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Cellular Construction of a Circadian Clock: Period Determination in the Suprachiasmatic Nuclei

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Summary

The circadian clock in the suprachiasmatic nuclei is composed of multiple, single-cell circadian oscillators (clock cells). We now test the hypothesis that the circadian period in behavior is determined by the mean period that arises from the coupling of clock cells with diverse circadian periods. For these studies, we monitored firing rate rhythms of individual suprachiasmatic nuclei neurons on fixed multielectrode plates and exploited the altered circadian periods expressed by heterozygous and homozygous *tau* mutant hamsters. The results show that circadian period in the whole animal is determined by averaging widely dispersed periods of individual clock cells. The data also demonstrate that the *tau* mutation affects circadian function in a cell-autonomous manner.

Introduction

Circadian rhythms in physiology and behavior are the external expression of an internal timing system or circadian clock (Pittendrigh, 1993). A genetically determined property of such clocks is the period (cycle length) manifested under constant conditions, which normally varies slightly from 24 hr. Circadian period is remarkably precise for each species and throughout the life history of individual animals. The genetic basis of circadian period is further supported by the phenotypic analyses of single gene mutations in organisms from cyanobacteria to mammals that show dramatic alterations in period (for reviews, see Dunlap, 1993; Takahashi, 1995). The discovery of the *tau* mutation in Syrian hamsters (Ralph and Menaker, 1988) and the *Clock* mutation in mice (Vitaterna et al., 1994) provides direct evidence that single gene mutations can alter circadian period in mammals.

The suprachiasmatic nuclei (SCN) of the anterior hypothalamus are the site of a master circadian clock in mammalian brain (reviewed in Reppert and Weaver, 1997). A seminal demonstration of the clock function of the SCN was made possible using the *tau* mutant hamster (Ralph and Menaker, 1988). The *tau* mutation shortens circadian period from ~24 hr to ~20 hr in homozygotes. *tau* mutant hamsters thus were used in SCN transplantation studies to show that the period of the restored rhythm is determined by the genotype of the donor, not the host (Ralph et al., 1990). Results from in vitro experiments with SCN slices have also shown that the *tau* mutation is expressed at the level of the circadian clock in the SCN (Davies and Mason, 1994).

The oscillatory machinery of the SCN is cell autonomous. This was discovered by Welsh et al. (1995) using a system for culturing dispersed SCN cells on fixed microelectrode arrays so that spontaneous action potentials could be recorded from individual neurons for weeks. With this system, SCN neurons in the same culture express circadian rhythms of widely different phases and different period lengths despite functional synapses. These data show that the SCN clock is composed of an ensemble of multiple circadian oscillators (clock cells).

Although progress is currently being made toward understanding the molecular mechanisms underlying circadian oscillations in mammals (King et al., 1997; Sun et al., 1997; Tei et al., 1997), the way in which a multioscillator circadian clock is constructed to elicit precisely timed output rhythms should not be ignored. Indeed, with the ability to record individual SCN neurons for days to weeks in vitro and to analyze the period and phase of their circadian rhythms (Welsh et al., 1995), we now have an unprecedented opportunity to address specific hypotheses concerning the cellular basis of clock construction within the SCN.

The initial study from our laboratory using fixed microelectrode plates showed that individual neurons dissociated from rat SCN express independently phased circadian firing rhythms with a mean circadian period (24.35 hr; Welsh et al., 1995) that is very similar to the mean period of behavioral rhythms (studied in constant darkness) in the same strain of rat (24.46 hr; Reppert and Schwartz, 1986). However, the standard deviation (SD) was 1.20 hr for clock cells versus 0.06 hr for behavior. Although measurement error could have contributed to the greater range of clock cell periods in culture, it is also possible that the circadian period in behavior expressed in vivo is determined by the mean period that arises from the coupling of a large population of single-cell circadian oscillators with diverse periods (Welsh, 1995).

We now test this hypothesis by monitoring firing rate rhythms of individual SCN neurons on fixed multielectrode plates and exploiting the altered circadian periods expressed by heterozygous and homozygous *tau* mutant hamsters. The results show that circadian period in the whole animal is determined by averaging widely dispersed periods of individual clock cells and demonstrate that the *tau* mutation is the result of a gene defect which affects cell-autonomous circadian function.

Results

tau Mutant Clock Cells Express a Wide Range of Circadian Periods

To test the hypothesis that the circadian period in behavior expressed by the whole animal is determined by

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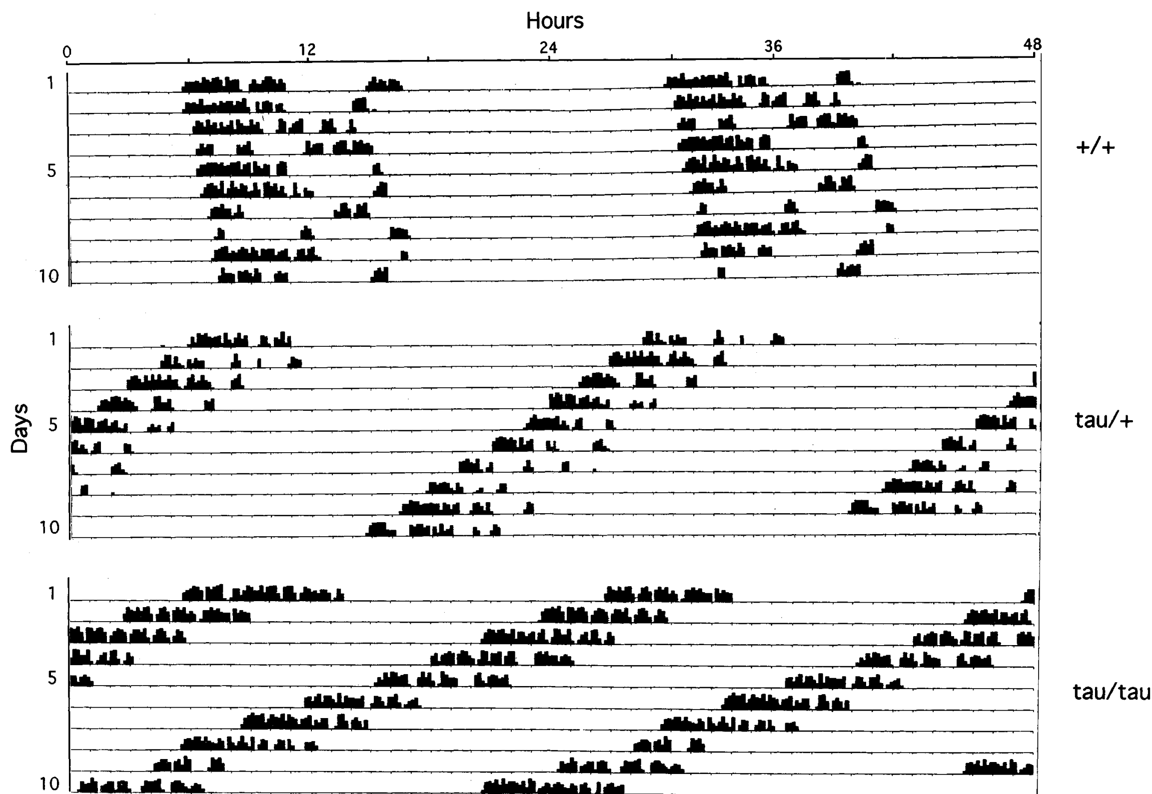

Figure 1. Circadian Rhythms in Wheel-Running Behavior for Wild-Type and *tau* Mutant Hamsters

Adult male wild-type (+/+), heterozygous *tau* mutant (*tau*/+), and homozygous *tau* mutant (*tau/tau*) hamsters were housed in a light–dark cycle for 3 weeks and then transferred to constant darkness. Wheel-running behavior was monitored by computer. The days of study are plotted side by side as well as vertically to facilitate visual assessment of circadian phase. The circadian periods depicted are 24.19 (+/+), 22.45 (*tau*/+), and 21.10 (*tau/tau*).

averaging a large population of clock cells with diverse periods, we cultured SCN neurons from wild-type (+/+), heterozygous (*tau*/+), and homozygous (*tau/tau*) *tau* mutant Syrian hamsters. The spontaneous, semidominant *tau* mutation was chosen for these studies because it consistently and reproducibly shortens circadian period in wheel-running behavior. Indeed, our studies of wheel-running behavior in *tau* mutant hamsters showed that the mutation shortens circadian period from 24.15 ± 0.06 hr (mean ± SD) in wild-type Syrian hamsters to 22.39 ± 0.28 hr in *tau* heterozygotes and to 20.75 ± 0.39 hr in *tau* homozygotes, when monitored in constant darkness (Figure 1); these data are consistent with those reported from other laboratories (Ralph and Menaker, 1988; Ralph et al., 1990).

For our clock cell recordings, SCN cells dissociated from 6 to 10 neonatal pups of the same genotype (wild-type, heterozygous *tau* mutants, or homozygous *tau* mutants) were combined for each culture on fixed multielectrode plates. A total of 38 clock cells were recorded from ten cultures, with 1–6 clock cells recorded from each culture. Starting at 2 weeks in culture, cells were recorded from active electrodes for 2 days, and those showing rhythmicity (16 to 26 hour periods; 50% of recorded cells) were selected for long-term recordings. Individual clock cells were recorded for at least 3 consecutive days for period determination; the mean (± SE) recording duration was 4.80 ± 0.30 days.

Clock cells from all three genotypes expressed robust circadian rhythms in culture (Figure 2); there were no obvious differences observed among the genotypes in the ratio of clock cells recorded nor in the amplitude or waveform of clock cell firing rhythms (data not shown). Our recordings showed that for each genotype, hamster clock cells in the same culture oscillate with a wide range of period lengths (Figure 3, left) similar to what has been reported in rats (Welsh et al., 1995). These period differences did not change over time in culture; they were the same from 2–10 weeks, which is inclusive of all culture ages used in these studies. Previous studies have shown that under our culture conditions there is no evidence for coupling or interactions among clock cells, despite the abundance of functional synapses (Welsh et al., 1995; Welsh and Reppert, 1996).

Clock cell periods ranged from 20.25–25.00 hr in cultures from wild-type hamsters, from 17.50–24.50 hr in cultures from heterozygous *tau* mutant hamsters, and from 16.50–21.75 hr in cultures from homozygous *tau* mutant animals. The range of circadian periods was much narrower for wheel-running behavior monitored for each of the three genotypes (Figure 3, right). The large variation of clock cell periods appeared to be intrinsic to clock cells because it was observed in all three hamster genotypes as well as in rats (Welsh et al., 1995). In addition, long-term recordings (≥ 6 days; n = 8) showed that individual clock cells manifest period

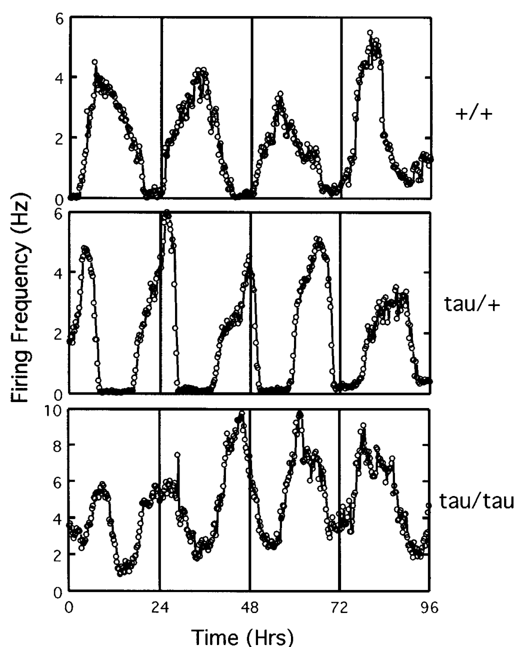

Figure 2. Circadian Rhythms of Firing Rates in Representative Clock Cells for Wild-Type and *tau* Mutant Hamsters

Neurons in cultures from wild-type (+/+), heterozygous *tau* mutant (*tau*/+), and homozygous *tau* mutant (*tau*/*tau*) hamsters were recorded at 15 min intervals on fixed multielectrode plates after 2 weeks in culture. Each point reflects the mean firing rate for a 5 min record. The circadian periods depicted are 23.75 (+/+), 21.00 (*tau*/+), and 19.75 (*tau*/*tau*).

lengths that never vary more than 1 hour over time in culture (data not shown). This is in contrast to the wide range of periods (5–7 hr) expressed among clock cells of a given genotype (Figure 3).

The *tau* Mutation Affects Circadian Function of Individual Clock Cells

In spite of the large variation of clock cell periods, the mean (± SD) period of clock cells in culture was distinctive for each genotype (23.43 ± 1.34 hr for wild-types, 20.97 ± 2.14 hr for heterozygotes, and 19.28 ± 1.69 hr for homozygotes; Kruskall-Wallis ANOVA $p = 0.001$ for significant effect of genotype) (Figure 4A). These results demonstrate that the period abnormality of the *tau* mutation is manifested at the single-cell level. The mean periods of wild-type, heterozygous, and homozygous clock cells correlated nicely with the respective circadian periods in behavior in the whole animal (Figure 4B).

Formal Model of the SCN as an Oscillator Network

We now outline a biologically plausible model of synchronization in the SCN that is consistent with our observations and predictive. Since SCN clock cells exhibit self-sustained, nearly sinusoidal oscillations (Figure 2), we model them as weakly nonlinear oscillators with a stable limit cycle (Winfree, 1967; Strogatz, 1994). We have no information about the relative connectivity patterns of the SCN cells in vivo, so no particular topology is assumed. If the coupling among oscillators is weak compared to the attractiveness of their limit cycle, or if the cells communicate via multiple pulses throughout the circadian cycle, one can prove (Ermentrout and Kopell, 1991) that the governing differential equations are accurately approximated by a simpler set of phase equations of the form

$$\dot{\theta}_j = \omega_j + \sum_i K_{ij} f(\theta_i - \theta_j), \qquad (1)$$

for $j = 1, \ldots, N$. Here θ_j is the phase of cell j in its circadian cycle, ω_j is its intrinsic frequency, N is the number of cells, the overdot denotes a time derivative, and the summation extends over all cells i that are coupled to cell j. The coupling matrix K_{ij} and the function f depend on the interactions between oscillators and are unknown, but in principle they can be derived from more detailed (ionic or biochemical) models or from experimental measurements.

Under certain assumptions, one can prove that a completely synchronized network will oscillate at a frequency given by the sample mean $\bar{\omega}$ of the frequencies in the population. Suppose that K_{ij} is any symmetric matrix ($K_{ij} = K_{ji}$) and f is any odd function ($f(-\phi) = -f(\phi)$). Then by summing Equation 1 over all j, one sees that any phase-locked solution ($\dot{\theta}_j = \dot{\theta}_k$ for all j,k) oscillates at the sample mean frequency $\bar{\omega}$. This result is consistent with our finding that for all three phenotypes the free-running behavioral period approximately equals the mean clock cell period. If the actual SCN network violates one or both of the mathematical assumptions for K_{ij} and f, the ensemble frequency need not equal the sample mean; it can be either higher or lower than the mean, depending on the detailed properties of K_{ij} and f (Ermentrout and Kopell, 1984). This may explain the differences observed between mean clock cell period and behavioral period for each of the three hamster genotypes (Figure 4B).

Discussion

The results indicate that the free-running period in vivo is determined by the mean period of clock cells in the SCN and further show that the interactions among clock cells in the whole SCN serve to coordinate or synchronize individual circadian clocks to generate a common circadian output. A striking aspect of the work presented is the wide range of circadian periods manifested by single clock cells for each of the three genotypes. With synchronization of individual clock cells in the whole SCN in vivo, a collective enhancement of precision is apparently achieved even though individual clock cell periods are not very accurate (i.e., they may differ widely from the mean value). It may be biologically too expensive to ensure accuracy of every clock cell or too unreliable to depend on a single pacesetting clock cell. How the periods of so many single clock cells can be so precise, yet inaccurate when compared to the ultimate circadian output from the collective SCN is difficult to explain at present in molecular terms.

Our proposed oscillator network model shows that a completely synchronized network will oscillate at a frequency close to the sample mean of the frequencies of the population. A prediction from this model is that the network of oscillators will exhibit a sharp threshold

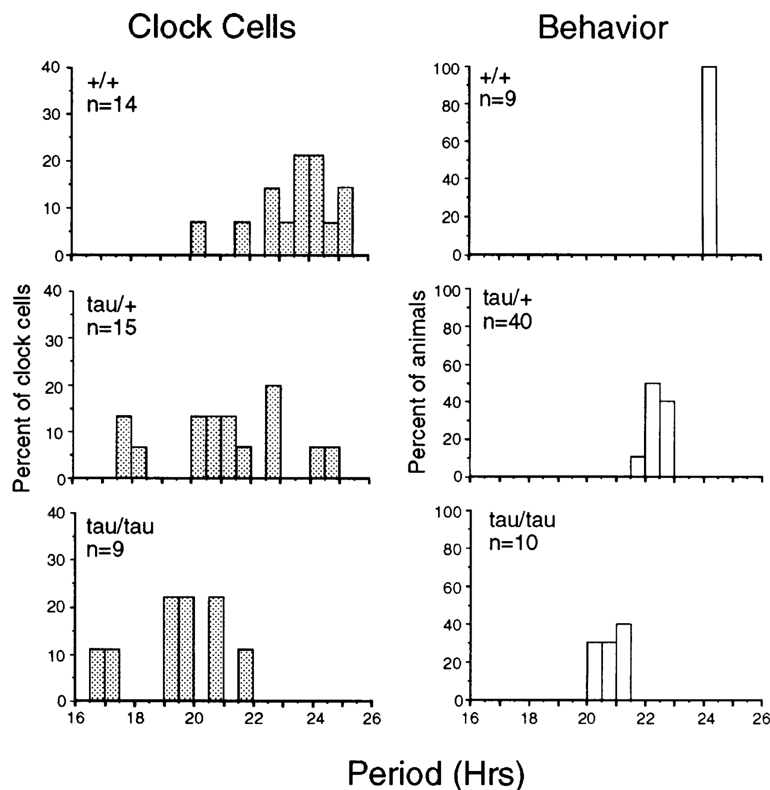

Figure 3. Range of Period Lengths for Each Genotype Is Greater for Clock Cells Than Behavior

Circadian periods of firing rate rhythms for individual clock cells (left, shaded vertical bars) or circadian period of wheel-running behavior for individual animals (right, open vertical bars) are plotted for wild-type (+/+), heterozygous *tau* mutant (*tau*/+), and homozygous *tau* mutant (*tau/tau*) hamsters.

for synchronization, analogous to a phase transition (Figure 5). The network is predicted to oscillate incoherently at weak coupling. As the coupling is increased, no synchronization occurs until a critical coupling is reached—then spontaneous partial synchronization occurs. The oscillators near the center of the distribution lock together in frequency, whereas the outlying oscillators continue to oscillate near their natural frequency. With further increases in the coupling, more of these outlying oscillators are pulled in, until eventually the whole system is synchronized. What probably happens in our culture system is that coupling is weak, below critical coupling, because of dilution of some diffusible substance necessary for coupling.

Although the precise nature of the coupling interaction among SCN neurons is not yet known, one reasonable neurochemical candidate for such coupling activity is γ-aminobutyric acid (GABA). It is known that most SCN neurons are interneurons and interact with each other within the SCN, and virtually all SCN neurons are GABAergic and respond to GABA (Card and Moore, 1984; van den Pol, 1986; Okamura et al., 1989; Mason et al., 1991). The recent discovery that GABA differentially affects SCN neuronal firing, depending on the time of day of application, supports the synchronizing potential of GABA (Wagner et al., 1997). Moreover, GABA analogs phase shift the circadian clock both in vivo and in vitro (Smith et al., 1989; Tominaga et al., 1994). It is also possible that diffusible substances other than GABA, such as nitric oxide or even neuropeptides, or a combination of these factors, serve as coupling factors among SCN neurons. Glia, through an insulating and/or a biochemical interaction, may also effect coupling among clock cells (see Welsh and Reppert, 1996).

A biological advantage of a coupled multioscillator system is the flexibility it provides in terms of entrainment of the circadian clock. Our oscillator network model predicts that when a portion of the oscillators in the network are phase-shifted, the whole network will resynchronize to a new phase. Therefore, only a subpopulation of clock cells need to respond to a specific entraining stimulus to generate a phase-shift in the whole network. This may explain the specificity of responses of the SCN clock to different phase-shifting stimuli (Klein et al., 1991). A specific subpopulation may respond to a specific stimulus at certain phases without requiring building such capacity in all clock cells. If this is true, then it should be possible to identify subpopulations of clock cells based on the resetting stimuli to which they respond.

A circadian clock composed of multiple oscillators with distributed periods also provides potential new insights into entrainment mechanisms, as external light-dark cycles need only to change the balance of interactions among clock cells so that the whole network will synchronize to faster or slower oscillators. It is interesting to note that the range of periods observed in individual clock cells correlates roughly with the limits for entrainment to non–24 hr light-dark cycles in the whole animal (Pittendrigh, 1974; Davis et al., 1983). This suggests that the range of clock cell periods expressed in vitro determines the range of entrainment expressed in vivo.

The cell autonomous nature of the *tau* mutation demonstrated here provides the strongest evidence to date that the product of the gene disrupted by the *tau* mutation encodes an essential clock element. We thus predict that the *tau* gene product is an actual gear of the

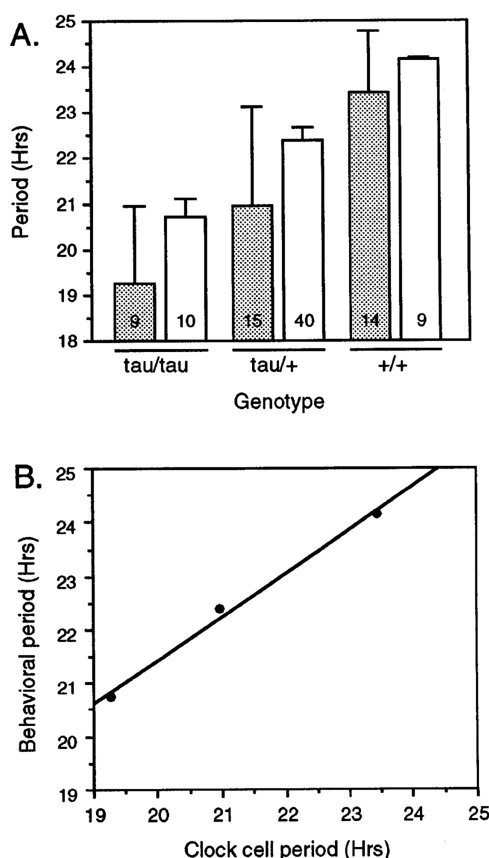

Figure 4. Mean Periods of Clock Cells Correlate with Respective Circadian Periods of Behavior in the Whole Animal

(A) Mean ±SD of clock cell periods (shaded vertical bars) and behavior periods (open bars) for wild-type (+/+), heterozygous *tau* mutant (*tau*/+), and homozygous *tau* mutant (*tau/tau*) hamsters. (B) Regression analysis of the data shown in (A). The regression line ($r^2 = 0.99$) is described by the formula
$y = 0.81x + 5.20$, where y = behavioral period and x = clock cell period (in hours).

clock, rather than being involved in secondary aspects of clock function (e.g., coupling or output). Our finding that the *tau* mutation affects circadian period in single clock cells also demonstrates the effectiveness of the single-cell recording system for assaying the cell-autonomous nature of mammalian circadian clock mutations. Defining the cell-autonomous nature of any circadian clock mutation now becomes a necessary prerequisite before considering the affected gene a candidate clock element.

Experimental Procedures

Animals

Our colony of *tau* mutant hamsters was derived from two male *tau/tau* hamsters generously provided by Fred C. Davis (Northeastern University, Boston). Wild-type LVG hamsters were purchased from Charles River Laboratories. Animals were housed in environmental compartments with a daily lighting cycle consisting of 14 hr light:10 hr dark (LD 14:10), except as noted. Dim red lights were on constantly during periods designated as darkness, including constant darkness. For cell culture experiments, offspring of known genotype were generated. Heterozygous pups were generated by crossing *tau/tau* males with +/+ females, while *tau/tau* and wild-type pups were generated by breeding parents of the same genotype.

Behavioral Analysis

Juvenile hamsters of either sex were used to monitor the circadian rhythms of locomotor behavior. Pups were housed individually in cages containing a running wheel. Wheel rotations were detected by a magnetic sensor, and data were continuously collected and stored using a Datacol III system (Minimitter Co.). For period analysis, the first 3–5 days after transfer from LD 14:10 to constant darkness were excluded, and data from the next 10–20 days in darkness were used. Activity records were plotted in the form of actograms, and the period of locomotor activity was determined from the slope of an eye-fitted line through the daily activity onset. To determine the reproducibility of period estimation by this method, actograms from a subset of animals were analyzed on two separate occasions. The period estimates were highly reproducible ($r^2 = 0.96$, $n = 11$). In no case was the deviation between the two period determinations greater than 0.5 hr.

Cell Culture

SCN cells were obtained from 1- to 3-day-old wild-type, heterozygous *tau* mutant, or homozygous *tau* mutant hamsters. Dissections were performed at midday, during the light portion of the light-dark cycle. Cylindrical punches of unilateral SCN were made from 400 μm coronal sections, using a 20-gauge needle. Cells were dissociated with papain and cultured as previously described (Welsh et al., 1995). Neuronal cell density was ~3000 cells per square millimeter. To control glia proliferation, cultures were treated 1–2 weeks after dissociation with 10 μM cytosine arabinoside (Sigma).

Multielectrode Plate Recordings

Multielectrode plates developed by Pine (1980) were used for extracellular recordings of action potentials from cultured neurons. For recordings, a multielectrode plate was fitted into a recess in a circuit board containing multiplexors and preamplifiers (Regehr et al., 1989). The entire circuit board assembly was maintained at 37°C inside a tissue culture incubator containing an atmosphere of 5% CO_2.

Extracellular voltage signals were amplified 10,000× and filtered to a bandwidth of 3 kHz. Amplified signals were analyzed by computer as described. Offline analysis eliminated low-amplitude noise and assigned clusters of spikes of similar amplitude and width to individual cells (Meister et al., 1994). Presence of a clear refractory period was used as criterion to assure that each spike cluster reflected activity of a single neuron (see Welsh et al., 1995). Circadian period was calculate by X^2 periodogram.

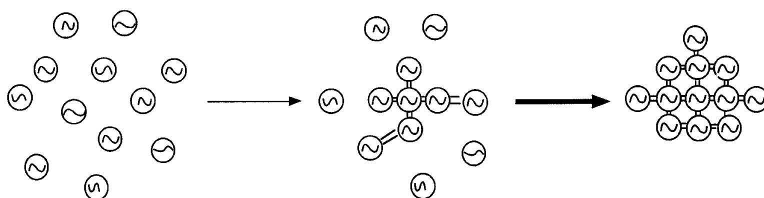

Figure 5. Oscillator Network Model

(Left) The SCN is comprised of multiple clock cells that express diverse circadian periods in culture. (Middle) With weak coupling (thin arrow), our network model predicts that clock cells with periods close to the collective mean are synchronized together first. (Right) With increased coupling strength (thick arrow), oscillators with more divergent periods are synchronized to the mean frequency until the whole population is synchronized.

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