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Synchronization and phase-resetting by glutamate of an immortalized SCN cell line

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Abstract

SCN 2.2 cultures were stably transfected with luciferase reporter constructs driven by Ca^{2+}/cAMP response element, E-box, or vasoactive intestinal peptide promoter to probe the circadian properties of this clock cell line. SCN 2.2 reporter lines displayed ~24-h rhythms of transcriptional activation after serum-shock. Serum-shocked cultures pulsed with glutamate exhibited phase-gated induction of phospho-CREB and of VIP, CRE, and E-box promoter activity. Glutamate-induced CRE promoter activity displayed restricted sensitivity to inhibitors of nitric oxide synthase and cGMP-dependent protein kinase. The temporal pattern of these sensitivities paralleled those of the SCN to light and glutamate during the night. Taken together, our data indicate that serum-shock can synchronize the circadian clock of SCN 2.2 cells to a state consistent with the day/night transition and, thus, establishes a temporal context for this cell line.

Keywords: cAMP; Circadian; Clock; CRE; CREB; E-box; Light; NOS; PKG; Suprachiasmatic nucleus; VIP

The complexity of interacting neuronal networks and cellular signaling pathways that regulate circadian rhythms has caused researchers to seek cell culture systems to complement analysis of the hypothalamic suprachiasmatic nucleus (SCN). This central mammalian pacemaker generates a near 24-h time base of circadian rhythms in behavior, physiology, and metabolism. The SCN is a nexus for the integration of external and internal signals that synchronize circadian rhythms with changing environmental and homeostatic conditions [1–3]. In addition to controlling organismic circadian rhythms, SCN outputs drive tissue-specific circadian oscillations within peripheral tissues throughout the organism [4,5]. Since circadian oscillations are properties of single SCN cells [6,7], various cell types have been utilized as clock models [2,8]. Whether a particular cell type exhibits central circadian clock or peripheral oscillator characteristics complicates the selection of an appropriate model system.

Satisfactory cellular models of the central pacemaker must express fundamental characteristics that distinguish the SCN from peripheral oscillators. These characteristics include: (1) spontaneous and predictable circadian rhythms in the absence of external stimuli, (2) temporally gated sensitivities to phase shifting stimuli, and (3) the ability to drive circadian oscillations in other cell types. Unlike the SCN clock, other brain regions and peripheral tissues generally require external signals to induce and maintain sustained rhythmic oscillations. These peripheral rhythms may be out-of-phase with those of the SCN [5]. Additionally, these slave oscillators exhibit tissue-specific phase relationships among oscillating elements that differ from the rhythmic expression patterns of the SCN [2,8–10]. Lesioning or surgically isolating the SCN abolishes circadian oscillations in other brain regions and body sites, emphasizing central pacemaker role of the SCN and the driven nature of peripheral tissues [4,11].

The SCN clock encompasses a dynamic system of regulatory mechanisms that respond differentially to signals that adjust circadian time. During the clock phase coinciding with environmental night, the SCN

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exhibits phase-resetting sensitivity to a number of stimuli, including light and its neurotransmitter, glutamate [12]. This sensitivity is gated and the SCN is unresponsive to these signals in the circadian day [13]. During the early portion of the night, light/glutamate induces delays in circadian rhythms. In contrast, during late night, light/glutamate advances the phase of circadian rhythms in the SCN.

The light/glutamate signal induces transcriptional activation in the SCN that is also gated by the circadian clock. Well-characterized transcriptional responses include increased levels of immediate early genes, such as *c-fos* [14–16], activating protein-1 complex (AP-1) [17,18] and Ca^{2+}/cAMP response element (CRE) [19–22] promoter sequences. Activation at CRE sites requires phosphorylation of ser-133, and possibly ser-142, of the CRE binding protein (CREB/PCREB), an event that is induced by light/glutamate during the night. Neither light nor glutamate induces PCREB nor activates CRE-mediated transcription in the subjective day of the SCN clock [19,20,22]. During nighttime, induction of CRE-dependent transcription by light parallels PCREB induction [22].

Early and late night induction of PCREB by light and glutamate appears to be dependent on the same initial signal transduction elements as in clock resetting: activation of *n*-methyl D-aspartate receptors (NMDA-R), Ca^{2+} influx, and activation of nitric oxide synthase (NOS) [23]. However, in early night the light/GLU pathway requires activation of ryanodine receptors that mediate Ca^{2+} release from intracellular stores [23]. In contrast, the late night light/glutamate phase-advance requires nitric oxide (NO)-mediated activation of guanylyl cyclase (GC) and subsequent stimulation of cGMP-dependent protein kinase (PKG). Both advances and delays are partially mediated by mitogen-activated kinase (MAPK) activation [24,25]. The causality of PCREB induction and CRE-dependent transcription in nighttime phase shifting of SCN circadian rhythms remains to be determined.

Antisense *mper1* oligodeoxyribonucleotides (ODNs) inhibit phase delays induced in the early night by light in the mouse and by glutamate in the SCN brain slice [26]. Therefore, *mper1* induction appears to be a required element of the early night phase advance. While the signaling pathways that induce *mper1* remain unclear, both the mouse and humans *mper1* promoters contain conserved segments that include five E-boxes, which are positively regulated by the CLOCK–BMAL1 complex, and four classic CREs, any of which may mediate light and glutamate signals [27–30].

In recent years, there have been numerous reports utilizing cell lines as models of circadian clocks [8,31,32]. Rhythmic expression of clock gene products has been demonstrated in several mammalian cell lines, including a variety of fibroblastic cell types as well as the E1A-immortalized embryonic SCN line (SCN 2.2). In most cell lines, robust stimulation (forskolin, 12-*O*-tetradecanoylphorbol 13 acetate, dexamethasone, serum, or vasoconstricting endothelin-1) induces only transient circadian expression patterns [8,31,32]. In contrast to other cell lines, the SCN 2.2 line is spontaneously rhythmic, expressing rhythmic 2-deoxyglucose, BDNF, and NT-3 secretion in vitro [33]. Derived from central circadian pacemaker tissue, SCN 2.2 cultures express elements of signaling pathways characteristic to the SCN [34]. Additionally, transplantation of these SCN 2.2 cells into an SCN-lesioned host restores circadian rhythmicity, whereas NIH/3T3 cells do not [33]. In a compelling recent report, SCN 2.2 cells were shown to drive circadian oscillations in co-cultured NIH/3T3 cells [35].

We investigated the SCN 2.2 cell line for expression of dynamic circadian pacemaker characteristics of the SCN. In pursuit of this goal, we stably transfected SCN 2.2 cells with luciferase reporter constructs driven by promoters known to operate under SCN clock regulation. We found that serum-shock predictably synchronized SCN 2.2 cells to express near 24-h rhythms of gene expression with relative phase characteristics similar to those demonstrated by the SCN. We employed our stable reporter lines to determine whether SCN 2.2 cultures exhibit circadian state-dependent, or gated, sensitivity to glutamate, the primary mediator of light-induced phase resetting in the SCN. The pattern of induction of reporter constructs and timing of sensitivities to inhibition of the light/glutamate signaling pathway indicate that serum-shock synchronizes SCN 2.2 cells to a specific state in the circadian cycle. This discovery is a requisite step towards fully establishing this cellular clock model for circadian studies.

Materials and methods

Cell culture. SCN 2.2 cultures (passage 12–18) and SCN 2.2 stable transfectants were plated at 30% confluence onto laminin-coated 12- or 24-well tissue culture plates (Gibco-BRL) and maintained in growth medium (GM: MEM, 10% FBS, 0.6% glucose, 2 mM L-glutamine, 2.5 μg/ml fungizone, and 100 μg/ml penicillin–streptomycin or 50 μg/ml gentamicin). Cultures were maintained in the dark in tissue culture incubators with sporadic light exposure during media changes.

Vectors. The stable transfection/luciferase reporter vector pGLH was prepared by inserting *Hpa*I/*Dra*I-cut p3'SS (Stratagene) containing the hygromycin resistance gene (hygR; 2752 bp band) into *Eco*47III-cut pGLb (Promega). The resulting luciferase reporter vector pGLH was 8349 bp. VIP reporter vector pGLHvipp was prepared by inserting 5.2 kb promoter sequence [36] of the human VIP gene (up to the *Hpa*II site of exon 1) (Fig. 1A) from *Bam*HI/*Bgl*II-cut ϕd2VIP5.2 (gift from J. Washeck) into *Bgl*II-cut pGLH. CRE reporter vector pGLHcre was prepared by hybridizing 5′-CTG ACG TCA TGA CGT CAT GAC GTC ATG ACG TCA TGA CGT CAT GAC GTC ATG ACG TCA TGA CGT CAC-3′ with 5′-TCG ACT GAC GTC ATG ACG TCA TGA CGT CAT GAC GTC ATG ACG TCA TGA CGT CAT GAC GTC ATG ACG TCA GAG CT-3′. The resulting double-stranded oligonucleotide contained eight CRE (Fig. 1B) sequences and was directly inserted into *Sst*I(*Sac*I)/*Xho*I-cut pGLH. E-box reporter vector pGLHebox was prepared by hybridizing 5′-CTT TAG CCA CGT GAC

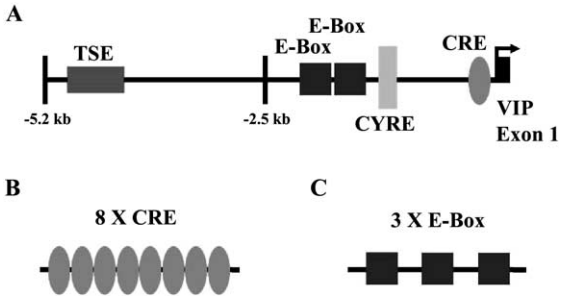

Fig. 1. Circadian reporter gene promoter elements. (A) The 5′-flanking region (5.2 kb) of the human VIP gene was inserted for SCN 2.2.*vip-luc* lines. TSE (tissue specifier element, red), E-box (blue), CRE (Ca^{2+}/cAMP response element, green), and CYRE (cytokine response element, yellow) [38]. (B) An artificial promoter with 8 adjacent CRE consensus sequences was used for SCN 2.2.*cre-luc* lines. (C) An artificial promoter containing three E-boxes and 6 bp flanking sequence (per E-box) from between exon 1A and exon 1B of the mouse *m per1* promoter was used for SCN 2.2.*ebox-luc* lines.

AGT GTA AGC ACA CGT GGG CCC TCA AGT CCA CGT GCA GGG AC-3′ and 5′-TCG AGT CCC TGC ACG TGG ACT TGA GGG CCC ACG TGT GCT TAC ACT GTC ACG TGG CTA AAG AGC T-3′. The resulting double-stranded oligonucleotide contained each of the three E-boxes and 6 bp flanking sequence per E-box, from between exons 1A and 1B of the mouse *per1* promoter (Fig. 1C) [27,28,30,37] and was directly inserted into *Sst*I(*Sac*I)/*Xho*I-cut pGLH. Primers were purchased from the Keck Biotech Center, University of Illinois.

Development of stable transfectants. Stably transfected SCN 2.2 cell lines expressing luciferase reporter activity were developed for each reporter construct. SCN 2.2.*vip-luc* stable transfectants were prepared by transfecting 30% confluent SCN 2.2 cultures on 60 mm dishes with pGLHvipp using lipofectamine reagent (Gibco-BRL). After 72 h, cultures were passaged and exposed to selective media (GM containing 100 μg/ml hygromycin) for approximately 15–30 days and individual clones were isolated using cloning cylinders. SCN 2.2.*cre-luc* and SCN 2.2.*ebox-luc* stable transfectants were prepared by transfecting 30% confluent SCN 2.2 cultures in 12-well tissue culture plates with pGLHcre or pGLHebox using DOSPER transfection reagent (Boehringer). After 72 h, cultures were passaged and exposed to selective media (GM containing 100 μg/ml hygromycin) for approximately 15–30 days while individual clones were isolated by serial dilution. Stably transfected clones were observed for growth characteristics in selective media, phenotype, and luciferase activity. Additionally, CRE-reporter lines were tested for luciferase induction after 6-h exposure to 10 μM 8-Br-cAMP.

Serum-shock synchronization protocol. SCN 2.2 cultures were grown to confluence on laminin-coated plates in GM. After 24–48 h at 100% confluence, culture media were replaced with serum-free neuronal medium (NM: 50% DMEM, 50% Ham's F12, N-2 supplement, 2.5 μg/ml fungizone, and 100 μg/ml penicillin–streptomycin or 50 μg/ml gentamicin) to promote differentiation. After 48–72 h in NM, SCN 2.2, and SCN stable transfectant cultures were exposed to a 2-h 50% fetal bovine serum (FBS) serum-shock in NM followed by a rinse with 50% DMEM/50% Ham's F12 media. Control cultures were treated the same in every way except that NM was used instead of the FBS pulse. Cultures were immediately returned to NM, then harvested at 4-h intervals for 72 h, and stored (−20 °C). Collection timepoints began immediately after serum-shock ($t = 0$ h). Due to the possibility of a phase-dependent response of SCN 2.2 cells to differing treatment, all cultures (within the same experiment) were split and exposed to media changes and serum-shock in parallel. This method differs from many commonly utilized protocols in which a portion of cultures are synchronized 12 h out-of-phase to allow convenient collection of samples. For additional consistency, splitting, media changes, and serum-shock were performed at the same time-of-day (±4 h) among separate experiments.

Glutamate treatment protocol. After serum-shock and release of cultures into NM, cultures were treated for 10 min with 10 μM glutamate or EBSS (control) at 3-h intervals for 24 h. For Western blot analysis, cultures were immediately collected by freezing. In experiments assessing luciferase induction, cultures were treated with glutamate (final concentration 10 μM) for 10 min followed by replacement of media with NM. Cultures were collected 6 h after each glutamate treatment to allow for luciferase mRNA/protein synthesis. To test for contribution of known downstream elements of the glutamate signaling pathway, L-NAME (final concentration 100 μM) or KT 5823 (final concentration 250 nM) were added to cultures 20 min prior to the glutamate pulse. Untreated control cultures were collected at 4-h intervals for 24 h after serum-shock to evaluate endogenous circadian oscillation of promoter activity in SCN 2.2.*vip-luc*, SCN2.2.*cre-luc*, and SCN 2.2.*ebox-luc* cell lines.

Western blot analysis. Cell extracts were prepared by adding boiling denaturing buffer (62.5 mM Tris–HCl pH 6.8, 0.5% SDS, 15% glycerol, 2.5% 2-mercaptoethanol, and 0.1% bromphenol blue) with inhibitor cocktail (1 mM EGTA, 5 mM EDTA, 2 mM sodium fluoride, 1 mM sodium orthovanadate, 10 mM glycerol phosphate, 200 μM sodium pyrophosphate, 5 μM mycocystin, 500 ng/ml leupeptin, 700 ng/ml pepstatin, 1 μg/ml aprotinin, and 40 ng/ml bestatin) onto frozen cell culture plates. Extracts were separated on 10% SDS–PAGE gels and transferred to nitrocellulose by standard protocols. Blots were probed with rabbit anti-PCREB (1:1000, UBI) primary antibody. This antibody was generated against a 14-amino acid sequence surrounding phospho-ser-133 of native CREB and shows cross-reactivity with PCREM. Immunoreactive bands were visualized using horseradish peroxidase-linked goat anti-rabbit secondary antibody (1:1000, Chemicon), SuperSignal Chemi-luminescent Substrate detection system (Bio-Rad), and Biomax MS film (Kodak). Protein loading was assessed by staining parallel gels using standard Coomassie blue protein staining protocols and these values were used to correct for loading variation.

Luciferase assays. Culture extracts were prepared by resuspending frozen SCN cultures by rocking (20 min at room temperature) with cell culture lysis buffer (CCLB, Promega) at approximately 25 μl CCLB/cm^2 cell culture surface area. Samples were centrifuged (2000*g* for 5 min) and supernatant was collected for luciferase or protein assay. Luciferase assays were performed using Luciferase Assay System (Promega) and measured on an MLX microtiter plate luminometer (Dynex Technologies) by luc-50 or luc-100 assay. Protein concentration of samples was determined by BCA Protein Assay Kit (Pierce) against bovine serum albumin standards in CCLB. Relative luciferase activity/μg protein was determined for each culture.

Data analysis. Single factor ANOVA analysis, data analysis, and graphing were performed using Microsoft Excel 2000. Less than 15% of sample data was removed from analysis due to methodological errors. Standard error of mean for glutamate induction experiments was determined by bootstrap function (repeated 1000×) with S-Plus 2000 statistical software. All figures were prepared using Adobe Photoshop 5.0.2. No level or contrast adjustments were performed on the presented PCREB Western data. Data management and statistical analysis for data sets was conducted in consultation with the University of Illinois Statistical Consulting Office.

Results

Characteristics of stably transfected luciferase reporter SCN 2.2 cell lines

To assess transcriptional activation in SCN 2.2 cultures, stable SCN 2.2 cell lines were created that

expressed luciferase reporter activity driven by one of the three different promoters (Figs. 1A–C). We utilized 5.2-kb human vasoactive intestinal peptide (VIP) promoter [36,38], eight CRE or three E-box elements to prepare each of these stable lines (SCN 2.2.*vip-luc*, SCN 2.2.*cre-luc*, and SCN 2.2.*ebox-luc* lines, respectively). Six SCN 2.2.*vip-luc,* 12 SCN 2.2.*cre-luc*, and 10 SCN 2.2.*ebox-luc* clones were initially isolated. Of these, three stable lines (SCN 2.2.*vip-luc-VO1*, SCN 2.2.*cre-luc-C63*, and SCN 2.2.*ebox-luc-E20*) grew rapidly in selective media, expressed varying luciferase activity and were qualitatively most similar in phenotype to the original SCN 2.2 cell line. Additionally, SCN 2.2.*cre-luc-C63* displayed 85% induction of CRE-dependent luciferase activity above control levels after 6 h of exposure to 10 μM Br-cAMP (cAMP analogue), a positive control for CRE induction ($n = 4$, data not shown). For all subsequent experiments, these lines were referred to as SCN 2.2.*vip-luc*, SCN 2.2.*cre-luc*, and SCN 2.2.*ebox-luc* lines.

Serum-shock induces rhythms of reporter gene expression in SCN 2.2 cultures

To evaluate whether circadian expression rhythms in SCN 2.2 cells are driven by the VIP, CRE, and E-box promoter constructs, luciferase activity was assessed in serum-shocked SCN 2.2.*vip-luc*, SCN 2.2.*cre-luc*, and SCN 2.2.*ebox-luc* lines. Within the same experiment, all SCN 2.2 cultures were prepared and collected in parallel to avoid the possible confounding effects of a circadian phase-dependent response to serum-shock. When SCN 2.2 cultures were exposed to 2-h control media change, the different constructs showed variations in luciferase activity but none showed significant (ANOVA, $p < 0.1$) circadian rhythmicity during the subsequent 72-h period (data not shown). However, after 2-h serum-shock, each of the reporter constructs exhibited significant near 24-h rhythms with distinct phase relationships for at least 2 cycles (Fig. 2).

In serum-shocked SCN 2.2.*vip-luc* cultures (Fig. 2A), highly significant circadian maxima of luciferase activity occurred at 32 and 56 h after release to NM (ANOVA, $p < 0.01$). These peaks were 4–5-fold greater than preceding minima. Another significant circadian maximum was also observed at 52 h after serum-shock (ANOVA, $p < 0.05$). In serum-shocked SCN 2.2.*cre-luc* cultures (Fig. 2B), the CRE-driven expression pattern was generally high, punctuated by significant circadian minima at 24 and 52 h after release to NM (ANOVA, $p < 0.01$). Another significant circadian minimum was also observed at 48 h after serum-shock (ANOVA, $p < 0.05$). These minima were approximately 2-fold less than surrounding maxima. Another minimum, 4 h after serum-shock, expressed significantly less luciferase activity than cultures collected 12 h later (16 h after serum pulse) (ANOVA, $p < 0.01$). Serum-shocked SCN 2.2.*ebox-luc* cultures (Fig. 2C) expressed significant circadian peak levels of luciferase activity at 20, 24, and 48 h after release to NM (ANOVA, $p < 0.01$). Variation between maxima and minima in SCN 2.2.*ebox-luc* cultures was approximately 2.5-fold.

Rhythms of circadian gene expression in the SCN maintain distinct relative phase relationships (Fig. 2D). The relative order of rhythmic expression is for CRE reporter expression to precede E-box and VIP peptide expression by approximately 4 and 16 h, respectively. Luciferase activity rhythms in SCN 2.2.*vip-luc*, SCN 2.2.*cre-luc*, and SCN 2.2.*ebox-luc* cultures maintained a consistent phase relationship after serum-pulse (Fig. 2E). The phase of CRE-dependent expression of the luciferase reporter preceded E-box and VIP reporter expression by 4–8 and 12–16 h, respectively. The relationship among these oscillations is consistent with expression patterns observed in the SCN and reveals that serum-shock can synchronize the SCN 2.2 cell line so that cultures express robust and stable circadian oscillations in transcription of promoters whose activity oscillates with defined phase-relationships in the native SCN.

Glutamate induction of PCREB is gated in serum-shocked SCN 2.2 cultures

To assess whether SCN 2.2 cultures exhibit gated sensitivity to an external stimulus, we tested for induction of PCREB by glutamate at 3-h intervals after serum-shock (Fig. 3A). Previous studies have reported that PCREB induction by light/glutamate pathways in rodent SCN occurs only at night [19,20]. In synchronized SCN 2.2 cells, PCREB immunoreactivity was observed at the expected molecular weight, 42 kDa, and at approximately 44/46 kDa in all samples. The 44/46 kDa band is likely to be CRE-modulating protein (CREM), which exhibits a closely related kinase-inducible domain that cross-reacts with anti-PCREB antisera. PCREB levels in SCN 2.2 cultures were enhanced by glutamate treatment over a 9-h period extending at 6, 9, 12, and 15 h after serum-shock ($n = 3$ for all timepoints) (Fig. 3B). In contrast, rat-1 fibroblasts failed to display PCREB-induction by glutamate at any time during the 24-h interval after serum-shock ($n = 2$ for all timepoints, data not shown). Because sensitivity of the native SCN to PCREB induction by glutamate is limited to the period during which the SCN is sensitive to phase-resetting by glutamate during the night, this 9-h period of PCREB induction in the SCN 2.2 cells may correspond to the nighttime circadian phase (±3 h surrounding the timepoints tested). This suggests that serum-shock synchronizes SCN 2.2 cells to a circadian state immediately preceding the onset of clock sensitivity to glutamate.

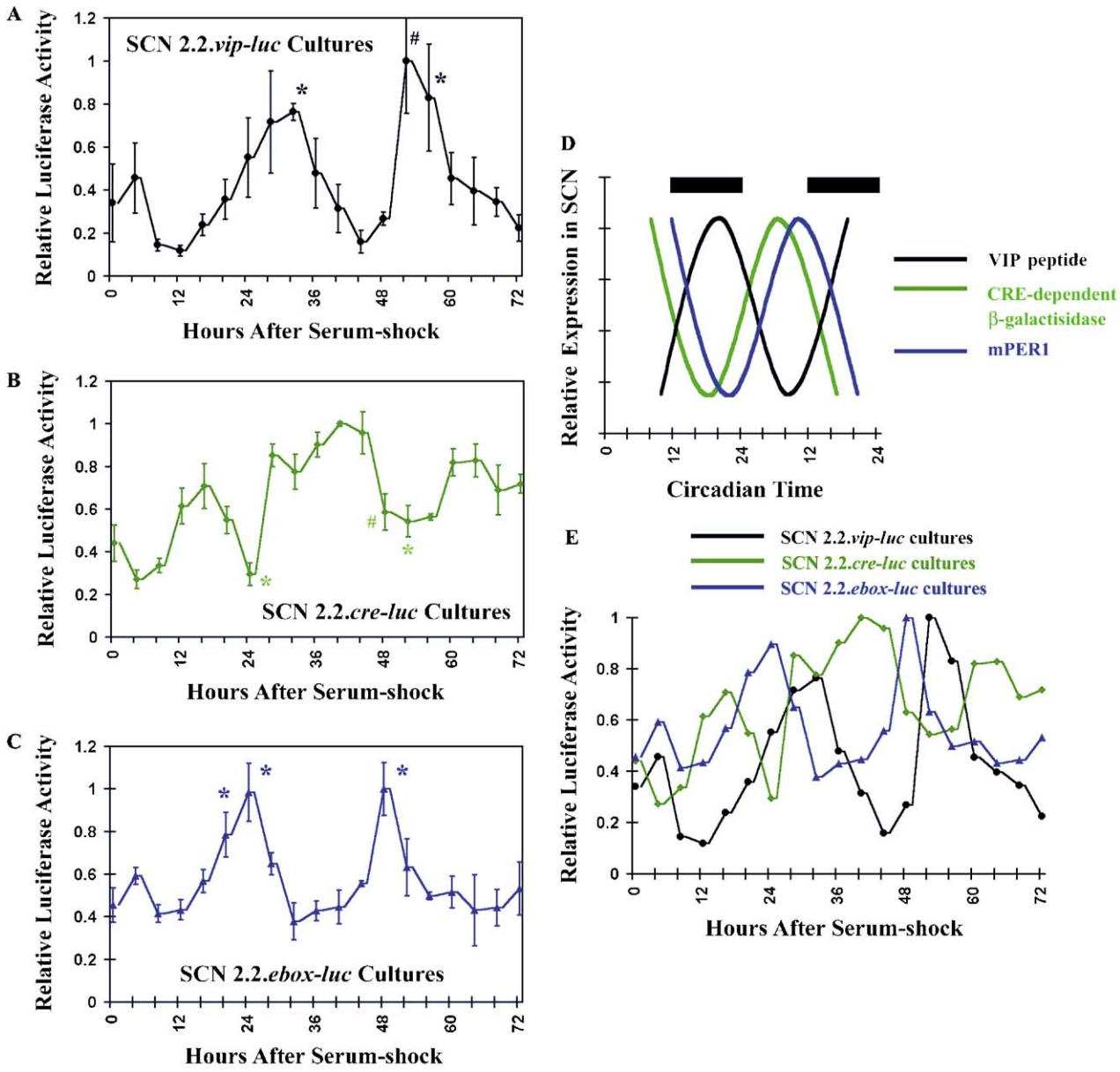

Fig. 2. Relative luciferase activity in differentiated stably transfected SCN 2.2 cultures after serum-shock. Data collection began immediately after removal of serum or control media and replacement with fresh serum-free NM ($n = 3$ to 4 for each data point). Symbols represent data sets with relative luciferase activity significantly (*, $p < 0.05$ or #, $p < 0.01$, single factor ANOVA) greater than cultures collected 12 h before and after. Error bars denote standard deviation. (A) SCN 2.2.*vip-luc* cultures (black). (B) SCN 2.2.*cre-luc* cultures (green). (C) SCN 2.2.*ebox-luc* cultures (blue). (D) Qualitative plot of relative levels of rat VIP peptide, [48] CRE promoter-dependent β-galactosidase activity [22] and mPER1 [42,45] versus circadian time in the SCN. Black bars above plot indicated night or subjective night phase of the animal. (E) Overlay of data from (A) to (C) to compare phase relationship of expression among stably transfected SCN 2.2 transfectants after serum-shock: SCN 2.2.*vip-luc* (black), SCN 2.2.*cre-luc* (green), and SCN 2.2.*ebox-luc* (blue).

Phase-dependent sensitivity to glutamate in SCN 2.2 cells is consistent with light-induced signaling in the SCN

To test the hypothesis that SCN 2.2 cultures are synchronized by serum-shock to the time preceding the onset of sensitivity of the SCN to glutamate, we examined the affects of glutamate signaling on CRE-dependent luciferase induction in serum-shocked SCN2.2.*cre-luc* cultures. Light-induced signaling pathways modulate the phase of SCN circadian rhythms only at night (Fig. 4A) [1]. Since nitric oxide synthetase (NOS) is a necessary element of the light/glutamate signaling cascade throughout the night [13], while activation of PKG is required only during the late night [23], inhibitors of these two enzymes were tested for their ability to modulate the glutamate response in serum-shocked cultures.

To define the profile of sensitivity of SCN 2.2 cultures to glutamate further, SCN 2.2.*cre-luc* cultures

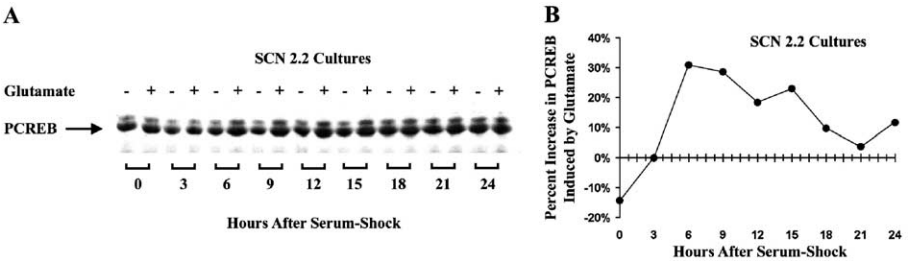

Fig. 3. Glutamate induces phosphorylation (ser133) of CREB (PCREB) in serum-shocked SCN 2.2 cultures. (A) Cultures were treated for 10 min with 10 μM Glu or vehicle control at 3-h intervals for 24 h after serum-shock. After treatment, cultures were immediately harvested for Western bolt analysis. A representative blot ($n = 3$) is displayed. (B) Graph represents data from Western blot in (A) after normalizing for protein concentration and determining relative amount of PCREB in control and Glu-treated samples. Induction of PCREB by glutamate (as a percentage of control culture PCREB levels) was plotted against time-of-treatment after serum-shock.

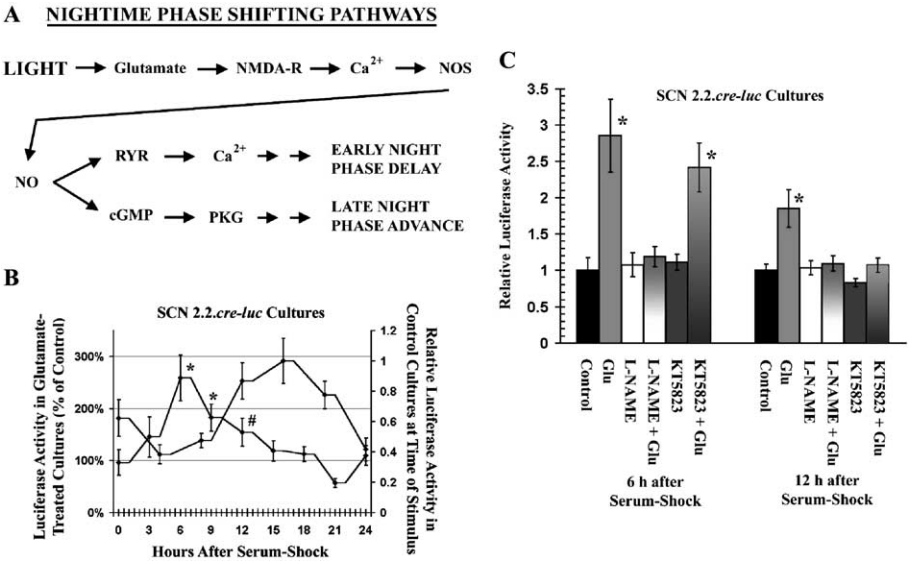

Fig. 4. Glutamate induces gene expression in native SCN and SCN 2.2 cultures. (A) Light induces phase delays and advances of circadian rhythms during the early and late night, respectively. (NMDA-R = ionotropic glutamate receptor, NOS = nitric oxide synthase, NO = nitric oxide, RyR = ryanodine receptor, PKG = cGMP-dependent protein kinase [1]). (B) Glutamate induces CRE-dependent luciferase activity in serum-shocked SCN 2.2.*cre-luc* cultures. Cultures were treated for 10 min with 10 μM glutamate or vehicle at 3-h intervals for 24 h after serum-shock. After treatment, media were replaced with fresh serum-free NM and cultures were collected 6 h after onset of treatment to assay luciferase activity. Percent induction (left axis, gray) of luciferase by glutamate (as a percentage of activity in control cultures) was plotted against time-of-treatment after serum-shock ($n = 3$ to 4). Error bars denote standard error of mean methods. Endogenous luciferase activity (right axis, black) in SCN 2.2.*cre-luc* cultures was plotted against time of extract collection (4-h intervals, 24 h) after serum-shock. Symbols represent data sets of glutamate-treated cultures with relative luciferase activity significantly different from control cultures (*, $p < 0.05$ or #, $p < 0.01$, single factor ANOVA). (C) Glutamate induction of luciferase activity in SCN 2.2.*cre-luc* cultures is mediated by differing signaling pathways, depending on time-of-treatment after serum-shock. At 6 and 12 h after serum-shock, cultures were treated with glutamate (10 μM, 10 min) or vehicle control. For inhibition experiments, cultures were pre-incubated with 100 μM L-NAME (NOS inhibitor) or 250 nM KT 5823 (PKG inhibitor) for 20 min prior to glutamate stimulus. Symbols represent data sets of cultures with relative luciferase activity significantly different from control cultures (*, $p < 0.05$ or #, $p < 0.01$, single factor ANOVA).

were exposed to glutamate at 3-h intervals after serum-shock. CRE-dependent luciferase activity induced by glutamate, as percent of control (vehicle treated) levels, was plotted against time of treatment after serum-shock of SCN 2.2.*cre-luc* cultures (Fig. 4B). Additionally, endogenous luciferase activity was measured at 4-h intervals levels in untreated cultures after serum-shock and plotted against the same timescale as glutamate-induced luciferase activity, but with relative luciferase levels on the abscissa (right). The relationship between time of exposure to glutamate, luciferase induction, and endogenous luciferase levels permitted visualization of the phase-response characteristics of glutamate response in SCN 2.2 reporter lines. SCN2.2.*cre-luc* cultures displayed significant CRE-induction by glutamate at 6 and 9 h (259% and 183% of control levels; $p < 0.01$, ANOVA) as well as at 12 h (155% of control levels; $p < 0.05$, ANOVA) after serum-shock. Additionally, there was a significant reduction in CRE activity when glutamate was applied at 21 h after serum-shock (57% of control levels; $p < 0.05$, ANOVA).

To evaluate whether the window of sensitivity to glutamate-induction of the CRE promoter in SCN 2.2.*cre-luc* cultures is equivalent to circadian night, the competitive NOS inhibitor, L-NAME, and selective PKG inhibitor, KT5823, were used to characterize the glutamate response at 6 and 12 h after serum-shock (Fig. 4C). A 20-min pretreatment of SCN 2.2.*cre-luc* cultures with 100 μM L-NAME completely blocked induced CRE expression when glutamate was applied at both 6 and 12 h after serum-pulse (Fig. 4C). In contrast, 20-min pretreatment with 250 nM KT5823 blocked glutamate induction only in cultures treated 12 h after serum-shock (Fig. 4C). L-NAME blocks the effects of glutamate in the native SCN in both early and late night [13]. Since KT5823 only affects the response to glutamate during late-night [23], the present data suggest that the circadian states of SCN2.2.*cre-luc* cultures at 6 and 12 h after serum-shock have signaling characteristics equivalent to the SCN at early and late night, respectively.

Glutamate induces E-box and VIP promoters in SCN 2.2 cultures

To determine whether E-box and VIP promoters were inducible by glutamate in a time-dependent manner, SCN 2.2.*ebox-luc* and SCN 2.2.*vip-luc* cultures were evaluated for 24 h after serum-shock for sensitivity to glutamate stimulation. E-box-dependent glutamate induction varied considerably over the 24-h (Fig. 5A). At 15 and 18 h after serum-shock of SCN 2.2.*ebox-luc* cultures, glutamate induced significant increase in E-box-dependent luciferase expression (157% and 138% of control levels, respectively; $p < 0.05$, ANOVA). This induction time preceded the highest observed endogenous E-box promoter-dependent luciferase activity by roughly 6 h. SCN 2.2.*vip-luc* cultures displayed two windows of sensitivity to glutamate induction of VIP promoter-dependent luciferase activity (Fig. 5B). These points occurred when glutamate was applied at 9 and 18 h (159% and 172% of control levels, respectively; $p < 0.01$, ANOVA) after serum-shock. Both sensitivities to induction of the VIP promoter occurred prior to the highest observed endogenous VIP-driven luciferase activity at 24 h after serum-shock in untreated SCN 2.2.*vip-luc* cultures. The glutamate-induction profile of both E-box and VIP promoter-driven luciferase expression in SCN 2.2 cultures appeared similar to the light-induction profile of mPER1 in the SCN, which shows maximal induction by light stimuli during the early and late night [39–44].

Discussion

To evaluate the usefulness of the SCN 2.2 cell line as a model for probing clock mechanisms, SCN 2.2 cultures were tested to determine whether they could be predictably synchronized to express characteristics of the SCN clock: circadian rhythms of transcription and restricted sensitivity to glutamate.

Our results suggest that 2-h serum-shock of parallel SCN 2.2 cultures resets these cells to a dusk-like circadian state. Rhythms of VIP, CRE, and E-box luciferase activity in stably transfected SCN 2.2 cultures maintain a consistent relative phase relationship for at least 72 h after serum-shock (Fig. 2). The phase of promoter expression in stably transfected SCN 2.2 cell lines after serum-shock is consistent with the predicted patterns based on circadian VIP, CRE, and *mPer1* (E-box) expression in the SCN (Figs. 2C and D). In the mouse SCN, reporter genes driven by the CRE promoter reach peak levels by CT 6 ± 4 h [22]. The endogenous oscillation of mouse *per1* mRNA peaks between CT 2 and CT 4, while maximum PER1 protein expression occurs approximately 6 h later at CT 10 ± 4 h [39–45]. Finally, VIP peptide levels in rat have been shown to rise through the night, peaking at CT 20 ± 6 h [46–49]. If endogenous circadian oscillations of *mper1* are primarily

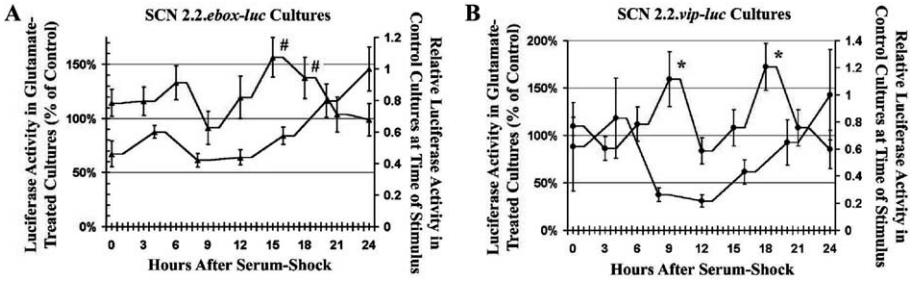

Fig. 5. Glutamate induces E-box and VIP promoters in serum-shocked SCN 2.2.*ebox-luc* (A) or SCN 2.2.*vip-luc* (B) cultures. For determination of glutamate induction, cultures were treated for 10 min with 10 μM glutamate or vehicle at 3-h intervals for 24 h after serum-shock. After treatment, media were replaced with fresh serum-free NM and cultures were collected 6 h after onset of treatment to assay luciferase activity. Percent induction ± SEM of luciferase by glutamate is plotted against time-of-treatment after serum-shock ($n = 3$ to 4) (left axis, gray). Endogenous luciferase activity in SCN 2.2.*cre-luc* cultures is plotted against time of extract collection (4-h intervals, 24 h) after serum-shock (right axis, black). Symbols represent data sets of glutamate-treated cultures with relative luciferase activity significantly different from time-yoked control cultures (*, $p < 0.05$ or #, $p < 0.01$, single factor ANOVA).

driven by the E-box promoter and synthesis of both PER1 and luciferase requires equivalent amounts of time, then CRE reporter expression should precede E-box and VIP peptide expression by approximately 4 and 16 h, respectively, in SCN-like tissue. These estimates do not take into account post-translational protein modification that is likely to effect the phase of VIP or PER1 but not luciferase expression. Furthermore, the difference between rat and mouse circadian systems may also contribute to error in the estimates of phase relationship. Nevertheless, the relative expression patterns of the reporter constructs in the SCN 2.2 cultures are as predicted.

In addition to providing evidence of phase relationships of specific promoter-driven oscillations, the comparison of promoter expression in SCN 2.2 cells with SCN tissue permits us to determine the relative circadian phase to which serum-shock resets cultures. VIP promoter-dependent luciferase activity peaked at 32 and 56 h after serum-shock (Fig. 2A). Since rat VIP peaks at CT 20 under LD [48], luciferase levels in serum-shocked SCN 2.2.*vip-luc* cultures suggest that serum-shock may reset cultures to a circadian state of approximately CT 10. Peaks of CRE-dependent luciferase activity occurred at approximately 16 and 40 h after release from serum-shock (Fig. 2B). As maximum CRE-dependent reporter gene expression in mouse occurs at CT 6 ± 4 h [22], our CRE reporter data suggest that serum-shock synchronized the SCN 2.2.*cre-luc* line to a CT 14-like circadian state. E-box-dependent expression in SCN 2.2 cultures peaked at 24 and 48 h after release from serum-shock (Fig. 2C). If PER1 circadian expression results significantly from an E-box-driven transcriptional event, then serum-shock would seem to reset SCN 2.2.*ebox-luc* cells to the equivalent of CT 10. The precision of these estimates is limited by the 4-h interval in collection of SCN 2.2 cultures as well as the resolution of published expression patterns in the SCN. However, taken together, these data are consistent with the notion that serum-shock resets SCN 2.2 cultures to a circadian state between CT 10 and CT 14, near the day-to-night transition of circadian rhythms in the SCN.

Considerable evidence indicates that the SCN 2.2 line behaves like a central pacemaker, generating spontaneous 24-h rhythms in metabolic activity and neurotransmitter expression as well as driving oscillations in SCN-lesioned rats and other cell lines [33,35]. Despite previous reports, we did not observe spontaneous luciferase rhythms in SCN 2.2 cultures that were not serum-shocked [33,35]. In our experiments, cells remained in culture after initial splitting for at least 6 days prior to control media change or serum-shock and subsequent collection for luciferase assay. During this time, individual culture dishes may have drifted out of phase with each other, contributing to variation among samples. In fact, both SCN 2.2.*vip-luc* and SCN 2.2.*cre-luc* control cultures show evidence of drifting circadian rhythms or possibly underlying ultradian rhythms (data not shown). This drift might have also contributed to variation in serum-shocked cultures if SCN 2.2 cells display circadian phase-dependent sensitivity to the serum-shock. For this reason, we attempted to maintain consistency by performing potentially phase-shifting events (media changes, etc.) at the same time-of-day for all experiments. However, despite these precautions, the circadian state of individual cultures and cells may have been variable prior to serum-shock. Until sources of variability have been identified, extreme stringency in manipulating these dynamic cell cultures is required.

Additionally, the serum-shocked SCN 2.2 cultures exhibit temporally restricted responses to glutamate. Glutamate is the primary chemical messenger transmitting the light signal from the eye to the SCN in vivo [13]. Sensitivity to light and glutamate is gated by the central pacemaker in the SCN so only nocturnal exposure induces PCREB formation and phase shifts the circadian clock. In SCN 2.2 cell lines, glutamate induction of PCREB and of CRE-dependent luciferase activity was restricted to a 6–9-h window of time, between 6 and 15 h after serum-shock. The gated response of the SCN 2.2 cell line to glutamate suggests that the period 6–15 h ± 3 h after serum-shock corresponds to the nighttime circadian phase of the SCN. Intriguingly, a 43% reduction in CRE-mediated luciferase activity was observed when glutamate was applied to cultures 21 h after serum pulse. This suggests that glutamate may inhibit CRE activity after the period following sensitivity to induction, possibly during the circadian day phase in the SCN. Finally, the window during which glutamate induced CRE-mediated luciferase expression preceded the endogenous rise of this reporter by roughly 9 h. This relationship between the window of promoter inducibility and endogenous circadian promoter activity may define a feedback relationship that changes with clock state. By this line of reasoning, the glutamate signal may induce levels of transcription that characterize the circadian state to which glutamate shifts the clock.

The signaling pathways that mediate glutamatergic phase-shifts in early and late night are regulated by both common and distinct elements [1] (Fig. 4A). In early and late night, PCREB is temporally specific in the SCN, a signaling cascade that includes light, glutamate, NMDA-R, Ca^{2+} influx, NOS, and NO [13]. None of these signals is effective in altering clock phase during the daytime. However, during the late-night, the pathway by which glutamate induces PCREB and advances clock phase requires PKG activity downstream of NO [23]. Utilizing these criteria to differentiate night domains from day, we found that the SCN 2.2 cultures express gated glutamate responses mediated by phase-dependent signal transduction pathways similar to those

observed in the native SCN (Fig. 4C). NOS activity was required for CRE-dependent transcription when glutamate was applied at 6 and 12 h after serum-shock. These data are consistent with circadian night in the intact SCN, when PCREB induction by light is NOS-dependent. In contrast, PKG activity was required only when glutamate was applied at 12 h, but not at 6 h, after serum-shock. Therefore, the period including 6 and 12 h (±4 h) after serum-shock transiently expresses multiple characteristics that correspond to the circadian states of early and late subjective night, respectively, in the SCN clock. Taken together, these data further suggest that serum-shock resets the SCN 2.2 cell line to a late day, dusk-like circadian state.

Phase-dependent responses of E-box- and VIP-dependent transcriptional induction by glutamate have not been reported in the intact SCN. However, both SCN 2.2.*ebox-luc* and SCN 2.2.*vip-luc* cultures displayed glutamate induction profiles characteristic of a gated response (Figs. 5A and B). Glutamate significantly enhanced E-box-dependent luciferase activity to 157% and 138% of control levels when applied at 15 and 18 h after serum-shock, respectively. These results are particularly interesting because they demonstrate that activation of the E-box promoter sequence in the cells is sensitive to glutamate and predict that the native SCN should show similar responsiveness.

VIP promoter induction by glutamate in SCN 2.2 cells exhibited two distinct windows of sensitivity to glutamate treatment. These occurred at 9 and 18 h (159% and 172% induction, respectively) after serum-shock (Fig. 5). When compared to CRE induction by glutamate and the circadian phase estimate for SCN 2.2.*cre-luc* cells, glutamate appeared to induce the VIP promoter during the equivalent of the middle of the night and at dawn. This demonstrates that induction of VIP promoter is gated by the clock in SCN 2.2 cells.

Published data on acute affects of light on VIP expression are contradictory. Although VIP oscillates in a circadian pattern in the SCN of animals in LD, its rhythmicity is lost when animals are kept under constant dark (DD) conditions [46,47,49]. Additionally, it is likely that somatostatin (SS) neurons from the dorsomedial SCN make synaptic contact with VIP neurons in the ventolateral SCN and suppress VIP rhythmicity under constant conditions since depletion of SS by cysteamine relieves suppression of VIP rhythmicity in SCN of rats in DD [50]. This may explain why SCN 2.2 cultures express circadian VIP promoter-driven luciferase under constant conditions, as this type of SS-mediated feedback may not be present in cell cultures. Despite the complexities of regulation of VIP expression in vivo, glutamate induction experiments in SCN 2.2.*vip-luc* cultures suggest that the two windows of sensitivity to glutamate may be regulated by separate promoter elements (Fig. 5B). The human VIP promoter contains several regulatory elements, some of which include: a tissue specifier element (TSE; POU homeodomain binding sites), 2 E-boxes, a cytokine response element (CyRE), and at least one CRE [38] (Fig. 1A). The temporal relationship between time of exposure to glutamate and effects on CRE, E-box, and VIP promoter expression suggests that VIP promoter induction by glutamate at 9 and 18 h after serum pulse may be separately mediated by CRE and E-box elements, respectively. This result is further evidence that the influence of light and glutamate on circadian gene transcription is gated through multiple signal transduction pathways during different phases of the circadian cycle.

Although the gated response of the SCN 2.2 cell line exhibits SCN-like characteristics, extrapolations about the SCN made from the cell line are limited in some respects. In the rat, extrinsic signals from a number of brain regions regulate SCN circadian rhythms and modulate phase-shifting stimuli. These are absent in cell culture. The native SCN can be anatomically subdivided into regions based on neuropeptide expression. These subdivisions of the SCN differ as primary targets of projections containing different chemical signals. Examples include glutamate and pituitary adenylyl cyclase-activating peptide (PACAP) colocalized in retinal ganglion cells projecting to the VIP neurons of the ventrolateral SCN [47,51]. As retinal ganglion cells are absent in SCN 2.2 cultures, glutamate treatment mimics one aspect of the light signal while omitting the issue of co-regulation by other pre-synaptic neurotransmitters. Also, the extent to which the range of SCN cell types are expressed within SCN 2.2 cultures remains to be fully defined.

The coordinating mechanism for relaying incoming signals to other regions of the SCN may involve neuronal and/or humoral cues [47]. However, how these cues are transmitted and synchronize the many cellular elements remains undefined. With the exception of VIP and VPAC2-R, it is unknown which coordinating mechanisms are expressed among cell types in differentiated SCN 2.2 cultures [34,52,53]. Because the SCN 2.2 cell line secretes several neuropeptides [33,35,52], tissue culture media become increasingly enriched with signaling factors as time in culture increases. These signaling factors may also affect the phase of cell line circadian rhythms, modulate experimental stimuli, and possibly contribute to the lack of observed oscillations in stably transfected cultures that were not serum-shocked. These caveats must be considered when executing experiments in the SCN 2.2 cell line and modeling regulatory mechanisms in the SCN.

In conclusion, we report the production of three stably transfected luciferase reporter expressing SCN 2.2 cell lines. In response to serum-shock, they generated promoter-dependent oscillations in VIP, CRE, and

E-box regulated transcription with similar phase relationships to those observed in SCN tissue. Serum-shocked SCN 2.2 cultures exhibited gated sensitivity to glutamate, a definitive characteristic of the SCN central pacemaker. To date, there have been no reports of any cell line exhibiting a circadian gated response to stimulus. The induction of CRE-dependent reporter activity by glutamate in this cell line requires signaling elements characteristic of nighttime light/glutamate signaling in the SCN. Additionally, both E-box and human VIP promoter elements were activated by glutamate. Based on these data, we conclude that these serum-shock conditions synchronize SCN 2.2 cultures to a circadian state consistent with the day/night transition, or dusk, in the SCN. This characterization of a circadian synchronization procedure that releases clock cells into a predictable state further establishes the utility of SCN 2.2 cells as a mammalian clock model.

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