Blockade of the V_{1b} receptor reduces ACTH, but not corticosterone secretion induced by stress without affecting basal hypothalamic–pituitary–adrenal axis activity

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Abstract

Vasopressin (AVP), produced in parvocellular neurons of the hypothalamic paraventricular nucleus, regulates, together with CRH, pituitary ACTH secretion. The pituitary actions of AVP are mediated through the G protein receptor V_{1b} (V_{1b}R). In man, hyperactivity of the hypothalamic–pituitary–adrenal axis has been associated with depression and other stress-related conditions. There are also clinical data suggesting a role for AVP in the dysfunctional HPA axis described in some depressed patients. In this study, we have investigated the effect of a recently synthesised selective antagonist of the V_{1b}R both on exogenous AVP-induced ACTH and corticosterone secretion, and on basal and stress-induced pituitary–adrenal activity. Adult male Sprague–Dawley rats treated with the V_{1b}R antagonist (Org, 30 mg/kg, s.c.) or vehicle (5% mulgofen in 0.9% saline,

2 ml/kg, s.c.). We found that blockade of the V_{1b}R reduced the increase in both ACTH and corticosterone secretion induced by AVP (100 ng, i.v.). The same treatment had no effect either on basal ACTH and corticosterone levels or on the ultradian or diurnal rhythms of corticosterone secretion. Acute administration of the V_{1b}R antagonist reduced ACTH secretion following both restraint and lipopolysaccharide, but did not antagonise the ACTH response to noise. The same treatment did not reduce corticosterone secretion in response to any of the three stressors used in this study. Our results confirm that this compound is an antagonist of the V_{1b}R in the rat, and that its ability to reduce stress-induced ACTH responses is stressor dependent with differential modulation of pituitary and adrenal responses.

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Introduction

Vasopressin (arginine vasopressin, AVP) and CRH are the two main neuropeptides regulating the hypothalamic–pituitary–adrenal (HPA) axis. AVP is a nonapeptide synthesised in parvocellular neurons of the paraventricular nucleus (PVN) of the hypothalamus, which, in synergy with CRH activates the release of pituitary ACTH. ACTH, in turn, potently stimulates the secretion of glucocorticoids (corticosterone in rats and cortisol in humans) from the adrenal cortex (Gillies *et al.* 1982, Rivier *et al.* 1984, Antoni 1993; Aguilera *et al.* 1994).

The effect of AVP on pituitary corticotrope cells is mediated through the activation of AVP V_{1b} receptor (V_{1b}R) subtypes (Jard *et al.* 1987, Aguilera 1994, Aguilera & Rabadan-Diehl 2000). Evidence for the involvement of AVP in the HPA axis response to stress includes the increased secretion of AVP into the pituitary portal circulation (de Goeij *et al.* 1991, 1992,

Chowdrey *et al.* 1995) increased AVP mRNA within the parvocellular part of the PVN (Makino *et al.* 1995, Ma *et al.* 1997a,b, Ma & Lightman 1998) and increased V_{1b}R density in the pituitary (Rabadan-Diehl *et al.* 1995, Aguilera & Rabadan-Diehl 2000) in rats exposed to chronic stress.

Dysfunction of the HPA axis is well described in the pathophysiology of stress-related disorders (Michael & Gibbson 1963, Holsboer 2000, 2001). Several clinical studies suggest that AVP could play an important role in the aetiology of these conditions and elevated plasma levels of AVP have been described in subjects suffering from depression (van Londen *et al.* 1997, 1998, 2001, de Winter *et al.* 2003) or post-traumatic stress disorders (de Kloet *et al.* 2007). Post-mortem studies have also shown increased numbers of AVP-expressing neurons in the PVN of depressed patients (Purba *et al.* 1996, Merali *et al.* 2006). In addition to evidence for abnormalities in AVP, there is also a suggestion that vulnerability to the development of depression could be due

to genetic variation in the human *V_{1b}*R gene (van West *et al.* 2004). Moreover, hyperactivity of the *V_{1b}*R has been found in melancholic depression (Dinan *et al.* 1999, 2004).

A number of preclinical studies support the role of AVP in the affective disorders. Studies where high (HAB) or low (LAB) anxiety-related behaviour rat lines were used also suggested that AVP could contribute to the marked dysregulation of the HPA system in the HAB rat (Keck *et al.* 2002). In these rats, overactivity of AVP was associated with increased anxiety-like behaviour that could be reduced with chronic treatment with the antidepressant citalopram (Jochum *et al.* 2007). The AVP-deficient Brattleboro rat has been shown to display an attenuated depression-like behaviour in forced swimming and sucrose preference tests (Mlynarik *et al.* 2007).

Although *V_{1b}*R knockout (KO) mice exhibit a normal corticosterone response to the acute psychological stress of restraint (Lolait *et al.* 2007a), they do have a reduction in the pituitary–adrenal response to more severe stressors, including forced swim and insulin-induced hypoglycaemia (Lolait *et al.* 2007a). Similarly, it has been shown using KO mice that the *V_{1b}*R is necessary for a normal HPA response to lipopolysaccharide (LPS) and ethanol (Lolait *et al.* 2007b).

There is therefore considerable interest in the possible role of a AVP antagonist in the treatment of mood disorders.

Blockade of the *V_{1b}*R using the *V_{1b}*R antagonist SSR149415 (Serradeil-Le Gal *et al.* 2002) has been shown to induce antidepressant and anxiolytic-like activity in several behavioural models (Griebel *et al.* 2002, Serradeil-Le Gal *et al.* 2002, 2005, Shimazaki *et al.* 2006, Ramos *et al.* 2006, Iijima & Chaki 2007, Breuer *et al.* 2008). Besides being localised in the anterior pituitary, *V_{1b}*R are also found in the central nervous system, in regions involved in the modulation of the stress response such as hippocampus, lateral septum and cortex (Lolait *et al.* 1995, Vaccari *et al.* 1998) suggesting that the anxiolytic and antidepressant effect of SSR149415 could also be mediated through the activation of central *V_{1b}*R.

SSR149415 is able to reduce, in rats, ACTH release induced by endogenous administration of *V_{1b}*R agonists (alone or in combination with CRH) and by various stressors (Serradeil-Le Gal *et al.* 2002, Ramos *et al.* 2006). However, an effect of SSR149415 on corticosterone release has not been reported.

The compound used in this study is a recently synthesised *V_{1b}*R antagonist that is able to bind to the human recombinant *V_{1b}*R with $a > 1000$ fold selectivity over other members of the AVP receptor subfamily and also over a broad range (> 60) of other receptors and enzymes (Presland *et al.* 2007, Craighead *et al.* 2008). Recent work from Craighead and colleagues has reported that this compound is an effective *V_{1b}*R antagonist *in vitro*, as demonstrated by its ability to reduce AVP-induced ACTH release from isolated rat anterior pituitary cells. Furthermore, the *in vivo* efficacy of Org was confirmed by the ability of both acute and chronic administration of Org to reduce, in rat, the ACTH release induced by various agonists of the *V_{1b}*R (Craighead *et al.*

2008). However, the effect of Org on both *V_{1b}*R agonist- and stress-induced corticosterone secretion has not been investigated. To further investigate whether this compound is an effective AVP antagonist *in vivo* we have investigated the effect of this compound on the time course of ACTH and corticosterone release following i.v. AVP administration in rat. We then studied its effect on basal ACTH and corticosterone levels, as well the circadian and ultradian patterns of corticosterone release. Finally, we investigated the effect of this *V_{1b}*R antagonist on the pituitary–adrenal response to various acute stressors.

Materials and Methods

Subjects

All experiments were conducted on male Sprague–Dawley adult rats (Harlan–Olac, Bicester, UK) weighing 250–300 g at the time of surgery. Animals were grouped, housed 4 to a cage and allowed to acclimatise to the housing facility for a minimum of 1 week prior to the start of experiments. Rats were maintained under standard environmental conditions (21 ± 1 °C) under a 14h light:10h darkness schedule (lights on at 0515 h) and food and water were provided *ad libitum* throughout the experiment. All animal procedures were approved by the University of Bristol Ethical Review Group and were conducted in accordance with Home Office guidelines and the UK Animals (Scientific Procedures) Act, 1986. All possible efforts were made to minimise the number of animals used and their suffering.

Surgery and blood sampling

Animals were anaesthetised with a combination of Hypnorm (0.32 mg/kg fentanyl citrate and 10 mg/kg fluanisone, i.m.; Janssen Pharmaceuticals, Oxford, UK) and diazepam (2.6 mg/kg i.p.; Phoenix Pharmaceuticals, Gloucester, UK). The right jugular vein was exposed and a silastic-tipped (i.d. 0.50 mm, o.d. 0.93 mm, Merck) polythene cannula (Portex, Hythe, UK) was inserted into the vessel until it lay close to the entrance of the right atrium. The cannula was pre-filled with pyrogen-free heparinised (10 IU/ml) isotonic saline. During the same surgery, a s.c. cannula, for drug administration, was inserted under the skin between the shoulder blades. The free ends of both cannulae were exteriorised through a scalp incision and then tunnelled through a protective spring that was anchored to the parietal bones using two stainless steel screws and self-curing dental acrylic. Following recovery, animals were housed in individual cages in a soundproof room. The end of the protective spring was attached to a mechanical swivel that rotated through 360 °C in a horizontal plane and 180° through a vertical plane allowing the rats to maximise freedom of movement. The cannulae were flushed daily with the heparinised saline to maintain patency. Blood samples were collected through the jugular vein cannula

either by hand or using an automated blood sampling (ABS) system as previously described (Windle *et al.* 1998). Blood samples collected by hand (0.2 ml) were stored in ice-cold eppendorf tubes containing 10 μ l EDTA (0.5 M; pH 7.4) and 10 μ l Trasylol (Aprotinin, 500 000 KIU/ml, Roche). Plasma was separated by centrifugation and then stored at -80°C until processed for ACTH and corticosterone measurements. Where the volume of the blood sample was less than 0.2 ml, only corticosterone was measured. At the end of each experiment rats were overdosed with 0.5 ml sodium pentobarbitone (Euthatal, 200 mg/ml; Merial, Harlow, UK).

Drug treatments

The V_{1b} R antagonist (Org, provided by Schering–Plough Corporation, Newhouse, UK,) was administered through the s.c. cannula in a 0.9% saline solution with 5% mulgofen (a detergent that improves solubility; GAF Ltd, Manchester, UK) at the dose of 10 mg/kg (Exp. 1) or 30 mg/kg (Exp. 1–4) using a volume of 2 ml/kg. In our study, we chose an administration route (via an s.c. cannula) that minimises any stress induced in the rat by the injection. Vehicle controls were injected with 0.9% saline solution with 5% mulgofen. Arginine (AVP, Sigma) was administered via the jugular vein cannula at 100 ng (~ 400 ng/kg) dissolved in 0.1 ml 0.9% saline. Both s.c. and i.v. injections were followed by injection of 0.2 ml heparin–saline to flush out the cannula and ensure that the entire volume of drug had been received by the animal.

Stress

Restraint was performed by enclosing the rats in cylindrical perspex restrainers (55 mm diameter) containing air holes to prevent overheating. The length of the restrainer was adjusted for the length of the rat in order to limit movement. Noise stress consisted of 10 min of white noise (96 dB). (LPS, *Escherichia coli*; 055:B5; 250 μ g/ml, Sigma) was dissolved in sterile saline (0.1 ml/rat) and injected via the jugular cannula.

Experimental procedures

Experiment 1. Effect of acute treatment with the V_{1b} R antagonist Org on AVP-induced ACTH and corticosterone secretion Experiments were performed between 0900 and 1300 h. Rats received either Org (10 and 30 mg/kg) or vehicle 30 min prior to injection with AVP or saline ($n=6-11$ for each group). Blood samples were collected by hand at the time points described in Fig. 1. Blood samples were processed for ACTH and corticosterone measurements as described below.

Experiment 2. Effect of acute treatment with the V_{1b} R antagonist Org on basal ACTH and corticosterone secretion Experiments were performed between 1600 and 1900 h, the time of expected ACTH and

Figure 1 Effect of the V_{1b} R antagonist Org on ACTH and corticosterone secretion induced by administration of exogenous AVP. Rats were injected with Org (10 and 30 mg/kg, s.c.) or vehicle (5% mulgofen in 0.9% saline, 2 ml/kg) 30 min prior administration of AVP (100 ng, i.v.) or saline (0.1 ml; $n=6-11$ /group). Data are expressed as mean $\pm$ S.E.M. of plasma ACTH (pg/ml, A) or plasma corticosterone (ng/ml, B) with respect to the time of AVP administration. Black arrows indicate Org or vehicle injection; grey arrows indicate AVP or saline injection. * $P<0.05$, effect of AVP, compared with saline, in rats treated with vehicle or Org. * $P<0.05$, effect of Org, compared with vehicle, in rats treated with AVP or saline.

corticosterone secretory peak levels. Rats received either Org (30 mg/kg, $n=7$) or vehicle ($n=7$). Blood samples were collected by hand as described above at the time points indicated in Fig. 2. Blood samples were processed for ACTH and corticosterone measurements as described below.

Experiment 3. Effect of acute treatment with the V_{1b}R antagonist Org on corticosterone diurnal rhythm and pulsatile pattern Experiments were performed between 1600 and 0700 h. Four days after surgery,

Figure 2 Effect of acute administration of the V_{1b}R antagonist Org on basal ACTH and corticosterone secretion. Sample collection started at 1600 h, the time of the expected hormone peak, immediately before administration of Org (30 mg/kg, s.c., $n=7$) or vehicle (5% mulgoferin in 0.9% saline, 2 ml/kg, $n=7$). Samples were collected for 3 h every 15 or 30 min. Data are expressed as mean $\pm$ S.E.M. of plasma ACTH (pg/ml, A) or plasma corticosterone (ng/ml, B) with respect to the time of day.

rats were connected to the ABS and blood samples were collected every 10 min. After collection of basal samples for 1 h, rats were injected with either Org (30 mg/kg, $n=7$) or vehicle ($n=7$). Blood samples were processed for corticosterone measurements as described below.

Experiment 4. Effect of acute treatment with the V_{1b}R antagonist Org on stress-induced ACTH and corticosterone secretion All experiments were performed between 0900 and 1300 h. Rats were injected with either Org (30 mg/kg) or vehicle 30 min prior to restraint ($n=9-10$ for each group), or noise ($n=8$ for each group) or acute administration of LPS ($n=6-8$ for each group). For each stressor a different group of animals was used. Blood samples were collected by hand prior to Org or vehicle administration and then before, during and after restraint or noise and before and after LPS administration. Blood samples were processed for ACTH and corticosterone measurements as described below.

Hormone measurements

ACTH levels were measured by IRMA using commercially available kits (DiaSorin Ltd, Wokingham, UK). For ACTH measurement, 80 μ l plasma were diluted in 120 μ l saline. Intra- and inter-assay coefficients of variation of the ACTH assay were 2.84 and 6.41% respectively.

Corticosterone levels were measured using RIA as previously described (Spiga *et al.* 2007). For blood samples collected by hand, 5 μ l of each plasma sample was diluted in 500 μ l of a citrate buffer and processed in triplicate. For blood samples collected using the ABS, samples were partially diluted by the ABS so that each sample consisted of 37.7 μ l blood diluted 1:5 in heparinised saline. For the assay, 50 μ l of each blood sample was further diluted into 50 μ l of a citrate buffer (pH 3.0) and samples were processed in duplicate. Samples were incubated overnight at 4 °C with 50 μ l of ¹²⁵I corticosterone tracer (Oxford Bio Innovation DSL Ltd, Oxford, UK) and 50 μ l of rabbit anti-rat corticosterone primary antibody (kindly donated by G. Makara, Hungary). On day 2, a charcoal/dextran solution was added to the samples, which were then centrifuged (15 min, 4000 r.p.m., 3.120 g, 4 °C) and aspirated before being loaded onto a γ -counter. Intra- and inter-assay coefficients of variation of the corticosterone assay were 16.65 and 13.30% respectively.

Statistical analysis

All statistical analyses were performed using SPSS 11.5 for Windows (SPSS Inc., Chicago, IL, USA). Data are expressed as mean $\pm$ S.E.M. One-way or two-way ANOVA with repeated measures was used to estimate the effect of Org (time $\times$ Org effect, exp.2 and exp.4) or Org and AVP (time $\times$ AVP effect, time $\times$ Org effect and time $\times$ AVP $\times$ Org effect, exp 1) on the time course of ACTH and corticosterone release. When appropriate, further comparisons were made

with Fisher's least significant difference *post hoc* test. In exp. 4, where a significant effect of time occurred (indicating a significant effect of the stressor) a paired sample *t*-test was used to identify the time at which the stressor effect occurred within each group. Moreover, in exp. 4, the area under curve (AUC) for the ACTH and corticosterone responses to stress were calculated for each group and analysed using Student's *t*-test. In exp. 3, the hormone profile for each animal was analysed for variation in pulse characteristics (AUC, mean daily corticosterone concentration and the number, amplitude and frequency of corticosterone pulses) using the PULSAR algorithm (Merriam & Wacher 1982). For the PULSAR algorithm, the following G values were employed: G1=5, G2=3, G3=2, G4=1.5 and G5=0.8, together with a peak splitting parameter of 5 (S.D. units). These values were obtained from visual inspection of the data, as recommended (Windle *et al.* 1998). Each PULSAR parameter were analysed using Student's *t*-test. Statistical significance was set at $P \leq 0.05$, except on the paired samples *t*-test where Bonferroni correction was used (exp. 4: $P \leq 0.006$, $P \leq 0.007$ and $P \leq 0.0055$ respectively for restraint, noise and LPS experiments).

Results

Experiment 1. Effect of acute treatment with the V_{1b}R antagonist Org on AVP-induced ACTH and corticosterone secretion

The effect of the acute administration of the V_{1b}R antagonist Org on the ACTH response to i.v. AVP is shown in Fig. 1A.

Two-way ANOVA revealed a significant effect of AVP (time×AVP effect: $F(6,258)=101.72$; $P<0.00001$), a significant effect of Org (time×Org effect: $F(6,258)=17.88$; $P<0.00001$) and a significant interaction between the two factors (time×AVP×Org effect: $F(6,258)=16.20$; $P<0.00001$) over the time course of ACTH release.

Acute administration of AVP induced a robust increase on ACTH secretion in rats treated with vehicle or Org. Pretreatment with Org had a significant inhibitory effect on the ACTH response to AVP and this effect was significant 5 min ($P<0.0001$) and 15 min ($P<0.005$) after AVP administration for both doses of Org. No effect of Org on ACTH release in rats treated with saline was observed.

The effect of the acute administration of the V_{1b}R antagonist Org on the corticosterone response to i.v. AVP is shown in Fig. 1B.

Two-way ANOVA revealed a significant effect of AVP (time×AVP effect: $F(6,342)=38.02$; $P<0.00001$), a significant effect of Org (time×Org effect: $F(6,342)=4.95$; $P=0.0012$) and a significant effect between the two factors (time×AVP×Org effect: $F(6,342)=3.56$; $P=0.01$) over the time course of corticosterone release.

Acute administration of AVP induced a robust increase on corticosterone secretion in rats treated with vehicle or Org. Pretreatment with Org had a significant inhibitory effect on

the corticosterone response to AVP and this effect was significant 30 min (10 and 30 mg/kg, $P<0.05$ and $P<0.0001$ respectively) and 45 min (30 mg/kg, $P<0.0005$) after AVP administration. No effect of Org on corticosterone release in rats treated with saline was observed.

Based on those results, the 30 mg/kg dose of Org was chosen for all further experiments.

Experiment 2. Effect of the V_{1b}R antagonist Org on basal ACTH and corticosterone secretion

The effect of Org on basal ACTH and corticosterone release was investigated during the diurnal peak (Fig. 2). No effect of treatment with Org on basal ACTH (Fig. 2A) and corticosterone (Fig. 2B) secretion was found.

Experiment 3. Effect of acute treatment with the V_{1b}R antagonist Org on basal corticosterone diurnal rhythm and pulsatile pattern

The mean corticosterone concentration of rats injected with Org or vehicle is represented in Fig. 3. The hormone profile for each animal was analysed for variation in pulse characteristics (AUC, mean daily corticosterone concentration and the number, amplitude and frequency of corticosterone pulses) using the PULSAR algorithm. No significant difference on any of the parameters analysed was found between the two experimental groups (Table 1).

Experiment 4. Effect of the V_{1b}R antagonist Org on stress-induced ACTH and corticosterone secretion

In all the stress experiments the time of Org administration with respect to the time of exposure to stress was based on the results from experiment 1.

Restraint

The effect of Org on the ACTH and corticosterone response to acute restraint is shown in Fig. 4.

Exposure to restraint increased ACTH release (time effect: $F(8,128)=43.844$; $P<0.0001$) both in rats treated with vehicle and Org. There was a significant effect of Org on ACTH response to stress (time×Org effect $F(8,128)=3.331$; $P=0.035$).

Restraint stress increased corticosterone secretion both in rats treated with vehicle and Org (time effect $F(8,136)=51.879$; $P<0.0001$). In contrast to the ACTH data, no effect of Org on restraint-induced corticosterone increase was found (time×Org effect: $F(8,136)=0.588$; $P=0.641$).

Noise

The effect of Org on ACTH and corticosterone secretion induced by acute noise stress is shown in Fig. 5.

Noise–stress induced a smaller (compared with restraint) increase in ACTH secretion in rats treated with both Org and

Figure 3 Effect of acute administration of the V_{1b} R antagonist Org on corticosterone diurnal rhythm. Data represented are mean $\pm$ S.E.M. blood corticosterone concentration for rats injected with Org (30 mg/kg, s.c., $n=7$) or vehicle (5% mulgofen in 0.9% saline, 2 ml/kg, s.c., $n=7$). Rats were connected to an automated blood sampling system and samples were collected every 10 min for 15 h. Injection time is indicated by black arrows; the grey bar represents the dark phase (1915–0515 h).

vehicle (time effect: $F(798)=63.394$; $P<0.0001$). No significant effect of Org on noise-induced ACTH increase was found (time $\times$ Org effect: $F(798)=0.681$; $P=0.481$).

Noise–stress increased corticosterone secretion in both Org and vehicle treated rats (time effect: $F(798)=92.968$; $P<0.0001$). No effect of Org on corticosterone secretion in response to noise was observed (time $\times$ Org effect: $F(798)=2.251$; $P=0.073$).

Lipopolysaccharide

The effect of Org on the ACTH and corticosterone response to LPS is shown in Fig. 6.

Intravenous injection of LPS increased ACTH in both Org and vehicle treated rats (time effect: $F(9117)=38.886$; $P<0.0001$). Acute treatment with Org reduced ACTH response to LPS (time $\times$ Org: $F(9117)=7.080$; $P<0.0001$).

Intravenous injection of LPS increased corticosterone secretion in both Org and vehicle treated rats (time effect: $F(9108)=100.539$; $P<0.0001$). In contrast to the ACTH

results, Org had no effect on the LPS-induced corticosterone increase (time $\times$ Org effect: $F(9108)=0.722$; $P=0.584$).

A summary of the effect of stress on ACTH and corticosterone secretion (expressed as AUC) and its modulation by Org is summarised in Table 2.

Discussion

Although there are extensive data on the regulation of AVP mRNA in parvocellular PVN neurones and on the synergistic actions of AVP and CRH in stimulating ACTH release, there is remarkably little information about the role of AVP or pituitary V_{1b} receptors in the regulation of normal circadian and ultradian activity of the HPA axis or on the integrated response to stress. We have used a novel and selective V_{1b} antagonist to assess the importance of AVP in HPA homeostasis.

The compound described in this study is an effective V_{1b} antagonist both *in vitro* and *in vivo* (Presland *et al.* 2007, Craighead *et al.* 2008), and this was confirmed by our data that

Table 1 Mean $\pm$ S.E.M. of PULSAR parameters measurements

	AUC (ng)	Mean blood corticosterone (ng/ml)	Number of pulses	Inter-pulse interval	Amplitude of pulses (ng/ml)	Frequency of pulses (pulse/h)
Vehicle	370.2 $\pm$ 54	25.4 $\pm$ 9	8.8 $\pm$ 1	1.5 $\pm$ 0.2	41.6 $\pm$ 6	0.64 $\pm$ 0.08
Org	384.3 $\pm$ 32	26.1 $\pm$ 3	10.8 $\pm$ 0.5	1.2 $\pm$ 0.04	45.3 $\pm$ 6	0.79 $\pm$ 0.04

Rats were treated with vehicle (5% mulgofen in 0.9% saline, 2 ml/kg, s.c., $n=7$) or Org (30 mg/kg, s.c., $n=7$). Parameters were measured between 1700 h, (time of vehicle or Org administration) and 0700 h (time of collection of the last samples; see Fig. 3).

Figure 4 Effect of the V_{1b} R antagonist Org on ACTH and corticosterone secretion induced by exposure to restraint stress. Rats were injected with Org (30 mg/kg, s.c., $n=9$) or vehicle (5% mulgofen in 0.9% saline, 0.2 ml/kg, $n=9-10$) 30 min prior exposure to restraint (60 min). Data are expressed as mean $\pm$ S.E.M. of plasma ACTH (pg/ml, A) or plasma corticosterone (ng/ml, B) with respect to the time of exposure to stress. Black arrow indicates Org or vehicle injection, shaded bar indicate restraint stress. $^{\#}P<0.05$, effect of stress, compared with basal value (0 min), in rats treated with vehicle or Org. $^{*}P<0.05$, effect of Org, compared with vehicle, in rats exposed to restraint.

clearly show that Org is able to reduce the effect of AVP on ACTH and corticosterone release. It was beyond the scope of the current study to perform dose-response relationships for Org or AVP, but we chose a dose of AVP that in pilot studies caused a pituitary–adrenal response of similar magnitude to that seen after noise stress. We found that the higher dose of

Figure 5 Effect of the V_{1b} R antagonist Org on ACTH and corticosterone secretion induced by exposure to noise stress. Rats were injected with Org (30 mg/kg, s.c., $n=8$) or vehicle (5% mulgofen in 0.9% saline, 0.2 ml/kg, $n=8$) 30 min prior exposure to white noise (10 min, 96 dB). Data are expressed as mean $\pm$ S.E.M. of plasma ACTH (pg/ml, A) or plasma corticosterone (ng/ml, B) with respect to the time of exposure to noise. Black arrows indicate Org or vehicle injection, shaded bars indicate noise. $^{\#}P<0.05$, effect of stress, compared with basal value (0 min), in rats treated with vehicle or Org.

Org used in this study (30 mg/kg) was, compared with the smaller dose of 10 mg/kg, more potent in reducing AVP-induced corticosterone secretion, whereas the ability of Org to reduce AVP-induced ACTH secretion was similar in both doses. These data are consistent with recently

Figure 6 Effect of the *V_{1b}*R antagonist Org on ACTH and corticosterone secretion induced by injection of lipopolysaccharide. Rats were injected with Org (30 mg/kg, s.c., *n* = 7–8) or vehicle (5% mulgofen in 0.9% saline, 0.2 ml/kg, *n* = 6–8) 30 min prior to administration of lipopolysaccharide (LPS, 250 µg, i.v.). Data are expressed as mean ± s.e.m. of plasma ACTH (pg/ml, A) or plasma corticosterone (ng/ml, B) with respect to the time of LPS injection. Black arrows indicate Org or vehicle injection, grey arrows indicate LPS injection. **P* < 0.05, effect of stress, compared with basal value (0 min), in rats treated with vehicle or Org. **P* < 0.05, effect of Org, compared with vehicle, in rats injected with LPS.

reported data showing an effect of this compound on reducing CRH/desmopressin (dDAVP)-induced ACTH release. Furthermore, the same authors also showed that this *V_{1b}*R antagonist was able to reduce both ACTH and corticosterone response to CRH in combination with the *V_{1b}*R agonist d[Cha]AVP (Craighead *et al.* 2008).

Table 2 Effect of the *V_{1b}*R antagonist Org on pituitary–adrenal response to stress

	Restraint	Noise	LPS
ACTH			
Vehicle	1276 ± 132	649 ± 53	7155 ± 602
Org	865 ± 114*	606 ± 36	4089 ± 206*
Corticosterone			
Vehicle	1255 ± 127	707 ± 78	2634 ± 168
Org	1187 ± 144	637 ± 55	2442 ± 230

Data are mean ± s.e.m. of area under curve (AUC) of ACTH (pg) or corticosterone (ng) of rats injected with Org (30 mg/kg, s.c.) or vehicle (5% mulgofen in saline, 0.2 ml/kg, s.c.) 30 min prior restraint (1 h), with noise (96 dB for 10 min) or lipopolysaccharide (LPS, 250 µg, i.v.). **P* < 0.05, effect of Org, compared with vehicle in rats exposed to stress.

*Effect of the *V_{1b}*R antagonist Org on basal pituitary–adrenal activity*

Org was injected in the late afternoon, at the time of the diurnal peak of HPA activity. We found no change in either ACTH or corticosterone secretion. This was not unexpected. Although AVP is a strong modulator of the response of the corticotrophs to CRH, AVP secretion is low under basal conditions (Gillies *et al.* 1982, Rivier *et al.* 1984, Antoni 1993). These data are consistent with the hypothesis for CRF to be primarily involved in the modulation of basal HPA axis activity, whereas AVP may be involved in conditions of stress.

We further investigated the effects of Org on basal HPA axis activity by studying the circadian (Jasper & Engeland 1991) and ultradian (Windle *et al.* 1998) rhythms of corticosterone secretion. Cortisol in man, and corticosterone in rodents, is released in a pulsatile manner throughout the 24-hour period and it is the variation in the amplitude and frequency of these pulses that determine the diurnal rhythm (Windle *et al.* 1998). Org had no effect on either the circadian rhythm or the pattern of pulsatile corticosterone release.

ACTH is also released in a pulsatile manner in the rat (Carnes *et al.* 1989), and shows a diurnal rhythm albeit with an amplitude which is considerably lower than that of corticosterone (Dallman *et al.* 1978, Kaneko *et al.* 1980, 1981). Unfortunately due to the large sample volumes needed for the assay of ACTH, we were limited in the frequency and duration of samples we were able to perform and thus we could not investigate the effect of Org on ACTH pulsatility or diurnal rhythm.

*Effect of the *V_{1b}* antagonist on stress-induced pituitary–adrenal activation*

We investigated the effect of Org on restraint – a physical and psychological stressor, exposure to white noise – a psychological stressor, and administration of LPS – an immunological stressor. Org reduced the ACTH responses to restraint and LPS but had no significant effect on the response to noise.

On the other hand, Org had no effect on the corticosterone response to any of these stressors.

With respect to the differential effects on ACTH secretion, it would certainly appear that the AVP antagonist was preferentially affecting the two stressors (restraint and LPS) which have the most pronounced effect on ACTH release. This could reflect the fact that AVP release in the portal circulation is increased only when there is a major activation of the HPA axis.

Our data are consistent with other studies where the inhibitory effect of an other V_{1b}R antagonist, SSR149415, was investigated on ACTH release in rats exposed restraint, although in the same study the effect of this compound on stress-induced corticosterone release was not described (Serradeil-Le Gal *et al.* 2002).

With respect to the differential effects of Org on stress-induced ACTH and corticosterone secretion, it is possible that, even following Org treatment, ACTH levels are still high enough to induce a normal corticosterone response to stress. However, a pituitary-independent modulation of adrenal corticosterone secretion cannot be excluded. There is certainly strong evidence supporting a role for sympathetic innervation in modulating adrenal glucocorticoid secretion (Engeland & Arnhold 2005). Splanchnic nerve transection results in decreased plasma corticosterone during the diurnal peak and this effect is associated with decreased adrenal sensitivity to ACTH.

Our data are consistent with evidence showing that AVP deficient rats display lower ACTH but normal corticosterone secretion in response to stress (Zelena *et al.* 2006, Mlynarik *et al.* 2007). However, our data contrast with the decrease in both ACTH and corticosterone response to LPS in V_{1b}R KO mice (Lolait *et al.* 2007a,b). Moreover, the same authors showed that mice lacking the V_{1b}R had an attenuated ACTH, but not corticosterone, response to acute restraint (Stewart *et al.* 2008).

Our data demonstrate that Org is an active V_{1b}R antagonist *in vivo* which has no effect on basal pituitary–adrenal activity but can reduce stress-induced ACTH secretion. In man there is a close concurrence between the pituitary secretion of ACTH and the adrenal secretion of cortisol (Henley *et al.* 2008). Since severe depression is frequently associated with hyperactivity of the HPA axis, a V_{1b} antagonist may have therapeutic potential in this condition and other stress-related disorders. Since any behavioural response might also be related to an effect on central V_{1b} receptors, further studies with the use of central (intraventricular or site specific) administration of V_{1b}R antagonists would also be of interest.

Declaration of interest

Some of the authors have conflict of interests, specifically: F Spiga, L Harrison and D Knight were funded by Schering–Plough Corporation; C MacSweeney, F Thomson and M Craighead are employed by Schering–Plough Corporation.

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