One might hypothesize that the cytosolic and vesicular sites are the same as those on the intact cell's membrane but behave as they do because the affinity of the sodium channel site for TTX is dependent on voltage or some other feature of the intact cell and because neither the vesicular nor the cytosolic sites have the same conformation as that of the intact cell's membrane site. Since the amount of membrane-bound STX does not change with isolation of the membrane in a variety of solutions, STX binding probably is not characterized by a similar dependence.

Alternatively, one might hypothesize that the cytosolic and intact membrane sites are fundamentally different. Then, if the sodium channel site affinity is lost during membrane isolation procedures, STX binding to vesicles could be due to adsorption of the cytosolic sites onto the vesicles. In both locations the difference in affinities for TTX and STX would be a property of the cytosolic sites. According to this hypothesis, the similarity of the number of sites in the intact cell's plasma membrane and in the derived vesicles is simply fortuitous. The hypothesis that the sites are related seems more likely to us.

In conclusion, we have found a tissue with either an exceptionally labile sodium channel or a cytoplasmic binding site with high affinity for STX. If the latter is true, as seems more likely, then we have found either an exception to STX's specificity for sodium channels or a soluble structure related to the sodium channel site.

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Splitting of the Circadian Rhythm of Activity Is Abolished by Unilateral Lesions of the Suprachiasmatic Nuclei

Abstract. *The circadian rhythm of activity in vertebrates often splits into two components after continuous exposure to constant light. This observation suggests that at least two circadian pacemakers underlie the activity rhythm. After unilateral ablation of the hypothalamic suprachiasmatic nuclei in hamsters, the splitting phenomenon was eliminated and a single rhythm of activity was established. The period of the new circadian activity rhythm differed from the periods of the split rhythm and that preceding the split. These results suggest an interaction between the bilaterally paired suprachiasmatic nuclei in the generation of the circadian rhythm of activity.*

Although circadian phenomena in the biological world have been described for centuries, the endogenous nature of circadian oscillators has been established only in the last 20 years. Elaboration of the biological basis of the circadian clock in multicellular organisms has been complicated by reports that both physiological and behavioral circadian rhythms are based on the interaction of more than one circadian pacemaker (*1, 2*). One of the strongest indications that there are multiple oscillators is provided by the observation that in many vertebrates the circadian rhythm of locomotor activity can dissociate, or "split," into two distinct bouts of activity (*1*). Any attempt to explain the biological basis for the generation of circadian rhythms in vertebrates must take into account the splitting phenomenon, although at present little is known about the physiological basis of splitting.

Splitting of the circadian locomotor rhythm occurs in various vertebrate species, including lizards, birds, and mammals (*1, 3*). In a study of the split rhythm in the golden hamster (*Mesocricetus auratus*), Pittendrigh and Daan (*1*) observed that (i) when splitting first occurs, the two activity components often free-run (that is, the activity bouts are not entrained) and have different circadian periods until they stabilize approximately 180° out of phase, and (ii) the attainment of the stabilized antiphase relationship between the bouts of activity is accompanied by a decrease in the circadian period (τ) of the split activity rhythm. On the basis of this splitting phenomenon and other observations, several investigators have suggested a system of at least two coupled oscillators as the underlying mechanism for the mammalian circadian pacemaker system (*1, 2, 4*).

Attention has focused on the hypothalamic suprachiasmatic nuclei (SCN) as the neural location of the circadian oscillators in mammals. The SCN receive a direct retinal innervation that relays information about the light-dark environment for the photic entrainment of endogenous circadian rhythms (*5*). Moreover, complete bilateral destruction of the SCN leads to the loss of a number of different circadian rhythms (*6, 7*). Although the SCN are a vital component of the circadian system, little is known about the complex neural interactions underlying the organization of the mammalian circadian system or how multiple circadian pacemakers might be coupled to each other to generate a single circadian pattern.

We have provided anatomical evidence that the two SCN are reciprocally innervated (*8*). This anatomical reciprocity indicates a physiological coupling, even though unilateral SCN lesions have shown little or no effect on a number of diverse circadian rhythms (*6, 7*); however, these studies had focused on entrainment and the generation of an intact circadian rhythm. We examined the effect of unilateral SCN ablation on split circadian activity patterns of golden hamsters maintained in constant light. The results suggest that both suprachiasmatic nuclei are required to produce the split activity pattern.

Animals were housed individually in cages equipped with activity wheels, and locomotor activity was recorded (*9*). Animals were maintained in constant light (*10*), and their activity records were examined periodically to determine when a split pattern of activity developed. Small electrolytic lesions aimed at a single SCN were made in 19 hamsters that had demonstrated many weeks of a split rhythm of locomotor activity (*11*). After the lesions were made, locomotor activity was recorded for several months in the same photic environment.

Because knowledge of the locus of the lesion was critical to the interpretation of the behavioral findings in this study, a rigorous histological procedure was used to determine the exact extent of the lesions. An injection of 4.0 μl of a 30 percent horseradish peroxidase (HRP) solution was given in the vitreous of the eye contralateral to the side of the lesion 24 to 48 hours before the animal was

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killed. The animals were perfused with 0.9 percent saline and then with 2.0 percent glutaraldehyde; serial 60-μm frozen sections were cut from the anterior septal region through the superior colliculus. Alternate sections were stained by the Nissl method or were reacted with tetramethylbenzidine for the histochemical demonstration of anterogradely transported HRP (*12*). Retinal terminals and processes infiltrate almost the entire three-dimensional boundaries of the hamster SCN and therefore these processes served as an ideal marker for indicating the completeness of the lesions (*12*). The histological analysis was performed without information about the animal's activity patterns.

The lesions aimed at a single SCN generated three distinct types of behavioral responses. In group A, the lesion abolished the split rhythm and a new single circadian rhythm of activity was established (N = 8) (Fig. 1A, top). Examination of the tissue sections from animals in this group indicated that all of the lesions were unilateral, with no damage to the contralateral SCN. In four animals, unilateral SCN ablation was 100 percent complete, with only minor damage to the surrounding neuropil (Fig. 1A, bottom). In two animals, the lesions destroyed approximately 50 percent of a single SCN, and in the other two animals, the lesions were just caudal to one of the SCN and the lesions extended to the ventral surface of the brain. In most instances, the locomotor activity was diffuse (Fig. 1) in comparison to the activity bouts before production of the lesion. The circadian period of the new activity rhythm was different from that of the split activity system in seven of the eight animals; five animals showed a decrease in τ and two showed an increase.

In group B, the lesion abolished the split activity rhythm and an ultradian or aperiodic pattern was established (N = 5) (Fig. 1B, top). The lesions in this group of animals were bilateral, completely ablating both SCN in four animals (Fig. 1B, bottom). Only a tiny portion of the lateral aspect of each SCN was spared in one animal, and this animal showed some indication of a very weak circadian rhythm. In group C, the lesion did not abolish the split activity pattern (N = 6) (Fig. 1C, top). Histological examination of these animals revealed that all the lesions were unilateral with centers anterior, lateral, or dorsocaudal to the SCN. The SCN were completely spared in three animals (Fig. 1C, bottom), and in three animals the damage was minor (< 20 percent). The circadian period was unaffected in five of these animals, and the sixth showed an increase in τ after surgery.

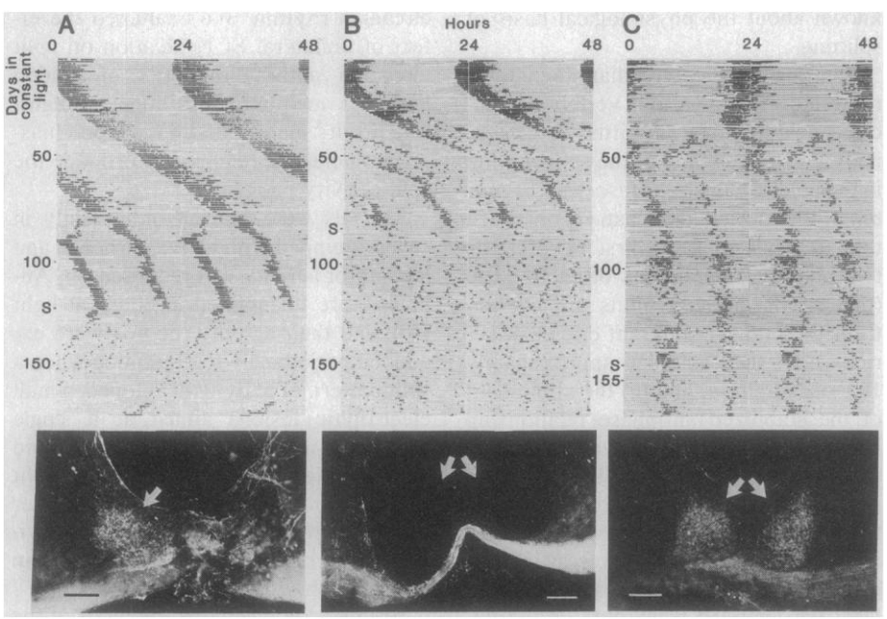

Fig. 1. Continuous records of wheel-running activity (top), and dark-field photomicrographs of coronal brain sections (bottom) of representative hamsters that were maintained in constant light for 172 to 174 days. The *S* to the left of each activity chart designates the day of surgery. The activity records have been plotted twice for a 48-hour time interval to facilitate the visual examination of the activity pattern. The arrows in the photomicrographs indicate either the HRP-filled retinal processes in the undamaged SCN (A and C) or the absence of SCN tissue (B); the calibration marks equal 100 μm. (A) No HRP-labeled retinal processes were found in the lesioned SCN, and the adjacent SCN was undamaged. This resulted in the abolition of the split condition and the induction of a single circadian rhythm of activity with a very short period. (B) The bilateral ablation of the SCN induced a noncircadian activity pattern. (C) A lesion that was dorsal and caudal to the SCN had no effect on the split activity rhythm.

The finding of a total disruption of the split circadian rhythm of activity in the five animals that had received bilateral SCN ablation (group B) substantiates a similar finding in a single rat that had shown split rhythms in drinking and feeding behavior (*13*). These results were expected since elimination of a large number of diverse circadian rhythms after bilateral SCN ablation has been well documented (*6, 7*). The finding in group C was also expected because, although damage to the SCN abolishes circadian rhythms, damage to the surrounding hypothalamic tissue is usually ineffective (*7*). Sham SCN lesions (no current passed through the electrode) do not alter the split condition in hamsters (*14*).

Our principal observation is that either complete (100 percent) or major (> 50 percent) destruction of a single SCN abolishes the split activity rhythm and results in the induction of a new single circadian activity (group A). Unilateral lesions immediately posterior to a single SCN have the same effect on splitting as unilateral destruction of the SCN. Van den Pol and Powley (*7*) observed that bilateral lesions posterior to the SCN produce the same effects on circadian rhythms as total SCN ablation. Moreover, deafferentation caudal to the SCN (but not anterior or lateral) also has the same effect on rhythmicity as total SCN ablation, presumably by destroying SCN efferents (*7, 15*). We conclude that the elimination of the split activity rhythm is a direct consequence of unilateral damage to the SCN or its neural connections.

The spontaneous occurrence of splitting in the hamster is always associated with a shortening of the period of the free-running activity rhythm (*1*) (Fig. 1). Therefore, the abolition of splitting and the induction of a single circadian activity pattern after unilateral SCN ablation could be expected to result in a lengthening of τ. On the contrary, there was a further decrease in τ in most animals after destruction of a single SCN. Thus, the ablation of a single SCN (or its efferents) often results in a period of activity rhythm that is different from the period of the rhythm when both SCN are functioning to generate either the single intact or the split rhythm. These results suggest that the period of the circadian activity rhythm also depends on an interaction between the two SCN.

There is now evidence that circadian

pacemakers are located in paired structures in bilaterally symmetric invertebrates, and the degree to which bilaterally paired oscillators are mutually coupled appears to be quite variable among species (*16, 17*). Hudson and Lickey (*18*) demonstrated that the two bilaterally symmetric circadian pacemakers in the eyes of *Aplysia* can become desynchronized from each other and free-run out of phase under constant lighting conditions. One interpretation of our data is that the bilaterally paired SCN might function as separate circadian oscillators in a manner similar to that of the paired oscillators of invertebrates. To extend this analogy further, the two SCN oscillators in the hamster might normally be coupled, but this coupling might be altered under certain environmental conditions (such as constant light), thus giving rise to the split condition.

Our results demonstrate the importance of the interaction of the SCN in the generation of circadian rhythms. One interpretation of this interaction consistent with the results is that each SCN has the capacity to serve as a circadian oscillator. Another possibility is that a set of interacting pacemakers may reside within each SCN, and the loss of the split rhythm may be a consequence of the total number of these oscillators destroyed; whether or not the destruction is unilateral may not be important. Experiments designed to sever the reciprocal neural connections between the SCN and partial bilateral SCN lesions in animals with split activity rhythms may clarify the role of the interaction between the SCN in the generation and maintenance of circadian rhythms.

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Spiroplasmas: Cultivation in Chemically Defined Medium

Abstract. *A chemically defined medium, CC-494, supports the cultivation in vitro of several spiroplasmas belonging to three distinct serogroups. Medium CC-494 supports the growth of flower spiroplasmas FS 23-6 and FS SR-3 and of honey bee spiroplasma HBS AS-576. The maximal populations of the two flower spiroplasmas and honey bee spiroplasma are comparable to those cultured in the undefined medium C-3G. The growth rate for all three spiroplasmas is slower in the defined medium.*

Spiroplasmas, which are organisms pathogenic to plants, insects, and several vertebrates, have been grown in media that, like those used for mycoplasma cultivation, are supplemented with one or more undefined constituents such as PPLO (pleuropneumo-like organismic) broth base, horse or fetal bovine serum, and yeast extract. These constituents are complex and difficult to replace with defined chemicals (*1*). Although completely defined media (*2*) have been developed for *Mycoplasma mycoides* and *Acholeplasma laidlawii*, no defined formulation has been reported for the culture of spiroplasmas. Knowing the chemical nature of the culture medium is important for determining the nutritional requirements, metabolic pathways, and biosynthetic capabilities of spiroplasmas and for characterizations of spiroplasma isolates. We now report the successful cultivation of spiroplasmas in a chemically defined medium.

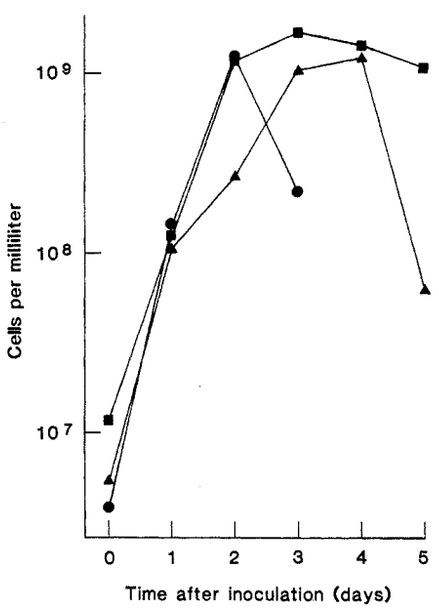

Fig. 1. Growth of FS 23-6 ●, FS SR-3 ▲, and HBS AS-576 (■) under optimal osmolarity.

Three strains of spiroplasmas, representing three of the six serogroups (*3, 4*), were used for the study. Flower spiroplasmas FS 23-6 (ATCC 29989) and FS SR-3 (ATCC 33095) were isolated from flowers of tulip trees in Maryland and Connecticut, respectively (*5*). Honey bee spiroplasma HBS AS-576 (ATCC 29416) was isolated from diseased bees (*6*). Routinely, the spiroplasmas were maintained in C-3G medium (*7*).

A defined medium was developed for the spiroplasma cultivation; CC-494 supports the growth of FS 23-6, FS SR-3, and HBS AS-576 (Table 1). The basal medium was prepared by mixing stock solutions (*8*) of the different fractions in Hepes. The lipid portion and bovine serum albumin (BSA) were prepared separately (*9*) and added to the basal medium in the ratio 1:4 (by volume). The *p*H of the basal medium and lipid-BSA portion was adjusted to 7.5 before mixing. The completed medium was then filter-sterilized (pore diameter, 0.45 μm), and 2.5-ml portions were dispensed into test tubes. Inoculum (30 μl) of each spiroplasma from a culture in the log phase of growth was added to each tube containing CC-494 medium, and the cultures were incubated at 31° ± 1°C. Subse-

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