Decrease of Endogenous Vasopressin Release Necessary for Expression of the Circadian Rise in Plasma Corticosterone: a Reverse Microdialysis Study

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Abstract

The mammalian suprachiasmatic nuclei (SCN) contain an endogenous pacemaker that generates daily rhythms in behavior and secretion of hormones. Previously we hypothesized that the SCN imposes its circadian rhythm on the rest of the brain through a rhythmic release of its transmitters in its target areas. In the present study we employed microdialysis-mediated intracerebral administration of vasopressin (VP) and its V_1 -antagonist to study the mechanisms underlying the circadian control of the release of the adrenal hormone corticosterone. Stress-free application of the VP V_1 -antagonist in the dorsomedial hypothalamus of freely moving, undisturbed animals during the middle of the light period (i.e. the trough of the corticosterone rhythm) caused an immediate increase of circulating plasma corticosterone levels. A similar administration of VP at the end of the light period completely prevented the diurnal rise in plasma corticosterone. These results indicate a pronounced inhibitory role for SCN-derived VP at the level of the dorsomedial hypothalamus with respect to the activity of the hypothalamo-pituitary-adrenal axis during the day period. Thus, the daily decline in VP release sets a specific time window for the occurrence of the daily corticosterone peak. On the other hand, during the dark period corticosterone levels are decreasing together with basal VP levels. Therefore, in addition to the inhibitory VP signal from the SCN, there is also the need for an excitatory SCN signal in order to accomplish the complete circadian profile of plasma corticosterone.

Introduction

In most mammals plasma corticosterone concentrations reach their zenith just prior to awakening and then abate to reach their nadir at the end of their activity period (1, 2). The persistent fluctuations of plasma corticosterone levels under constant conditions, with a near 24-h (i.e. circadian) rhythm, proved the endogenous nature of this rhythm (3, 4). In the mid sixties, lesion experiments (5, 6) indicated that afferent projections important for the circadian control of corticosterone release originated from a medial anterior hypothalamic region. After recognition of the direct retinal projection to the suprachiasmatic nuclei (SCN) in the anterior hypothalamus (7, 8) a number of lesion and/or knife cut studies have confirmed the importance of the SCN for a circadian pattern of activity in the hypothalamo-pituitary-adrenal (HPA) system (9–12). However, twenty years later the nature of the circadian rhythmic information generated and the mechanism of transduction of this rhythmic information from the SCN to the HPA system is still unknown.

Vasopressin (VP) levels in the CSF and VP mRNA abundance in the SCN show a circadian rhythm with high levels in the early day period (13–15). CSF-VP levels probably reflect the net result of VP diffusion after its release in the different SCN target areas (16). That VP is indeed released in a rhythmic fashion in the

SCN target areas is evidenced by our previous microdialysis study, showing a circadian pattern in the amounts of VP recovered from the extracellular fluid surrounding the SCN (17). In a first series of experiments we reached the conclusion that the main signal from the SCN to the HPA axis is an inhibitory one (18–20). We provided evidence that the VP-containing fibres in the SCN projection towards the PVN/DMH target area form an essential component of this inhibitory message (18, 19). Although these experiments showed a clear inhibitory effect of VP at the level of the dorsal hypothalamus on the HPA axis, the significance of these data for the circadian regulation of the HPA-axis could not be established due to the HPA-activating side effects of the intracerebral infusion procedure. Similar problems had also been reported with infusions in the PVN and preoptic area of the hypothalamus. Saline infusions in the PVN induced grooming behavior (21) and control injections in the preoptic area almost invariably induced changes in body temperature (22). For these latter experiments administration via a microdialysis probe proved to be a significant improvement (22). Accordingly, the present experiments were initiated to establish the role of VP in the circadian control of corticosterone release using an infusion technique that would not interfere with basal HPA-activity. Undisturbed local intracerebral delivery of VP and its antagonist

was accomplished by reverse dialysis, i.e. introduction of substances into (instead of removal from) the extracellular space via a microdialysis probe, as described previously (23).

Results

Experiment 1

Mean corticosterone levels varied between 10 ng/ml at the trough and 112 ng/ml at peak value (Fig. 1). Basal values were apparent from 2 h before daybreak until at least 6 h into the day period (i.e. CT22 to CT6). From CT6 onwards plasma corticosterone levels increased to reach their zenith with the onset of darkness. Decrement of the elevated corticosterone concentrations was completed shortly before the end of the dark period. Mean plasma corticosterone values at the three overlapping timepoints were always higher in the first than in the corresponding last sample of a run, but we detected no significant differences (CT6.5, $P=0.11$, CT14.5, $P=0.07$ and CT22.5, $P=0.16$).

Experiment 2

Several hours perfusion (from CT2–CT8) of microdialysis probes aimed at the DMH with Ringer did not affect corticosterone values as compared to basal values obtained in the control animals of Exp. 1 (Fig. 2). Changing the perfusion medium in the dialysis probe from Ringer to Ringer plus VP-antagonist (50 ng/ μ l) caused an immediate and rapid increase of circulating plasma corticosterone concentrations. Peak levels in plasma corticosterone were obtained 40 min after the VP-antagonist first

entered the microdialysis probe. Plasma corticosterone concentrations then decreased and reached control values 40 min after the change of VP-antagonist dialysis back to Ringer again.

Administration of VP (50 ng/ μ l) tended to decrease plasma corticosterone values as compared to the Ringer perfused group. But in a separate ANOVA (Ringer- vs VP-perfused) the difference just missed significance ($F(1, 9)=4.08$, $P=0.074$).

However, the plasma corticosterone values of the Ringer group showed a significant (paired t -test) increase at 80, 100 and 120 min following calculated entrance of VP into the brain as compared to the first time point sampled ($t=-20$), whereas the VP-infused groups showed no such increase. Plasma corticosterone values of both Ringer- and VP-infused animals remained close to the range of plasma corticosterone concentrations (mean $\pm$ SEM) observed in the non-infused control animals of Exp. 1 (area between dashed lines in Fig. 2).

Experiment 3

In a number of cases plasma corticosterone concentrations were already elevated in the first (i.e. $t=0$) blood sample, probably due to the repeated use of the animals. Elevated levels of circulating corticosterone clearly affect the subsequent reaction of the HPA-system (24, 25), and final analysis was therefore performed only with experiments of which the plasma corticosterone concentration of the $t=0$ sample was below 50 ng/ml (69% and 65% of the VP and V_1 -antagonist applications, respectively). Dialysis of Ringer during the last 4 h of the day period did not affect the endogenous circadian rise of corticosterone. Peak values

FIG. 1. Mean plasma corticosterone levels of 10 male rats during a 24-h light/dark period. Animals were sampled at 3 different occasions during 9 subsequent hours starting at either CT6.5, CT14.5 or CT22.5.

FIG. 2. Effect of Ringer (○); VP (50 ng/μl; ▲) and V₁-antagonist (50 ng/μl; ■) infusions at CT6.5 to CT7.5 on plasma corticosterone values. Infusions were performed via microdialysis probes in the DMH. MANOVA indicated clear effects of infusion ($F(2, 14) = 50.3$, $P < 0.001$), time ($F(7, 98) = 10.4$, $P < 0.001$) and 'infusion $\times$ time' ($F(14, 98) = 11.2$, $P < 0.001$). Dotted lines indicate corticosterone levels of control animals in Exp.1 (Mean $\pm$ SEM). * $P < 0.05$ as compared to Ringer and VP; ♦ $P < 0.05$ vs VP.

were very much similar to those obtained in the non-implanted control animals of Exp. 1 (Fig. 3). ANOVA indicated a pronounced effect of time on the amount of corticosterone released by the Ringer perfused group ($F(5,35) = 5.97$, $P < 0.001$). Plasma corticosterone levels were significantly elevated at CT8.5, 9.5 and 11.5 as compared to CT 7.5. On the other hand, none of the VP dialyzed groups showed a significant effect of time on the amount of corticosterone released ($F(5,25) = 1.28$, $P = 0.304$; $F(5,25) = 1.45$, $P = 0.241$; $F(5,30) = 1.09$, $P = 0.387$ for 25, 50 and 75 ng VP/μl, respectively), which indicates an absence of the endogenous diurnal increase. Separate ANOVAs on the different VP concentrations revealed that infusion of both the 25 and 50 ng VP/μl dosage, but not 75 ng VP/μl, differed significantly from the Ringer infusion ($F(1,12) = 8.12$, $P = 0.015$, $F(1,12) = 14.41$, $P = 0.003$ and $F(1,13) = 2.85$, $P = 0.115$, respectively).

Delivery of VP V₁-antagonist to the DMH in different concentrations caused a clear dose-dependent increase of plasma corticosterone levels (Fig. 4). Although both the 1 (data not shown) and 10 ng V₁-antagonist/μl perfusions caused an increase of mean plasma corticosterone levels, no significant effects were detected. With the intermediate concentrations, i.e. 25 ng and 50 ng V₁-antagonist/μl, clearly significant differences were found at $t = 40$ and $t = 60$. The highest dose of antagonist used (75 ng V₁-antagonist/μl) yielded an equivocal effect: although the mean levels were clearly increased at all time points after the VP-antagonist had entered the brain, due to the large variation in the reaction of the individual animals the difference reached significance only at timepoint $t = 20$ (Fig. 4D).

Discussion

Methodological considerations

Using an improved method for intracerebral infusions we provided evidence that (the circadian pattern of) VP secretion from SCN terminals is strongly implicated in the establishment of a diurnal rhythm of corticosterone secretion. Daily variations in plasma corticosterone concentrations in the rat, with a peak before the onset of activity, have been observed for a long time (26, 27) and described in a large number of different species. However, the endogenous release pattern of this adrenal hormone is easily disturbed by stimuli that disrupt the homeostasis of the animal. A large variety of stimuli is able to cause either large excursions of corticosterone or inhibit its daily rise (5, 28, 29). Therefore, in order to study the endogenous nature of the rise in plasma corticosterone at the end of the light period, it was essential that all experimental procedures could be performed without disturbing the animal. Contrary to previous results (30, 31) in our hands, the basal levels of corticosterone measured in cannulated and individually housed animals were not elevated as compared to those in animals sacrificed immediately after removal from their home cages. In spite of the continuous attachment to a metal collar and counterbalancing beam our curve is very much similar to those described recently by others using either jugular cannulation, tail vein sampling or decapitation (2, 32, 33).

Previous studies indicated that after invasive surgery a recovery time of one week is necessary for the animal to normalize its circadian rhythms of body temperature, locomotor activity, feed-

FIG. 3. Effect of VP infusions (A = 25 ng/ μ l; B = 50 ng/ μ l; C = 75 ng/ μ l) in the DMH during the last 4 h of the light period (CT7.5–CT11.5) on the endogenous rise of plasma corticosterone. MANOVA indicated clear effects of both infusion ($F(3, 23) = 4.32$, $P = 0.015$) and time ($F(5, 115) = 2.89$, $P = 0.017$) but not the interaction effect ($F(15, 115) = 1.44$, $P = 0.139$). Dotted lines indicate plasma corticosterone values of control animals in Exp. 1 (Means $\pm$ SEM). Ringer, $n = 8$; VP-25, $n = 6$; VP-50, $n = 6$; VP-75, $n = 7$. ** $P < 0.05$ as compared to CT7.5 and CT8; * $P < 0.05$ as compared to CT7.5; Ringer $\circ$; VP $\blacktriangle$.

ing behavior (34, 35), and probably plasma corticosterone. However, microdialysis experiments are predominantly performed on the first 2 days after implantation due to the rapid decrease in recovery of the dialysis membrane (36). Pilot experiments showed that the longevity of the probes was increased considerably by postponing its first usage (37). This finding is in line with other studies showing that tissue reactions to the mere presence of the probe, i.e. without perfusion, were minimal (36, 38). As shown by the Ringer-dialyzed controls in the present study (Fig. 3) plasma corticosterone levels indeed regained their normal circadian rhythm within one week after implantation of an intracerebral dialysis probe.

Control perfusions with the Ringer vehicle showed that with reverse dialysis it is indeed possible to deliver transmitters in the PVN/DMH area without any side effects of the administration itself on the basal activity of the HPA-axis (Figs 2 & 4). The endogenous rise in plasma corticosterone during the dialysis of Ringer at the end of the light period (Fig. 3) also shows that the dialysis procedure itself does not interfere with the diurnal rise in plasma corticosterone, and thus does not artificially suppress

corticosterone levels during midday perfusions. On the other hand, diffusion of sufficient amounts of peptide outside the probe is evidenced by the effects of VP and its V_1 -antagonist (Figs 2–4).

Vasopressin suppression of corticosterone release

High levels of VP released from SCN terminals during the light period coincide with basal levels of plasma corticosterone, as is clearly illustrated in Fig. 5. Therefore, we hypothesized that the increased VP release during the first part of the light period is responsible for the trough in corticosterone levels at this time of the day, whereas the arrest of the inhibitory VP signal at the end of the light period enables the rise of corticosterone secretion at the onset of the dark period (18, 19). Our initial results already indicated the inhibitory influence of VPergic neurons in the SCN upon the HPA-axis. In addition, these experiments showed that the elevated corticosterone levels in SCN-lesioned rats are not due to a decreased clearance. The rapid decrease of the increased plasma corticosterone levels upon infusion of VP in the PVN/DMH area of SCN-lesioned animals indicated a similar half-life time as in control animals (18). But, these first results

FIG. 4. Effect of V₁-antagonist infusions at different doses (A, B, C and D respectively 10, 25, 50 and 75 ng/μl) in the DMH at CT6–7 on plasma corticosterone levels. MANOVA indicated clear effects of infusion ($F(5, 47)=3.27$, $P=0.013$), time ($F(3, 141)=24.8$, $P<0.001$) and the interaction of 'infusion vs time' ($F(15, 141)=2.03$, $P=0.017$). Dotted lines indicate plasma corticosterone values of control animals in Exp. 1 (Means $\pm$ SEM). The number of animals used for the Ringer infusion was 14 and for the different dosages of V₁-antagonist (i.e. 1 (not shown), 10, 25, 50 and 75 ng/μl) the N was 4, 8, 12, 9 and 7, respectively. * $P<0.05$ as compared to Ringer. Ringer $\circ$; V₁-antagonist $\blacktriangle$.

were confounded by the concomitant stress-evoked release of corticosterone (18). The present experiments, however, unequivocally show the inhibitory effect of VP on the release of corticosterone at the level of the DMH. In the first place, extension of the endogenous period of VP release by infusion of (synthetic) VP during the last 4 h of the light period prevented the circadian rise of corticosterone at this time of the day (Fig. 3). Furthermore, blockade of V₁-receptors by applying different concentrations of the VP-antagonist d(CH₂)Tyr(Me)AVP in the DMH during the middle of the light period caused clear dose-dependent increases in plasma corticosterone levels (Fig. 4), revealing the inhibitory nature of endogenously released VP at this time of the day. The dose-dependency of both the increases of plasma corticosterone upon administration of the V₁-antagonist and inhibition of the endogenous rise in plasma corticosterone by application of VP confirm the physiological significance of the observed effects (Figs 3–4).

Clearest effects were attained with the intermediate doses (i.e. 25 and 50 ng/μl). At the highest dose both the effects of VP and its antagonist were not unequivocal, resulting in an inverted

U-shaped dose-response curve. Similar dose-dependent stimulatory and inhibitory effects have been described previously for VP (39–41). One hour after implantation *in vivo* transfer of VP outside the dialysis probe has been calculated to be 0.5% (23). By including a post-operative recovery time of several days after implantation this recovery declines even further, although this decline is considerably attenuated by postponing the first perfusion of the probe (36, 37). Therefore, the effective doses delivered in the present study are expected to be less than 25 nM, i.e. 0.1% of 25 ng/ml. This concentration is lower than the ones used in most infusion studies (1–100 μM) (40, 41), and only a little higher than the concentration of VP expected to saturate the V₁ receptors ($K_d=3$ nM). However, especially with the higher doses used it may be expected that in the vicinity of the probe much higher concentrations are reached due to the limited diffusion in the interstitial fluid. Accumulation of VP and the V₁-antagonist, especially in the first hour after onset of perfusion, probably explains the sometimes opposite effects of the highest doses used (Figs 3C & 4D).

These results indicate that by changing its VP input to the

FIG. 5. Composite drawing showing the temporal relation between plasma corticosterone levels and VP release by SCN terminals. Corticosterone data are the same as in Fig. 1 and data on VP release are derived from our previous study (17).

PVN/DMH area the SCN can control the timing of the endogenous corticosterone peak. *In vitro* (42, 43) and *in vivo* (17) results showed that indeed VP release by SCN fibers is changing with a 24-h rhythm within the surroundings of the SCN. Recently, the high (non-rhythmic) intranuclear release of intrinsic magnocellular VP elements prevented assessment of a diurnal VP input into another SCN target area, i.e. the PVN (17). Therefore, up till now it has not been possible to monitor a diurnal pattern of VP release in SCN projection areas other than the SCN itself. But measurement of daily changes in VP levels in the CSF are suggestive of a concomitant change of VP output by SCN fibers in all its target areas (13, 15, 16). These data together with previous data on the origin of the VPergic PVN/DMH innervation from the SCN (44, 45), and the abundant presence of V_{1A} receptor mRNA in the DMH (46, 47) indicate a physiological role for the VPergic SCN projection to the DMH in the control of corticosterone release.

Stimulation of corticosterone release

The question remains whether the mere presence or absence of the inhibitory VP signal is sufficient to create a circadian corticosterone rhythm. Indeed, the simultaneous appearance of both low corticosterone levels and minimal VP release during the dark period (Fig. 5) shows that VP cannot be the only factor responsible for low corticosterone release. Furthermore, also the high levels of corticosterone attained with the V_1 -antagonist, i.e. higher than the endogenous peak, indicate that VP is not the only factor governing basal corticosterone release. In 1987 Cascio *et al.* reached the conclusion that the SCN has a stimulatory effect on

the release of corticosterone since SCN-lesioned animals showed basal levels of plasma ACTH and corticosterone but no circadian rise in the afternoon. However, these findings were based on only two sampling points (i.e. AM and PM) and more frequent sampling has shown a clear increase in the basal secretory activity of the adrenal gland during the light period in SCN-lesioned animals (11, 12, 18, 20), confirming the inhibitory nature of the SCN. Still, the fact that SCN-lesioned animals do not exhibit plasma corticosterone levels continuously at peak concentrations (i.e. 100–150 ng/ml) indicates the existence of an additional stimulatory input from the SCN. Most likely this stimulatory factor also shows a circadian release pattern with increased activity during the second half of the light period. Infusion of the VP-antagonist at CT6 reveals the full strength of this stimulatory input, whereas during the endogenous corticosterone peak the release of VP (albeit decreasing (Fig. 5)) allows only a partial expression. This scenario also explains the higher levels of corticosterone reached during infusion of VP-antagonist as compared to those of the normal circadian rise. Absence of this stimulatory factor during the dark period would also explain decreasing corticosterone levels in this period unless the absence of substantial VP release. Probable transmitter candidates for this stimulatory SCN signal are VIP/PHI and GRP. Both transmitters are present in SCN projections towards the PVN/DMH complex (45, 48) and infusion studies have shown release of corticosterone upon ICV or PVN application of either VIP/PHI or GRP (49–51). Furthermore, the rhythm of VIP synthesis and release by the SCN is opposite to that of VP, i.e. increasing activity towards the end of the light period (52, 53). Current experiments

at our laboratory are aimed at elucidating the role of endogenously released VIP and GRP in the PVN/DMH area in the release of corticosterone.

Conclusions

The present study shows that endogenously released VP in the DMH inhibits HPA-axis activity during the light period, and that the absence of VP release in the DMH is a prerequisite for the occurrence of the circadian rise in corticosterone before the onset of darkness. These data therefore provide compelling evidence that the decrease of VP release from SCN terminals in the DMH at the end of the light period sets a time window for the endogenous rise in plasma corticosterone. However, apart from this inhibitory VP signal there is need for an additional stimulatory SCN signal in order to explain the complete circadian profile of plasma corticosterone levels.

Materials and methods

Male Wistar rats (TNO Zeist, The Netherlands) were used in all experiments. They were kept in a temperature-controlled environment (20–22 °C) on a 12 h light/12 h dark schedule (lights on at 07.00 h or 04.00 h (only the VP-dialyzed animals of Exp. 3)). Before the start of experiments animals were allowed to acclimatize to the lighting schedule for at least 3 weeks with 4–6 animals per cage. During experiments animals were housed in individual cages (38 × 26 × 16 cm), with food and water available *ad libitum*.

Experimental animals for dialysis and blood sampling studies were provided with a microdialysis probe and a silicone catheter in the right atrium when their bodyweight was >300 g. The microdialysis probe was stereotactically implanted with its U-shaped end positioned just lateral to the DMH (coordinates with flat skull: 2.8 mm caudal to bregma; 1.6 mm lateral to the midline; 8.0 mm below the brain surface; with a 10° angle to the sagittal plane). The dialysis probe consisted of a dialysis fiber (molecular weight cut-off 6 KDa, 3 mm in total length), glued into the ends of parallel lengths of 25-gauge stainless steel tubing. The loop of the probe was positioned in the rostrocaudal direction along the DMH. The jugular venectomy was performed according to the method of Steffens (54). We secured the probe- and atrial outlet to two stainless steel screws inserted in the skull with dental cement.

After implantation of the microdialysis probe and the jugular catheter at least 1 week was allowed for postoperative recovery before experiments. During this period, the animals were accustomed to the experimental conditions in Plexiglas cages designed to allow blood sampling and longterm dialysis in unrestrained animals. All experiments were performed in the animals' own home cages. After each sample a transfusion of an equal volume of heparinized donor blood was given to avoid diminution of the blood volume with related changes in hemodynamics. Donor blood was obtained from undisturbed rats with permanent heart catheters (55, 56).

Microdialysis mediated administration of VP was preferred over the previously used microinfusions (18). A great advantage of the microdialysis technique is that no fluid is introduced into the extracellular space of the brain and no moving fluid is in contact with brain tissue. Both events will inevitably activate CRF-containing neuroendocrine 'motorneurons' in the case of PVN/DMH infusions and thus cause increased corticosterone levels. Previous studies have shown that both VP and its V_1 -antagonist can be administered effectively to a specific brain area via reverse dialysis in free moving animals (23, 57, 58).

Experiment 1

Experiments began with establishing the circadian profile of plasma corticosterone in our experimental conditions: i.e. solitary housing, jugular cannulation, permanent presence of an experimenter. In addition, these animals were permanently connected to the blood sampling catheter which was protected by a metal collar and kept free from the animals by means of a counterbalanced beam. In this way all manipulations occurred

outside the cages and no handling was necessary in the hours preceding the start of an experiment. The complete circadian profile was determined by taking 0.25 ml blood samples once every hour from 10 rats during 9 consecutive hours on 3 different occasions. The 3 runs of 9 h, with the first sample of each run overlapping with the last sample of the previous run, were completed in 2 weeks. Different runs started at CT6.5, CT14.5 and CT22.5 (CT12 being the beginning of the dark period).

Experiment 2

This experiment was designed to establish the feasibility of the reverse dialysis technique as a new tool for local delivery of VP or its antagonist into the brain without activation of the HPA-axis by the infusion procedure *per se*. One-hour microinfusions of VP (50 ng/μl) or the V_1 -antagonist (50 ng/μl) were performed during the circadian trough of the corticosterone rhythm (i.e. CT 6–8). Four h earlier the microdialysis probe was connected to an extended piece of tubing and a Ringer perfusion was started (200 μl/hour). Lagtime from syringe to microdialysis probe was 40 min. Blood samples were taken every 20 min starting at CT6.10 (i.e. 20 min before the calculated entrance of VP or the V_1 -antagonist into the brain) until 60 minutes after ending the 1-h infusion. Ringer-perfused controls were treated in the same way, including the change of syringes at CT5.50 and CT6.50. All animals ($n=5-6$) were used only once, at 6 days after implantation of the dialysis probe.

Experiment 3

Experiment 3 served to test the capacity of VP infused in the DMH to prevent the circadian rise in plasma corticosterone and the dose-response curve for this effect. A dose-response curve for the V_1 -antagonist-induced rise of plasma corticosterone at CT6 was also established. Experiments were performed 6–9 days after implantation of the dialysis probe, each animal receiving one infusion on 2 or 3 separate days. The start of the experiments was similar to that of experiment 2, i.e. animals were connected to extended pieces of tubing and a Ringer perfusion was started 3–4 h before the start of either the VP or the V_1 -antagonist administration. In order to block the circadian rise of plasma corticosterone, VP was dialyzed for 4 h, from CT7.30 onwards, with VP doses of 0, 25, 50 or 75 ng/μl. Blood samples were taken at $t=0, 30, 60, 120, 180$ and 240 min after calculated entrance of VP in the brain. V_1 -antagonist dialysis was performed from CT6–CT7, with doses of either 0, 1, 10, 25, 50 or 75 ng/μl and blood samples taken every 20 min for one h, starting at CT6.

Histology

At the completion of the experimental protocol, animals were anesthetized with pentobarbital (Nembutal; 60 mg/kg i.v.), and brains were removed after decapitation. Brains were subsequently blocked, frozen, sectioned (40 μm) through the region of the PVN/DMH, and stained with cresyl violet. Subsequent analysis of probe placement showed that in all cases the dialysis membrane was positioned just lateral to the DMH similar as shown previously (59).

Measurement of corticosterone

Plasma samples were assayed for corticosterone by RIA (ICN Biomedicals, Costa Mesa, CA, USA). From the samples 10 μl was taken and diluted in 4 ml assay buffer. The inter- and intra-assay coefficients of variance were 7% and 4%, respectively, with a detection limit of 1 ng/ml.

Materials

Vasopressin and the vasopressin V_1 -antagonist (Manning compound) were obtained from Saxon Biochemicals GMBH (Hannover, Germany). All other chemicals were obtained from Merck (Darmstadt, Germany).

Calculations and statistical procedures

All results are expressed as mean $\pm$ SEM. Multivariate analysis of variance (MANOVA) with repeated measures design was used to determine whether the effects of the different infusates on plasma corticosterone concentrations differed significantly. In cases where MANOVA detected a significant effect this was followed by the Student Newman-Keuls post-hoc procedure to assess the specific timepoint of these differences (Figs. 2 & 4). With respect to the diurnal peak in corticosterone release (Fig. 3) the significance of the diurnal variation (time dependency) was assessed using a one-way ANOVA with repeated measures. If significant, ANOVA was followed by a Student's *t*-test (paired) to establish the time points

that differed from 'time 0' values. For multiple comparisons the Bonferroni correction was used. $P=0.05$ was accepted as a level of significance.

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References

- Dallman MF, Strack AM, Akana SF, *et al.* Feast and famine—Critical role of glucocorticoids with insulin in daily energy flow. *Front Neuroendocrinol* 1993; **14**: 303–347.
- De Boer SF, Van Der Gugten J. Daily variations in plasma nor-adrenaline, adrenaline and corticosterone concentrations in rats. *Physiol Behav* 1987; **40**: 323–328.
- Fischman AJ, Kastin AJ, Graf MV, Moldow RL. Constant light and dark affect the circadian rhythm of the hypothalamic–pituitary–adrenal axis. *Neuroendocrinology* 1988; **47**: 309–316.
- Takahashi K, Inoue K, Takahashi Y. Parallel shift in circadian rhythms of adrenocortical activity and food intake in blinded and intact rats exposed to continuous illumination. *Endocrinology* 1977; **100**: 1097–1107.
- Slusher MA. Effects of chronic hypothalamic lesions on diurnal and stress corticosteroid levels. *Am J Physiol* 1964; **206**: 1161–1164.
- Halász B, Slusher MA, Gorski RA. Adrenocorticotrophic hormone secretion in rats after partial or total deafferentation of the medial basal hypothalamus. *Neuroendocrinology* 1967; **2**: 43–55.
- Moore RY, Lenn NJ. A retinohypothalamic projection in the rat. *J Comp Neurol* 1972; **146**: 1–9.
- Hendrickson AE, Wagoner N, Cowan WM. An autoradiographic and electron microscopic study of retino–hypothalamic connections. *Z Zellforsch* 1972; **135**: 1–26.
- Moore RY, Eichler VB. Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. *Brain Res* 1972; **42**: 201–206.
- Cascio CS, Shinsako J, Dallman MF. The suprachiasmatic nuclei stimulate evening ACTH secretion in the rat. *Brain Res* 1987; **423**: 173–178.
- Abe K, Kroning J, Greer MA, Critchlow V. Effects of destruction of the suprachiasmatic nuclei on the circadian rhythms in plasma corticosterone, body temperature, feeding and plasma thyrotropin. *Neuroendocrinology* 1979; **29**: 119–131.
- Szafarczyk A, Ixart G, Malaval F, Nouguié-Soule J, Assenmacher I. Effects of lesions of the suprachiasmatic nuclei and of p-chlorophenylalanine on the circadian rhythms of adrenocorticotrophic hormone and corticosterone in the plasma, and on locomotor activity of rats. *J Endocr* 1979; **83**: 1–16.
- Uhl GR, Reppert SM. Suprachiasmatic nucleus vasopressin messenger RNA: circadian variation in normal and Brattleboro rats. *Science* 1986; **232**: 390–393.
- Schwartz WJ, Coleman RJ, Reppert SM. A daily vasopressin rhythm in rat cerebrospinal fluid. *Brain Res* 1983; **263**: 105–112.
- Reppert SM, Artman HG, Swaminathan S, Fisher DA. Vasopressin exhibits a rhythmic daily pattern in cerebrospinal fluid but not in blood. *Science* 1981; **213**: 1256–1257.
- Schwartz WJ, Reppert SM. Neural regulation of the circadian vasopressin rhythm in cerebrospinal fluid: A pre-eminent role for the suprachiasmatic nuclei. *J Neurosci* 1985; **5**: 2771–2778.
- Kalsbeek A, Buijs RM, Engelmann M, Wotjak CT, Landgraf R. In vivo measurement of a diurnal variation in vasopressin release in the rat suprachiasmatic nucleus. *Brain Res* 1995; **682**: 75–82.
- Kalsbeek A, Buijs RM, Van Heerikhuizen JJ, Arts M, Van Der Woude TP. Vasopressin-containing neurons of the suprachiasmatic nuclei inhibit corticosterone release. *Brain Res* 1992; **580**: 62–67.
- Kalsbeek A, Buijs RM. Peptidergic transmitters of the suprachiasmatic nuclei and the control of circadian rhythmicity. In: Joosse J, Buijs RM, and Tilders FJH, eds. *Progress in Brain Research*, Vol. 92, *The Peptidergic Neuron*, 1992; 321–333. Elsevier, Amsterdam.
- Buijs RM, Kalsbeek A, Van Der Woude TP, Van Heerikhuizen JJ, Shinn S. Suprachiasmatic nucleus lesion increases corticosterone secretion. *Am J Physiol* 1993; **264**: R1186–R1192.
- Van Erp AMM, Kruk MR, Semple DM, Verbeet DWP. Initiation of self-grooming in resting rats by local PVH infusion of oxytocin but not α -MSH. *Brain Res* 1993; **607**: 108–112.
- Quan N, Blatteis CM. Microdialysis: A system for localized drug delivery into the brain. *Brain Res Bull* 1989; **22**: 621–625.
- Engelmann M, Ludwig M, Landgraf R. Microdialysis administration of vasopressin and vasopressin antagonists into the septum during pole-jumping behavior in rats. *Behav Neural Biol* 1992; **58**: 51–57.
- Akana SF, Dallman MF, Bradbury MJ, *et al.* Feedback and facilitation in the adrenocortical system — unmasking facilitation by partial inhibition of the glucocorticoid response to prior stress. *Endocrinology* 1992; **131**: 57–68.
- De Souza EB, Van Loon GR. Stress-induced inhibition of the plasma corticosterone response to a subsequent stress in rats: A nonadrenocorticotropin-mediated mechanism. *Endocrinology* 1982; **110**: 23–33.
- McCarthy JL, Corley RC, Zarrow MX. Diurnal rhythm in plasma corticosterone and lack of diurnal rhythm in plasma compound F-like material in the rat. *PSEBM* 1960; **104**: 787–789.
- Guillemin R, Dear WE, Liebelt RA. Nychtemeral variations in plasma free corticosteroid levels in the rat. *PSEBM* 1959; **101**: 394–395.
- Seggie JA, Brown GM. Stress response patterns of plasma corticosterone, prolactin, and growth hormone in the rat, following handling or exposure to novel environment. *Can J Physiol Pharmacol* 1975; **53**: 629–637.
- Donnerer J, Lembeck F. Different control of the adrenocorticotropin–corticosterone response and of prolactin secretion during cold stress, anesthesia, surgery, and nicotine injection in the rat: involvement of capsaicin-sensitive sensory neu. *Endocrinology* 1990; **126**: 921–926.
- Fagin KD, Shinsako J, Dallman MF. Effects of housing and chronic cannulation on plasma ACTH and corticosterone in the rat. *Am J Physiol* 1983; **245**: E515–E520.
- Greco AM, Gambardella P, Sticchi R, D'Aponte D, De Francis P. Circadian rhythms of hypothalamic norepinephrine and of some circulating substances in individually housed adult rats. *Physiol Behav* 1992; **52**: 1167–1172.
- Kwak SP, Morano MI, Young EA, Watson SJ, Akil H. Diurnal CRH messenger RNA rhythm in the hypothalamus—Decreased expression in the evening is not dependent on endogenous glucocorticoids. *Neuroendocrinology* 1993; **57**: 96–105.
- Ishikawa M, Ohdo S, Watanabe H, Hara C, Ogawa N. Alteration in circadian rhythm of plasma corticosterone in rats following sociopsychological stress induced by communication box. *Physiol Behav* 1995; **57**: 41–47.
- Farr LA, Campbell-Grossman C, Mack JM. Circadian disruption and surgical recovery. *Nurs Res* 1988; **37**: 170–175.
- Drijfhout WJ, Grol CJ, Westerink BHC. Microdialysis in the rat pineal gland, monitoring the innervation and hormonal response of a peripheral gland. In: Louilot A, Durkin T, Spampinato U, Cador M, eds. *Monitoring Molecules in Neuroscience. Proceedings of the 6th International Conference on in vivo Methods*, Seignosse, 1994: 367–368.
- Landgraf R, Horn T, Neumann I, Pittman QJ, Ludwig M. Effects of osmotic stimulation of the supraoptic nucleus on central and peripheral release of vasopressin and on baro- and thermoregulation. In: Pleschka K, Gerstberger R, eds. *Integrative and Cellular Aspects of Autonomic Functions: Temperature and Osmoregulation*. Paris: John Libbey Eurotext, 1994: 419–428.
- Kalsbeek A, Buijs RM. Inhibitory control of the suprachiasmatic nuclei on both corticosterone and melatonin release patterns as revealed by *in vivo* microdialysis. In: Buijs RM, Kalsbeek A, Mirmiran M, Pennartz CMA, Romijn HJ, eds. *Hypothalamic Integration of Circadian Rhythms, Progress in Brain Research*, Vol. 95, Amsterdam: Elsevier Science Publishers BV: 1995.
- Woodroffe MN, Sarna GS, Wadhwa M, *et al.* Detection of interleukin-1 and interleukin-6 in adult rat brain, following mechanical injury, by *in vivo* microdialysis: evidence of a role for microglia in cytokine production. *J Neuroimmunol* 1991; **33**: 227–236.

- 39 Diamant M, De Wied D. Differential Effects of Centrally Injected AVP on Heart Rate, Core Temperature, and Behavior in Rats. *Am J Physiol* 1993; **264**: R51–R61.
- 40 Hennessey AC, Whitman DC, Albers HE. Microinjection of arginine-vasopressin into the periaqueductal gray stimulates flank marking in syrian hamsters (*Mesocricetus auratus*). *Brain Res* 1992; **569**: 136–140.
- 41 Wang ZX, Ferris CF, De Vries GJ. Role of septal vasopressin innervation in paternal behavior in prairie voles (*Microtus ochrogaster*). *Proc Natl Acad Sci USA* 1994; **91**: 400–404.
- 42 Earnest DJ, Sladek CD. Circadian rhythms of vasopressin release from individual rat suprachiasmatic explants *in vitro*. *Brain Res* 1986; **382**: 129–133.
- 43 Gillette MU, Reppert SM. The hypothalamic suprachiasmatic nuclei: circadian patterns of vasopressin secretion and neuronal activity *in vitro*. *Brain Res Bull* 1987; **19**: 135–139.
- 44 Hoorneman EMD, Buijs RM. Vasopressin fiber pathways in the rat brain following suprachiasmatic nucleus lesioning. *Brain Res* 1982; **243**: 235–241.
- 45 Kalsbeek A, Tecler-Mariam-Mesbah R, Pévet P. Efferent projections of the suprachiasmatic nucleus in the golden hamster (*Mesocricetus auratus*). *J Comp Neurol* 1993; **332**: 293–314.
- 46 Ostrowski NL, Lolait SJ, Young WS. Cellular localization of vasopressin V1a receptor messenger ribonucleic acid in adult male rat brain, pineal, and brain vasculature. *Endocrinology* 1994; **135**: 1511–1528.
- 47 Szot P, Bale TL, Dorsa DM. Distribution of messenger RNA for the vasopressin V1a receptor in the CNS of male and female rats. *Mol Brain Res* 1994; **24**: 1–10.
- 48 Watts AG, Swanson LW. Efferent projections of the suprachiasmatic nucleus: II. Studies using retrograde transport of fluorescent dyes and simultaneous peptide immunohistochemistry in the rat. *J Comp Neurol* 1987; **258**: 230–252.
- 49 Gunion MW, Tache Y, Rosenthal MJ, *et al*. Bombesin microinfusion into the rat hypothalamic paraventricular nucleus increases blood glucose, free fatty acids and corticosterone. *Brain Res* 1989; **478**: 47–58.
- 50 Olsen L, Knigge U, Warberg J. Gastrin-releasing peptide stimulation of corticotropin secretion in male rats. *Endocrinology* 1992; **130**: 2710–2716.
- 51 Alexander LD, Sander LD. Vasoactive intestinal peptide stimulates ACTH and corticosterone release after injection into the PVN. *Regul Pept* 1994; **51**: 221–227.
- 52 Inouye SIT, Shibata S. Neurochemical organization of circadian rhythm in the suprachiasmatic nucleus. *Neurosci Res* 1994; **20**: 109–130.
- 53 Yang J, Cagampang FRA, Nakayama Y, Inouye SIT. Vasoactive intestinal polypeptide precursor messenger RNA exhibits diurnal variation in the rat suprachiasmatic nuclei. *Mol Brain Res* 1993; **20**: 259–262.
- 54 Steffens AB. A method for frequent sampling blood and continuous infusion of fluids in the rat without disturbing the animal. *Physiol Behav* 1969; **4**: 833–836.
- 55 Wiersma J, Kastelijn J. A chronic technique for high frequency blood sampling/transfusion in the freely behaving rat which does not affect prolactin and corticosterone secretion. *J Endocr* 1985; **107**: 285–292.
- 56 Wiersma J, Kastelijn J. Haematological, immunological and endocrinological aspects of chronic high frequency blood sampling in rats with replacement by fresh or preserved donor blood. *Lab Animals* 1986; **20**: 57–66.
- 57 Engelmann M, Bures J, Landgraf R. Vasopressin administration via microdialysis into the septum interferes with the acquisition of spatial memory in rats. *Neurosci Lett* 1992; **142**: 69–72.
- 58 Engelmann M, Landgraf R. Microdialysis administration of vasopressin into the septum improves social recognition in Brattleboro rats. *Physiol Behav* 1994; **55**: 145–149.
- 59 Kalsbeek A, Drijfhout WJ, Westerink BHC, Van Heerikhuizen JJ, Van Der Woude T, Van Der Vliet J, Buijs RM. GABA receptors in the region of the dorsomedial hypothalamus of rats are implicated in the control of melatonin. *Neuroendocrinology* 1995; **63**: 69–78.