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

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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 LD 12:12h and under constant dim light conditions. 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 organization of behavioral activity, which is weak in voles, and may be lost under constant dim light conditions. In the ventrolateral SCN, under LD 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 in ventrolateral C-FOS expression 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 are 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. (E-mail correspondence: D.Veen@surrey.ac.uk).

Key words: C-Fos, Vole, Suprachiasmatic nucleus, SCN, Photoperiod

Introduction

The suprachiasmatic nuclei of the hypothalamus (SCN) form the main circadian pacemaker in mammals, governing rhythms in physiology and behavior (Stephan & Zucker, 1972; Moore & Eichler, 1972). One of the functions of the SCN is entrainment of circadian rhythms to the external light-dark (LD) environment. Photoc 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 the 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 polypeptide 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), both in response to light perceived by rods and cones and that perceived by specialized melanopsin-containing retinal ganglion cells (Lupi et al., 2006). 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.

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 behavioral 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 SCN c-Fos rhythms in constant conditions (constant dark: DD and constant light: LL) and in LD cycles. Under constant 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 h 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 External Time (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 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.

Based on 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, in which 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 dorsomedial 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 diurnal 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 h later, evenly distributed over the whole SCN (Katona et al., 1998). As Schwartz et al. (1994) already noted, c-Fos immunoreactivity patterns in the vSCN seem to be species-specific, and might be coupled to the intrinsic properties of the circadian system. In order to address the comparative differences seen in c-Fos expression, we studied c-Fos expression in the Common vole.

c-Fos in Common voles

In the common vole (*Microtus arvalis*), circadian and ultradian behavioral rhythms are simultaneously expressed in overall activity (Daan & Slopeema, 1978), where the circadian activity component is less rigid than the ultradian component (Gerkema & Daan, 1985). The strength of the circadian activity component varies between individuals and under constant light conditions about 30% of the animals lose their circadian organization of activity completely (Gerkema et al., 1990, 1994). The strength of circadian organization of behavior in voles has been correlated to protein kinase C α and vasopressin in the SCN (Jansen et al., 1999, 2000, 2003), but there is no correlation with vasoactive intestinal polypeptide in the SCN of voles (Jansen et al., 2007). These differences in correlations between behavior and neuropeptide systems indicate a difference in the circadian system in voles, which can be related to functional aspects of the SCN subdivisions to which these neuropeptides correspond.

Because voles that remain rhythmic under constant conditions show less rigid circadian organization of behavior than, for instance mice and rats, we are interested in differences in the general circadian activity of the SCN in these animals under entrained and free-running conditions. Moreover, the previously demonstrated correlation between

strength of circadian rhythmicity and the differential effects in the SCN raise the question whether differences in general SCN activity are region-specific.

We have studied C-FOS expression in the SCN as a marker of general transcriptional activity in the SCN of the vole under entrained and free-running conditions to compare these species-specific expression patterns to those of other rodent species. In doing so, we aim to add to our understanding of C-FOS expression in the SCN. We see distinctly different expression profiles in the vlSCN and dmSCN, supporting the notion of the differential functions attributed to these SCN subdivisions. The vole can be both diurnally and nocturnally active due to its strong ultradian nature (Hoogenboom et al., 1984), sampling light conditions throughout the day. We thus wished to investigate the effect of different photoperiods on c-Fos expression. Because of the different C-FOS expression seen in the subdivisions under LD 12:12 in line with different functions of the vlSCN and dmSCN, we focus on the vlSCN since it is predominantly involved in entrainment to light. In our case, it would be ambiguous to include the dmSCN (expressing vasopressin and protein kinase C α , which are correlated to the intrinsic expression of circadian rhythmicity and driving of SCN output). We therefore focussed on C-FOS expression in the vlSCN under a long photoperiod (LD 16:8) in comparison to LD 12:12. Interestingly, in the vole we see similar C-FOS profiles in the vlSCN in reaction to LD 12:12 and LD 16:8. This is different from what has been reported for other species.

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 an attached nest box (17x11x13 cm), with wood shavings as cage bedding. Running wheels were available throughout the experiment and food (Hopefarms mouse pellets) and tap water were available *ad libitum*. Cages were placed in a sound-attenuated, climate-controlled room (temperature $22 \pm 1^\circ\text{C}$; 70% relative 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 free-running circadian activity patterns. Experiments were approved by the Animal Experimentation Committee of the University of Groningen (DEC No. 2594) and experiments were performed in agreement with the standards of this journal (Touitou et al., 2006).

Running wheel activity was recorded on an event recording system, storing activity counts in 2 min intervals. Using the locomotor activity data, animals were categorized as rhythmic, weakly rhythmic, or arrhythmic by both arbitrary visual inspection and by Chi-square (χ^2) periodogram analyses (Sokolove & Bushell, 1978). Only animals that were categorized as rhythmic, both by visual inspection (when a circadian component in behavior 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 in different LD conditions were studied. Group A was kept in LL (~2 Lux). Group B was maintained in LD 12:12, and group C in LD 16:8. To prevent the animals from 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 h before the onset of dawn were included to determine whether C-FOS could anticipate lights on and 3 h after lights on to see expression after direct 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 midday and midnight. If 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 the number of individuals studied per time point.

The resulting time points when animals were assessed are thus as follows. In LL animals, they corresponded to internal times (InT; Daan et al., 2002, defining activity onset as 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 the 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 h 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 h. Coronal brain sections of the SCN and surrounding tissue were cut at 20 or 25 μ m 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 min. After rinsing thrice in 0.01 M PBS, sections were pre-incubated with normal goat serum (5%) for 30 min. 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 h 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 min and 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 h. 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 min and then processed with diaminobenzidine (DAB)-H₂O₂ (30 mg DAB in 100 ml PBS and 0.01% H₂O₂) 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 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 both SCN subregions. 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 the 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 the 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 points 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 & Slopeema, 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 $7.87\mu\text{m}$. Using the optical fractionator method, the total number of C-FOS immunoreactive (ir) cells in the 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 the number of C-FOS-ir cells from 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. The 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 coincided with the moment of alteration of light conditions, at dawn and dusk. At ExT 5.5, C-

FOS-ir cell counts were 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 showed significant variation with time (One-Way ANOVA on ranks, $P < 0.01$), but the profile was distinctly different from that of the vlSCN of the same animals. The number of C-FOS-ir cells in the dmSCN ranged from 13 (ExT 0) to 1246 (ExT 12). Only one peak was 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 the 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). The number of vlSCN C-FOS-ir cells ranged from 23 (ExT 3) to 2404 (ExT 6). The numbers of C-FOS-ir cells differed 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 peaked 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 was at the same external time point as in LD 12:12. In the case of LD 16:8, the peak at ExT 6 followed 2 h after the shift from dark to light, and no peak in the number of C-FOS-ir cells in the vlSCN of voles was seen at the alteration from dark to light.

C-FOS immunoreactivity in the SCN in LL

The numbers of C-FOS-ir cells in the vlSCN of animals in LL were between 192 (InT 6) and 555 (InT 18) as 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 did 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).

Discussion

C-Fos expression under LD 12:12 conditions

The dmSCN showed 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 expectation 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 due to its 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 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 up-regulated 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 the

Arvicanthis, mouse, and rat peak 2-4 h thereafter, at ExT 14-16, (Dardente et al., 2004; Cagampang et al., 1994). In the case of the vole, the weak circadian behavioral 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 behavior 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 two 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 begin their rise 30 min 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 (Table 1).

LL

A rhythm was not detected in C-FOS immunoreactivity in the vlSCN under continuous light conditions. While this is inconsistent with most findings under LL immediately after LD entrainment (Aronin et al., 1990; Earnest et al., 1992; Sumová & Illnerová, 2005), it is consistent with findings in the mouse, Syrian hamster, and rat after a prolonged period of darkness (Guido et al., 1999; Schwartz et al., 2000; Colwell & Foster, 1992). When considering the similarities between vlSCN c-Fos expression under prolonged continuous light conditions, then the prolonged absence of daily LD changes (darkness for Guido et al., 1999; Schwartz et al., 2000; Colwell & Foster, 1992 and dim light for our study in the voles) results in an absence of c-Fos rhythmicity in the vlSCN. The c-Fos rhythms in the vlSCN seen

in complete darkness or light immediately after entrainment in LD may still result from the previous light-dark schedule.

Adding to the DD data, the absence of rhythms in C-FOS immunoreactivity in our voles after prolonged LL suggests that continuous light does not keep those rhythms going, which are seen immediately after release in constant conditions. Although it has been shown that light information is perceived by the eye and can readily induce C-FOS expression in the vlSCN (Lupi et al., 2006), continuous illumination does not change the absence of rhythmic 'spontaneous' C-FOS expression in the vlSCN.

Most studies showing no rhythm in vlSCN c-Fos under constant darkness, do however report c-Fos expression in dmSCN (Chambille et al., 1993; Guido et al., 1999; Schwartz et al., 2000), but Colwell & Foster (1992) report almost no C-FOS in the whole SCN of the mouse under DD. In the vole we see low levels of C-FOS rhythms in the dmSCN, but no rhythmicity after a prolonged duration in constant light. In the studies summarized in Table 1 reporting c-Fos expression in the dmSCN immediately after release from LD in DD or LL, c-Fos expression in the dmSCN is often seen to peak at early subjective day. Releasing rats into LL instead of DD phase delays the peak in dmSCN by 4 h after 1.5 days in constant light conditions (Sumová & Illnerová, 2005). The absence of such a peak in the vole might be the result of prolonged absence of LD, but such a peak is also seen after a prolonged duration in DD (Chambille et al., 1993; Guido et al., 1999; Schwartz et al., 2000). The absence of rhythmicity in dmSCN C-FOS immunoreactivity in the vole may also be related to the less stringent circadian rhythms of activity in the species. While all our animals show clear circadian rhythm in behavior, the vole is more ultradian than circadian. The simultaneous exhibition of circadian and ultradian activity rhythms may contribute to the 'loss' of strong circadian rhythms in the dmSCN, the output of the central circadian pacemaker, in LL.

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

Light entrains the SCN and while the dorsomedial subdivision of the SCN is involved in output of the SCN (Leak & Moore, 2001), and as we have shown here may be affected by behavioral state, the ventrolateral subdivision receives retinal input from the eye and is involved in entrainment of the SCN to light (Reviewed in Moore et al., 1996). Because our result suggests that in the vole, time of lights-on in LD 12:12 is preceded, possibly even anticipated (consider ExT 5.5), by a rise in C-FOS immunoreactivity in the vlSCN, we wished to establish whether this was a time-of-day effect, or an entrainment effect. 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 by 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 (Figure 4), i.e., with different phase relationships relative to lights-on. In the work by Illnerová and colleagues (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 hamsters in response to a light pulse during the subjective night is rapid and short lasting, reaching peak values after 30 min, returning to normal within 2 h (Kornhauser et al., 1990). In our long photoperiod experiment, a large peak in C-FOS-ir cells

is seen 2 h after the onset of light. At this time, lower levels were to be expected if the peak was light induced. In rats entrained to a LD 12:12, peak expression of C-FOS is seen only 2 h 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 to 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 (τ) under constant conditions and to be a part of the 'core clock', not the input pathway.

Our vole data corroborate the heterogeneous nature of *c-Fos* expression under different properties in at least two aspects of the SCN. This heterogeneity underlines the notion of a dynamic organization 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.

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Figure legends

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 cells 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 of total immunoreactive cells per subdivision were established using the formula: $N_{\text{per nucleus}} = ((1/\text{area sampling fraction}) * (1/\text{section sampling fraction}) * (1/\text{tissue sampling fraction}))$.

Figure 2

Representative double-plotted qualitative actogram of an animal kept under dim light for four weeks and subsequently entrained for two weeks to a light dark cycle of 16 h light and 8 h dark.

Figure 3

Photomicrograph of immunocytochemical c-Fos staining in the SCN of a vole (taken at Ext 12, LD 16:8). Note that both the vSCN and the dmSCN show clear nuclear c-Fos staining.

Figure 4

Number of c-Fos immunoreactive cells in the vISCN (left) and dmSCN (right) of common voles entrained to a 12 h light and 12 h dark (upper row), 16 h light and 8 h of dark (middle row), or continuous dim light (bottom row). Both the vISCN and dmSCN show significant variation over time in 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 neighboring 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 significant variation over time (One-Way ANOVA on ranks, $P < 0.005$). Post hoc testing revealed a significant peak at ExT 6; 2 h after lights on (vs. ExT 4 by the Mann-Whitney test, $P < 0.05$ and vs. ExT 9 by 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 in 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.