Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 7090–7094, August 1992
Neurobiology

Disruption of circadian regulation by brain grafts that overexpress Alzheimer β/A4 amyloid

(Alzheimer dementia/circadian rhythms/genetic transfection/suprachiasmatic nuclei)

BARBARA TATE*†‡§, KAREN S. ABOODY-GUTERMAN†¶, ALISON M. MORRIS†‖, ELISABETH C. WALCOTT†‡**, RONALD E. MAJOCHA*†‡, AND CHARLES A. MAROTTA*†‡§††

*Department of Psychiatry and ††Neuroscience Program, Harvard Medical School, Boston, MA 02115; †Neurobiology Laboratory, Psychiatry Service, Massachusetts General Hospital, Boston, MA 02114; and ‡Mailman Research Center, McLean Hospital, Belmont, MA 02178

Communicated by Seymour S. Kety, May 7, 1992 (received for review December 15, 1991)

ABSTRACT **Alzheimer disease patients exhibit irregularities in the patterns of normally circadian (daily) rhythms. Alzheimer-type pathology has been reported in the hypothalamus and in the suprachiasmatic nuclei, the putative site of the circadian oscillator. We examined the relationship between the neuropathology of Alzheimer disease, as modeled by an animal system, and circadian dysregulation by grafting genetically transformed cells that overexpress β/A4 amyloid into the suprachiasmatic nuclei of adult rats. Grafts of β/A4-positive cells, but not of control cells, significantly altered the pattern of activity of implanted rats. Although experimental conditions included light–dark cycles that normally tend to drive rats to 24-h rhythms, animals with grafts of β/A4-positive cells showed abnormally high levels of activity during the light phase in addition to a disrupted circadian pattern. Periodogram analysis demonstrated significant rhythms outside of a circadian range. The body temperature rhythm of these animals was also weak 6 weeks after grafting; however, unlike activity patterns, body temperature regained a circadian period by 8 weeks after cell implantation. These data indicate that disruption of circadian activity is a behavioral measure of the consequences of β/A4 accumulation in brain implants.**

Alzheimer disease (AD) is a progressive dementing illness of the elderly, characterized by a dramatic decline in cognitive function (1). However, the behavioral abnormalities of AD are not limited to cognitive deficits. Irregular patterns of normally circadian (24 h) rhythms are also observed and the severity of dementia has been correlated with the degree of disturbance in the circadian rest–activity rhythm (2). As a consequence, the circadian pattern of sleep–wake cycles (3) and activity (4) that are disrupted in AD hinder clinical management (5). Studies on the interaction of cognition and the circadian system suggest that abnormal rhythms may contribute to the decline in mental function in AD (6). However, the magnitude of disruption displayed by different rhythms is selective in AD patients since the locomotor rhythm is affected, while the body temperature rhythm appears to remain intact (3, 7).

The suprachiasmatic nuclei (SCN) of the hypothalamus, the putative central sites of regulation of circadian rhythms in mammals, appear to be compromised in AD. Alzheimer-type pathology, including amyloidotic senile plaques, has been reported in the surrounding hypothalamus (8, 9). The nucleus itself contains neurofibrillary tangle-bearing neurons (10) accompanied by dramatic decreases in the volume and the cell number (11).

The relationship between the neuropathology of AD and circadian dysregulation is currently not described. Hypothalamic pathology, in addition to the intranuclear degeneration, may contribute to the circadian abnormalities of the AD victim. We explored the issue of circadian dysregulation by implanting genetically transformed cells that overexpress the β/A4-C-terminal region of the amyloid precursor protein (APP) (12) into the SCN of adult rats. Similar to the described disruption of circadian rhythms in AD patients, rats with grafts of β/A4-positive cells displayed persistent disruption of circadian locomotor rhythm and but not of body temperature rhythm.

MATERIALS AND METHODS

Animals. Two-month-old Sprague–Dawley female rats (Charles River Breeding Laboratories) were used in the following experiments. Animals were individually housed in standard laboratory cages in light- and temperature-controlled rooms with lighting schedules of 12 h of light and 12 h of darkness and were provided food and water ad libitum.

Cells. Detailed methods for the preparation of transfected PC12 cells used in these studies have been described (12). Briefly, PC12 cells were permanently transformed with cDNA corresponding to the 1.1-kilobase β/A4-C-terminal region of the human APP obtained after *Eco*RI digestion (13). The cDNA sequence codes for 97 APP amino acids representing 40 amyloid amino acids followed by the C terminus of APP. The presence of the insert was confirmed by Southern and Northern blot analyses (unpublished data). Amyloid overexpression was verified by immunocytochemical staining with anti-amyloid antibody (see *Results*) (12, 14). Untransfected PC12 cells (normal control, NN cells), PC12 cells transfected with the vector only with no APP DNA (V120 cells), and PC12 cells transfected with the A4 C-terminal region of the APP (AC127 cells) were used for grafting experiments.

Grafted Rats and Control Groups. PC12 cells were harvested for stereotaxic injection by rinsing in Hanks' basic salt solution followed by brief trypsin treatment and washing with Dulbecco's modified Eagle's medium (DMEM). Cells were counted and resuspended in DMEM at a concentration of 50,000 cells per μl. Cells were drawn into the lumen of a Hamilton syringe, which was then affixed to a stereotaxic instrument (Kopf Instruments, Tujunga, CA). The animals receiving grafts were sedated with diazepam and anesthetized with sodium pentobarbital. In addition, the animals

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: AD, Alzheimer disease; SCN, suprachiasmatic nuclei; APP, amyloid precursor protein.

§To whom reprint requests should be addressed at: Neurobiology Laboratory, Bulfinch 4, Massachusetts General Hospital, Boston, MA 02114.
¶Present address: Mt. Sinai School of Medicine, New York, NY 10029.
‖Present address: Duke University School of Medicine, Durham, NC 27710.
**Present address: Department of Psychobiology, University of California, Irvine, CA 92717.

were given atropine to aid in breathing. Anesthetized animals were placed in the stereotaxic instrument, a midline incision was made in the skin of the head, and two burr holes were drilled in the skull to permit bilateral injections into the SCN. The needle containing cells was stereotaxically lowered into the brain and 2 μl of cells was extruded from the needle for each injection. After a delay of several minutes to allow the cells to disperse, the needle was slowly withdrawn and the skin was sutured closed. Stereotaxic coordinates for the SCN as measured from bregma were 1.3 mm anterior, ±0.1 mm lateral, and 9.1 mm ventral.

After surgery, animal rhythms were monitored. Grafted animals consisted of the following three groups, named for the cell type grafted: NN, V120, or AC127. Two groups of ungrafted control rats were also monitored. One group of ungrafted controls received sham surgery, during which DMEM without cells was injected (vehicle). A second group of control rats did not receive stereotaxic surgery but did receive intraperitoneal implants of radio transmitters (UNOP) similar to the other groups of rats. Behavioral and body temperature data were collected for up to 8 weeks after surgery, at which time animals were sacrificed by cardiac perfusion; brains were removed, sectioned, and histologically examined to document the location of the graft.

Activity and Body Temperature Data Collection and Analysis. Activity and body temperature data were simultaneously collected from each animal by an intraperitoneally implanted radio transmitter (Mini-mitter, Sunriver, OR) placed in the anesthetized animal at the time of stereotaxic surgery. A radio receiver placed under the cage of each animal accumulated activity and temperature data and transmitted it to a computer programmed with a data collection and analysis system (DATAQUEST III; Datasciences, Minneapolis). The data were stored in 10-min bins. The level of gross motor activity was compared between ungrafted and grafted rats to ensure that the presence of the graft had not compromised the animals prior to evaluation of the pattern or rhythms of activity and body temperature. Graphic representation of activity and body temperature data (actograms) were generated for each animal using circadian data analysis software (CIRCADIA, Behavioral Cybernetics, Cambridge, MA).

Statistical Analysis. Two weeks of activity data and 1–2 weeks of body temperature data collected during weeks 3–8 after surgery were analyzed for the presence of a circadian rhythm (a rhythm with a period of 24 h) by power spectrum analysis using a fast Fourier transform (DATAQUEST III). Power spectrum values at the period corresponding to 24 h are higher if the variation in the data are due to a repeating cycle with a frequency of 24 h and lower as the frequency of the repeating cycle within the data moves further from a period of 24 h. Mean power spectrum values for each of the groups of animals were compared by an ANOVA followed by a Tukey protected *t* test. The presence in the data of significant periodicities and the frequency of those periodicities was also determined by periodogram analysis (CIRCADIA). Analyses were run for the identical data sets that were analyzed by power spectrum analysis.

Histology. Animals were transcardially perfused with 4% (wt/vol) paraformaldehyde. Brains were serially sectioned at 42-μm on a cryostat and processed for immunohistological (15) and histochemical staining. The location of the graft was accessed by immunostaining brain sections with antibodies to tyrosine hydroxylase (16) and counterstaining with cresyl violet. Additionally, grafts of some animals were detected with a monoclonal antibody to a membrane ganglioside, TC6 (17).

RESULTS

Cell Types. Of the three PC12 cell types used for grafting experiments, the permanently transfected cell line containing the β/A4-C-terminal APP DNA insert (AC127) was shown to contain and express the proper insert by Northern and Western blot analyses (18). Overexpression of the β/A4 antigen in AC127 was confirmed by immunocytochemical staining of cells using a mixture of monoclonal antibodies to amino acids 1–28 of β/A4 (14). As shown in Fig. 1, immunostained AC127 transfected cells contained relatively increased levels of β/A4 antigen. V120 control cells exhibited a lower intensity of immunostain that was similar to nontransfected control NN PC12 cells.

Grafted Animals. Control groups included five rats that had received sham surgery with vehicle only and seven rats that had received no stereotaxic surgery but that were otherwise exposed to the same environmental conditions as all other groups. Seven animals with NN grafts, six animals with V120 grafts, and eight animals with AC127 grafts were included in the behavioral analysis. After implantation, the three cell types exhibited a similar appearance in the SCN as indicated by immunostained and cresyl violet-counterstained brain

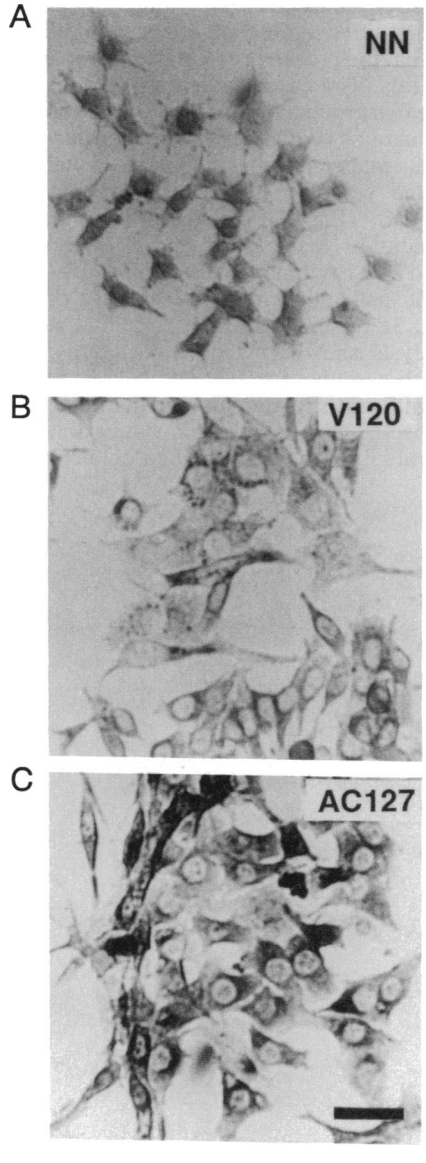

FIG. 1. Immunostaining of control and transfected PC12 cells. Light micrographs of cells that were immunostained with a mixture of three monoclonal antibodies to the β/A4 region of the APP (14). (*A*) NN cells show only light background staining. (*B*) V120 cells containing the transfecting vector without an amyloid insert also show light background staining. (*C*) AC127 cells transfected with vector DNA containing the region from A4 to the C terminus of the APP are darkly immunostained. (Bar = 20 μm.)

sections. A representative photomicrograph is shown in Fig. 2. Tumor growth occurred in only one NN and one V120 animal; they were included in the behavioral analyses since <50% of the total SCN was affected. None of the AC127 animals developed tumors. The SCN extends in a rostral–caudal direction through the anterior hypothalamus. Most grafts occupied the middle region of each nucleus and few grafts were located at either rostral or caudal sites. Graft sites in different animals were not distributed differently among the three cell types. Within groups of rats containing a single cell type, the location of the graft did not have a significant effect on the activity or body temperature rhythms of animals, as demonstrated by ANOVAs.

Behavioral Evaluations. The effects of SCN grafts on rat behavior were monitored by continuous recordings from radio transmitters implanted in freely moving animals housed in standard cages under lighting conditions of 12 h of light and 12 h of darkness. Transmitters measured two circadian rhythms, activity and body temperature. Mean motor activity levels did not differ significantly between groups ($P > 0.01$), indicating that the presence of a graft did not alter the overall activity of rats. The circadian pattern of activity was monitored in the five groups of animals described above, as shown by 24-h actograms (Fig. 3). All groups of rats except AC127 displayed the main nocturnal activity bout with regular times of onset and cessation and decreased relative levels of daytime activity (Fig. 3 *A–D*). In contrast, although experimental conditions included light–dark cycles that tend to drive rats to 24-h rhythms, animals with grafts of AC127 cells showed unusually high levels of activity during the light phase (Fig. 3*E*) and a disrupted circadian pattern. Periodogram analysis showed significant rhythms in the AC127 animals with periods of <22 h and >25 h; no other groups showed significant rhythms outside the range of 23–25 h.

Power Spectra. Power spectrum values at a period of 24 h were generated for the activity data collected over 2-week periods at weeks 3–6 and weeks 6–8. The group of animals receiving AC127 cells had a significantly lower mean power spectrum value than all other groups ($P < 0.05$), indicating that the AC127 group failed to show a major peak in the power spectrum at a period of 24 h (Fig. 4*A*) at both the early and late time periods. Animals receiving amyloidotic transfected cells grafted into the anterior hypothalamus, but not adjacent to the SCN failed to show behavioral abnormalities (data not shown).

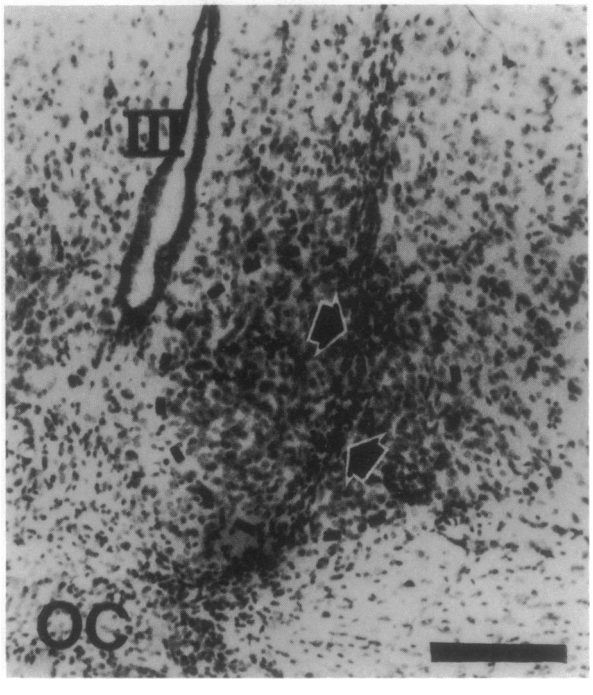

FIG. 2. Representative photomicrograph of the V120-cell graft in the SCN region (42-μm section) 6 weeks after surgery, immunostained with anti-tyrosine hydroxylase and counterstained with cresyl violet. The darkly immunostained cluster of PC12 cells (arrows) is visible as it extends dorsal–ventrally through the midline of the SCN. The margins of the SCN are outlined (broken circle). III, third ventricle; OC, optic chiasm. (Bar = 100 μm.)

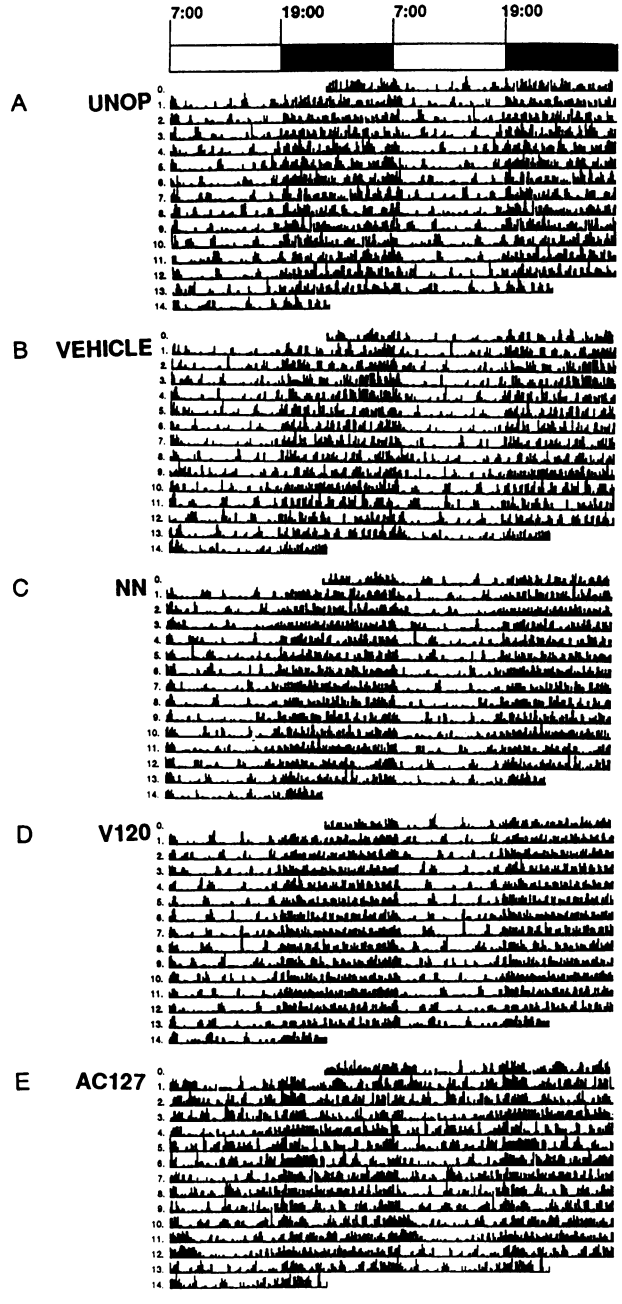

FIG. 3. Continuous record of activity data (actograms) for control and grafted rats for a 14-day period 4–6 weeks after surgery. Activity events are plotted as vertical bars with the height of the bar proportional to the magnitude of the activity value. Each horizontal line represents a 24-h period, with consecutive days arranged vertically; the data are plotted twice for clarity. The light/dark schedule is indicated at the top of the record. Lights were activated at 0700 and turned off at 1900. UNOP, animals given radio transmitters but no stereotaxic surgery; VEHICLE, animals stereotaxically injected with cell media only; NN, animals given grafts of normal control PC12 cells; V120, animals given grafts of vector-control cells; AC127, animals given grafts of β/A4-overproducing cells.

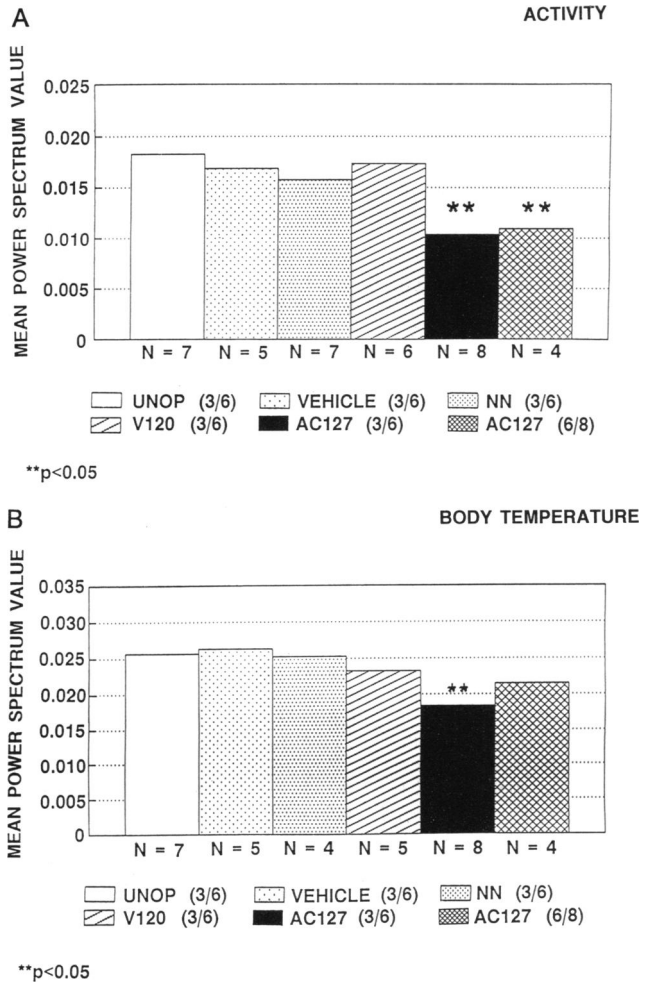

FIG. 4. Mean power spectrum values for circadian activity and temperature rhythms of grafted and ungrafted rats. Power spectrum values indicate the relative strength of the rhythm when the period is assumed to be 24 h. The identity of animal groups with and without grafted cells are indicated. All values were determined 3–6 weeks (3/6) after surgery except that AC127 animals were also examined after 6–8 weeks (AC127 6/8). Power spectrum values were determined for activity rhythms (*A*) and for body temperature (*B*). Temperature data were available for all animals used for activity data analysis except for three animals that had received control cell grafts and one animal that had received a graft of V120 cells. Statistical analyses (ANOVA followed by the Tukey protected *t* test) showed a significant difference in circadian activity between the rats grafted with AC127 cells and all other groups (**, $P < 0.05$). Unlike the AC127 group, there was no change in the activity or the body temperature rhythms of the implanted control groups 6–8 weeks after grafting.

Power spectrum values were also calculated for the body temperature rhythm data for the same two time intervals. Similar to the results for circadian locomotor activity, the mean power spectrum values for body temperature (Fig. 4*B*) was significantly lower in the AC127 group during the early time interval (3–6 weeks after surgery). However, unlike the persistent disruption of locomotor activity, circadian temperature regulation recovered and a 24-h rhythm was restored 6–8 weeks after grafting.

DISCUSSION

Unlike rats in control groups, animals grafted with cells overexpressing the β/A4-C-terminal region of the APP showed persistent alterations in circadian activity, but not temperature rhythms, with significant deviations from a 24-h cycle. The SCN oscillator function is robust and is moderately resistant to mechanical damage (19, 20). Upon histological examination of the implanted rat brains, the SCN was found to be intact in animals with circadian dysfunction, indicating that mechanical damage could not account for the observed behavioral effects. Further, localized tumor growth at graft sites could not have accounted for the results since the β/A4-positive cells, unlike two control cell grafts, did not produce tumors.

Disruption of circadian activity was more severe than the dysregulation of the body temperature rhythm. After transient interruption, the body temperature rhythm of the AC127 animals recovered a circadian pattern by 8 weeks after grafting and did not differ significantly from other groups at this time point. Although it has been observed that grafts of PC12 cells in the brain of adults rats die over the course of several weeks (21), the contribution of cell loss to recovery of the body temperature rhythm does not account for the continued disruption of the activity rhythm. Mechanisms underlying a differential effect of grafts on the activity versus the temperature rhythm are not clear. Temperature and activity rhythms may be controlled by separate oscillators, a theory consistent with earlier studies that separated the two functions by means of lesions applied to the SCN (22). Body temperature rhythm and the activity/sleep–wake cycles appear to be regulated by neuroanatomically distinct oscillators that reside close to or within the boundaries of the SCN (22).

Concurrent investigations on the PC12 cell lines used here have shown that they secrete one or more factors with neurite-stimulating activity (18, 23, 24). The hypothesis that PC12 cells engineered to overexpress β/A4 may secrete biologically active substances is supported by earlier observations (25, 26). The biological activity of β/A4 and homologous peptides has been observed in several experimental systems (27–29). The mechanism by which the AC127 cells grafted into the SCN disrupt circadian rhythmicity may be related to the altered secretory products of these cells. Further study is necessary to elucidate the products. The possible mechanism of disruption of the activity rhythm was studied by examining the restoration of rhythmicity in SCN-lesioned animals by grafts of SCN tissue. Data derived from transplantation studies using various experimental approaches implicate either the formation of neural connections between host and graft or humoral influences as the primary mechanism by which SCN implants restore circadian function (30). Grafting studies, that are analogous to those of the present report, indicated that SCN cell suspensions were capable of restoring behavioral rhythms in the absence of apparent neuroanatomic reorganization (31).

The process by which SCN cells generate a circadian rhythm is not known but may involve intercellular communication (32), possibly by means of gap junction channels (33). Disruption of intercellular information transfer within the SCN by the presence of β/A4-positive cells or their products could result in the loss of normal circadian rhythmicity. Consistent with this hypothesis are recent studies that demonstrated that the APP and/or amyloid may modulate cognitive behavior of the rat (34, 35), possibly through a role in intercellular communication.

Transplants can be positioned at restricted neuroanatomic sites and consequent behavioral disturbances can be related with specificity to the disruptive influence of the graft. We conclude, therefore, that implants to the SCN of permanently transfected PC12 cells that overexpress the β/A4 antigen, to levels that are immunologically detectable, are responsible for disrupting the ability of rats to generate a normal circadian activity pattern. These and similar biobehavioral studies may ultimately facilitate our understanding of neuropathologic mechanisms that interrupt normal performance and functioning of AD patients.

We thank Mark Kim Lee for technical assistance. This research was supported by Grant P01 AG 02126 from the National Institutes of Health, the Axelrod Family Fund, Milton Fund Awards from Harvard University, and an award from the Metropolitan Life Foundation.

1. Katzman, R. (1986) *N. Engl. J. Med.* **314,** 964–973.
2. Witting, W., Kwa, I. H., Eikelenboom, P., Mirmiran, M. & Swaab, D. F. (1990) *Biol. Psychiatry* **27,** 563–572.
3. Prinz, P. N., Vitaliano, P. P., Vitiello, M. V., Bokao, A., Raskind, M., Perskind, E. & Gerber, C. (1982) *Neurobiol. Aging* **3,** 361–370.
4. Satlin, A., Teicher, M. H., Lieberman, H. R., Volicer, L. & Rheume, Y. (1991) *Neuropsychopharmacology* **5,** 115–126.
5. Sanford, J. R. (1975) *Br. Med. J.* **3,** 471–473.
6. Jolles, J. (1986) *Prog. Brain Res.* **70,** 15–39.
7. Satlin, A., Teicher, M. H., Lieberman, H. R., Volicer, L. & Rheume, Y. (1990) *Neurobiol. Aging* **11,** 337.
8. McDuff, T. & Sumi, S. M. (1985) *Neurology* **35,** 123–126.
9. Saper, C. B. & German, D. C. (1987) *Neurosci. Lett.* **74,** 364–370.
10. Stopa, E. G., Tate-Ostroff, B., Walcott, E. C., Majocha, R. E. & Marotta, C. A. (1989) *J. Neuropathol. Exp. Neurol.* **48,** 327.
11. Swaab, D. F., Fliers, E. & Partiman, T. S. (1985) *Brain Res.* **342,** 37–44.
12. Marotta, C. A., Chou, W.-G., Majocha, R. E., Watkins, R. & Zain, S. B. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 337–341.
13. Zain, S. B., Salim, M., Chou, W.-G., Sajdel-Sulkowska, E. M., Majocha, R. E. & Marotta, C. A. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 929–933.
14. Majocha, R. E., Benes, F. M., Reifel, R. L., Rodenrys, A. M. & Marotta, C. A. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 6182–6186.
15. Majocha, R. E., Marotta, C. A. & Benes, F. (1985) *Can. J. Biochem. Cell Biol.* **63,** 577–584.
16. Jaeger, C. B. (1987) *Ann. N.Y. Acad. Sci.* **495,** 334–353.
17. Chou, D. K. H., Dodd, J., Jessel, T. M., Costello, C. E. & Jungalwala, F. B. (1989) *J. Biol. Chem.* **264,** 3409–3415.
18. Majocha, R. E., Tate, B., Ventosa-Michelman, M. & Marotta, C. A. (1991) *Soc. Neurosci. Abstr.* **17,** 1105.
19. Mosko, S. & Moore, R. Y. (1979) *Neuroendocrinology* **29,** 350–361.
20. Yanovski, J. A., Rosenwasser, A. M., Levine, J. D. & Adler, N. T. (1990) *Brain Res.* **537,** 216–226.
21. Freed, W. J., Patel-Vaidya, U. & Geller, H. M. (1987) *Exp. Brain Res.* **63,** 557–566.
22. Satinoff, E. & Prosser, R. A. (1988) *J. Biol. Rhythms* **3,** 1–22.
23. Majocha, R. E., Zain, S. B., Chou, W.-G., Tate-Ostroff, B., Ventosa-Michelman, M. & Marotta, C. A. (1989) *Soc. Neurosci. Abstr.* **15,** 684.
24. Marotta, C. A., Majocha, R. E., Tate-Ostroff, B., Chou, W.-G. & Zain, S. B. (1990) *J. Neuropathol. Exp. Neurol.* **49,** 332 (abstr. 213).
25. Yankner, B. A., Villa-Komaroff, L. & Neve, R. L. (1988) *Soc. Neurosci. Abstr.* **14,** 896.
26. Yankner, B. A., Dawes, L. R., Fisher, S., Villa-Komaroff, L., Oster-Granite, M. L. & Neve, R. (1989) *Science* **245,** 417–420.
27. Yankner, B. A., Duffy, L. K. & Kirschner, D. A. (1990) *Science* **250,** 279–282.
28. Whitson, J. S., Glabe, C. G., Shintani, E., Abcar, A. & Cotman, C. W. (1990) *Neurosci. Lett.* **110,** 319–324.
29. Whitson, J. S., Selkoe, D. J. & Cotman, C. W. (1989) *Science* **243,** 1488–1490.
30. Ralph, M. R. & Lehman, M. N. (1991) *Trends Neurosci.* **14,** 362–366.
31. Bos, N. P. A. & Mirmiran, M. (1990) *Brain Res.* **511,** 158–162.
32. Rosbash, M. & Hall, J. C. (1989) *Neuron* **3,** 387–398.
33. Bargiello, T. A., Saez, L., Baylies, M. K., Gasic, G., Young, M. W. & Spray, D. C. (1987) *Nature (London)* **328,** 686–691.
34. Doyle, E., Bruce, M. T., Breen, K. C., Smith, D. C., Anderton, B. & Regan, C. M. (1990) *Neurosci. Lett.* **115,** 97–102.
35. Flood, J. F., Morley, J. E. & Roberts, E. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 3363–3366.