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Immortalized Suprachiasmatic Nucleus Cells Express Components of Multiple Circadian Regulatory Pathways

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We undertook an extensive antigenic characterization of the SCN 2.2 cell line in order to further evaluate whether the line expresses components of circadian regulatory pathways common to the hypothalamic suprachiasmatic nucleus (SCN), the central circadian clock in mammals. We found that differentiated SCN 2.2 cultures expressed a broad range of putative clock genes, as well as components of daytime, nighttime, and crepuscular circadian regulatory pathways found within the SCN *in vivo*. The line also exhibits several antigens that are highly expressed in a circadian pattern and/or differentially localized in the SCN relative to other hypothalamic regions. Expression of a broad complement of circadian regulatory proteins and putative clock genes further support growing evidence in recent reports that the SCN 2.2 cell line is an appropriate model for investigating the regulation of central mammalian pacemaker.

Key Words: **period; cryptochrome; casein kinase; PACAP; PKA; PKC; PKG; NOS; I1; VGF.**

The central biological pacemaker in mammals lies in the hypothalamic suprachiasmatic nucleus (SCN). The SCN drives circadian behavior, expresses rhythmic gene expression and integrates external stimuli in order to synchronize molecular timekeeping mechanisms with changing environmental conditions. Receptivity to phase shifting stimuli is gated by the circadian state of the central pacemaker. This gating behavior is most clearly demonstrated by the sensitivity of the SCN to light during the night phase, but not during the day phase. All photic, social and hormonal influences on the circadian clock are integrated at the cellular level through multiple signaling pathways within the SCN.

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The complexity of interacting signaling pathways involved in regulation of the SCN has encouraged researchers to seek cell culture systems to complement *in vivo* analysis of the mammalian circadian clock. Whether a cell line exhibits central or peripheral circadian clock characteristics complicates selection of an appropriate model system. To provide useful applications for central mammalian pacemaker research, cell line models must exhibit persistent self-sustained oscillations in circadian gene products, restore animal circadian rhythms in SCN-lesioned hosts and exhibit time-dependent responses to stimuli through regulatory pathways that characterize SCN function. Rhythmic gene expression has been demonstrated in NIH/3T3 fibroblasts, Rat-1 fibroblasts and spontaneously immortalized embryonic mouse fibroblasts after synchronizing signals (1–5). However, there is no published evidence that these lines express spontaneous, self-sustained rhythms or rescue rhythms in SCN-lesioned animals (5, 6). In contrast, another cell line derived from fetal rat SCN, the SCN 2.2 cell line, exhibits endogenous spontaneous circadian rhythmicity *in vitro* (5, 7). We have focused on the SCN 2.2 line to further assess its usefulness as a model system for studying the mammalian central circadian pacemaker.

The SCN 2.2 cell line is a pluripotent and immortalized line of SCN progenitor cells from fetal rat hypothalamic tissue that exhibits both neuronal and glial morphologies in culture (5, 7). Neuronal cell types express several peptide neurotransmitters found in the SCN. These include vasoactive peptide (VIP), arginine-vasopressin (AVP), and somatostatin (SMT). Additionally, the line lacks oxytocin- (OXY) and corticotropin-releasing factor- (CRF) staining, which are found in the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus but not in the nearby SCN (7). Like the SCN *in situ*, SCN 2.2 cultures generate robust twenty-four hour rhythms in brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT3) content

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as well as 2-deoxyglucose (2-DG) uptake. The phase relationship of these rhythms in culture match those seen in adult SCN tissue *in vivo* where 2-DG uptake peaks approximately twelve hours out-of-phase with peak levels of BDNF content (5). Finally, SCN 2.2 grafts into the third ventricle of SCN-lesioned animals rescue circadian locomotor rhythms (5) and are capable of driving rhythms in NIH/3T3 co-cultures (6). It is, therefore, of interest to determine whether the SCN 2.2 cell line expresses other SCN characteristics such as clock gene products and components of day, night and crepuscular regulatory pathways exhibited by the SCN *in vivo*.

In recent years, circadian research has demonstrated that interactions among several putative clock gene products constitute positive and negative feedback loops that, at least partially, comprise the molecular mammalian circadian clock. Three mammalian *Period* genes (*Per1/2/3*) have been identified (8–13) as well as mammalian *Timeless* (*Tim*) (14–16), which is most likely the ortholog of the *Drosophila Timeout* gene (17, 18). Positive regulatory genes *Clock* (*Clk*) and *bmal1* (the mammalian homolog of *Drosophila Cycle*) (19, 20) as well as two *Cryptochrome* genes, *Cry1* and *Cry2*, have also been identified (21, 22). Even the classic *tau* mutation in hamsters, which shortens circadian period, has been mapped to the caseine kinase 1ϵ (CK1ϵ, the mammalian homolog of the *Drosophila* clock gene *Doubletime*) gene (23, 24). Although mammalian clock research has advanced greatly in recent years, the aforementioned genes and models of clock mechanism are most likely incomplete.

Regulation of the mammalian circadian cycle can be subdivided into three major elements: daytime, nighttime and crepuscular (25). The SCN exhibits phase-advancing sensitivity to dark pulses, cAMP analogues, activation of cAMP-dependent protein kinase (PKA) and pituitary adenylyl cyclase activating peptide (PACAP) during the day (26–29). In opposition to daytime regulation, phase-shifting sensitivity in the SCN to light, cGMP analogues, cholinergic and glutamatergic stimuli is limited to the nighttime (30–32). The crepuscular domains, corresponding to dusk and dawn, are windows of sensitivity to the pineal hormone melatonin, which phase advances circadian rhythms in the rat SCN at both times (33). Interestingly, many of the phase shifting signals in different regulatory domains share common signal transduction components. However, while it seems unlikely that phase-shifting pathways are identical in the central pacemaker and peripheral oscillators, this possibility has not been directly addressed by circadian researchers (4, 25).

This study complements recent reports on the SCN-like character of the SCN 2.2 cell line through a further investigation of the antigenic characteristics of the line in comparison with rat SCN tissue. Our analysis focused on the expression of putative clock gene products as well as components of day, night and crepuscular regulatory pathways exhibited by the SCN *in vivo*. Additionally, SCN 2.2 cultures were screened for several marker proteins whose expression within the hypothalamus is relatively high or differentially localized in the SCN.

MATERIALS AND METHODS

Cell culture. SCN 2.2 cultures (passage 12–18) were plated at 30% confluence onto laminin-coated (Gibco BRL) tissue culture flasks (25 cm^2) or coverslips 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 (Gibco BRL)]. Cultures were maintained in the dark in tissue culture incubators with sporadic light exposure during media changes. After cultures reached appropriate cell density (100% confluent for Western/RT-PCR, 30–50% for immunocytochemistry) media was replaced with a defined 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 (Gibco BRL)] (7). Cultures remained in NM for 48–72 h prior to collection at 2-h intervals over 24 h and pooled during extract preparation.

SCN tissue. SCN tissue was isolated from 8-week-old Long-Evans rats for comparative analysis of SCN 2.2 cultures. Animals that had been maintained on a 12-h light/12-h dark lighting schedule were sacrificed by decapitation at 2-h intervals for 24 h. This procedure was accomplished during the dark phase of the light-dark cycle using dim red light. SCN tissue was immediately dissected, pooled and frozen for protein extraction. For analysis of mRNA expression, SCN tissue samples were collected at 2-h intervals during the 12-h light phase of the light-dark cycle, pooled and frozen prior to RNA isolation.

Protein extracts. Total cellular protein was extracted by lysis of frozen SCN 2.2 cultures and SCN tissue with boiling denaturing buffer (62.5 mM Tris–HCl pH 6.8, 0.5% SDS, 15% glycerol, 2.5% 2-mercaptoethanol, 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, 40 ng/ml bestatin). Protein concentration was determined by Bradford analysis of parallel samples (34).

Western analysis. Equivalent amounts of SCN tissue and SCN 2.2 cell culture protein extracts were separated on SDS–PAGE gels and transferred to nitrocellulose by standard protocols. Blots were probed with primary antibody (room temp or 4°C; 2–18 h). Immunoreactive bands were visualized using horseradish peroxidase-linked goat anti-rabbit (1:1000, Chemicon), goat anti-mouse (1:1000, Zymed), horse anti-mouse (1:1000, Vector) or rabbit anti-chicken (1:1000, Chemicon) secondary antibodies and SuperSignal Chemiluminescent Substrate detection system (Bio-Rad) and Biomax MS film (Kodak).

Primary antibodies. Mouse anti-Ca^{2+}/calmodulin-dependent protein kinase (CKII) (1:2000, Calbiochem), mouse anti-CKIV (1:5000, Transduction Labs); mouse anti-Casein Kinase 1ϵ (CK1ϵ) (1:1000, Transduction Labs); rabbit anti-heme oxygenase 2 (HO-2) (1:1000, StressGen); affinity purified rabbit anti-hMOP3 (1 μg/ml, Alpha Diagnostic International); rabbit anti-protein phosphatase inhibitor 1 (I1) (1:500, gift from A. Nairn); rabbit anti-Ca^{2+}/cAMP-dependent protein kinase (PKA) (1:1000, gift from M. Uhler); rabbit anti-cGMP-dependent protein kinase (PKG) (1:500, Gillette lab); anti-glutamic acid decarboxylase 65/67 (GAD65/67) (1:1000); mouse anti-protein kinase Cα (PKCα) (1:500, Transduction Labs); mouse anti-PKCβ (1:500, Transduction Labs); mouse anti-PKCγ (1:500, Transduction Labs); rabbit anti-mitogen-activated protein kinase (MAPK) (1:5000,

UBI); affinity purified rabbit anti-mammalian CLOCK (CLK) (1 μg/ml, ADI); rabbit anti-mammalian CRYPTOCHROME 1 (CRY1) (1:1000; ADI); rabbit anti-mCRY2 (1:2000; ADI); rabbit anti-mammalian PERIOD 1 (PER1) (1:2000, ADI); rabbit anti-mammalian PER2 (1:2000, ADI); rabbit anti-mammalian PER3 (1:2000, ADI); rabbit anti-NMDA receptor (2 μg/ml, Chemicon); rabbit anti-neuronal nitric oxide synthase (NOS) (1:500, Transduction Labs); goat anti-ryanodine receptor (1:1000, UBI) and, rabbit anti-VGF (1:2000, gift from A. Levi).

RT-PCR analysis. Total RNA was prepared from SCN tissue and SCN 2.2 cultures using TRIZOL reagent (Gibco BRL). Reverse transcription reactions were performed using Superscript II RT (Gibco BRL). Standard PCR protocols were performed using *Taq* polymerase (Gibco BRL). After agarose gel analysis, bands containing the expected RT-PCR product were cut out and purified by Compass DNA purification kit (American Bioanalytical). Identity of PCR products was verified by DNA sequencing (Keck Biotech Center, University of Illinois).

Primers. Rat *timeless* (*rtim*): *rtim sense* 5′-AGG GAC TTA CCA CAA GGA-3′ (200-217 of GenBank 6755788), *rtim RT/antisense* 5′-AGT CGT CCA TGC CAC TGA-3′ (718-735 of GenBank 6755788), *rtim* expected product: 535 bp. Melatonin receptor 1 (MT1): *MT1 sense* 5′-ACC TGC AAA CC-3′ (476-485 of GenBank AF130341), *MT1 RT/antisense* 5′-ACA AAA ACT ACA AAC ATG GT-3′ (704-724 of GenBank AF130341), *MT1* expected product: 248 bp. Melatonin receptor 2 (MT2): *MT2 sense* 5′-ATT TCT TTG TG-3′ (111-120 of GenBank AF141863), *MT2 RT/antisense* 5′-ACA AAC ACT GCG AAC ATG GT-3′ (339-359 of GenBank AF141863), *MT2* expected product: 248 bp. Pituitary adenylyl cyclase-activating peptide (PACAP) receptor 1 (PAC1-R): *PAC1-R sense* 5′-TTT CAT CGG CAT CAT CAT CAT CAT CCT T-3′ (1056-1077 of GenBank Z23279), *PAC1-RT/antisense* 5′-CCT TCC AGC TCC TCC ATT TCC TCT T-3′ (1345-1321 of GenBank Z23279), *PAC1-R* expected products: 280 bp (*PAC1-R-s*), 364 bp (*PAC1-R-hip*), 364/361 bp (*PAC1-R-hop1*/*PAC1-R-hop2*), 448/445 bp (*PAC1-R-hiphop1*/*PAC1-R-hiphop2*). VIP/PACAP receptor 2 (VPAC2-R) *VPAC2-R sense* 5′-ATG GAT AGC AAC TCG CCT TTC TTT AG-3′ (886-911 of GenBank 8394531), *VPAC2-R RT/antisense* 5′-GGA AGG AAC CAA CAC ATA ACT CAA ACAG-3′ (1211-1184 of GenBank 8394531), *VPAC2-R* expected product: 325 bp (35–37). Primers were purchased from the Keck Biotechnology Center, University of Illinois.

Image analysis. All figures were prepared using Adobe Photoshop 5.0.2. Image contrast was adjusted to improve visualization of gels with high background levels.

RESULTS

Expression of putative clock genes. SCN 2.2 cultures maintained in serum-free N2 media for 48–72 h were tested for expression of putative clock genes found in the rat SCN. For Western analysis, cell line samples were pooled (2-h intervals over 24 h) and compared against pooled SCN tissue collected at 2-h intervals from rats throughout the 12-h light/12-h dark lighting cycle. Both SCN tissue *ex vivo* and SCN 2.2 cell cultures expressed a PER1 immunoreactive band at the expected molecular weight of 136 kDa (Fig. 1A). An additional PER1 cross-reactive band at 145 kDa was expressed in both extracts and may represent phosphorylated/modified PER1 protein. Single protein bands were found at 136 kDa for PER2 (Fig. 1B) and at 120 kDa for PER3 (Fig. 1C) in SCN tissue and SCN 2.2 extracts. Although SCN and SCN 2.2 extracts expressed equivalent amounts of both PER1 and PER2, SCN 2.2 culture expressed barely detectable PER3 compared rat SCN tissue. In the absence of reliable antisera against rat TIM (rTIM), RT-PCR analysis of the amino-terminal (200–735 of GenBank 6755788) of *rtim* was performed on SCN 2.2 RNA. *rTim* was the only putative clock gene tested by RT-PCR. The expected single 535 bp product was gel-extracted and sequenced to confirm identity as *rtim* (Fig. 1D). CLK was identified as a prominent immunoreactive band with a molecular size of 97 kDa, although expression was barely detectable in SCN 2.2 extracts compared to rat SCN (Fig. 1E). This relative difference in CLK expression may be correlated with the low PER3 levels observed in SCN2.2 cells, but did not appear to impact on PER1 or PER2 expression because similar levels of these proteins were detected in SCN tissue and cell line (Figs. 1A–1C). In comparison, BMAL, which forms heterodimers with CLK that bind to E-box sites, was equivalently expressed as a 69 kDa band in both the SCN and SCN 2.2 extracts (Fig. 1F). CRY1 and CRY2 were detected in both samples at expected molecular weights of 68 and 67 kDa, respectively (Figs. 1G and 1H). Additional cross-reactive bands were also detected in both extracts at 60 kDa for CRY1 and at 50 and 55 kDa for CRY2. The lower MW bands, recognized by both CRY antibodies, may represent proteolytic breakdown products. Similar to PER3 and CLK, both CRY1-immunoreactive bands were less intense in SCN 2.2 cells compared to rat SCN. Lastly, CKIε immunoreactive bands at 44 kDa were highly expressed in both SCN tissue and cell line (Fig. 1I).

Receptor expression. Melatonin signaling has been demonstrated to be involved in crepuscular, or dusk/dawn, regulation of circadian rhythms (33). The melatonin signal is transmitted to the SCN via two G protein-linked receptors, MT1 and MT2 (38). We utilized RT-PCR analysis to probe for *MT1* and *MT2*. As *MT1* and *MT2* are closely related, primers were designed against a significantly dissimilar region near the amino-terminal of both receptors (*MT1:* 467-485 of AF130341; *MT2:* 111-120 of AF141863). DNA sequencing of the expected 248 bp RT-PCR products for both primer sets revealed that the SCN 2.2 cell line expresses both *MT1* and *MT2* mRNA (Figs. 2A and 2B). Further analysis using *in vivo* melatonin binding and MT1/MT2 antisera will be necessary to determine whether the SCN 2.2 cell line expresses either receptor protein.

Because of the critical contribution of NMDA/glutamate ionotropic receptor (NMDAR) activation to the light/glutamate phase-shifting signal at night (31), we probed for expression of a contributory subunit of these receptors. Identical type 1 NMDAR (NMDAR1)-reactive bands at approximately 100 and 105 kDa were found in both SCN tissue and SCN 2.2 extracts. These masses correspond to the predicted molecular weight

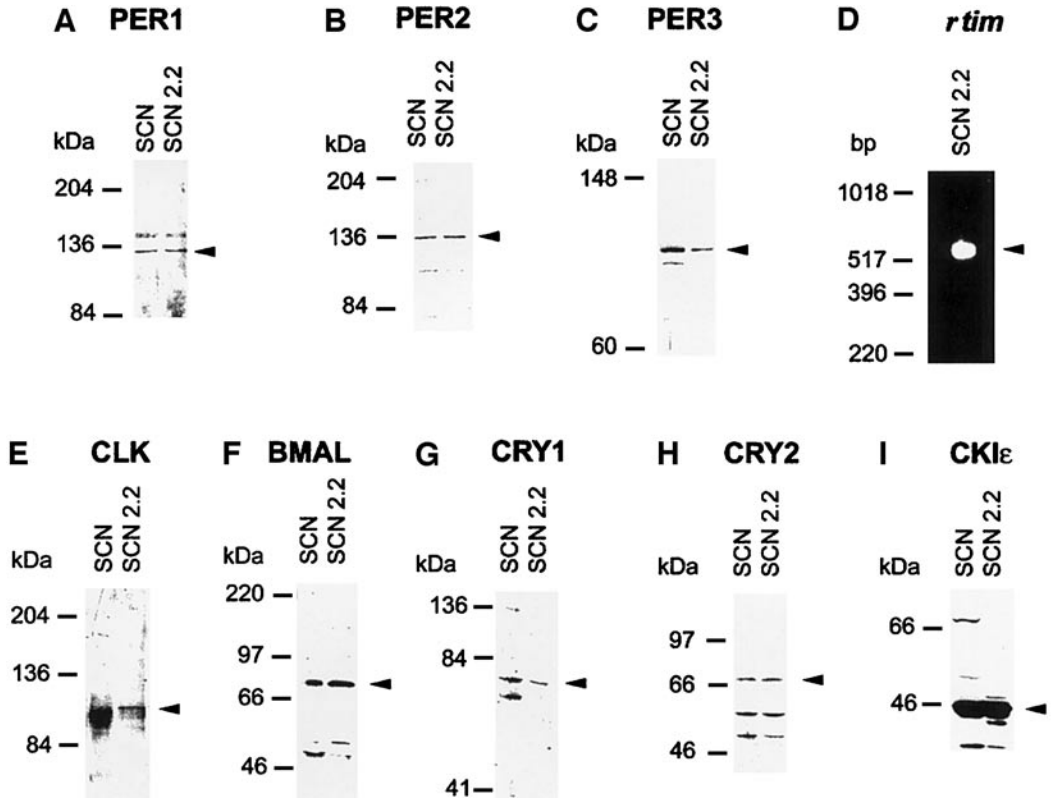

FIG. 1. Expression of putative clock genes in SCN tissue samples and differentiated SCN 2.2 cultures. Samples were probed by: A, Western analysis for PER1; B, Western analysis for PER2; C, Western analysis for PER3; D, RT-PCR analysis of SCN 2.2 for *rtim* mRNA; E, Western analysis for CLK; F, Western analysis for BMAL/MOP3; G, Western analysis for CRY1; H, Western analysis for CRY2; I, Western analysis for CKIε. ◂ Indicates expected protein or RT-PCR product bands.

of several NMDAR1-a and NMDAR1-b isoforms based on mRNA sequence (Fig. 2C). Additionally, cell line extracts expressed an immunoreactive band of equivalent intensity at 110 kDa that was only marginally detectable in SCN extracts. This band may be a NMDAR1 precursor peptide or one of the larger NMDAR1 variants. Further investigation for NMDAR2 subunits and characterization of the glutamate response by SCN 2.2 cells will indicate whether this line expresses a glutamate stimulus pathway similar to the SCN.

The light-activated signaling pathway that induces clock resetting during the early night involves Ca^{2+}-induced Ca^{2+}-release via intracellular ryanodine receptor (RYR)-regulated stores downstream from NMDAR activation by glutamate (39). Blots probed with anti-RYR antisera indicate that both SCN tissue and SCN 2.2 cell line expressed large proteins at significantly higher than 220 kDa (Fig. 2E). The expected MW of RYR is >350 kDa, which extends beyond our largest available MW marker at 220 kDa. Antisera also revealed cross-reactive bands at approximately 250 kDa that were most likely spectrin, which is also recognized by the anti-RYR antibody used in this study (L. Faiman, unpublished results). Additional lower-molecular weight bands were observed in cell line extracts and may represent breakdown products or other related proteins.

Because of the prominent role of PACAP in signal transmission from the eye to the SCN in resetting of the circadian clock mechanism during the day (29, 40, 41), SCN tissue and SCN 2.2 cell cultures were examined for expression of PACAP type 1 receptor (PAC1-R, previously PVR1). PAC1-R is a G protein-linked seven-pass transmembrane receptor that activates adenylyl cyclase (AC) and/or phospholipase C (PLC) signaling pathways (35, 36, 42). Of the eight PAC1-R isoforms, six can be differentiated using primers surrounding the PAC1-R cassette insertion region that modulates AC and PLC activity. Products with no cassette (PAC1-Rs), one cassette (PAC1-Rhip, PAC1-Rhop1 and PAC1-Rhop2) or two cassettes (PAC1-Rhiphop1 and PAC1-Rhiphop2) were expected respectively at 280, 364/361 or 448/445 bp (35, 36). We utilized these size variations to determine PAC1-R isoform expression by the SCN and SCN 2.2 cell line.

Bands for PAC1-R with one insert (approximately 364 bp) and without insert (approximately 280 bp) were found in both SCN and cell line RNA (Fig. 2F). A nonspecific 220 bp band was also observed in both

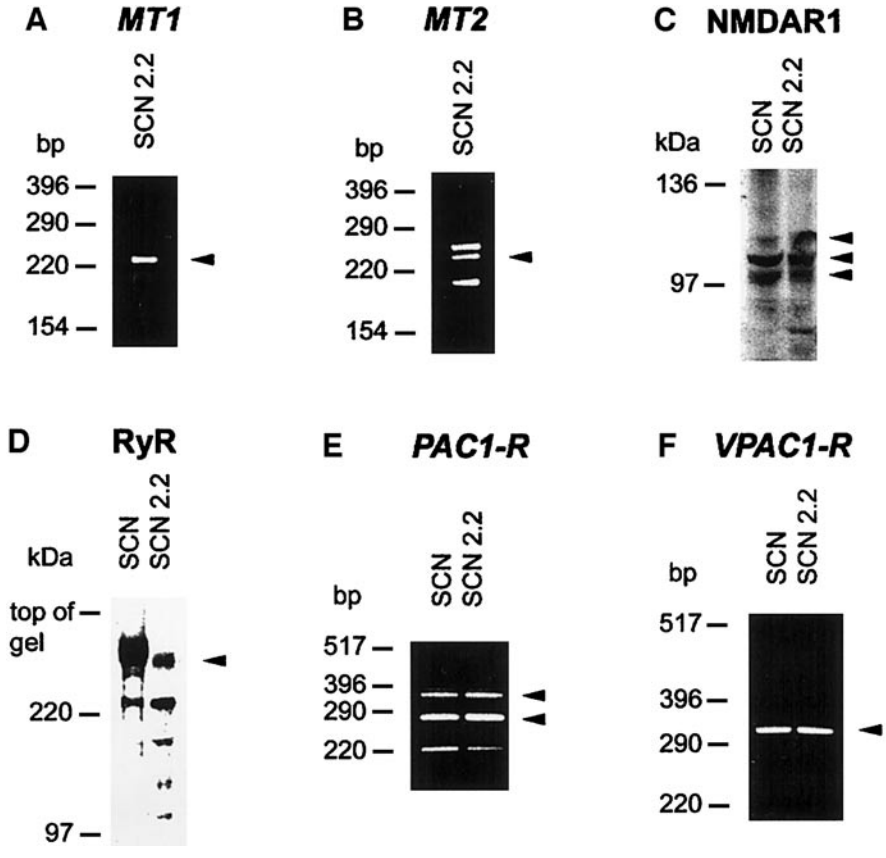

FIG. 2. Expression of receptors in SCN and differentiated SCN 2.2 cultures. Samples were evaluated by: A, RT-PCR analysis of SCN 2.2 RNA for *MT1;* B, RT-PCR analysis of SCN 2.2 RNA for *MT2;* C, Western analysis for NMDAR1; D, Western analysis for RyR; E, RT-PCR analysis for PAC1-R/PVR1/VIPR3; F, RT-PCR analysis for VPAC2-R/PVR2/VIPR2. ◂ Indicates expected protein or RT-PCR product bands.

samples. DNA sequencing confirmed the identity of the 280 bp band as PAC1-R. Double cassette insertions were not detected, yet might have been present but selected against during amplification. Cassette-specific primer sets will be required to differentiate between hip, hop1 and hop2 PAC1-R inserts.

Additionally, VIP/PACAP receptor type 2 (VPAC2-R, previously PVR2/VIP2R) was also analyzed in SCN tissue and cell line. VPAC2-R binds PACAP and VIP with equal affinity inducing AC activity (35, 36, 42). Whether VPAC2-R stimulates Ca^{2+} influx and PLC activity in mammalian tissue remains unclear (42). VPAC2-R mRNA was found by RT-PCR analysis in both samples (Fig. 2G). The expected 325 bp amplification products were also verified as VPAC2-R by DNA sequencing.

Kinase expression. Specific kinases that transduce ligand-receptor activation signals into changes of cellular state through phosphotransferase activity have been demonstrated to be necessary components of critical clock-resetting pathways. The first kinase system discovered to alter circadian state was PKA (27). Both SCN tissue and SCN 2.2 cell line expressed immunopositive bands at 54 kDa for the catalytic subunit of PKA (Fig. 3A). We have previously observed PKA immunoreactivity at this molecular weight in SCN extracts (43).

We also assayed for expression for PKG, a prominent regulator of light (39, 44) and acetylcholine (32) signaling pathways in the SCN. Anti-pan-PKG antisera, which was produced in our lab against bovine brain PKG, was used to probe SCN tissue and cell line extracts. Immunoreactive bands were observed in both SCN and cell line extracts at approximately 75 kDa (Fig. 3B) suggesting expression of a PKG type I isoform (PKGIα or PKGIβ; expected MW 76 or 78 kDa, respectively). Due to limited resolution with SDS–PAGE, isoform-specific antisera will be required to determine which PKGI subtype is expressed by the SCN and SCN 2.2 cell line. While immunoreactive bands corresponding to PKGII (expected MW 87 kDa) were not observed in either extract (Fig. 3B), this isoform has recently been identified in the SCN (45). It is likely that our antisera had insufficient affinity for PKGII or that the window of expression for this protein is very brief and therefore not evident in our extracts.

We probed protein blots for CKII and CKIV, candidate mediators of light-induced phase shifting signals

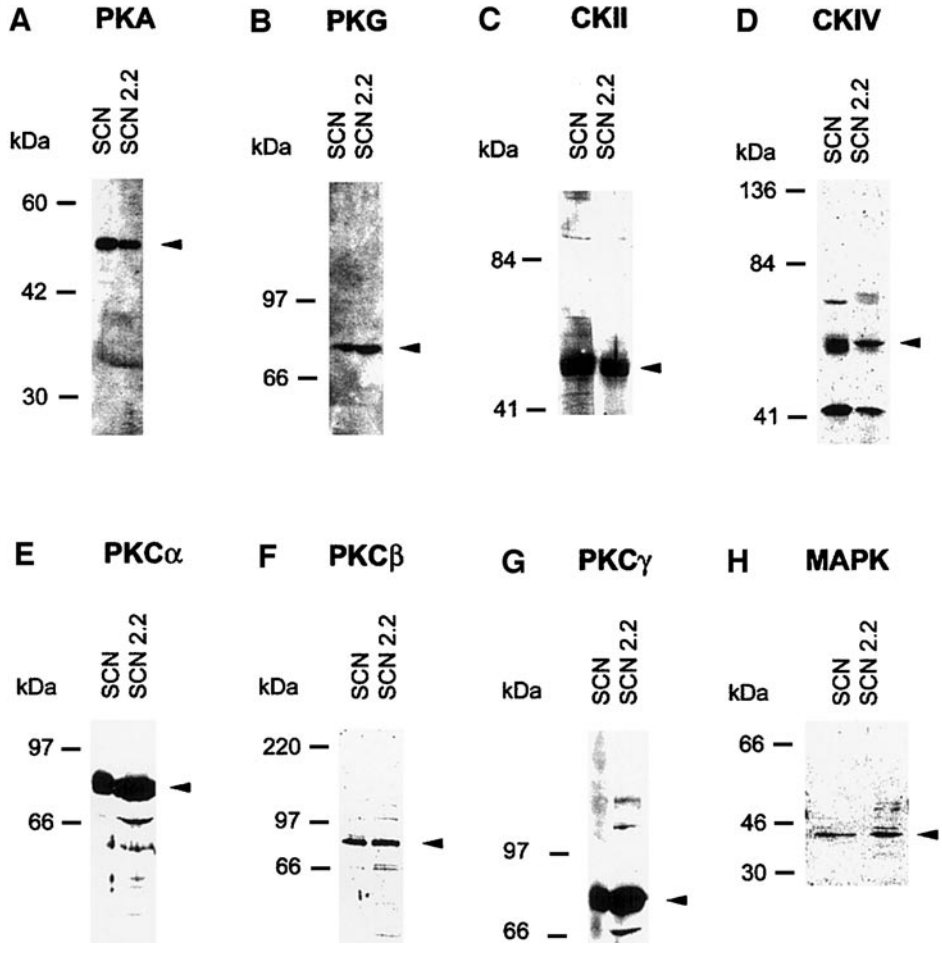

FIG. 3. Expression of kinases in SCN and differentiated SCN 2.2 cultures. Samples were probed for: A, PKA; B, PKG; C, CKII; D, CKIV; E, PKCα; F, PKCβ; G, PKCγ; H, MAPK. ◂ Indicates expected protein or RT-PCR product bands.

in the SCN (46). Both SCN and SCN 2.2 cultures expressed CKII (Fig. 3C) and CKIV (Fig. 3D) at roughly equal levels and expected sizes (MW 52 kDa and 60 kDa, respectively). Additionally, due to the role of PKC in mediating melatonin signaling (33), we tested three of eleven known PKC isoforms. Both SCN tissue and SCN 2.2 cell line expressed PKCα, PKCβ and PKCγ at equivalent levels as well as at the expected MW of 80–82 kDa for each isoform (Figs. 3E–3G). A single immunoreactive band representing MAPK, a nighttime regulator of light-induced phase-shifting, was observed at approximately 44 kDa in SCN tissue and SCN 2.2 cell line (Fig. 3H). Previously, MAPK-immunopositive bands at 42 and 44 kDa have been reported in mouse SCN tissue (47).

Expression of other signaling enzymes and SCN marker peptides. Additional known contributors to time-of-day gated signaling cascades were evaluated in the immortalized SCN cells. Bands against neuronal NOS, a mediator of nighttime phase shifts induced by light (31), were evident at 155 kDa in SCN 2.2 extracts, consistent with SCN tissue and previous reports (Fig. 4A) (48). During the circadian night-phase, activation of heme oxygenase-2 (HO-2) mimics cGMP phase shifting and is downstream of acetylcholine signaling (49). HO-2 was expressed in both extracts at an expected

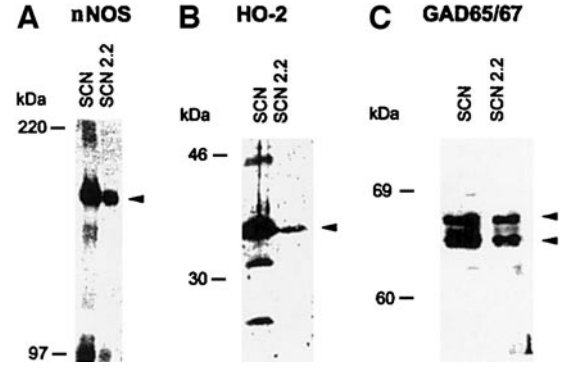

FIG. 4. Expression of signaling enzymes in SCN and differentiated SCN 2.2 cultures. Samples were probed for: A, nNOS; B, HO-2; C, GAD65/67. ◂ Indicates expected protein bands.

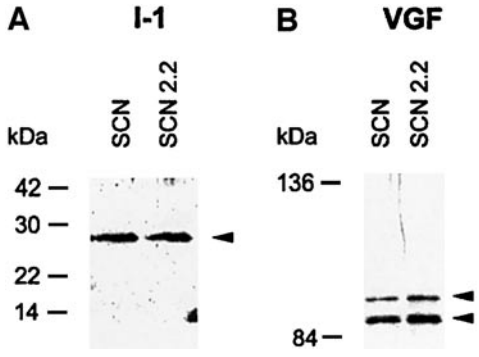

FIG. 5. Expression of SCN marker proteins in SCN and differentiated SCN 2.2 cultures. Samples were probed for: A, I-1; B, VGF. ◂ Indicates expected protein bands.

MW of 36 kDa, but levels were 3–5-fold higher in the SCN than in the cell line (Fig. 4B).

GABA is found in virtually all SCN cell bodies (50) and is known to modulate light-induced phase shifts (51–53). Additionally, because GAD isoforms exhibit circadian variation of expression in the SCN (54), we examined the SCN 2.2 cell line with anti-GAD65/67 antisera. GAD isoforms were detected at 65 and 67 kDa in roughly equal amounts within each type of extract, although SCN levels of both enzyme subtypes were higher than those found in SCN 2.2 cells (Fig. 4C).

Western analysis using antisera against I-1, which is highly expressed by the SCN but not in other regions of the hypothalamus (55, 56), revealed robust expression of a 28 kDa band in both SCN tissue and cell line (Fig. 5A). Cross-reactivity with closely related DARPP-32 was not detected in either type of extract (55, 56). Additionally, VGF, which is strongly expressed in the SCN, but only weakly in the neighboring magnocellular paraventricular (PVN) and supraoptic nuclei (SON) of the hypothalamus was also used as a SCN marker peptide (57–59). Both SCN tissue and SCN 2.2 cells expressed VGF-immunoreactive bands at 90 kDa (Fig. 5B). A second 100 kDa cross-reactive band was also evident in both sample types. The 100 kDa band may be a pre-VGF peptide, modified VGF or possibly another closely-related protein (57–59).

DISCUSSION

Gene product expression common to SCN tissue and SCN 2.2 cell line. The SCN 2.2 cell line expressed gene products characteristic of the SCN tissue from mature rats. These included putative clock gene products, circadian regulatory components and SCN marker proteins. Based on the equivalent loading of protein from SCN tissue and SCN 2.2 cultures on each blot, most of the assayed proteins were expressed at roughly equal levels in both the cell line and SCN tissue.

Although nine putative clock-associated gene products were found in the SCN 2.2 cell line, several differences were observed between expression patterns in the SCN tissue and cell line. Relative levels of CLK were higher in the SCN than in SCN 2.2 cultures. Decreased expression of this positive transcriptional regulator had no apparent effect on PER1 or PER2 levels but may contribute to lower PER3 in SCN 2.2 cells. Interestingly, SCN tissue was also distinguished by higher levels of CRY1 as well as an additional 60 kDa band that was not detected in the cell line. Decreased CRY1 levels may offset the effects of diminished CLK expression in the cell line because CRY1 acts as negative regulator of E-box promoters. This may explain why SCN and cell line express equivalent PER1 and PER2 levels. Despite these differences, SCN 2.2 cultures still express robust rhythms of BDNF, NT-3, *mPer1-luc* transgene and metabolic activity, indicating that the lower CLK and CRY1 levels do not impede circadian cycling (5, 6). Although CRY2 levels were roughly equal in both rat SCN and SCN 2.2 cell line, additional unexpected bands were found in both extracts at approximately 50 kDa and 55 kDa. Cross-reactive bands were generally observed when using antisera against most clock genes in both SCN and cell line extracts. These bands may be proteolytic breakdown products or non-specific cross-reactivity due to common sequences found in the peptides used to generate antisera. However, it is possible that these bands may represent alternate clock gene isoforms or splice variants.

SCN 2.2 cell cultures also expressed daytime regulatory elements of circadian clock function. RT-PCR analysis revealed expression of PAC1-R isoforms in both SCN and cell line RNA. Although double cassette insertions of PAC1-R were not detected, mRNA for these receptor isoforms may be present at very low levels in both tissues. Additionally, VPAC2-R mRNA was expressed in both the SCN and SCN 2.2 cell line. The presence of type 1 and 2 PACAP receptors indicates that PACAP could modulate both cAMP and PLC pathways in both the intact SCN and SCN 2.2 cell line. Observed expression of PKA catalytic subunit in SCN and SCN 2.2 cultures (54 kDa) significantly differed from the 40–42 kDa generally observed in bulk rat brain extracts and predicted by PKA (α, β or γ subtypes) amino acid sequence. Expression of this molecular weight variant of PKA also supports the "SCN-like" character of the SCN 2.2 line. Although the SCN 2.2 line did express PKA levels equivalent to that observed in the SCN (43), whether this variant plays a specific role in circadian regulation remains to be determined.

While dark-phase and crepuscular regulators of SCN circadian function were found in the SCN 2.2 cell line, expression of several gene products in this line were of particular interest. It is significant that both SCN tis-

sue and SCN 2.2 cell line were found to express PKG type 1, as determined by molecular weight. This result is noteworthy because PKG expression is rare in cell lines (60–64). Furthermore, expression of neuronal NOS is similarly rare in cell lines, but was also observed in SCN 2.2 cultures. Finally, it is somewhat surprising that RT-PCR analysis revealed expression of both *MT1* and *MT2* melatonin receptor mRNA in SCN 2.2 cells since 2-iodomelatonin binding, a standard indicator of melatonin receptor expression, has not been reported in this line. Analysis of protein expression for receptor subtypes and of downstream elements in melatonin signal transduction may clarify the issue of whether these mRNA are translated into functional melatonin receptors in SCN 2.2 cells.

Our results with various marker proteins also indicate that the SCN 2.2 cell line consists of SCN-like, rather than general hypothalamic, cell types. Within the hypothalamus, I-1 is predominately expressed by the SCN with only sparse immunoreactivity in other regions (55, 56). SCN 2.2 cultures expressed I-1 bands comparable to those in SCN tissue. Interestingly, when phosphorylated by PKA, I-1 is a potent inhibitor of serine/threonine protein phosphatase-1 (PP-1). Additionally, I-1 is dephosphorylated by Ca^{2+}/calmodulin-dependent protein phosphatase 2B (PP2B). Therefore, I-1 is a node for cross-regulation by cAMP and Ca^{2+} signaling pathways and may play a role in integrating convergent signals that mediate circadian regulation (55, 56).

VGF was also found in both tissue and cell line extracts. Although VGF was initially characterized as a gene product induced in PC12 cells by nerve growth factor (NGF) (58), this peptide is strongly expressed by both the ventrolateral and dorsomedial SCN, but only weakly in the neighboring magnocellular paraventricular and supraoptic nuclei (57–59). This also supports the "SCN-like," rather than general hypothalamic, character of the SCN 2.2 cell line.

Lastly, this cell line expressed the GAD 65/67 enzyme, which produces the inhibitory neurotransmitter GABA. GABA is found in all SCN cell bodies and is the most intensely expressed neurotransmitter within the SCN (50). GABA may modulate photic phase shifting signals (51–53) and GABA-producing enzymes, GAD 65 and GAD 67, exhibit circadian variation of expression in the SCN (54).

Central clocks, peripheral oscillators and cell lines. Studies of mammalian clock genes have been accompanied by a growing recognition of significant differences between the central circadian pacemaker and peripheral biological oscillators. The central circadian clock, localized to the SCN, displays spontaneous and predictable circadian expression patterns in the absence of external stimuli. The central clock drives peripheral oscillators via neural and/or humoral signals (65, 66). Peripheral tissues, as found in liver and skeletal muscle, require zeitgebers (synchronization signals) to induce and maintain sustained rhythmic oscillations. Additionally, peripheral oscillators display PER gene oscillations that are phase-delayed 3–9 h relative to the SCN (4, 65). In SCN-lesioned rats, RNA oscillations in peripheral tissues are abolished (67). Furthermore, circadian rhythms of *PER1* promoter-driven luciferase in rat liver and skeletal muscle dampen within 2–7 cycles after removing the tissue from animal and maintaining it in culture (68). These data suggest that peripheral oscillators are driven, directly or indirectly, by signals from the central circadian clock in the SCN (65, 66). The basis for the functional differences between the central clock and peripheral oscillators remains to be determined (4).

Therefore, to provide a valid model for the central circadian pacemaker in the SCN, a cell line must display rhythmic gene expression, rescue circadian rhythms in SCN-lesioned animals and express circadian regulatory pathways that characterize the SCN. Rhythmic expression of several circadian gene products in response to serum-shock or other potent stimuli has been demonstrated in NIH/3T3 cells, rat-1 fibroblasts and spontaneously immortalized embryonic mouse fibroblasts (1–4, 65). Consistent with the derivation of these lines from peripheral tissues, these circadian oscillations dampen within a few days (4, 65). Furthermore, many of these cell lines do not express known circadian regulatory components of the central pacemaker or are relatively uncharacterized in this regard.

In contrast, the SCN 2.2 line is characterized by properties that define the central circadian pacemaker located in the SCN (5, 7). SCN 2.2 cultures are capable of endogenously generating robust 24-h rhythms of brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT3) content as well as 2-deoxyglucose (2-DG) uptake without exogenous stimulation. The phase relationships among these rhythms in culture are similar to those seen in adult SCN tissue *in vivo* where 2-DG levels peak 12 h out-of-phase with peak levels of BDNF expression (5).

Neural transplantation and co-culture studies also demonstrate conservation of clock properties in the SCN 2.2 cell line (5, 6). Circadian rhythms of wheel-running activity are abolished in SCN-lesioned rats. However, when SCN 2.2 cells are grafted into the third ventricle near the SCN-ablation site, circadian locomotor rhythms are restored within 4 to 10 days (5). The strength of the restored rhythm positively correlates with SCN 2.2 cell survival in the graft. Grafts of immortalized mesencephalic cells or of NIH/3T3 fibroblasts both fail to rescue circadian rhythmicity in SCN-lesioned animals (7). Of particular interest is the recent finding that SCN 2.2 cells act as central pace-

makers in driving circadian oscillations in NIH/3T3 co-cultures (6).

The extent to which expression of known circadian regulatory components in cell lines resembles that found in the native SCN remains undetermined. Some putative clock genes, such as the *Per* genes, are expressed in several mammalian cell lines. Furthermore, one would also expect that ubiquitously expressed neuronal elements would be found in cell lines of neural origin. However, previous studies have indicated that some circadian regulatory components found in SCN 2.2 cells are rarely expressed in cell culture, even in lines derived from neurons. Primary examples of this are PKG and nNOS, both of which are uncommon to cell lines (60–63). Additionally, phosphatase inhibitor 1 (I1) is not normally observed in NIH/3T3 fibroblasts (69). Finally, although melatonin receptors are found in some cell lines, they have not been previously reported in NIH/3T3 cells, rat-1 fibroblasts or SCN 2.2 lines (70–72).

In summary, the present investigation of SCN antigens in differentiated SCN 2.2 cultures complements recent reports and indicates that this cell line generates an array of signaling molecules and gene products similar to the SCN *ex vivo.* These phenotypic characteristics of SCN 2.2 cells, coupled with their ability to generate spontaneous circadian oscillations, to restore rhythmicity to SCN-lesioned hosts and to drive oscillations in NIH/3T3 cells (5, 6), supports this line's utility as a promising model for distinguishing distinct regulatory mechanisms underlying the central pacemaker and for identifying signaling molecules that drive oscillations in peripheral tissues.

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