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Research Report

Expression of circadian rhythmicity correlates with the number of arginine-vasopressin-immunoreactive cells in the suprachiasmatic nucleus of common voles, *Microtus arvalis*

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Abstract

We have studied the number of arginine-vasopressin (AVP)-immunoreactive (IR) cells in the suprachiasmatic nuclei (SCN) in common voles, *Microtus arvalis*, with a strong ($n = 18$), weak ($n = 10$) or absent ($n = 9$) expression of circadian rhythmicity. Spontaneous expression of rhythmicity was assessed from records of wheel running activity and general activity, measured with passive infra-red detection during 4 weeks of continuous low light (LL) conditions. Subsequently, 20 voles were perfused in LL. After additional exposure to a 12:12 h light–dark (LD) cycle during 14 days, the other 17 voles were perfused in the early morning. AVP-positive neurons were visualized immunocytochemically with a polyclonal IgG antibody. AVP-stained cell somata were present predominantly in the dorsomedial SCN, whereas terminals, contacting both AVP positive and negative neurons, were distributed throughout the SCN. In LL conditions, AVP-staining intensity of neurons was low in all 20 voles. Also the number of AVP-IR cells in the SCN in brain sections obtained in LL conditions was low and not different for the three categories of rhythmicity. In LD conditions, the staining intensity of AVP positive SCN neurons was low in rhythmic animals, high in non-rhythmic animals not expressing, and intermediate in animals weakly expressing circadian rhythmicity. The number of AVP positive cells in LD conditions showed a negative correlation with the propensity of animals to lose circadian rhythmicity under LL conditions. Differences in AVP-IR were found solely in the dorsomedial SCN, and neither in the rostral and caudal aspects of the SCN nor in the area adjacent to the SCN. These results suggest that the AVP-neurons of the SCN form part of a discrete neuronal substrate which functionally correlates with the expression of circadian rhythmicity.

Key words: Suprachiasmatic nucleus; Circadian rhythm; Vasopressin; Arginine-vasopressin; Immunoreactivity; Vole

1. Introduction

In the past decade, the circadian clock function of the suprachiasmatic nuclei (SCN) in the hypothalamus of mammals has become established [16]. Originally, afferent pathways from the retina have guided to the SCN as a clock and it is now largely clarified how environmental light conditions entrain the circadian pacemaker system [17,18]. The efferent connections of the pacemaker have been described neuroanatomically [1,26,33,34,35] and these should also include the efferent pathways to the observed, overt circadian patterns in physiology and behaviour. We are, however, far

from understanding how and what kind of a circadian signal arrives at what effector targets.

One of the major peptidergic gene products of the SCN is the neuromodulator arginine-vasopressin (AVP). AVP shows circadian rhythmicity, in the AVP content of SCN-tissue [23,28], and in AVP release from the SCN into the CSF [22]. In vitro, a circadian release into perfusate medium was produced by SCN explants [7], SCN-slices [13], and by cultured SCN-neurons [19]. Thus it is shown that AVP release increases at the beginning of the (subjective) day, in a light–dark (LD) cycle and in constant darkness (DD), whereas in constant bright light the amplitude of the AVP rhythm decreases [22].

Immunocytochemically, AVP producing cells in the SCN have been identified [27,31], mainly in the dorsomedial aspect of the SCN. The number and distribu-

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tion of AVP-immunoreactive (AVP-IR) cells varies between species [24]. Many of the AVP-IR cells project to cells within the SCN [4,5,29,32,33]. AVP-containing fibres leaving the SCN have terminals in other hypothalamic regions [15,32,33], especially in the paraventricular area of the hypothalamus [33]. Application of tetrodotoxin affects the AVP production in SCN explants, but not the clock mechanism of the SCN [8]. In all, the role of AVP-IR cells in the expression of circadian rhythmicity is not known. The evidence mentioned above seems, however, not incompatible with a part of AVP in the efferent system [2,32].

The herbivore microtine rodents of the new and old world are characterized by a rigid ultradian (2–4 hour) rhythm in feeding behaviour [9,10]. By contrast, the circadian component is less dominant in the temporal organisation of vole behaviour, compared with murine rodents [11,12]. In the Eurasian common vole, *Microtus arvalis*, circadian rhythmicity shows a large interindividual variability in its stability. The expression of circadian rhythmicity even disappears spontaneously in continuous darkness (DD) and low light (LL) conditions in a quarter of the individuals [10,12]. Preliminary data revealed a considerable variation in AVP-staining-intensity and number of AVP-positive neurons in the SCN of common voles housed in a 12:12 h LD cycle and perfused in the early morning. Such interindividual variability in both the expression of circadian rhythmicity and the AVP system of the SCN raises the question of a possible role of AVP and its neuronal substrate, and could facilitate a functional evaluation of the efferent side of the circadian system. For this reason we assessed the number of AVP-IR cells in the SCN in intact common voles with different degrees of spontaneous expression of circadian rhythmicity. In the initial experiment, we kept the animals in continuous low light (LL) intensity. In a second experiment, we exposed animals to an additional LD cycle, that followed the LL treatment.

2. Materials and methods

2.1. Animals and maintenance

Thirty-seven adult male laboratory-reared common voles (*Microtus arvalis*) were used (initial body weight 15–30 g, minimum age 4 months). The vole colony maintained in Haren was based on individuals trapped in the Lauwersmeer (53°20'N;6°16'E). Animals were housed in lucite cages (25×25×30 cm) equipped with a running wheel (diameter 20 cm) and connected with a lucite nest box (17×11×13 cm) covered with a plywood top and provided with wood shavings. Ten cages were installed in a climate room; cages were neither visually nor acoustically separated from each other. The animals were kept at constant temperature (19°±1°C) and humidity (70%±5%). Food (Hopefarms mouse pellets) and tap water were available in the running wheel cage ad libitum. In addition, the animals received some endive, carrot and apple at irregular, about weekly intervals.

2.2. Experimental procedure

LL experiment

Two groups of 10 animals were consecutively kept in a 12:12 h light/dark (LD) cycle (lights on 7:00 h) during two weeks. Following this LD regime the voles stayed during 4 weeks in constant low light conditions (LL; 2±1 lux) after which the animals were killed for immunocytochemistry. Voles were taken out of the recording cages between 9:00 h and 12:00 h, just before perfusion and the phase of the freerunning rhythm at which the perfusion took place was assessed in the rhythmic animals. In the animals in which circadian rhythmicity had disappeared this phase was estimated, as far as possible, by extrapolation from parts of the initial record where circadian rhythmicity was still detectable.

LD experiment

Two groups of 9 and 8 individuals were consecutively exposed to light conditions as in the LL experiment; additionally, they were subjected to a second LD treatment (12:12 h; lights on 7:00 h) for two weeks. Voles were taken out of the recording cages between 9:00 h and 12:00 h, just before the perfusion.

2.3. Activity recording and analysis

Two types of activity were recorded: (a) revolutions of the running-wheel via a microswitch and (b) general locomotor activity by passive infra-red (PIR) sensors (ELRO SA-209). These activities were recorded on separate channels of an Esterline-Angus event recorder (chart speed 2.34 cm/h). The number of these behavioral events per 2 min interval were recorded separately on a PDP-based data-logging device (Kraüssling systems).

The presence of circadian rhythmicity in individual animals was assessed by visual inspection of the actograms of total activity and wheel running, using criteria from Gerkema et al. [12]. These actograms were constructed by plotting the wheel running and general locomotor activity data, on a daily basis with successive days beneath each other. Chi-square (χ^2) periodogram analysis [25] was applied to the records obtained in the last ten days of LL conditions. Significance of peak values of rhythmicity index Q_p ($P < 0.05$) above the interpolated level of ultradian multiple peaks in the range of 22–26 h [12] was used as an indication of circadian rhythmicity.

A quantitative estimation of the degree of rhythmicity was achieved by calculating ΔQ_p , the difference between the maximal Q_p obtained for the record of an animal in the circadian range (22–26 h) and the $Q_{p=0.05}$ value of the period concerned. The parameter ΔQ_p thus indicates the degree of expression of the circadian periodicity, independent of the actual length of the circadian period, and allows evaluation of the interindividual variability of period length.

2.4. Immunocytochemistry

The animals received 0.02 ml/g body weight 0.2% sodium pentobarbital i.p. and were transcardially perfused with 0.9% saline containing 3.5% heparin 500 IU/ml for 1 min, followed by 150 ml phosphate-buffered fixative (consisting of 3–4% paraformaldehyde + 0.05% glutaraldehyde, pH 7.4) at a perfusion rate of 10 ml/min. The brains were removed and cryo-protected overnight in 30% buffered sucrose at 4°C. The brain was cut at the level of the hypothalamus into 20 μ m frontal sections on a cryostat. For immunocytochemistry, the free floating brain sections were rinsed in phosphate-buffered saline (pH 7.4), pre-incubated with normal goat serum (5%) and incubated with primary polyclonal IgG antibody rabbit anti-AVP (Truus '86; 1:1000) [3,6] overnight at 4°C. Subsequently, the sections were thoroughly rinsed again, preincubated with normal goat serum

(5%), and incubated with goat anti-rabbit IgG (Zymed; 1:200) for 2 h at room temperature. After rinsing, the sections were exposed to rabbit peroxidase antiperoxidase (PAP; Dakopatts; 1:500) for 2 h at room temperature. Triton X-100 (0.5%) was added during all incubation steps. Finally, the sections were processed with diaminobenzidine. We quantified AVP-positive cells in the SCN by counting light microscopically the number of AVP-IR neurons in every second, frontal section containing the SCN. Like Bult et al. [3], we discerned six regions of the SCN. The counts in the sections were assigned to these six anatomically distinguished regions, equally distributed along the rostro-caudal axis within the SCN. For each animal and for each SCN-region a score was obtained, based on one or the mean of two to three sections.

3. Results

All 37 voles used in the experiments showed synchronised circadian activity rhythms during the LD 12:12 regimes. In contrast, the activity of the voles differed with respect to the occurrence of circadian patterns in LL. Among those animals that showed a free running circadian activity the period length of the rhythm varied from 23 to 25 h. Fig. 1 shows typical examples of differences in the expression of circadian rhythmicity in the common vole kept in LL. Visual inspection of such actograms of wheel running activity by three independent investigators resulted in uniform judgement of the presence or absence of a circadian rhythm. Three categories were distinguished: voles that showed strong, weak or no circadian rhythms (Fig. 1). Basically, wheel running activity and general locomotor activity as measured with PIR sensors resulted in similar temporal patterns. However, the wheel running activity was mainly restricted to the (subjective) night, and thus formed the better indicator of circadian modulations, compared with general activity that contained also rather extended ultradian patterns in (subjective) daytime.

The visual assignment of individual records to three categories according to the degree of expression of circadian rhythmicity was confirmed by the Chi-square periodogram analysis. Of the 20 animals kept in LL conditions, 12 showed clear circadian rhythmicity, five had weakly expressed circadian rhythmicity, and three showed no circadian rhythmicity. The ΔQ_p value differed significantly between animals with strong circadian rhythmicity and those with weak ($P < 0.0005$; Mann-Whitney one-tailed test) or absent ($P < 0.01$) expression of circadian rhythmicity. ΔQ_p did not differ significantly between animals with weak and absent circadian rhythmicity, but this comparison is affected by the low number of the latter category (Table 1). In the LD experiment we aimed at equal numbers of animals in each of these categories. After recording 17 animals, it appeared that we had obtained these three groups with strong ($n = 6$), weak ($n = 5$) or absent ($n = 6$) rhythmicity, following visual criteria described

Fig. 1. Double-plotted actograms of wheel running activity in three common voles, kept in continuous low light conditions. A shows a clear free running of the circadian rhythm with a period shorter than 24 h. In B, the circadian rhythmicity fades away gradually, although a weak circadian period shorter than 24 h is still distinguishable. C shows a complete loss of circadian rhythmicity. Panels show the last 20 days of activity in constant conditions. A, B and C represent the categories of strong, weak and no expression of circadian rhythmicity, respectively.

by Gerkema et al. [12]. The ΔQ_p values of the three groups differed here according to the visual judgements (Table 1). There was no difference in the strength of expression of circadian rhythmicity in the two groups of voles visually scored as rhythmic in the LL and LD experiment with respect to their ΔQ_p values (Table 1).

In the LL experiment the phase of perfusion did not affect the number of AVP-IR cells for a range of circadian times (CT = 12 is onset of activity) from CT = 1 to CT = 19. Similarly, in the LD experiment

the number of AVP-IR cells did not vary systematically over the small range of CT of perfusion (4–8 h).

AVP-immunoreactivity in the SCN of voles was present in the cell somata and associated proximal dendrites of small, densely packed neurons (longest diameter ranging from 8 to 12 μm) predominantly localized in the dorsomedial portion (Figs. 2 and 3). Only rarely were some AVP-positive somata encountered in the ventrolateral subdivision of the SCN (see double arrows in Fig. 3C). Numerous immunopositive terminals were distributed throughout the SCN, albeit more frequently in the upper part of the SCN (Fig. 3). High-power light-microscopical inspection revealed that AVP-positive terminals were frequently contacting both AVP-positive and AVP-negative SCN neurons. In the area adjacent to the SCN, large (longest diameter ranging from 20 to 30 μm), multipolar AVP-labelled neurons (arrows in Fig. 3) and fibres were scattered throughout the region. The AVP-stained fibres most likely arise from AVP-positive neurons of the SCN.

The density of AVP-immunostaining differed between LL and LD conditions. Under LL, no clear differences were found between voles with strong or absent expression of circadian rhythmicity (Fig. 2A,B). Only few faintly stained AVP-positive cells and terminals were present in the SCN. Under LD conditions clear differences in AVP-staining intensity became apparent between voles with and without expression of circadian rhythmicity (Fig. 2C,D). This difference in staining-intensity was present throughout the entire antero-posterior axis of the SCN (Fig. 3). In contrast,

no differences in staining-intensity between both categories of voles were observed in AVP-positive neurons in the area adjacent to the SCN (see arrows in Fig. 3). The group of voles characterised by weak circadian rhythmicity showed intermediate levels in AVP-staining intensity (data not shown).

The number of AVP-positive neurons in the three categories of voles were quantified at six levels of the SCN. In all voles, AVP-IR neurons were most abundant in the medial part of the SCN (Table 1, Fig. 4). Comparison of all voles from the LL experiment (groups A, B and C) revealed no significant difference in number of AVP-positive neurons (Table 1). Voles with strong expression of circadian rhythmicity (group D) in the LD experiment did not differ in the number of AVP-IR neurons from all voles from the LL experiment (Table 1). Between the three categories of voles from the LD experiment (groups D, E and F) significant differences in the number of AVP-positive neurons were found (Table 1, Fig. 4). In the rostral part of the SCN (level 2), voles with strong expression of circadian rhythmicity had significantly lower numbers of AVP-IR neurons compared with the other two categories (Table 1, Fig. 4). Conversely, the number of AVP-IR neurons in the medial and posterior part of the SCN (levels 3, 4 and 5) was significantly higher in voles without expression of circadian rhythmicity than in the other two categories (Table 1, Fig. 4). AVP-IR neurons in voles without expression of rhythmicity therefore outnumbered those in voles with strong rhythmicity significantly at levels 2, 3, 4 and 5 (Table

Table 1

The number of AVP-positive cells (means $\pm$ S.E.M.) at six frontal levels of the SCN assessed under continuous low light (LL) and in light/dark (LD) conditions for the groups of voles with different degrees of circadian rhythmicity (strong, weak or absent)

Group	Expression of circadian rhythm	n	Final light conditions	ΔQ_P	Number of AVP-IR cells at six coronal levels of the SCN (rostral = 1, caudal = 6)					
					1	2	3	4	5	6
A	strong	12	LL	850.4	51.50	119.82	136.03	103.65	77.23	33.50
(S.E.M.)				112.0	14.48	19.36	15.52	15.20	13.28	7.05
B	weak	5	LL	143.2	30.90	114.90	190.10	116.40	70.90	30.40
(S.E.M.)				34.4	10.63	10.67	23.16	37.40	24.62	9.86
C	absent	3	LL	181.8	20.00	107.83	127.50	102.50	65.00	21.25
(S.E.M.)				79.5	9.39	28.15	36.78	37.17	24.95	14.98
D	strong	6	LD	979.2	55.08	93.42	116.56	111.67	93.42	44.58
(S.E.M.)				176.2	18.17	28.08	44.76	42.05	40.66	19.01
E	weak	5	LD	231.8	85.10	180.03	172.50	119.10	80.70	50.80
(S.E.M.)				38.1	11.34	19.29	16.54	15.08	12.21	13.52
F	absent	6	LD	114.4	105.25	214.50	249.08	236.42	180.83	83.78
(S.E.M.)				26.5	29.06	20.82	10.35	5.91	17.78	10.41
Statistic comparisons between groups										
A–D				–	–	–	–	–	–	–
D–E				**	–	*	–	–	–	–
D–F				**	–	**	*	*	*	–
E–F				**	–	–	**	**	**	–

ΔQ_P : difference between χ^2 periodogram rhythmicity index Q_P and the $\chi^2_{(P < 0.05)}$ value (means $\pm$ S.E.M.). In the lower part of the table the statistic comparisons between the groups (A–F) are summarized: – no significance, * $P < 0.05$, ** $P < 0.01$ Mann–Whitney U -test (one-tailed).

1). For all categories of voles, no significant differences were found at levels 1 and 6.

4. Discussion

Circadian organisation of wheel running behaviour disappeared within 4 weeks of constant low light conditions in 24% of the common voles ($n = 37$). This degree of lability of the circadian organisation seems characteristic for this rodent species. Previous experiments in constant darkness resulted in an identical proportion of loss [10,12]. A dichotomy of rhythmic and non-rhythmic animals, as suggested by Gerkema and

Daan [10], appears too rigid. In 10 of 28 animals scored as rhythmic, only weak traces of the original circadian pattern could be observed.

In all voles examined, AVP-IR cells were exclusively clustered in the dorsomedial aspect of the SCN (dm-SCN). This distribution differs from that in the house mouse where there are two subdivisions of AVP-IR cells in the SCN: dorsomedial and ventrolateral (vl-SCN) [3]. More generally, the localisation of AVP-IR cells in the SCN appears species-specific. The dominating dm-SCN distribution in voles resembles the situation in the golden hamster [4] and ground squirrel [21], but deviates from the rat [27,30], rabbit, cat and tree shrew [24], showing at least a second, prominent, vl-SCN centred concentration of AVP-IR cells.

Fig. 2. High power photomicrographs of the density of AVP-immunoreactivity in the middle portion of the SCN of non-rhythmic (A, C) and strongly rhythmic (B, D) voles under constant low light conditions (LL; A, B) and under a 12:12 h light/dark cycle (LD; C, D). Only some faintly AVP-positive somata are present in the SCN of both non-rhythmic and rhythmic voles when kept under LL (arrows in A and B, respectively). However, a significant difference in cellular AVP-immunoreactivity becomes apparent between non-rhythmic and rhythmic voles 14 days (arrows in C and D, respectively) after transfer from LL to LD conditions. Besides AVP immunoreactivity in cell somata, numerous positive terminals (arrowheads) are present in the SCN, impinging upon either AVP-positive neurons or immunonegative neurons. Although clear AVP-positive boutons are present in the SCN of all voles under both light conditions, AVP-stained terminals are most prominent in number and staining-intensity in non-rhythmic voles under LD conditions (C). Bars in A–D = 30 μ m.

Fig. 4. Quantification of the number of AVP-positive cells within the SCN in voles with differential expression of circadian rhythmicity (CR) at 6 different levels. Voles were kept in LD conditions after 4 weeks of LL (groups D, E and F: see Table 1 for additional details).

The SCN of rat and mice contain an interconnected population of vasopressinergic neurons, capable of synchronized activity [5,29]. A similar interconnection of the vasopressinergic neurons in the SCN of voles is indicated by the observation of numerous darkly AVP-positive terminals impinging upon AVP-positive neurons. The distribution of AVP-IR terminals observed in the SCN of voles suggests that the majority of local AVP-positive terminals contact AVP-negative neurons. In the common vole, the observed interindividual differences in the number of AVP-IR cells of the SCN thus only concerned the dm-SCN. This interindividual variability was spontaneous and not selected for, as we used individuals from a non-inbred colony. In contrast, Bult et al. [2,3] obtained similar interindividual variability by using selection lines for thermoregulatory nest-building behaviour. This variability was built up by parallel changes in the dm-SCN and vl-SCN.

The differences between numbers of AVP-IR cells in voles obtained for the three categories of rhythmicity varied with respect to the six coronal levels of the SCN, and were significant in the medial sections only. Voles that showed an intermediate expression of circadian rhythmicity also had an intermediate number of AVP-IR cells in the medial sections, thus strengthening the correlation between AVP neurons and rhythmicity. These results suggest a discrete neuroanatomical substrate of circadian rhythmicity; differences in the pattern of activity might even be identified at the

level of the circadian pacemaker already. The results suggest furthermore a potential role for AVP in the efferent pathway leading to circadian patterns in behaviour. This interpretation seems in conflict with the observation that AVP deficient mutant Brattleboro rats still show circadian rhythmicity [14,20]. For these mutant rats it was shown that AVP cannot operate as an indispensable factor in the expression of circadian activity patterns. The Brattleboro model does not exclude, however, that in genetically intact animals AVP plays a crucial part in the circadian organisation of behaviour.

Besides a difference in the number of AVP-immunoreactive cells, we obtained in this study also a difference in the staining-intensity for AVP in those cells, depending on the rhythmic state of the voles. The difference in staining intensity appeared to be solely restricted to the cells in the SCN, since no differences were found in staining intensity in AVP-positive neurons outside the SCN. The differences in intensity of AVP immunoreactivity in SCN cells in voles of the three levels of circadian rhythmicity most likely reflects differences in AVP protein content of the SCN cells. These differences show up in LD conditions, but are marginally detectable in LL, where nearly no AVP immunoreactivity was present in any animal. A decrease by approximately half in the mean level of AVP content in the rat SCN was reported already after 3 days under LL conditions [28].

The similar numbers in AVP-IR cells for different CT of perfusion of rhythmic voles under continuous LL situations suggests that the number of detectable AVP-positive cells did not vary with circadian phase. This is in contrast to the temporal pattern of cellular AVP content of the SCN reported in other species [22,23,28]. In our experiment, all AVP-IR cells were affected equally by LL conditions, thus suggesting a homogeneity in response in the population of AVP-IR cells, but this result leaves the possibility that the AVP content of these cells may vary with CT.

Interestingly, the differences in numbers of AVP-IR cells were revealed in this study in LD conditions only, where all categories of voles showed well synchronised circadian rhythmicity; these results are similar to the preliminary findings mentioned earlier. These differences in AVP immunoreactivity are correlated with a disposition to lose rhythmicity, as expressed in a previ-

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Fig. 3. Low power photomicrographs depicting the differences in the density of AVP immunoreactivity in the SCN of a non-rhythmic (A–D) and a strongly rhythmic (E–H) vole. Coronal sections at levels 2 (A, E), 3 (B, F), 4 (C, G) and 6 (D, H) display the anterior to posterior distribution of AVP-positive neurons, fibres and terminals. The density of AVP immunoreactivity in non-rhythmic voles is higher than in rhythmic voles. AVP-positive neurons in the peri-SCN region do not differ in staining-intensity (arrows in A, B, E and G) between non-rhythmic and rhythmic voles, indicating an SCN-specific reduction in AVP immunoreactivity in rhythmic voles. The double arrows in C point at the main difference in the localization of AVP-immunoreactivity in the SCN between voles and mice, i.e. the absence of the ventro-lateral subdivision of AVP-positive neurons in voles. 3V, third ventricle; OC, optic chiasm. Bar = 60 μ m.

ous LL, and obviously not masked in LD, in contrast to the synchronised rhythmicity.

The presumed role of AVP in the efferent pathway leading to a circadian pattern of behaviour could suggest that high levels of AVP correlate with a strong circadian organisation. As such one should expect that the rhythmic voles contain higher levels of AVP-IR in the SCN, especially in the early subjective day. The current data show unambiguously the opposite: the voles lacking circadian rhythmicity have high numbers of AVP-IR cells, heavily stained compared with the circadian voles. Presuming there is no difference in the level of AVP-synthesis between voles with and without circadian rhythmicity, the present results suggest that a lower rate of AVP-release takes place in non-rhythmic voles. If AVP functions as a circadian messenger, one could speculate that this messenger is stuck into the SCN cells of these voles. Alternatively, if there is no difference in the rate of AVP-release between the different categories of voles, a higher level of AVP-synthesis in the voles without circadian activity patterns may cause the difference.

To our knowledge this is the first study of a natural population of animals showing a spontaneous variability in the expression of circadian rhythmicity which is reflected in a neuronal substrate, represented by the AVP-IR cells of the SCN. This model of the common vole opens a new perspective in the analysis of the functional role of AVP-IR neurons and AVP itself in the organisation of the circadian clock in relation to its efferent pathways.

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