Immortal Time: Circadian Clock Properties of Rat Suprachiasmatic Cell Lines

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Cell lines derived from the rat suprachiasmatic nucleus (SCN) were screened for circadian clock properties distinctive of the SCN in situ. Immortalized SCN cells generated robust rhythms in uptake of the metabolic marker 2-deoxyglucose and in their content of neurotrophins. The phase relationship between these rhythms in vitro was identical to that exhibited by the SCN in vivo. Transplantation of SCN cell lines, but not mesencephalic or fibroblast lines, restored the circadian activity rhythm in arrhythmic, SCN-lesioned rats. Thus, distinctive oscillator, pacemaker, and clock properties of the SCN are not only retained but also maintained in an appropriate circadian phase relationship by immortalized SCN progenitors.

Recent progress has unveiled the identities and distribution of putative molecular components of the mammalian circadian clock. Orthologs of the *Drosophila period* gene, m*Per1*, m*Per2*, and m*Per3*, and the mouse *Clock* gene express transcripts that are localized and regulated by light within the known site of the circadian pacemaker in the hypothalamic SCN (*1*–*5*). However, the distribution and circadian expression of transcripts encoded by these genes are not restricted to the SCN or to known components of the vertebrate clock (*2*, *6*, *7*). The widespread spatial pattern of molecular oscillations leads to at least two critical questions: Is the oscillatory behavior of clock-related genes in peripheral, nonclock tissues strictly indicative of their function as components of the circadian pacemaker mechanism, and if rhythmicity in the periphery persists independent of SCN regulation, then what is the function of these molecular oscillations within the SCN? In essence, what specific properties distinguish an "oscillator," a "pacemaker," and a "clock"?

To explore this issue, we generated immortal cell lines from the anlage of the rat SCN. Similar to other neural cell lines, immortalized SCN cells are characterized by the conservation of many biochemical properties that distinguish mature parental cell types. For example, these lines express neurotrophins and neuropeptides found within the SCN in vivo (*8*). If immortalized SCN progenitors also retain the distinctive functional properties of the SCN, then indices of their cellular activity should oscillate with a circadian periodicity and recovery of circadian behavioral rhythmicity should occur after their transplantation into arrhythmic, SCN-lesioned animals.

Because rhythmic utilization of 2-deoxyglucose (2-DG) is a well-documented circadian property of SCN activity (*9*, *10*), immortalized cells were assessed for evidence of oscillatory behavior in this index of cellular metabolism (*11*). The capacity of immortalized cells to generate circadian rhythms in the expression of brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) was also examined concurrently (*12*) because these neurotrophins are found in the SCN and BDNF levels oscillate on a circadian basis in vivo (*13*, *14*). Glucose utilization in immortalized SCN cells was characterized by robust circadian rhythmicity in both the uptake of 2-DG and phosphorylation to 2-DG-6-phosphate (2-DG-6P) for two cycles in vitro (Fig. 1A). Throughout both cycles, 2-DG-6P concentrations were consistently maintained at 70 to 80% of the values for 2-DG uptake. The circadian profiles of 2-DG and 2-DG-6P concentrations in immortalized cells were contemporaneous, with peak values at 68 and 92 hours after plating of the cultures. Maximal concentrations of 2-DG and 2-DG-6P were two to three times greater than the corresponding minimum for both rhythms. In contrast to the rhythmic profiles of 2-DG uptake and 2-DG-6P concentrations, accumulation of labeled free 2-DG and glycogen remained at constant basal concentrations in immortalized cells. SCN2.2 cells also exhibited circadian fluctuations in BDNF and NT-3 content, with a three- to sixfold difference between peak and minimum levels of these neurotrophins (Fig. 1B). The circadian patterns of BDNF and NT-3 content were expressed in an inverse phase relationship; when BDNF content reached peak values, NT-3 levels were near their minimum. The circadian maxima in NT-3 content recurred 8 hours in advance of the rhythmic crest in BDNF levels. Despite the differences in their circadian profiles, the rhythms in glucose utilization and neurotrophin expression were invariably locked in the same phase relationship with regard to each other and the time of plating across different experiments. On three separate occasions, the inaugural crests in NT-3 and BDNF levels and glucose utilization occurred at 48, 56, and 68 hours, respectively, after cell plating, with recurrent peaks every 24 hours thereafter. The 12-hour antiphase relationship between the

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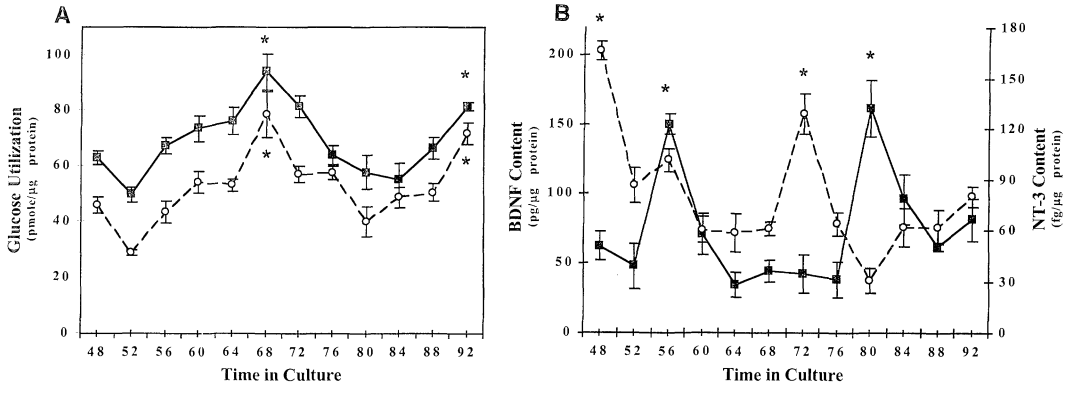

Fig. 1. Circadian patterns of glucose utilization and neurotrophin expression in confluent cultures of SCN2.2 cells (N = 5). **(A)** 2-DG (solid line, ■) uptake and conversion to 2-DG-6P (dashed line, ○). **(B)** BDNF (solid line, ■) and NT-3 (dashed line, ○) content. Symbols denote mean (±SEM) determinations at 4-hour intervals. Asterisks indicate sampling intervals in (A), during which 2-DG and 2-DG-6P values were significantly greater ($P < 0.05$) than those observed during the three preceding intervals, and in (B), during which peak values for BDNF and NT-3 content were significantly greater ($P < 0.05$) than the three succeeding minima.

rhythms of glucose utilization and BDNF content in immortalized SCN cells is similar to that observed in the SCN in vivo; the rat SCN is characterized by maximal 2-DG uptake at circadian time (CT) 6 (*9*) and peak BDNF content around CT 18 (*14*). Any potential association between the growth cycle and oscillatory behavior of SCN2.2 cells is unlikely because their generation time of 28 hours is distinctly longer than the circadian periodicity of the glucose utilization and neurotrophin rhythms. Furthermore, this cell line is distinguished by the arrest of proliferative activity and DNA synthesis upon establishment of contact between neighboring cells in confluent cultures (*8*).

The neural transplantation technique was also used to examine circadian pacemaker and clock properties of SCN2.2 cells in vivo (*15*). In these experiments, immortalized cells were grafted near the ablation site in SCN-lesioned rats exhibiting arrhythmicity or ultradian rhythms in their wheel-running activity for at least 6 weeks (N = 10). The circadian rhythm of wheel-running behavior was restored within 4 to 10 days after transplantation in five of the arrhythmic hosts receiving SCN2.2 cell grafts (Fig. 2A). In these animals, the free-running period of the restored rhythms (mean = 24.02 ± 0.02 hours) was typically shorter than that observed before ablation of the SCN (mean = 24.12 ± 0.09 hours). Complete destruction of the host SCN was confirmed by the absence of immunostaining for vasoactive intestinal polypeptide (VIP), gastrin-releasing peptide (GRP), and arginine vasopressin (AVP) (*16*). Functional correlates of SCN2.2 cell grafts in promoting the recovery of circadian rhythmicity were correlated with graft viability and the expression of SCN-like phenotype. Animals with restored circadian behavior were distinguished by viable grafts expressing neuropeptides or neurotrophins found in the SCN in vivo (*13*, *17*). These SCN2.2 cell grafts were located in the host third ventricle region as a single aggregate containing small clusters of VIP-, GRP-, AVP-, or BDNF-immunopositive perikarya (Fig. 3). In contrast, immortalized cell grafts that failed to restore circadian rhythmicity in the remaining SCN-lesioned rats (N = 5) were characterized by low cell survival or were devoid of SCN-like elements. The restoration of circadian wheel-running behavior was not observed in SCN-lesioned animals that received control transplants of either E1A-immortalized mesencephalic cells (N = 6) or NIH 3T3 fibroblasts (N = 6; Fig. 2B), although viable grafts were confirmed in subsequent histological analysis.

Like other cell lines (*7*) and many peripheral insect and mammalian tissues (*6*, *18*), our immortalized cells derived from the SCN generate circadian rhythms in vitro and thus will be valuable in studying the molecular mechanisms for circadian oscillations. However, our immortalized SCN cells are distinguished by their capacity to confer rhythmicity to the organism, similar to the circadian pacemaker function of the SCN in vivo. These results underscore fundamental distinctions between a circadian oscillator and clock. Whereas an "oscillator" is

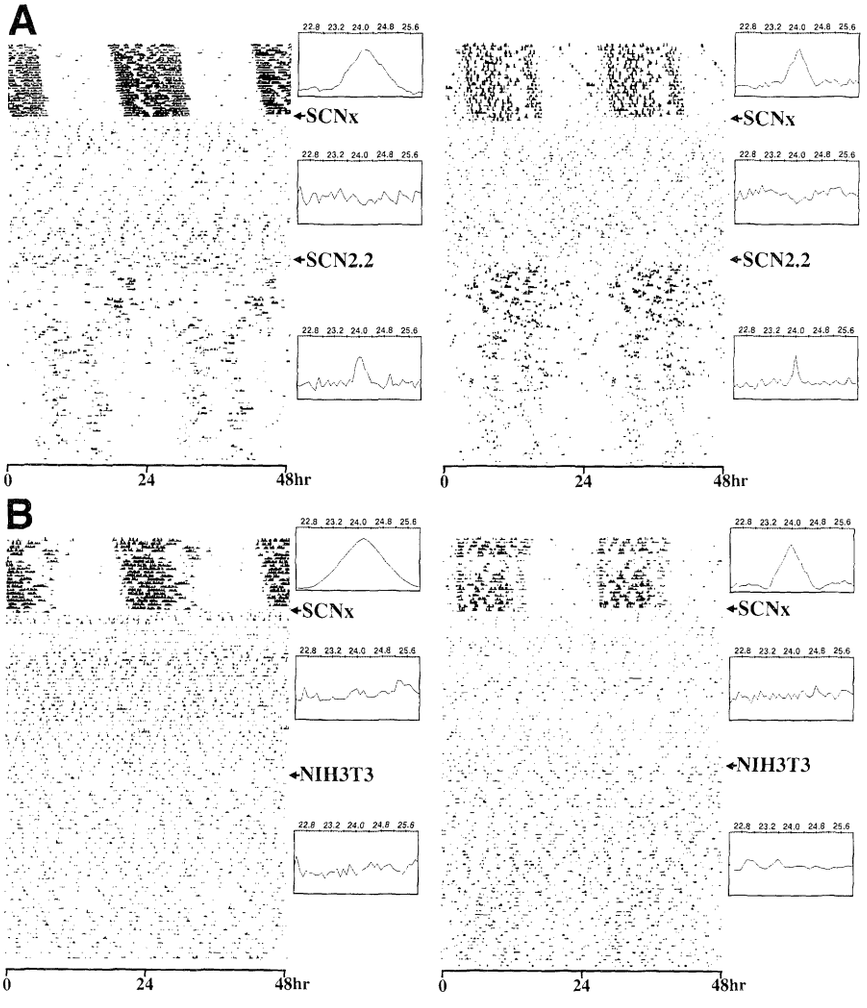

Fig. 2. (**A**) Restoration of circadian wheel-running activity in two SCN-lesioned rats by grafted SCN2.2 cells. In both intact hosts, the endogenous period of the activity rhythm was 24.1 hours in constant dim illumination. The activity patterns were rendered arrhythmic by bilateral SCN ablation (SCNx), and circadian rhythmicity (period = 24.0 hours) was later restored in both animals by SCN2.2 cell grafts. (**B**) Activity records of two lesioned, arrhythmic rats receiving grafts of NIH 3T3 cells. Intact hosts exhibited activity rhythms with periods of 24.1 and 24.0 hours. Activity rhythms of both animals were abolished by SCN lesions, and this arrhythmicity persisted after transplantation of NIH 3T3 cells. Periodogram analyses of the data during the last 15 to 25 days of the intact, lesioned, and grafted intervals are shown on the right of each record.

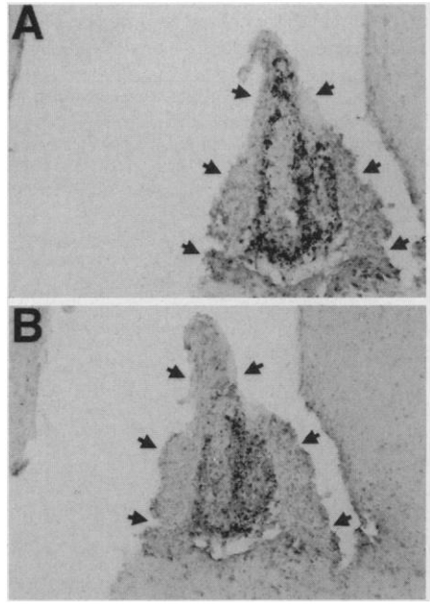

Fig. 3. Photomicrographs depicting immunocytochemical localization of VIP (**A**) and GRP (**B**) in transplanted SCN2.2 cells located within the third ventricle of an SCN-lesioned host. Arrows delineate graft location.

merely a device that oscillates, a "clock" represents "[a]ny instrument for measuring or indicating time" and a "pacemaker" is defined as a process or substance that "regulates" the timing of other events (*19*). Although the capacity to oscillate is a widely distributed property, the restoration of circadian rhythmicity in SCN-lesioned, arrhythmic hosts by immortalized SCN cells but not NIH 3T3 mouse fibroblasts implies that only oscillators derived from the SCN act as pacemakers and have the capability to impose their rhythmicity on mammalian behavior. How these oscillators in the SCN drive rhythms in behavior is unclear at this point, but there is increasing evidence indicating that the SCN secretes a diffusible factor that at least in part contributes to this rhythmic efflux (*20*). Perhaps one of these factors is a neurotrophin such as BDNF or NT-3, based on their rhythmic expression in immortalized SCN cells and the SCN in vivo (*14*).

References and Notes

1. U. Albrecht, Z. S. Sun, G. Eichele, C. C. Lee, *Cell* **91**, 1055 (1997).
2. L. P. Shearman, M. J. Zylka, D. R. Weaver, L. F. Kolakowski, S. M. Reppert, *Neuron* **19**, 1261 (1997).
3. Y. Shigeyoshi *et al.*, *Cell* **91**, 1043 (1997).
4. Z. S. Sun *et al.*, *ibid.* **90**, 1003 (1997).
5. H. Tei *et al.*, *Nature* **389**, 512 (1998).
6. M. J. Zylka, L. P. Shearman, D. R. Weaver, S. M. Reppert, *Neuron* **20**, 1103 (1998).
7. A. Balsalobre, F. Damiola, U. Schibler, *Cell* **93**, 929 (1998).
8. D. J. Earnest *et al.*, *J. Neurobiol.*, in press.
9. W. J. Schwartz, in *Suprachiasmatic Nucleus: The Mind's Clock*, D. C. Klein, R. Y. Moore, S. M. Reppert, Eds. (Oxford Univ. Press, New York, 1991), pp. 144–156.
10. G. C. Newman and F. E. Hospod, *Brain Res.* **381**, 345 (1986).
11. The SCN2.2 cell line is derived from fetal progenitors of the rat SCN (embryonic day 15) immortalized with the adenovirus E1A gene (*8*). Cells derived from a single passage were expanded onto multiple dishes (60 mm) coated with mouse laminin and maintained in minimum essential medium containing 10% fetal bovine serum, glucose (2 μg/ml), and L-glutamine (292 μg/ml) under constant temperature (37°C) and 5% CO_2. At 4-hour intervals for 2 days, confluent cultures ($N = 5$) were incubated for 1 hour with ^{14}C-labeled 2-DG (0.2 mCi/ml; American Radiological Company, St. Louis, MO). Fractional products of 2-DG metabolism were measured with the methods described by Newman and colleagues [G. C. Newman, F. E. Hospod, C. S. Patlak, *J. Cereb. Blood Flow Metab.* **10**, 510 (1990)]. Fractions derived from 2-DG, 2-DG-6P, free 2-DG, and glycogen compartments were placed in scintillation vials in triplicate, dried before addition of scintillant, and then counted on a Beckman scintillation counter.
12. Portions (25 μl) of recovered protein from these samples were also assayed in triplicate for BDNF and NT-3 content by enzyme-linked immunosorbent assay (*2*). The intra-assay and interassay coefficients of variation were less than 5 and 10%, respectively. BDNF and NT-3 levels were quantified within the linear range of their standard curves (1 to 250 ng/ml and 4.7 to 300 pg/ml, respectively). The antibodies to BDNF and NT-3 in these assays show less than 2% cross reactivity with structurally similar neurotrophins (at 10 μg/ml). Determinations of ^{14}C-labeled 2-DG uptake and neurotrophin content were normalized for sample protein content as measured by the bicinchoninic acid method (Pierce). This analysis was replicated on three separate sets of cultures. Time-dependent alterations in glucose utilization and neurotrophin levels were identified with one-way analysis of variance, and differences between determinations at distinct time points were tested post hoc for significance with the Newman-Keuls sequential range test.
13. F.-Q. Liang, F. Sohrabji, R. Miranda, B. Earnest, D. Earnest, *Exp. Neurol.* **151**, 184 (1998).
14. F.-Q. Liang, R. Walline, D. J. Earnest, *Neurosci. Lett.* **242**, 89 (1998).
15. Male adult Sprague-Dawley rats (150 to 200 g) were housed in individual cages, and wheel-running activity was continuously recorded with Dataquest IV software (Data Sciences, St. Paul, MN). Animals were exposed to a standard 12 hour light:12 hour dark photoperiod (LD) for 7 to 10 days and then maintained under dim constant light (LL; 5 to 10 lux). After 2 to 4 weeks of baseline recording, animals were anesthetized (Xylazine, 2 mg/kg; Ketamine, 10 mg/kg), and with the use of stereotaxic coordinates, SCN lesions were generated by current injection (4 mA for 15 s) through a Teflon-coated tungsten wire (0.203 mm). Animals exhibiting a loss of circadian rhythmicity in their activity behavior for 6 to 8 weeks received transplants of either SCN2.2 cells, E1A-immortalized mesencephalic progenitors, or NIH 3T3 fibroblasts. Colonies of these cell lines were harvested by enzymatic (0.125% trypsin) disaggregation and immediately transplanted as aggregates of 200,000 cells in Hanks' balanced salt solution into the periventricular third ventricle region of anesthetized recipients. Some cultures were preincubated with the carbocyanine dye DiI (12 μg/ml) to allow selective identification of transplanted cells in host brain sections by fluorescence microscopy. Activity records during SCN intact, postlesion, and posttransplantation intervals were separately analyzed for evidence of circadian rhythmicity with χ^2 periodogram (Tau) and fast Fourier transform analyses. After behavioral analysis, animals were killed with sodium pentobarbital (3.0 mg/kg) and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer. Animal care and procedures were performed in compliance with state law, federal statute, and NIH policy.
16. Brains were prepared for histological processing as described previously (*13*). Coronal sections (30 μm) were separately processed for immunocytochemical analysis with antibodies to GRP, VIP (1:1500 and 1:5000, respectively; Peninsula Laboratories, Belmont, CA), AVP (1:10,000; Arnel Labs, New York), or BDNF (1:500; Promega, Madison, WI). No immunostaining for these antigens was observed within the host brain or grafts when the primary antisera was omitted or preincubated with homologous antigen (10^{-5} M), except for occasional light staining in the area of gliosis surrounding the lesion site.
17. R. Y. Moore, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **42**, 2783 (1983).
18. J. D. Plautz, M. Kaneko, J. C. Hall, S. A. Kay, *Science* **278**, 1632 (1997).
19. *American Heritage Dictionary of the English Language* (Houghton Mifflin, Boston, MA, 1979).
20. R. Silver, J. LeSauter, P. A. Tresco, M. Lehman, *Nature* **382**, 810 (1996).

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Horizontal Propagation of Visual Activity in the Synaptic Integration Field of Area 17 Neurons

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The receptive field of a visual neuron is classically defined as the region of space (or retina) where a visual stimulus evokes a change in its firing activity. At the cortical level, a challenging issue concerns the roles of feedforward, local recurrent, intracortical, and cortico-cortical feedback connectivity in receptive field properties. Intracellular recordings in cat area 17 showed that the visually evoked synaptic integration field extends over a much larger area than that established on the basis of spike activity. Synaptic depolarizing responses to stimuli flashed at increasing distances from the center of the receptive field decreased in strength, whereas their onset latency increased. These findings suggest that subthreshold responses in the unresponsive region surrounding the classical discharge field result from the integration of visual activation waves spread by slowly conducting horizontal axons within primary visual cortex.

The average size of the minimal discharge field (MDF) in area 17 neurons is ~2° of visual angle (for the representation near the area centralis) when it is mapped with a small spot or slit of light (*1*, *2*). The strength of the spiking response results from the amplification of the feedforward thalamo-cortical drive by a local recurrent intracortical loop that preserves the retinotopic mapping of visual input onto cortex (*1*, *3*). However, firing responses to stimuli presented within the MDF can also be modulated by the concomitant stimulation of its surround, over a region up to 10° of relative eccentricity (*4*, *5*). These

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