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Development of the mouse suprachiasmatic nucleus: determination of time of cell origin and spatial arrangements within the nucleus

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Abstract

The suprachiasmatic nucleus (SCN) in mammals functions as the principal circadian pacemaker synchronizing diverse physiological and behavioral processes to environmental stimuli. It consists of heterogeneous populations of cells with unique spatial organization that can vary among species, but are commonly discussed within a framework of two principal regions, the ventrolateral or dorsomedial halves of the nucleus or in other instances the core and shell. In both hamsters and rats, cells of different SCN regions have been shown to have different developmental histories. Using bromodeoxyuridine as a marker of cell division, the present study investigated the time of SCN cell origin in mice (C57BL/6) and their settling patterns within the nucleus. Results show that SCN cytogenesis occurs between embryonic days 12 and 15 and is complete 5 days prior to birth. Cells born on embryonic day 12 are mainly confined to a ventrolateral region of the mid-SCN, whereas cells produced later on embryonic days 13.5 and 14.5 form a cap around the cells produced first and extend into the posterior and anterior ends of the nucleus. These results suggest an ordered spatiotemporal program of SCN cytogenesis whereby a mid-SCN core is born first followed by a surrounding shell of later-born cells. Variation in cytogenesis could affect the relative sizes of different SCN regions and, thereby, affect its function. The relative contributions of a highly ordered program of cytogenesis and intercellular interactions after post-mitotic cells leave the germinal epithelium remain to be determined.

Keywords

SCN; neurogenesis; circadian; C57BL/6

1. Introduction

Daily rhythmicity is widespread in behavior and physiology, the most obvious being the sleep/wake or activity/rest rhythms of humans and other animals. The timing of behavioral or physiological states with respect to the environment is achieved through the entrainment of a central circadian pacemaker in the hypothalamus, the suprachiasmatic nucleus (SCN). The SCN receives direct retinal input and is required for daily rhythms expressed by the animal as

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a whole. This central clock is a bilaterally represented nucleus that lies just lateral to the third ventricle at the base of the anterior hypothalamus [16]. In rats, each nucleus comprises approximately 10,000 neurons [10,21] plus glia in the ratio of about 3:1 neurons to astroglia [10]. Isolated SCN neurons spontaneously generate circadian rhythms, and many SCN neurons appear to have this capacity [6,32].

The anatomical boundaries of the SCN, defined by cell density, correspond well with functional markers such as 2-deoxyglucose utilization [26], c-fos expression [17], and the expression of genes involved in the generation of circadian oscillations (“clock genes”) [9]. Accordingly, although other tissues and cells are known to express endogenous molecular oscillations, SCN neurons are functionally distinct from surrounding hypothalamic neurons. Although they are all equally part of an anatomically and functionally defined nucleus, SCN neurons are themselves heterogeneous. They differ with respect to the expression of specific neurotransmitters and neuropeptides [21,31], their efferent and afferent connections [1,22], and their neurophysiological firing patterns [14]. In addition, isolated SCN cells express different circadian periods [13,32] and, within the nucleus, different individual cells or groups of cells oscillate with different phases [25,33].

The unique properties of SCN neurons and the differences among them must have functional significance related to properties such as the period expressed by the pacemaker as a whole, the magnitude of phase-shifts, or the ability of the pacemaker to regulate multiple functions. Little is known about how SCN organization arises during development or how the fates of SCN cells are specified, yet it is likely that abnormal regulation of circadian rhythms would result from either genetic or environmental disruption of these processes. For example, mice in which the optic primordia are resorbed between embryonic days 11.5 and 13.0 have anatomically abnormal SCN and, in some cases, abnormal circadian rhythms [29].

A starting point for understanding the regulation of SCN development is to determine when and how SCN cells are produced. SCN neurogenesis has been described for several mammals, in particular rats [3] and hamsters [4,7]. These studies described the ages when SCN cells are “born” (undergo their final mitotic division) and the pattern in which different regions of the SCN are produced. Altman and Bayer [2] also followed the migration of newly produced cells and traced their origin to the germinal epithelium of the ventral third ventricle. Hamster SCN neurogenesis is similar, although studies in hamsters used greater spatial and temporal resolution [7] and looked at the production of identified cell types [4]. Considering the growing importance of mice in many areas of physiology, including circadian rhythms, it is surprising that a detailed analysis of SCN neurogenesis has not been reported in this species. Neurogenesis of the mouse hypothalamus has been performed using tritiated thymidine [27], but without focus on the SCN. One study examined genesis of hypothalamic cells that express the neuropeptide, vasopressin (VP), including VP cells in the SCN [23], but comparisons with other regions of the SCN were not included. The present study was undertaken to provide a detailed description of the temporal and spatial patterns of SCN cytogenesis in the most commonly used mouse strain for circadian studies, C57Bl/6. Dividing cells were labeled with the thymidine analogue, bromodeoxyuridine (BrdU) and labeled cells were visualized by immunocytochemistry. Results show that SCN neurogenesis in C57Bl/6 mice is similar to that in other rodents, and provide new insight into the ways different regions of the SCN are formed.

2. Results

2.1. SCN cytogenesis mainly occurs between E12 and E14.5

The timing of SCN cell generation was determined by plotting percent heavily BrdU labeled cells (HBL cells – Fig. 1) in the nucleus as a function of embryonic age (Fig. 2). There was a significant variation in % HBL cells in the SCN among the different age groups ($p < 0.0001$,

ANOVA). Few SCN cells were heavily labeled between E10 and E11.5, but then increased significantly by E12. The proportion of HBL cells in the SCN continued to rise over the next two days, reaching a peak on E13.5–E14. Thereafter, the % HBL cells declined and by E15.5 was similar to E11.5. Representative sections from three ages are shown in Figure 3.

2.2. SCN cytogenesis is heterochronous along the rostrocaudal and mediolateral axes

The largest changes in the % HBL cells occurred at different ages in the anterior (aSCN), middle (mSCN) and posterior (pSCN) regions of the nucleus (Fig. 4). For example, the % HBL cells increased significantly in the mSCN between E11.5 and E12 but not in either the aSCN or pSCN. In the aSCN and pSCN % HBL was not significantly different from E11.5 until E14.5 and E13.5, respectively. Examination of mSCN sections labeled at E12 revealed a difference in labeled cells between the dorsomedial (dmSCN) and ventrolateral (vlSCN) regions (see Experimental Procedure). This is expressed quantitatively by the significant increase in % HBL cells in the vlSCN between E11.5 and E12 (Fig. 5). In contrast, the smaller increase in the dmSCN at this age was not significant. A difference between vlSCN and dmSCN was reversed in the % HBL change that occurred between E13 and E13.5, the increase this time being significant in the dmSCN (Fig. 5). Sections of mSCN from E12 and E13.5 mice in Figure 3 illustrate the difference in labeling between the vlSCN and the dmSCN.

Both the pSCN and aSCN appeared to lag slightly the mSCN in the onset of substantial cytogenesis, primarily due to the early production of the vlSCN in the mSCN. All levels of the SCN showed relatively high levels of cytogenesis on E13.5 and E14, with greater production of the dmSCN (Fig. 5). Cells destined for the aSCN tended to be produced last (Fig. 4).

2.3. SCN cells show a complex settling pattern during nucleus formation

The results suggest the early production of a vlSCN in the mSCN followed by increased production of dmSCN during the overall peak of cytogenesis (E13.5 – E14). The continued production of some cells destined for both the dmSCN and vlSCN suggests, however, a spatiotemporal pattern more complex than a strict ventrolateral to dorsomedial gradient. Figure 6 shows SCN sections from brains labeled at three different ages (E13, E13.5, and E14) after the early production of vlSCN (E12). These sections show a commonly observed labeling pattern in which HBL cells are found in both dmSCN and vlSCN, especially the most lateral margin, but a distinct ventral region is nearly devoid of labeled cells. This suggests that a ventral region is produced first, primarily in the mSCN, followed by the production of cells dorsal, and lateral to it as well as in the anterior and posterior SCN. Taken together, these results suggest that an SCN “core” is produced first followed by the production of a “shell” of cells that surround it.

3. Discussion

The present study is the first to describe the spatiotemporal formation of the SCN in C57BL/6 mice. We used bromodeoxyuridine (BrdU) immunocytochemistry to determine when mouse SCN cells are born and how they settle in the nucleus. Accordingly, SCN cytogenesis occurred over three days, between E12 and E15, and was complete 5 days prior to birth. This observation parallels that seen in both rats [3] and hamsters [7,4]. In rats, SCN cell production occurs over 3 days (E14 to E17) and is complete 5 days prior to birth. Similarly, the duration of SCN cytogenesis in hamsters is 3 days (E10–E13) and is complete 3 days before birth. Based on comparative stages of development in humans and mice, human SCN cytogenesis is likely to occur during the 8th week of gestation [24]. Not surprisingly, if the germinal epithelium of the third ventricle in mice is disrupted during the time of SCN cytogenesis, this leads to gross morphological disruptions of the nucleus and of circadian rhythms [29].

Similar to the formation of rat and hamster SCN, as well as of hypothalamic nuclei in general [2,7], the SCN shows heterochronic waves of cytogenesis. In the present study, two gradients of cytogenesis were observed: a ventrolateral-to-dorsomedial gradient, most evident in middle sections, and, to a lesser extent, a caudal-to-rostral gradient. Cells in the middle SCN were born first on E12, whereas peaks of HBL cells were seen in posterior and the most anterior regions on E13.5 and E14, respectively. Closer observation of middle SCN sections (Fig. 6) suggested a more complex spatiotemporal settling pattern of cells. Cells born first on E12 settled in a ventral core, while those born later settled in a shell around the core, including the lateral margin, a dorsomedial cap, and the posterior and anterior ends of the nucleus. The observation that the anterior SCN had the most late-produced cells could mean that the shell is more represented in the anterior SCN, i.e. the core is situated slightly more posterior within the nucleus.

A spatiotemporal program of SCN formation in which a central core is produced before surrounding cells, as reported here for C57BL/6 mice, was not explicitly described in earlier reports of rat and hamster neurogenesis. Observations in those reports are, however, consistent with such a program. In hamsters, a ventrolateral-to-dorsomedial gradient of cell production was described, with the addition of late-produced cells on the lateral margin of the nucleus [7]. Thus, late-produced cells actually included both a dorsomedial cap and a lateral margin, similar to a shell as described here. In rats as well, an additional late-produced ventral groups of cells were described [2,3]. As discussed below, the organization of the mature SCN has been described as consisting of a core and shell based on the distributions of cells with specific characteristics, such as neuropeptide content. This is most striking in hamsters where a cluster of cells expressing calbindin (CaB) define a central core [28]. If a core defined in this way is produced first during SCN development, then cells expressing CaB should be among the earliest to be produced. This prediction is consistent with observations by Antle et al. [4] in which labeling with BrdU was combined with neuropeptide immunohistochemistry. They found that CaB-expressing cells, as well as other cells found in the core, were produced early in SCN neurogenesis. Vasopressin (AVP)-expressing cells, typically described as part of an SCN shell [21,5], were produced throughout neurogenesis and continued to be produced after the core-defining cells. Similarly, Okamura et al. [23] reported that production of AVP-expressing cells in the SCN reached a peak on E14, an age that corresponds to the late-produced shell in our study.

The early-formed core and late-produced shell observed here in mice could correspond to the core and shell that have been functionally defined for the adult SCN in several mammalian species, including mice [1,21,20,5]. These SCN regions are structurally and functionally heterogeneous based on different neuropeptide expression, photic input and sensitivity to light, expression pattern of clock and clock-driven genes and cytological features in general [11, 34,15]. Distinct roles for these two regions in the regulation of circadian rhythms have been proposed [30,5]. As recently discussed by Morin et al. [22], the definitions of SCN regions, including core and shell, vary among species and depend on the criteria used. Similarly, there is considerable overlap in the timing of cell production for different regions of the SCN. Nevertheless, the near absence of late-produced cells from a central region of the mouse SCN defines a region that is likely to correspond with structural and functional distinctions in the mature nucleus. The development of specific cell types will be necessary to test this hypothesis. An interesting question that is not addressed by the current study is whether late-produced cells ever settle in the core region, but migrate out or die as the SCN matures.

The present results show that cells born at a particular time do not become randomly distributed within the mature SCN; cells that are born late do not become part of the core but rather form a cellular cap that spans the entire nucleus. It is not known whether the fate of a cell born at a particular time (for example a cell expressing a particular peptide) is already determined when

it is born, or is determined by the micro-environment into which it migrates. It is possible that, once produced, a core of early cells organizes the fates of cells that are born subsequently. A test of this would be to force cells into an environment without a previously produced core. In several studies SCN neurons have been dispersed in culture during development, disrupting their normal spatial relationships, and cells with different characteristic phenotypes were observed [32]. In all cases; however, the cells were dispersed after SCN formation was complete. In these instances the fates of cells could have been determined before or after they became postmitotic. Thus, it remains unclear whether disruption of cell-cell interactions during the initial migration of SCN cells would eventually affect their fates.

Altman and Bayer [2] observed the migration of labeled neurons in rat hypothalamus and concluded that the SCN develops in the third wave of neurogenesis of the diencephalon from a region they called the suprachiasmatic primordium – a specialized zone of the ventral, internal germinal layer of the third ventricular neuroepithelium. They proposed that different regions of the SCN arise from different parts of the epithelium. Even if core and shell SCN cells arise from different parts of the germinal neuroepithelium, their fates could subsequently be determined by their postmitotic environment.

Circadian pacemaker properties are known to be affected by intercellular interactions. In particular, the number of cells and the proportions of different cell types affect period and the stability of rhythms [8,19,12,18]. If functionally different cells are produced at different times, as the present results indicate [4], then small changes in the duration or intensity of proliferation of SCN progenitors at different times in development could be one source of variation (both within and between species) in the overall function of the nucleus.

In general, the SCN is now recognized as a highly organized structure. The present results show that, at a gross level, a program of cytogenesis is correlated with nuclear organization, suggesting that the regulation of cytogenesis is critical for specific aspects of circadian timing. The relative contributions, however, of a highly ordered program of cytogenesis and intercellular interactions after postmitotic cells leave the germinal epithelium remain to be determined.

4. Experimental procedure

4.1. Animals and housing conditions

Female C57BL/6 mice (*Mus musculus*, Jackson Laboratory, Bar Harbor ME), 5 – 6 weeks old, were maintained in 14:10 light/dark (LD) cycle (lights on 0800 hrs) at 21°C with water and food provided *ad libitum*. Breeding was carried out at light onset (0800 – 0900 hrs) and mating was confirmed by checking the presence of vaginal plugs at 0900 hrs. This marked the beginning of embryonic day one (E1).

4.2. BrdU injections

Pregnant mice were given single intraperitoneal injections (i.p.) of bromodeoxyuridine (BrdU, 50 µg/g body weight – Sigma, dissolved in saline containing 0.007N NaOH) from E10 through E15, when development of hypothalamic nuclei was estimated to occur (see Shimada and Nakamura 1973). Accordingly, at least two dams received BrdU injections at regular 12-hour intervals from E10 through E15.5. This injection schedule provided better resolution than previous mouse studies. BrdU was administered either an hour after lights on (i.e. 0900 hrs) or an hour before lights off (i.e. 2100 hrs).

4.3. Tissue collection

Pups were collected on postnatal day 24 (P24) $\pm$ 3 days, anesthetized with sodium pentobarbital (70 mg/kg body weight, i.p.) and killed by intracardial perfusion with 70% ethanol. Tissues were then histologically processed for paraffin embedding. Serial coronal 7 μ m sections were obtained for the SCN region (rostrocaudally).

4.4. BrdU immunocytochemistry (ICC)

Sections were washed in xylene to dissolve paraffin, re-hydrated in decreasing concentrations of ethanol, blocked with 0.5% solution of 30% H₂O₂ (Sigma) and washed with 0.01M PBS. NaOH (0.07N) was used to partially hydrolyze dsDNA into single strands. Tissues were then blocked with 2% normal horse serum (*Vectastain Elite*), incubated with mouse monoclonal anti-BrdU antibody (Becton-Dickinson; diluted 1:75 in 0.01M PBS containing 0.3% Triton X) in a humidified chamber at room temperature. This was followed by incubation in biotinylated horse anti-mouse IgG (*Vectastain Elite*; diluted 1:600) and then with avidin-bound peroxidase complex (*Vectastain Elite*; diluted 1:400) at room temperature. The peroxidase signal was revealed with a solution of 30% H₂O₂ containing diaminobenzidine (DAB, 0.5 mg/ml). Thionin was used as a counterstain in order to define the SCN boundaries. Finally, slides were histologically treated and coverslipped for microscopic analysis.

4.5. Statistical analysis

To determine the timing of SCN cell generation, different regions of the nucleus (i.e. anterior, middle, posterior, dorsomedial and ventrolateral) were analyzed for heavily BrdU-labeled cells (HBL) for each age of injection. In 3-week old mice, the length of the nucleus in the caudo-rostral axis was approximately 350 μ m (about 50 sections, each section being 7 μ m thick). A section used to represent the anterior SCN was selected as that which was approximately 10% in from the most rostral section (on average, 5 sections in), and posterior sections were selected as those that were approximately 10% in from the most caudal section. In addition, selection was based on the general quality of the sections, as well as on the cross-sectional shape and size of the SCN, aided by an ocular grid (500 $\times$ 500 μ m²). To determine whether SCN cytogenesis followed an outside-in gradient (i.e. lateromedial gradient) the middle SCN was further divided diagonally into two equal regions – dorsomedial and ventrolateral (Fig. 7). Anterior regions filled approximately 6 $\times$ 10⁴ μ m² grid area, while the areas of middle and posterior SCN regions were approximately 13.5 $\times$ 10⁴ μ m² and 6.9 $\times$ 10⁴ μ m², respectively. For a given anterior, middle or posterior section the total number of cells in the SCN were on average 199 $\pm$ 7, 403 $\pm$ 9 and 172 $\pm$ 7, respectively (mean $\pm$ SEM, n = 53).

To determine the timing of SCN cell generation, percent HBL cells was evaluated for each region of the SCN. Percent HBL cells was calculated as number of HBL in a given region of the SCN divided by the total number of cells in that region. Using camera lucida, the boundaries were drawn around the SCN, and all cells (i.e. labeled and unlabeled with BrdU) were counted within the boundaries. Two populations of BrdU-labeled cells were distinguished: heavily-labeled (HBL) were those in which nuclei were 50 – 100 % intensely filled with chromogen, and lightly-labeled (LBL) cells were those in which the chromogen filled less than 50% of the nucleus (Fig. 1). HBL cells are those likely to have left the cell cycle after the next mitosis, while LBL cells might have had re-entered the cell cycle, further diluting BrdU content.

Eight to nine mice (4–5 per litter) were initially examined for each day of injection, but only those animals in which the total number of cells could be clearly seen and counted in the SCN were included in the analysis. Ultimately, at least two mice were analyzed for each SCN area and age of injection, with a range of 2–7 and an average of four. Percent counts of HBL cells for a particular section were averaged across animals of the same treatment group and expressed as mean % HBL cells $\pm$ standard error of the mean (SEM). Data were plotted as mean % HBL

cells (or simply % HBL cells), against embryonic age for different regions of the SCN. Statistical analysis across embryonic ages was carried out using one way analysis of variance (ANOVA). When the overall ANOVA was significant, Student's t-test was used to compare specific ages and was used to compare the dmSCN and vlSCN at particular ages.

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List of abbreviations

aSCN	anterior region of suprachiasmatic nucleus
BrdU	bromodeoxyuridine
CaB	calbindin
dmSCN	dorsomedial region of suprachiasmatic nucleus
HBL	heavily-labeled BrdU cells
mSCN	middle region of suprachiasmatic nucleus
pSCN	posterior region of suprachiasmatic nucleus
vlSCN	ventrolateral region of suprachiasmatic nucleus

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Figure 1. Photomicrograph showing nuclear BrdU labeling in SCN cells. Arrow heads indicate heavily BrdU-labeled (HBL) cells likely to have undergone their last round of cell division at the time of BrdU administration. Full arrows indicate lightly BrdU-labeled (LBL) cells which may have undergone additional cycles of cell division following BrdU injection. (Scale bar, 50 μ m).

Figure 2.

A plot of % HBL cells in the SCN following BrdU injection at different gestational ages ($P < 0.0001$, ANOVA). Few HBL cells were present in the nucleus prior to E12 ($p = 0.05$, E11.5 vs E12.0). The % HBL cells was maximal between E13.5 and E14 and decreased thereafter returning to initial low levels by E15.5.

Figure 3.

Photomicrographs of HBL cells in the SCN along the rostrocaudal axis following BrdU injections on E12, E13.5, and E14. On E12, more HBL cells are seen in the middle section, as compared to anterior and posterior sections. An increase in % HBL cells is apparent in the pSCN on E13.5 and in the aSCN on E14. Arrows identify the SCN; V3, third ventricle; oc, optic chiasma. In the mSCN and pSCN from E13.5, a piece of optic nerve became folded under the oc during embedding (Scale bar, 200 μ m).

Figure 4.

A plot showing heterochronous cytogenesis in the SCN along its caudorostral axis. A) % HBL cells in aSCN showing a peak on E14; B) % HBL cells in mSCN showing two peaks, first on E12 followed by another on E13.5; and C) % HBL cells in pSCN with a peak on E13.5. The first value significantly different from that at E11.5 ($p < 0.05$) was E12.0, E13.5, and E14.5 for mSCN, pSCN, and aSCN respectively.

Figure 5.

Plots of % HBL cells in two regions of the mSCN at different embryonic ages. The vISCN ($p < 0.05$) but not the dmSCN ($p > 0.05$) showed a significant increase in % HBL between E11.5 and E12. Between E13.0 and E13.5 a change in % HBL was significant in the dmSCN ($p < 0.05$) but not in the vISCN ($p > 0.05$).

Figure 6.

Photomicrographs of BrdU labeling in the SCN on E13, E13.5, and E14. HBL cells can be seen in the dorsomedial area as well as the lateral margin. A central region in the ventral part of the nucleus is almost devoid of labeled cells (C, core) and is surrounded by areas with labeled cells (S, shell). V3, third ventricle; oc, optic chiasma. (Scale bar: 100 μ m).

Figure 7.
Schematic representation of the two mSCN subdivisions that were separately analyzed for % HBL at different ages. dm, dorsomedial; vl, ventrolateral; oc, optic chiasma; v3, third ventricle.