Chapter 6

Circadian rhythms of C-FOS expression in the suprachiasmatic nuclei of the common vole (*Microtus arvalis*)


Submitted
Abstract

The suprachiasmatic nuclei of the hypothalamus (SCN) are the master circadian clock in mammals. Transcriptional activity in this master clock has a marker in the immediate-early gene c-Fos. Within the SCN, distinct differences in c-Fos in the ventrolateral and the dorsomedial SCN have been reported for rodent species such as rats, mice and hamsters. We studied C-FOS expression in the common vole (Microtus arvalis) SCN under constant dim light and in an LD 12:12h. In the vole dorsomedial SCN rhythmic C-FOS expression was seen in LD with a clear peak in the middle of the light period. Under constant dim light conditions we report constitutive, non-rhythmic expression of C-FOS in the dorsomedial SCN. This pattern is consistent with circadian organisation of behavioural activity, which is weak in voles, and may be lost under constant dim light conditions. In the ventrolateral SCN, under light-dark conditions we observed a rise in C-FOS expression prior to lights-on followed by peak expression at lights-on. Another peak was seen at lights-off. In an additional experiment we subjected animals to LD 16:8 to test the hypothesis that the dawn and dusk peaks change phase along with the photoperiod. The peak in C-FOS expression did not shift with the time of lights on, but remained at the same External Time 6. The results would be consistent with the interpretation that in the vole, c-Fos expression reports transcriptional activity associated with an internal, gating process, more likely than with an external effect of light.
Circadian rhythms of C-FOS expression in the suprachiasmatic nuclei

Introduction

The suprachiasmatic nuclei of the hypothalamus form the main circadian pacemaker in mammals, governing rhythms in physiology and behaviour (Stephan & Zucker, 1972, Moore & Eichler, 1972). One of the functions of the SCN is entrainment of the circadian rhythms to the external light-dark (LD) environment.

Photic information received by the eye is transmitted directly to the SCN via the retinohypothalamic tract which terminates at the ventrolateral part of the SCN (Reviewed in Moore et al., 1996). The dorsomedial part (dmSCN) does not receive direct retinal input. The dmSCN, also called shell (Moore et al., 2002), shows an overrepresentation of vasopressin (Vandesande et al., 1975), and with the majority of efferents of the SCN originating from the shell, this indicates its role in the output of the SCN (Leak & Moore, 2001). The ventrolateral part of the SCN (vlSCN), also referred to as the core (Moore et al., 2002), consists mainly of cells expressing vasoactive intestinal peptide or gastrin releasing peptide (Van den Pol & Tsujimoto, 1985).

Light induced c-Fos mRNA and C-FOS protein expression in the SCN has been reported in species such as rat, mouse, hamster and even the blind mole rat (Rea, 1989, Colewell & Foster, 1992, Kornhauser et al., 1990, Vuillez et al., 1994). The proto-oncogene ‘cellular Fos’ (c-Fos, Curran et al., 1983) is classified as an immediate-early gene. The nuclear C-FOS protein binds with a transcript from the “Jun” gene family, forming the activator protein 1, a transcription factor binding to a binding site in the promoter region, activating transcription (for review see, Curran & Morgan, 1995). Because of the rapid appearance of c-Fos and the subsequent activation of transcription of target genes (such as vasopressin; Yoshida et al., 2006), it is seen as an early marker for transcriptional activity.

Light induced c-Fos

Light information reaches the SCN and elicits immediate-early gene expression, including c-Fos, with peak levels of mRNA expression as soon as 30 minutes after the light pulse followed by a steady decrease back to base levels within 2-6 hours (Kornhauser et al., 1990, Schwartz et al., 2000). c-Fos expression in the vlSCN as a result of a light pulse is only seen at circadian phases where the system is capable of phase shifting in response to the light pulse given (Rusak et al., 1990, 1992) i.e., only during the subjective night. This window of sensitivity is referred to as a ‘gating’ mechanism (Bendová et al., 2004, Sumová et al., 1995).

c-Fos under constant lighting conditions

The literature (table 1) concerning c-Fos expression in the SCN of nocturnal rodents is somewhat ambiguous. Besides light induced c-Fos expression as associated with behavioural phase shifts, the SCN exhibits other c-Fos transcription patterns. Table 1 lists a number of studies and summarizes their findings on these c-Fos rhythms. A primary distinction can be made between on SCN c-Fos rhythms in constant conditions (constant dark; DD and constant light; LL) and in light-dark cycles. Under con-
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<tr>
<td>Imm DD (LD 12:12)</td>
<td>Wistar rat</td>
<td>Peptide, rhythmic, no peak at InT 8</td>
<td>Rhythmic. Peak at InT 8</td>
<td>Sumová et al., 1998</td>
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<td>Peptide, no measurable differences between InT 10 v 22</td>
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<td>Peptide, rhythmic, peak at InT 8</td>
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<td>Aronin et al., 1990</td>
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<tr>
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<td>Low level expression, not rhythmic</td>
<td>Rhythmic, peak at InT 8 v through InT 0</td>
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<td>Imm DD (LD 12:12)</td>
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<td>Imm DD (LD 14:10) Syrian hamster</td>
<td>mRNA</td>
<td>N/A</td>
<td>Rhythmic. High mRNA from InT 5.5 to 10</td>
<td>Guido et al., 1999</td>
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<td>Low level expression, not rhythmic</td>
<td>Rhythmic. Increase from InT 4-8 through InT 22 to 2</td>
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<tr>
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<td>Peptide</td>
<td>Low level expression, not rhythmic</td>
<td>Rhythmic, increase from InT 6-8 through InT 20 to 6</td>
<td>Sumová et al., 2000</td>
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<tr>
<td>DD</td>
<td>C57BL/6J mouse</td>
<td>Peptide, random staining at dorsal border of SCN</td>
<td></td>
<td>Colwell &amp; Foster, 1992</td>
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<td>DD</td>
<td>Syrian hamster</td>
<td>Peptide, high levels during subjective day (InT 13 &amp; 17) v night (InT 20 &amp; 1)</td>
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<td>Guido et al., 1999</td>
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<tr>
<td>DD</td>
<td>Syrian hamster</td>
<td>Peptide, N/A</td>
<td>Significantly elevated levels during subjective day from InT 6-10</td>
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<td>Rhythmic mRNA and peptide high during subjective day and low during subjective night</td>
<td>Schwartz et al., 2000</td>
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<td>Daytime higher than nighttime (InT 10 v 22)</td>
<td>Earnest et al., 1990</td>
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<tr>
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<td>dmSCN</td>
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<td>Subjective night expression increased versus LD 12:12</td>
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<td>Peptide</td>
<td>Rhythmic. High at night (InT 20, 24 &amp; 3) v day (InT 8, 12 &amp; 16)</td>
<td>Earnest et al., 1992</td>
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<td>Imm LL (LD 12:12)</td>
<td>Wistar rat</td>
<td>Peptide</td>
<td>Rhythmic. Peak at InT 2 to 6</td>
<td>Sumová &amp; Illnerová, 2005</td>
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<td>LD 8:16</td>
<td>Wistar rat</td>
<td>Peptide</td>
<td>Decrease at ExT 17 and increase at ExT 7.5 14.5 hrs low levels in dark period</td>
<td>Jáč et al., 2000</td>
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<td>LD 12:12</td>
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<td>Peptide</td>
<td>Daytime higher than nighttime (ExT 10 v. 22)</td>
<td>Earnest et al., 1990</td>
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<td>LD 12:12</td>
<td>Sprague-Dawley rat</td>
<td>Peptide</td>
<td>Expression observed from ExT 7/8 to 16/17</td>
<td>Aronin et al., 1990</td>
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<td>Peptide</td>
<td>Peak at ExT 11</td>
<td>Kononen et al., 1990</td>
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<tr>
<td>LD 12:12</td>
<td>C57BL/6J mouse</td>
<td>Peptide</td>
<td>C-Fos peptide detected between ExT 7-11</td>
<td>Colwell &amp; Foster, 1992</td>
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<tr>
<td>LD 12:12</td>
<td>Sprague-Dawley rat</td>
<td>Peptide</td>
<td>Rhythmic, peak at ExT 8N/A</td>
<td>Schwartz et al., 1994</td>
</tr>
<tr>
<td>LD 16:8</td>
<td>Wistar rat</td>
<td>Peptide</td>
<td>Decrease at ExT 20 and increase at ExT 4 8 hrs low levels in dark period</td>
<td>Jáč et al., 2000</td>
</tr>
<tr>
<td>SPP (12:12)</td>
<td>Sprague-Dawley rat</td>
<td>Peptide</td>
<td>Short peak at ExT 8</td>
<td>Schwartz et al., 1994</td>
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</table>

Literature citings for spontaneous c-Fos expression in ventrolateral (vl) and dorsomedial (dm) SCN under (immediate (imm.)) DD, LL after LD, continuous dark conditions (DD) and under light-dark (LD) conditions and skeleton photoperiods (SPP) entrainment.
stant conditions spontaneous c-Fos expression is seen predominantly in the dmSCN, although there are species specific differences. In the dmSCN, in DD, c-Fos peptide and mRNA are reported to peak at the time of previous lights on, or a few hours later (Sumová et al., 1998, 2000, 2005, Guido et al., 1999, Schwartz et al., 2000). Alternatively, a peak in C-FOS expression in the dmSCN is reported at 7 hours into the subjective day (Chambille et al., 1993). Several reports state that no circadian rhythm in vlSCN c-Fos peptide or mRNA expression is seen under constant dark conditions (Schwartz et al., 1994, Sumová et al., 1998, 2000, 2005). When housed in continuous light, C-FOS immunoreactivity in the vlSCN is high around midnight (Earnest et al., 1992) or several hours later (Sumová et al., 2005). Earnest et al. (1990) state that in DD, the dmSCN exhibits higher levels of spontaneous C-FOS peptide, while in LL the vlSCN c-Fos levels are higher.

c-Fos under LD conditions

Under LD conditions c-Fos is predominantly expressed in the vlSCN, the same area where light pulse induced C-FOS peptide expression is seen (Schawartz et al., 1994). Peaks in vlSCN C-FOS peptide expression are reported at ExT 8 -11 (Kononen et al., 1990, Colwell & Foster, 1992, Schwartz et al., 1994) under LD 12:12 or around dawn in short and long photoperiods (Jác et al., 2000). In general terms, c-Fos in the vlSCN is high during the light period and low in the dark (Earnest et al., 1990, Aronin et al., 1990, Kononen et al., 1990, Colwell & Foster, 1992, Schwartz et al., 1994). Some studies on dmSCN c-Fos expression under LD conditions report on dmSCN c-Fos expression, while others (Jác et al., 2000) report almost identical expression profiles in the vlSCN and dmSCN peptide levels. Earnest et al. (1990) report high levels of dmSCN c-Fos in the dark period.

Overlooking these studies, it seems possible that under LD conditions, c-Fos is also expressed in the dmSCN, but not discerned because of a much lower level of expression. Support for this notion is found in the mouse, where in the dmSCN c-Fos immunoreactivity under DD is reported only in the ‘dorsal border’ of the SCN (Colwell & Foster, 1992). Also, in the rat “...the film exposure time required for demonstrating dorso-medial cfos mRNA expression at CT 0.5 (= InT 6.5) in darkness was at least six times longer than the exposure time required for demonstrating ventrolateral cfos mRNA expression in light during a light–dark cycle...” (Schwartz et al., 2000).

An effect of photoperiod on C-FOS expression is seen both in the vlSCN and dmSCN. In rats, the morning rise of spontaneous C-FOS levels in the vlSCN and the dmSCN is advanced under a long photoperiod (Jác et al., 2000, Sumová et al., 2000). The main difference in C-FOS peptide expression between different photoperiods is the morning increase in expression, an evening decrease is less distinct. The daytime period of elevated levels of C-FOS in the rat SCN shortens and lengthens with photoperiod (Jác et al., 2000, Sumová et al., 2000).

From these studies, the current state of knowledge on c-Fos expression in the SCN of nocturnal mammals remains ambiguous. A range of peak times is indicated for vlSCN c-Fos expression, and the duration of daytime high levels of expression varies. In diur-
nal rodents, the picture is complicated further. In the diurnal four-striped field mouse, peak C-FOS immunoreactivity in DD is found predominantly in the dorsal SCN at InT 8 (‘morning’ peak) and 16 (‘evening’ peak) (Schumann et al., 2006). In Arvicanthis niloticus, one peak in peptide expression at ExT 9 is seen in LD 12:12, after which levels steadily decrease until 24 hours later, evenly distributed over the whole SCN (Katona et al., 1998). As Schwartz et al. (1994) already noted, c-Fos immunoreactivity patterns in the vlSCN seems to be species specific, and might be coupled to the intrinsic properties of the circadian system. To add further information to the functional interspecific comparison, we set out to analyze c-Fos expression in the common vole, a rodent that can be both diurnal and nocturnal (Hoogenboom et al., 1984) and has a weak circadian system (Gerkema et al., 1990)

**c-Fos patterns in common voles**

In the common vole (*Microtus arvalis*) circadian and ultradian rhythms are simultaneously expressed in overall behavioural activity. These rodents come out of their burrows to feed in an ultradian rhythm. Transitions of light and dark entrain both circadian and ultradian rhythms and about 30% of the animals lose circadian rhythmicity in constant dim light conditions (Gerkema et al., 1994). We compared C-FOS immunoreactivity in both the vlSCN and dmSCN of animals exhibiting circadian rhythmicity under entrainment (LD 12:12) and in prolonged free run (constant dim LL) conditions. We also investigated C-FOS rhythms under a long photoperiod (LD 16:8) to address the question whether C-FOS patterns reflect external Zeitgeber properties.
Material and methods

Animals and housing

Adult common voles were taken from our breeding colony in Haren (the Netherlands) based on individuals trapped in the Lauwersmeer (53°20'N:6°16'E). Animals were individually housed in translucent cages (25x25x30 cm) with a nestbox (17x11x13 cm) attached, with wood shavings as cage bedding. Running wheels were available throughout the experiment and food (Hopefarms mouse pellets) and tap water was available ad libitum. Cages were placed in a sound attenuated, climate controlled room (temperature 22 ± 1°C; 70% humidity). All animals were initially kept under LD 12:12 (L 250-350 lux, depending on cage placement, D = dim red light) for two weeks, and then for four weeks in dim light (LL; ~2 lux) in order to establish freerunning circadian activity patterns.

Running wheel activity was recorded on an Event Recording System, storing activity counts in 2-minute intervals. Using the locomotor activity data, animals were categorized as rhythmic individuals and weakly rhythmic or arrhythmic individuals by both arbitrary visual inspection and by Chi-square (χ2) periodogram analyses (Sokolove & Bushell, 1978). Only animals that were categorized as rhythmic individuals both by visual inspection (when a circadian component in behaviour could be seen) and by periodogram analysis (when a significant circadian period was seen) were included in the experiment.

Protocols

Three groups of animals, kept at different light-dark conditions are included. Group A was kept in LL (~2 Lux). Group B was maintained in LD 12:12, group C in LD 16:8. To prevent the animals form getting a light pulse, all animals were sacrificed under the lighting conditions in which they were at that time. Group A was sacrificed at the conclusion of the four week period of dim light during which the rhythmicity category was established.

Initially animals were taken at (projected) dusk and dawn and mid (subjective) day and night. In the case of the animals entrained to a LD 12:12 time points 0.5 hour before the onset of dawn were included to see whether C-FOS could anticipate lights on and 3 hours after lights on to see expression after directly light induced C-FOS expression is reduced. When a dawn anticipating peak in vlSCN C-FOS expression was seen in LD 12:12, we sought to confirm this in the vlSCN of animals entrained to LD 16:8, again just prior dawn, at dawn and dusk and mid day and night. When under LD 16:8 no peak was found to anticipate dusk and dawn transitions in the vlSCN, we included time points at the external time of the LD 12:12 dawn peak C-FOS expression. In order to correct for inter assay differences, reference time points had to be included in each assay, leading to differences in number of individuals per time point.

The resulting time points at which animals were taken are thus as follows. In LL animals were taken at internal times (InT; Daan et al., 2002, defining activity onset as
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InT 18) 6 (n = 6), 12 (n = 6), 18 (n = 6) and 0 (n = 6). Animals in group B were sacrificed at external time (ExT; Daan et al., 2002, defining middle of the dark period as ExT 0) 0 (n = 4), 5.5 (n = 6), 6 (n = 6), 9 (n = 6), 12 (n = 6) and 18 (n = 7). Group C was kept in LL for four weeks and then entrained to a LD 16:8. After two week these animals were sacrificed at ExT 0 (n = 4), 3.5 (n = 3), 4 (n = 6), 6 (n = 3), 9 (n = 3), 12 (n = 10), 16 (n = 4), and 20 (n = 6).

Immunocytochemistry

Animals were deeply anesthetized with an overdose of sodium pentobarbital (100 mg/kg, intraperitoneal) and killed by decapitation followed by a quick dissection of the brain. Brains were fixed for 6 hours in 4% paraformaldehyde in 0.1 M phosphate buffer. After fixation brains were rinsed thrice in 0.05 M phosphate buffer in 0.9% NaCl (PBS, pH 7.4). Before sectioning, brains were cryoprotected in 30% sucrose in PBS for 24 hours. Coronal brain sections of the SCN and surrounding tissue were cut at 20 or 25 μm and kept in PBS with 0.1% NaN3 at 4 °C until further processing.

Before immunocytochemical staining sections were rinsed in 0.01 M PBS and quenched in 0.45% H2O2 in 0.01 M PBS for 30 minutes. After rinsing thrice in 0.01 M PBS, sections were pre-incubated in with normal goat serum (5%) for 30 minutes. Free floating sections were kept in primary antibody rabbit anti-Fos (polyclonal IgG (Santa Cruz Biotechnology); 1:8000 in 0.01M PBS + 0.5% Triton-X-100 and 1% Goat serum in PBS) for 48 hours at 4 °C. After rinsing thrice with 0.01 M PBS, sections were exposed to the secondary antibody biotinylated goat anti-rabbit (IgG F(ab)’2 fraction (Zymed); 1:200 in 0.01M PBS + 0.5% Triton-X-100 + 1% goat serum) for 90 minutes and subse-

Figure 1

Schematic representation of the quantification method, OC = optic chiasm, 3V = third ventricle. On the left, the dissector frame subdivided in small squares enclosed 25% of the visual field. Only cell completely within the squares, or partially crossing the thick line were counted. On the right, the diagonal line from the ventromedial corner to the dorsolateral corner is shown that was used to make a distinction between the ventrolateral (vlSCN) and dorsomedial (dmSCN) SCN. Counts if total immunoreactive cells per subdivision were established using the formula: N_{per\,nucleus} = ((1/area\,sampling\,fraction) \times (1/section\,sampling\,fraction)) \times (1/tissue\,sampling\,fraction)).
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Subsequently rinsed thrice in 0.01 M PBS. After rinsing sections were kept in a horse radish peroxidase – streptavidin conjugate (Zymed; 1:200 in 0.01M PBS and 0.5% Triton-X-100) for 1 hour. After rinsing thrice in 0.01 M PBS, sections were pre-incubated in 0.05 M TRIS-HCL (pH 7.6 in water) for 10 minutes and then processed with diaminobenzidine (DAB)-H$_2$O$_2$ (30 mg DAB in 100 ml PBS and 0.01% H$_2$O$_2$) under visual guidance. After immunocytochemical staining, sections were rinsed thoroughly in 0.01M PBS, mounted, air dried and coverslipped.

Quantification

Sections were quantified blind to time of sacrifice, using an optical fractionator protocol based on Jansen et al. (1998). In short, in the microscopic field at a magnification of 1000x, a dissector frame was fitted around a single SCN. The dissector frame was subdivided in small squares with a distance of 27 µm, which was equal to the length/width of a single square. In a surface area of 54 µm * 54 µm only one quarter (25%) is counted, hence the area sampling fraction was 0.25. Only one out of three sections was stained for c-Fos, setting the section sampling fraction at 0.33. The tissue sampling fraction for 20 µm and 25 µm samples was set at 0.61 and 0.69, respectively. A distinction was made between vlSCN and dmSCN by drawing a diagonal line in the microscopic field from the ventromedial corner to the dorsolateral corner (see figure 1). This line was used as the separation between the both SCN sub regions. Different staining batches (due to post-hoc additions of time points) within a group were pooled by including standard time points within each set and equalizing counts between sets.

Statistical analysis

Differences in number of sections between animals were controlled for by calculating totals per animal based on the maximum number of sections for one animal within a batch. Using a Kruskal-Wallis One Way ANOVA on ranks, variation over the day in number of C-FOS immunoreactive cells was tested. Because the dataset was not equidistant, when a significant variation over time was found within a SCN region under LL or LD, the number of C-FOS positive cells at all time point were compared to their adjacent time points using a Mann-Whitney rank sum test, or a t-test as its parametrical equivalent when applicable.
Results

A representative double plotted actogram of vole overall activity is shown in figure 2. Voles show both circadian and ultradian activity patterns (e.g., Daan & Slopsema, 1978), which are also visible in this actogram. After sacrificing the animals, immunocytochemical staining of C-FOS was carried out in sections containing the SCN. Nuclear staining of C-FOS was seen and clearly distinguishable from the background staining (See figure 3 for representative photomicrograph). The average nucleus size did not differ significantly (p < 0.05, unpaired t-test) between samples and was on average 7.87μm. Using the optical fractionator method, total number of C-FOS immuno-
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Figure 4

Number of c-Fos immunoreactive cells in the vlSCN (left) and dmSCN (right) of common voles entrained to a light dark cycle of 12 hours light and 12 hours dark (upper row), 16 hours of light and 8 hours of dark (middle row) or continuous dim light (bottom row). Both the vlSCN and the dmSCN show significant variation over time for animals housed under LD 12:12 (one way ANOVA on ranks, $P < 0.001$ both cases). Asterisks are placed at time points at which levels of c-Fos expression are significantly different from the levels of both neighbouring time points (Mann-Whitney test or t-test when applicable; $\alpha = 0.05$). Numbers of immunoreactive cells in the ventrolateral SCN of common voles housed under LD 16:8 show a significant variation over time (One Way ANOVA on ranks, $P < 0.005$). Post hoc testing revealed a significant peak at ExT 6; 2 hours after lights on (vers. ExT 4 (Mann-Whitney test, $P < 0.05$) and vers. ExT 9 (t-test, $P = 0.001$)). The asterisk between ExT 20 and 0 indicates a significant decrease in c-Fos expression (t-test, $P < 0.05$). No significant variation over time is seen for levels of c-Fos expression in the vlSCN or dmSCN of common voles housed under continuous dim light. Gray areas represent the periods of dim light levels; error bars indicate SEM of the mean.
reactive (ir) cell in dmSCN and vlSCN were established. Profiles of numbers of total C-FOS-ir cell counts in the vlSCN and dmSCN are shown in figure 4 for voles kept in LD 12:12, LD 16:8 or dim light.

**C-FOS immunoreactivity in the SCN of voles in LD 12:12**

C-FOS-ir cell counts in the vlSCN of voles housed in LD 12:12 conditions showed significant variation over the day (One Way ANOVA on ranks, \( p < 0.001 \)) and average counts ranged from 6 (ExT 0) to 1874 (ExT 6). By comparing number of C-FOS-ir cells form individual time points with counts of adjacent time points, we could identify two peaks in C-FOS expression: at ExT 6 and 18. The peak in C-FOS expression at ExT 6 was preceded by a significant increase in C-FOS expression at ExT 5.5. Numbers of C-FOS-ir cells at ExT 6 were significantly larger than those of the preceding time point (ExT 5.5; t-test, \( p < 0.005 \)) as well as the following time point (ExT 9; t-test, \( p < 0.001 \)). Also at ExT 18 the C-FOS-ir cell count was significantly larger than that of the preceding and following time points (ExT 12 and 0, Mann-Whitney test; \( p < 0.05 \) and \( p < 0.01 \) respectively). Both peaks at ExT 6 and 18 are at the moment of alterations of light conditions, at dawn and dusk. At ExT 5.5, C-FOS-ir cell counts are significantly higher than at ExT 0 (Mann-Whitney test, \( p < 0.05 \)), but lower than ExT 6 (t-test, \( p < 0.005 \)).

C-FOS-ir cell counts in the dmSCN in LD 12:12 (see figure 4 top right panel) also show significant variation with time (One Way ANOVA on ranks, \( p < 0.01 \)), but the profile is distinctly different from that of the vlSCN of the same animals. Number of C-FOS-ir cells in the dmSCN range from 13 (ExT 0) to 1246 (ExT 12). Only one peak is seen in number of C-FOS-ir cells at ExT 12 and one trough at ExT 0, which is significantly higher than the counts at ExT 9 and 18 (t-test, \( p < 0.005 \) and \( p < 0.05 \) respectively).

**C-FOS immunoreactivity in the SCN of voles in LD 16:8**

To check whether peaks in C-FOS-ir cell counts in the vlSCN coincided with alterations between dark and light phases, voles were exposed to LD 16:8. The profile of C-FOS-ir cells is shown in figure 4 (middle row). Number of vlSCN C-FOS-ir cells range from 23 (ExT 3) to 2404 (ExT 6). Numbers of C-FOS-ir cells differ significantly over time (One Way ANOVA on ranks, \( p < 0.005 \)). One peak is seen at ExT 6 and a significant decrease in C-FOS-ir cells is seen between ExT 20 and ExT 0. The number of C-FOS-ir cells significantly peaks at ExT 6, which is significantly higher than the counts at ExT 4 (Mann-Whitney test, \( p < 0.05 \)) and at ExT 9 (t-test, \( p = 0.001 \)). The peak seen in C-FOS immunoreactivity in the vlSCN in LD 16:8 is at the same external time point as in LD 12:12. In the case of LD 16:8, the peak at ExT 6 follows two hours after the shift from dark to light, and no peak in the number of C-FOS-ir cells in the vlSCN of voles is seen at the alteration from dark to light.
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C-FOS immunoreactivity in the SCN in LL

Numbers of C-FOS-ir cells in the vlSCN of animals in LL are between 192 (InT 6) and 555 (InT 18) and are shown in figure 4 (bottom left panel). No significant differences over time could be established in the number of C-FOS-ir cells (One Way ANOVA on ranks, P > 0.05). Also the number of C-FOS-ir cell counts in the dmSCN does not show differences over time (One Way ANOVA on ranks, P > 0.05). Counts in the dmSCN of voles housed in dim light ranged from 154 (InT 0) to 717 (InT 12) C-FOS-ir cells and are shown in figure 4 bottom right panel.
Circadian rhythms of C-FOS expression in the suprachiasmatic nuclei

Discussion

LD 12:12

The dmSCN had rhythmic C-FOS expression in LD 12:12, with a peak value at noon (ExT 12). Such a rhythm has not been reported in other species in entrainment by either complete or skeleton photoperiods (see table 1). This is possibly related to the fact that levels of c-Fos expression in the dmSCN can be both low and highly localized in comparison to the vlSCN c-Fos expression under entrained conditions under light-dark (Colwell & Foster, 1992, Schwartz et al., 2000).

The vole in contrast shows high levels of dmSCN C-FOS immunoreactivity and a clear rhythm under LD 12:12. This is intriguing, in particular since no rhythm was observed in dim LL. This is opposite to the general idea that c-Fos expression in the dmSCN is rhythmic under constant conditions, and not observed under entrained conditions. c-Fos expression in the dmSCN may well be related to the output of the SCN and is generally rhythmic both under entrained conditions (but often not noticed by the very low level) as well as under constant conditions.

A predominant neuropeptide in the dmSCN is vasopressin (Vandesande et al., 1975). Although vasopressin and C-FOS are not abundantly found to be expressed in the same cells in rats (Sumová et al., 2000, Schwartz et al., 2000), c-Fos in the dmSCN could be indicative of transcriptional activity coupled to vasopressin. c-Fos is part of the activator protein (AP-1) complex and recently it has been shown that the vasopressin gene contains an AP1-like element and that the transcription of vasopressin is upregulated by different AP1 complexes including C-FOS (Yoshida et al., 2006). In voles vasopressin immunoreactivity decreases during the day and rises at night (Jansen et al., 2003). Here, C-FOS immunoreactivity in the dmSCN is high at ExT 12. SCN vasopressin mRNA levels in Arvicanthis, mouse and rat peak two to four hours thereafter at ExT 14-16, (Dardente et al., 2004, Cagampang et al., 1994). In the case of the vole, the weak circadian behavioural rhythm is possibly the result of weak circadian transcriptional activity in the dmSCN. We have shown before that in the mouse the coupling between SCN-vasopressin dynamics and circadian rhythmicity in behaviour is not at the level of transcriptional activity, but downstream of this process (Van der Veen et al., 2005).

The ventrolateral part of the vole SCN shows 2 peaks in C-FOS peptide expression under LD 12:12 conditions. The ‘morning’ peak is maximal at lights-on, but numbers of C-FOS-ir cells are already rising 30 minutes before dawn. A second, ‘evening’ peak is seen at lights-off. The peak in vlSCN C-FOS expression at dawn is consistent with the literature on other species (Kononen et al., 1990, Colwell & Foster, 1992, Schwartz et al., 1994), but the timing in the case of the common vole is early in comparison to the timing seen other rodents (see table 1).
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In the vole vlSCN, the absence of circadian variation in C-FOS immunoreactivity under continuous light conditions is consistent with other species (Guido et al., 1999, Schwartz et al., 2000, Colwell & Foster, 1992, Chambille et al., 1993). These same studies do however report c-Fos expression in dmSCN. c-Fos expression in the dmSCN of hamsters and rats in DD is high in the early subjective day (Guido et al., 1999, Schwartz et al., 2000). Chambille et al. (1993) report a peak in whole SCN C-FOS immunoreactivity at InT 13; i.e., 7 hours into the subjective day in constant darkness. We see cells expressing C-FOS in the vole dmSCN under constant dim light conditions, but no significant circadian pattern is seen. Most studies on c-Fos expression under constant conditions have been made in animals coming from entrained conditions and released in constant conditions for just one or two cycles to get rid of masking effects of light (table 1). In these studies, c-Fos expression in the dmSCN is often seen to peak at early subjective day (Sumová et al., 1998, 2000, 2005). Releasing rats into LL instead of DD phase delays the peak in dmSCN by 4 hours after 1.5 days in constant light conditions (Sumová et al., 2005). Still, a rhythm is seen in dmSCN c-Fos expression under constant conditions. The absence of rhythmicity in the vole may be related to the weak circadian rhythms of activity in the species. Loss of circadian rhythmicity in behaviour might be a result of the absence of rhythmic transcriptional activity in the dmSCN, although we must note that for this experiment, only animals that showed clear behavioural circadian rhythms in LL were used.

Relationship between C-FOS expression in the SCN of voles and daylength

In the vole, lights-on in LD 12:12 is preceded, possibly even anticipated (consider ExT 5.5) by a rise in c-FOS immunoreactivity (thus transcriptional activity) in the vlSCN, the light-input side of the SCN. The peak of C-FOS expression in the vole vlSCN just after lights on in LD 12:12 may be indicative of a mechanism whose activity coincides with the dark to light transition. Such activity in the SCN might be associated with a mechanism tracking dawn, as postulated in a dual oscillator mechanism coding for daylength (Daan et al., 2001). In rats, the morning rise of spontaneous c-Fos expression in the vlSCN and the dmSCN is advanced under a long photoperiod (Jác et al., 2000, Sumová et al., 2000). Also in in vitro multiple unit electrical activity in hamsters a morning (and evening) peak has been observed around projected dawn (and dusk). When the photoperiod before culturing is changed, the peaks in multiple unit activity shifted accordingly (Jagota et al., 2000). In our study, the morning peak in C-FOS expression occurred at the same external time in LD 12:12 and LD 16:8 (figs. 4 and 5), i.e. with different phase relationships relative to lights-on. In the Illnerová lab (Jác et al., 2000, Sumová et al., 2000) an earlier rise in spontaneous C-FOS immunoreactivity was found in a long photoperiod than in a short photoperiod in the vlSCN and dmSCN in rats. c-Fos mRNA levels in hamster in response to a light pulse during the subjective night is rapid and short lasting, reaching peak values after 30 minutes and returning to back to normal within two hours (Kornhauser et al., 1990). In our long photoperiod experiment, a large peak in C-FOS-ir cells is seen 2 hours after the onset of light. At this time
lower levels were to be expected if the peak where light induced. In rats entrained to a LD 12:12, peak expression of C-FOS is seen only two hours after onset of light, and C-FOS levels remain elevated during the full light period (Schwartz et al., 1994).

*c-Fos* expression in the vlSCN as a result of a light pulse, is only seen in the time window when the circadian system is capable of phase shifting in response to the light pulse given (Rusak et al., 1990, 1992). This process is termed ‘gating’ (Bendová et al., 2004, Sumová et al., 1995). Perhaps in the vole under LD 16:8 the SCN is not receptive for light input and a rise in transcriptional activity is not observed at ExT 4 when the lights are turned on. In this case, ‘gating’ of SCN sensitivity could interfere with a process tracking dawn (Daan et al., 2001). We do not know which transcriptional processes are marked by C-FOS immunoreactivity expression in this case. Hence caution should be taken when interpreting these data. There are clear differences in *c-Fos* expression between species. We conclude that a process in the vlSCN of the vole does not seem dependent on photoperiod. Such processes are expected to be coupled to length of the natural day, or the intrinsic period (tau) under constant conditions and to be a part of the ‘core clock’, not the input pathway.

Our vole data corroborate the heterogeneous nature of the c-Fos expression under different properties in at least two aspects of the SCN. This heterogeneity underlines the notion of a dynamic organisation of the SCN (Morin, 2007) in which c-Fos might report on different processes between species, or that these processes have different dynamics across species.