Research report

In vivo measurement of a diurnal variation in vasopressin release in the rat suprachiasmatic nucleus

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Abstract

Diurnal changes in the intranuclear release of vasopressin (VP) and oxytocin (OT) in the suprachiasmatic (SCN), paraventricular (PVN) and supraoptic nuclei (SON) of the rat were studied by means of brain microdialysis. A significant diurnal variation in VP release in the SCN was detected, with the highest levels occurring during midday and a trough around midnight. OT release from the SCN was below detection limit. The release of neither of these neurohypophysial peptides showed diurnal variations within the PVN or SON.

Keywords: Oxytocin; Circadian rhythm; Microdialysis; Supraoptic; Paraventricular

1. Introduction

In mammals the primary pacemaker for generation and entrainment of circadian rhythms is located in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus. Main characteristic of the endogenous pacemaker is the generation of a nearly 24-h (i.e. circadian) rhythm of electrical, metabolic and secretory activities. In vitro a diurnal oscillation of SCN activity has been demonstrated by several authors, by recording neuronal firing rates [2,16], vasopressin (VP) [12,35,61], and recently VIP [53] release in the medium. On the other hand, in vivo monitoring of the rhythmic changes in SCN neuronal activity has proven much more difficult. The first in vivo data for a rhythmic change in SCN metabolic activity were derived from the uptake of [¹⁴C]2-deoxyglucose [47,49,50]. But with this technique no longitudinal studies can be performed since every animal only provides one sampling point. Also, in vivo monitoring of daily changes in the firing rate of SCN neurons has proven very difficult [21,27]. Therefore, to date, the best in vivo estimate of SCN activity is provided by measurement of VP levels in the cerebrospinal fluid (CSF) [48,51]. However, also the CSF sampling technique has considerable drawbacks. Since

the transmitter of interest measured in the CSF will be derived from different sources, additional (lesion) experiments are needed [22,51]. Next to the poor spatial resolution, also resolution in time is low since the small quantity of CSF only permits a restricted number of samples to be taken, especially in small animals [26].

The present study was initiated in order to improve both the spatial and temporal resolution for measurement of SCN activity by application of the intracranial microdialysis technique to follow intranuclear VP release. Microdialysis has rapidly been established as an effective technique for in vivo measurement of neuropeptide release. Recently, microdialysis has been successfully applied to measure local release of several peptides, including VP and oxytocin (OT) in the paraventricular (PVN) and supraoptic nuclei (SON) [19,29,38]. Initially, application of the microdialysis technique has been hampered by the low release rates and the relatively poor recovery of the high molecular weight neuropeptides. Since the amount of VP contained in the SCN is much lower than that reported for PVN and SON [39,63], the present study started to examine the feasibility of measuring basal VP release from the SCN. Subsequently, the origin of the VP measured was ascertained by comparing VP release from the SCN with that of other hypothalamic nuclei, i.e. PVN and SON. Furthermore, for comparison, OT was measured in the dialysates.

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2. Materials and methods

Male Wistar rats (300–350 g) were used in all experiments. Rats were housed in a temperature-controlled room with a 12:12 h light/dark cycle (lights on at 07:00 h). Food and water were provided ad libitum.

Rats were anaesthetized with Halothane (SCN and SON) or Hypnorm (PVN; 0.1 ml/100 g b.wt.) and a microdialysis probe was stereotactically implanted with its U-shaped tip aimed at either the right SCN (1.4 anterior to bregma, 1.1 mm lateral from midline and 9.2 beneath the surface of the skull), SON (–1.1 mm, 1.8 mm, 9.1 mm) or PVN (–1.8 mm, 2.0 mm, 8.0 mm). The probes were secured with dental cement onto two stainless steel screws inserted in the skull. The dialysis probe consisted of a dialysis fiber (molecular weight cut-off 6000 Da, 2.5 mm in total length), glued into the ends of parallel pieces of 25-gauge stainless steel tubing. The loop of the probe was positioned in the rostrocaudal direction along the target nuclei. After surgery, the rats were housed in separate cages. After a recovery period of two or three nights (at least 36 h) microdialysis was performed in conscious, freely behaving animals, which apparently did not express discomfort during the dialysis. Sampling was performed according to techniques that were validated thoroughly in a number of previous *in vivo* and *in vitro* studies [14,28,29,31,36,38] confirming that it reliably reflects peptide fluctuations in the extracellular fluid of a distinct brain area. In short, experiments were started in the morning and 2.5 h before collection of the first sample animals were connected to the syringe pumps via a piece of 80 cm PE20 tubing. The probe was perfused continuously with Ringer at a flow rate of 200 μ l/h. Sequential 1-h samples were collected from a 10 cm outflow tubing (volume 10 μ l, i.e. 3 min delay) directly into Eppendorf vials. The dialysis membrane prevents large molecules (e.g. enzymes) from diffusing into the dialysate. Therefore, in contrast to e.g. push–pull perfusion, degradation of peptides is minimal during the 1-h sampling period [26]. Afterwards, all samples were immediately stored at –20°C until assayed. Sample collection proceeded for 36 h with dim red light during the dark period.

At the end of the experiment rats were decapitated, and their brains frozen on dry ice. After cryostat sectioning and Cresyl violet staining brain sections were mounted onto microscope slides and evaluated for proper probe placement.

Dialysates were lyophilized and their OT and VP contents measured without extraction by sensitive and specific radioimmunoassays. The antisera raised in rabbits showed negligible cross-reaction with related peptides, including

Fig. 1. Coronal sections of the hypothalamus showing the placement of the different dialysis probes used in the present study. Δ , SCN (Groups 1 and 2); $\blacktriangle$, Group 3; $\bullet$, PVN; $\square$, SON.

VP and OT. Therefore, eventual degraded VP/OT molecules present in the dialysate would not cross-react with our highly specific antibodies. Hence, only authentic VP/OT molecules were detected. Synthetic VP and OT obtained from Ferring (Sweden) and Bachem (Switzerland), respectively, were used as standards. 125 I-labelled peptides were purchased from New England Nuclear (Boston, MA). The minimal detection limit of both assays was in the range of 0.1 pg/sample with half-maximal displace-

ment of [125 I]VP occurring at approximately 3 pg VP standard and [125 I]OT occurring at approximately 4 pg OT. The intra- and inter-assay coefficients of variation were between 7% and 10%, and 9% and 13%, respectively.

Significance of diurnal variation (time dependency) was assessed using a one-way analysis of variance with repeated measures. Subsequently, data points were subjected to a statistical analysis by Cosinor analysis on the assumption that circadian rhythmicity was present. The 24-h

Fig. 2. Diurnal fluctuations in VP release within the SCN under LD conditions. A: VP-output depending on placement of the dialysis probe (—, Group 1 (in the SCN ($n = 7$)); ---, Group 2 (just dorsal to the SCN ($n = 6$)); Group 3 (outside the SCN ($n = 8$))). B: VP-release pattern of animal #37.

cosine curve was used to calculate circadian rhythm parameters, including mesor, amplitude and acrophase [1,7,33,40,42]. The Student's *t*-test was used for the assessment of diurnal peak times in VP release.

3. Results

Histological investigation of the probe placement showed a clear correlation between VP recovery and distance of the probe to the SCN (Fig. 1). In seven out of the 20 animals mean levels of VP detected in the 60-min sample of SCN dialysates were > 1.5 pg. In all these 'group 1' animals the tip of the dialysis probe was located in the SCN. One animal (#37) was exceptional in that it showed mean levels of 5.2 pg/h, in a few instances resulting in a VP output of over 10 pg/h (Fig. 2b). In an additional 6 animals (group 2) showing mean hourly levels of approximately 1.0 pg/h (0.94–1.14) the tip of the dialysis probe was placed just dorsal to the SCN. Output of VP by the SCN, as measured in group 1, exhibited a clear diurnal rhythm with peak levels occurring during daytime and a nadir in the night. In group 2 animals the diurnal variation was considerably smaller (Fig. 2). Mean VP levels of the eight animals whose dialysis probe was outside the SCN (group 3) varied between 0.1 and 1.0 pg/h with no indication of a diurnal rhythm (Fig. 2). In this case, the probe was located either between the bilateral SCN (2 times) or in the third ventricle (2 times). In another four animals the dialysis probe was located dorsolateral from the SCN.

As shown in Table 1, analysis of variance showed a clear effect of time on the amount of VP released by the SCN in groups 1 (probe in SCN), 2 (probe just dorsal to the SCN) and in groups 1 and 2 combined. In order to check whether the pronounced rhythm of #37 was not

solely responsible for the significant results, statistical analyses were also performed without data from #37. In no instance was the level of significance changed. Student's *t*-tests indicated that for groups 1 and 2 combined, all timepoints during the first light period differed from the trough in VP release at CT 20 and 21. Peak levels were dampened during the second light period, but 8 timepoints remained different from the previous trough (see * in Fig. 2a). Cosine fitting, too, indicated a clear diurnal variation in groups 1, 2 and the combination of the two groups, with acrophases between CT6–8 and an amplitude of 40%. In group 3 (probe outside the SCN) ANOVA indicated no daily variation.

In four animals with probe placements within the SCN (mean levels 1.1, 1.1, 2.1 and 2.6 pg VP/h) the OT content of the dialysates was also measured. OT levels in SCN dialysates were in most cases below the detection limit of the assay. Only in 13 out of the total of 144 samples a measurable quantity of OT (mean 0.76 ± 0.22 pg/h) was found. All 13 OT-containing samples were found in the two animals with the lowest output of VP, i.e. 1.1 pg/h, and were dispersed throughout the light/dark cycle.

Mean levels of VP in the 1-h dialysates of probes aimed at the PVN varied between 2.5 and 4.2 pg (Fig. 3). The majority of the animals showed hourly levels between 1.0 and 3.0 pg/h. One animal (#9), showed a mean output of 7.3 pg/h. In all animals the hourly PVN dialysate was also assayed for its OT content. As shown in Fig. 3A mean output varied between 2.5 and 5.0 pg/h. However, one animal (#3) showed an exceptionally high output of OT with hourly levels mostly above 10 pg/h. VP and OT recovery were clearly correlated with the position of the dialysis probe. The highest recovery rate was attained when the tip of the probe was located just lateral to the magnocellular part of the PVN (Fig. 1).

Table 1

Statistical analysis of the diurnal variation in intranuclear vasopressin and oxytocin release of hypothalamic nuclei

	(1)	(1 ^a)	(2)	(1 + 2)	(1 + 2 ^a)	(3)	PVN-VP	PVN-VP ^a	PVN-OT	PVN-OT ^a	SON-VP	SON-OT
(A) ANOVA												
<i>F</i>	2.58	2.94	2.03	3.29	3.43	0.90	0.77	0.51	1.20	1.24	0.71	0.81
df	35,210	35,175	35,175	35,420	35,420	35,425	35,245	35,210	35,245	35,210	35,105	35,140
<i>P</i>	< 0.001	< 0.001	0.002	< 0.001	< 0.001	0.631	0.827	0.991	0.216	0.177	0.897	0.759
<i>n</i>	7	6	6	13	12	8	8	7	8	7	4	5
(B) Cosinor												
<i>r</i> ²	0.50	0.26	0.47	0.59	0.42	0.169	0.04	0.111	0.06	0.01	0.001	0.01
<i>F</i>	16.8	5.88	14.8	23.3	12.12	3.35	0.77	2.06	1.06	0.19	0.03	0.16
<i>P</i>	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.05	> 0.05	0.05	> 0.05	> 0.05	> 0.05	> 0.05
Mesor (pg/h)	2.30	1.92	0.96	1.67	1.44	0.51	3.29	3.43	5.12	2.18	3.49	1.94
Amplitude (%)	40	38	38	40	31	29	3.6	4.7	3.5	2.3	2.3	2.0
Acrophase (h)	6.6	7.9	7.3	6.8	7.7	6.7	6.8	6.7	6.2	4.4	10.1	13.5

Given are the results of (A) one-way (TIME) ANOVA and (B) Cosinor analysis (period = 24 h) on vasopressin release in the SCN (groups 1–3) and vasopressin and oxytocin release in PVN and SON.

^a Without animals #37, #9 and #3 respectively. Amplitude is equal to one-half the total extent of the sinusoid and is expressed as a percentage relative to the daily mean value.

VP and OT release within the SON were measured in 5 animals (Fig. 3B). With the exception of one animal (#126) the mean hourly VP output of the SON varied between 4 and 6 pg. In #126 only one sample was above the detection limit. The dialysis probe in this animal was located in the most dorsal region of the SON. In the same dialysate samples the OT content was also measured. Mean output of OT by the SON was very constant and varied between 1.5 and 2.5 pg/h.

Analysis of variance showed no significant effect of

time on the amount of either of these neurohypophysial peptides released within the SON or PVN area.

4. Discussion

The present investigation is the first to apply the technique of *in vivo* brain microdialysis to explore the pattern of VP release in the SCN. Our results show that apart from extracellular levels of 5-hydroxyindoleacetic acid (5-HIAA)

Fig. 3. Intranuclear release pattern of VP and OT during a 36-h period in the PVN (A) and SON (B).

and excitatory amino acids [17] also peptides are released in sufficient amounts in the SCN to be measured by microdialysis. Provided that the microdialysis probe is positioned very close to the SCN a mean amount of 1–2 pg/h VP can be recovered. The results gathered in this way are consistent with previous *in vitro* and *in vivo* results showing a marked daily variation in VP release by the SCN with a pronounced peak during (subjective) daytime.

The mean amount of VP recovered was clearly correlated with the distance of the microdialysis probe to the target nucleus. Only if the tip of the microdialysis probe was placed within the SCN a clear VP signal (i.e. mean amount more than 1.5 pg/h) could be picked up. If the dialysis probe was more than 200 μm dorsal or lateral to the SCN the mean recovery was less than 1.0 pg/h. In this group of animals only Cosinor analysis could detect a significant rhythm with an amplitude of 30%, probably due to the consistently low VP levels during nighttime. The strict correlation between probe placement and the strength of the VP signal picked up already provides a first bit of proof for the site specificity (i.e. SCN) of the diurnal fluctuation in the VP signal. In order to check further the site specificity of the effects observed, two additional controls were performed. In the first place microdialysis probes were also aimed at two other VP-containing nuclei in the hypothalamus, i.e. the PVN and SON. The mean amount of VP recovered in these nuclei was about three-fold the amount found in the SCN. However, in contrast to the results in the SCN, VP release in PVN or SON showed no pronounced daily variations (i.e. maximal amplitude 5% of the daily mean). As a final control OT release was also measured in all three structures. Previous anatomical studies have shown an OT-containing cell population in the PVN and SON but not in the SCN [4,54–56]. In accordance with these anatomical data OT recovery in the SCN was below detection limit (i.e. < 0.1 pg) in more than 90% of the samples, whereas in the nearby SON the mean amount of OT recovered was about 2 pg/h. The regional specificity and restricted diffusion of intranuclearly released VP and OT is also indicated by the one dialysis probe located in the most dorsal part of the SON. The lack of VP measured by this probe is in line with anatomical data showing a preferential location of VP-containing neurons in the ventral part of the SON and a dorsal rim of OT neurons [46,56,60]. Altogether these experiments show that the VP signal picked up in the SCN is not confounded by diffusion of either VP or OT released in nearby magnocellular structures if the dialysis probe is located exactly in or immediately adjacent to the SCN. In addition it shows that the diurnal fluctuation in the intranuclear release of VP is a phenomenon specific for the SCN. In this respect the rhythms in VP and glutamate release [17] seem to be more localized to the SCN area than the previously reported release of serotonin [18].

The diurnal rhythm in the central release of VP was

initially described by Reppert et al. [44]. In a series of experiments Reppert et al. showed that rhythmic changes in VP concentrations in the CSF could be found in a number of species, i.e. cat, rat and monkey [41,45,48]. Irrespective of whether these species were active during the day or at night, peak and trough of the central VP rhythm were always found during day- and nighttime, respectively. Additional lesion experiments suggested that VP released from SCN fiber terminals was largely responsible for the rhythm found in the CSF [22,51]. Subsequent *in vitro* experiments confirmed the intrinsic capability of the SCN to generate a circadian rhythm in VP secretion [9,12,13,16,35,61]. The results of the present experiments extend the data described above in showing that VP is also released in a rhythmic fashion within the nucleus of its origin.

Together with anatomical data showing a dense VP-containing local innervation of the SCN [3,10,25] and electrophysiological data on the responsiveness of SCN neurons to VP [30,32,52], the present results suggest an important role for locally released VP in the maintenance of circadian activity. Contrary to the initial results of Liou and Albers [30] that neither V1 nor V2 antagonists were able to block the spontaneous increase in SCN neural activity, the recent report of Mihai et al. [32] revealed a decrease in spontaneous discharge rate of SCN neurons by bath application of a V1-antagonist *in vitro*. The attenuation of the antagonist-induced decline between subjective day and night phase indicates a changing endogenous excitatory VP tone, and is compatible with the presently reported peak secretion of VP during daytime. Since the peak in V1a receptor mRNA expression occurs at night [65], the fluctuating VP tone may primarily involve a change in peptide secretion and not receptor expression. Therefore, the cycling release of VP might serve not only to transmit the message of the circadian oscillator to other areas of the CNS such as the HPA-axis [3,23,24], but also to synchronize and amplify the pacemaker output within the SCN. A positive feedback of endogenously released VP has recently been described for the SON [62].

The lack of a clear rhythmicity in locally released VP or OT from the PVN and SON is in accordance with most of the previous experiments. Burbach et al. [6] reported a lack of diurnal variation in VP and OT mRNAs in rat PVN and SON. Earlier Uhl and Reppert [59] reported that they could not find any day–night differences in VP mRNA levels in PVN and SON. Recently, Cagampang et al. [8] confirmed the lack of variation in VP mRNA levels in the SON during a 24-h cycle. On the other hand, measurement of VP peptide levels in punched-out PVN and SON did show diurnal variations [39,63]. As regards VP and OT levels in the peripheral circulation, results are not unequivocal. But in those cases where a diurnal variation was reported, peak levels were found during the dark period in general [15]. Therefore, during basal conditions daily changes in the peripheral demands of the magnocellular system are pri-

marily reflected in its peptide content but not in intranuclear release or mRNA levels. Only physiological challenges such as dehydration and lactation cause clear changes in both mRNA levels and intranuclear release of the magnocellular VP and OT system [5,31,37,64].

The present demonstration of *in vivo* measurement of VP release within the SCN for more than 24 h and with a relatively high resolution (i.e. 1 h) opens the possibility of a new series of experiments to study the functional significance of the intranuclear release of peptidergic SCN transmitters. Thus, in addition to VP, also the dynamic release pattern of other SCN transmitters such as somatostatin, substance P and neuropeptide Y may now be monitored by microdialysis [11,43,57,58]. Furthermore, measurement of SCN secretory activity by microdialysis can be easily combined with behavioural observations and/or measurement of hormonal release patterns [14,19,20,34,36,38].

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