or reduced amplitude of individual cells **(Fig. 3)**. To distinguish between these possibilities, we monitored VIP effects on the PER2::LUC rhythms in individual SCN neurons from a slice using an ultra-sensitive CCD camera. Vehicle-treated SCN cells maintained their synchrony throughout the recording (Rayleigh test, before vehicle: $p < 0.05$, $r = 0.87$, after vehicle: $r = 0.84$; **Fig. 4A, B**).150 nM or 10 μM VIP administration reduced the synchrony in individual neurons (Rayleigh test, 150 nM VIP, before VIP: $p < 0.05$, $r = 0.92$ after VIP: $r = 0.51$; 10 μM VIP, before VIP: $p < 0.05$, $r = 0.74$, after VIP: $r = 0.37$; **Fig. 4C-F**). The maintenance of synchrony in

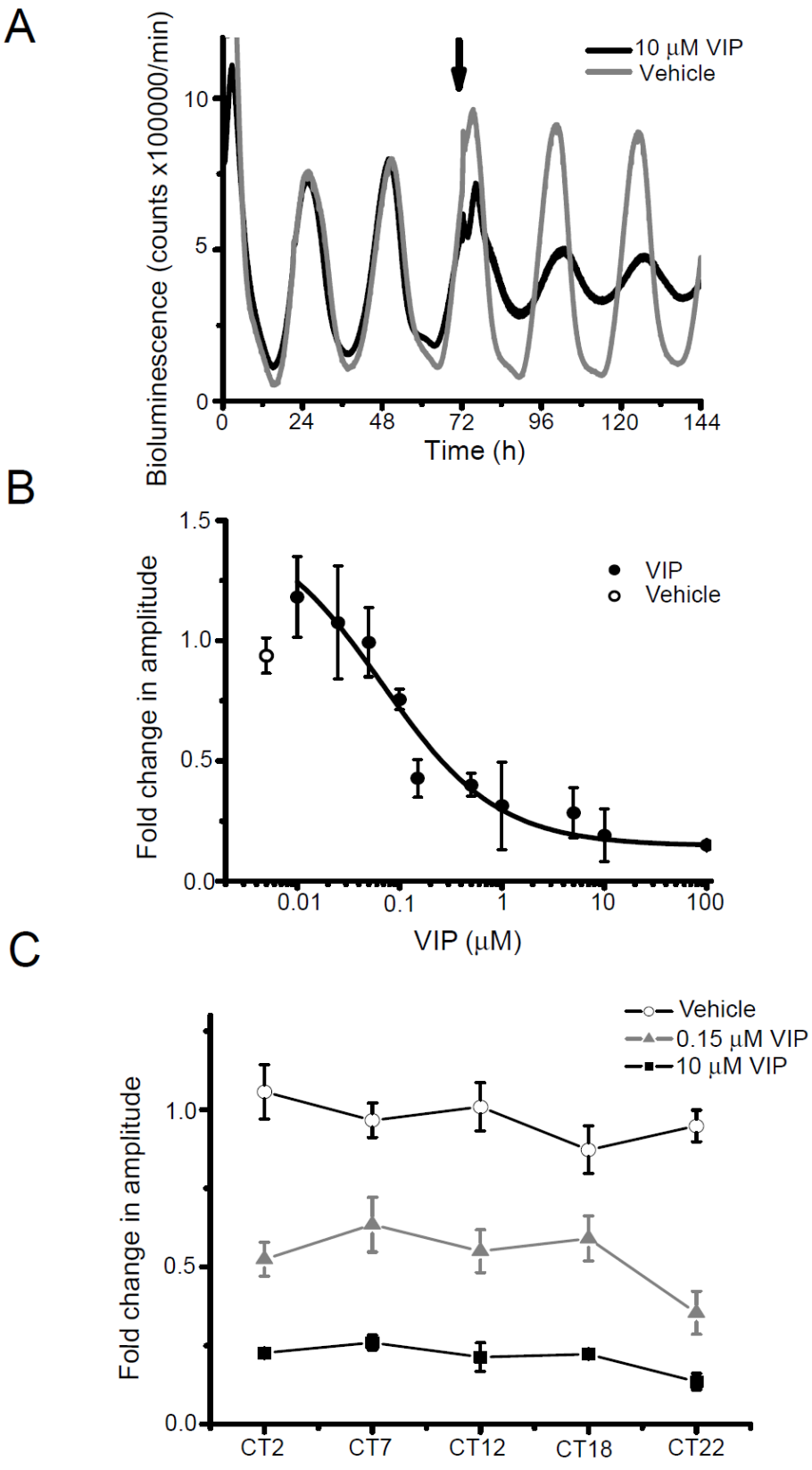

A
10 μM VIP
Vehicle
Bioluminescence (counts x100000/min)
0
5
10
0
24
48
72
96
120
144
Time (h)
B
VIP
Vehicle
Fold change in amplitude
0.0
0.5
1.0
1.5
0.01
0.1
1
10
100
VIP (μM)
C
Vehicle
0.15 μM VIP
10 μM VIP
Fold change in amplitude
0.0
0.5
1.0
CT2
CT7
CT12
CT18
CT22

Figure 1. VIP reduced the amplitude of circadian rhythms in the SCN. (A) Representative bioluminescence traces from SCN explants treated with 10 μM VIP (black) or vehicle (gray) near the peak of PER2-driven bioluminescence (CT12, arrow). Each PER2::LUC rhythm was normalized to the peak prior to treatment. **(B)** The dose-dependent amplitude reduction (mean ± SEM; n = 3-5 cultures at each dose) by VIP application at CT 12. Above 150 nM VIP and below 10 μM VIP, the amplitude reduction of the PER2::LUC rhythm increased linearly with logarithmic increases in VIP concentration. Data were fitted with a logistic function (black line). Amplitude was measured as the trough-to-peak magnitude on the rising phase 48 h after VIP application. **(C)** VIP application reduced the amplitude of SCN rhythms similarly at all circadian phases. The amplitude reduction of PER2::LUC rhythm (mean ± SEM) was higher following 10 μM VIP (squares) than 150 nM VIP (triangles; n = 20 and n = 16 respectively) at all times, but did not vary with the time of VIP application ($p > 0.05$, $F_{2,59} = 22.76$, n = 74, Two-way ANOVA). Vehicle (open circles) did not reduce the amplitude of ensemble PER2 rhythm at any time.

vehicle-treated culture and reduction of synchrony in VIP-treated culture was consistent in multiple cultures (*r* value before vehicle: 0.93 ± 0.01, after vehicle: 0.91 ± 0.07, n = 2; before 150 nM VIP: 0.84 ± 0.03, after 150 nM VIP: 0.51 ± 0.07, n = 5; before 10 μM VIP: 0.77 ± 0.03, after 10 μM VIP: 0.25 ± 0.12, n = 2). Fold change in top-to-bottom amplitude of individual cells with VIP administration was not significantly different from that with vehicle application ($p > 0.05$, $F_{1,750} = 3.2$, One-way ANOVA, Scheffé post hoc; **Fig. 5**). Therefore, ensemble PER2 rhythm by VIP can be explained by the reduced synchrony among the SCN population.

VIP modulates circadian amplitude *in vivo.*

These *in vitro* results led us to test if VIP plays a role in modulating circadian amplitude *in vivo*. Because prolonged constant light (LL) has been reported to desynchronize rhythms among SCN cells and produce arrhythmic locomotor behavior (Ohta *et al.* 2005), we recorded wheel running from wild-type (C57BL/6, n = 18) and VIP-deficient (*Vip-/-*, n = 15) mice maintained in constant light (LL) for about 40 days . Time-series plots of wild type mice showed a marked reduction of peak-to-trough amplitude in LL compared to in DD (LL / DD ratio: 0.2 ± 0.04; **Fig. 6A, C** and **E**), while *Vip-/-* mice did not (LL / DD ratio: 0.94 ± 0.17, ***: $p < 0.0005$, comparison of fold change in amplitude between wild type and *Vip-/-* mice, One way ANOVA; **Fig. 6B, D** and **E**). Notably, 40 % of *Vip-/-* mice even improved their circadian amplitude in LL, compared to in DD (6 out of 15). Therefore, VIP plays a critical role in reducing the amplitude of locomotor activity rhythms in response to light.

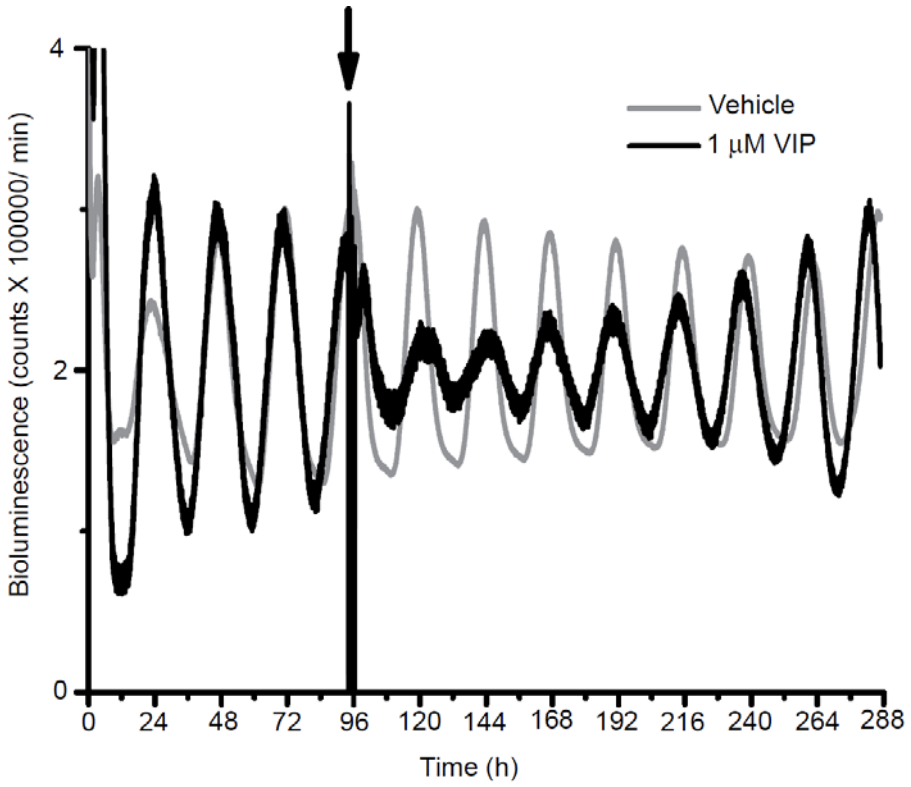
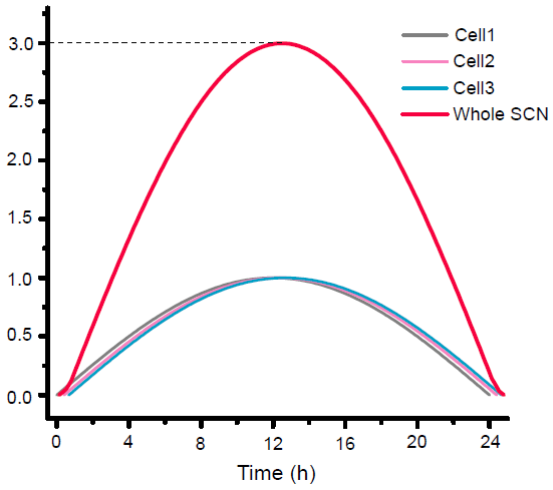
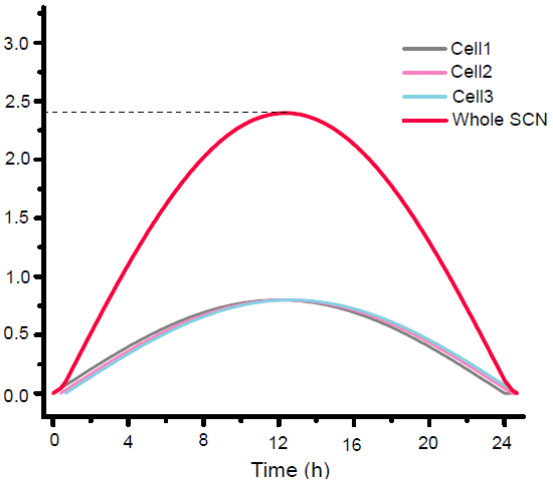


Figure 2. The amplitude reduction by VIP was transient. The top-to-bottom amplitude of PER2::LUC rhythm initially reduced by 1 μM VIP application at CT12 (black arrow), but gradually recovered back. Note that the amplitude of VIP- and vehicle-treated rhythms is almost identical after 6 days (240 hours from the start of the recording) from the VIP application.

A


Cell1
Cell2
Cell3
Whole SCN
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
4
8
12
16
20
24
Time (h)

B


Cell1
Cell2
Cell3
Whole SCN
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
4
8
12
16
20
24
Time (h)

C

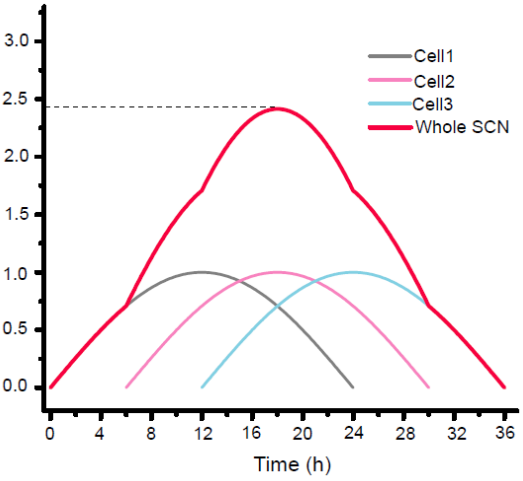

Cell1
Cell2
Cell3
Whole SCN
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
4
8
12
16
20
24
28
32
36
Time (h)

Figure 3. Damping or desynchrony may explain the amplitude reduction of circadian rhythms in the whole SCN. Schematics assume that the whole SCN is composed of three cells. **(A)** The amplitude of the rhythm in the whole SCN (a hot pink line) is high because the three cells oscillate with high amplitude and in synchrony (gray, pale pink, and sky blue line respectively). **(B** and **C)** The amplitude of rhythm in the whole SCN reduces when the three cells are damping **(B)** or desynchronized **(C)**.

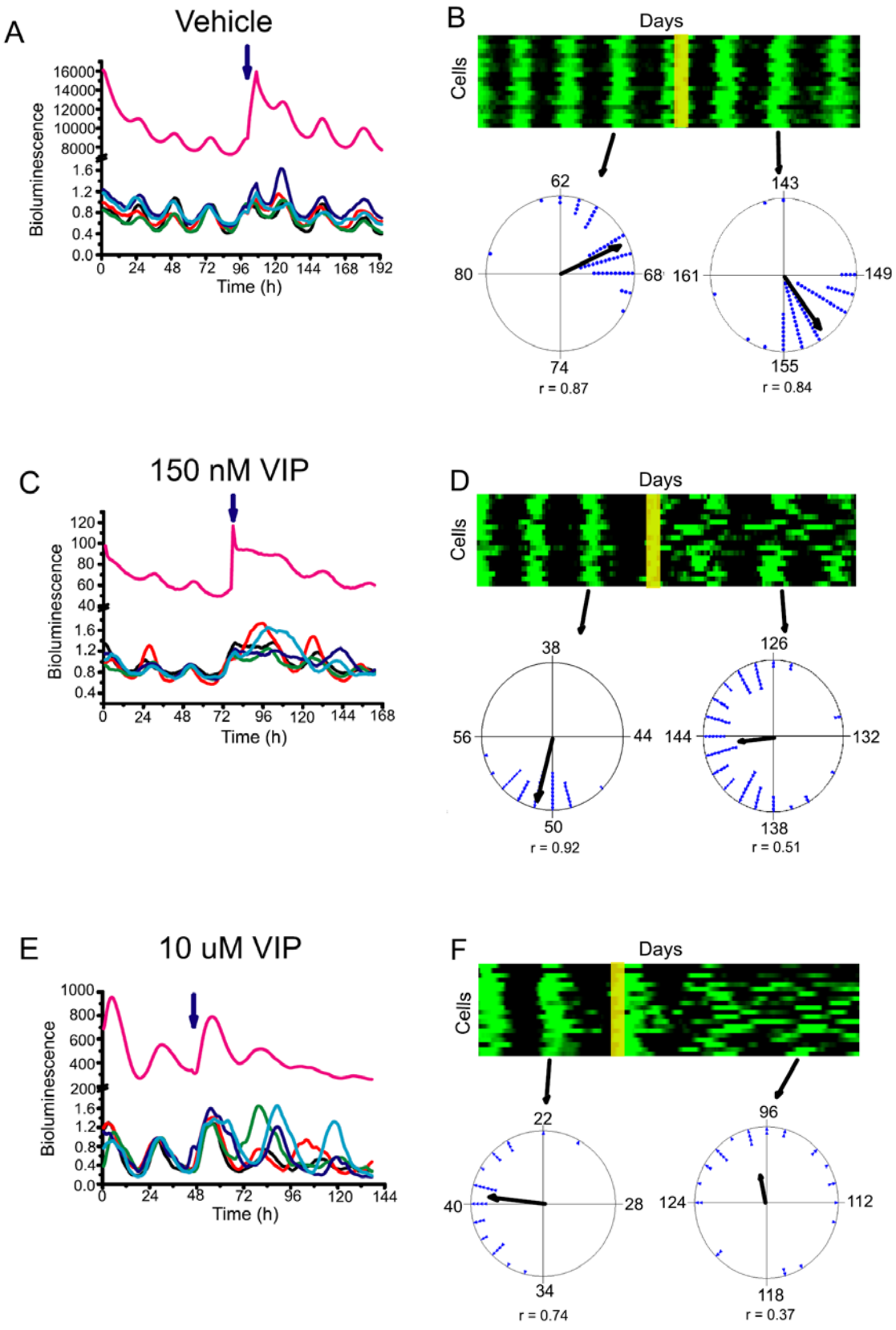

A
Vehicle
Bioluminescence
Time (h)
B
Days
Cells
62
80
68
74
r = 0.87
143
161
149
155
r = 0.84
C
150 nM VIP
Bioluminescence
Time (h)
D
Days
Cells
38
56
44
50
r = 0.92
126
144
132
138
r = 0.51
E
10 uM VIP
Bioluminescence
Time (h)
F
Days
Cells
22
40
28
34
r = 0.74
96
124
112
118
r = 0.37

Figure 4. VIP reduced the synchrony among SCN cells. (A) PER2::LUC traces of 5 randomly selected cells with vehicle application (blue arrow) remained rhythmic and synchronized so that the ensemble PER2::LUC rhythm of the slice (pink line) did not change amplitude during the recording. **(B)** A raster plot shows the daily increase (green) and decrease (black) in PER2 expression from 20 representative cells in the same SCN slice treated with vehicle (yellow bar). Two Rayleigh plots show distribution of phases among cells (n = 140) in this SCN on a day before and a day after vehicle administration, respectively. Each dot represents the time of daily peak PER2 expression for one cell. Note that the length of the mean vector (*r*) did not change following the treatment indicating that the cells remained synchronized. **(C)** PER2::LUC traces of 5 randomly selected cells with 150 nM VIP application at CT12 (blue arrow) remained rhythmic, but their synchrony was reduced. Note that the gene expression rhythm amplitude of individual cells was not reduced after VIP application. **(D)** A raster plot shows clear reduction of synchrony of PER2 expression rhythm from 20 representative cells in the same SCN slice with 150 nM VIP application at CT12 (yellow bar). Two Rayleigh plots show that the length of the r was reduced after VIP application indicating cells are less synchronized (n = 82). **(E)** PER2::LUC traces of 5 randomly selected cells with 10 μM VIP application at CT2 (blue arrow) was not synchronized but their rhythm amplitude was not. **(F)** A raster plot of 20 representative PER2::LUC traces and two Rayleigh plots (n = 30) show clear reduction of synchrony after VIP application.

VIP speeds up the entrainment of animals to changes in light schedule.

Our simulation results predict that VIP-induced reduction of synchrony could help the animal to entrain faster to changes in light schedule. To test this possibility directly, we implanted cannula (C57BL/6, n = 26) aimed at the SCN. After a week in a 12 h/12 h light/dark schedule (light on from 7:00 am to 7:00 pm), mice received 20 or 200 pmole VIP or vehicle (aCSF) at their Zeitgeber time (ZT) 3-4 (10:00-11:00 am). Light was off briefly after that and on again at 11:00 pm corresponding to an 8 hr-advanced onset (light on from 11:00 pm to 11:00 am). We chose to deliver VIP at ZT 3 as a time when it had been shown to produce minimal shifts (Piggins *et al.* 1995; An *et al.* 2011). The animals injected with VIP shifted rapidly their onset of activity and required fewer days to entrain than the vehicle-injected animals (mean of VIP-injected animals: 2.93 ± 0.37 days, n = 14; vehicle-injected animals: 5.75 ± 0.83 days, n = 12, ***: $p < 0.005$, two sample independent T-test; **Fig. 7**). These results suggest that VIP reduced the circadian amplitude of the behavioral rhythm and sped entrainment following a large advance in the light schedule.

VIP speeds up the entrainment of the SCN to changes in temperature schedule.

Next, we wanted to determine whether VIP also accelerates entrainment to the changes in cycles of temperature cycle in the SCN level. To do this, we located SCN cultures in 12 h/ 12 h temperature cycle (at 6:00 am warm to 36.5 °C, at 6:00 pm cool to 35 °C) for 8 days. At the 8^{th} cycles, we applied 10 μM VIP or vehicle at circadian time (CT) 3 (4:00 pm) to minimize the phase shift by VIP (An *et al.* 2011). 2 hours after lowering the temperature at 6:00 pm, we raised the temperature at 8:00 pm to advance the cycle by 10

hours (at 8:00 pm warm, at 8:00 am cool). The temperature oscillated with the schedule for 9 days and stayed at 36.5 °C for 4 days. We assumed that an SCN culture entrains if the peak PER2 expression has a stable phase relation for 48 h with the temperature cycle and continued it until the end of the temperature cycle. SCN cultures with vehicle treatment, took longer days to entrain to the 10h advanced temperature cycle than cultures with VIP treatment (n = 5 each; Fig. 8). Notably, the phase of the peak PER2 expression in VIP-treated cultures were more clustered (mean phase: 17.23 ± 0.5, Rayleigh test, $p < 0.005$, $r = 0.8$, n = 5) than vehicle-treated ones (mean phase: 12.57 ± 1.74, $p > 0.05$, $r = 0.25$, n = 5) at the end of the new temperature cycle (day 18^{th}). On average, VIP-treated cultures entrained to the new temperature cycle after 4.2 ± 0.37 days from the beginning of the advanced schedule while vehicle-treated cultures entrained after 9.6 ±2.09 days (*: $p < 0.05$, two sample independent T-test, Figure 5F). Therefore, together with the behavioral data these data suggest that VIP double the speed of the entrainment to a new environmental cycle.

Discussion

We found that VIP accelerated the entrainment of behavioral rhythms of the animal and PER2 gene expression rhythms of SCN explants (Fig. 7 and 8).This acceleration may be explained by a phase advancing effect of VIP (Piggins *et al.* 1995; Watanabe *et al.* 2000; Reed *et al.* 2001; An *et al.* 2011). VIP is known to shift PER2 gene expression rhythms in the SCN and behavioral rhythms minimally in early subjective day (ZT or CT3-4) (Piggins *et al.* 1995; An *et al.* 2011), when we applied VIP to the SCN cultures or

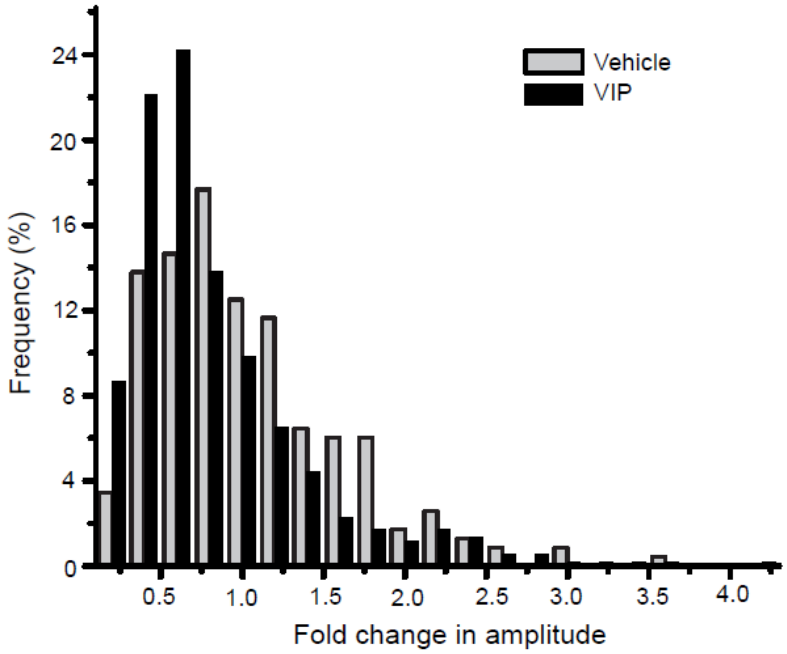


Figure 5. VIP did not reduce the amplitude of PER2 rhythm in individual SCN cells. A histogram shows the distribution of SCN cells according to their top-to-bottom amplitude change. The amplitude was measured two cycles after the vehicle (gray) or VIP (black) was divided with the amplitude before the treatment. If the fold change in amplitude is smaller than 1, it means that the amplitude of a cell's PER2 rhythm was reduced after the treatment. Note that the distribution of the VIP-received cell populations (n = 520 cells from 5 SCN slices) is similar to that of vehicle-received cells (n = 232 cells from 2 SCN slices).

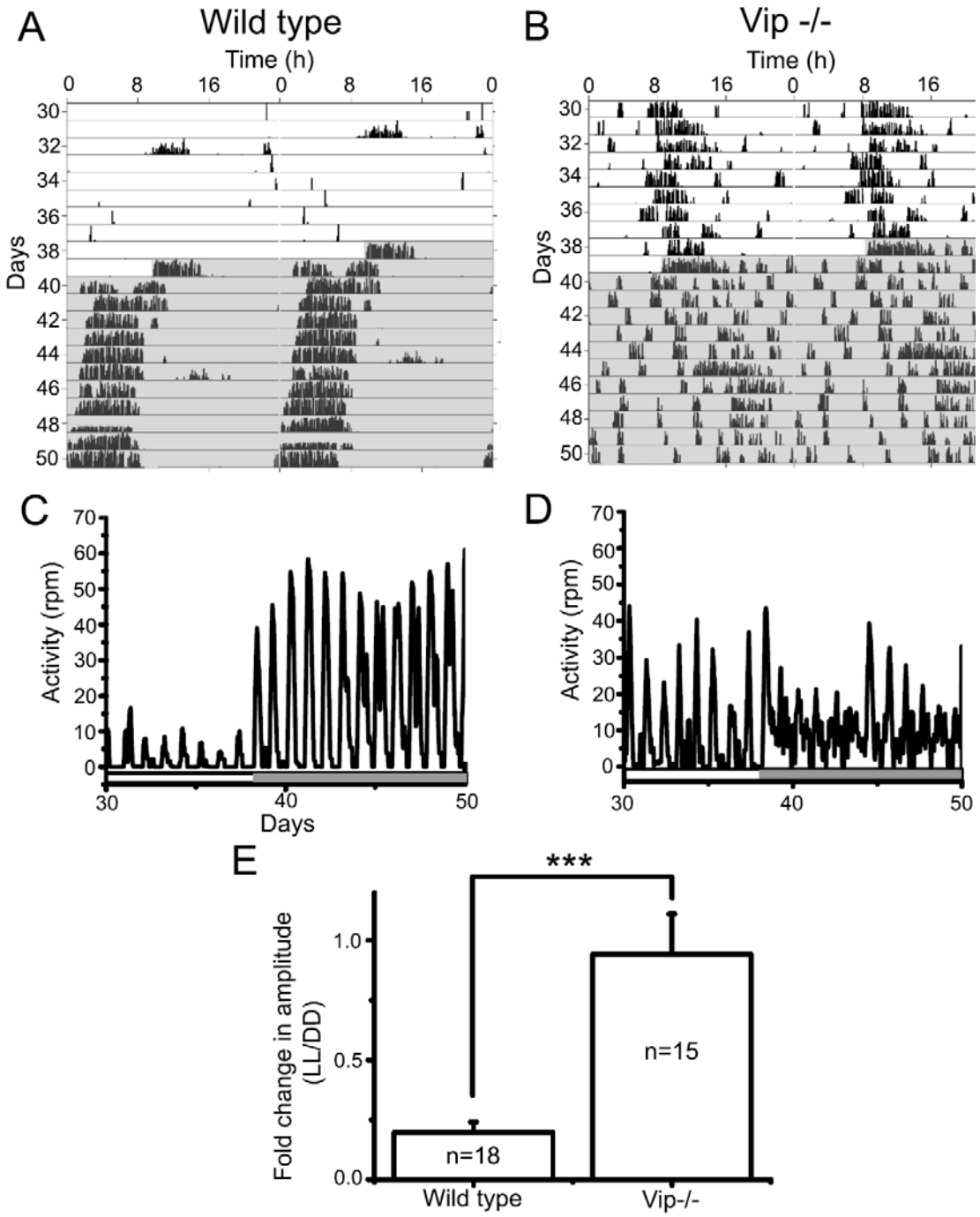

A
Wild type
Time (h)
0 8 16 0 8 16 0
Days
30 32 34 36 38 40 42 44 46 48 50
B
Vip -/-
Time (h)
0 8 16 0 8 16
Days
30 32 34 36 38 40 42 44 46 48 50
C
Activity (rpm)
0 10 20 30 40 50 60 70
30 40 50
Days
D
Activity (rpm)
0 10 20 30 40 50 60 70
30 40 50
E
***
Fold change in amplitude (LL/DD)
0.0 0.5 1.0
n=18
n=15
Wild type
Vip-/-

Figure 6. VIP mediated the amplitude reduction of locomotor rhythms in LL. (A and **B)** Representative actograms of wild type and VIP knockout (*Vip-/-*) mice kept in LL for about 40 days (darkness shown as gray). Note that the wild-type mouse expressed multiple daily peaks of activity compared to the more consolidated activity of the *Vip-/-* mouse. Cage changes were done in days 32, 39, and 45. **(C** and **D)** Time series plots of the same actograms reveal that the wild-type mouse showed low-amplitude rhythms in LL and high amplitude rhythms in DD compared to the weak circadian rhythms in LL and DD of the *Vip-/-* mouse. The bar at the bottom of each plot shows the times of lights on (white) and off (gray). **(E)** The fold change in the peak-to-trough amplitude of daily locomotion in wild-type animals was reduced dramatically in LL compared to DD, but did not change in *Vip-/-* mice (mean ± SEM, n indicates the number of mice, ***: $p < 0.0005$, two sample independent T-test).

animals. Also, VIP reduced both the synchrony among the SCN and the amplitude of ensemble gene expression rhythm throughout the day **(Fig. 1)**, whereas VIP-induced shifts showed large delays from mid-subjective day to late-subjective night and small advances from late subjective night to early subjective day (An *et al.* 2011). Based on these data, therefore, we conclude two things: 1) the faster entrainment by VIP is due to the reduction of synchrony among the SCN population and 2) this acceleration can occur throughout the day. Therefore, VIP effects, shown here, differ from the phase-dependent acceleration of entrainment by sildenafil (Agostino *et al.* 2007) or long-term suppression of aderenal corticosterone (Kiessling *et al.* 2010). The both treatments promoted the entrainment in a phase advancing light schedule, but had no or worsening effect in a phase delaying schedule.

VIP application reduced the synchrony among the SCN population and the amplitude of gene expression rhythms in the whole SCN. In uncoupled cells such as astrocytes (Marpegan *et al.* 2009) or *Vip-/-* SCN neurons (Aton *et al.* 2005), VIP augmented the amplitude of ensemble PER2 rhythms and the synchrony among them. Therefore, different coupling states may determine whether VIP application increases or decreases synchrony. The intracellular action of VIP including PER induction (Nielsen *et al.* 2002; Marpegan *et al.* 2009; An *et al.* 2011) or receptor internalization(Schulz *et al.* 2004; Langer & Robberecht 2007) , may be possibly involved in this mechanism. Alternatively, VIP action may depend on its concentrations. Wild type SCN cultures express and release VIP into their medium, whereas astrocyte cultures or *Vip-/-* SCN neuronal cultures do not. VIP may act as a synchronizing agent when there is little or noVIP whereas it may act as

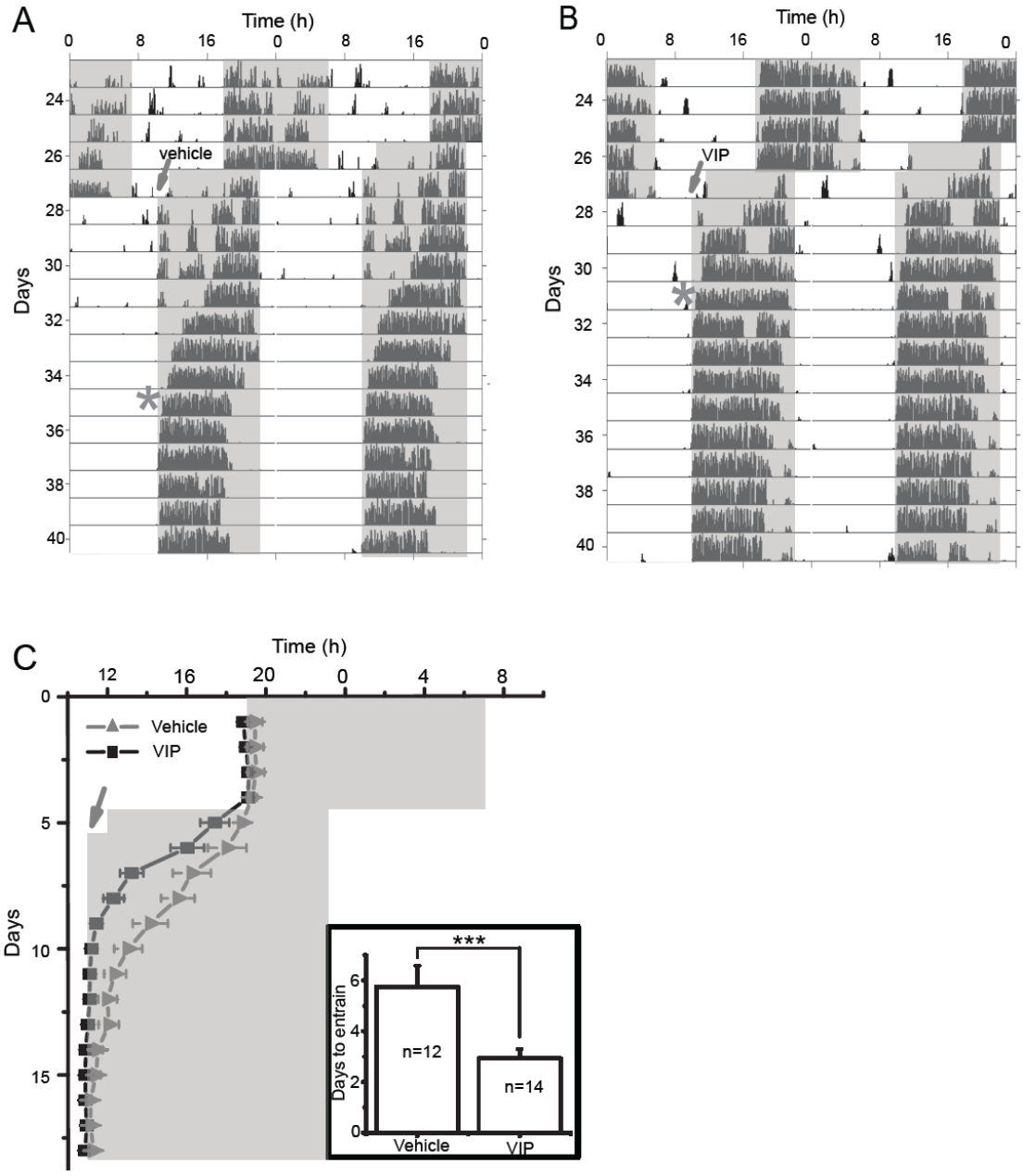

A
Time (h)
0
8
16
0
8
16
0
Days
24
26
28
30
32
34
36
38
40
vehicle
*
B
Time (h)
0
8
16
0
8
16
0
Days
24
26
28
30
32
34
36
38
40
VIP
*
C
Time (h)
12
16
20
0
4
8
Days
0
5
10
15
Vehicle
VIP
***
Days to entrain
6
4
2
0
n=12
n=14
Vehicle
VIP

Figure 7. VIP accelerated the entrainment to phase-advancing entraining schedule *in vivo*. (A and **B)** Representative actograms of vehicle- or VIP-injected mice followed by an 8h-advance in light schedule. Animals were in 12 h/12 h light schedule for a week, where light was on from 7 am. After an injection at ZT3-4 (arrow), the light onset was advanced by 8 h (11 pm). Phase shift of behavioral rhythm and PER2 expression induced by VIP at early subjective day is known to be minimal. Note that the vehicle-injected mouse took longer days to entrain (the first entrained day: day 36^{th}, asterisk, 8 days from the injection day) while the VIP-injected mouse quickly entrained to the new light schedule (the first entrained day: day 31^{th}, asterisk, 3 days from the injection day). **(C)** A group summary of daily activity onset of vehicle- or VIP-injected animals (mean ± SEM). After the injection (arrow), the activity onset of VIP-injected animals (n = 14) shifted more quickly following the 8 h advance in light schedule than that of vehicle-injected animals (n = 12). Inset shows a group summary of days required to entrain to an 8h-advanced light schedule. VIP-injected group significantly entrained faster than vehicle-injected group (mean ± SEM, ***: $p < 0.005$, two sample independent T-test, VIP-injected group: 2.93 ± 0.37 days, n =14; vehicle-injected group: 5.75 ± 0.83 days, n = 12).

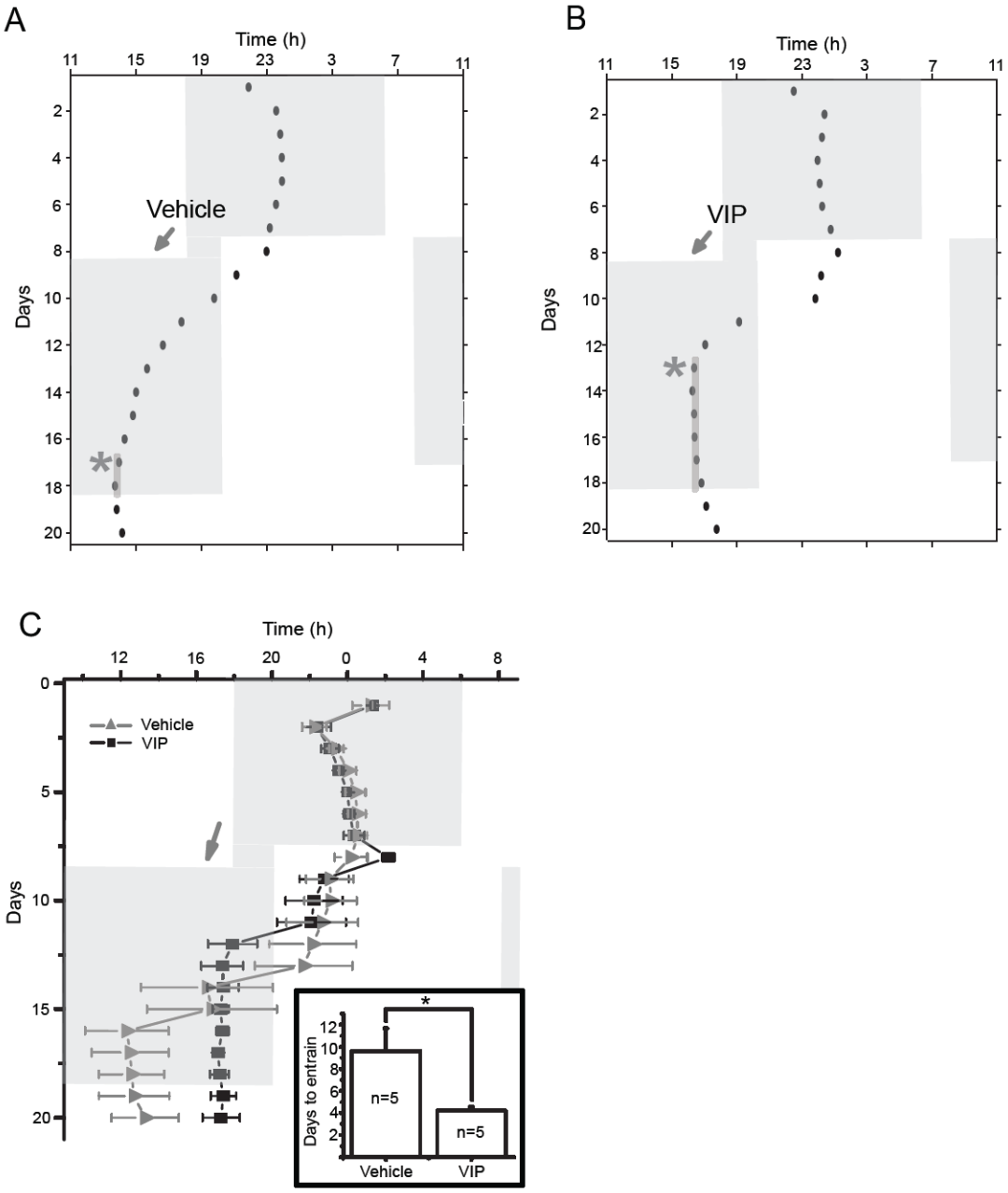

A
Time (h)
11 15 19 23 3 7 11
Days
2 4 6 8 10 12 14 16 18 20
Vehicle
*
B
Time (h)
11 15 19 23 3 7 11
Days
2 4 6 8 10 12 14 16 18 20
VIP
*
C
Time (h)
12 16 20 0 4 8
Days
0 5 10 15 20
Vehicle
VIP
*
Days to entrain
12 10 8 6 4 2
n=5
n=5
Vehicle
VIP

Figure 8. VIP accelerated the entrainment to phase-advancing entraining schedule *in vitro*. (A and **B)** Actograms of daily peak PER2 expression of SCN cultures with vehicle- or 10 μM VIP application followed by a 10 h-advanced temperature cycle. Initially, cultures were entrained to 12 h/12 h temperature fluctuations for 8 days (gray: 35 °C, white: 36.5 °C). At day 8, vehicle or 10 μM VIP was applied at CT3 (4:00 pm). After becoming cool at 6 pm, temperature became warm at 8 pm to advance the temperature cycle by 10 hours. The culture was assumed to entrain to the new cycle when the phase for consecutive 4 days had a stable phase angle with temperature cycle. Note that the vehicle-treated bioluminescence trace required 9 days to entrain to the new temperature cycle (the first entrained day: day 17^{th}, asterisk) while VIP-treated trace quickly entrained (the first entrained day: day 13^{th}, asterisk). The vertical line represents days when these cultures had a stable phase relation with the temperature cycle. **(C)** A group summary of daily peak phase of PER2 rhythm (mean ± SEM). White and gray areas represent warm (36.5 °C) and cool (35 °C) temperature respectively. After the treatment (arrow), all VIP-treated cultures (n = 5) drastically altered their phase, quickly entrained to the new temperature cycle than vehicle-treated cultures (n = 5). Note the bigger error bars in vehicle-treated case, indicative of poor synchrony among cultures. Inset shows the days required to entrain in the vehicle- and VIP-applied groups. VIP-applied cultures entrained significantly faster than vehicle-treated ones (mean ± SEM, *: $p < 0.05$, two sample independent T-test, VIP-injected group: 4.2 ± 0.37 days, n =5; vehicle-injected group: 9.6 ± 2.09 days, n = 5).

a desynchronizing agent with the large amount of VIP. This idea is also supported by the dose-dependency of synchrony reduction **(Fig. 4)** and the increased amplitude of ensemble gene expression rhythm with 10-50 nM VIP application **(Fig. 1B)**.

We also found that VIP modulates the reduction of behavioral rhythm amplitude in LL. Top-to-bottom amplitude of behavioral rhythm of wild type animals dramatically reduced at the end of LL but that of *Vip-/-* did not **(Fig. 6)**. Since the reduced synchrony among the SCN neurons caused the amplitude reduction of circadian rhythms in the whole SCN, and the SCN generates output rhythms, the amplitude reduction in behavioral rhythms might be caused by the reduced synchrony in the SCN. Notably, LL appears to have the similar effects with VIP. In LL, some of mice become arrhythmic, and their SCN neurons are desynchronized (Ohta *et al.* 2005). In addition, mice in LL for only one day show a big shift in behavioral rhythms by 15 hours, which may facilitate to entrain to an altered entraining cycle (Chen *et al.* 2008). Therefore, we expect that illumination by a strong light before a travel would be an alternative way to alleviate jet lag.

Interestingly, 40 % of our *Vip-/-* mice became rhythmic at the end of LL protocol **(Fig. 5)**. We assumed that a secondary synchronizing agent may act to drive rhythmicity only in LL without VIP. Notably, it was shown that elevated K^+ concentration or gastrin releasing peptide (GRP) temporarily restored the PER2 gene expression rhythms from *Vipr2-/-* SCN explants (Maywood *et al.* 2006). Thus, it is intriguing to test whether potassium chloride (KCl) or GRP injection temporarily recovers the synchrony in *Vip-/-* or *Vipr2-/-* animals, and the synchrony among the SCN neurons is actually improved in

these rhythmic animals. Alternatively, external or internal noises such as routine cage changes or little fluctuations in light intensity or gene expression might have induced spontaneous behavioral rhythms in *Vip-/-* animals at the end of LL. One simulation predicts the emergence of synchrony from arrhythmic population with noise in light illuminations(Ullner *et al.* 2009). Also, noise in gene expression is suggested to drive the irregular circadian rhythms of PER2 expression in *Bmal1-/-* SCN explants, which is thought be arrhythmic (Ko *et al.* 2010). Like the simulation showing the role of the noise in *Bmal-/-* SCN, simulations reconstructing the emergence of rhythmicity in behavior of *Vip-/-* animals would provide valuable information to the circadian researchers.

Taken together, we revealed that VIP addition leads to faster entrainment via reduction in synchrony among SCN population and in theamplitude of ensemble gene expression in the SCN. Future works will focus on categorizing the SCN cells depending on their response to VIP to understand the VIP actions in detail. We conclude that VIP facilitates the entrainment by reducing synchrony among the circadian oscillators in the SCN, and it can be used as a potential therapy for jet lag and day night shift works.

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Chapter 4.

Spatiotemporal distribution of VIP receptor, VPAC2R in the SCN

This chapter includes the manuscript:

An S., Tsai C., and Herzog E.D. Spatiotemporal distribution of VIP receptor, VPAC2 in the SCN. In preparation.

Abstract

The suprachiasmatic nucleus (SCN) in the hypothalamus is a master circadian pacemaker in mammals. The SCN neurons must synchronize each other and to the ambient entraining cues to coordinate daily rhythms in physiology and behavior. Vasoactive intestinal polypeptide (VIP) and its major receptor in the SCN, vasoactive intestinal polypeptide receptor type 2 (VPAC2R) play a role in maintaining circadian synchrony as coupling factors. It is unknown where and when VPAC2R expression exists in the SCN. It has been predicted based on mRNA and transgene expression that VPAC2R may be expressed rhythmically and in a subset of SCN cells. Here, we examined VPAC2R abundance using a specific antibody. VPAC2R was expressed broadly in the brain and most highly in the SCN with stronger expression in dorsomedial area of the rostral and medial SCN. VPAC2R immunoreactivity heavily overlapped with AVP and VIP cells in the SCN and was mainly observed along dendrites and cell bodies, but not along axons. This regional localization and signal intensity did not change with time of day in a12 h/12 h light/ dark or in constant darkness. These results suggest that VPAC2R mediates circadian synchrony throughout the day via broad, constitutive expression in the SCN.

Introduction

The suprachiasmatic nucleus (SCN) acts as a master circadian pacemaker generating various physiological rhythms in mammals. Though individual SCN neurons harbor cell-autonomous feedback machinery, they depend on neuropeptides to reliably express the clock genes (Yamaguchi *et al.* 2003; Aton *et al.* 2005; Maywood *et al.* 2006). Neuropeptides and their efferent projections play a major role in synchrony among SCN neurons, conveying photic information and coordination of SCN with the rest of the body (Vosko *et al.* 2007).

Previous research suggests the roles of VIP-VPAC2R in circadian synchrony by conveying timing information. VIP is released rhythmically in constant conditions, and its release is stimulated by light (Shinohara *et al.* 1993; Shinohara *et al.* 1995). Animals lacking VIP (*Vip-/-*) or VPAC2R (*Vipr2-/-*) have defects in rhythm generation. They show fragmented locomotor activity in constant conditions (Harmar *et al.* 2002; Colwell *et al.* 2003; Aton *et al.* 2005) and arrhythmic body temperature, hormonal release, and heart rate (Loh *et al.* 2008; Schroeder *et al.* 2011). In the SCN, neurons are arrhythmic or lost synchrony among them in firing rate and gene expression rhythms (Aton *et al.* 2005; Maywood *et al.* 2006). These results suggest that the VIP-induced rhythm generation of and synchrony among the SCN neurons is critical to generation of rhythmic behaviors. Loss of VIP signaling also impairs the delivery of light information in the SCN. *Vip-/-* or *Vipr2-/-* mice have aberrant responses to light including the smaller phase shift and altered phase relation with light (Harmar *et al.* 2002; Colwell *et al.* 2003). *Vipr2-/-* mice fail to induce gene expressions following light stimulus, for instance, *Period (Per)*, c-

FOS, and phosphorylated extracellular signal-regulated kinases 1/2 (Harmar *et al.* 2002; Hughes *et al.* 2004). Importantly, VIP entrains *in vitro* and phase shifts the PER2 gene expression and firing rate rhythms (Reed *et al.* 2001; An *et al.* 2011). Therefore, VIP may mediate circadian communication and synchrony by the rhythmic release into the SCN, ability to entrain the SCN, and requirement for the rhythmic behaviors. It is not known how and when VIP acts to synchronize SCN neurons.

Vasoactive intestinal polypeptide (VIP), a 28-amino acid neuropeptide is a candidate synchronizing factor. In the SCN, cells in the ventrolateral area generate VIP and project mainly to the dorsomedial SCN, where arginine vasopressin (AVP) cells are located. The wide distribution of VIP projection in the SCN suggests their direct communication with all SCN cells. VIP binds to secretin receptor family (class B) of G-protein coupled receptor (GPCR), which includes vasoactive intestinal polypeptide 1 (VPAC1R), vasoactive intestinal polypeptide 2 (VPAC2R), and pituitary adenylate cyclase-activating polypeptide type 1 receptor (PAC1R). mRNA signal of all three receptors are widely expressed throughout the brains (Usdin *et al.* 1994). In the SCN, however, a strong VPAC2R mRNA signal, moderate PAC1R mRNA signal, and no VPAC1R signal is present (Usdin *et al.* 1994; Cagampang *et al.* 1998c; Kalamatianos *et al.* 2004). Biochemical studies reveal that PAC1R preferentially binds to pituitary adenylate cyclase-activating polypeptide to VIP (Vosko *et al.* 2007). Therefore, VPAC2R appears to mainly mediate VIP signaling in the SCN.

VIP peptide and VPAC2R mRNA expression exhibit a juxtaposed pattern in the SCN. VIP-ergic neurons are mainly located in ventrolateral area, which receives photic inputs directly from intrinsically photosensitive retinal ganglion cells (Kalamatianos *et al.* 2004; Hattar *et al.* 2006). VIP-ergic neurons project their dense fibers throughout the SCN, especially the dorsomedial area, where vasopressin-expressing (AVP-ergic) neurons are located (Abrahamson & Moore 2001). mRNA profile of VPAC2R (Kalamatianos *et al.* 2004), binding assay with a VPAC2R-specific agonist (Harmar *et al.* 2002) and exogenous expression of β-gal driven by human VPAC2R promoter (Kallo *et al.* 2004) implicate its main expression in the dorsomedial SCN. The β-gal expression overlaps with ~50 % AVP-ergic cells and ~30 % VIP-ergic cells (Kallo *et al.* 2004). Though the mRNA and transgene results raise interesting insights about VPAC2R, they have not identified critical information yet. For instance, it is not known whether VIP acts as a paracrine signal by crossing synapses or an endocrine signal to travel large distances.

Since VIP signaling generates rhythmic behaviors, it is intriguing whether VPAC2R oscillates in the SCN in a circadian day. Previous results of the temporal *Vipr2* expression show, at least, diurnal oscillations, but are not consistent each other. In light-dark (LD) cycle, VPAC2R mRNA peaks at the late subjective night only (Shinohara *et al.* 1999) or at the late subjective day and night (Cagampang *et al.* 1998c). In constant darkness (DD), the expression may show no rhythm (Shinohara *et al.* 1999) or peaks at late subjective day and night (Cagampang *et al.* 1998a). Although we assume that VPAC2R is rhythmically expressed in the SCN following the mRNA profile, the temporal profile of VPAC2R can be different from that of *Vipr2*. For instance, the PER2

gene expression rhythm is about 6 hour delayed than its mRNA rhythm (Field *et al.* 2000). A recent VIP PRC of PER2 expression rhythm exhibits that the SCN responds to VIP differently at different time of the day, suggesting the circadian regulation of amount, or, at least, properties of VPAC2R such as intracellular localization, affinity to the VIP.

Here, we characterized expression profile of VPAC2R in the SCN with a specific antibody (Schulz *et al.* 2004). We found moderate to weak VPAC2R expression in various brain areas and strong expression in the SCN. We found VPAC2R expression from rostral to caudal axis with more intensive expression in dorsomedial area. Consistent to this finding, an intense overlap of VPAC2R expression with AVP-ergic cells and VIP-immunoreactive cells or projections was observed. Intracellular expression of VPAC2R was mainly along cell bodies and dendrites not along axons. We found that VPAC2R expression does not oscillate in circadian or diurnal manner. We conclude that VPAC2R presents broadly in the SCN throughout a day to mediate circadian synchrony in the SCN.

Materials and Methods

ANIMALS. Male C57BL/6 mice, VPAC2R knockout mice (*Vipr2-/-*), PERIOD2::LUCIFERASE knockin (PER2::LUC) mice were housed individually in 12 h/12 h light/ dark cycle. For the experiments in DD, animals were transferred to light-tight, ventilated chamber for 2 days. All procedures were approved by the Animal Care and Use Committee at Washington University and conformed to National Institutes of Health (NIH) guidelines.

TISSUE PREPARATION. For the circadian measurement, 40 Animals were housed in 12 h/12 h LD or DD before perfusion at 6 different zeitgeber time (ZT) or circadian time (CT) respectively. After anesthesia with 2.5 % avertin, animals were perfused intracardially with 0.9 % saline followed by 4 % paraformaldehyde (PFA) solution. During the period without a light (ZT12-24 and CT), anesthesia and perfusion were done in the dim red light. Brains were kept in the PFA solution for 24 hours, then transferred to 30 % sucrose phosphate buffered (PBS) solution for 3 days until the brains sunk. Brains were quickly frozen with 2-methylbutane maintained at -35 °C and stored at -80 °C for further usage. 6 series of coronal sections were obtained with a cryostat (CM1850; Leica, Maryland Heights, MO) and stored in Watson's cryoprotectant solution, pH 7.2, at -20 °C at least for 48 hrs before proceeding immunohistochemistry. For the analysis of intracellular localization, dissociated SCN neuronal cultures were used. SCN punches were taken from coronal hypothalamic slices of C57BL/6 pups (p1-5) and cells were digested with papain at 37 °C for 40 minutes. After brief centrifugation, papain solution was replaced with media and cells were triturated by gentle pipetting. Cells were plated on a poly-D-lysine (Sigma, Saint Louis, MO) and laminin (Sigma, Saint Louis, MO)-covered glass coverslip with a density of 3,000 cells / mm^2. After a week from the surgery, cells were fixed with chilled 4 % PFA solution for 15 minutes.

IMMUNOSTAINING.

1. Co-staining of VPAC2R with VIP/ AVP or axonal/dendritic marker

Series of free-floating sections or cell cultures were rinsed with PBS three times followed by incubation with 3 % Triton X for 30 minutes at 37 °C. After wash with PBS, samples

were incubated with blocking solution (10 % non-fat milk, 10 % BSA, 0.015 % Tween-20) for 1 h at room temperature followed by addition of 1: 1,000 rabbit anti-VPAC2 antibody (AbCam). Then, samples were incubated with 1:200 donkey anti-rabbit Cy2 for 2 h at room temperature. For double-staining with VPAC2R antibody, samples were co-incubated with 1:50 mouse anti-AVP antibody (a gift from Dr. H. Gainer) or 1: 2,000 chicken anti-MAP2 antibody (dendritic marker, AbCam) or 1: 2,000 mouse anti-Tau1 antibody (axonal marker, Millipore) overnight at 4 °C. In case of a double-staining using antibodies raised from the same species, two separate immunostainings were performed to prevent cross talk. Samples were sequentially incubated with VPAC2R antibody, 1:200 donkey anti-rabbit Cy2, and then incubated with 1: 2,000 rabbit anti-VIP antibody (Immunostar), 1:200 goat anti-rabbit Cy3. At the end of the staining, every sample was incubation with 4',6-diamidino-2-phenylindole (DAPI) for 10 minutes to distinguish the boundary of the SCN. Sections were mounted on slides (12-550-14; Fisher Scientific), dried overnight, and coverslipped with Permount (SP15-500; Fisher Scientific). In each experiment, SCN-containing sections from VPAC2 knockout mice and sections without the incubation of primary antibody were used as negative controls.

2. Staining for VPAC2R as a function of time of day

After permeablization with Triton X and washes, series of sections collected at different ZTs or CTs were incubated with 10% H_2O_2 in PBS at 4 °C for 30 minutes. Sections were washed with PBS, blocked for 1 h and incubated with 1:15,000 anti-VPAC2R antibody at 4 °C for 2 days with agitation. Following 3 time wash in PBS, sections were incubated with 1:200 biotinylated goat anti-rabbit antibody (Standard ELITE ABC kit, Vectastatin,

Vector labs, Burlingame, CA) at 4 °C for 1 h. Then, sections were incubated with an avidin-biotin complex (Vector Labs). Sections were rinsed 3 times with PBS, rinsed with 50 mM Tris-HCl, and then incubated for 10 minutes with 0.05 % 3,3'-diaminobenzidine, 0.01 % H_2O_2, 0.03 % $NiCl_2$ in 50 mM Tris-HCl. Sections were rinsed with PBS overnight, and wet mounted onto glass Superfrost slides (Fisher) the next day. The mounted sections were dehydrated in series of ethanol and xylene solutions.

IMAGE PROCESSING. Digitized images were taken using an epifluorescent microscope (Retiga 1350EX; QImaging, Burnaby, British Columbia, Canada), or a confocal microscope (Nikon A1, Imaging core, Department of Biology), or slide scanner (Nanozoomer Digital Pathology, Hamamatsu, Alafi NeuroImaging Laboratory, Department of Neurology) under the fluorescent or bright light illumination. For brightness and contrast, the images were processed with ImageJ (NIH, Bethesda, MD). To quantify the circadian intensity of VPAC2R expression, pixel intensity of VPAC2R immunoreactivity was averaged in the bilateral SCN area after being subtracted from the background (brain areas outside of the SCN).

DATA ANALYSIS. Day and night difference of VPAC2R expression was assessed by one-way ANOVA with a Schéffe post hoc (Origin 7.0, OriginLab, Northampton).

Results

Specificity of the VPAC2R antibody

We tested the specificity of three antibodies against VPAC2R: rabbit anti-VPAC2R (antibody generated using C-terminus of VPAC2R, AbCam), goat anti-VPAC2R (SantaCruz), mouse anti-VPAC2R (antibody generated using N-terminus of VPAC2R, AbCam) by a comparison between wild type and *Vipr2-/-* brain sections. The rabbit anti-VPAC2R showed an intense staining on the wild type SCN but not on the *Vipr2-/-* SCN above the background staining **(Fig. 1)**. The other two antibodies had almost equal intensity of staining in both wild type and *Vipr2-/-* SCN (data not shown). We concluded that only the rabbit anti-VPAC2R specifically recognizes the VPAC2R among them and used it for further study.

VPAC2R levels were highly abundant in the SCN.

We found a strong VPAC2R expression in the SCN along the rostral to caudal axis **(Fig. 2)**. In middle to rostral sections, VPAC2R immunoreactivity was higher in dorsomedial area. We also detected moderate to weak VPAC2 expression in various brain areas including hippocampus, indusium gresium, olfactory bulb, and arcuate nucleus **(Fig. 1)** of hypothalamus, bed nucleus of the stria terminalis (BNST) in the amygdala, ventrolateral thalamus, and cerebellum. Thus, VPAC2R was expression throughout the brain with the highest intensity in the SCN.

VPAC2R was detected in VIP and AVP neurons.

We examined if VPAC2R expression overlaps with the expression of two neuropeptides, VIP and AVP. Sections stained with VPAC2R and AVP or VIP showed a clear overlap with these two neuropeptides **(Fig. 3)**. Notably, VPAC2R expression overlapped strongly

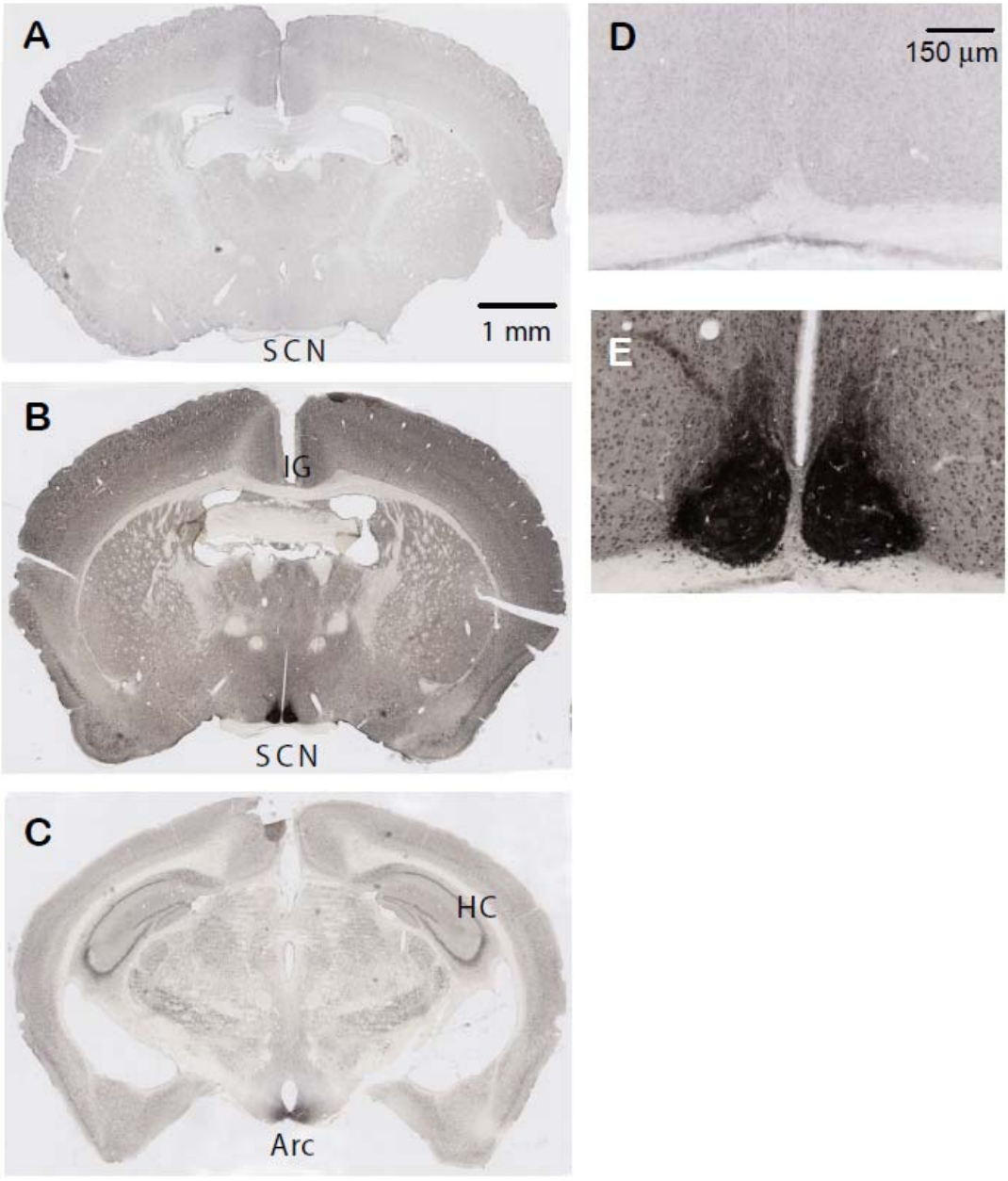
A
1 mm
SCN
B
IG
SCN
C
HC
Arc
D
150 µm
E

Figure 1. VPAC2R expression existed in various brain areas with the strongest expression in the SCN. (A and **D)** *Vipr2-/-* brain section shows little or no VPAC2R immunoreactivity when it is stained it with the rabbit anti-VPAC2R antibody. **(B, C,** and **E)** Brain sections of wild type mice exhibit a modest to strong signal of VPAC2R expression. Note that the intense staining in the SCN **(B, E)**, moderate to weak staining in the cortex **(B, C)**, indisium griseum **(B)**, hippocampus **(C)**, and arcuate nucleus **(C)**. All the sections were illuminated in the same intensity using a bright field microscope and the slide scanner.

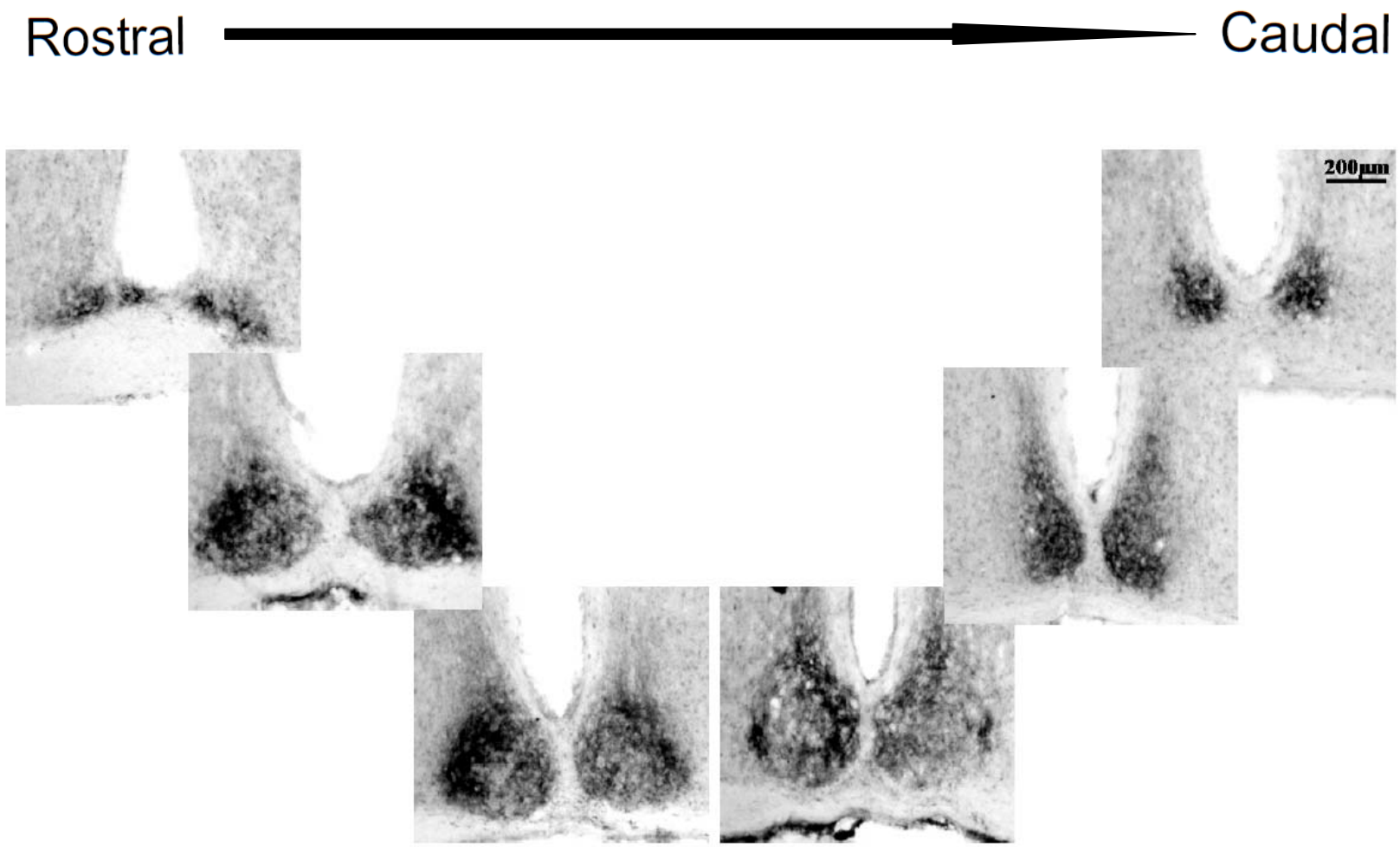
Rostral
Caudal
200μm

Figure 2. VPAC2R expression broadly existed in the SCN. VPAC2R immunoreactivity was present throughout the rostral to caudal axis of the SCN. Note that stronger dorsomedial expression of VPAC2R than ventrolateral one in the medial SCN (the second to the forth images from the left).

with AVP cell bodies and VIP projections in the dorsomedial area. On the other hand, the overlap between VPAC2R expression and VIP cell bodies in the ventrolateral area was relatively weak. We also detected VPAC2R labeling in other areas that did not express VIP or AVP in the SCN slice and in most of cells in dispersed SCN neuronal cultures. Therefore, we concluded that VPAC2R is expressed in most, if not all, SCN cells.

VPAC2R was expressed primarily in dendrites and cell bodies.

To identify the role of VPAC2R in synaptic transmission, we examined the intracellular localization of VPAC2R by staining dispersed SCN cultures in low density with VPAC2 antibody and either dendritic (MAP2) or axonal (Tau-1) antibody. VPAC2R mainly overlapped with the dendritic marker on somata and dendrites of neurons, while it barely overlapped with the axonal marker **(Fig. 4)**. We confirmed a strong VPAC2R expression in soma and dendrites, which receive synaptic transmission.

VPAC2R was detected throughout the day.

To examine whether VPAC2R expression is rhythmic, we collected the 2-3 brains from mice housed in 12 h/12 h light/dark cycle (LD) or in constant darkness (DD) at 6 different time points over a circadian day and measured the SCN VPAC2R expression level. The average pixel intensity of the VPAC2R staining in medial, bilateral SCN was determined after subtracting the staining intensity of the brain area outside of the SCN. The VPAC2R expression was not significantly different at day and night in LD (day: ZT1-10, night: ZT12-22; $p > 0.05$, $F_{1,15} = 0.64$, One-way ANOVA, Schéffe post hoc)

and DD (day: CT1-11, night: CT12-22; $p > 0.05$, $F_{1,16} = 0.22$, One-way ANOVA, Schéffe post hoc; **Fig. 5**). We conclude that the SCN expresses VPAC2R constitutively.

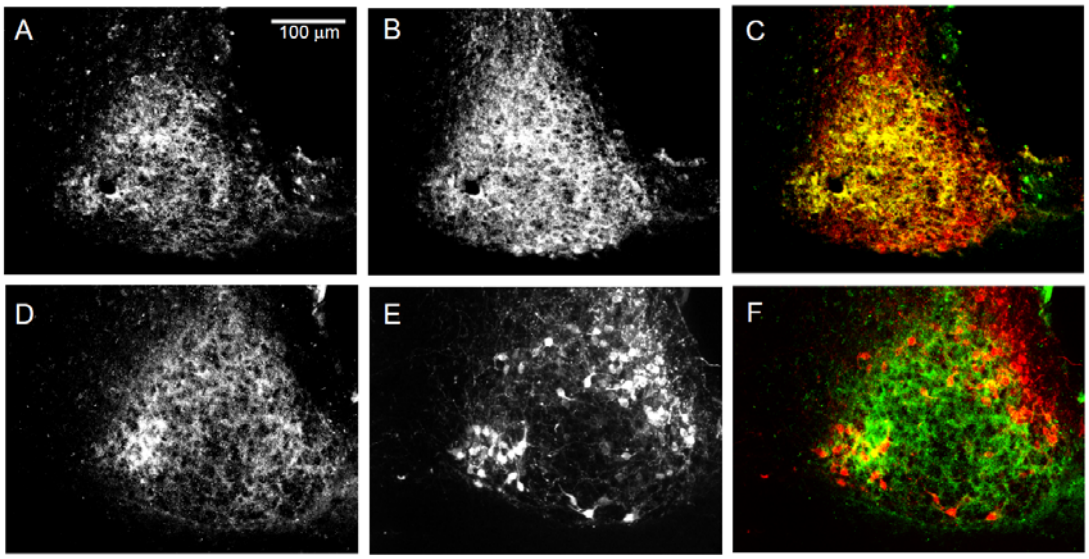

Figure 3. VPAC2R colocalized with VIP and AVP in the SCN. Repesentative images of SCN stained for VPAC2R **(A-D)**, and VIP (B) or AVP **(E)**. The composite images show the extensive overlap (yellow) between VPAC2R (green) and VIP (red), and AVP (red) expression (**C** and **F**).

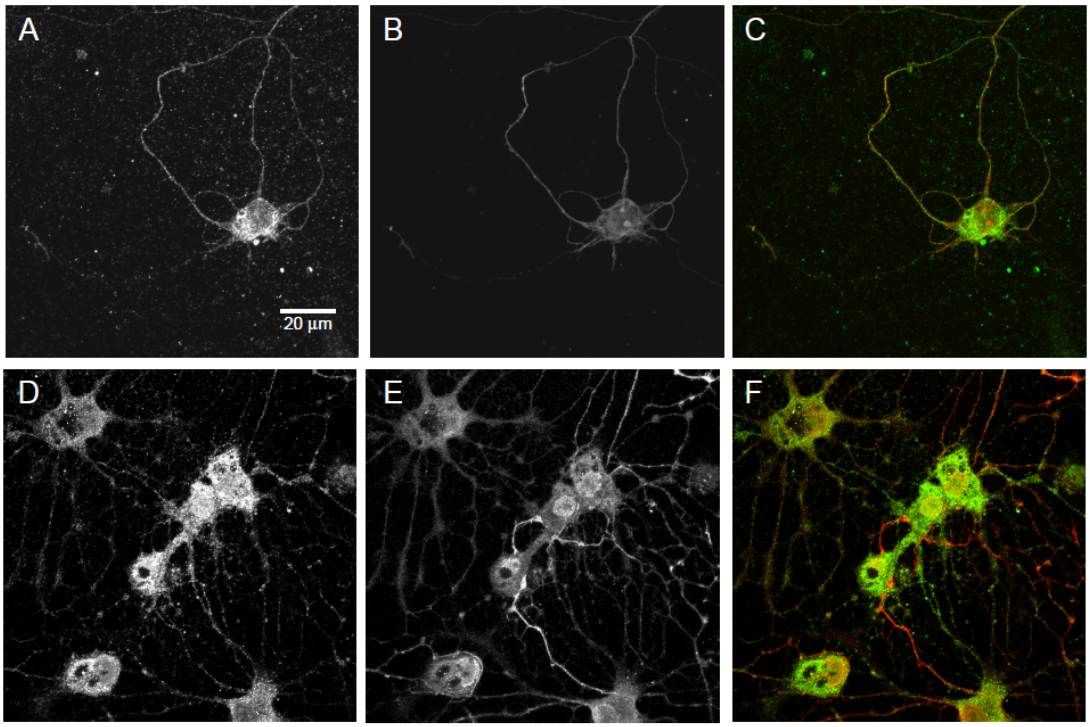

Figure 4. VPAC2R colocalized with dendritic markers but not with axonal markers in the SCN. Representative images of dissociated SCN neurons stained for VPAC2R (A and D) and MAP2, a dendritic marker protein **(B)**, or Tau-1, an axonal marker protein **(E)**. Note that VPAC2R (green) staining colocalizes with MAP2 (red) along cell body and projections (**C**) but does not overlap with Tau-1 (red) staining **(F)**.

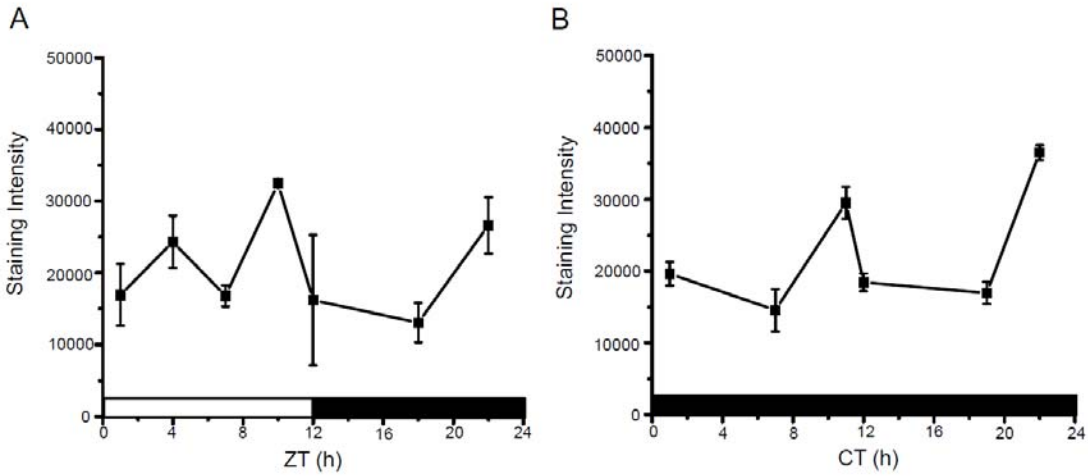

Figure 5. VPAC2R expression did not vary temporarily in either (A) 12h/ 12h LD or (B) constant darkness. The average intensity of VPAC2R expression in bilateral SCN was measured in medial SCN. Data points represent the mean ± SEM of 3 brains per each time point. The result suggests that VPAC2R expression be not fluctuating between day and night in LD ($p > 0.05$, $F_{1,15} = 0.64$, One way ANOVA, Scheffé post hoc) or in DD ($p > 0.05$, $F_{1,16} = 0.22$, One way ANOVA, Scheffé post hoc).

Discussion

VPAC2R delivers VIP-mediated signaling in various mouse brain areas.

Using a specific antibody against VPAC2R, we found weak to moderate staining throughout the brain, and intensive staining in the SCN in wild type mice. We confirmed the specificity with no staining of VPAC2R in the *Vipr2-/-* mice brains. The strongest signal of the SCN is consistent to the previous results of intense *Vipr2* mRNA throughout the SCN (Usdin *et al.* 1994; Kalamatianos *et al.* 2004) and transgene expression of VPAC2R (Kallo et al. 2004). Notably, the VPAC2R expression in the SCN heavily overlaps with the immunoreactivity of VIP neurons and their projections.

In addition to the SCN, we also found the proximity of VPAC2R expression to the areas of VIP expression. For instance, VIP cell bodies have been found in the retina, olfactory bulb, and cerebral cortex (Staun-Olsen *et al.* 1985; Gall *et al.* 1986; Okamoto *et al.* 1992). VIP projections have been observed in the lateral geniculate nucleus, hippocampus, amygdala, and throughout the medial hypothalamus to paraventricular nucleus of the thalamus (Abrahamson & Moore 2001; Heintz 2004). We confirmed the VPAC2R expression in or near some of these areas. Also, we found VPAC2R expression in the indusium griseum, hippocampus, midbrain, and cerebellum. The VIP application to these areas is shown to have effects including the modulation of firing rates (Yang *et al.* 2009), learning and memory (Gozes *et al.* 1993; Yamaguchi & Kobayashi 1994), and neurotrophic action (Cavallaro *et al.* 1996), supporting our findings. We observed a fairly intense expression in the arcuate nucleus, which confirms the role of VIP in neuroendocrine and lactation (Harney *et al.* 1996; Smith *et al.* 2000; Gerhold *et al.* 2001).

VPAC2R expression outside the SCN suggests the VIP-VPAC2R signaling in non-circadian functions or the influence of VIP projections from these areas.

Notably, some brain areas with high VIP-immunoreactivity expressed VPAC2R only weakly. For instance, we saw low expression of VPAC2R in the BNST in spite of its strong VIP immunoreactivity. This result indicates that some of the VIP signaling outside of the SCN is received by other receptors including VPAC1R. Also, VPAC2R immunoreactivity of the hypothalamus or thalamus was not as strong as the *Vipr2* mRNA signal (Usdin *et al.* 1994; Kalamatianos *et al.* 2004), which suggests post-translational modification of VPAC2R expression. We did not observe any brain areas showing low VIP and high VPAC2R staining, indicating the regionally restricted expression of VPAC2R compared with VIP expression. These data suggest that VIP may use any individual receptor or combination of different ones in the VIP/PACAP receptor family outside of the SCN.

VPAC2R generates coupling among SCN cells.

We found VPAC2R expression in all SCN cells, including VIP-ergic and AVP-ergic neurons. Since both VIP and AVP genes have CRE (Hahm *et al.* 1999; Kim *et al.* 2001), and VIP signaling increases intracellular cAMP (Rea 1990; An *et al.* 2011), VPAC2R-mediated cAMP increase would facilitate the upregulation of both neuropeptides. VPAC2R expression and VIP projections on AVP cells suggest the dorsomedial SCN receives inputs from the ventrolateral SCN through VIP-VPAC2R signaling. Notably, the dorsomedial SCN loses synchrony when it is separated from the ventrolateral SCN

(Yamaguchi *et al.* 2003; Buhr *et al.* 2010). Therefore, VPAC2R may mediate coupling among and autoregulation of the SCN neurons.

VPAC2R is similar to *Drosophila* PDFR.

These results share similarities with the studies of a synchronizing neuropeptide, pigment dispersing factor and its receptor, Though VIP and PDF do not have a sequence homology in their genes, they function similarly. Both neuropeptides are released to generate robust rhythms and synchronize clock cells. PDF- or PDFR-null flies are arrhythmic in constant conditions with desynchronized clock neurons (Renn *et al.* 1999; Lin *et al.* 2004), which parallels in *Vip-/-* or *Vipr2-/-* animals (Harmar *et al.* 2002; Colwell *et al.* 2003; Aton *et al.* 2005; Maywood *et al.* 2006). Both VPAC2R and PDFR belong to the class B GPCR family, increasing intracellular cAMP level with the binding to its ligand (Shafer *et al.* 2008; An *et al.* 2011). Like the VPAC2R expression in all SCN neurons, PDFR is expressed in most of clock neurons including PDF-positive neurons in the *Drosophila* brain (Shafer *et al.* 2008; Im & Taghert 2010). We conclude that both VIP and PDF contribute the robustness of the oscillator by regulating and autoregulating the output of clock cells.

VPAC2R integrates circadian timing inputs.

VPAC2R staining is present mainly along the cell body and dendrites. Therefore, a VPAC2R-expressing cell would integrate inputs from multiple VIP cells and determine the dominant circadian rhythms. As a class B of GPCR, VPAC2R activates adenylate cyclase, protein kinase A, or phospholipase C and D, and in turn, increases cAMP and

Ca^{2+} (Nielsen *et al.* 2002; Meyer-Spasche & Piggins 2004; Langer & Robberecht 2007; An *et al.* 2011). These second messenger molecules would upregulate the activity of various transcription factors (Ding *et al.* 1997), leading to the modulation of circadian firing rates (Reed *et al.* 2002; Aton & Herzog 2005), inhibitory synaptic transmission (Itri & Colwell 2003; Itri *et al.* 2004), and gene expression in the SCN (An *et al.* 2011). Interestingly, *Vipr2-/-* animals lose circadian gene expression rhythms of c-FOS and p-ERK in the SCN. Based on these data, we conclude that VPAC2R functions to integrate circadian timing information from VIP-ergic neurons for cyclic gene expression and firing rates.

VPAC2R is expressed throughout the circadian day.

VIP entrains PER2 expression rhythm in SCN explant cultures with a stable phase relation as predicted from the phase response curve (An *et al.* 2011). This raises the possibility that cyclic VPAC2R abundance induces the differential response to VIP over the circadian day. Diurnal variation of VPAC2R mRNA levels also supports this possibility (Cagampang *et al.* 1998c; Shinohara *et al.* 1999). In contrast to the previous results, we found that VPAC2R immunoreactivity does not oscillate in the SCN. This result may be due to the post-translational modification of VPAC2R or too low amplitude of VPAC2R rhythms. Alternatively, the receptor affinity to VIP or receptor regulation such as phosphorylation could be rhythmic. Perhaps, oscillations in VPAC2R may not need to be rhythmic. Rather, the rhythms in release of a synchronizing factor or expression of downstream molecules may be sufficient to regulate the SCN functions.

The constitutive expression of VPAC2R is somewhat consistent to our VIP PRC since VIP shift PER2 gene expression rhythms throughout the day. The continuous expression of VPAC2R would be beneficial based on our recent data, showing the constitutive receptivity to VIP helps the SCN to recover from jet lag by VIP administration at any time point. Taken together, we conclude that constitutive VPAC2R expression in all SCN neurons mediates circadian synchrony among the SCN neurons.

Future directions

We found that various cell types in the SCN express VPAC2R throughout the circadian day. Since our immunolabeling data disagreed with the mRNA data, we will find possible mechanisms, which regulate properties of VPAC2R rhythmically. For instance, we will measure VIP-induced VPAC2R internalization of dissociated SCN neurons plated in a low density. Since surface VPAC2R levels may be regulated by the phosphorylation (Langer & Robberecht 2007), we can measure the phosphorylation of VPAC2R using Western blots at different time of the day, instead. Rhythmic variation in internalization or phosphorylation of VPAC2R would indicate the circadian positioning of VPAC2R to the membrane, which may be one of the underlying mechanisms of the time-dependent phase shifts by VIP.

In addition, we found the strong VPAC2R staining in the dorsomedial SCN, and the relatively weak staining in ventrolateral SCN. In the medial SCN, it was notable that the moderate staining at the center was surrounded by a circle of strong VPAC2R immunostaining. Interestingly, neurons in the center of the SCN exclusively express a

neuropeptide, calbindin in hamster (Antle & Silver 2005), and it is possible that calbindin-expressing neurons have weak VPAC2R expression. Also, we have not quantified how much AVP-ergic and VIP-ergic neurons contribute to the VPAC2R expressing populations. Since different neuropeptides have various functions inside and of the output of the SCN (Antle & Silver 2005), it is worthwhile to characterize the VPAC2R expression in different neuropeptidergic cells. We will count the cells expressing different neuropeptides including AVP and VIP, besides, monitoring their VPAC2R expression in dispersed SCN cultures.

Finally, we will identify the light-mediated regulations of VPAC2R. Since VIP release is induced by the light in the SCN (Shinohara *et al.* 1993), VPAC2R expression may be also regulated by light. Recently, it was shown that constant light causes arrhythmic behavior and desynchrony among the SCN (Ohta *et al.* 2005), and now we have evidence that VIP modulates these events. These results suggest that VIP or VPAC2R may be depleted in constant light, phenocopying *Vip-/-* or *Vipr2-/-* mice. To test this possibility, we will house mice in light tight chamber, collect their brains after two different light protocols, a light pulse in constant darkness or the constant light, and measure their VIP- or VPAC2R immunoreactivity in the SCN.

In summary, we screened several antibodies against VPAC2R, and discovered an antibody specific to VPAC2R. Due to the lack of a specific antibody, the anatomical characterization of VPAC2R has not been extensively studied. Using the antibody, we

identified spatiotemporal expression of VPAC2R in SCN tissues and individual neurons, and hope to examine the three possibilities near future.

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