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Antiphase Oscillation of the Left and Right Suprachiasmatic Nuclei

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An unusual property of the circadian timekeeping systems of animals is rhythm "splitting," in which a single daily period of physical activity (usually measured as wheel running) dissociates into two stably coupled components about 12 hours apart; this behavior has been ascribed to a clock composed of two circadian oscillators cycling in antiphase. We analyzed gene expression in the hypothalamic circadian clock, the suprachiasmatic nucleus (SCN), of behaviorally "split" hamsters housed in constant light. The results show that the two oscillators underlying the split condition correspond to the left and right sides of the bilaterally paired SCN.

Daily biological rhythms are governed by an innate timekeeping mechanism, a circadian clock. Such clocks have been localized to discrete sites and, in mammals, to the bilaterally paired SCN in the hypothalamus (*1*). The clock in the SCN is composed of multiple autonomous single-cell oscillators (*2*); within each cell, interacting transcription and translation feedback loops constitute the clock's actual oscillatory mechanism (*3*). It is less well understood how individual SCN cells interact to create an integrated tissue pacemaker that accounts for the circadian behaviors of whole animals.

One long-standing model of the rodent circadian clock describes it as a complex pacemaker consisting of two mutually coupled oscillators (*4*). The evidence for this idea rested initially on a phenomenon known as "splitting," which has been most extensively studied in hamsters exposed to constant light, in which an animal's single daily bout of locomotor (wheel-running) activity dissociates into two components that each free-run with different periods until they become stably coupled 180° (about 12 hours) apart. Circadian rhythms of drinking (*5*), body temperature (*6*), luteinizing hormone secretion (*7*), and SCN electrophysiological activity (*8*) also split along with locomotion. Theoretical (*9*) and experimental (*10–12*) analyses suggest that the antiphase coupling of two equivalent oscillators is likely to be responsible for this phenomenon.

In this report, we present evidence that the two putative oscillators underlying the split condition correspond to the left and right sides of the paired SCN. This idea has been raised previously (*9*), supported by the observation that unilateral SCN lesions in split hamsters abolish behavioral splitting and produce a single bout of locomotion (*13*), although this interpretation is compromised by nonspecific surgical effects (*14*). We therefore assayed clock activity in unlesioned hamsters by measuring the expression of *Per1*, *Per2*, and *Per3*, which are homologous to the *Drosophila Per* gene that forms part of the clock's core oscillatory mechanism. The three mammalian *Per* genes encode mRNAs that oscillate with a circadian rhythm in the SCN (*3*), with high levels during both the light phase of a light-dark (LD) cycle and the subjective day in constant darkness and with low levels during both the dark phase and the subjective night. The cyclic expression of these genes is believed to be central to normal clock function in mammals, at least for *Per1* and *Per2* (*15*, *16*).

Wheel-running activity was continuously recorded in male golden hamsters maintained in constant light. About 60% of our hamsters develop stably split activity rhythms after 6 to 8 weeks in this lighting regimen (Fig. 1A). Individual animals were killed at various circadian times, and their brains were processed for in situ hybridization with antisense cRNA probes to each *Per* subtype and film autoradiography (*17*). When tissue sections were examined from behaviorally split hamsters killed during either of the two inactive phases of their locomotor activity cycles, *Per* gene expression within the SCN was markedly asymmetric (Fig. 1B), with hybridization signal observed unilaterally on either the left or right sides of the SCN. This asymmetry was especially striking for *Per1*, less so for *Per2*, and barely discernable for *Per3*. In each animal, the more pronounced hybridization signal for all three *Per* genes was on the same side of the SCN, although randomly on the left or the right.

The magnitude of left-right asymmetry was quantitated from the autoradiographs by calculating an optical density (OD) ratio [OD from the "high" (whether left or right) half-SCN/OD from the "low" half-SCN] (Fig. 1C). The ratio for *Per1* peaked about 6 to 8 hours after the offset of the last locomotor activity bout; for *Per2*, the asymmetry was less prominent and appeared delayed by about 2 to 4 hours relative to *Per1*. This relation between *Per1* and *Per2* resembled that in the SCN of animals entrained to an LD cycle (or free-running in constant darkness), in which the peak level of *Per2* lagged that of *Per1* by several hours (*18–20*). In two groups of behaviorally split hamsters killed at either the 6- or 10-hour time points in Fig. 1C, the rank order of OD ratios was *Per1* (3.84 ± 0.35) > *Per2* (2.16 ± 0.35) > *Per3* (1.25 ± 0.12) at the 6-hour point, whereas it was *Per2* (1.83 ± 0.30) > *Per1* (1.54 ± 0.14) > *Per3* (1.17 ± 0.08) at the 10-hour point (mean ± SE) ($n = 5$ animals per group; for *Per1* and *Per2*, the 95% confidence interval of these means was greater than 1.00). In hamsters maintained in constant light but with unsplit locomotor activity rhythms, *Per* hybridization signal was present on both sides of the SCN and unilateral expression was not observed (OD ratio for *Per1* of 1.10 ± 0.06 for three animals killed in the middle of the inactive phase of their locomotor activity cycles).

These findings indicate that the left and

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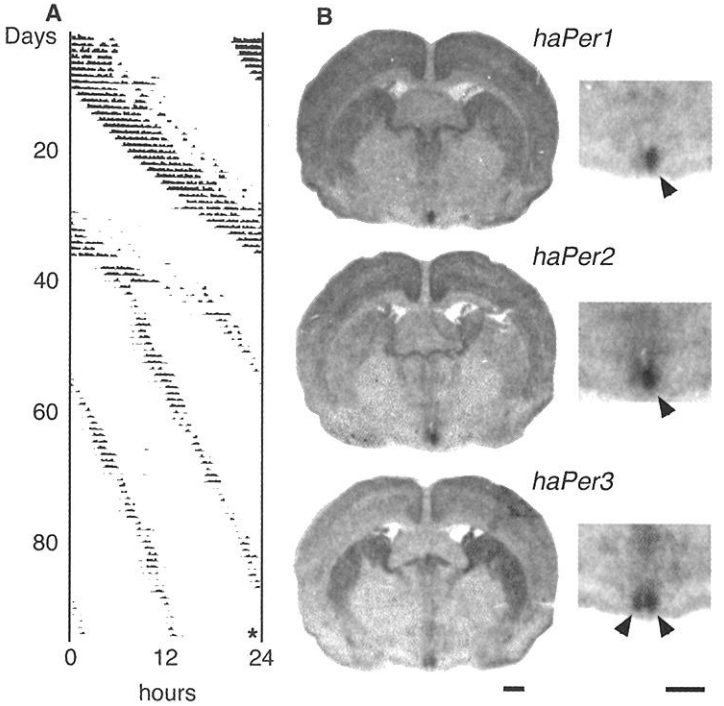

Fig. 1. SCN *Per* gene expression in the behaviorally split hamster. (**A**) Locomotor (wheel-running) activity record of a representative hamster maintained in constant light; around day 40, the animal's single daily bout of activity dissociated into two split components stably coupled about 12 hours apart. (**B**) Adjacent coronal brain sections through the SCN of the hamster recorded in (A), killed at the phase denoted [asterisk in (A)] and processed for in situ hybridization. The SCN (arrowheads) is magnified at right. Scale bars, 1 mm. (**C**) Magnitude of left-right asymmetry of SCN *Per* gene expression in individual behaviorally split hamsters killed at different circadian times; all three genes were measured in single animals.

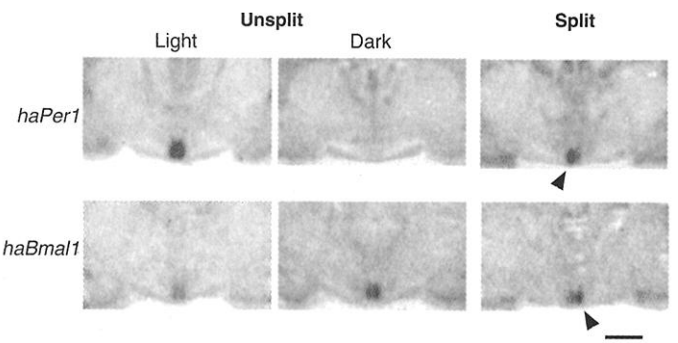

Fig. 2. SCN *Per1* and *Bmal1* mRNA levels cycle in antiphase. Coronal brain sections through the SCN were processed for in situ hybridization. Arrowheads on adjacent sections show *Bmal1* preferential expression on the side of the SCN contralateral to high *Per1* expression. Scale bar, 1 mm.

right sides of the SCN of behaviorally split hamsters do not oscillate together in phase. It is possible that the left and right sides each continue to oscillate with a ~24-hour circadian period but that each side is cycling oppositely in antiphase, thereby generating the overt ~12-hour split rhythm of locomotor activity. To test this hypothesis, we analyzed the expression of another gene, *Bmal1*, which encodes a basic helix-loop-helix transcription factor involved in the regulation of *Per* (*3*). In the SCN of normal, unsplit animals entrained to an LD cycle (or free-running in constant darkness), *Bmal1* mRNA levels cycle in antiphase to *Per1*, with high *Bmal1* and low *Per1* levels during the dark phase and vice versa during the light phase (*21*, *22*) (Fig. 2). In a group of behaviorally split hamsters killed 6 to 8 hours after the offset of the last locomotor activity bout (OD ratio for *Per1* of 2.97 ± 0.62, $n = 4$ animals), *Bmal1* was preferentially expressed on the side of the SCN contralateral to high *Per1* expression (OD ratio for *Bmal1*, relative to *Per1*, of 0.80 ± 0.04, $n = 4$ animals; the 95% confidence interval of this mean was less than 1.00) (Fig. 2). Thus, mRNAs characteristic of subjective day (*Per1*) and subjective night (*Bmal1*) are simultaneously expressed on opposite sides of the paired SCN of behaviorally split hamsters. *Per1* is rapidly induced in the SCN of normal, unsplit animals in constant darkness after they are acutely exposed to light during the subjective night (*3*); in behaviorally split hamsters in constant light, this response is missing on the side of the SCN expressing the relatively high *Bmal1* level. We suspect that the molecular mechanism(s) mediating this unresponsiveness are likely to be critical for the generation and maintenance of the split condition.

The split condition also affects molecules outside the clock's core autoregulatory loop. The gene encoding the peptide neurotransmitter arginine vasopressin (AVP) is rhythmically transcribed in the SCN by the same mechanism that regulates the transcription of *Per1* (*23*), and, like *Per1*, it is asymmetrically expressed in behaviorally split hamsters (Fig. 3B). AVP gene expression in the left and right supraoptic nuclei was symmetrical in these hamsters, consistent with the noncircadian regulation of the gene in these nuclei by osmotic stimuli (*23*, *24*). The immediate-early gene c-*fos* was also unilaterally expressed in the SCN of behaviorally split animals, on the side ipsilateral to both *Per1* and AVP (Fig. 3C). In normal, unsplit hamsters, c-*fos* is rapidly induced by nighttime light in the caudal and ventral parts of the SCN, whereas it exhibits spontaneous rhythmicity (with high levels during subjective day) in the rostral and dorsal parts of the SCN (*25*, *26*). Immunohistochemistry for the c-Fos protein (*27*) in behaviorally split hamsters revealed a unilateral rostral/dorsal distribution (Fig. 3, D and E) supporting our subjective-day assignment to the half-SCN with high *Per*, AVP, and c-*fos* gene expression.

There are precedents for the presence of dual, bilaterally symmetrical, and dissociable circadian oscillators in the nervous systems of mollusks (*28*), insects (*29*–*31*), and crabs (*32*). In normal rodents with unsplit locomotor activity rhythms, there is evidence to suggest that SCN tissue is functionally organized into two oscillatory components (*33*, *34*) that might underlie photoperiodic time measurement (*4*). We do not yet know how these two components are aligned with respect to the SCN's left-right axis. Data from SCN lesions (*35*) and transplants (*36*) suggest that bimodal locomotor activity rhythms in hamsters can arise from a unilateral SCN.

In summary, splitting appears to be the consequence of a paired SCN that has become reorganized into two oppositely phased, left-

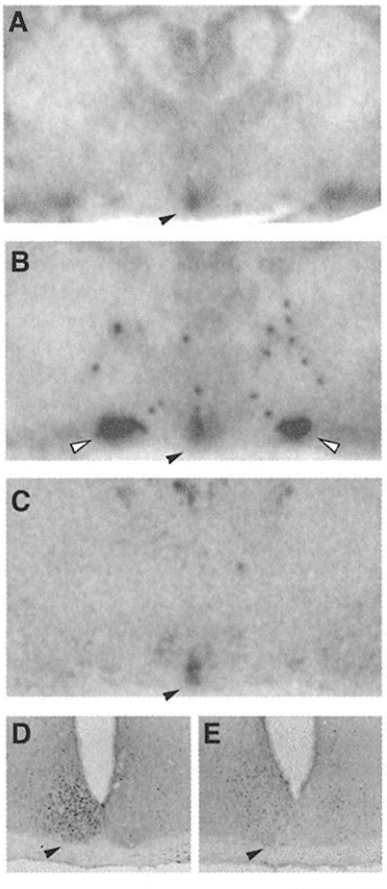

Fig. 3. SCN AVP and c-*fos* gene expression in the behaviorally split hamster. Coronal brain sections through the SCN were processed for in situ hybridization. SCN AVP mRNA levels were relatively high [black arrowhead in (**B**)] on the side of the SCN ipsilateral to high *Per1* expression [arrowhead in (**A**)]; AVP levels in the supraoptic nuclei were symmetrical [open arrowheads in (B); black dots in the autoradiograph are small clusters of magnocellular AVP perikarya]. SCN c-*fos* mRNA levels were relatively high [arrowhead in (**C**)] on the same side as *Per1* and AVP, and immunohistochemistry for the c-Fos protein showed a unilateral rostral [arrowhead in (**D**)] and dorsal [arrowhead in (**E**)] distribution. Scale bars: 1.1 mm, (A) to (C); 400 μm, (D) and (E).

and right-sided circadian oscillators. This is a unique neural state, a "split" brain without surgical bisection. Because AVP is a neurotransmitter controlled by the SCN clock, and because AVP and other SCN efferents project ipsilaterally to their targets (*37*), other bilaterally represented brain regions outside the SCN may also be running on antipodal time.

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Integration of Multiple Signals Through Cooperative Regulation of the N-WASP–Arp2/3 Complex

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The protein N-WASP [a homolog to the Wiskott-Aldrich syndrome protein (WASP)] regulates actin polymerization by stimulating the actin-nucleating activity of the actin-related protein 2/3 (Arp2/3) complex. N-WASP is tightly regulated by multiple signals: Only costimulation by Cdc42 and phosphatidylinositol (4,5)-bisphosphate (PIP_2) yields potent polymerization. We found that regulation requires N-WASP's constitutively active output domain (VCA) and two regulatory domains: a Cdc42-binding domain and a previously undescribed PIP_2-binding domain. In the absence of stimuli, the regulatory modules together hold the VCA-Arp2/3 complex in an inactive "closed" conformation. In this state, both the Cdc42- and PIP_2-binding sites are masked. Binding of either input destabilizes the closed state and enhances binding of the other input. This cooperative activation mechanism shows how combinations of simple binding domains can be used to integrate and amplify coincident signals.

Many cellular processes are controlled by networks of interacting signaling pathways (*1, 2*). For example, during directed cell motility, multiple pathways converge to precisely target actin polymerization to the cell's leading edge. Little is known, however, about the molecular mechanisms by which the relevant signaling proteins integrate these multiple inputs to yield a coordinated response.

WASP and its homolog N-WASP link