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MELATONIN AFFECTS NUCLEAR ORPHAN RECEPTORS mRNA IN THE RAT SUPRACHIASMATIC NUCLEI

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Abstract—The pineal hormone melatonin nocturnal synthesis feeds back on the suprachiasmatic nuclei (SCN), the central circadian clock. Indeed, daily melatonin injections in free-running rats resynchronize their locomotor activity to 24 h. However, the molecular mechanisms underlying this chronobiotic effect of the hormone are poorly understood. The endogenous circadian machinery involves positive and negative transcriptional feedback loops implicating different genes (particularly *period (Per) 1–3, Clock, Bmal1, cryptochrome (Cry) 1–2*). While CLOCK:BMAL1 heterodimer activates the rhythmic transcription of *per* and *cry* genes, the PER and CRY proteins inhibit the CLOCK:BMAL1 complex. In previous studies, we observed that the immediate resetting effect of a melatonin injection at the end of the subjective day on the SCN circadian activity did not directly involve the above-mentioned clock genes. Recently, nuclear orphan receptors (NORs) have been presented as functional links between the regulatory loops of the molecular clock. These NORs bind to a retinoic acid receptor–related orphan receptor response element (RORE) domain and activate (RORα) or repress (REV-ERBα) *bmal1* expression. In this study, we investigated whether melatonin exerts its chronobiotic effects through transcriptional regulation of these transcription factors. We monitored *rorα*, *rorβ* and *rev-erbα* messenger RNA (mRNA) expression levels by quantitative *in situ* hybridization, up to 36 h following a melatonin injection at circadian time (CT) 11.5. Results clearly showed that, while *rorα* was not affected by melatonin, the hormone partially prevented the decrease of the *rorβ* mRNA expression observed in control animals during the first hours following the injection. The major result is that the *rev-erbα* mRNA expression rhythm was 1.3±0.8-h phase-advanced in melatonin-treated animals during the first subjective night following the melatonin administration. Moreover, the *bmal1* mRNA expression was 1.9±0.9-h phase-shifted in the second subjective night following the melatonin injection. These results clearly suggest that the NOR genes could be the link between the chronobiotic action of melatonin and the core of the molecular circadian clock.

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Abbreviations: CCG, clock-controlled gene; cRNA, complement RNA; CRY, cryptochrome; CT, circadian time; mRNA, messenger RNA; NOR, nuclear orphan receptor; PER, period; RIA, radioimmunoassay; RORE, retinoic acid receptor-related orphan receptor response element; SCN, suprachiasmatic nuclei; TF, transcription factor.

Key words: SCN, melatonin, rev-erbα, ror, bmal1, *in situ* hybridization.

Circadian rhythmicity is an essential property of behavioral and physiological processes and is organized around biological clocks. These clocks express rhythmic processes whose endogenous period, observed in free-running conditions, is around 24 h but entrained exactly to 24 h either by photic or nonphotic cues (Challet et al., 2003). In mammals, the master daily clock lies in a single structure, the hypothalamic suprachiasmatic nuclei (SCN) (Rusak and Zucker, 1979; Ralph et al., 1990). The SCN, precisely synchronized to 24 h by the light/dark cycle, through the retino-hypothalamic tract, distribute their circadian rhythmicity to the CNS structures and exert a control on most of the day/night biological rhythms (for review: Kalsbeek and Buijs, 2002; Perreau-Lenz et al., 2004). The SCN control especially the synthetic activity of the pineal gland, a neuroendocrine tissue that transduces the SCN circadian nervous message into a humoral message: the synthesis of the melatonin hormone (Reiter, 1993). Indeed, melatonin is secreted at night by the pineal gland and provides a daily and a seasonal message to all tissues expressing melatonin receptors (for review: Simonneaux and Ribelayga, 2003; Pévet et al., 2006). Elevated levels of high-affinity melatonin receptors are also expressed in the SCN (Vanecek et al., 1987), but their precise physiological significance is still unknown (Liu et al., 1997). However, the expression of these receptors is a strong argument to suggest a physiological feedback of the pineal hormone onto the central master clock. (Lincoln and Clarke, 1994; Cassone, 1990; Reiter, 1991, 1993; Wittkowski et al., 1999; Morgan, 2000; Von Gall et al., 2005).

Over the past decades, acute stimulation of the circadian system by exogenous melatonin has been extensively studied. Long term effects have been described, such as the synchronization of the free-running locomotor activity by daily melatonin injections (Redman et al., 1983; Cassone et al., 1986, 1987; Pitrosky et al., 1999; Slotten et al., 1999). Immediate effects have also been highlighted. Indeed, in the late subjective day, a single melatonin injection inhibits the SCN metabolic activity (Cassone et al., 1988; Cassone, 1990), and instantly phase-shifts the clock activity *in vivo* (Warren et al., 1993). Moreover, melatonin injected at pharmacological doses at circadian time (CT) 11 phase-advances the light-induced expression of *c-fos* in the rat SCN (Sumova and Illnerova, 1996), as well as the locomotor activity, in the following hours (Sharma et al., 1999). *In vitro*, at physiological concentrations, melatonin applications on SCN slices inhibit the neuronal electrical

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activity (Shibata et al., 1989; van den Top et al., 2001), but also phase-advance the SCN circadian neuronal activity rhythm (McArthur et al., 1991; Gillette and McArthur, 1996). These melatonin effects on the circadian clock may involve melatonin binding to its well-described SCN receptors. Indeed, mice with melatonin receptor deletion were unable to phase-shift their locomotor activity after a single pharmacological melatonin injection (Dubocovich et al., 2005). However, the molecular mechanisms underlying these effects of exogenous melatonin on SCN endogenous rhythmicity are not yet clarified. Over the past few years, the molecular bases of the SCN circadian mechanisms have been established by the identification of "*clock*" genes. The clock proteins interact in positive and negative transcriptional/posttranslational regulatory loops. The positive regulatory loop involves two basic helix—loop–helix/PAS domain-containing transcription factors CLOCK and BMAL1. The CLOCK and BMAL1 heterodimer binds to an E-box (Reppert and Weaver, 2002; Maywood et al., 2003) upstream of period (*per*), cryptochrome (*cry*) and clock-controlled gene (*ccg*) coding sequences, and consequently stimulates their messenger RNA (mRNA) transcription. The resulting PER and CRY proteins dimerize to enter the nuclei of SCN neurons where CRY2 represses the CLOCK-BMAL1 stimulation on clock and *ccg* transcription (Hogenesch et al., 1998; Jin et al., 1999; Kume et al., 1999; for review: Hirota and Fukada, 2004). So far, numerous and differential regulations of the clock genes have been demonstrated in response to both photic and nonphotic cues (Challet et al., 2003). These complex mechanisms of clock gene regulation could be the key events to understanding how the SCN are synchronized to either external (light/dark cycle) or internal cues (such as locomotor activity, feeding). Among the so-called nonphotic cues, the melatonin hormone is of special interest because of its implication in the clock resetting. In order to characterize the initial steps of the SCN response to acute melatonin treatment, we previously monitored *per1, per2, per3*, *cry1* and *bmal1* mRNA levels after a single pharmacological melatonin injection at CT 11.5, a condition known to have a chronobiotic action on the clock, but no immediate action was observed (Poirel et al., 2003a). Recently, genes belonging to the nuclear orphan receptor (NOR) family have been implicated in the regulatory pathways of the molecular endogenous clock. *Rev-erbα*, member of the NOR family (Forman et al., 1994; Zamir et al., 1996, 1997; Preitner et al., 2002), exhibits a circadian mRNA transcription rhythm in the SCN. The REV-ERBα protein which is a transcription factor (TF), was shown to inhibit *bmal1* transcription *via* three independent RORE (retinoic acid receptor–related orphan receptor response element) sequences (Preitner et al., 2002), while BMAL1 enhances *rev-erbα* expression through the well-known E-box sequence, located upstream of the gene promoter. The orphan nuclear receptor REV-ERBα couples the negative limb of the molecular oscillator to its positive limb and provides a molecular basis for a negative feed-back within the positive limb. Other TF members of the NOR family, RORα and RORβ, may be implicated in the control of the clock. While both of them display circadian mRNA transcription rhythm within the SCN (Sumi et al., 2002; Sato et al., 2004), RORα activates *bmal1* transcription through the RORE sequence (Nakajima et al., 2004; Sato et al., 2004; Akashi and Takumi, 2005). RORα is supposed to compete with REV-ERBα to bind to the RORE sequences of the *bmal1* promoter (Sato et al., 2004). RORβ, which was suggested to be redundant to RORα (Akashi and Takumi, 2005) was shown to be restricted to the CNS, specifically localizing to areas involved in the sensory information processing (Park et al., 1996, 1997). Additionally, *rorβ* mRNA expression localization is restricted to anatomical substrates of the circadian system, such as the retina, the SCN and the pineal gland. The overall distribution pattern of *rorβ* suggests that this NOR could regulate genes; the products of this gene play a role in the context of sensory input integration as well as in the context of circadian timing system (Schaeren-Wiemers et al., 1997). Moreover, *rorβ* mutant mice present the *vacillans* phenotype with an extended period of free-running rhythm, which suggests a potential role for RORβ in the molecular clock (Andre et al., 1998).

In the present study, we investigated whether melatonin chronobiotic effects could be correlated to rapid transcriptional regulations of TF members of the nuclear receptor family. Therefore, we monitored, by quantitative *in situ* hybridization, the rat *rorα*, *rorβ*, and *rev-erbα* mRNA expression patterns in the SCN during the first hours up to 36 h after a single s.c. melatonin injection, an experimental condition known to immediately affect the SCN circadian clock. Moreover as these factors regulate *bmal1*, we also followed the *bmal1* mRNA expression rhythm the first subjective day and the second subjective night after the melatonin injection.

EXPERIMENTAL PROCEDURES

Animals

All experiments were performed in accordance with the "Principles of Laboratory Animal Care" (U. S. National Institutes of Health publication no. 86-23, revised 1985) and with the French national laws. All efforts were made to minimize animal suffering and to use the minimal number of animals. Young adult male Wistar rats (200–250 g, animal husbandry R. Janvier, Le genest-St-Isle, France) were kept from birth in a 12-h light/dark regimen (a dim red light was kept permanently on throughout the 24-h period). Food and water were provided *ad libitum*. For all the experiments reported in the present study, the animals were transferred to constant darkness (dim red light) for 2 days prior to the melatonin injection. On the second day of constant darkness, at CT 11.5, one group of animals was s.c. injected with a melatonin solution (1 mg/kg; Sigma, St Quentin Fallavier, France) and the other group with the vehicle solution only (NaCl 9% and ethanol 5‰). To investigate putative effects of melatonin on the *rorα*, *rorβ* and *rev-erbα* mRNA transcription pattern, animals were killed at different time points throughout the 24 h following the melatonin injection. The time points of the killings are mentioned within the Results section. For each experimental time point, five animals were used.

The animals were killed by decapitation. Brains were rapidly dissected out, frozen on dry ice and maintained at −20 °C until sectioning. Serial coronal sections (20 μm thickness) of the brain region containing the SCN were cut on a cryostat, mounted onto