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TEMPORAL MODULATION OF THE CANONICAL CLOCKWORK IN THE SUPRACHIASMATIC NUCLEUS AND OLFACTORY BULB BY THE MAMMARY PHEROMONE 2MB2 IN PRE-VISUAL RABBITS

L. TREJO-MUÑOZ,[a] E. NAVARRETE,[a] R. MONTÚFAR-CHAVEZNAVA[b] AND I. CALDELAS[a]*

[a] *Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Ciudad Universitaria, 04510, Distrito Federal, Mexico*

[b] *Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Ciudad Universitaria, 04510, Distrito Federal, Mexico*

Abstract—During the early stages of development, the olfactory system plays a vital role in the survival of altricial mammals. One remarkable example is the *Oryctolagus cuniculus*, whose mother–young interaction greatly depends on the 2-methylbut-2-enal (2MB2) pheromone that triggers nipple search and grasping behaviors. Olfactory stimulation with 2MB2 regulates the expression of the core body temperature and locomotor activity rhythms in rabbit pups, indicating the modulation of the circadian system by this volatile cue. To address this issue, in the present study, we determined the effect of stimulation with pulses of 2MB2 on the molecular circadian clockwork in the suprachiasmatic nucleus (SCN) and in the main olfactory bulb (MOB). For this purpose, 7-day-old rabbits were stimulated with distilled water (CON), with ethyl isobutyrate (ETHYL) or with the pheromone (2MB2) at different times of the cycle, and 1 h later, the expression of the activity marker C-FOS and of the clock proteins PER1, CRY1 and BMAL1 was evaluated in the SCN and in the three layers of the MOB. The clock proteins were abundantly expressed in both structures; nevertheless these showed diurnal rhythmicity only in the MOB, confirming that central pacemakers exhibit a heterochronical development of the molecular clockwork. C-FOS expression in the SCN and in the MOB was modulated by exposure to ETHYL and to 2MB2 only when these stimulants were presented at ZT00 and at ZT18. In contrast, the clock proteins were essentially modulated by 2MB2 at ZT00 and at ZT06 in both structures. In addition, the PER1 and CRY1 proteins exhibited differential responses to stimulation in the three layers of the MOB. For the first time, we report a modulatory and time-dependent effect of the mammary pheromone 2MB2 on the expression of the core clock proteins in the SCN and in the MOB in rabbits during pre-visual stages of development.

Key words: clock proteins, main olfactory bulb, suprachiasmatic nucleus, mammary pheromone, non-photic synchronization, circadian rhythms.

INTRODUCTION

Almost all biological functions in living organisms are temporally organized in fluctuations close to 24 h, which are known as circadian rhythms. In mammals, a biological pacemaker, which is in the suprachiasmatic nucleus (SCN), orchestrates this temporal organization. This hypothalamic structure is constituted of cells capable of generating self-sustained oscillations in their electrical, metabolic and molecular activities (Schwartz and Gainer, 1977; Welsh et al., 1995; Maywood et al., 2003). In addition, the SCN coordinates the rhythmicity of the entire organism through the communication of rhythmical signals to peripheral oscillators (Review in Herzog, 2007).

The generation of circadian rhythms is achieved by a molecular clockwork that is composed of a set of genes and proteins called clock genes (Lowrey and Takahashi, 2004). The current model of the mammalian molecular clock consists of a series of autoregulatory feedback loops In the core loop, the transcription factors CLOCK and brain and muscle ARNT-like 1 (BMAL1) heterodimerize through the PAS binding domain and initiate transcription through a union with E-box regulatory sequences contained in the target genes Period (*Per1, Per2* and *Per3*), Cryptochrome (*Cry1* and *Cry2*) and clock-controlled genes (CCGs), among others. In the cytoplasm, PER and CRY proteins accumulate and form a complex that translocates into the nucleus, where these proteins repress their own transcription through an interaction with the CLOCK:BMAL1 heterodimer. Post-translational modifications of PER and CRY, such as phosphorylation and ubiquitination, regulate their stability in the cytoplasm. A new transcription cycle is initiated when the heterodimer PER-CRY is repressed by proteolytic degradation. Additionally, the CLOCK:BMAL1 complex activates the transcription of CCGs, such as the retinoic acid-related orphan nuclear receptors (*Rev-erbα* and *Rorα*), which compete to bind to the retinoic acid-related orphan receptor response elements (ROREs) within the *Bmal1* promoter. *Bmal1* transcription is negatively regulated by REV-ERB (α and β), whereas ROR (α, β and γ) exerts opposite effects (Review in Hastings and Herzog, 2004; Ko and Takahashi, 2006). The transcriptional regulation feedback loop of these genes takes 24 h to complete a

*Corresponding author. Tel: +52-5556223148; fax: +52-5555503893.
E-mail address: caldelas@biomedicas.unam.mx (I. Caldelas).

Abbreviations: 2MB2, 2-methylbut-2-enal; BMAL1, brain and muscle ARNT-like 1; C-FOS, FOS proto-oncogene protein; CCGs, clock-controlled genes; CRY1, cryptochrome gene 1; ETHYL, ethyl isobutyrate; IRC, immunoreactive cells; MOB, main olfactory bulb; PER1, period gene 1; SCN, suprachiasmatic nucleus.

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cycle, which generates an endogenous temporal regulation that controls output genes and affects physiological and metabolic processes (Reppert and Weaver, 2002).

In the last decade, almost all clock genes have been shown to be rhythmically expressed in mammalian cells and in tissues, such as the liver, cornea, kidney, pituitary gland and fibroblast cells, among others (Herzog, 2007). Nevertheless, the daily oscillations of clock gene expression in these structures show dampening with time, suggesting that temporal cues originated from the SCN are crucial for peripheral oscillations (Abe et al., 2002; Izumo et al., 2003). With the exception of the main olfactory bulb (MOB), this structure exhibits autonomous circadian rhythmicity in *Per1*:luc gene expression and in the firing rate (Granados-Fuentes et al., 2004a,b). Moreover, the MOB displays time-regulated responses to odorants that do not depend on the hypothalamic circadian pacemaker because mice bearing SCN lesions exhibit clear circadian rhythms in olfactory discrimination (Granados-Fuentes et al., 2004a). The 24-h fluctuations in olfaction are lost in mice lacking *Bmal1* or both *Per1* and *Per2* genes, indicating that olfactory discrimination rhythm depends on the expression of canonical clock genes (Granados-Fuentes et al., 2011). However, the biological function of the MOB as a pacemaker remains unclear.

The circadian timing system in mammals is functional beginning at embryonic stages. The neurogenesis of the SCN occurs during the last third of gestation in rodents and in primates; clock gene expression in the rat SCN is evident as early as embryonic day 19 (Ohta et al., 2002, 2003). Synaptogenesis and the maturation of the retinohypothalamic pathway occur postnatally (Davis et al., 1990). During this early stage of development, the circadian system is sensitive to non-photic cues, primarily those cues arising from the mother (Ohta et al., 2002, 2003; Review in Reppert and Weaver, 2002). One remarkable example of the relevance of maternal cues as entraining agents of circadian system during the early stages of development occurs in the European rabbit (*Oryctolagus cuniculus)*. Through evolution, this species has acquired a specific maternal behavior consisting of a single and brief nursing episode throughout the day (Zarrow et al., 1965). From the first days of life, newborn rabbits are capable of predicting and anticipating the arrival of the doe (Hudson and Distel, 1982; Jilge and Hudson, 2001; Trejo-Muñoz et al., 2012). The mother's single nursing episode is a strong synchronizing signal for the pup, entraining behavioral (Hudson and Distel, 1982; Jilge, 1993, 1995), physiological (Caldelas et al., 2007) and molecular parameters (Caldelas et al., 2005, 2007, 2009; Caba et al., 2008). Changes in the nursing schedule induce phase shifts in the body temperature rhythm and in the SCN clock gene expression in newborn rabbits (Caldelas et al., 2009).

Olfactory signals are vital for the survival of altricial mammals. In rabbits, the volatile chemical cues originating from the lactating female abdomen and from milk produce a state of arousal in pups, modulating mother–pup interactions, determining nipple location, suckling and orientation to the nest (Hudson and Distel, 1983; Schaal et al., 2003). Several volatile compounds present in the rabbit milk were recently identified (Schaal et al., 2003). Among these volatile compounds were hexan-2-one, 2-methylpropan-1-ol, butan-1-ol, D, L limonene, butanoic acid, decanal, pyridine and 2-methyl-but-2-enal (2MB2). 2MB2 meets all of the criteria to be considered a pheromone because this semiochemical is capable of triggering a stereotypical pattern of nipple search behavior in newborn rabbits (Coureaud et al., 2001; Schaal et al., 2003). The responsiveness to 2MB2 does not seem to be derived from prenatal or postnatal learning processes and is species-specific; odors such as vanillin, linalool and ethyl isobutyrate do not produce this behavioral activation (Coureaud et al., 2006).

In a recent report by our group, we have shown that the mammary pheromone acts as a non-photic cue for the circadian system of newborn rabbits because the daily olfactory stimulation with 2MB2 or with rabbit milk can entrain the daily patterns of locomotor activity and core body temperature in artificially fed newborn rabbits. These observations demonstrated that the circadian system is sensitive and is feasible to entrain the 2MB2 pheromone during pre-visual stages of development (Montúfar-Chaveznava et al., 2013). To understand the mechanisms underlying this non-photic synchronization, the aim of the current study was to determine the effect of the olfactory stimulation with the mammary pheromone 2MB2 on the molecular circadian clockwork of the two central pacemakers, the SCN and the MOB, of newborn rabbits maintained in constant light.

EXPERIMENTAL PROCEDURES

The experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 86-23, revised 1996) and to the Treatment of Animals in Research guidelines of the Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México (UNAM). The protocol was reviewed and approved by the Animal Care and Use Committee of the Instituto de Investigaciones Biomédicas, UNAM, before the study was conducted (Permit Number: 098).

Animals

We used a Chinchilla strain of domestic rabbits (*O. cuniculus*) that were bred and maintained at the Instituto de Investigaciones Biomédicas, UNAM. Pregnant rabbits were housed in individual stainless steel cages (120 × 60 × 45 cm) and maintained on a 16:8-h light:dark cycle (the lights turned on at 08:00 h). The room temperature was controlled at 20 ± 2 °C, with a relative humidity between 40% and 60%; rabbit chow (Conejo Ganador, Malta Cleyton, México) and water were available *ad libitum*.

Four days before the programed date of parturition, an artificial burrow was placed in the cage that contained the pregnant rabbits. The burrow (28 × 29.5 × 30 cm high) was made of opaque polyvinyl chloride and contained a 14-cm diameter entrance. Sterile hay was placed in each maternal cage for building nests. The day of parturition was defined as postnatal day (P) 0. The