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RAPID COMMUNICATION

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Different patterns of circadian oscillation in the suprachiasmatic nucleus of hamster, mouse, and rat

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Abstract Although spontaneous neural firing in the mammalian suprachiasmatic nucleus is accepted to peak once during mid-subjective day, dual activity peaks have been reported in horizontal brain slices taken from hamsters. These two peaks were interpreted as new evidence for the theory of dual circadian oscillators and raised the expectation that such activity would be found in other circadian model systems. We examined hamster, mouse, and rat slices in both coronal and horizontal planes and found a second peak of activity only in hamster horizontal preparations. This raises interesting questions about the relative circadian physiology of these important experimental animals.

Keywords Circadian rhythm · Hamster · Mouse · Rat · Suprachiasmatic nucleus

Abbreviations *CT* circadian time · *SCN* suprachiasmatic nucleus

Introduction

The hypothalamus is a central integrator of many physiological variables, such as motor activity, sleep, body temperature and feeding behaviors. In the ventral hypothalamus, the suprachiasmatic nucleus (SCN) was identified in 1972 as a nucleus critical for imposing circadian rhythmicity on mammalian physiology and behaviors (Moore and Eichler 1972; Stephan and Zucker 1972). Later, spontaneous electrical activity recorded from a deafferented SCN "island" in behaving rats was found to produce an oscillation with a ~24-h period, peaking at midday (Inouye and Kawamura 1979). In 1982, three independent laboratories demonstrated that the population of neurons within the SCN collectively express a circadian rhythm of electrical activity in vitro (Green and Gillette 1982; Groos and Hendriks 1982; Shibata et al. 1982). These reports all used coronal brain slices to study the SCN electrical rhythm. Coronal slices have been the preparation of choice to study SCN rhythmicity, and the electrophysiology in this orientation is well documented (Shibata et al. 1982; Gillette 1986; Ding et al. 1998; Biello and Dafters 2001; Meyer-Spasche et al. 2002) and consistent among different species, such as hamster (Yannielli and Harrington 2000), mouse (Akiyama et al. 1999) and rat (Tischkau et al. 2000). As a result, comparison of SCN rhythmicity in different slice orientations has not been systematically examined. However, it has long been understood that the heterogeneous nature of the SCN may be reflected by differences in clock behavior when examined in different planes of section (Gillette 1991).

Investigations of SCN neuroanatomy and neurochemistry have established that this nucleus contains subregions of differing cytoarchitecture (van den Pol 1991; Abrahamson and Moore 2001), neuromodulators (Abrahamson and Moore 2001), and clock gene expression (de la Iglesia et al. 2000; Hamada et al. 2001). Functional distinctions among these subregions are just beginning to be realized, and it remains to be determined how these subregions are organized to produce output circadian rhythms. Recently, Jagota et al. (2000) published a report that multi-unit recordings of hamster SCN from horizontal slices produced a two-peak activity pattern. The peaks showed sensitivity to prior photoperiod and were independently shifted in response to nighttime glutamate application. The data were interpreted as the expression of morning and evening

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oscillators long proposed by Pittendrigh and Daan (1976) and support the hypothesis that the plane of section could alter clock coupling and expose the presence of more than one oscillator (Dunlap 2000; Jagota et al. 2000; Daan et al. 2001). We sought to understand whether this activity pattern was observable across mammalian species, thus proving a general feature of SCN organization. We here report that rat and mouse SCN in horizontal slices exhibit only a single peak of activity, similar in timing and amplitude to those seen in coronal SCN slices. Comparisons to hamster brain slices suggest that the two-peak circadian rhythm in horizontal slices may be a phenomenon specific to hamster circadian clock organization.

Materials and methods

Animal entrainment: rats

SCN-containing slices were prepared from 6- to 12-week-old inbred Long-Evans rats (LE/BluGill, http://pga.mcw.edu/pga-bin/strain_desc.cgi). Rats were entrained to a daily 12:12 h light:dark (LD) cycle. Circadian time (CT) in slices was projected from the light schedule to which the rats were entrained, where time of "lights on" was designated as CT 0. Subjective day corresponded to CT 0–12, and subjective night was defined as CT 12–24.

Animal entrainment: hamsters

Male golden Syrian hamsters (Harlan, Indianapolis, Ind., USA) were housed in pairs. Because hamsters undergo hormonal and reproductive changes under a 12:12 h LD cycle, animals were entrained to a 14:10 h LD schedule for a minimum of 10 days prior to sacrifice and preparation of brain slices. As with rats, circadian time was projected from the preceding light schedule. On a 14:10 h light-dark schedule, either lights-on time or onset of activity will differ from the times of an animal on a 12:12 h LD schedule. By convention, time of "lights off" was designated as CT 12. This set time of "lights on" at CT 22.

Animal entrainment: mice

C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, Me., USA) or were kindly donated by Dr. Karl Obrietan (Ohio State University). Mice were introduced to a 12:12 h LD schedule for a minimum of 2 weeks prior to sacrifice and SCN recordings. CT was projected from the entrainal lighting cycle, with time of "lights on" designated as CT 0 and time of "lights off" at CT 12.

Brain slice preparation and single-unit recording

Animals were sacrificed by cervical dislocation (mice) or guillotine (rats and hamsters) during subjective daytime, and coronal brain slices (500 μm) were prepared using a tissue chopper. Horizontal brain slices (400–450 μm) were prepared with a vibrating tissue slicer. Brain slices were maintained in a brain slice chamber perfused with Earle's Essential Balanced Salt Solution, supplemented with 24.6 mmol l^{-1} glucose, 26.2 mmol l^{-1} $NaHCO_3$, and 2.5 mg l^{-1} gentamicin, and saturated with 95% O_2/5% CO_2 at 37°C, pH 7.4. In the case of mice, slices were kept at 34°C. Salt solution and gentamicin were both purchased from Sigma (St. Louis, Mo., USA).

The single-unit recording method has been described previously (Tischkau et al. 2000) and is summarized as follows. A glass microelectrode was positioned over the SCN and advanced into the tissue until an electrical signal from a single neuron with a signal:noise ratio of at least 2:1 was isolated. Neuron activity was observed for stability and then counted for 4 min using LabView software (National Instruments, Austin, Tex., USA). The electrode was then advanced until another cell was isolated or until the microelectrode passed through the entire slice thickness. The electrode was then repositioned arbitrarily to sample single-unit activity from another location within the SCN. To avoid the possibility that right and left SCN could be out of phase with each other (de la Iglesia et al. 2000; Schaap et al. 2001), data from each experiment were collected solely from the right or left SCN, but never both. Single-unit firing rates from a single experiment were grouped into 2-h running averages using 15-min time lags. Time-of-peak for each experiment was visually determined to be the symmetrically highest point of neuronal activity along the time axis. Peak time determinations were made independently by two of the authors (P.W.B. and P.T.L.), and the average of these two measurements was used for analysis of data.

Results

In rat coronal slices, peak electrical activity of the SCN has been established to occur ca. CT 7 of the animal's subjective day (Tischkau et al. 2000; Biello and Dafters 2001; Prosser 2001; Meyer-Spasche et al. 2002) and can persist for at least two circadian cycles. Figure 1 illustrates the pattern of SCN brain slice ensemble activity for hamster, mouse, and rat in both coronal and horizontal planes of section. These single unit recordings demonstrate the persistence of SCN circadian rhythms in vitro in both coronal and horizontal planes of section.

Like the rat, both mouse and hamster SCN generated single midday peaks of activity when prepared as coro-

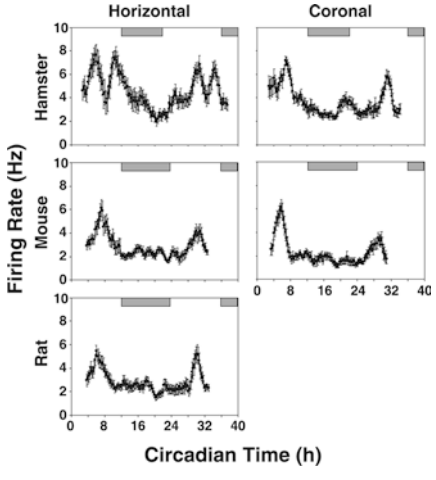

Fig. 1 Representative single-uni t recordings from coronal and horizontal brain slices of hamsters, mice, and rats demonstrate the reproducibility of electrical activity peaks in two orientations and their persistence for two circadian cycles in vitro. The suprachiasmatic nucleus (SCN) from horizontal mouse and rat brain slices produces only one peak of circadian activity, whereas horizontal hamster slices produce two peaks. The SCN from coronal slices exhibits one peak in all three species. Data are presented as mean ± SE. *Dark bar* indicates subjective night

nal sections. Peak activity occurred at CT 6.25 ± 0.23 for mouse ($n = 3$) and CT 6.56 ± 0.83 h ($n = 3$) for hamster. In the horizontal orientation, however, rat and mouse SCN produced single-peak activity while hamster SCN produced two-peak activity, as reported in multi-unit recordings (Jagota et al. 2000). The rat SCN peaked near CT 7 (CT 6.81 ± 0.14 h, $n = 8$). These results from horizontal slices are similar to those from coronal slices published previously by different investigators from our lab using the LE/BluGill rat (Chen et al. 1999; Tischkau et al. 2000; Hunt et al. 2001). Mouse SCN activity peaked at CT 6.61 ± 0.21 ($n = 4$). The two peaks in hamster horizontal SCN occurred at CT 6.76 ± 0.24 h and CT 10.09 ± 0.13 h ($n = 4$).

In these experiments, rats and mice were entrained to a 12:12 h LD schedule prior to sacrifice, while hamsters were entrained to a longer 14:10 h light exposure. To determine if these horizontal slice results were reflective of the animals' prior photoperiod, mice on a 12:12 h LD schedule were compared to mice entrained to a 14:10 h LD background. Mice subjected to this longer light exposure also exhibited single-peak SCN activity (P.T. Lindberg and M.U. Gillette, unpublished results).

Figure 2 illustrates the distribution of time-of-peak for all the recordings with considerations for species and slice plane of section. Because a second peak has not been previously observed in untreated single-unit recording, we were unable to generate an independent probability of its occurrence, and were therefore unable to apply conventional statistics. However, because the second peak was observed in every horizontal hamster slice ($n = 4$) and in no other preparation (total $n = 22$), we conclude that the occurrence of the dual peak by chance alone is unlikely.

Discussion

While past investigations have reported a spontaneous circadian rhythm of SCN neuronal activity that peaks near midday in coronal and parasagittal slices from rat (Tischkau et al. 2000; Kim et al. 2001; Prosser 2001), the present report is the first to investigate the circadian behavior of SCN activity across three mammalian species and two planes of section. SCN in both coronal and horizontal hypothalamic slice preparations from rat and mouse exhibited a single peak of activity measured by averaging single-units that was like the peak observed with multi-unit recordings of rat SCN in vivo or in vitro (Inouye and Kawamura 1979; Tcheng and Gillette 1996; Liu et al. 1997; Meijer et al. 1998). We found variation in one species, the golden hamster that depended on plane of section.

Whereas the hamster SCN in coronal section generated an oscillation with a single peak, in the horizontal brain slice it exhibited bimodal peak activity. This pattern of SCN activity is not an artifact of sampling or recording method. In both single-unit (this report) and multi-unit recordings (Jagota et al. 2000), a single hamster SCN in a horizontal slice exhibits two peaks of activity. It is notable that the shape and precise timing of the activity peaks differ between these single-unit data, and the multi-unit data compiled by Jagota et al. (2000). Those multi-unit recordings measured the onset and offset of increased activity, whereas single-unit activity reports time of peak. As such, the peak activity times gathered from multi-unit recording appear longer and flatter than peaks from single-unit firing-rate averages. Although the average time of peak seen in single-unit experiments is captured between the average onset and offset reported from multi-unit experiments (Jagota et al. 2000), it is not clear that the activity profile of these two methods is identical. For these reasons, comparing profiles between the two methods is difficult. However, the core finding of a second peak is reliable across protocols.

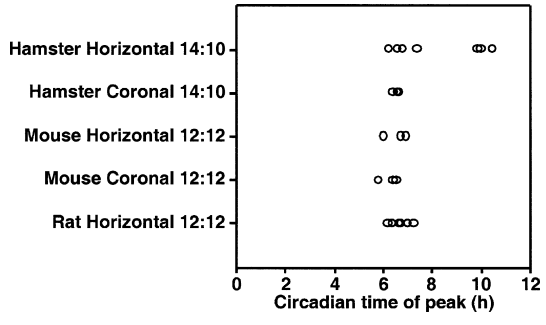

Fig. 2 Single-unit activity recordings reveal time-of-peak results for hamster, mouse and rat SCN in horizontal and coronal planes of section. Hamster SCN from horizontal slices produce two peaks of circadian activity, seen as clusters of data around circadian time 7 and 10

Comparative functional anatomy

The basis of this effect of plane of section on the circadian rhythm must reside in the functional anatomy of the hamster SCN. Understanding of the intrinsic functional organization of the SCN is at an early stage; nevertheless, general patterns are emerging. Vasoactive intestinal peptide (VIP)- or arginine vasopressin (AVP)-expressing neurons occupy identifiable core and shell subregions, respectively, in species ranging from rodents to humans (Moore et al. 2002). Distributions of other neuropeptides (somatostatin, gastrin-releasing peptide, cholecystokinin, corticotrophin-releasing factor) vary markedly among species (Albers et al. 1992; Morin 1994; Moore and Silver 1998). Across all three species studied here, additional organizational differences exist with respect to the dimensions of the SCN, the topography of retinal innervation (Moga and Moore 1997; Abrahamson and Moore 2001; Muscat et al. 2003) and the expression and distribution of key calcium-buffering proteins (Moore 1983; Albers et al. 1992; LeSauter et al. 1999). The horizontal brain slice largely preserves the optic tract and attendant retinohypothalamic

innervation, but may sever the dorsal-most extent of the taller hamster SCN. Functions of this shell region are unknown beyond housing vasopressinergic output neurons in all three species.

Notable among behavioral and physiological characteristics of the circadian system that distinguish the hamster are strong photoperiodic control of reproductive state and propensity toward splitting of behavioral rhythms into two components that occur in antiphase (Pittendrigh and Daan 1976). This reordering of behavioral rhythms into two circadian components is paralleled by realignment of molecular rhythms such that peak expression alternates between the left and right SCN (de la Iglesia et al. 2000). Distinct from splitting, the two peaks observed in the hamster SCN in the horizontal slice both appear in subjective daytime, separated by $\sim$3 h, and they are expressed within a single SCN.

Most studies examining SCN clock properties in slices have used the coronal plane of section. However, the present study suggests that by examining the SCN in different planes of section and in different species, we may identify components of complex tissue-level oscillations, and insights may emerge as to how organismic rhythms are generated and orchestrated as outputs from the SCN. This possibility emphasizes the importance of comparative, cross-species studies in evaluating the neural substrates of the organization of complex behaviors. Differences among species become particularly important when considered in light of the findings of Schwartz and colleagues (Jagota et al. 2000) which, considered alone, make a convincing case that separate oscillations in electrical activity might represent separate circadian oscillators.

Possible substrates for divergence

The question remains as to why the hamster SCN in horizontal section exhibits two activity peaks. It has been suggested (Dunlap 2000) that some coupling factor, which would normally suppress the emergence of a second oscillation, had been removed during preparation of the horizontal slice of hamster SCN. This putative factor might have escaped section in both mouse and rat for a number of reasons. The hamster SCN is taller than either the mouse or the rat (Lydic et al. 1982; Cassone et al. 1988); thus, a coupling factor removed during horizontal section from the dorsal SCN or subparaventricular zone of hamster might be spared in the case of mouse or rat. Alternatively, the putative coupling factor might be more distributed in the mouse and rat. Such a factor not only could underlie the single oscillation in horizontal rat and mouse slices, but could also result in divergent behavioral responses to constant light stimulation.

Species differences in the functional organization of the SCN could be manifest at a number of levels. They might take the form of different intra-SCN neuronal couplings or circuits, different relationships among SCN subregions or even different couplings with extra-SCN brain regions. It follows that if a putative coupling factor determines the expression patterns of adaptive changes in behaviors under circadian control, such as seasonal breeding or crepuscularity, then such a factor would comprise an important component of the organism's circadian clock. Additionally, circadian gene or protein expression within the SCN may be anatomically different between species, which may result in different patterns of circadian activity when examined in a horizontal slice preparation. For example, in examining the endogenous rise of *Per1*, the pattern of mRNA localization in the hamster SCN (Hamada et al. 2001) is most intense in and near the midline, unlike that of rat (Yan et al. 1999) or mouse (Sun et al. 1997). To our knowledge there are no reports examining circadian protein expression in horizontal slices. Further research linking gene expression and SCN output across mammalian species and in different planes of section will be important to our understanding of pacemakers or coupling factors.

We are left with a spectrum of possible substrates for divergence between the hamster SCN and the central clocks of other rodents. Either the hamster possesses circadian machinery not developed in the other species, or there has been an alteration in the manner by which it expresses that machinery, both behaviorally in the animal and electrically in a horizontal brain slice. In the case of the former, the current data are the first clues of such uniqueness in the golden hamster, and the physiological role of such machinery must be explained. In the case of the latter, the current data suggest that a locus within the dorsal SCN or subparaventricular zone may contribute to differential expression of rhythms due to its salient morphology. As research continues to elucidate the functional organization of the SCN, it will be important to remain cognizant that a component of the SCN that is tightly coupled in one species might be less so in another.

During the final revisions of this paper, it has come to our attention that horizontal rat slices expressing a *Per1-luc* reporter construct also exhibit a single peak of bioluminescence, further supporting the results found by single-unit recording (E. Herzog, personal communication).

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