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EXPRESSION OF *Tgfα* IN THE SUPRACHIASMATIC NUCLEI OF NOCTURNAL AND DIURNAL RODENTS

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Abstract—Transforming growth factor alpha (TGFα) in the suprachiasmatic nuclei (SCN) has been proposed as an inhibitory signal involved in the control of daily locomotor activity. This assumption is based mainly on studies performed in nocturnal hamsters. To test whether the transcriptional regulation of *Tgfα* can be correlated with the timing of overt activity in other species, we compared Tgfα expression in the SCN of nocturnal Swiss mice and of diurnal *Arvicanthis* housed under a light/dark cycle (LD) or transferred to constant darkness (DD). In agreement with data on hamsters, *Tgfα* mRNA levels in the mouse SCN showed peak and trough levels around (subjective) dawn and dusk, respectively, roughly corresponding to the period of rest and activity in this species. In contrast, in *Arvicanthis* housed in DD, the circadian rhythm of SCN *Tgfα* was similar to that of the mice in spite of opposite phasing of locomotor activity. Furthermore, in *Arvicanthis* exposed to LD, *Tgfα* mRNA levels were constitutively high throughout the day. A tonic role of light in the regulation of *Tgfα* in *Arvicanthis* was confirmed by an increased expression of *Tgfα* in response to a 6-h exposure to light during daytime in animals otherwise kept in DD. In conclusion, this study shows that, contrary to what is observed in mice, *Tgfα* mRNA levels in the SCN of *Arvicanthis* do not match timing of locomotor activity and are modulated by light.

Key words: circadian rhythm, *Arvicanthis*.

Many organisms display circadian rhythmicity in most aspects of physiology and behavior. In mammals, these rhythms are controlled by an endogenous pacemaker located in the suprachiasmatic nuclei (SCN) of the hypothalamus. Ablation of the SCN abolishes rhythmicity at both behavioral and hormonal levels (Moore and Eichler, 1972; Stephan and Zucker, 1972). Furthermore, the behavioral rhythmicity in SCN-lesioned animals can be restored by grafting neonatal SCN (Ralph et al., 1990; Silver et al., 1990; Aguilar-Roblero et al., 1994).

Because SCN-lesioned animals grafted with tightly encapsulated SCN still show a reasonable recovery of their circadian locomotor activity, it was proposed that this rhythm may be controlled by the SCN through, at least in part, humoral signals (Silver et al., 1996). The nature of the humoral factors secreted by the SCN that would be responsible for the reinstatement of this rhythm, as well as the location of putative targets of such molecules, have been a large matter of discussion. On one hand, the specific target(s) of the SCN for the control of the locomotor activity rhythm are still unclear even though the implication of the subparaventricular zone (SPVZ) of the hypothalamus has been proposed in that respect (Lu et al., 2001; Schwartz et al., 2004). On the other hand, several molecules released by the SCN have been proposed as humoral signals involved in the control of daily locomotor activity. These include transforming growth factor alpha (TGFα, Kramer et al., 2001) and prokineticin 2 (PK2, Cheng et al., 2002) which may act in a concerted manner in nocturnal species to suppress locomotor activity during the day and restrict the active phase of locomotor activity to the night.

Tgfα is expressed in the SCN of several species including Syrian hamster (Kramer et al., 2001; Li et al., 2002; Jobst et al., 2004), mouse (Van der Zee et al., 2005) and rhesus macaque (Ma et al., 1992). In the Syrian hamster, *Tgfα* expression appears to be circadian with higher values during the subjective light phase (Kramer et al., 2001). This pattern is consistent with a role for TGFα as an inhibitor of locomotor activity. This hypothesis is also supported by the fact that i.c.v. infusions of TGFα in this species during the night inhibit wheel-running activity as well as other active behaviors (Kramer et al., 2001; Snodgrass-Belt et al., 2005).

Diurnal and nocturnal species display oppositely phased patterns of locomotor activity, mostly restricted to daytime or nighttime, respectively (Smale et al., 2003). If TGFα plays a direct role in the control of locomotor activity, one would predict that the phase of its expression would be reversed in the SCN. Considering that *Tgfα* is involved in the timing of daily locomotor activity, we thus sought to clarify the circadian expression pattern of *Tgfα* in the SCN of both a nocturnal (*Mus musculus*) and a diurnal (*Arvicanthis ansorgei*) species and assess possible effects of light on *Tgfα* expression.

EXPERIMENTAL PROCEDURES

Animals

Adult male Swiss mice (*Mus musculus*, Charles River, Lyon, France) and male and female Sudanian grass rats (*Arvicanthis*

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Abbreviations: CT, circadian time; DD, constant darkness; EGFR, epidermal growth-factor receptor (or ErbB1); LD, light/dark cycle; PCR, polymerase chain reaction; PK2, prokineticin 2; SCN, suprachiasmatic nuclei; SPVZ, subparaventricular zone; TGFα, transforming growth factor alpha; ZT, zeitgeber time.

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ansorgei) from our breeding colony were kept in a standard 12-h light/dark cycle (LD12:12), with food and water provided *ad libitum*. A dim red light was on during the whole cycle. Under deep anesthesia with isoflurane, animals were killed by decapitation. Brains were dissected and quickly frozen in pre-cooled isopentane and stored at −80 °C. All procedures used in animal experimentation complied with the French National law, implementing the European Communities Council Directive 86/609/EEC. All efforts were made to minimize the number of animals used and their suffering.

The first experiment aimed at determining the pattern of expression of *Tgfα* and *Per1* in the SCN of mice and of *A. ansorgei* exposed to LD or to constant darkness (DD). *Per1* is a clock gene critical for photic resetting (Albrecht et al., 1997) that is highly expressed in the SCN of mice (Albrecht et al., 1997; Sun et al., 1997; Tei et al., 1997) and *A. ansorgei* (Caldelas et al., 2003). In the present experiment, two days before kill, half of the mice and half of the *A. ansorgei* were transferred to DD. Five mice and five male *A. ansorgei* were killed every 4 h throughout the LD and DD cycles starting at ZT2 (zeitgeber time in LD) or CT2 (circadian time in DD). ZT (or CT) 0 represents the (projected) time of lights on.

The second experiment aimed at clarifying the putative effects of light on expression of *Tgfα* in the SCN of *A. ansorgei*. For this experiment, 16 female *A. ansorgei* were placed in DD two days prior to the manipulation. Eight animals (control group) remained in this DD condition, while eight individuals (light-pulsed group) were exposed to a short (1 h) light-pulse at CT6. Four animals from both DD control and light-pulsed groups were then killed at either CT7 (1 h after the onset of the light pulse) or CT8 (2 h after the light pulse).

The third experiment had two aims: first confirming the results obtained in experiment 1 by choosing two representative time points and second, obtaining further insights on light regulation of *Tgfα* in the SCN of *A. ansorgei*. For this experiment, a group of eight female *A. ansorgei* was kept in LD and four animals were killed at ZT8 and ZT16. Another group of eight animals was placed in DD for two days. Half of them received a long (6 h) light-pulse starting at CT2, while the other half remained in DD. These eight animals were then killed at CT8. The ZT16 point was a control group during nighttime for assessing day–night differences in females and comparing them with data of the first experiment.

Radioactive *in situ* hybridization

Twenty-micrometer coronal sections were made on a cryostat and mounted as series on gelatin-coated slides. These slides were then air-dried and stored at −20 °C until hybridization. *rPer1* and *mTgfα* riboprobes were used. The *rPer1* clone was a gift from Prof. H. Okamura (Department of Anatomy and Brain Science, Kobe University School of Medicine, Kobe, Japan). For *mTgfα*, cDNA fragments were-amplified by reverse-transcriptase (RT) polymerase chain reaction (PCR). PCR products of the expected sizes were cloned into the pCR-Script Amp SK(+) cloning vector (Stratagene, La Jolla, CA, USA). Identity and orientation of the cloned PCR fragments were confirmed by restriction analysis and sequencing (AGOWA Sequencing Services, Berlin, Germany). This probe covers nucleotides 116–559 of GenBank accession number NM_031199.

Specificity of the hybridization signal was assessed by use of the corresponding sense probes. Synthesis of probes and hybridization procedure have been thoroughly described elsewhere (Tournier et al., 2003). Briefly, sections were hybridized with 1.10^6 c.p.m. of probe per slide for one night at 54 °C after dehydration. The day after, slides were treated with RNaseA at 30 °C then washed in sodium citrate buffer and submitted to several rinses in buffers of increasing stringency. After dehydration, slides were exposed with ^{14}C standards to an autoradiographic film (BioMax MR, Kodak; Sigma, St. Louis, MO, USA) for 4 days. Relative optical density (ROD) in SCN was measured using NIH-Image J software with OD standards and background subtracted. For each probe and each animal, quantification was performed on the two SCN on three sections.

Statistical analysis

Data are presented as mean±standard error of the mean (S.E.M.). Two-way analyses of variance (ANOVA) with time and lighting condition or with time and treatment as factors and Tukey's post hoc tests were performed on absolute values.

RESULTS

Experiment 1: expression of *Per1* and *Tgfα* in the SCN

SCN of M. musculus in LD and DD. Hybridization signals obtained for *Per1* and *Tgfα* in mice at their respective peak and trough times of expression in DD are shown in Fig. 1. Left panels of Fig. 2 show daily (in LD cycle) and circadian (in DD) expression of *Per1* and *Tgfα* in the SCN of mice. The expression of *Per1* was rhythmic in both LD

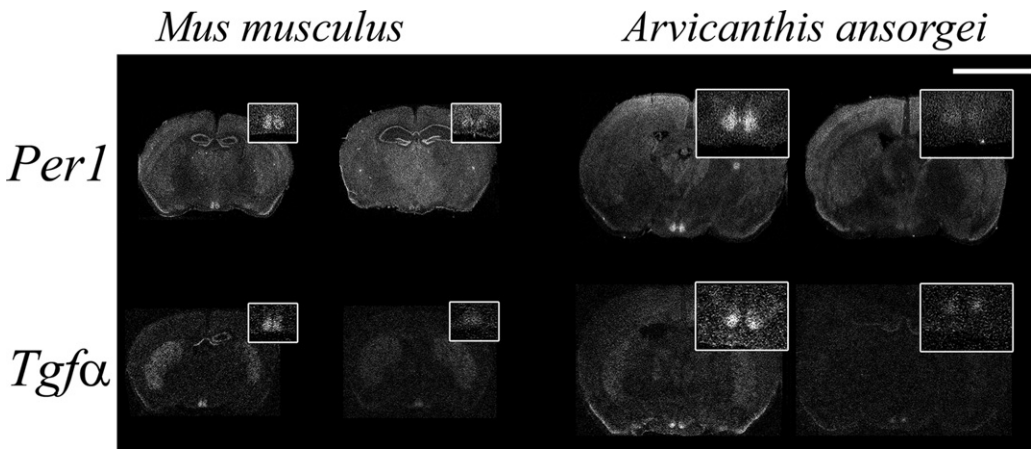

Fig. 1. Expression of *Per1* and *Tgfα* in the SCN of *Mus musculus* and *Arvicanthis ansorgei* transferred to DD. The sections presented here for *Per1* correspond to high (CT4) and low (CT20) values in both species. The time-points chosen for *Tgfα* also represent high (CT0) and low (CT8) values in both species. Scale bar=4 mm.