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Pharmacological aspects of corticotrophinergic and vasopressinergic function tests for HPA axis activation

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CHAPTER 3

A pharmacological tool to assess vasopressinergic co-activation of the hypothalamus–pituitary–adrenal (HPA) axis more integrally in healthy volunteers

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Abstract

BACKGROUND Pharmacological function tests consisting of 100µg hCRH (corticorelin) and 10µg dDAVP (desmopressin) mimic endogenous HPA-axis activation. However, physiological CRH concentrations preclude informative vasopressinergic co-activation (using dDAVP) and independent quantification of both corticotrophinergic (using hCRH) and vasopressinergic (using dDAVP) activation is limited due to administration on separate occasions.

OBJECTIVES This randomized, double-blind, placebo-controlled, partial five-way crossover study in healthy males and females (six: six) examined whether (1) concomitant administration of dDAVP and hCRH provides more informative vasopressinergic co-activation than dDAVP alone and (2) whether the administration of dDAVP followed two hours later by hCRH can quantify both vasopressinergic and corticotrophinergic activation on a single test day.

RESULTS 10µg dDAVP combined with 10µg and 30µg hCRH caused dose-related ACTH- and cortisol-release which was larger than with 10µg dDAVP alone and respectively comparable to and greater than that induced by 100µg hCRH. 10µg dDAVP alone demonstrated limited ACTH-release while the effects of 100µg hCRH two hours later were three times as large. ACTH and cortisol released by 10µg dDAVP returned to baseline prior to 100µg hCRH administration and dDAVP did not influence the response to subsequent hCRH administration.

CONCLUSIONS Dose-related vasopressinergic co-activation of the HPA-axis was induced by combining 10µg dDAVP with 10µg and 30µg hCRH. 10µg dDAVP combined with 10µg hCRH induced the potentially most informative vasopressinergic co-activation since it is not restricted by ceiling- or flooring-effects. The hCRH-response was not affected by prior dDAVP, allowing for a practical function test examining both HPA-activation routes on the same day.

Introduction

The hypothalamic neuropeptides corticotrophic release hormone (CRH) and vasopressin (AVP) modulate activation of the hypothalamus-pituitary-adrenal axis (HPA), releasing adrenocorticotrophic hormone (ACTH) from pituitary corticotrophes and subsequently cortisol from the adrenal cortices. Under physiological circumstances, CRH acts as major neuroendocrine secretagogue via pituitary CRH_1 receptors (CRH_1) (inducing corticotrophinergic activation) while AVP has weak neuroendocrine properties at pituitary vasopressin 3 receptors (V_3) (inducing vasopressinergic co-activation) (Ring, 2005; Scott and Dinan, 1998). However, following acute psychological and/or physical stress, AVP synergistically releases ACTH in the presence of increased levels of CRH, inducing an up to 30 times higher ACTH release compared to the effects of CRH alone (vasopressinergic co-activation) (DeBold et al., 1984; Favrod-Coune et al., 1993; Lamberts et al., 1984). After disappearance of a stressful insult, the HPA axis returns to basal activity via cortisol feedback and AVP again resumes its role as minor activator (de Kloet et al., 2005; Holsboer, 2000). In some stress-related psychiatric disorders, failure of the HPA axis to "reset" after stress has been attributed to chronic vasopressinergic hyperactivity or sustained vasopressinergic co-activation (Ring, 2005; Scott and Dinan, 1998; Holsboer et al., 1984; Holsboer and Ising, 2008; Scott et al., 1999b). A pharmacological function test quantifying vasopressinergic co-activation would therefore be useful to examine functional vasopressinergic disturbances of the HPA axis.

The synthetic analogue of AVP, desmopressin (dDAVP), is frequently used as a pharmacological function test for vasopressinergic functionality (Craighead et al., 2008). Infusing 10 µg dDAVP over 60 seconds induces a neuroendocrine response at the V_3 receptor, without causing clinically relevant confounding effects via other AVP receptors (Jacobs et al., 2009). However, ACTH and cortisol release induced by dDAVP alone is small and (frequently) variable (Dinan and Scott, 2005; Scott et al., 1999b; Jacobs et al., 2009). Without addition of hCRH, 10 µg dDAVP administered as a bolus over one minute induces a maximal V_3-mediated ACTH release of 16 ng/l, which is virtually equal to the increase (15 ng/l) induced by 30 µg dDAVP administered incrementally over one hour (Jacobs et

al., 2009). These increases are small compared to corticotrophinergic HPA activation with intravenous (i.v.) human corticotrophic release hormone (hCRH) or oral administration of the serotonergic precursor agent 5-hydroxtrypthophan (5-HTP) (Dinan et al., 1999; Scott et al., 1999b) (Smarius et al., 2008). At maximally tolerated doses, these interventions induce maximal serum cortisol concentrations (C_{MAX}) that are roughly 90 – 100% higher than those induced by 10 µg dDAVP alone. In these experiments, the effects of dDAVP alone seem to be limited by the low levels of endogenous CRH. Thus, since AVP is a CRH-dependent co-activator of the HPA axis, it might be better to study vasopressinergic co-activation by administration of dDAVP in combination with hCRH.

Corticotrophinergic (via CRH_1) and vasopressinergic (via V_3) HPA axis activation have been quantified previously, most frequently by investigating the effects of dDAVP and hCRH separately on three (including placebo) different study days (Dinan et al., 1999; von Bardeleben and Holsboer, 1988). Since such an approach would be unfeasible in most studies in (depressed) patients, the current study examined whether it would be possible to address the most important aspects of pituitary ACTH-release (vasopressinergic co-activation and corticotrophic responsiveness alone) in a single short study occasion. Independent evaluations of each route would require an adequate washout period, to ensure that V_3 stimulation does not affect subsequent CRH_1 stimulation (or vice versa). A minimal washout period after a dDAVP challenge would at least require a full return of ACTH levels to baseline, which according to previous studies occurs after 90 minutes (Jacobs et al., 2009; Scott et al., 1999a).

To examine these issues, we performed a single trial consisting of two separate experiments divided by two hours. In experiment A we examined whether vasopressinergic co-activation could be quantified by simultaneously adding small amounts of hCRH (either 10 µg or 30 µg hCRH) to a vasopressinergic function test consisting of 10 µg dDAVP in healthy volunteers. Experiment B examined the effects of hCRH alone, and potential interaction effects of prior dDAVP administration on corticotrophinergic activation by administration of 100 µg hCRH two hours later. To compare the two routes of ACTH-activation, the ACTH-release by 100 µg hCRH alone in experiment B was used as benchmark for the effects of the combinations in experiment A. This dose was chosen since 100 µg

hCRH has often been used in the literature, whereas the effects of lower doses (such as those used in experiment A in combination with dDAVP) were unknown. This attempt to characterize both routes of ACTH-stimulation on the same day had several practical reasons. The design restricted the burden for subjects and the amount of blood sampling, and is anticipated to be more acceptable than separate study days for patients in future clinical trials.

Methods

Study design

The study protocol was approved by the Medical Ethics Committee of Leiden University Medical Centre and performed according to Good Clinical Practice and International Conference on Harmonisation guidelines. A randomized, double blind, placebo-controlled, partial five-way crossover trial was performed in 12 healthy volunteers. Study medication was administered intravenously over 30 seconds, at two time points separated by two hours (t=0min and t=120min). In this way, two different experiments could be performed on the same day (*Figure 1*). Experiment A examined modulation of vasopressinergic co-activation by concomitant administration of different doses of hCRH with dDAVP at t=0min. Experiment B examined the effect of corticotrophinergic activation alone, and any carry-over effects of vasopressinergic co-activation after two hours, by first administering dDAVP or placebo followed by hCRH at t=120min. The main pharmacodynamic (PD) outcome measures for both experiments were changes in the concentrations of serum ACTH, cortisol and prolactin. Also, adverse events (AE's) were recorded to assess the safety and tolerability of concomitantly administering dDAVP and hCRH.

Volunteers

Twelve healthy volunteers (six male and six female) participated in the study. After providing their written informed consent volunteers received a full medical examination during a pre-study screening. Volunteers were excluded from study participation if (1) using more than 4 units alcohol on average per day, (2) smoking

more than 5 cigarettes per day, (3) using any drug or substance within one week before the first dosing, (4) using any drug or substance known to influence the metabolism of dDAVP in the month preceding the trial (5) demonstrating an ADAMTS-13 deficiency or presenting with a personal or family history of hypercoagulability as reflected by a diagnosis of coagulation factor deficiency (eg. Factor V Leiden mutation, APC resistance) or previous pulmonary embolism, deep venous thrombosis or other (arterial) cardiovascular disease, (6) presenting with a personal or family history of renal disease including diabetes insipidus and symptoms such as polydypsia, (7) presenting with a personal or a first degree family history of a clinical significant psychiatric disorder according to DSM-IV. No xanthine containing foods or beverages, tobacco or alcohol were allowed during the stay on the research unit. Concomitant medication other than paracetamol was not permitted during the study period.

dDAVP and hCRH

Identical syringes containing either dDAVP (Octostim®) or 0.9% NaCl as placebo were prepared for administration by the Department of Clinical Pharmacy and Toxicology of the Leiden University Medical Centre (LUMC). hCRH (Ferring CRH®) was provided by the Department of Clinical Pharmacy and Toxicology of the LUMC and identical syringes containing either hCRH or hCRH solvent as placebo were prepared for administration on-site at CHDR by trained staff not directly involved in the trial. Due to hCRH's rapid degradation after preparation, a maximal limit of 6 minutes was allowed between its preparation and administration to the volunteers. If any delay caused transgression of this limit, hCRH was prepared again and administered within 6 minutes.

Study days

Volunteers arrived at the Centre for Human Drug Research (CHDR) on the evening preceding each study day. On admission urinary screening was performed for drug use on all volunteers (tetrahydrocannabinol (THC), cocaine, morphine,benzodiazepines, 3,4-methylenedioxymethamphetamine (MDMA)/metamphetamine and

amphetamines) using the OnCall™ Test, ACON laboratories, Inc. Rapid Assays for Drug Abuse (Instruchemie Hilversum B.V. the Netherlands). In all females hCG was qualitatively measured in urine using the 'On Call' test device (Acon Laboratories Inc, San Diego, CA 92121, USA). Volunteers went to bed at 23.00 and were woken up around 8.00 the next morning after which a standardized breakfast was served. Two cannulas were inserted into the antecubital vein of each arm for blood sampling and intravenous administration of study medication. This occurred at least 1 hour preceding the first PD blood sampling to allow any puncture-induced HPA axis activation to return to baseline. Fluid intake was restricted to a total of 900 ml until the first urination infusion and only water was administered on fixed time points during the study day. Administration of the intravenous infusions was performed under hospital conditions and a research physician attended all study days. At the end of a study day, volunteers were discharged from the research unit only after having produced urine.

Biochemical measurements and vital signs on study days

1.2ml venous blood was collected in prechilled EDTA collection tubes for the determination of plasma ACTH on -60, -10 before and 5, 10, 15, 20, 25, 30, 35, 40, 50, 60, 75, 90, 120, 125, 130, 135, 140, 145, 150, 155, 160, 170, 180, 195, 210, 240, 270, 300, 360, 390, 420 and 510 minutes after the administration of the infusion at t=0h. Samples were immediately placed on ice, processed within 30 minutes and stored at -80°C. Samples were analyzed within six weeks using the Immulite 2500 Analyzer (Siemens, Germany) at the Laboratory for Clinical Chemistry, Leiden University Medical Centre (LUMC), Leiden, the Netherlands. 1.2ml venous blood was collected in serum collection tubes for the determination of serum cortisol and prolactin on -60, -10 before and 10, 20, 30, 40, 50, 60, 75, 90, 120, 130, 140, 150, 160, 180, 195, 210,240, 300, 360, 390, 420 and 510 minutes after start of the incremental infusion. Samples were stored for 30 – 45 minutes at ambient temperature to allow coagulation, subsequently centrifuged for 15 minutes at 2000 x g and stored at -20 °C. Samples were analyzed using Perkin Elmer AutoDelfia at Organon Development GmbH, Department of Bioanalytics, Waltrop, Germany. Blood pressure and pulse rate were measured at fixed

timepoints using the Nihon-Kohden (BSM-1100) or Colin (Pressmate BP-8800) blood pressure apparatus. Electrocardiogram (ECG) recordings were made at -30 and 180 min after infusion.

Pharmacodynamic (PD) analysis

The PD endpoints (ACTH, cortisol, prolactin) for 12 volunteers were log-transformed and analyzed by mixed model analyses of variance (using SAS PROC MIXED) with treatment, time, gender, gender-by-treatment and treatment-by-time as fixed effects, with subject and subject-by-time as random effect, and with the average baseline value as covariate. Since the trial consisted of two experiments, the PD effects were analyzed over two different time periods. To examine the effects of 10 µg and 30 µg hCRH on 10 µg dDAVP-induced vasopressinergic HPA axis activation (experiment A), PD effects were analyzed over the period 0 to 90 min. This analysis period was based on a previous investigation of the PD effects of 10 µg dDAVP, showing that ACTH and cortisol had returned to placebo levels within 90 minutes after its administration. To examine the effect of 100 µg hCRH on corticotrophinergic HPA axis activation and the effect of vasopressinergic HPA axis activation using 10 µg dDAVP two hours earlier (experiment B), PD effects were analyzed over the period 120 to 510 min since 100 µg hCRH was administered at t=120min. To put the vasopressinergic-corticotrophinergic co-activation in perspective, the mean maximal concentrations (C_{MAX}) of ACTH and cortisol of 100 µg hCRH in experiment B were compared to those of 10 µg and 30 µg hCRH with 10 µg dDAVP in experiment A. Neuroendocrine results were presented as estimates of percentual difference from placebo, with 95% confidence intervals and mean maximal concentrations (C_{MAX}). Adverse events were registered from spontaneous reports and hourly inquiries.

Results

Subject disposition and demographic data

Thirteen volunteers were screened after having provided informed consent. Twelve volunteers received study medication of which one dropped out due an AE not related to study medication. The mean

age of the volunteers was 21 years (range 18-27 years), which was similar for males and females. Females had a mean weight of 65kg (60-67kg) and mean height of 1.73m (range 1.68-1.84m). Males had a mean weight of 80kg (range 63-99kg) and mean height of 1.88 (range 1.83-1.90m).

Adverse events

All AE's were of mild to moderate intensity, transitory in nature and had dissipated within 12 hours after drug administration. AE's were predictable based on the side-effect profiles of hCRH and dDAVP and no subjects discontinued participation directly due to related adverse effects. Since the side-effects of both drugs are very much alike, it was not possible to readily identify the causating agent when dDAVP and hCRH were administered simultaneously. At any rate, the administration of dDAVP and hCRH together, either simultaneously or sequentially did not influence the intensity or occurrence of AE's. The most commonly occurring AE's were headache (2/12 subjects) for placebo; facial flushing (10/12 subjects), palpitations (4/12 subjects), dizziness (2/12 subjects) and headache (1/12 subjects) for 100 µg hCRH; facial flushing (8/12 subjects), palpitations (3/12 subjects), dizziness (2/12 subjects) and headache (2/12 subjects) for 100 µg hCRH preceded by 10 µg dDAVP; facial flushing (6/12 subjects), palpitations (1/12 subjects) and headache (1/12 subjects) for 10 µg dDAVP combined with 10 µg hCRH and facial flushing (8/12 subjects), headache (3/12 subjects), palpitations (2/12 subjects) and dizziness (1/12 subjects) for 10 µg dDAVP combined with 30 µg hCRH.

Neuroendocrine effects

In experiment A, ACTH increased by +34.6(8.7, 66.7,p=0.0075)%, (C_{MAX}19.3ng/l) with 10 µg dDAVP; by +154 (105, 213,p<0.0001)%, (C_{MAX}56.7ng/l) after 10 µg dDAVP combined with 10µg CRH; and by +242(177, 322,p<0.0001)%, (C_{MAX}75.2ng/l) following 10 µg dDAVP combined with 30µg hCRH (Table 1; Figures 2 and 3). Cortisol increased similarly by +18.2(-2.4, 42.9)%, (C_{MAX}114.2 ng/ml) with 10 µg dDAVP; by +76.7(46.1, 114)%, (C_{MAX}172 ng/ml) after 10 µg dDAVP combined with 10µg hCRH; and by +113(76.5, 158)%,

(C_{MAX}192 ng/ml) following 10 µg dDAVP combined with 30µg hCRH. (Table 1; Figure 4).

In experiment B, ACTH increased by +88.0(58.4, 145),p<0.0001)% (C_{MAX}59.2 ng/l) after 100µg hCRH, and by +104(70.5, 145.,p<0.0001)% (C_{MAX}70.4 ng/l) with 100 µg hCRH preceded by 10 µg dDAVP. The difference between these two responses was not statistically significant [+8.7(-9.0, 29.9)% p=0.340)%] (Table 2; Figures 2 and 5). Cortisol increased similarly by +40.0(25.5, 56.1),p<0.0001)% (C_{MAX}196 ng/ml) following 100 µg hCRH and by +45.7 (30.2, 63.1),p<0.0001)% (C_{MAX}209 ng/ml) after 100 µg hCRH preceded by 10 µg dDAVP. This difference was also not statistically significant [+4.1 (-6.7, 16.1),p=0.425)%]. (Table 2; Figure 6).

The ACTH increases after 10 µg or 30 µg hCRH combined with 10 µg dDAVP of respectively 154% and 242%, were larger than the increases of 88.0% and 104% following 100 µg hCRH.

Discussion

AVP is believed to enhance CRH_1-associated cAMP production in pituitary corticotrophes by stimulating V_3, potentiating the effect of CRH and leading to a synergistic release in ACTH and subsequently cortisol (Scott and Dinan, 1998; DeBold et al., 1984; Favrod-Coune et al., 1993). A full pharmacological characterisation of this co-activation, including a quantitative description of the individual and (supra)additive components of the system, would have required a complete assessment of all interactions between dDAVP and hCRH, including different doses. Although undoubtedly useful, such a study would be very difficult to perform, even in healthy volunteers and much more so in patients. The current study was not designed for these purposes, but it provided clear indications for a dose-related co-activation, and showed that the effects of low doses of hCRH are boosted by dDAVP to the levels of a much higher dose of hCRH alone. Administration of 10 µg dDAVP alone induced a maximal ACTH concentration of 19.3 ng/l. The addition of 10 µg hCRH produced an ACTH-peak of 56.7 ng/l, which did not differ much from the effect of a ten-fold higher dose of 100 µg hCRH alone (59.2 ng/l). The combination with 30 µg hCRH caused a C_{MAX} of 75.2 ng/l, which was on average even larger than with 100 µg hCRH. Very similar results were found for cortisol, where the maximal

levels induced by dDAVP alone or combined with 10 µg and 30 µg hCRH were 60%, 90% and 98% of those produced by 100 µg hCRH alone. This clearly shows that dDAVP potentiates the pituitary release of ACTH caused by exogenous hCRH in a dose-related manner. The results also show that the effects of 10 µg dDAVP alone are limited to a C_{MAX} of 19.3 ng/l, possibly reflecting the relatively low endogenous hCRH levels in healthy volunteers during the morning.

Instead of administering 100 µg hCRH in a separate treatment arm, it was given two hours later than 10 µg dDAVP. This relatively short washout period was mainly chosen to investigate whether a vasopressinergic and corticotrophinergic function test could be combined in half a day, in order to reduce the burden for patients in future clinical trials. Previous studies showed that ACTH-release following dDAVP alone return to baseline within 90 min (Jacobs et al., 2009; Scott et al., 1999a), and this was confirmed in the current study for dDAVP combined with hCRH. A longer washout duration could also have introduced differences related to diurnal fluctuations, which were probably less important within this late morning two-hour period. The results show that the vasopressinergic effects of a single dose of 10 µg dDAVP are small and have disappeared after two hours. Such a dose does not have a significant interaction with 100 µg hCRH administered 120min later in healthy volunteers. This allows for a fairly independent assessment of the corticotrophic component of ACTH-stimulation, after determination of the vasopressinergic co-activation using a dDAVP-challenge earlier the same day. The difference in ACTH- and cortisol release induced by hCRH with or without preceding dDAVP was on average less than 10%. Although this difference is statistically non-significant, a small augmentation of the hCRH effect by administering dDAVP two hours earlier is not fully precluded. However, this potential carry-over effect is no more than a fraction of the effects of the concomitant administration of dDAVP and hCRH. It should be realized however that the optimal challenge interval to avoid confounding carry-over effects still needs to be confirmed in patients. In patients with vasopressinergic hyperactivity the effects of dDAVP alone will be determined by both V_3 receptor hypersensitivity and (increased) endogenous CRH-levels. Under these circumstances, the carry-over effects may be larger and therefore might require a longer wash-out period between

subsequent administrations. On the other hand, co-administration of dDAVP and hCRH in the present trial pharmacologically mimicked
a vasopressinergic hypersensitivity analogous to that expected in patients. Despite a clear augmentation of the maximal ACTH and cortisol responses in experiment A, their return to baseline levels remained unaffected and there was no detectable influence on the effects of hCRH in part B. In practice therefore, 10 µg dDAVP with 10 µg or 30 µg hCRH followed by hCRH alone after two hours, is also expected to provide useful information both on vasopressinergic and corticotrophinergic activity of the HPA-axis in patients. Moreover, the application of a function test in this form in patients could be used to detect unexpected interactions between V_3 and CRH_1 by comparing baseline values and normative responses.

An important goal of this study programme is to develop a reliable and practical pharmacological function test to quantify vasopressinergic co-activation. Such a function test could be used to demonstrate treatment effects of novel pharmacological agents modulating the V_3 receptor (Spiga et al., 2008b; Spiga et al., 2008a), and ultimately it can be used to detect functional differences betweens (patient) groups. In this regard, it is important to induce co-activation that is neither too small nor too large (Gijsman et al., 2004). If a function test would produce suboptimal stimulation, it would be difficult to detect differences between patient groups or treatment effects. On the other hand, maximal or supramaximal stimulation may obscure changes or differences in vasopressinergic sensitivity by means of ceiling effects. The ideal dose combination to quantify vasopressinergic/corticotrophinergic co-activation can only be determined from the full dose-response curves of hCRH and dDAVP and their combinations. Although such data are not available, our studies provide indications that 10 µg dDAVP with 10 µg hCRH may actually constitute a reasonable combination. We showed that the 10 µg dDAVP function test (in the presence of physiological CRH concentrations) induces small maximal cortisol concentrations of 120 ng/ml in the present study and 140 ng/ml in a previous study (Jacobs et al., 2009). A dose of 30 µg hCRH administered alone has previously been reported to induce maximal cortisol concentrations of 134 ng/ml (Oelkers et al., 1988). Considering the C_{MAX} of 196 ng/ml after the 100 µg dose in the current study,

50 µg hCRH should produce a readily detectable HPA-activation above baseline, but clearly lower than that caused by 100 µg. Addition of 10 µg hCRH to 10 µg dDAVP increases cortisol levels to 172 ng/ml, which is still below E_{MAX}-values in healthy volunteers found with other pharmacological function tests. For example, the administration of 100 µg hCRH previously demonstrated maximal serum cortisol concentrations around 210 ng/ml (Dinan et al., 1999; Scott et al., 1999b). Also, the non-specific serotonin agonist meta-chlorophenylpiperazine (mCPP) 0.5 mg/kg administered i.v. lead to a cortisol C_{MAX} 228.4 ng/ml (Gijsman et al., 1998) and the direct serotonin precursor agent 5-hydroxytryptophan (5-HTP) 300mg administered orally induced a cortisol C_{MAX} of 219 ng/ml (Smarius et al., 2008). Higher doses of hCRH have not been administered due to undesirable aspecific stress effects, while mCPP and 5-HTP are not tolerated at higher doses due to serotonergic side-effects. Cortisol release (at least via the CRH_1 receptor) therefore appears to be limited to approximately 210 - 230 ng/ml in this setting. Thus, a combination of 10µg dDAVP with 10 µg hCRH is expected to constitute a good function test to assess co-activation. The vasopressinergic contribution to the HPA-response can be determined by comparing the ACTH- and cortisol-effects of the combination of 10 µg dDAVP and 10 µg hCRH to those of 50 µg hCRH alone, administered two hours later. This being said, it should be borne in mind that our recommendation is putative and the application of pharmacokinetic-pharmacodynamic (PK-PD) modelling techniques in future will have to point out the validity of this approach.

In conclusion, we demonstrated dose-dependent vasopressinergic co-activation when combining hCRH with dDAVP. These findings form a basis for the further development of a pharmacological function test to assess vasopressinergic co-activation of the HPA-axis. This tool should be able to establish functional differences between different patient groups, and to demonstrate modulatory effects of innovative drug such as V_3-antagonists. Furthermore, administering dDAVP followed by hCRH at least two hours later presents a practical procedure to independently examine vasopressinergic co-activation and corticotrophinergic activation of the HPA axis.

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Table 1 Average plasma ACTH (ng/l) and serum cortisol (ng/ml) for the period 120–510 min for 100 mg hCRH and for 100 mg hCRH preceded by 10 mg dDAVP: estimated means (back transformed least square means [LSM]) for placebo, 100 mg hCRH and 100 mg hCRH preceded by 10 mg dDAVP. Estimated difference (%) with 95% confidence interval from placebo for 100 mg hCRH and 100 mg hCRH preceded by 10 mg dDAVP; and estimated difference (%) with 95% confidence interval from 100 mg hCRH for 100 mg hCRH preceded by 10 mg dDAVP

	Back transformed least Square Means (LSM)						**Estimated difference with 95% Confidence interval (%)**					
	Placebo (n=12)		10µg dDAVP (n=12)	10µg dDAVP combined with 10 µg hCRH (n=12)	10µg dDAVP combined with 30µg hCRH (n=12)	100 µg hCRH (n=12)						
Parameter	0- 90 min	120-510 min	0- 90 min	0- 90 min	0- 90 min	120-510 min	10µg dDAVP vs placebo	10µg dDAVP combined with 10 µg hCRH vs placebo	10µg dDAVP combined with 30 µg hCRH vs placebo	100 µg hCRH (120-510min) vs placebo (120-510min)	10µg dDAVP combined with 10 µg hCRH vs 10µg dDAVP	10µg dDAVP combined with 30 µg hCRH vs 10µg dDAVP
Plasma ACTH (ng/l)	11.2	13.4	15.1	28.4	38.3	25.3	34.6 (8.7, 66.7) p=0.0075	153.5 (105.1, 213.4) p<0.0001	241.9 (177.1, 321.8) p<0.0001	88.0 (58.4, 145.1) p<0.0001	88.4 (48.0, 139.7) p<0.0001	154.0 (99.4, 223.5) p<0.0001
Serum cortisol (ng/ml)	77.3	80.4	91.3	136.6	164.9	112.5	18.2 (-2.4, 42.9) p=0.0849	76.7 (46.1, 113.6) p<0.0001	113.4 (76.5, 158.0) p<0.0001	40.0 (25.5, 56.1) p<0.0001	49.6 (20.4, 85.9) p=0.0006	80.7 (45.3, 124.7) p<0.0001
Serum prolactin (ng/l)	5.1	5.3	4.8	4.9	5.1	5.2	-5.5 (-15.2, 5.3) p=0.297	-2.6 (-12.6, 8.5) p=0.622	0.7 (-9.6, 12.1) p=0.899	-1.9 (-15.4, 13.7) p=0.790	3.0 (-9.1, 16.8) p=0.633	6.5 (-5.9, 20.6) p=0.309

Table 2 **Average plasma ACTH (ng/l) and serum cortisol (ng/ml) for the period 120 to 510 min for 100 µg hCRH and for 100 µg hCRH preceded by 10 µg dDAVP: Estimated means (back transformed least Square Means) for placebo, 100µg hCRH and 100 µg hCRH preceded by 10µg dDAVP. Estimated difference (%) with 95% confidence interval from placebo for 100µg hCRH and 100µg hCRH preceded by 10 µg dDAVP; and estimated difference (%) with 95% confidence interval from 100 µg hCRH for 100µg hCRH preceded by 10 µg dDAVP.**

	Back transformed least Square Means (LSM)			**Estimated difference with 95% Confidence interval (%)**		
Parameter	Placebo (n=12)	100 µg hCRH (n=12)	100 µg hCRH preceded by 10 µg dDAVP (n=12)	100 µg hCRH vs placebo	100 µg hCRH preceded by 10 µg dDAVP vs placebo	100 µg hCRH vs 100 µg hCRH preceded by 10 µg dDAVP
Plasma ACTH (ng/l)	13.4	25.3	27.5	88.0 (58.4, 145.1)% p<0.0001	104.4 (70.5, 145.1) p<0.0001	8.7 (-9.0, 29.9) P=0.340
Serum cortisol (ng/ml)	80.4	112.5	117.2	40.0 (25.5, 56.1)% p<0.0001	45.7 (30.2, 63.1) p<0.0001	4.1 (-6.7, 16.1) P=0.452

Figure 1 **Schematic representation of the study design: a five-way crossover study consisting of two different experiments.**

Treatment	*t = 0 min*	*t = 120 min*
1	10µg dDAVP + 10 µg hCRH	placebo
2	10µg dDAVP + 30µg hCRH	placebo
3	placebo + placebo	placebo
4	10µg dDAVP + placebo	100µg hCRH
5	placebo + placebo	100µg hCRH

Experiment A
t = 0 min
placebo + placebo
10µg dDAVP + placebo
10µg dDAVP + 10µg hCRH
10µg dDAVP + 30µg hCRH

Experiment B	
t = 0 min	*t = 120 min*
placebo + placebo	placebo
placebo + placebo	100 µg hCRH
10 µg dDAVP + placebo	100 µg hCRH

Figure 2 **Average concentration time profile of plasma adrenocorticotropic hormone (ACTH) with SD error bars (ng/l) for experiments A and B combined (closed circle: placebo followed by placebo; open triangle: placebo followed by 100 mg hCRH; open circle: 10 mg dDAVP followed by 100 mg hCRH; closed square: 10 mg dDAVP combined with 10 mg hCRH followed by placebo; open square: 10 mg dDAVP combined with 30 mg hCRH followed by placebo).**

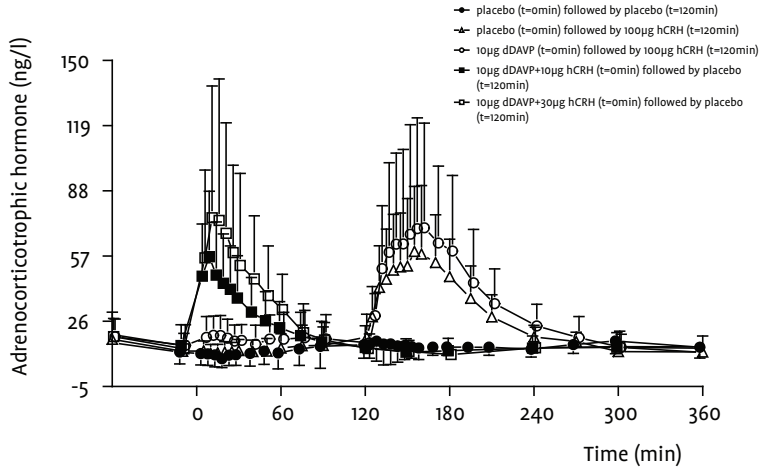


Figure 3 Average concentration time profile of plasma adrenocorticotropic hormone (ACTH) with SD error (ng/l) bars for experiment A (closed circle: placebo; open circle: 10 mg dDAVP; closed square: 10 mg dDAVP combined with 10 mg hCRH; open square: 10 mg dDAVP combined with 30 mg hCRH; grey open triangle: 100 mg hCRH superimposed from administration point at t = 120 min in experiment B).

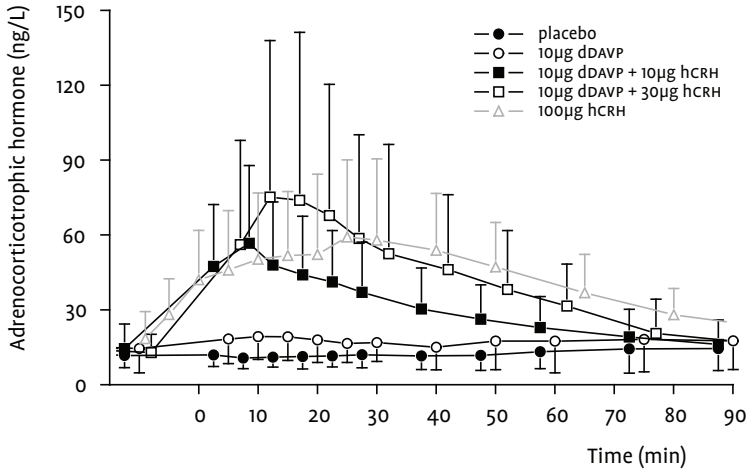


Figure 4 Average concentration time profile of serum cortisol with SD error (ng/ml) bars for experiment A (closed circle: placebo; open circle: 10 mg dDAVP; closed square: 10 mg dDAVP combined with 10 mg hCRH; open square: 10 mg dDAVP combined with 30 mg hCRH; grey open triangle: 100 mg hCRH superimposed from administration point at t = 120 min in experiment B).

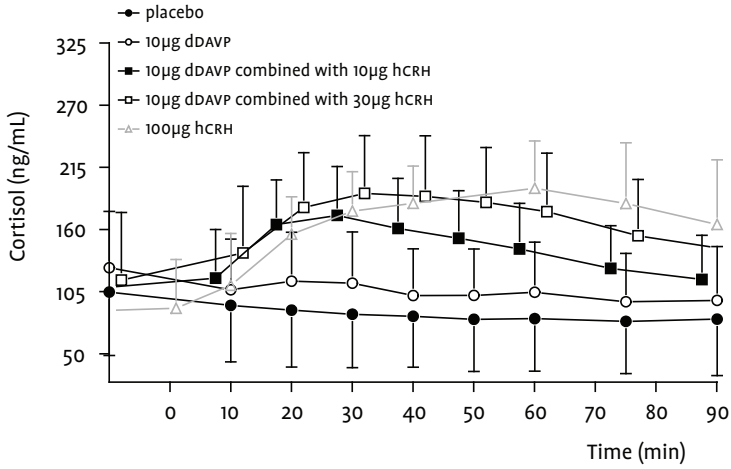


Figure 5 Average concentration time profile of plasma adrenocorticotropic hormone (ACTH) with SD error (ng/l) bars for experiment B (closed circle: placebo followed by placebo; open triangle: placebo followed by 100 mg hCRH; open circle: 10 mg dDAVP followed by 100 mg hCRH).

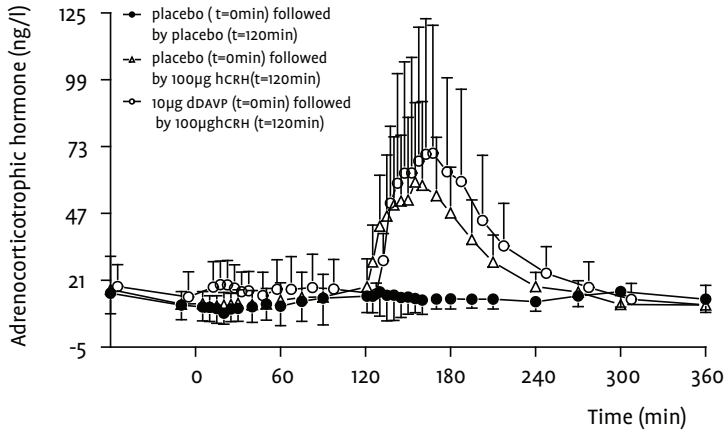
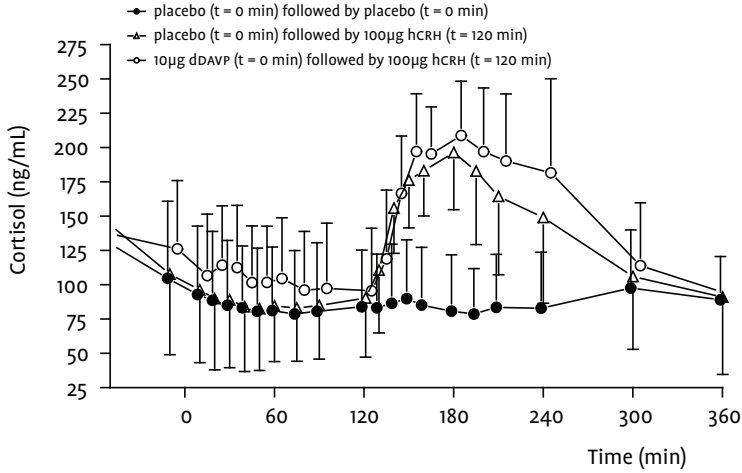


Figure 6 Average concentration time profile of serum cortisol with SD error (ng/ml) bars for experiment B (closed circle: placebo followed by placebo; open triangle: placebo followed by 100 mg hCRH; open circle: 10 mg dDAVP followed by 100 mg hCRH).



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