Endogenous vasopressin contributes to hypothalamic–pituitary–adrenocortical alterations in aged rats

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Abstract

The ageing process in animals and humans is thought to be accompanied by a gradual impairment of corticosteroid receptor function, which is reflected by increased pituitary–adrenocortical hormone secretion at baseline and a number of aberrant neuroendocrine function test results. The latter include the ACTH and corticosteroid responses to a combined dexamethasone (DEX)/corticotropin-releasing hormone (CRH) challenge. The excessive hormonal response to this test among aged individuals has been taken as indirect evidence of enhanced endogenous arginine vasopressin (AVP) release, which – together with peripherally administered CRH – is capable of overriding DEX-induced ACTH suppression. The current study was designed to explore the role of endogenous AVP in mediating excessive hypothalamic–pituitary–adrenocortical (HPA) activity in ageing. The combined DEX/CRH test was administered to aged (22–24 months old) Wistar rats and the effect of the AVP type 1 (V1) receptor antagonist, $d(CH_2)_5Tyr(Me)AVP$, on ACTH release was studied. Infusion of the V1 receptor antagonist after DEX pretreatment and before CRH administration prevented the CRH-induced rise in ACTH secretion in comparison with vehicle-treated aged rats (area under the concentration–time curve: 699 ± 479 versus 2896 ± 759; $P<0{\cdot}01$). This difference was absent in young (3 months old) control rats.

In situ hybridization showed an increased number of AVP mRNA-expressing neurons in the parvocellular but not the magnocellular, portion of the hypothalamic paraventricular nucleus in DEX-pretreated aged rats. The number and synthetic activity of parvocellular neurons expressing CRH mRNA was also increased. We have concluded that the increased HPA activity in aged rats involves enhanced synthesis and release of AVP from parvocellular neurons, possibly secondary to impaired corticosteroid receptor function.

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Introduction

Increased activity of the hypothalamic–pituitary–adrenocortical (HPA) system is a frequent concomitant of ageing. This led to the hypothesis that the neuroendocrine changes seen are causally involved in the development of both age-associated cognitive decline and stress-related psychiatric disorders, such as depression (for reviews see Holsboer & Barden 1996, Lupien & McEwen 1997). It has been postulated that these age- or disease-associated changes are induced by a synergistic action of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) at pituitary corticotropic cells (von Bardeleben *et al.* 1985, von Bardeleben & Holsboer 1991), but only indirect evidence exists so far for a functional synergism between CRH and AVP as a crucial mechanism underlying excessive adrenocorticotropin (ACTH) and corticosteroid release in dexamethasone (DEX)-pretreated aged humans and animals (von Bardeleben & Holsboer 1991, Hatzinger *et al.* 1996).

The current study was designed to elucidate the role of endogenous AVP in age-related alterations of the HPA system in rats. The combined DEX/CRH challenge was used as a paradigm because this test is an extremely sensitive measure for changes in the HPA system regulation in both humans and rats (Heuser *et al.* 1994*a*, Hatzinger *et al.* 1996). The mechanisms underlying the excessive secretion of ACTH and corticosteroids in aged individuals are believed to involve impaired corticosteroid receptor function in the limbic system (Sapolsky *et al.* 1986, Reul *et al.* 1991, Rothuizen *et al.* 1993). Since DEX, which suppresses ACTH, is rapidly exported from the brain by a transporter system of the blood–brain barrier (Schinkel *et al.* 1995), it has very limited access to brain tissues and, thus, should rather exert a pituitary than a central

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site of action in its suppressive effect on pituitary–adrenal activity (de Kloet *et al.* 1975, Meijer *et al.* 1998). Thus, at a functional level, a low dose of DEX poorly substitutes for depletion of the endogenous corticosteroid receptor ligands from the brain and, therefore, in this tissue, may cause a condition resembling that of adrenalectomy or corticosteroid receptor antagonism (van Haarst *et al.* 1996). In response to this intervention, an enhanced release of corticotropic secretagogues from the hypothalamus, including CRH and AVP, occurs. Hence, in the context of the combined DEX/CRH test, this would result in inadequate suppression of ACTH and cortisol/corticosterone by DEX and, through the joint action of increased endogenous AVP and exogenous CRH, in excessive ACTH and corticosteroid secretion (Antoni 1986, von Bardeleben & Holsboer 1989, Heuser *et al.* 1994*b*, Hatzinger *et al.* 1996).

To elaborate this functional role of endogenous AVP we administered systemically an AVP receptor (V1) antagonist, expecting that this intervention would abolish the DEX/CRH-induced pituitary–adrenocortical activation in aged rats. The antagonist used is able to distinguish between V1 and V2 AVP receptors, but not between the V1 receptor subtypes (Thibonnier *et al.* 1997). AVP that is released into portal blood vessels (Herman *et al.* 1989) may originate not only from parvocellular, but also from magnocellular neurons of the hypothalamic paraventricular nucleus (PVN) (Holmes *et al.* 1986, Antoni *et al.* 1990, Wotjak *et al.* 1996). To define the brain area from which AVP acting at the pituitary of aged rats is derived, and to investigate possible age-associated alterations in the responsiveness of paraventricular CRH and AVP neurons to the inhibitory action of DEX, we used *in situ* hybridization of CRH mRNA and AVP mRNA in DEX-pretreated aged and young animals. Preliminary results have been presented in abstract form (Hatzinger *et al.* 1997).

Materials and Methods

Animals

The animal studies were performed according to the Bavarian law (Germany) for the care and use of laboratory animals. Experiments were carried out on young adult (379 ± 10 g body weight, 3 months old, $n=19$) and aged (672 ± 18 g body weight, 22–24 months old, $n=28$) male Wistar rats (Charles River, Sulzfeld, Germany). Rats were housed under standard laboratory conditions with a 12 h light:12 h darkness cycle (lights on at 0600 h; 22 ± 1 °C, 60% humidity); pelleted food and water were available *ad libitum*. Two weeks before the experiment, all rats were allowed to adapt in the laboratory, housed singly, and were handled daily to reduce non-specific stress during the experiments.

Surgery and blood sampling

Five days before testing, the jugular vein was chronically catheterized under halothane anesthesia for subsequent drug infusion and blood sampling. The catheter was exteriorized on the neck of the animal, filled with sterile saline (0·9% NaCl) containing gentamicin (30 000 IU/ml; Centravet, Bad Bentheim, Germany), closed with a stylet and flushed once after 3 days.

On the day of the experiment at 0900 h, the jugular vein catheter was connected via 50 cm PE-50 tubing to a 1 ml sterile plastic syringe filled with heparinized saline (30 IU/ml). At selected time-points, blood samples (0·2 ml) were collected on ice in EDTA-coated tubes containing aprotinin (10 μl/tube; Bayer AG, Leverkusen, Germany) and replaced by the same volume of sterile saline (0·9% NaCl). After centrifugation (4 °C, 5000 r.p.m., 5 min), the plasma was stored at − 20 °C (corticosterone) and − 80 °C (ACTH) respectively, until assay.

DEX/CRH test in vehicle- or V1 antagonist-treated young and aged rats

The DEX/CRH test was performed in young ($n=19$) and aged ($n=28$) rats as previously described (Hatzinger *et al.* 1996). In brief, DEX (30 μg/kg, dissolved in 0·9% saline; 0·5 ml/kg; Merck, Darmstadt, Germany) was administered i.v. during the diurnal trough of the rats' HPA system at 1200 h. For monitoring the effects of DEX treatment on basal plasma concentrations of ACTH and corticosterone during the diurnal acrophase, three 0·2 ml blood samples were collected at 1800, 1900 and 1930 h. At 1945 h, either vehicle (0·9% saline, 0·5 ml/kg; 9 young and 13 aged rats) or the AVP V1 receptor antagonist $d(CH_2)_5Tyr(Me)AVP$ (10 μg/kg dissolved in 0·9% sterile saline, 0·5 ml/kg; Dr M Manning, Toledo, OH, USA; 10 young and 15 aged rats) was injected i.v. An additional blood sample was taken at 2000 h, and thereafter CRH (50 ng/kg, 0·5 ml/kg) was injected. To assess the CRH-stimulated ACTH and corticosterone levels, further blood samples were taken at 2010, 2030, 2050 and 2110 h.

The next morning, 2 ml blood was withdrawn from the jugular vein for measurement of general laboratory parameters, including hematology (leukocytes, hemoglobin, red blood cell (RBC) count, volume of packed RBC, platelets), blood chemistry (sodium, potassium, chlorine, calcium, phosphate, creatinine, liver enzymes, amylase), and thyroid function (thyroid-stimulating hormone). Afterwards, the animals were killed by decapitation under light halothane anesthesia and the brains of four young and six aged vehicle-treated rats were quickly removed, frozen in prechilled *n*-methylbutane on dry ice and stored at − 80 °C for subsequent *in situ* hybridization histochemistry. In all animals, the pituitary, adrenals and internal organs were visually examined to exclude from

statistical analysis animals with pituitary or adrenal tumours or with other internal diseases.

Hormone assays

For estimation of plasma ACTH and corticosterone concentrations, commercially available radioimmunoassay kits (ICN, Costa Mesa, CA, USA) were used. The intra- and interassay coefficients of variation were below 7 and 10% respectively.

Recordings of blood pressure and heart rate in young and aged rats

To monitor the effects on the cardiovascular system of the V1 antagonist used, the femoral vein and artery of urethane-anesthetized young ($n=7$, 3 months old) and aged ($n=10$, 18 months old) Wistar rats (Charles River, Montreal, Quebec, Canada) were catheterized for drug infusion and blood pressure recording respectively. Sixty minutes after surgery, basal blood pressure and heart rate were recorded (Statham pressure transducer) for at least 30 min. Then vehicle (0·5 ml/kg, 0·9% saline) was administered i.v. to all rats. After another 30 min, the V1 antagonist was infused (10 μg/kg, 0·5 ml/kg) and recordings were continued for 45–60 min.

In situ *hybridization histochemistry*

In situ hybridization histochemistry was performed essentially as previously described (Wisden & Morris 1994). Briefly, consecutive frozen cryostat sections of the rat hypothalamus (14 μm) were alternately mounted on poly-L-lysine-coated slides for subsequent hybridization of either AVP or CRH mRNA. Sections were lightly fixed in ice-cold 4% paraformaldehyde, dehydrated and stored in 96% ethanol at 4 °C. To detect both AVP and CRH mRNAs, two synthetic oligonucleotides were used: 5′-CAT GGC GAG CAT AGG TGG GCA CTG CGT GCA GC-3′ (complementary to the cDNA sequence of AVP, corresponding to nucleotides 229–260; Ivell & Richter 1984) and 5′-CAG TTT CCT GTT GCT GTG AGC TTG CTG AGC TAA CTG CTC TGC CCT-3′ (cDNA of CRH, nucleotides 496–543; Jingami *et al.* 1985). According to a computer database search, neither oligonucleotide showed internal complementarity or resembled any other known sequence. Probes were 3′-end-labeled with [α-^{35}S]dATP (>1000 Ci/mmol; Amersham International plc. Amersham, Bucks, UK) by terminal deoxynucleotidyl transferase (Boehringer-Mannheim, Mannheim, Germany). Sections were then hybridized with the labeled probes in hybridization buffer (50% formamide, 4 × SSC, 10% dextran sulphate, 5 × Denhardt's, acid–alkali-cleaved salmon sperm DNA; Wisden *et al.* 1994) at 42 °C for 20 h. After hybridization, slides were washed in 1 × SSC at 55 °C for 30 min, rinsed in 1 × SSC, and 0·1 × SSC, dehydrated through graded alcohols and dried at room temperature. Slides were apposed to X-ray film (Kodak Biomax) for 7–10 days, thereafter dipped in photographic emulsion (diluted 1:1 with water; Kodak, NTB-2) and exposed for 6 weeks. After developing, sections were stained with hematoxylin–eosin and coverslipped.

To control for specificity of the oligonucleotide binding, some sections were preincubated with a 50-fold excess of the cold oligonucleotides before adding the radioactive labeled probes. Under these conditions, no signals were registered within the respective nuclei.

For evaluation of the hybridization signals, sections of the same level of the PVN were chosen according to the autoradiograms, the brain atlas of Paxinos & Watson (1986), and histological criteria (prominent part of magnocellular and parvocellular neurons respectively). For objective evaluation, the images of at least three sections per animal were grabbed by a computer-assisted image analyzer (Optimas-Bioscan) fitted with a Zeiss Axioplan microscope and a Sony CCD camera. The images were overlapped with predefined masks, which enclosed either the magnocellular or the parvocellular part of the PVN (according to Kiss 1988, Kiss *et al.* 1991). Subsequently, the number of positively labeled neurons (number of silver grains over the cell at least twice as high as the background) and the number of silver grains per positively labeled cell were counted by a person blind to the sections' coding. The number of silver grains was counted for one to, if possible, six positively labeled neurons randomly chosen from each image.

Statistical analysis

Results are reported as means ± S.E.M. After CRH administration, ACTH and corticosterone responses were computed as the area under the concentration–time curve (AUC, arbitrary units) corrected for basal concentration (averaged plasma concentration of ACTH and corticosterone, respectively, between 1800 and 1930 h) using trapezoidal integration according to Forsythe *et al.* (1969). The maximal increase of ACTH and corticosterone levels after CRH administration (time between 2000 and 2110 h) is reported as delta value (maximal concentration corrected for basal concentration). Additionally, the ratios of the AUCs for ACTH and corticosterone were calculated as a crude estimate of adrenal responsiveness to ACTH (pituitary–adrenal ratio).

To evaluate the *in situ* hybridization, the averaged number of positively labeled neurons per animal and the mean number of silver grains per cell and animal were calculated.

Statistical analysis was performed with a statistical software package (GB-Stat version 5·4; Dynamic Microsystems, Silver Spring, MD, USA). Statistical significance was determined by one-way ANOVA for

repeated measurements followed by Fisher's least significant difference (LSD) protected *t*-test (treatment response), two-way ANOVA for repeated measurements (age × treatment response, and for comparisons of basal, delta and AUC values) followed by Fisher's LSD protected *t*-test, or the Mann–Whitney U test (for comparisons of the number of positively labeled cells and silver grains). Statistical significance was accepted if $P<0{\cdot}05$.

Results

Animals

Six of the aged animals had to be excluded from statistical analysis due to tumours found on visual examination (liver, kidney, testis, pituitary or skin). In the remaining animals (aged: $n=22$; young: $n=19$), adrenal wet weight was significantly higher in the aged rats than in the young rats ($76 \pm 4{\cdot}0$ mg vs $57 \pm 4{\cdot}0$ mg; $P<0{\cdot}01$), whereas there was no difference in pituitary wet weight (aged: $21 \pm 3{\cdot}0$ vs young: $16 \pm 2{\cdot}0$ mg; $P=0{\cdot}35$). However, if these values were corrected for body weight, a significant decrease of adrenal weight/kg body weight was demonstrated in the aged compared with the young animals (113 ± 5 vs 52 ± 10 mg/kg; $P<0{\cdot}01$). Pituitary weight/kg body weight was not changed significantly in aged rats (aged: 34 ± 5 vs young: 43 ± 3 mg/kg; $P=0{\cdot}138$). Routine laboratory investigations did not reveal any differences between young and aged animals.

DEX/CRH test in young and aged rats treated with vehicle

After DEX treatment the release of ACTH in response to CRH was significantly higher in the aged rats than in the young rats (two-way ANOVA, age × time: $P=0{\cdot}027$), which was also reflected by significantly higher AUC values ($P<0{\cdot}05$) (Figs 1 and 2, Table 1). Similarly, the maximal rise of the corticosterone concentration in plasma after CRH stimulation was significantly higher in the aged than in the young rats (two-way ANOVA, age × time: $P=0{\cdot}0002$), with significantly higher values at both 2030 and 2110 h (Figs 1 and 2), although the differences in the AUC between the two groups of rats were not statistically significant ($P=0{\cdot}64$).

Although the pituitary–adrenal ratio tended to be higher in the aged animals ($0{\cdot}90 \pm 0{\cdot}37$) than in the young animals ($0{\cdot}53 \pm 0{\cdot}12$; $P=0{\cdot}94$), this difference failed to reach statistical significance, indicating that the global adrenal responsiveness was not significantly altered in the aged rats.

DEX/CRH test in aged rats treated with vehicle or V1 antagonist

After DEX treatment, basal ACTH and corticosterone levels between 1800 and 1930 h did not differ significantly between the groups of aged rats treated subsequently with either vehicle or the V1 antagonist (Fig. 1, Table 1). Administration of vehicle or the V1 antagonist had no immediate effect (first 15 min) on ACTH or corticosterone secretion (Fig. 1). However, application of the V1 antagonist abolished or reduced the CRH-stimulated increase in both ACTH and corticosterone secretion seen in the vehicle-treated aged animals (two-way ANOVA, time × treatment: $P<0{\cdot}0007$ and $P<0{\cdot}004$ respectively). This effect was also reflected by significantly lower delta values for ACTH ($P<0{\cdot}05$), and significantly lower AUC values for both ACTH ($P<0{\cdot}01$) and corticosterone ($P<0{\cdot}05$) (Table 1).

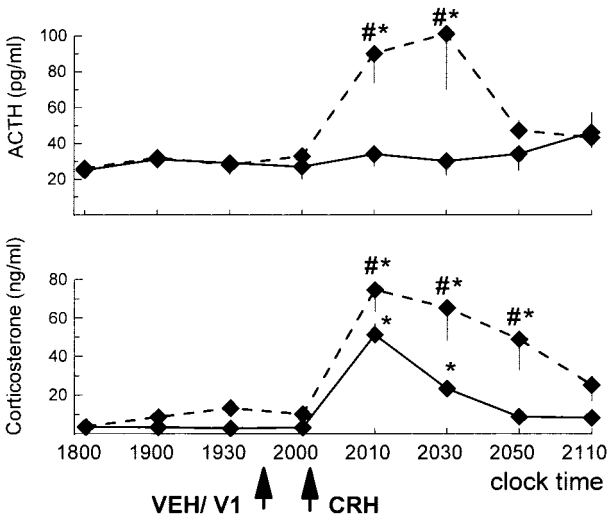

Figure 1 Plasma ACTH and corticosterone concentrations of vehicle-treated (broken lines, $n=11$) and V1 antagonist-treated (solid lines, $n=11$) aged Wistar rats (22–24 months old) between 1800 and 2110 h during the combined DEX/CRH test. All rats were pretreated with DEX (30 µg/kg i.v.) at 1200 h. Vehicle (VEH, saline 0·9% i.v.) or V1 antagonist (10 µg/kg i.v.) were injected at 1945 h (arrow); i.e. 15 min before CRH was administered (50 ng/kg i.v.) at 2000 h (arrow). Values are means ± S.E.M. #$P<0{\cdot}01$ vs corresponding values in V1 antagonist-treated rats (two-way ANOVA, treatment × time, ACTH: $P=0{\cdot}0007$, corticosterone: $P=0{\cdot}0037$ followed by Fisher's LSD protected *t*-test). *$P<0{\cdot}01$ vs corresponding baseline levels (two-way ANOVA for repeated measurements, factor time, ACTH: $P=0{\cdot}0002$, corticosterone: $P<0{\cdot}0001$ followed by Fisher's LSD protected *t*-test).

DEX/CRH test in young rats treated with vehicle or V1 antagonist

After DEX treatment, basal ACTH and corticosterone levels between 1800 and 1930 h did not differ significantly between the groups of young rats treated subsequently with either vehicle or the V1 antagonist ($P=0{\cdot}51$; Fig. 2, Table 1). Administration of vehicle or the V1 antagonist *per se* had no immediate effect (first 15 min) on ACTH or corticosterone secretion (Fig. 2). Furthermore, the

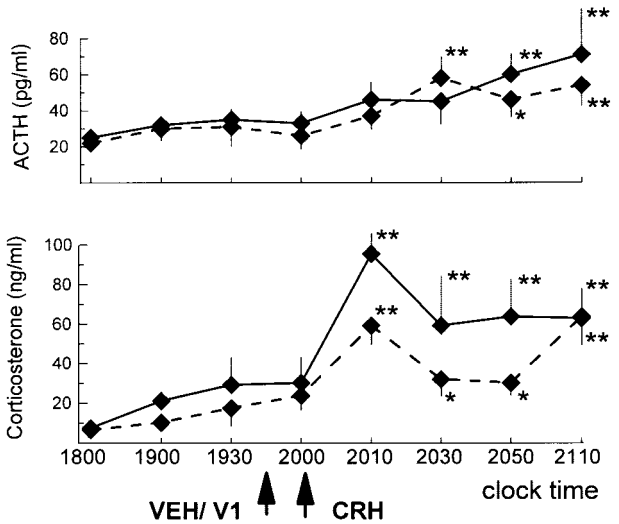

Figure 2 Plasma ACTH and corticosterone concentrations of vehicle-treated (broken lines, $n=10$) and V1 antagonist-treated (solid lines, $n=9$) young adult Wistar rats (3 months old) during the combined DEX/CRH test as described in the legend to Fig. 1. Values are means ± S.E.M. There were no significant differences between groups (two-way ANOVA, treatment × time, ACTH: $P=0{\cdot}7371$, corticosterone: $P=0{\cdot}0775$). *$P<0{\cdot}05$ and **$P<0{\cdot}01$ vs corresponding baseline levels (two-way ANOVA for repeated measurements, factor time, both ACTH and corticosterone: $P<0{\cdot}0001$ followed by Fisher's LSD protected *t*-test).

CRH-stimulated secretion of ACTH and corticosterone did not differ between the vehicle- and the V1 antagonist-treated young rats (two-way ANOVA, factor time × treatment: $P=0{\cdot}74$ and $P=0{\cdot}08$ respectively; Fig. 2, Table 1).

Cardiovascular effects of V1 antagonist administration in young and aged rats

Basal systolic (aged: 93·0 ± 5·2 vs young: 113 ± 10·3 mmHg) and diastolic blood pressure (59·7 ± 3·2 vs 69·7 ± 6·7 mmHg) did not differ significantly between the groups of urethane-anesthetized rats. Administration of the V1 antagonist affected systolic and diastolic blood pressure to a similar extent in the two groups of rats within 5–10 min after i.v. infusion, with a maximal response after 10 min (delta systolic, aged: − 12 ± 2·3, young: − 19 ± 5·3 mmHg; delta diastolic, aged: − 7·5 ± 1·7, young: − 12·0 ± 3·6 mmHg; two-way ANOVA, factor time: $P<0{\cdot}01$ both) and no significant difference between groups. Similarly, infusion of the V1 antagonist affected the heart rate to a similar extent in the two groups (aged: from 278 ± 15·7 to 288 ± 18·3; young: from 342 ± 21·3 to 351 ± 23·4 beats/min; factor time: $P<0{\cdot}0002$) with no significant difference between groups.

Expression of AVP and CRH mRNAs within the PVN of young and aged rats

The number of magnocellular neurons of the PVN synthesizing AVP did not differ significantly between the young adult and aged animals (Fig. 3A, $P=0{\cdot}52$). The number of silver grains per magnocellular neuron tended to be higher, but also showed greater variability in aged than in young animals (Fig. 3B, $P=0{\cdot}14$). In contrast, the number of parvocellular neurons positively labeled for AVP mRNA was significantly higher in aged rats (Fig. 3C, $P=0{\cdot}019$), whereas the number of silver grains per parvocellular neuron did not differ between the two groups of animals (Fig. 3D, $P=0{\cdot}29$) (Fig. 4).

Table 1 Basal (between 1800 and 1930 h), delta (peak value corrected for baseline) and AUC (area under the concentration–time curve corrected for baseline) values of ACTH and corticosterone in plasma during the combined DEX/CRH test in young adult and aged male Wistar rats treated with vehicle (VEH) or the AVP receptor 1 antagonist (V1). Values are means ± S.E.M.

	Young rats		**Aged rats**	
	VEH ($n=10$)	V1 ($n=9$)	VEH ($n=11$)	V1 ($n=11$)
ACTH				
Basal (pg/ml)	27·5 ± 6·78	30·3 ± 3·30	28·7 ± 4·76	28·1 ± 1·70
Delta (pg/ml)	40·3 ± 10·6	62·1 ± 21·0	101 ± 31·3#	23·4 ± 10·5*
AUC	1370 ± 483	1590 ± 408	2900 ± 759#	699 ± 479**
Corticosterone				
Basal (ng/ml)	9·55 ± 4·68	18·2 ± 6·52	8·45 ± 2·08	3·17 ± 0·85
Delta (ng/ml)	63·7 ± 11·9	97·1 ± 14·6	78·9 ± 14·5	48·8 ± 6·67†
AUC	2290 ± 465	3630 ± 871	3130 ± 836	1290 ± 232*†

Rats chronically implanted with a catheter in the jugular vein 5 days before testing were treated with DEX (30 µg/kg) at 1200 h and basal blood samples were taken between 1800 and 1930 h. VEH or V1 antagonist (10 µg/kg) was infused at 1945 h, before CRH (50 ng/kg) was administered at 2000 h. *$P<0{\cdot}05$ and **$P<0{\cdot}01$ vs VEH-treated aged rats; #$P<0{\cdot}05$ vs VEH-treated young rats; †$P<0{\cdot}05$ vs V1-treated young rats (two-way ANOVA followed by Fisher's LSD protected *t*-test).

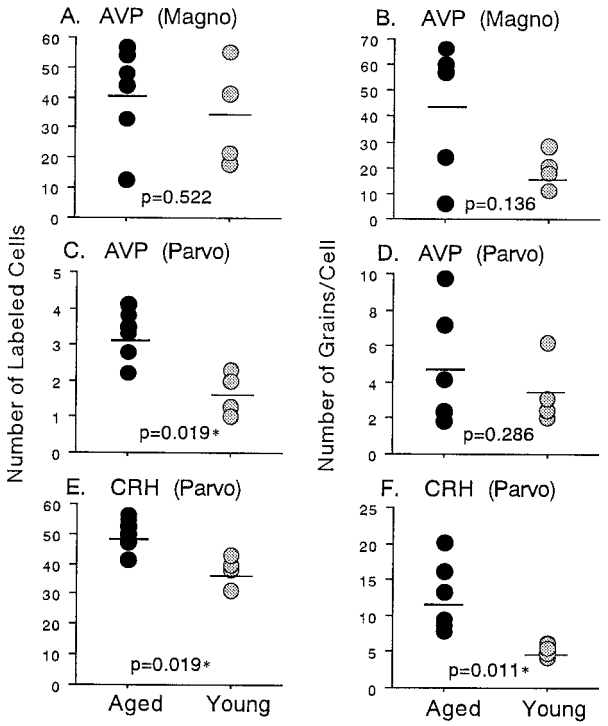

Figure 3 Quantitative analysis of *in situ* hybridization signals (AVP and CRH mRNAs) of parvocellular (Parvo) and magnocellular (Magno) divisions of the PVN of aged (solid circles, $n=6$, 22–24 months old) and young adult (shaded circles, $n=4$, 3 months old) male Wistar rats pretreated with DEX (30 µg/kg i.v.) 20 h earlier. The left side of the panel (A, C and E) shows the number of cells expressing AVP or CRH mRNA, whereby each circle represents the mean number of cells in three to six slices from a given rat, the slices having been selected from identical regions of the PVN in each rat. The right side (B, D and F) shows the number of silver grains (hybridization signal for AVP and CRH mRNA respectively) per cell. Each circle represents the mean number of silver grains per cell in the same slices evaluated for cell numbers. Horizontal bars reflect mean values for each group. Statistical comparisons between aged and young rats were performed using the Mann–Whitney U test. *$P<0{\cdot}05$.

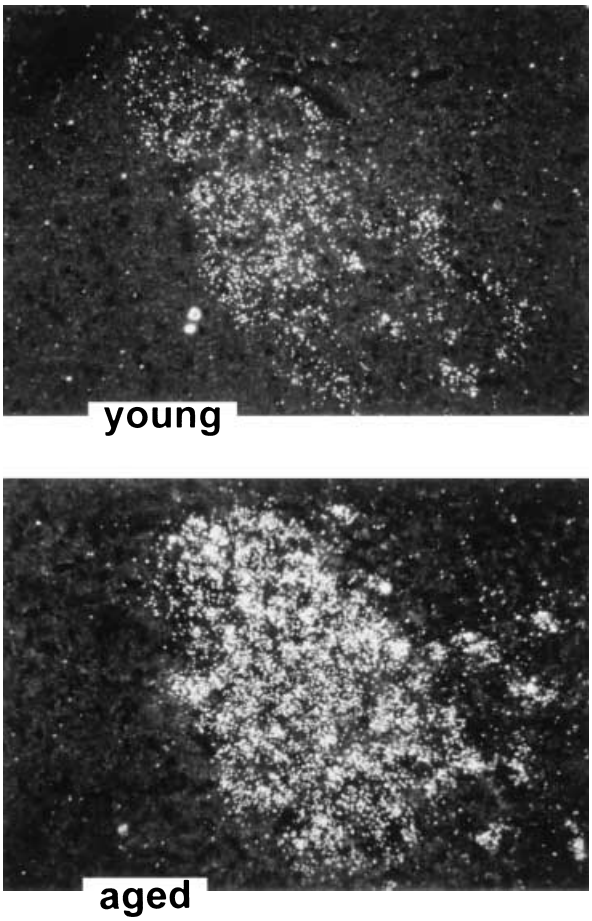

Figure 4 Photomicrographs show AVP mRNA hybridized in the PVN of young (top) and aged (bottom) male Wistar rats pretreated with DEX (30 µg/kg i.v.) 20 h earlier. AVP mRNA levels are increased in the parvocellular region of the nucleus. Magnification × 15.

With respect to CRH, the number of parvocellular neurons of the PVN positively labeled for CRH mRNA and the number of silver grains per parvocellular neuron were significantly higher in the aged animals (Fig. 3E and F, $P=0{\cdot}019$ and $P=0{\cdot}011$ respectively).

Discussion

The current study has explored the origin and functional role of endogenous AVP in producing excessive release of ACTH and corticosteroids when CRH is administered to DEX-pretreated aged rats. When DEX was given during the diurnal trough (1200 h) of the rats' HPA system activity, CRH administered at 2000 h (when HPA activity is gradually declining) could override the suppressive effect of DEX on ACTH secretion in aged, but not young, Wistar rats, which is in accordance with our previous results (Hatzinger *et al.* 1996). The major finding of the present study is that the ACTH increase can be abolished by peripheral administration of a V1 receptor antagonist; this is the first direct evidence that the enhanced activity of AVP-expressing neurons is functionally involved in age-dependent HPA hyperactivity.

Previous findings in aged human controls (Heuser *et al.* 1994*b*), aged depressed patients (von Bardeleben & Holsboer 1991) and aged rats (Hatzinger *et al.* 1996, Heroux *et al.* 1991, Tizabi *et al.* 1992), together with the equivalent pituitary–adrenal ratios in aged and young rats obtained in the present study provide indirect evidence for a hypothalamic rather than a pituitary or adrenocortical origin of age-related HPA dysregulation. A synergistic effect between CRH and AVP has been suggested by a

large number of studies in which these neuropeptides were co-administered (Gillies *et al.* 1982, von Bardeleben *et al.* 1985, Antoni *et al.* 1990). Moreover, as in chronic stress in rats (de Goeij *et al.* 1992, Lightman *et al.* 1993), an increased number of AVP- and CRH-expressing neurons has been found in human senescence (Hoogendijk *et al.* 1985, Raadsheer *et al.* 1994). The findings reported here of increased expression of CRH mRNA and AVP mRNA in the parvocellular part of the PVN in DEX-pretreated aged rats, together with our neuroendocrine results support the view that, in ageing, enhanced hypothalamic AVP activity may lead to increased functional action of AVP at corticotropic cells.

The reason why this phenomenon is observed in aged rats only is not yet clear, but, most likely, age-dependent changes in corticosteroid receptor-mediated signaling are involved. Glucocorticoids inhibit HPA system activity primarily via glucocorticoid receptors (GRs) located within the parvocellular part of the PVN and on anterior pituitary corticotropes and via a co-ordinated negative feedback action of mineralocorticoid receptors and GRs in the hippocampus (Reul & de Kloet 1985, de Kloet *et al.* 1993). There is compelling evidence that the function of hippocampal corticosteroid receptors gradually becomes impaired with increasing age (Reul *et al.* 1991, van Eekelen *et al.* 1991, Rothuizen *et al.* 1993). Reduced corticosteroid capacity leading to insufficiently suppressed hypothalamic CRH and AVP gene expression is likely, as the transcriptional activity of both genes is highly sensitive to minor changes in circulating glucocorticoids (Davis *et al.* 1986, Kovács *et al.* 1986). Importantly, neurons that either express AVP or CRH or co-express the two neuropeptides also express high levels of GRs (Fuxe *et al.* 1985, Herman 1993). The ligand-mediated activation of GRs would be sufficient to suppress AVP and CRH expression, provided that GR signaling is effective (Sawchenko 1987, Knapp *et al.* 1995). Thus, our data showing increased hypothalamic neuropeptide mRNA expression after DEX pretreatment are in line with the view of impaired corticosteroid receptor function in ageing.

In the aged rats studied here, enhanced AVP mRNA expression was restricted to parvocellular neurons. The contribution of magnocellular AVP to HPA activation is still a matter of debate, although there is increasing evidence that magnocellular neurons are capable of releasing AVP into portal blood vessels, either while passing through the median eminence or by short portal vessels from the posterior pituitary (Holmes *et al.* 1986, Antoni *et al.* 1990). However, our finding suggests that, at least under the current experimental conditions, the age-related AVP enhancement is restricted to the parvocellular neurons.

In theory, the effect induced by the V1 receptor antagonist could have been contaminated because the synthetic drug might have caused stress-related changes via systemic cardiovascular actions. However, this seems rather unlikely because there were no age-related differences in the blood pressure or heart rate response to the synthetic peptide. Nevertheless, besides AVP, a possible involvement of oxytocin has to be considered. Recently, an *in vitro* study showed that oxytocin is able to trigger ACTH release from corticotropes via action at V1 receptors (Schlosser *et al.* 1994). However, so far, the role of oxytocin in HPA system regulation is controversial (Fliers *et al.* 1985, Silverman *et al.* 1990, Neumann *et al.* 1999).

In summary, we have demonstrated that the enhanced ACTH secretion following CRH in DEX-pretreated aged rats involves activation of V1 receptors, since the ACTH response can be abolished by co-administration of a V1 receptor antagonist. Furthermore, expression of AVP mRNA is increased in the parvocellular part of the PVN of DEX-pretreated aged rats. Thus, the resulting increased release of AVP into portal vessels would, together with exogenous CRH, mediate the escape of ACTH and corticosterone from DEX-induced HPA suppression. Circumstantial evidence supports the view that the primary cause of this abnormality may be due to corticosteroid receptor malfunctioning. In view of the clinical data derived from aged humans (Holsboer & Barden 1996), these findings can probably be extrapolated to clinical conditions.

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