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Temazepam Triggers the Release of Vasopressin into the Rat Hypothalamic Paraventricular Nucleus: Novel Insight into Benzodiazepine Action on Hypothalamic–Pituitary–Adrenocortical System Activity During Stress

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We investigated the influence of a representative classical benzodiazepine on the regulation of the hypothalamic–pituitary–adrenocortical (HPA) axis activity both under basal conditions and stress. Adult male Wistar rats were intravenously administered with temazepam (0.5, 1, and 3 mg/kg body weight) and plasma concentrations of corticotropin (ACTH) and vasopressin (AVP) were measured in blood samples collected via chronically implanted jugular venous catheters. Simultaneously, the release of AVP within the hypothalamic paraventricular nucleus (PVN) was monitored via microdialysis. Plasma AVP levels remained unaffected by the different treatment conditions. Temazepam blunted the stressor exposure-induced secretion of ACTH in a dose-dependent manner. Concurrently, and also in a dose-dependent manner temazepam enhanced the intra-PVN release of AVP, known to originate from magnocellular neurons of the hypothalamic neurohypophyseal system. Furthermore, temazepam did not affect the *in vitro* secretion of ACTH from the adenohypophyseal cells. Taken together, the results of this study suggest that temazepam modulates the central nervous regulation of the HPA axis by altering intra-PVN AVP release. An increasingly released AVP of magnocellular origin seems to provide a negative tonus on ACTH secretion most probably via inhibiting the release of ACTH secretagogues from the median eminence into hypophyseal portal blood.

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INTRODUCTION

Changes in hypothalamic–pituitary–adrenocortical (HPA) system regulation have repeatedly been demonstrated to be critically involved in the pathogenesis of affective disorders such as major depression (Keck and Holsboer, 2001; Keck *et al*, 2005). In fact, a restoration of normal HPA system function has been shown to be a prerequisite for remission (Holsboer, 2000). 'Classical' benzodiazepines are known to act in a sedative and anxiolytic manner but also to normalize the HPA axis activity (Schuckit *et al*, 1992; Korbonits *et al*, 1995). Among the signals which seem to contribute to HPA axis regulation at the level of the hypothalamus, vasopressin (AVP) seems to play an important role (Wotjak *et al*, 2002). AVP is synthesized not only in magnocellular neurons, which are part of the hypothalamic–neurohypophysial system (HNS), but also in parvocellular neurons of the paraventricular nucleus (PVN). The latter neurons project to the external layer of the median eminence, where it is released from nerve terminals in response to appropriate stimuli (eg physical stress) and enters fenestrated portal capillaries. Transported to the anterior pituitary, AVP then stimulates the secretion of corticotropin (ACTH) synergistically with corticotropin-releasing hormone (CRH) (Antoni, 1993). ACTH, in turn, triggers the release of cortisol/corticosterone (CORT) from the adrenal glands which mediates a variety of adaptive

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responses to stress. Previous studies investigating the effects of temazepam on HPA axis regulation relied on the measurement of ACTH and CORT in the general circulation and extrapolated the altered plasma levels to the putative underlying central effects. Korbonits *et al* (1995), for example reported an inhibition of the CRH-induced release of ACTH and CORT in healthy volunteers, which was taken as evidence that temazepam acts via a reduction of hypothalamic AVP release.

The present study was designed to investigate the effects of different dosages of temazepam on HPA system regulation in male Wistar rats under basal conditions and in response to 5 min of forced swimming, a combined physical and emotional stressor. *In vivo* microdialysis was used to monitor the intrahypothalamic release patterns of AVP from magnocellular neurons in the PVN. Intra-PVN release of AVP into the extracellular compartment has been suggested to have an inhibitory effect on the secretion of ACTH from the anterior pituitary (Wotjak *et al*, 1996; Wotjak *et al*, 1998). To investigate simultaneous changes in the release patterns of ACTH and AVP into the peripheral blood, blood samples were taken via chronically implanted jugular venous catheters. Plasma ACTH reflects the activity of the central nerval components of the HPA axis, that is, the secretory activity of the parvocellular neurons, whereas plasma AVP reflects the activity of the HNS, that is, the activity of axon terminals of magnocellular neurons projecting to the posterior pituitary. We extended these studies by analyzing the influence of temazepam on both basal and stimulated ACTH release from primary rat anterior pituitary cell cultures to control for possible direct effects of the drug on corticotrope cells.

MATERIALS AND METHODS

In Vivo Studies

Animals. The animal studies were conducted in accordance with the Guide for Care and Use of Laboratory Animals of the Government of Bavaria and the guidelines of the NIH. The experiments were performed on young adult male Wistar rats ($n = 59$; 3 months old; 320 ± 20 g body weight) that were housed in groups of six in the breeding unit of the Institute under standard laboratory conditions (12 h light : 12 h dark cycle with lights on at 0700, $22 \pm 1^{\circ}$C, 60% humidity, pelleted food and water *ad libitum*) for at least 1 week after delivery from the supplier (Charles River, Sulzfeld, Germany).

Surgery. All surgical procedures were performed under halothane anesthesia and aseptic conditions (Keck *et al*, 2000, 2002).

Implantation of microdialysis probese. The implantation of microdialysis probes was essentially performed as described in detail elsewhere (Horn and Engelmann, 2001). Briefly, 3 days before the experiment, a U-shaped microdialysis probe (dialysis membrane: molecular cutoff of 18 kDa; Hemophan™, Gambro Dialysatoren, Hechingen, Germany) was implanted stereotaxically according to the atlas of Paxinos and Watson (Paxinos and Watson, 1986) with their tips aimed at the right PVN (1.5 mm caudal to bregma, 1.8 mm lateral to midline, 8.9 mm beneath the surface of the skull, angle of 10° to avoid sagital sinus damage). The probe was secured with dental cement to two stainless-steel screws inserted into the bone.

Implantation of jugular venous catheters. Immediately after implantation of the microdialysis probe, the jugular vein was chronically catheterized for subsequent blood sampling as described elsewhere (Keck *et al*, 2001). The catheter was exteriorized at the neck of the animal and filled with sterile saline containing gentamicin (30 000 IU/rat; Gentamycin, Centravet, Bad Bentheim, Germany). Approximately 0.2 ml of the gentamycin solution was also infused into the animal.

Forced swimming. The forced swimming apparatus consisted of a cylindrical Plexiglas tank that was 40 cm high and 18 cm in diameter. The cylinder was filled with tap water (23°C) up to a level of 25 cm. Rats were transferred to the Plexiglas tank from their home cages, forced to swim in the apparatus for 5 min, gently dried with a towel and returned to their home cages. The water was changed after each animal. During forced swimming, the behavior was recorded and subsequently analyzed. The following parameters were measured (Wotjak *et al*, 1998): Struggling as the time spent in movements of the fore limbs with breaking the surface of the water (eg scratching along the wall). Swimming as duration spent in fore and hind limb movements without breaking the water's surface with the fore limbs. Floating, defined as the behavior during which the animal uses just enough limb movement to keep afloat, with no struggling in the trunk.

Experimental protocol. The animals were divided into the following groups (temazepam: 0.5 mg/kg $n = 13$, 1 mg/kg $n = 14$, 3 mg/kg $n = 21$; vehicle $n = 11$). At 0700 on the experimental day, jugular venous catheters were connected via 50-cm PE-50 tubing to plastic syringes filled with sterile heparinized saline (30 IU/ml; Heparin, Ratiopharm, Ulm, Germany). Simultaneously, microdialysis probes were connected to a microinfusion pump via PE-20 tubing and perfused with sterile Ringer's solution (3.3 µl/min) for 3 h without sampling to adapt to the equipment (Keck *et al*, 2000). To monitor intra-PVN release of AVP under basal conditions, after administration of temazepam or vehicle and in response to forced swimming, eight consecutive 30-min microdialysates were sampled from the right PVN. Microdialysates were directly collected in Eppendorf tubes and immediately stored at −80°C until measurement. During collection of the third microdialysis sample, temazepam or vehicle were administered intravenously. Animals were subjected to 5 min of forced swimming during the fifth dialysis period. To simultaneously monitor the release of ACTH and AVP into blood five blood samples were taken. Two samples were drawn during the second and fourth microdialysis period and another three 5, 15, and 60 min after forced swimming. Blood samples were collected in prechilled tubes containing EDTA and a protease inhibitor (10 µl aprotinin; Trasylol™, Bayer, Germany) and centrifuged (5 min, 4000 r.p.m., 4°C). Plasma samples were stored at −20°C until measurement. Figure 1 illustrates the experimental protocol.

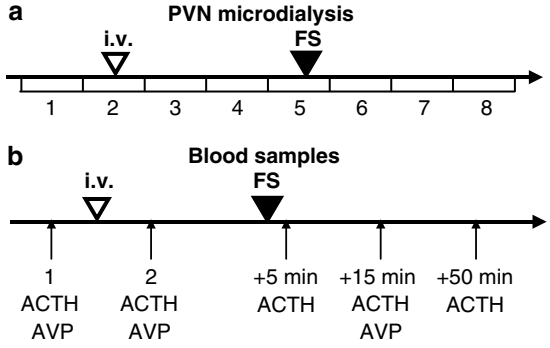

Figure 1 Experimental protocols. (a) PVN microdialysis and (b) jugular venous catheter blood sampling in relation to i.v. drug infusion (white triangle) and a 5 min forced swimming session (black triangle, FS). (a) Temazepam or vehicle were infused during collection of microdialysis sample 2; the 5 min of forced swimming session at 23°C was performed during collection of microdialysis sample 5. (b) Blood sample 1 was collected 45 min after beginning of the experiment, sample 2 45 min after application of temazepam or vehicle.

Postmortem analysis and histology. Animals were killed by an overdose of halothane at the end of the experiments. Brains were removed, frozen in prechilled *n*-methylbutane on dry ice and stored at −80°C. For histological analysis of the placement of the microdialysis probes, 25 µm coronal cryostat sections were cut and stained with cresyl violet. Furthermore, we visually inspected in all animals the pituitary, adrenals, and other defined internal organs (thymus, etc) to ensure that all animals used were free from tumors.

Treatment of blood samples and radioimmunoassays. Plasma ACTH concentrations were measured using a commercially available radioimmunoassay kit according to the protocol of the manufacturer (Biochem, Freiburg, Germany; sensitivity: <1.0 pg/ml). The intra-assay coefficient of variation was 7%. AVP was measured in lyophilized dialysates by a highly sensitive and selective radioimmunoassay (detection limit: 0.1 pg/sample; crossreactivity of the antisera with other related peptides, including oxytocin, was <0.7%; for a detailed description see Landgraf and Neumann (2004).

Rat pituitary cell culture. For cell culture, material and reagents, except where stated, were purchased from Flow Laboratories (Meckenheim, Germany), Gibco BRL Life Technologies (Eggenstein, Germany), Seromed (Berlin, Germany), Sigma Chemical Co. (St Louis, MO, USA), Falcon (Heidelberg, Germany) or Nunc (Wiesbaden, Germany).

Primary rat pituitary cell cultures were established as previously described (Renner *et al*, 1995). In brief, pitutaries of male Sprague–Dawley rats were washed in preparation buffer (137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 15 mM HEPES, 10 mM glucose, 2.5 mg/l amphotericin-B, 10^5 U/l penicillin/streptomycin, pH 7.3) and cut into small pieces. The tissue fragments were mechanically and enzymatically dispersed in preparation buffer containing 1000 U/ml collagenase (Worthington Biochemical Corp., Lakewood, NJ), 4 g/l BSA, 10 mg/l DNAase II, 1 g/l trypein inhibitor, 2 g/l hyaluronidase. The dispersed cells were washed by repetitive centrifugation and resuspension, and were then finally resuspended in DMEM culture medium (pH 7.3) supplemented with 10% heat-inactivated fetal calf serum, 2.2 g/l $NaHCO_3$, 10 mM HEPES, 2 mM glutamine, 10 ml/l nonessential amino acids (Biochrom AG, Berlin, Germany), 10 ml/l MEM vitamins (Biochrom AG, Berlin, Germany), 5 mg/l insulin, 5 mg/l transferrin, 2.5 mg/l amphotericin-B, 10^5 U/l penicillin/streptomycin, 20 µg/l sodium selenit, and 30 pM T_3 (Henning, Germany). Cell viability was consistently over 90% as assessed by acridine orange/ethidium bromide staining. Cells were plated in 48-well plates (100 000 cells/well in 0.5 ml culture medium) and incubated for 2–3 days in a 5% CO_2 atmosphere at 37°C until the cells had attached to the plates.

Hormone stimulation in vitro. For ACTH stimulation, the initial culture medium was removed, the cells were washed with phosphate-buffered saline and stimulation medium (DMEM pH 7.3, supplemented with 0.5 g/l BSA, 2.2 g/l $NaHCO_3$, 10 mM HEPES, 2 mM glutamine, 30 mg/l ascorbic acid) was added. CRH, AVP, and temazepam were diluted in a small amount of stimulation medium and were applied to the cell cultures alone or in combination at various concentrations as indicated. After a stimulation period of 3 h, ACTH was measured in the cell culture supernatants by RIA using an N-terminal-specific antibody as previously described (Arzt *et al*, 1993).

Statistics. Results are represented as means ± SEM. Statistical analysis was performed with a statistical software package (GB-Stat version 6.0, Dynamic Microsystems, Silver Spring, MD, USA). Statistical significance was determined between groups by two-way analysis of variance (ANOVA) (treatment × time) for repeated measures. When appropriate, Fishers LSD test for *post hoc* comparison was used. A value of $p<0.05$ was considered statistically significant.

RESULTS

In Vivo Studies

Experimental animals were included in the statistical analysis of the intra-PVN release of AVP only if the microdialysis probes were found to be correctly localized within the right PVN and visual examination confirmed no organ pathology *post mortem*. In total, 54% of microdialysis probes were implanted correctly ($n=32$). Furthermore, in some of the animals the jugular venous catheter failed to work properly which resulted in missing blood samples or blood samples with a reduced volume allowing the measurement of either AVP or ACTH only. These animals were also excluded from the statistical analysis of plasma ACTH and/or AVP values.

Figure 2a illustrates the impact of temazepam treatment on the intrahypothalamic PVN release of AVP. A two-way ANOVA revealed a significant treatment × time interaction effect ($F_{21,196}=1.91$; $p<0.02$). According to the subsequent *post hoc* analysis temazepam stimulated the release of this

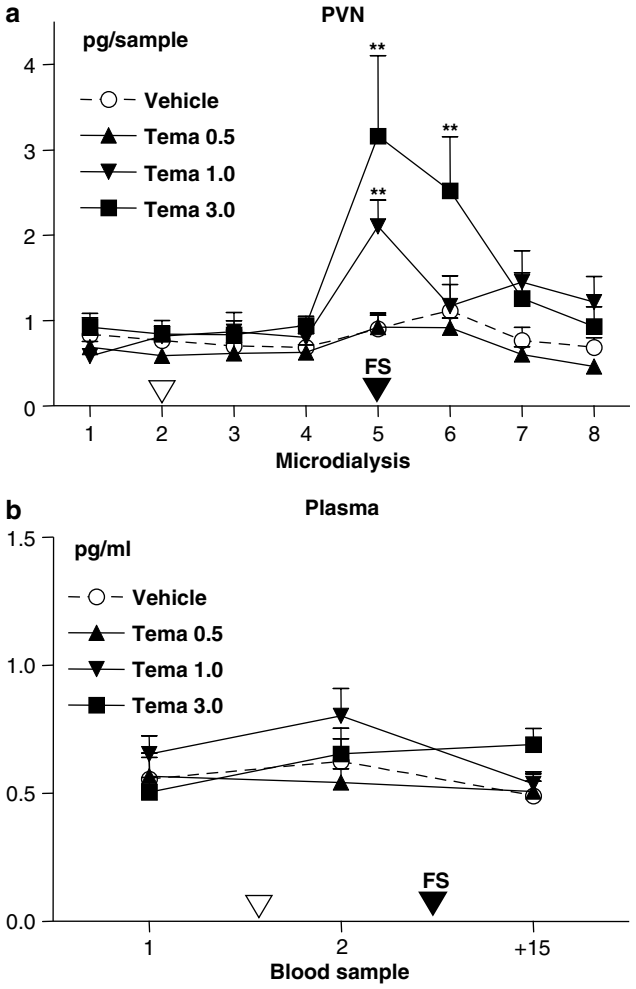

Figure 2 Temazepam increases dose dependently stress-induced intra-PVN, but not plasma AVP release. Effects of intravenous infusion (white triangle) of 0.5 mg/kg (A: $n=8$; B: $n=12$), 1.0 mg/kg (A: $n=6$; B: $n=9$) and 3.0 mg/kg (A: $n=12$; B: $n=18$) temazepam (Tema) and vehicle (A: $n=6$; B: $n=9$) as well as forced swimming (black triangle, FS) on the concentration of AVP (a) in consecutively collected 30-min microdialysates samples from the PVN and (b) in blood samples. Data are means + SEM. $^{**}p<0.01$ *vs* the same sample of all other treatments and $p<0.01$ *vs* samples 1–4 of the same treatment (Fisher's LSD *post hoc* test).

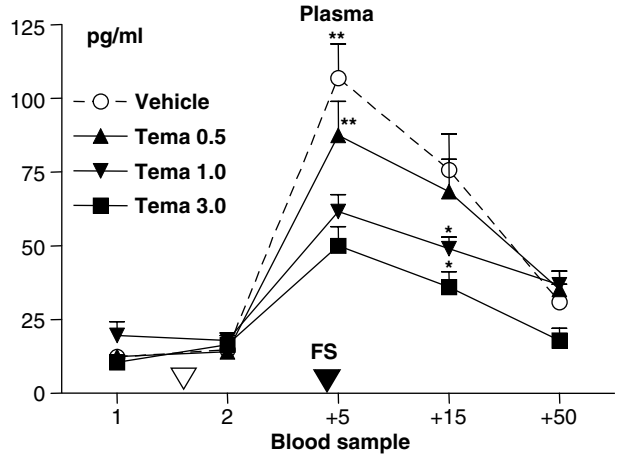

Figure 3 Temazepam reduces dose dependently stress-induced increase in plasma ACTH. Effects of intravenous infusion (white triangle) of 0.5 mg/kg ($n=11$), 1.0 mg/kg ($n=14$), and 3.0 mg/kg ($n=16$) temazepam (Tema) and vehicle ($n=8$) as well as forced swimming (black triangle, FS) on plasma ACTH. Data are means + SEM. $^{*}p<0.05$ and $^{**}p<0.01$ *vs* the same sample of all other treatments; for all treatments: samples + 5 min and + 15 min are $p<0.01$ *vs* samples 1 and 2 of the same treatment (not shown, Fisher's LSD *post hoc* test).

neuropeptide into the PVN in a dose-dependent manner. In contrast, as measured via jugular venous catheters, neither temazepam nor forced swimming had a statistically significant influence on the secretion of AVP from the posterior pituitary into the peripheral blood (treatment × time interaction, $F_{6,90}=1.17$, $p=0.33$; Figure 2b).

As shown in Figure 3, forced swimming caused a significant increase in plasma ACTH 5 and 15 min after stress (treatment × time interaction, $F_{12,180}=6.05$, $p<0.0001$). A subsequent analysis provided evidence that temazepam treatment reduced the forced swimming-induced ACTH increase in a dose-dependent manner.

Analysis of the behavior monitored during forced swimming failed to provide significant differences between control animals and all dosages of temazepam used in either of the parameters measured (struggling, swimming, floating; $p>0.05$, data and statistics not shown).

Table 1 Effects of Temazepam on the Secretion of ACTH from Primary Rat Anterior Pituitary Cell Cultures

		CRH stimulation	AVP stimulation
	Basal	**10 nM**	**1.0 μM**
Basal	8.51 ± 0.44	37.29 ± 3.11	15.21 ± 1.22
Temazepam 0.1 μM	7.92 ± 0.46	33.02 ± 2.28	13.75 ± 0.61
Temazepam 1.0 μM	7.85 ± 0.24	32.13 ± 3.22	14.50 ± 0.97
Temazepam 10 μM	7.97 ± 0.81	34.73 ± 1.61	13.92 ± 1.04
Propylenglycol	8.97 ± 0.37	38.85 ± 2.91	14.71 ± 0.91

Data are means ± SEM.

In Vitro Studies

A two-way ANOVA revealed that treatment with temazepam failed to significantly affect the basal and CRH- or AVP-stimulated secretion of ACTH from primary rat anterior pituitary cell cultures. Also, treatment with propylenglycol did not affect ACTH secretion ($p>0.05$, statistics not shown, Table 1).

DISCUSSION

The results of the present study demonstrate that temazepam affects the activity of the HPA axis in response to stressful stimuli. Furthermore, we show that the drug increased intra-PVN release of AVP while leaving plasma neuropeptide levels unchanged. As demonstrated by our *in vitro* studies, temazepam failed to significantly influence basal or CRH- or AVP-stimulated ACTH release from corticotrope cells.

Temazepam blunted the activity of the HPA system in response to forced swimming in a dose-dependent manner. This is in line with previous studies which were predominantly based on the analysis of plasma corticosterone concentrations (Bruni *et al*, 1980; Schürmeyer *et al*, 1988; Owens *et al*, 1989, 1993; Kalogeras *et al*, 1990; Breier *et al*, 1991, 1992; Schuckit *et al*, 1992; Torpy *et al*, 1993, 1994; Rohrer *et al*, 1994; Korbonits *et al*, 1995; Arvat *et al*, 1998, 1999; Skelton *et al*, 2000). However, in contrast to corticosterone, changes in plasma ACTH can be directly linked to the secretory activity of the parvocellular neurons of the PVN: AVP and CRH are secreted as releasing hormones from these neurons into the pituitary portal blood to synergistically stimulate the secretion of ACTH from the anterior pituitary (Aguilera and Rabadan-Diehl, 2000; Aguilera *et al*, 2001). We, therefore, tested the possibility whether or not temazepam might directly act at the level of the anterior pituitary. In our hands, drug administration failed to alter ACTH secretion *in vitro* (Kalogeras *et al*, 1990). Thus, there is good evidence that the observed changes in ACTH release induced by temazepam after stressor exposure are due to an action of the benzodiazepine at the brain level. In this context, different lines of evidence suggest that temazepam might act on $GABA_A$ receptors expressed by parvocellular PVN neurons (Makara and Stark, 1974; Olschowka, 1987; Calogero *et al*, 1988; Meister *et al*, 1988; Hillhouse and Milton, 1989; Tsagarakis *et al*, 1990; Stotz-Potter *et al*, 1996). Unfortunately, there are no tools available to allow for measurement of portal blood concentrations of AVP and CRH in freely moving rats. Therefore, it is impossible to determine whether the effects of temazepam are based on a decreased secretion of AVP or CRH or both from parvocellular neurons into the portal blood.

Our results confirm that, at the defined time points, plasma AVP concentrations remain unchanged in response to forced swimming in control animals (Wotjak *et al*, 1998; Engelmann *et al*, 2000). Therefore, and given the various reports about a GABAergic control of the release of AVP from the posterior pituitary (Bisset and Chowdrey, 1980; Knepel *et al*, 1980; Iovino *et al*, 1982, 1983; Unger *et al*, 1983; Wible *et al*, 1985a, b; Chowdrey and Bisset, 1988; Chiodera *et al*, 1989; Roberts and Robinson, 1991; Magnusson and Meyerson, 1993), one would have expected to measure reduced plasma AVP levels after temazepam treatment. However, at the dosages used here, temazepam failed to influence plasma AVP concentrations under basal and stress conditions. This renders the possibility unlikely that AVP release from the axon terminals of magnocellular HNS neurons is inhibited by temazepam and is unlikely to have modulated the ACTH response observed after temazepam (Wotjak *et al*, 2002; Engelmann *et al*, 2004).

This is the first study using *in vivo* microdialysis to monitor the influence of temazepam on the intrahypothalamic release of AVP under basal conditions and in response to defined stressor exposure. In the extracellular fluid of the PVN, AVP is most likely derived from dendrites and somata of local magnocellular neurons (Wotjak *et al*, 2001). Temazepam enhanced the release of AVP within the PVN in a dose-dependent manner. This is particularly striking with respect to the suggested action of benzodiazepines to reinforce the inhibitory tonus of GABA on neuronal activity. Although magnocellular neurons have been shown to express $GABA_A$ receptors (Magnusson and Meyerson, 1993; Fenelon and Herbison, 1995; Fenelon *et al*, 1995) recent findings suggest that not only the peripheral but also intrahypothalamic release of AVP both under basal conditions and in response to forced swimming is predominantly controlled by the inhibitory amino-acid taurine rather than GABA (Engelmann *et al*, 2001). Thus, our data confirm that under both basal and stress conditions dendritic/somatic release of AVP is controlled by other (inhibitory) signals than GABA. Whether temazepam might have acted directly to alter taurine release or whether an action of the drug in more remote brain areas might have contributed to the increase in intra-PVN release of AVP requires further investigation.

Released from magnocellular neurons at the level of the hypothalamus, AVP has been proposed to act as a neuromodulator to facilitate the return of the HPA axis to basal levels via interaction with local V1 receptors (Wotjak *et al*, 1996, 2002). Therefore, the results of the present study suggest that temazepam affects HPA axis via at least two different mechanisms: having an inhibitory effect directly on parvocellular PVN neurons and, as shown here, by stimulation of the somatic dendritic release of AVP from magnocellular PVN neurons. This fits well with the hypothesis that at the level of the hypothalamus AVP of HNS origin seems to play an important role in controlling HPA axis activity (Engelmann *et al*, 2004). The dissociation between the increased release of AVP from somata and dendrites at the level of the PVN while the secretion from the axon terminals of the same neuronal population remained virtually unchanged has been reported earlier and illustrates a remarkable feature of magnocellular neurosecretoric control (Wotjak *et al*, 1998).

Although the dose dependency in the increase of intra-PVN AVP mirrors the dose dependency in ACTH levels, a statistical analysis of ACTH and PVN AVP levels just failed to provide a significant correlation ($R^2 = 0.13$; $p = 0.09$). This may be due to the relatively low number of animals analyzed. However, the differences seen in both intra-PVN and ACTH response are unlikely to be due to an altered perception (and/or interpretation) of the stressor caused by temazepam as we failed to monitor differences in the acute behavioral stressor response. Thus, the present results encourage further studies about the role AVP plays after its release into the extracellular fluid of the PVN in response to temazepam treatment under stress conditions. This concerns in particular a detailed analysis of the receptor subtype via which AVP seems to act at the level of the PVN. Furthermore, subsequent studies should focus on the action of locally administered AVP antagonists to selectively interfere with the increasingly released neuropeptide in response to temazepam treatment and the consequences of this treatment on ACTH secretion. Based on the results of the present study, a partial restoration of the ACTH response by intra-PVN AVP antagonist treatment would be expected (Wotjak *et al*, 1996).

Taken together, we provide evidence that temazepam increases AVP release from magnocellular neurons in the PVN in response to defined stressor exposure in a dose dependent manner. This release into the extracellular fluid of the hypothalamus AVP has been suggested to facilitate

the return of the HPA axis to basal levels. Thus, temazepam seems to act in a dual way to reduce HPA axis activity: directly via $GABA_A$ receptors and indirectly by increasing the intrahypothalamic concentrations of AVP. Furthermore, the results of the present study support the hypothesis that AVP acting at the level of the hypothalamus plays an important role in HPA axis regulation.

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