Vasopressin immunoreactivity and release in the suprachiasmatic nucleus of wild-type and \textit{tau} mutant Syrian hamsters

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Despite the prominent role of the Syrian hamster (\textit{Mesocricetus auratus}) as a model system in studies of circadian rhythms, no data are available about the temporal dynamics of the neuropeptide vasopressin (AVP), a major output system of the suprachiasmatic nucleus (SCN). We studied the hamster SCN-AVP system \textit{in vivo} across the light period and \textit{in vitro} using long-term organotypic SCN cultures. We additionally compared wild-type and \textit{tau} mutant hamsters with an endogenous circadian period of \textasciitilde20h and \textasciitilde24h, respectively. No differences in the number of SCN-AVP neurons were found between the two genotypes of hamsters studied at three time points across the light period of the circadian cycle. A significantly higher level of AVP content, however, was found in the SCN of wild type compared to \textit{tau} mutant hamsters at the beginning and in the middle of the light period (ZT0 and ZT6), but not at the end of the light period (ZT12). SCN AVP cell number and content decreased significantly across the light period in wild-type hamsters, but not in \textit{tau} mutants. A significantly higher rate of AVP release per 24h was observed from \textit{tau} mutant SCN compared to wild-type SCN. Robust circadian oscillations in AVP release were not found in either type of hamster. These results suggest that the SCN-AVP system of hamsters, irrespective of genotype, is relatively weak compared to other species. Moreover, the \textit{tau} mutation seems to influence the SCN-AVP system primarily by enhancing the rate of AVP release.
Introduction

The suprachiasmatic nucleus (SCN) of the laboratory rat and the Syrian hamster have been model systems in the field of chronobiology (Hastings et al., 1996; Reuss, 1996) since the discovery in the early ‘70s of this nucleus as the main circadian oscillator (Moore and Eichler, 1972; Stephan and Zucker, 1972). Final, unequivocal proof for this role of the SCN was provided by transplantation experiments using tau mutant hamsters (Ralph et al., 1990). Most detailed anatomical and notably physiological data of the SCN are derived from the rat, but most behavioral observations are obtained from the hamster because of its high cycle-to-cycle circadian timing accuracy. Although the location in brain, cytoarchitecture and retinal afferents of the SCN are identical in a wide variety of mammals including rat and hamster (Cassone et al., 1988), minor differences in neuropeptidergic organization were observed between these species (Moore and Silver, 1998). The neuropeptide vasopressin (AVP), a major output system of the SCN, is abundantly expressed in both species (Sofroniew and Weindl, 1980; Card and Moore, 1984; 1991; Van den Pol and Tsujimoto, 1985; Kalsbeek et al., 1993; Ueda et al., 1983; Wollnik and Bihler, 1996; Dubois-Dauphin et al., 1990). These studies show that SCN-AVP neurons are more densely packed in rat than in hamster, and a separate group of AVP neurons in the ventro-lateral subdivision of the SCN such as particularly present in mouse SCN (Bult et al., 1992) is more pronounced in hamster (Card and Moore, 1984; 1991) compared to rat. AVP-immunoreactivity (AVPir) fluctuates in the rat SCN across the light-dark cycle, with high levels at the beginning of the light period (Zeitgeber Time - ZT 0) and lower levels at the beginning of the dark period (ZT 12) (Okamura, 1996). Hamsters kept under a natural LD cycle in April/May expressed more SCN-AVPir in the middle of the light period compared to the middle of the dark period (Schilling and Nürnberger, 1998). To our knowledge, however, changes of AVPir in hamster SCN across the light period has not been reported in literature. Most studies describe detailed anatomical characteristics of AVPir with no information of time of day (Dubois-Dauphin et al., 1990; Card and Moore, 1984; Miller et al., 1996; Sofroniew and Weindl, 1980; Ueda et al., 1983; Kalsbeek et al., 1993), or restricted the analysis to a small part of the LD cycle (Bult and Smale, 1999).

AVP is released from the SCN during the light period, and protein levels are restored during the dark period (reviewed in van Esseveldt et al., 2000). Rhythmic AVP levels derived from the SCN have been measured in the cerebral spinal fluid of rats, guinea pigs, rabbits, sheep, goats, cats, and rhesus monkeys (see for review Miller et al., 1996). AVP release from rat SCN has been thoroughly studied using a variety of techniques,
such as acute SCN slices (Earnest and Sladek, 1986; 1987), microdialysis (Kalsbeek et al., 1995) or long-term organotypic SCN cultures (Shinohara et al., 1995). Micropunches of rat SCN revealed essential the same AVP rhythm as the other methods (Inouye, 1996). Surprisingly, however, no AVP release data are available from hamster SCN.

Here, we studied AVPir in the SCN across the light period and AVP release from long-term organotypic SCN cultures in Syrian hamsters (Mesocricetus auratus). Additionally, we compare normal, wild-type (tau +/+ ) hamsters with a circadian period of ~24h with tau mutant (tau -/- ) hamsters with a circadian period of ~20h (Ralph and Menaker, 1988). Tau is a single, semi-dominant mutation in the gene encoding for casein kinase I (Lowrey et al., 2000), a critical component of the mammalian circadian clock. Levels of AVP mRNA are significantly reduced in the SCN of tau -/- compared to wild-type hamsters (Scarbrough and Turek, 1996). This difference may hint at a major impact of this mutation on SCN-AVP regulation at the protein level as well.

Materials and methods

Animals

Male Syrian hamsters (Mesocricetus auratus) were obtained from a breeding stock at the Zoological Laboratory, Haren, The Netherlands. Wild-type (tau +/+ ) and homozygote tau (tau -/- ) mutant hamsters (F2 generation) were obtained from crossings between heterozygous parents (F1 generation; Oklejewicz et al. 1997). All hamsters (age 5-6 months) were housed individually in cages (l x w x h = 40 x 24 x 15 cm) in a climate controlled room at 23 ± 0.5 ºC. Food (standard Hope farms® pellets) and water were available ad libitum. Tau +/- hamsters were entrained to a light-dark cycle of 12h light and 12h dark (LD 12:12) and tau -/- were kept in LD 10:10 for at least 14 days prior to the experiment. Principles of laboratory animal care (NIH publication No. 86-23, revised 1985) were followed, and experiments were approved by the Animal Experimentation Committee of the University of Groningen (Dec. No. 2091 & 2093).

Experimental protocol

Adult hamsters were used at three time points across the light period to establish whether the level of AVP-immunostaining changes during the light period. 32 hamsters (13 tau +/+ and 19 tau -/- ) were sacrificed at the beginning (ZT0) of the light period, and similar numbers at ZT6 and ZT12, respectively.
AVP immunocytochemistry

Animals were deeply anesthetized with an overdose of sodium pentobarbital (900 mg/kg, i.p.), followed by a quick dissection of the brain. Thereafter, brains were immersion fixed for 24 hr in 4% paraformaldehyde at 4 °C. Subsequently, brains were stored in phosphate buffer with 0.1% sodium azide. Before sectioning on a cryostat, brains were cryoprotected in 30% sucrose phosphate buffer for 24 hr. Coronal brain sections were cut at a thickness of 25 µm, and collected in phosphate buffered saline (PBS, pH 7.4) and stored in this medium with 0.1% NaN₃ at 4 °C until further processing. Brain sections were rinsed in PBS and pre-incubated with 0.1% H₂O₂ for 20 min, followed by three rinses with PBS. Thereafter, brain sections were pre-incubated with 5% normal goat serum and subsequently incubated overnight with the primary rabbit anti-vasopressin antibody (1:100; ICN) at room temperature. Sections were preincubated again with normal goat serum (5%), and incubated with biotinylated goat anti-mouse (1:200; Zymed) for 2 hr at room temperature. After rinsing with PBS, the sections were exposed to HRP-conjugated Streptavidin (1:200; Zymed) for 2 hr at room temperature. Triton-X-100 (0.5%) was added during all incubation steps. Finally, the sections were processed with diaminobenzidine (DAB)-H₂O₂ (30mg DAB/100ml PBS) under visual guidance.

Quantitative analyses of AVP immunoreactivity

AVPir was quantified in two complementary ways by counting AVP cells and by image analysis. Quantitative analyses of either way described below were performed “blind” to the type of hamster. Cell counts in brain sections were performed as described earlier (Bult et al., 1992; Gerkema et al., 1994; Van der Zee et al., 1999). In short, AVP-positive SCN cells were counted (left and right hemisphere were summed) in two sections containing the medial SCN in the rostro-caudal axis. Optical densities (ODs) of AVPir were determined in the SCN. The OD in the SCN was measured in the same two brain sections used for cell counting. The OD is expressed in arbitrary units corresponding to gray levels using a Quantimet 600 image analysis system. The value of background labeling was measured in a hypothalamic area devoid of AVP cell bodies, fibers or punctae. The OD of the area of interest was related to the background value by the formula \[
\frac{(OD_{area} - OD_{background})}{OD_{background}},
\]
thus eliminating the variability in background staining among sections.

Preparation of organotypic cultures

Pup SCN explants were cultured according to the organotypic slice culture technique using the roller-drum method (Gähwiler, 1994; Van der Zee et al., 2000). In short, pups
of 7 days old were deeply anesthetized with an overdose of sodium pentobarbital (900 mg/kg, i.p.) before decapitation. The brains were quickly removed and a block of hypothalamic tissue containing the bilateral SCN was cut coronally in 400 µm thick slices using a tissue chopper. Slices containing the SCN were trimmed and placed in Gey’s balanced salt solution and cooled for two hours at 5 °C. The slice was embedded in a plasma clot (10 µl chicken plasma and 10 µl thrombin) on a coverslip and placed in a culture tube with 700 µl of culture medium (25% horse serum, 45% Eagle’s basal medium with 62 mM D-glucose and 4.16 mM NaHCO3, and 30% Hanks’ balanced salt solution with 4.16 mM NaHCO3). The SCN slice was cultured at 36 °C with rotation (12 revolutions/h). In all cases, the medium was replaced once or twice a week. The morphological development of the cultures was followed daily by light microscopic inspection. Drawings as well as photomicrographs were made by phase-contrast microscopy. After ten days of culturing, samples were collected from each culture by changing the sample medium (700 ml, consisting of 10% horse, 60% Eagle’s basal medium, and 30% Hanks’ balanced salt solution) every two hours for two days (25 time points). The collected medium was immediately frozen in liquid nitrogen and stored at –20 °C until analysis.

Radioimmunoassay for AVP

240 µl of each sample was analyzed with a highly sensitive radioimmunoassay (RIA) for AVP according to Watanabe et al. (1988), with minor modifications. In short, we used rabbit anti AVP (“Peter”, 3-10-1980; The Netherlands Institute for Brain Research, Amsterdam) in a final dilution of 1:16.000. For each tube, 50 µl of Sac-cel solid phase second antibody coated cellulose suspension (IDS, England) was used as precipitation reagents. Standard curves ranged from 0.25 to 64 pg AVP per 50 µl and the assay had a detection limit of 0.25 pg/ml. Cross reactivity of “Peter” besides AVP was 0.04% with vasotocin, and <0.01% with oxytocin. 240 µl of all samples was analyzed in triplicate (80 µl per tube). The interspecific assay coefficient of variation was 12%. To be able to compare the total amount of AVP release between cultures analyzed in different RIAs, a “calibration RIA” was performed. In this RIA four samples of each culture (with values close to the average AVP release of that culture) were taken and analyzed in one RIA. The outcome of the AVP concentration in the calibration RIA was then used to calibrate all cultures. RIA data were further analyzed with different approaches to determine whether peak values in AVP release, circadian periods and rhythmicity were present. The number of peaks was determined by calculating the standard deviation of the average of the relative values within an individual assay. When a relative value exceeded the average plus > 2 standard deviations, it was considered to be a significant peak value.
Statistical analyses

Differences in AVP release between the genotypes were tested for statistical significance by a Mann-Whitney U-test. Differences in AVP OD measures were tested by two-way analysis of variance followed by a post hoc Tukey test (Sokal and Rohlf, 1995). A probability level of p < 0.05 was used as an index of statistical significance. All tests were two-tailed, and all data are presented as means ± standard error unless mentioned otherwise.

Results

Spontaneous AVP release in vivo

Spontaneous, endogenous AVP release is maximal during the first half of the (subjective) light period, and drops at the end of the (subjective) light period (see Introduction). To test whether indications for spontaneous AVP release could be demonstrated immunocytochemically, \( \text{tau}^{+/-} \) and \(-/-\) hamsters entrained to a 12:12 and 10:10 LD cycle, respectively, were killed at ZT 0, 6, and 12. The expression of AVPir for both genotypes at these time points is shown in Figure 9.1. AVP cells were predominantly found in the dorsomedial part of the SCN, whereas AVP positive fibers and terminals were predominantly present in the dorsomedial and ventrolateral parts of the SCN. The parvocellular AVP cells of the SCN were moderately to weakly stained, compared to the densely stained hypothalamic magnocellular AVP cells of the paraventricular nucleus, supraoptic nucleus and nucleus circularis. AVP-positive fibers and notably terminals, however, were densely stained in the SCN like seen in other hypothalamic regions.

Cell counts of AVPir neurons in the medial SCN revealed that the number of detectable AVPir neurons did not differ between the two genotypes at any of the time points studied (Figure 9.2A). However, the number of AVP neurons in \( \text{tau}^{+/-} \) hamsters was significantly higher at ZT 0 compared to the other two time points (p<0.01). No such ZT-dependent difference was observed in \( \text{tau}^{-/-} \) mutant hamsters (p>0.5). OD measures (representing AVP content) in the same brain sections containing the medial SCN as studied for the number of cells revealed a significant difference between the genotypes at ZT0 and 6, but not at ZT12 (p<0.05; Figure 9.2B). AVP content differed significantly between ZT0 and 12 in \( \text{tau}^{+/-} \) hamsters (p<0.05; Figure 9.2B), but not between ZT6 and the other time points. In contrast, no significant differences in AVP content across the light period were found in \( \text{tau}^{-/-} \) hamsters.
Development and AVP release from SCN organotypic cultures

The finding of differences in spontaneous AVP release across the light period between the two genotypes raised questions about the regulation of AVP release, which could be studied in more detail in vitro. Following explantation of the SCN at postnatal day 7, the morphology of the cultures of tau -/- and +/+ hamsters was studied by means of phase contrast microscopy on a daily basis until the beginning of sampling 12 days later. In all cultures a clear morphological organization in ependymal zone, neuronal zone, and dispersed cell zone was seen as described in literature for SCN organotypic cultures.

![Figure 9.1](image-url) AVPir in the SCN of wild-type (tau +/+; a,b,c) and tau mutant (tau -/-; d,e,f) hamsters at the beginning (ZT0), middle (ZT6), and end (ZT12) of the light period. Scale bar = 210µm.
of other species (see Van der Zee et al., 2000, and references therein). No consistent differences in development were observed between the genotypes, and the development was similar to that described previously for wild-type hamsters with the exception that these cultures developed more towards 1 to 3 layers of cells in the neuronal zone compared to 3 to 5 observed previously (Van der Zee et al., 2000). In the majority of cultures (7 out of 9 \( \text{tau}^{+/-} \), and 6 out of 9 \( \text{tau}^{-/-} \) cultures) a circulation of fluid in the ependymal zone was present.

AVP release was measured over 50 h, with 2 h intervals. Two cultures of each genotype were discarded from analyses because they were (partly) lost during the sampling procedure. Representative examples of 50 h AVP release profiles of both genotypes are presented in Figure 9.3. The presence of significant peak values revealed no differences between \( \text{tau}^{+/-} \) and \(-/-\) hamsters. Using statistical criteria for the presence of a peak in AVP release of >1, 1.5, or 2 standard deviations above average levels of AVP release, all cultures but one \( \text{tau}^{-/-} \) culture at >2 standard deviations had significant peak values. The number of peaks, depending on the criterion used, is shown in Table 9.1. No significant differences were found in number of peaks between the genotypes.

After a calibration procedure (see Materials and methods), the total amount of AVP release was obtained by summation of all AVP sample concentrations. Total AVP release was significantly higher in \( \text{tau}^{-/-} \) than in \( \text{tau}^{+/-} \) cultures (2.22-fold; \( p<0.01 \)). Because the endogenous circadian rhythm of \( \text{tau}^{-/-} \) hamsters is \( \sim 4 \) h shorter than that of \( \text{tau}^{+/-} \) hamsters, also \textit{in vitro} (Davies and Mason, 1994), a correction was performed to see whether the total amount of AVP still differed when expressed per predicted circadian period. Also after this correction, \( \text{tau}^{-/-} \) hamsters release significantly more AVP (1.85-fold; \( p<0.05 \)).
Figure 9.3 Representative examples of AVP release (in per cent of the mean level) of three wild-type (left panel; a,b,c) and three tau mutant (right panel, d,e,f) hamsters; dashed line represents 1.5 standard deviation.

Table 9.1 Analyses of AVP release patterns from organotypic SCN cultures.

<table>
<thead>
<tr>
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<th>tau +/- (n=7)</th>
<th>tau -/- (n=7)</th>
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<tbody>
<tr>
<td>averaged number of significant peaks per culture:</td>
<td></td>
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<tr>
<td>based on &gt;1 standard deviation</td>
<td>2.86 ± 0.37</td>
<td>3.00 ± 0.47</td>
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<tr>
<td>based on &gt;1.5 standard deviations</td>
<td>2.00 ± 0.33</td>
<td>2.14 ± 0.37</td>
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<tr>
<td>based on &gt;2 standard deviations</td>
<td>1.14 ± 0.16</td>
<td>1.00 ± 0.24</td>
</tr>
<tr>
<td>total amount of AVP released over 50 h (in pg)</td>
<td>406.07 ± 76.57</td>
<td>902.14 ± 102.23**</td>
</tr>
<tr>
<td>AVP released per predicted cycle (in pg)</td>
<td>194.92 ± 36.76</td>
<td>360 ± 40.89*</td>
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</table>
Discussion

Conclusions

The major findings reported here are (1) the significantly higher level of AVPir in the SCN of wild-type compared to \textit{tau} mutant hamsters at ZT0, (2) the absence of a decrease in SCN-AVP cell number and content across the light period in \textit{tau} mutant hamsters in contrast to wild-types and (3) the significantly higher rate of AVP release per unit of time from \textit{tau} mutant SCN compared to wild-type SCN in the absence of robust circadian oscillations in AVP release in both genotypes.

Features of the hamster SCN-AVP system

The results of AVPir reveal some of the dynamic properties of the hamster SCN-AVP system across the light period. In general, the number of immunocytochemically detectable peptidergic SCN neurons is a direct reflection of the total amount of protein (such as AVP) stored in the somata (Shinohara \textit{et al.}, 1993; Jansen \textit{et al.}, 2000; Van der Zee \textit{et al.}, subm.). The number of AVP cells did not differ between \textit{tau} +/+ and \textit{tau} -/- hamsters. Therefore, the higher number of cells at ZT0 in \textit{tau} +/+ hamsters is most likely due to accumulation of newly synthesized and not yet released AVP, while AVP levels in the cell bodies of \textit{tau} -/- SCN (which have a two-fold higher rate of AVP release (as shown \textit{in vitro}) remain lower. Across the light period, AVP cell numbers in \textit{tau} +/+ hamsters drop between ZT0 and 6 and AVP OD values (reflecting AVP content) between ZT6 and 12. This time lack of alterations in AVP content can be explained by transport of AVP from the somata to the axon and terminals, depleting the cell body of AVP (and hence reducing the weakly stained cells below immunocytochemical detection) whereas total AVP content does not change clearly yet. Later in time, AVP is released from the SCN, causing the drop in AVP content between ZT6 and 12. A similar time lack between these two SCN-AVP parameters has been observed in mouse SCN (Van der Zee \textit{et al.}, subm.).

AVP mRNA fluctuates in a circadian fashion in wild-type hamsters (Scarborough and Turek, 1996; Duncan \textit{et al.}, 2001), and a significant reduction in the amount of AVP mRNA has been observed in \textit{tau} mutant hamsters comparing circadian times 4 and 16 (Scarborough and Turek, 1996). The \textit{tau} -/- hamsters, however, had a lower amplitude at these both time points. It should be noted that Scarborough and Turek used Charles River’s hamsters where wild-types and mutants were bred separately, whereas we used hamsters of the Wright’s strain with both genotypes bred from heterozygous crossings. Nevertheless, based on these mRNA data one could expect to find time-dependent
changes at the protein level in both types of hamsters. *Tau* -/- hamsters, however, do not show any sign of changes in protein content over time. This absence in *tau* mutant hamsters may indicate that a two-fold higher rate of AVP release from their SCN neurons is too high to fully replenish AVP levels at ZT0 as seen in wild-type hamsters. Although it remains to be determined that AVP mRNA fluctuates in our *tau* -/- hamsters, it also raises the possibility that AVP mRNA translation (or AVP feedback on its own production) differs between the genotypes.

Species comparison

Shimomura and Menaker (1994) suggested that the coupling between individual oscillators of the circadian clock might be weakened by the *tau* mutation. The higher amount of AVP released by *tau* mutant hamsters could be a compensatory action to enhance internal synchronization of individual SCN cells, since AVP appears to play an important role in determining the power of circadian rhythms (Ingram *et al*., 1996). In contrast to vole, mouse, and rat long-term organotypic SCN cultures (Jansen *et al*., 2000; Van der Zee *et al*., submitted), no clear circadian fluctuation in AVP release was observed in either genotype of hamster. This suggests that the AVP system in hamster SCN is less robust than that of the other rodents. Moreover, the absence of AVP reduction across the light period suggests that specifically *tau* mutant hamsters have a weak SCN-AVP system. If SCN-AVP mediates rhythmicity of locomotor activity in hamsters, it would imply that *tau* mutant hamsters have a weaker circadian organization of behavior. Indeed, *tau* mutant hamsters do show a less precise rhythm with higher fragmentation of the rhythm (Osiel *et al*., 1998). Similar differences in precision of activity onset have been observed in our *tau* mutant hamsters compared to wild type hamsters (Daan and Oklejewicz, 2001), both with a similar genetic background.

The expression of circadian fluctuation in AVP release from hamster organotypic cultures is relatively weak, irrespective of type of hamster. In SCN cultures obtained from (rhythmic) voles (Gerkema *et al*., 1999; Jansen *et al*., 2000), rats (Shinohara *et al*., 1995; Jansen *et al*., 2000), or mice (Van der Zee *et al*., submitted) explanted at similar postnatal ages generally reveal clearer circadian AVP release profiles. This difference cannot be attributed to a poorly developed SCN-AVP system of the hamster at the age of explanting (postnatal day 7), since the hamster SCN develops at least as early (Delville *et al*., 1994) or even earlier (Romero and Silver, 1990) than that of rat (de Vries *et al*., 1981; Isobe *et al*., 1995), mouse (Herzog *et al*., 2000) or vole (K. Jansen, unpublished observations). It may, however, be related to the relatively lower numerical density of AVP neurons in hamster SCN (at least of those used in our laboratory where
wild-type and tau mutant hamsters were bred from heterozygote tau mutant parents) compared to the other species. Analyzed in brain sections, mice, voles, and rats have approximately 8, 5, and 4 times more AVP neurons in the mid-SCN region compared to wild-type hamsters at ZT0; Gerkema et al., 1994; Bult et al., 1992; Wollnik and Bihler, 1996). In monolayered organotypic SCN cultures, mice voles and rats have approximately 4, 2, and 1.5 times more AVP neurons (Van der Zee et al., 2000; submitted). Additionally, poor AVP release rhythms of hamster SCN cultures may also be due to phase-angle differences in AVP release rhythms among individual AVP cells. It has been shown that circadian firing rhythms of individual SCN cells in culture can be out of phase (Welsh et al., 1995).

The observation of the deviant nature of the SCN-AVP system of the tau mutant hamster may indicate that casein kinase I (Lowrey et al., 2000) influences temporal dynamics of AVP. From this point of view it is worthwhile to further scrutinize the impact of this mutation on peptide regulation at the protein level, for example for vaso-active intestinal polypeptide, somatostatin, or other SCN derived neuropeptides.
Infradian timing