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Gradual reappearance of post-hibernation circadian rhythmicity correlates with numbers of vasopressin-containing neurons in the suprachiasmatic nuclei of European ground squirrels

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Abstract European ground squirrels (*Spermophilus citellus*) in outside enclosures show suppressed circadian rhythmicity in body temperature patterns during the first days of euthermia after hibernation. This may reflect either gradual reappearance of circadian rhythmicity following suppressed functioning of the circadian system during hibernation, or it may reflect transient days during re-entrainment of the circadian system which, during hibernation, has drifted out of phase with the environmental light-dark cycle. Here we report that animals kept under continuous dim light conditions also showed absence of circadian rhythmicity in activity and body temperature in the first 5–15 days after hibernation. After post-hibernation arrhythmicity, spontaneous circadian rhythms re-appeared gradually and increased daily body temperature range. Numbers of arginine-vasopressin immunoreactive neurons in the suprachiasmatic nuclei correlated positively with individual circadian rhythmicity and increased gradually over time after hibernation. Furthermore, circadian rhythmicity was enhanced rather than suppressed after exposure to a light-dark cycle but not after a single 1-h light pulse (1,700 lux). The results support the view that the functioning of the circadian system in the European ground squirrel is suppressed during hibernation at low temperatures and that it requires several days of euthermia to resume its summer function.

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Abbreviations *AVP* arginine-vasopressin · *AVPir* AVP-immunoreactive · *ct* circadian time · *LD* light-dark · *PAF* paraformaldehyde · *PB* phosphate buffer · *PBS* phosphate-buffered saline · *SCN* suprachiasmatic nuclei · *SON* supraoptic nuclei

Introduction

In a previous study we reported the absence or suppression of circadian fluctuations in body temperature in the European ground squirrel during the first days of euthermia after hibernation (post-hibernation arrhythmicity) under semi-natural conditions (Hut et al. 2001). This loss may be caused either by changed intrinsic properties of the circadian system that suppressed its function in the course of hibernation, or by interference between internally generated rhythms and a direct response of body temperature to daily environmental rhythms (masking) during the process of re-entrainment in the first days after hibernation.

Suppressed circadian system hypothesis

Post-hibernation arrhythmicity may be indicative for a suppressed functioning of the circadian pacemaking system caused by long periods of extreme low body temperatures during hibernation. The functioning of the circadian system during mammalian hibernation is still under debate, and may vary between species, with torpor bout duration, and with body temperature during torpor (Körtner and Geiser 2000; Hut et al. 2001; Oklejewicz et al. 2001). Persistence of circadian body temperature oscillations during torpor has been shown in golden-mantled ground squirrels (Grahn et al. 1994) and bats (Menaker 1959), and a circadian effect on the timing of arousals has been claimed in several other mammals

(Strumwasser 1959; Pohl 1961, 1967, 1987, 1996; Daan 1973a; Twente and Twente 1987; Canguilhem et al. 1994; Grahn et al. 1994; Wollnik and Schmidt 1995; Waßmer and Wollnik 1997; Körtner et al. 1998; Körtner and Geiser 2000). However, some of the evidence is not convincing and absence of circadian rhythmicity during hibernation has also been demonstrated (Waßmer and Wollnik 1997; Thomas 1992; Pohl 1987; Oklejewicz et al. 2001). In the European ground squirrel we did not find circadian fluctuations in torpor body temperature either under semi-natural conditions (Hut et al. 2001) or constant dim light conditions at 5 °C (A.M. Strijkstra and G.M. ten Hoopen, unpublished observations). Circadian oscillations persisting at low body temperatures during hibernation are of interest due to their presumed ecological function (Körtner and Geiser 2000; Hut et al. 2001) and because of the question of temperature compensation in homeotherms (Rawson 1960). Many hibernators may not have a need for a functional circadian system during hibernation and therefore may lack the physiological mechanisms to maintain circadian oscillations during hibernation. European ground squirrels remain sequestered in their hibernacula for 6–7 months of hibernation (Millesi et al. 1999) and appear to lack the need for a functional circadian system during hibernation. Post-hibernation arrhythmicity might then simply reflect the gradual re-appearance of circadian oscillations in a pacemaker that has not been functional during hibernation.

Re-entrainment hypothesis

Alternatively, the circadian system may continue to function during hibernation. The observed post-hibernation arrhythmicity in body temperature may be attributed to interference between the endogenous circadian influence and exogenous light-dark (LD) cycle on body temperature during re-entrainment to the environmental LD cycle. The European ground squirrel is deprived of daily LD cycles during 6–7 months in its hibernaculum. If the circadian system continued to oscillate, it would probably end up out of phase with the daily LD cycle and would consequently need to re-entrain after hibernation. During the transient days of re-entrainment, the activity pattern may be influenced directly by the LD cycle (masking). This may temporarily lead to a reduction in overt body temperature amplitude when the internally generated body temperature rhythm and the direct effect of the environment on body temperature are out of phase. The expectation would be that this reduction would gradually decrease when the process of post-hibernation re-entrainment causes the internal and external rhythms to be in phase with each other.

To distinguish between these hypotheses we studied post-hibernation activity and body temperature rhythms under continuous dim light conditions. This procedure rules out any external, daily effects on activity or body temperature. Post-hibernation rhythmicity under these conditions would support the hypothesis that the functioning of the circadian system had deteriorated during hibernation.

In addition to body temperature and activity, we studied arginine-vasopressin (AVP) immunostaining in the ground squirrel suprachiasmatic nuclei (SCN) during post-hibernation euthermia. AVP is present in SCN cells of ground squirrels (Reuss et al. 1989; Schindler and Nürnberger 1990; Smale et al. 1991) and has been implicated as a key neuropeptide in the control of the circadian pacemaker in the mammalian SCN over behavioural rhythms in mice (Bult et al. 1993), rats (Wollnik and Bihler 1996), and voles (Gerkema et al. 1994; Jansen et al. 1999). Brattleboro rats, lacking AVP production, remain circadian rhythmic, but circadian rhythmicity is more pronounced in heterozygotes as compared to homozygotes (Ingram et al. 1996). This indicates that the SCN probably signals its circadian rhythmicity by more than one mechanism, and that AVP is a good candidate for one of these output signals from the mammalian circadian pacemaker. In addition to the possible output function of AVP production on the SCN, there may also be an internal function. SCN neurons in acute brain slices from Brattleboro rats increase their firing rate when treated with AVP (Ingram et al. 1996). Hence, AVP content in the SCN after post-hibernation arrhythmicity might be indicative of regaining function by the SCN.

In a second experiment, we investigated the effects of light on circadian activity patterns after hibernation. The suppressed body temperature rhythm in the first few days after hibernation under natural conditions was measured in animals that were probably exposed to sunlight during this period (Hut et al. 2001). This light exposure may have an effect on the circadian system. Re-appearance of circadian rhythmicity by a single light pulse has been shown in spontaneously arrhythmic *Clock* mutant mice (Vitaterna et al. 1994). We applied both a single light pulse and an LD cycle after hibernation and analysed the effects on circadian rhythmicity under continuous dim light conditions.

Materials and methods

Animals and housing

European ground squirrels (*Spermophilus citellus*) used in this study originated from a population near Vienna, Austria (Millesi et al. 1999). The animals were individually housed in cages (length × width × height = 48 × 28 × 50 cm) with an external nest box (15 × 15 × 15 cm) in a climate-controlled room. Food (rabbit breeding chow; Teurlings, Waalwijk, The Netherlands) and tap water were available ad libitum. Ambient temperatures in the climate-controlled room were recorded to the nearest 0.18 °C on several locations in the room. No daily variations in ambient temperature fluctuations were detected in the climate room and any differences were due to position of the thermistors (± 1.1 °C) rather than due to the regulation of ambient temperature (< ± 0.2 °C). All animals used in this study hibernated and showed torpor bouts

of normal durations (8–16 days) after the lowering of the ambient temperature to 3.5 °C.

Recordings

Body temperature patterns were recorded every 48 min for 264 days by loggers (Onset Computer Cooperation, Mass., USA; customised Stowaway Tidbit temperature loggers, 12.8 g; range: –5 °C to 40 °C; accuracy: 0.18 °C). The loggers were covered with three layers of Elvax coating (Minimitter) before implantation. The animals were anaesthetised with halothane for intraperitoneal implantation and removal of the loggers. Locomotor activity was continuously recorded with passive infrared detectors, connected to a PC-based event-recording system that stored the number of events in 2-min bins.

Experiment 1: post-hibernation circadian rhythms under continuous dim light and AVP staining in the SCN

Experimental conditions

Animals (4 females, 12 males) were placed in the climate-controlled room on November 8 1997. Ambient temperature was gradually lowered from 20.0 °C to 3.5 °C, in three steps of similar duration and temperature decrease, from November 14–21 1997. Light conditions were changed from a 12-h:12-h LD (300–400 lux) cycle to continuous dim light (<0.1 lux) on November 21 1997. To mimic natural temperature conditions to some extent, ambient temperature was gradually raised from 3.5 °C to 25 °C, in four steps of similar duration and increase, from March 22–29 1998. This successfully terminated hibernation in four animals; the rest of the animals had done so before the temperature rise. Activity was recorded in all animals; body temperature was recorded in 11 males.

Brain material

At different intervals after the end of hibernation, nine males and one female were killed by an intraperitoneal injection of 2 ml 0.2% sodium-pentobarbital. Individuals were killed during the first half of their active phase [mean = circadian time (ct) 4.7; SEM = 1.4 circadian hours; centre of gravity of activity (Kenagy 1980) was defined as ct 6]. The brains were collected after 6 min transcardial perfusion with heparinised saline followed by a 20-min 4% paraformaldehyde (PAF) perfusion in 0.1 M phosphate buffer (PB). The heads of the animals and the perfusion solutions were kept at 0 °C for standardisation with other experiments (Deelman et al. 1998; Strijkstra 1999). The mid region of the brain, containing the SCN, was dissected and post-fixated in 4% PAF solution (in 0.1 M PB) for 2 h and then stored in 0.1 M PB at 4 °C until sectioned. The brain region containing the SCN was cut coronally in 25-μm slices on a cryostat and every fourth section was collected for AVP immunocytochemical staining.

Free floating sections were rinsed with 0.05 M phosphate-buffered saline (PBS), pre-incubated with normal sheep serum (5%), and then incubated with a mouse monoclonal AVP-anti body (1:200, PS41; supplied by Dr. H. Gainer, Bethesda, MD) overnight at 4 °C. After rinsing, sections were exposed to biotinylated sheep anti-mouse Ig (Amersham Pharmacia Biotech, Calif.; 1:200) for 2 h at room temperature. Subsequently, the sections were rinsed again in PBS and incubated with Streptavidin-Horseradishperoxidase (Zymed, Calif.; 1:200) for 1 h at room temperature. The sections were thoroughly rinsed in PBS and Tris buffer (0.05 M, pH 7.4) and the protein-antibody complex was visualised with diaminobenzidine (30 mg in 100 ml Tris buffer, pH 7.4) as chromogen and 0.01% H_2O_2 for initiation of the staining reaction. Finally, sections were rinsed in PBS, mounted, air dried, and coverslipped for light microscopy and stereology. Stereological counting of all available SCN sections resulted in an estimation of total number of immunoreactive AVP (AVPir) neurons in the SCN for each individual (West 1993; Madeira et al. 1995; Jansen et al. 1998).

Data treatment

Activity and body temperature data were used for describing the spontaneous development of post-hibernation circadian rhythms under continuous dim light conditions. A measure for circadian rhythmicity, ΔQ_p-value (Gerkema et al. 1994), was calculated using the difference between the level of the dominant peak in a χ^2-periodogram analysis (Sokolove and Bushell 1978) and its accompanying significance level, using $\alpha = 0.001$ with Bonferroni correction (Sokal and Rohlf 1995). Spontaneous development of post-hibernation rhythmicity was assessed over time by a running periodogram analysis over 7 days. The step size was 1 day, and the calculated statistic describing the strength of rhythmicity in the circadian range was the peak ΔQ_p-values for periods between 20 h and 28 h. Standard periodogram analyses for periods between 20 h and 28 h were performed on 10-day intervals for correlation of circadian rhythmicity with the number of AVPir SCN cells. Correlations were tested using Pearson's correlation coefficient. The daily range of body temperature was calculated as the difference between maximal and minimal body temperature reached within a 24-h period.

Experiment 2: effects of light on post-hibernation circadian rhythmicity

Experimental conditions

Animals used in this experiment (six females, five males) were individuals other than those used in Experiment 1. From these 11 animals, 3 females and 4 males were implanted with body temperature loggers October 22 1998, after which they were placed in a climate-controlled room. Light conditions were changed from a 12-h:12-h LD cycle to continuous dim light (<0.1 lux) on November 26 1998. Ambient temperature was gradually lowered from 20.0 °C to 3.5 °C, in three steps of similar duration and temperature decrease, from November 16 1998 to November 22 1998. To preserve similarity with Experiment 1, ambient temperature was gradually raised from 3.5 °C to 25 °C, from March 22 1999 to March 29 1999, in four steps of similar duration and temperature increase. This terminated hibernation in those seven animals that had not done so before the temperature rise. This treatment induced greater similarity in the timing of the end of hibernation between animals and facilitated further experimentation. In this experiment we aimed to establish whether light would either stimulate or suppress the post-hibernation development of circadian rhythmicity. We first applied a single light pulse, and then an LD cycle.

Light pulse

Light pulses and sham light pulses were applied 8–17 days after the end of hibernation. Expression of circadian rhythmicity before application of the light pulse was matched between the experimental (exposed to a light pulse) and the control group (not exposed to a light pulse). Experimental and control animals were moved to another climate room and exposed, respectively, to a light pulse or a sham light pulse. The animals remained in their home cages with the entrance to the nest box blocked during the pulse. Light pulses were provided by two normal tube lights (1-h 1,700 lux at the bottom of the cage; Osram L40 W/20SA tube lights) at 50 cm above the cage floor. Instead of a light pulse, the control group was exposed to the normal dim red light conditions (<1 lux; 1 h).

LD cycle

The LD cycle (L:D = 12 h:12 h) was applied to all animals participating in Experiment 2 from April 20 1999 until April 29 1999. The LD cycle started 22–38 days after the end of hibernation for each individual animal. Light was provided by two normal tube lights (1,700 lux at the cage floor; Osram L40 W/20SA tube lights) at 50 cm above the cage floor. The animals had access to their nest boxes during the application of the LD cycle.

Data treatment

Daily body temperature range was calculated as in Experiment 1. Circadian rhythmicity in body temperature and activity was assessed by calculating ΔQ_p-values based on χ^2-periodogram analyses on 7 days data to increase the temporal resolution. Periods between 20 h and 28 h were tested using $\alpha = 0.001$ and Bonferroni correction (Sokal and Rohlf 1995). The effect of the light pulse application on the difference in body temperature range or rhythmicity in body temperature and activity, between 7 days before the light pulse and 7 days after the light pulse, was tested using the two sample *t*-test comparing the control group and the experimental group. Since all animals were exposed to the LD cycle, we tested the effect of the LD cycle on rhythmicity in activity and daily body temperature range by paired comparisons (paired *t*-test), testing the changes within the individual. Mann-Whithey *U*-test for group comparisons was used whenever the data did not allow for parametric testing (i.e. unequal variances).

Results

Development of circadian rhythmicity after hibernation

All ground squirrels used in this study showed 5–15 days of circadian arrhythmicity in locomotor activity and body temperature after the end of hibernation. After this phase of post-hibernation arrhythmicity, circadian rhythmicity spontaneously reappeared and gradually increased in amplitude and stability. Examples of this process are shown for animals from both experiments (Figs. 1, 2, 5, 7). The spontaneous development of post-hibernation circadian rhythmicity under continuous dim-light conditions is quantified for locomotor activity and body temperature as the increase of the ΔQ_p-value over time and for body temperature as the increase in daily range and ΔQ_p-value (Fig. 3). ΔQ_p in activity and body temperature rose until post-hibernation day 10–12, after which it fluctuated at high levels. Daily body temperature range increased from 1.8 °C at post-hiber-

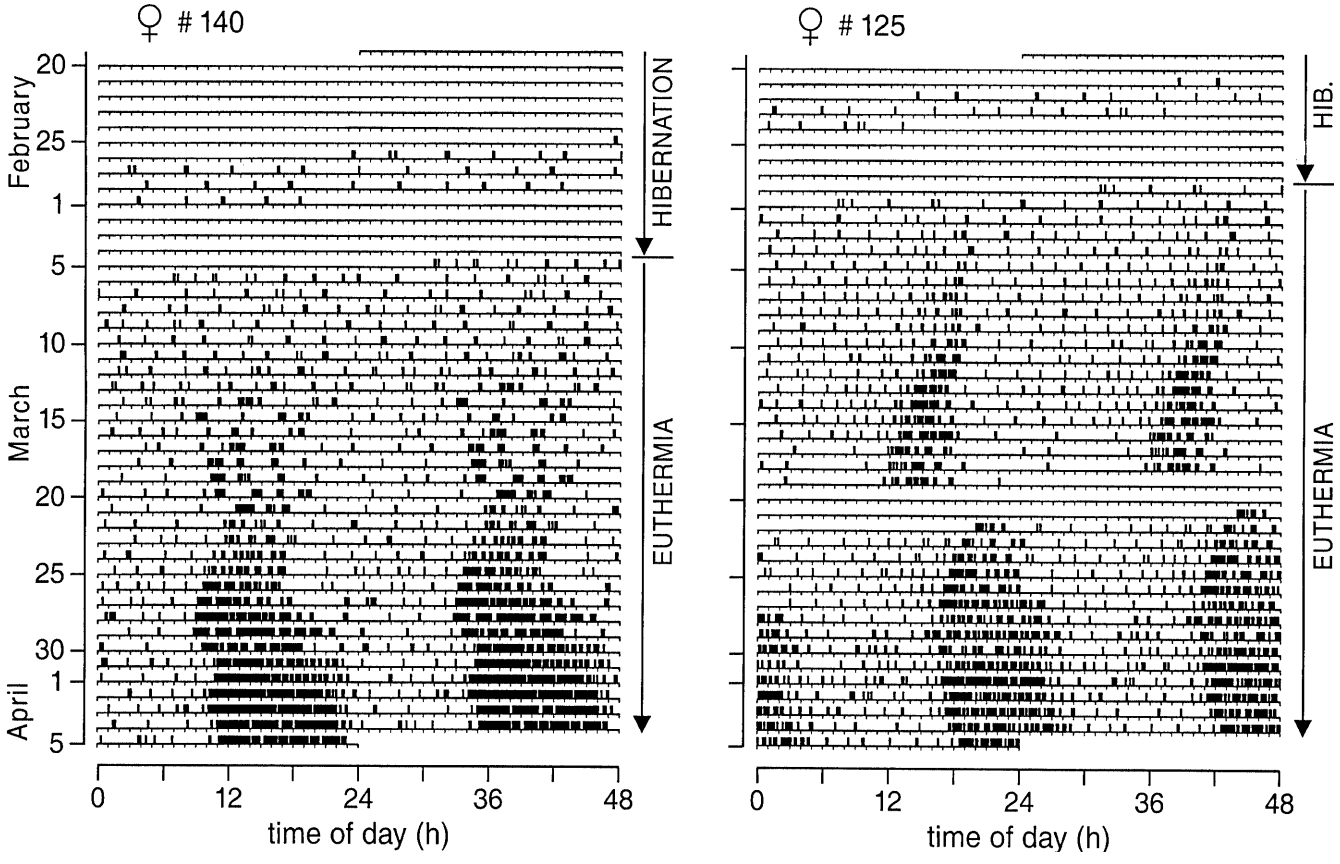


Fig. 1 Double plotted actogram indicating spontaneous re-occurrence of circadian rhythmicity in locomotor activity in two animals (**A** female no. 140, **B** female no. 125) after hibernation. Date is increasing from top to bottom, time of day (h) is indicated on the horizontal axes. One *horizontal line* in the plot indicates time for 2 consecutive days, activity is plotted as a *vertical mark* for each 2-min bin. Hibernation phase and post-hibernation euthermia are indicated at the *right hand side* of the plots. Torpor bouts are evident from the lack of activity during periods longer than a day. Note the phase shift, period change, and amplitude decrease in the circadian activity rhythm after a short torpor bout (March 19–22) during the post-hibernation euthermic phase in female no. 125. This may indicate effects of torpor and low body temperatures on the functioning of the circadian system

nation day 1, to about 3.8 °C after post-hibernation day 20. The data in Fig. 3 are shown as population averages, but, as the error bars indicate, there are considerable individual differences in the duration of post-hibernation arrhythmicity. As a result, a small but positive ΔQ_p-value may be due to a minority of the animals showing significant circadian rhythms. It is difficult to compare ΔQ_p-values based upon activity or body temperature data because of their different sampling intervals (2 min and 48 min, respectively) and different accuracy compared to the typical range of a cycle (range = ~0–50 counts, accuracy = 1 count; and range = ~2–3 °C, accuracy = 0.18 °C, respectively).

Experiment 1: post-hibernation circadian rhythms under continuous dim light and AVP staining in the SCN

We found substantial variation between animals in AVPir staining intensity in the SCN (Fig. 4). AVP has not been described in this species before. AVPir neurons were present in the dorsal and medial portions of the SCN throughout the rostro-caudal axis. Fibres and, notably, terminals were found in the same SCN region. In addition to AVP immunostaining in the SCN, the supraoptic nuclei (SON) were densely stained, and AVPir cells could also be found in the paraventricular nuclei of all the animals (Fig. 4A, E). This indicates that differences between the animals in SCN-AVP immunoreactivity are area specific. The two representative examples chosen for illustration of SCN-AVP staining had the third lowest and the third highest estimation for the number of AVPir SCN neurons (Fig. 4). Post-hibernation activity patterns (Fig. 5) of both animals are different for the 10 days before the animals were killed for brain analyses. The example of an animal with a weak circadian rhythm (Fig. 5; female 113, ΔQ_p on activity over final 240 h = 121) had a relatively small number of AVPir SCN neurons (257). The example of an animal with a strong circadian rhythm (Fig. 5, female 131, ΔQ_p on activity over final 240 h = 350) had a high number of AVPir SCN neurons (1075). The number of AVPir SCN neurons correlated positively with the time spent in post-hibernation euthermia (Fig. 6A; $r^2 = 0.44$, $P = 0.04$), indicating a gradual return of AVP production in SCN neurons after hibernation. The number of AVPir SCN neurons was also positively correlated with ΔQ_p assessed from the prior 240-h activity recording (Fig. 6B; $r^2 = 0.51$, $P = 0.02$) and body temperature recording (Fig. 6C; $r^2 = 0.54$, $P = 0.06$). The latter correlation is on the verge of significance, which may partly be due to the fact that body temperature was only recorded in seven of the ten animals analysed for AVP expression, while activity recordings were available for all ten animals. None of the parameters presented in

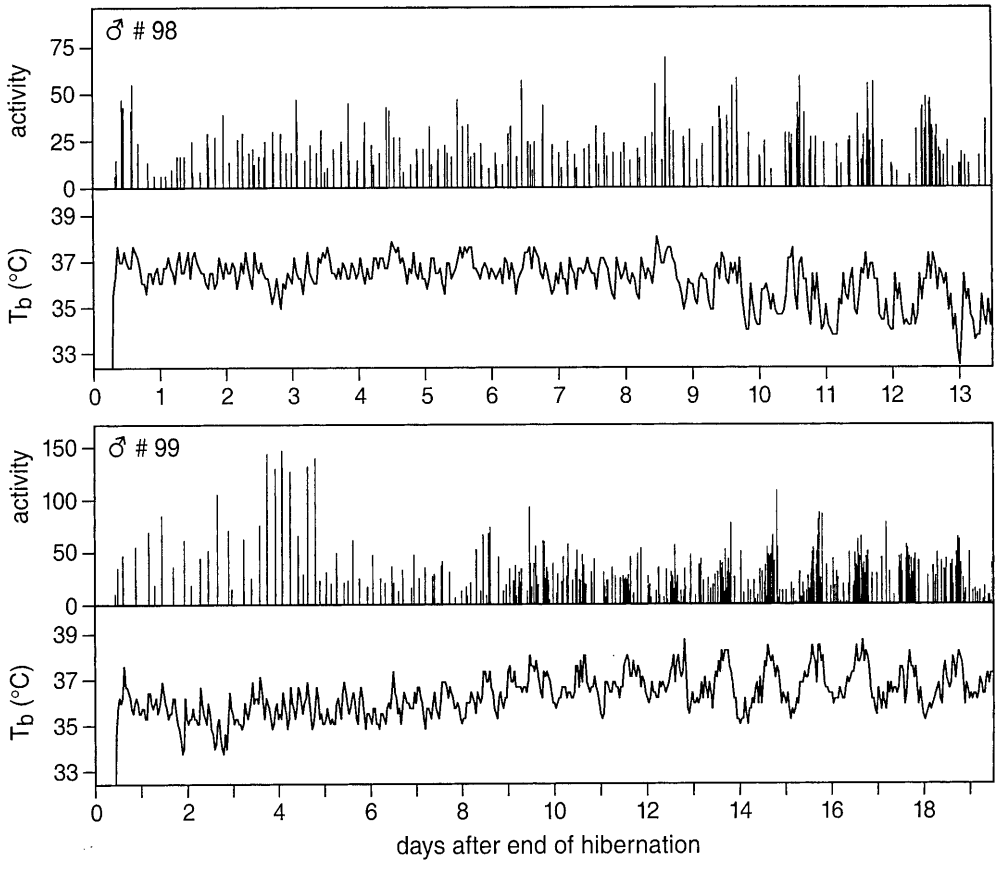


Fig. 2 Strip charts of body temperature (°C) and activity patterns (per 2-min bin) in two animals after hibernation (*top two panels*: male no. 98; *lower two panels* male no. 99). Time course (days after the end of hibernation) is indicated *horizontally*

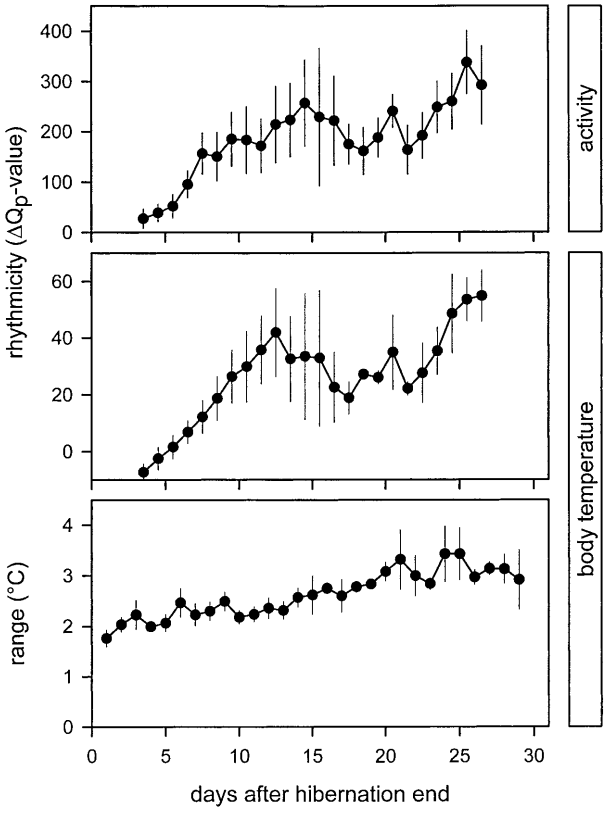


Fig. 3 Spontaneous development of circadian rhythmicity (ΔQ_p-value) in activity and body temperature patterns (*upper* and *middle panel*) and daily body temperature range against time (days after hibernation end). *Error bars* indicate SEM

Fig. 6 showed significant correlation with the optical density of the AVPir staining in the SON (days after hibernation: $P=0.26$; ΔQ_p activity: $P=0.71$; ΔQ_p body temperature: $P=0.35$), indicating that post-hibernation increase in neuronal AVP content is specific for the SCN.

Experiment 2: effects of light on post-hibernation circadian rhythmicity

Locomotor activity

Control animals showed a spontaneous, gradual increase in post-hibernation circadian rhythmicity (Fig. 3, 8), similar to that of untreated animals under continuous dim light (Fig. 1). Circadian rhythmicity in locomotor activity was strongly expressed during and after the application of an LD cycle, but there was little or no effect after the application of a single light pulse (Fig. 7, 8A). The effects of both the light pulse and the LD cycle were tested using ΔQ_p-values as an indicator of the expression of circadian rhythmicity in locomotor activity. Due to missing activity data, ΔQ_p could not be calculated in one animal after the light pulse and in two other animals after the LD cycle. On average, ΔQ_p-values were slightly increased following the light pulse. However, the increase in ΔQ_p was not significantly different from the control group (Fig. 8; increase in ΔQ_p: control = 116, experimental = 278; Mann-Whitney-*U* test $n = 10$, $P=0.11$). The difference was transient and there was no difference in rhythmicity between both groups during the 7 days before the application of the LD cycle (Fig. 8A; two sample *t*-test: $t=1.18$, $df=9$, $P=0.27$). ΔQ_p within the individual increased during the LD cycle when compared to the ΔQ_p before the LD cycle (Fig. 8A; paired *t*-test: $t=4.71$, $df=10$, $P=0.0008$). ΔQ_p remained enhanced after application of the LD cycle when compared to ΔQ_p before the LD cycle (Fig. 8A; paired *t*-test: $t=3.68$, $df=8$, $P=0.006$).

Body temperature

During the first period after hibernation there was no significant rhythmicity in body temperature for periods in the circadian range between 20 h and 28 h (Fig. 8B). Unlike rhythmicity in activity, rhythmicity in body temperature was seemingly enhanced after the light pulse application, but this effect was not significant (Fig. 8B; increase in ΔQ_p: control = 36, experimental = 97; Mann-Whitney-*U* test: $n=6$, $P=0.38$). Within the individual there was no increase in rhythmicity when ΔQ_p before the LD cycle is compared with ΔQ_p during the LD cycle (Fig. 8b; paired *t*-test: $t=0.6$, $df=6$, $P=0.57$). Also the rhythmicity in body temperature after the LD cycle was not enhanced when compared to before the LD cycle (Fig. 8b; paired *t*-test: $t=2.1$, $df=6$, $P=0.11$).

The application of a 1-h light pulse did not increase the body temperature range (Fig. 8C; increase in range: experimental group = 0.16 °C, control group = 0.27 °C; two sample *t*-test: $t=0.99$, $df=5$, $P=0.38$). Daily body temperature range data from animals that were exposed to a light pulse and animals that were not exposed to a light pulse were pooled in order to analyse the effect of the LD cycle to which all animals were exposed. During the LD cycle the range of body temperature was higher than before (Fig. 8; paired *t*-test: $t=3.76$, $df=5$, $P=0.01$). After releasing the animals in continuous dim red light conditions, daily body temperature range was still significantly enhanced when compared to the temperature range before the LD cycle was applied (Fig. 8; paired *t*-test: $t=3.48$, $df=5$, $P=0.02$).

Discussion

European ground squirrels show reduction or absence of circadian rhythmicity in body temperature for several days after hibernation under natural conditions (Hut et al. 2001). Here we demonstrate that ground squirrels showed an even longer period of circadian arrhythmicity in activity and body temperature (Figs. 1, 2, 3) under

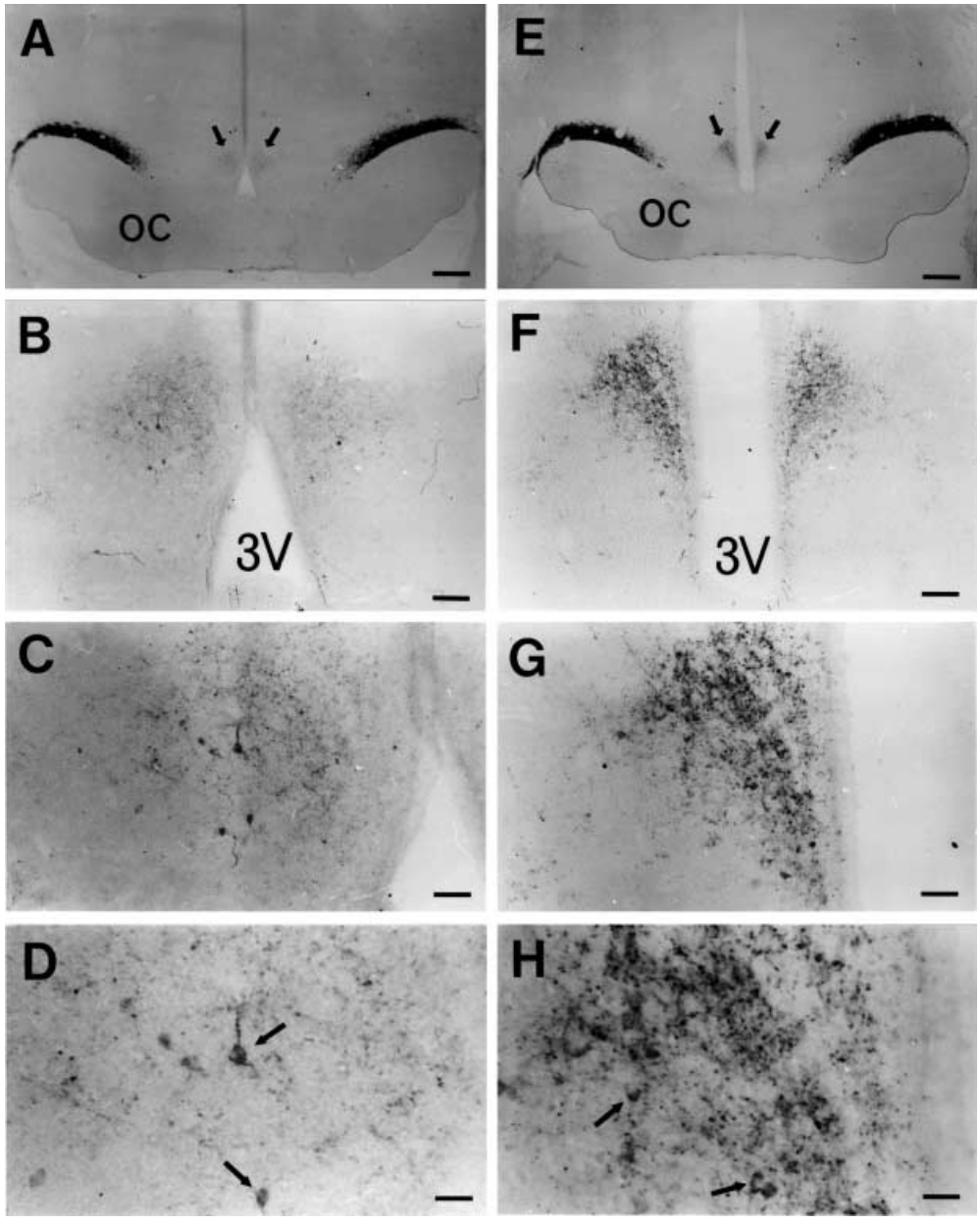


Fig. 4A–H Photomicrographs of AVP-immunoreactivity in the SCN of an arrhythmic (left panels male no. 113) and rhythmic (right panels male no. 131) ground squirrel. An overview of the ventral part of the hypothalamus is shown in **A** and **E**. Dense arginine vasopressin (AVP)-immunostaining is seen in the supraoptic nucleus on top of the optic chiasm (*OC*), and relatively weak labelling in the suprachiasmatic nuclei SCN (indicated with *arrows*). The SCN is shown with increasing magnification in **B**–**D** and **F**–**H**. Note the difference in AVP-positive terminals and varicosities in the SCN of both animals, while no such difference is seen in the supraoptic nucleus, or the AVP-immunoreactive (AVPir) fibres running along the wall of the third ventricle (**B**, **F**). *Arrows* in **D** and **H** point at AVP-positive cell bodies. (*3V* third ventricle). Scale bars in **A**, **E** = 400 μm; in **B**, **C** = 80 μm; in **C**, **G** = 40 μm; in **D**, **H** = 20 μm. Animals correspond to the example actograms shown in Fig. 5. Stereological estimations of total number of AVPir neurons in the SCN: female no. 113: 257; female no. 131: 1075

continuous dim light conditions in the laboratory. This indicates that post-hibernation arrhythmicity is an intrinsic property of the circadian system and not the result of transient days during re-entrainment to the LD cycle after hibernation under natural conditions. These results are consistent with the hypothesis that the circadian system has partly discontinued its function during hibernation and requires several days of post-hibernation euthermia to resume its oscillation.

Correlations with numbers of AVPir neurons in the SCN

We found a dense AVP immunocytochemical staining in the dorso-medial part of the SCN in euthermic European ground squirrels. Similar patterns in global AVP immunoreactivity within the SCN were also found in euthermic Richardson's ground squirrels (Reuss et al. 1989; Schindler and Nürnberger 1990), thirteen-lined ground squirrels (Reuss et al. 1989), and golden-mantled ground squirrels (Smale et al. 1991).

Output signals from the SCN are necessary for conveying timing signals to other brain regions and to regulate circadian organisation of behaviour and physiology. AVP is considered to be a major output system of the SCN targeting other brain regions, synaptically and non-synaptically. In addition, Ingram et al. (1996) concluded that AVP may amplify the circadian rhythm of SCN neurons via the V1 receptor pathway. Correlations of AVP with expression of circadian rhythms have been found most clearly in spontaneously arrhythmic common voles. In the vole, the content and release of AVP correlated strongly with the state of

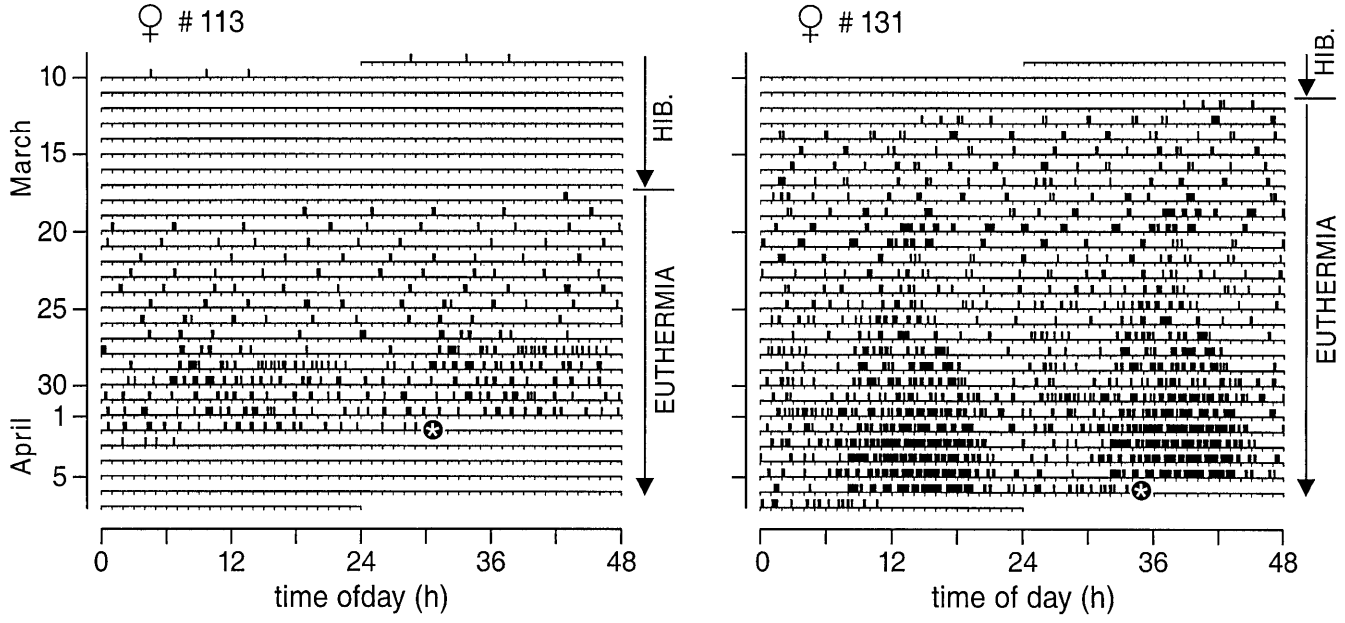


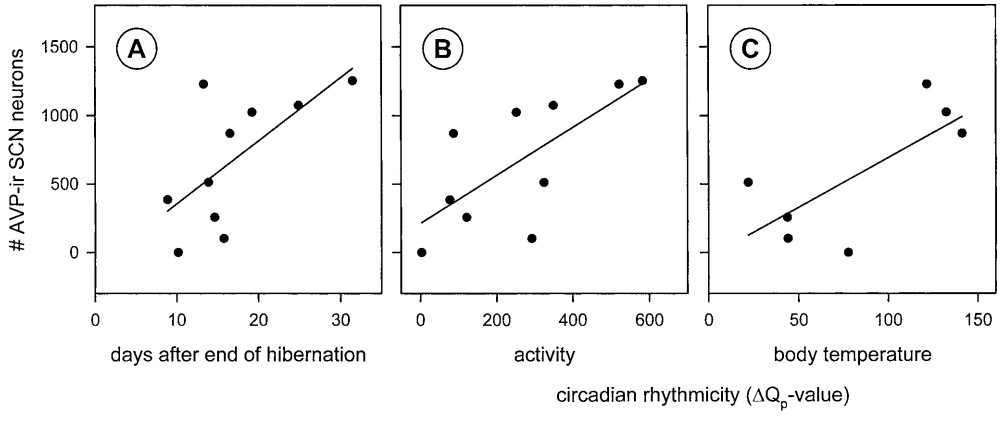
Fig. 5 Actograms of two animals used for the analyses of AVPir in the SCN during development of circadian rhythmicity after hibernation. A relative arrhythmic animal (female no. 113; ΔQ_p over final 240 h = 121) and a rhythmic animal (female no. 131; ΔQ_p over final 240 h = 350). An *asterisk* indicates when the animal was taken from its cage for a sodium-pentobarbital injection and brain perfusion. See Fig. 4 for AVP staining in the SCN and the estimated number of AVPir SCN neurons of these two animals

rhythmicity of locomotor behaviour (Gerkema et al. 1994; Jansen et al. 1999; Jansen et al. 2000). Furthermore, precision and presence of circadian rhythmicity in locomotor activity declined with increasing age in voles, which correlates with a severe loss of AVP in the SCN of old voles (Van der Zee et al. 1999). A role of AVP in controlling aspects of circadian organisation of behaviour has also been reported in other species. In rats and house mice, numbers of AVP-positive cells in the SCN correlate with a variety of parameters of circadian organisation of locomotor activity (Bult et al. 1993; Wollnik and Bihler 1996). Moreover, AVP injections induced a higher amplitude of the circadian sleep-wake rhythm in rats, whereas AVP antagonists reduced this amplitude (Kruisbrink et al. 1987; Arnauld et al. 1989). We found that AVP content in the SCN of ground squirrels around ct 4 gradually increases over several days following hibernation and that this increase was associated with the spontaneous recovery of behavioural rhythmicity (Fig. 6). This adds another correlation to the evidence for a role of AVP in the link between pacemaker and overt rhythm. It also supports our interpretation that post-hibernation arrhythmicity is attributed to dysfunction of the circadian system at the level of the SCN.

The SCN during hibernation

Post-hibernation arrhythmicity may be a reflection of the effects of prolonged periods of extreme low body temperatures on the physiology of the SCN. Although

Fig. 6 Total number of AVP-ir neurons in the SCN against the time interval between the end of the last torpor bout and the perfusion of the animals (**A** Pearson's correlation coefficient: $r = 0.66$; $P = 0.037$). The total number of AVPir neurons in the SCN plotted against circadian rhythmicity (ΔQ_p-value) in locomotor activity (**B** Pearson's correlation coefficient: $r = 0.71$; $P = 0.021$) and body temperature (**C** Pearson's correlation coefficient: $r = 0.73$; $P = 0.06$), calculated over 240 h before the animals were dissected



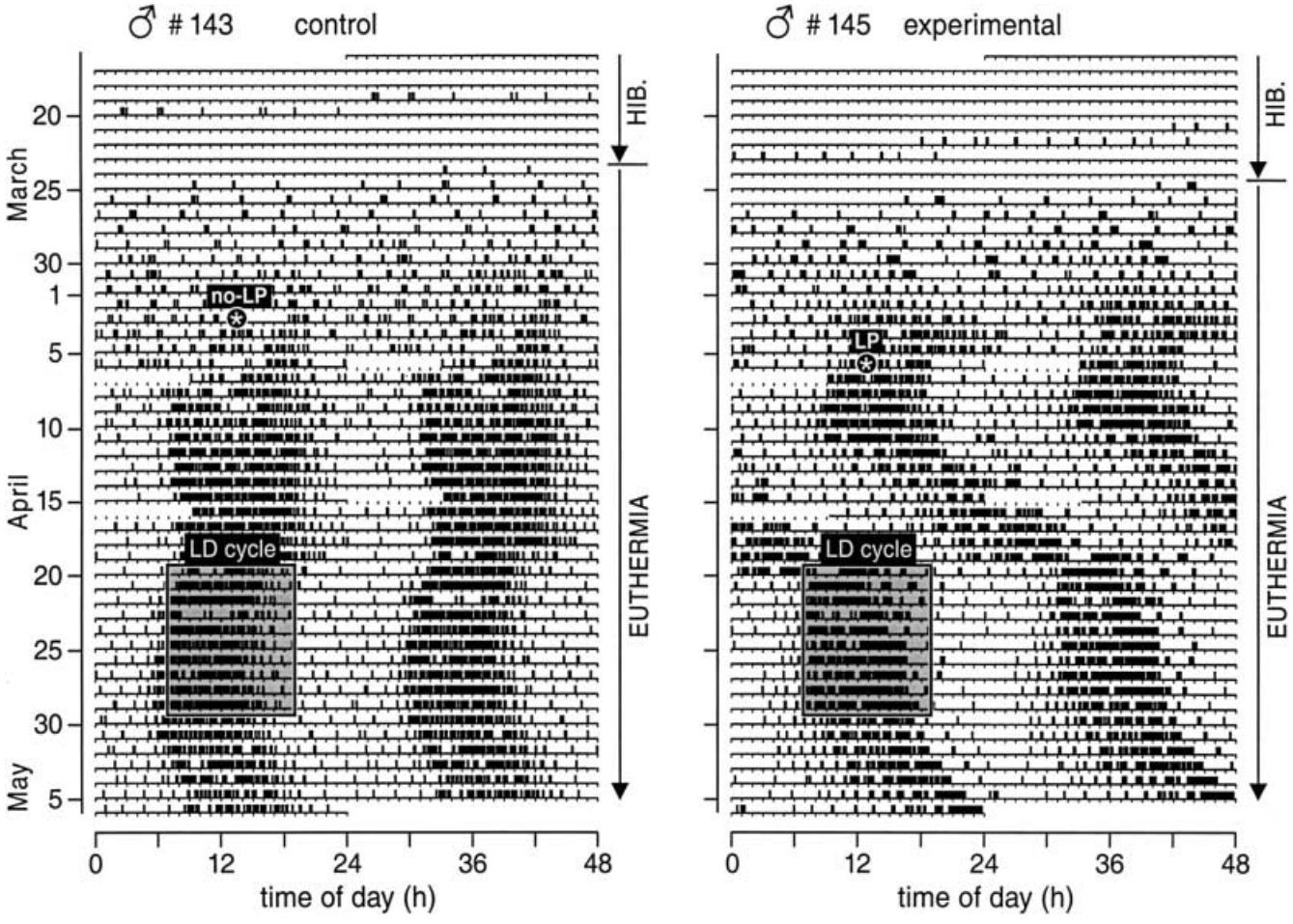


Fig. 7 Activity data plotted in standard actogram format (see Fig. 1) from two example animals in the second experiment [*left panel* male no. 143 (control); *right panel* male no. 145 (experimental)]. On the *left side* of each actogram the experimental treatments are indicated. An *asterisk* indicates the timing of the light pulse (experimental group) or sham light pulse (control group). The *shaded square* indicates the timing of the light phase during the LD cycle (applied in all animals)

some studies have shown a circadian influence on the timing of arousal episodes (Daan 1973a; Grahn et al. 1994) or body temperature during torpor (Menaker 1959, 1961; Grahn et al. 1994), the data presented here and in some other studies are in line with the hypothesis that the circadian system damps out after several days at low body temperatures (Hut et al. 2001; Oklejewicz et al. 2001; for review see Körtner and Geiser 2000). This discrepancy may be partly explained by the relatively high body temperatures at which circadian effects during hibernation were measured: > 8–10 °C (Menaker 1959), > 3–10 °C (Menaker 1961), > 12 °C (Daan 1937a) and > 10 °C (Grahn et al. 1994). In brain slices of golden-mantled ground squirrels, no electrical activity of SCN neurons was detected below 16.6 °C (Miller et al. 1994) and a cessation of spontaneous firing of posterior thalamic neurons was found in torpid golden-mantled ground squirrels with a body temperature below 15–18 °C (Krilowicz et al. 1988, 1989). These studies indicate a possible loss of communication between SCN neurons at low body temperatures, which would weaken the coupling strength between different oscillating neurons within the SCN. As a result, the oscillating neurons within the SCN may gradually become desynchronised.

Quantification of labelled deoxyglucose uptake (R2DGU) in torpid golden-mantled ground squirrels revealed elevated uptake levels in the SCN in comparison with other brain areas, suggesting a metabolic active SCN (Kilduff et al. 1989). This is only seemingly in contrast with reduced electrical activity at low temperatures and a possibly internally desynchronised SCN during torpor. Elevated R2DGU may be the result of temperature-compensated molecular processes underlying the circadian clockwork, but the long incubation times (24 h) used in this study do not allow for any assessment of phase or degree of synchronisation between neurons and hence provides no evidence that the SCN is functioning as a circadian pacemaker during torpor. In this context it is important to note that the basis for circadian oscillations in mammalian SCN neurons is an intra-cellular molecular loop (King and Takahashi 2000) which is not affected by loss of synaptic signalling between SCN neurons (Schwartz et al. 1987; Earnest et al. 1991; Schwartz 1991; Shibata and Moore

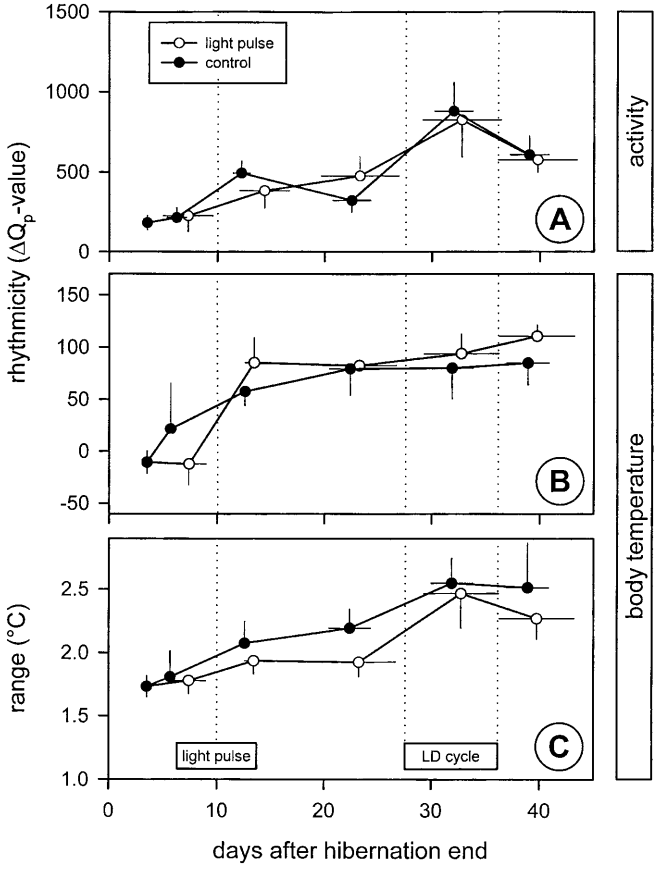


Fig. 8A–C Development of circadian rhythmicity after hibernation for animals in Experiment 2, treated with a light pulse (*open circles*, $n=6$ for activity data, $n=4$ for temperature data) or sham light pulse (*closed circles*, $n=5$ for activity data, $n=3$ for temperature data). Average timing of the light pulse and the LD cycle, to which all animals were exposed, are indicated with a *dotted line*. Panels show development of: **A** circadian rhythmicity in activity patterns (ΔQ_p), **B** circadian rhythmicity in body temperature (°C) and **C** circadian body temperature range (°C). Measures are calculated over six intervals of 7 days. *Error bars* indicate SEM

1993). However, when intercellular synchronisation between these molecular loops deteriorates, the output signal of the SCN as the main circadian pacemaker may gradually decrease and eventually become arrhythmic depending on body temperature during torpor and the duration of the torpor bout.

Alternatively, only the output mechanism of the circadian pacemaker may be affected, while the molecular loop and the intercellular synchronisation within the pacemaker (SCN) may still be intact. Obviously studies on the cellular level of the SCN in hibernation are needed to give more insight in this problem, but it may be appropriate to indicate here that the same signals that serve as output may also serve to synchronise cells within the SCN. In such a case, the distinction between internal pacemaker signals and output signals may become artificial.

Indicative for torpor-induced changes on the level of the pacemaker is the change in phase, period and amplitude of the circadian activity rhythm observed in one individual after a 3-day torpor bout during post-hibernation euthermia (Fig. 1B). The phase shift here can be explained by an increased free-running circadian period of about 27 h during these few days of torpor (Hut et al. 2001), but changes in amplitude and tau after this torpor bout may be caused by the lability of the circadian pacemaker during torpor. Also, the highly variable circadian periods observed in marginal temperature oscillations in ground squirrels during torpor (Grahn et al. 1994) are suggestive of labile circadian pacemakers. Synchronisation of SCN cells may not be completely restored during the short arousal episodes during hibernation, causing the circadian system to be fully desynchronised at the end of hibernation and resulting in post-hibernation arrhythmicity.

We hypothesise an important role for AVP in (re-establishing) synchronisation of individual SCN neurons. SCN neurons are richly endowed with AVP receptors (V1a receptors; Young et al. 1993). AVP neurons form soma-somatic appositions and have extensive synaptic relationships with other (AVP) neurons in both ipsilateral and contralateral SCN, indicating a mechanism for strong intercellular coupling (Van den Pol and Gorcs 1986; Castel et al. 1990). AVP-induced excitation within the SCN in combination with this elaborated intrinsic SCN connectivity has been postulated to determine the power of circadian rhythms (Ingram et al. 1996). Therefore, the increase of AVP immunoreactivity in the SCN during the first 2 weeks after the end of hibernation suggests a gradual increase of AVP activity, resulting in an increase in the amplitude of the AVP output signal as well as enhancement of synchronisation between SCN cells. The power of SCN output systems may therefore be inextricably related to synchronisation of individual SCN pacemaker neurons. Overall, the effect of increased AVP immunoreactivity in the SCN is indicative of a gradual enhancement in stability and amplitude of this major output system of the circadian system.

Response to light

Besides a spontaneous increase in circadian rhythmicity, the rhythmicity of the activity and body temperature was not additionally enhanced by a 1-h light pulse of 1,700 lux (Fig. 8A, B). The data suggest a response to the light pulse in body temperature rhythmicity (Fig. 8B), but this increase was not significant. However, when interpreting these results, it should be considered that the low number of animals used for the body temperature measurements reduced the power of statistical testing. Both during and after the LD cycle, circadian rhythmicity of activity and daily body temperature range were significantly enhanced. This indicates that circadian rhythmicity can be enhanced by light during post-hibernation arrhythmicity, but only after the application of an LD cycle or alternatively after stimulus durations of longer than 1 h of similar light intensity.

This may translate to the natural situation where circadian rhythmicity is expressed only after the ground squirrels have perceived several LD cycles during post-hibernation euthermia. Indeed, we found a gradual increase in daily body temperature range only a few days after emergence from hibernation (Hut et al. 2001).

European ground squirrels spend about 7 months in complete darkness during hibernation (Ružić 1978). It is likely that the animals have no information on the time of day at the end of hibernation and their first emergence in spring may theoretically occur at any time of day. Indeed, strictly nocturnal bats have been observed to fly from their hibernacula in full daylight just after hibernation (Daan 1973b). The European ground squirrel is adapted to be active only during daytime (Hut et al. 1999, 2000), thus it would be beneficial to synchronise its activity phase as quickly as possible with the light phase of the day. A lower amplitude of the circadian pacemaker will generally imply higher responsiveness towards the entraining zeitgeber stimulus (Hau and Gwinner 1994; Gwinner 1996). Thus, fewer transient days will be needed for re-entrainment when the amplitude of the oscillator is reduced.

In short, we suggest that the lack of AVP expression in SCN neurons following hibernation is indicative, at least partly, of a dysfunctional or desynchronised circadian pacemaker. This suppressed functioning of the circadian system seems to be associated with hibernation and may be induced by low brain temperatures during torpor bouts in hibernation. Synchronisation of individual pacemaking neurons within the SCN and its AVP output system may be restored slowly over the course of 5–15 days of post-hibernation euthermia. In turn, this would result in a low amplitude circadian oscillator facilitating re-entrainment in the first days following hibernation. From this perspective, post-hibernation arrhythmicity might be viewed as an adaptive strategy to reduce the time needed for synchronising activity to the preferred phase of the day.

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