Neuroscience 144 (2007) 395–401

DIURNAL PATTERN OF CLOCK GENE EXPRESSION IN THE HYPOTHALAMUS OF THE NEWBORN RABBIT

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Abstract—In the European rabbit (*Oryctolagus cuniculus*) nursing acts as a strong non-photic synchronizer of circadian rhythmicity in the newborn young. Rabbits only nurse for a few minutes once every 24 h and previous studies have shown that the pups, blind at birth, display endogenous circadian rhythms in behavior and physiology entrained by this regular daily event. As a further step toward understanding the neural organization of the rabbit's early circadian system, we investigated the expression of clock genes in the suprachiasmatic nucleus of the hypothalamus (SCN; the principal circadian pacemaker in adult mammals) across the pups' 24-h day. We used 43 pups from seven litters maintained in constant darkness and entrained non-photically by nursing at the same time each day until P7. After nursing on day 7, pups were killed in the dark at 3-h intervals so as to obtain eight groups (n=5–6 pups/group) distributed evenly across the 24 h before the next scheduled nursing. Profiles in the expression of the clock genes *Per1*, *Per2*, *Cry1* and *Bmal1* were determined using *in situ* hybridization in brain sections through the hypothalamus at the level of the SCN. We report for the first time: 1) that *Per1*, *Per2*, *Cry1* and *Bmal1* are all expressed in the SCN of the newborn rabbit, 2) that the expression of *Per1*, *Per2* and *Bmal1* but not *Cry1* shows diurnal rhythmicity similar to that in adult mammals, and 3) that the expression of *Per1*, *Per2* and *Bmal1* is consistent with the strong entraining effect of nursing found in previous studies. Unexpectedly, and contrasting somewhat to the pattern in the SCN, we also found diurnal rhythmicity in the expression of *Cry1* and *Bmal1* but not of *Per1* in the anterior ventromedial hypothalamic nucleus. Overall, our findings suggest that the SCN is a functional part of the newborn rabbit's circadian system and that it can be entrained by non-photic cues associated with the mother's daily nursing visit.

Key words: circadian rhythms, non-photic entrainment, suprachiasmatic nucleus, anterior ventromedial hypothalamic nucleus, development, mammals.

Circadian rhythmicity is a fundamental characteristic of organisms, which helps ensure that vital functions occur in an appropriate and precise temporal sequence and in accordance with cyclic environmental changes (Pittendrigh, 1981). In mammals, exposure to a cyclic environment already occurs *in utero*, where fetuses are subject to a variety of non-photic stimuli originating from the mother including fluctuations in hormones, nutrients and uterus motility (Morgan et al., 1992; Reppert, 1995; Ninomiya-Alarcón et al., 2004). The main circadian pacemaker, located in the hypothalamic suprachiasmatic nuclei (SCN), is already functional before birth (Reppert and Schwartz, 1984; Reppert and Uhl, 1987; Shibata and Moore, 1987; Davis et al., 1990) and can be entrained by such non-photic cues (Reppert and Schwartz, 1983, 1986; Shibata and Moore, 1988; Reppert, 1995). This has led to the suggestion that for altricial young, non-photic cues provided by the mother are the main environmental signals synchronizing their circadian system until their eyes open, they have left the nest and are able to use the photic cues so important for adult circadian function (Reppert, 1995).

A notable example of such maternal influence is provided by the European rabbit (*Oryctolagus cuniculus*). Immediately after giving birth to the altricial young in a dark underground nest (or laboratory nest box), the mother leaves and only returns once approximately every 24 h to nurse for 3–5 min (Zarrow et al., 1965; Hudson and Distel, 1982, 1989; Hudson et al., 1999; Jilge and Hudson, 2001; Bautista et al., 2005). A variety of studies has shown that this visit is a strong synchronizing signal for the pups. In response to imposed nursing schedules pups display diurnal rhythms in general arousal (Hudson and Distel, 1982, 1989; Jilge, 1993, 1995; Pongrácz and Altbäcker, 1999), body temperature (Jilge et al., 2000, 2001), plasma prolactin (Alvarez et al., 2005, 2006) and corticosterone levels (Rovirosa et al., 2005), serum and liver metabolites (Escobar et al., 2000), and c-Fos expression in thalamic and hypothalamic nuclei (Allingham et al., 1998).

These rhythms appear adaptive, preparing pups for the competitive scramble for nipples accompanying nursing (Drummond et al., 2000; Bautista et al., 2005). Shortly before the mother's arrival the pups become increasingly active, uncover from the nest material (Hudson and Distel, 1982, 1989; Jilge, 1993, 1995), and their body temperature and levels of plasma corticosterone rise (Jilge et al., 2000; Rovirosa et al., 2005). These changes persist even in pups fasted for up to 72 h (Hudson and Distel, 1982, 1989; Jilge, 1993; Jilge et al., 2000, 2001), a procedure which they readily survive. However, although newborn rabbits clearly possess a functional circadian system sensitive to and entrained by maternal non-photic cues, it is not known how these cues act on the pups' circadian system, and more particularly on the SCN.

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Abbreviations: AVHN, anterior ventromedial hypothalamic nucleus; *Cry1*, cryptochrome gene 1; *Cry2*, cryptochrome gene 2; P, postnatal day; *Per1*, period gene 1; *Per2*, period gene 2; *Per3*, period gene 3; SCN, suprachiasmatic nucleus.

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doi:10.1016/j.neuroscience.2006.09.020

Reppert and Weaver (2001, 2002) recently suggested that rhythmicity in the SCN is generated by two interconnected feedback loops that drive the rhythmic expression of a set of so-called clock genes, including the *Period* genes *Per1*, *Per2* and *Per3*, the *Cryptochrome* genes *Cry1* and *Cry2*, and the transcription factors *Dec1*, *Dec2*, *Clock* and *Bmal1* (Reppert and Weaver, 2001, 2002; Honma et al., 2002; Okamura, 2004). These genes are expressed abundantly in the SCN both of nocturnal (Reppert and Weaver, 2001; Honma et al., 2002) and diurnal (Caldelas et al., 2003) mammals, expression of several is modulated by both photic and non-photic cues (Challet et al., 2003), and mutations of some produce arrhythmicity in rodents maintained in constant darkness (Reppert and Weaver, 2001, 2002). Additionally, several studies have shown that clock gene expression is a particularly high resolution marker of temporal changes in SCN activity accompanying phase shifts of the circadian pacemaker (Zylka et al., 1998; Miyake et al., 2000; Tamaru et al., 2000). To our knowledge, however, there is no information on the expression of these genes in the SCN of newborn rabbits. It is therefore the aim of this study to describe the expression of *Per1*, *Per2*, *Cry1* and *Bmal1* in the hypothalamus of newborn rabbits maintained on a 24-h nursing schedule. We chose these genes because they are known to be major components of the molecular circadian clockwork (Reppert and Weaver, 2001, 2002).

EXPERIMENTAL PROCEDURES

Throughout the study, animals were kept and treated in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 86-23) revised 1996, and the guidelines for the treatment of animals in research of the Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México. Experiments were conducted using the minimum number of animals needed to obtain statistically meaningful results, and taking care to minimize suffering.

Animals

We used chinchilla-strain domestic rabbits (*Oryctolagus cuniculus*) bred and maintained at the Instituto de Investigaciones Biomédicas, UNAM. Data were obtained from 43 pups from seven litters, each litter from a different female mated with a different male. Mothers were housed in individual stainless steel cages (120×60×45 cm high) that could be divided in two equal-sized compartments by a sliding door. They were kept on an 18-h light/6-h dark cycle (lights on at 10 a.m.) to approximate natural conditions for this long-day breeder (Hudson and Distel, 1990). Ambient temperature was maintained at 20±2 °C, relative humidity at 40–60%, and rabbit chow (Conejos Engorda, Malta Cleyton, Mexico) and tap water were available continuously. For nest building, straw and an opaque acrylic box (28×29.5×30 cm high), closed except for a 14 cm diameter entrance, were placed in the females' cages 4 days before term.

Experimental treatment

After parturition, which lasts only a few minutes in the rabbit (Hudson et al., 1999), the pups were weighed and the mothers allowed access to the nest box for 6 h. The sliding door was then closed, separating mothers from their young, and a lightproof plastic cover placed over the box. The day of parturition was considered postnatal day 0 (P0). The next day (P1), 30 min before lights-on but in dim red light (maximum 10 lux in the colony room and 2 lux in nest boxes; light meter YK-10LX, Lutron, Electronic Enterprise Co., Taipei, Taiwan) the cover was removed from the box taking care not to disturb the pups, and the sliding door opened to allow the mother access to the young to nurse. Such a low level of illumination is unlikely to have affected the pups as their eyes are still closed at this age, central projections of the visual system are immature (Rapisadi et al., 1975), and to obtain a response to light in the SCN of altricial mammals, high intensity illumination of long duration is needed (900 lux for 1 h in mouse; Llamosas et al., 2000). Ten minutes later, after the mother had finished nursing and had left the pups, the box was again covered, the sliding door closed and the red light turned off until the same time next day. This procedure was repeated until P7. On P7 randomly selected individuals were killed at 0, 3, 6, 9, 12, 15, 18, and 21 h post nursing to form eight groups of five to six pups each. No more than two pups from the same litter contributed to any time point. Pups were removed from the box in dim red light and immediately killed in a CO_2 chamber, weighed and decapitated. Brains were then removed under normal light, frozen in isopentane and stored at −80 °C. Sixteen-micrometer transversal sections through the anterior hypothalamus at the level of the SCN were cut in a cryostat, mounted on gelatinized slides and *in situ* hybridization was performed.

In situ hybridization

To obtain temporal profiles of clock gene expression, *Per1*, *Per2*, *Cry1* and *Bmal1* riboprobes against mouse were used, generated from plasmid kindly provided by Dr. Steven M. Reppert (Massachusetts General Hospital and Harvard Medical School, USA). The antisense and sense cRNA probes (Fig. 2) were generated by *in vitro* transcription (Maxiscript kit, Ambion Inc., Austin, TX, USA) using [^{35}S]UTP as radiolabel. Pre-hybridization, hybridization and post-hybridization were performed as described previously (Caldelas et al., 2003), except that sections were hybridized overnight at 54 °C as we have found that this lower temperature reduces background expression. After high stringency post-hybridization washes and dehydration, the sections were exposed to Kodak Biomax film (Eastman Kodak, Rochester, NY, USA) for 15 days at room temperature, together with microscale standards (Amersham, Arlington Heights, IL, USA). Sections were processed for expression of the four genes, each gene in a single run.

The hybridization signal in the SCN and adjacent anterior ventromedial hypothalamic nucleus (AVHN; Shek et al., 1986), which also showed strong gene expression, was quantified using the image analysis program Microcomputer Image Device (Imaging Research Inc., St. Catherines, ON, Canada). For this, the sections were digitalized (Canon scanner 9950F; Canon, Tokyo, Japan) and the contrast enhanced and set to a standard gray scale using the program Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA, USA). Signal values are expressed as relative optical density, with levels of expression calculated from the difference between signal measured within a 3,142 μm^2 ellipse within the SCN and within the AVHN in comparison with an adjacent hypothalamic area showing only background expression (Fig. 1). Individual mean values were obtained from three sections per brain.

Statistical analysis

To evaluate rhythmicity in gene expression, cosinor analyses were performed using different trial period lengths to test the hypothesis that rhythm amplitudes differed from zero (Nelson et al., 1979). For this, we fitted periodic senoidal functions to the values for expression of each of the genes across the eight time points using the formula $f(t)=M+A\cos(\omega t+\theta)$, where $f(t)$ is the level of gene expression across time, the mesor M is the value about which the