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Behaviour of pigs given corticotrophin-releasing hormone in combination with flumazenil or diazepam

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Abstract

The possible involvement of an endogenous benzodiazepine (BZ) inverse agonist in the activational effects of corticotrophin-releasing hormone (CRH) on behaviour was examined in pigs given porcine CRH (75 μg) intracerebroventricularly (i.c.v.) and the BZ antagonist flumazenil (FLU; 0.09 mg/kg) intravenously (i.v.). In Experiment 1, behaviour was recorded for 75 min in pigs ($n=5$) given i.v. FLU or saline (SAL) followed by i.c.v. CRH or vehicle (VEH). Significant changes in arousal, posture and oro-nasal activity were induced by CRH, whereas FLU alone had no effect but appeared to reduce some responses to CRH. In Experiment 2, behaviour was observed for 60 min in pigs ($n=6$) given i.c.v. CRH followed by i.v. FLU, VEH or diazepam (DZ; 0.2 mg/kg). Behavioural responses to CRH, however, were unaffected by FLU, whereas certain aspects of arousal, posture and oro-nasal activity were reduced by DZ; a higher dose of DZ (0.3 mg/kg) given before CRH tended to enhance these inhibitory effects. All treatments also produced similar increases in plasma cortisol. Taken together with previous findings, the negligible effect of FLU in this study suggests that endogenous ligands for the BZ binding site on the $GABA_A$ receptor are of little importance in regulating the behavioural actions of CRH in swine.

Keywords: Pigs; CRH; Behavior; Flumazenil; Diazepam; Cortisol

Intracerebroventricular (i.c.v.) administration of corticotrophin-releasing hormone (CRH) in pigs, as in other species, has pronounced activational effects on behaviour and markedly increases plasma corticosteroid concentrations [13,18–20,23]. However, although the central mechanisms underlying these anxiogenic effects are poorly understood, rodent studies suggest that CRH may act by causing central disinhibition. This is envisaged to involve the release of an inverse agonist for the benzodiazepine (BZ) binding site on the $GABA_A$ receptor [4]. The endogenous neuromodulator is believed to be chemically similar to the betacarbolines, drugs that seem to induce anxiety in rodents [6].

Studies of CRH mechanisms in swine are predicated upon the need to increase understanding of the effects of stress on the welfare of this economically important species. Accordingly, recent research has compared the behavioural actions of porcine CRH and BZ inverse agonists in pigs. The first study [20] found that octadecaneuropeptide (ODN), a peptide derived from the putative endogenous anxiogen [5], had no activational effect at the i.c.v. dose administered. Subsequently, a comparison of the effects of CRH and betacarbolines in pigs indicated that the behavioural responses differed [21]. Hence, these findings are at variance with those reported in rodents.

The possible involvement of endogenous betacarboline-like agents in the response to CRH can be examined using the BZ antagonist flumazenil (FLU [12]). The prediction is that FLU will diminish the activational effects of CRH by preventing the inverse agonist from binding to the BZ receptor. However, because FLU may also have BZ agonist or inverse agonist activity in certain situations [8,25], there is the possibility that any reduction in behaviour may simply reflect the agonist (i.e. anxiolytic) properties of the drug. On the other hand, the combination of FLU and a submaximal dose of CRH may permit any inherent inverse agonist (i.e. anxiogenic) effects to be identified.

To the authors' knowledge, interactions between FLU and CRH have only been examined previously in rats, and

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on three occasions. Using conflict procedures, it was found that the CRH-induced decrease in punished responding for food could be reversed by peripheral [1] or central [4] administration of FLU. However, FLU did not antagonize the action of CRH in elevated plus maze or social interaction tests [7]. In view of these conflicting data, effects of FLU in an ungulate species such as the pig, in which the responses to CRH have been well documented, might provide useful additional information on this subject.

Pigs adapted to human contact and tested in their home cages in the absence of food show a variety of behavioural reactions to i.c.v. CRH (100 μg [20]). Hence, the aim of the present study was to use this approach to determine whether the behavioural response to a lower dose (75 μg) of CRH would be affected by intravenous (i.v.) administration of FLU. The FLU treatment used was slightly in excess of the amount required to reverse BZ overdosage in man (5 mg; 0.07 mg/kg [11]).

Two experiments were conducted. The first investigated the behavioural effects of CRH and FLU when administered alone or in combination. The second re-examined the interaction between CRH and FLU using a different injection protocol, and also investigated the effect of diazepam (DZ) in CRH-treated pigs.

1. Methods

The animals used in Experiment 1 were six prepubertal Large White boars weighing approximately 30 kg. They were housed in individual metabolism cages equipped with operant panels for the delivery of food (20 g) and water (20 ml) reinforcements on a fixed ratio (FR 5:1 and FR 2:1, respectively) schedule. When the pigs had learned to press the panels, food availability was signalled by a buzzer and limited to 3.5 h/day (09:00 to 12:30 hours), whereas water was continuously available. The animals were regularly handled so that they adapted to human contact. They were then surgically prepared under closed circuit halothane anaesthesia, using sterile precautions, with a catheter in the jugular vein and a cannula in the lateral cerebral ventricle. Venous catheters were flushed daily with sterile heparinised saline (SAL) and the placement of the i.c.v. cannulae was verified by the demonstration of a drinking response to angiotensin II (AII, 580 ng in 350 μl SAL). All animals drank (water reinforcements; mean ± sem, 52.0 ± 13.8) with a latency of about 30 s.

Porcine CRH (Asn^{40} [10]) was prepared by the Babraham Microchemical Facility on a P.E. Biosynthesis 'Pioneer' peptide synthesizer, using fMOC chemistry, and characterised by N-terminal sequencing, mass spectrometry and HPLC. The peptide was dissolved in sterile water (vehicle, VEH) and stored in aliquots at −30°C. Each i.c.v. injection consisted of 75 μg CRH in 400 μl VEH followed by 300 μl SAL. FLU (R0 15-1788), a gift from Hoffmann-La Roche, Welwyn Garden City, UK, was prepared, as required, by dissolving 3 mg in 0.5 ml DMSO, to which, 1.5 ml of SAL was added; each i.v. FLU injection was followed by a further 2 ml SAL. This volume of DMSO has previously been shown to have no behavioural or endocrine effect when given i.v. to pigs [21].

The test protocol involved a 2-ml i.v. injection of SAL or FLU followed 15 min later by i.c.v. administration of VEH or CRH at the start of the 75-min observation period. Two pigs were tested each afternoon in consecutive recording sessions and all animals received each of the four treatments. These were given in the order FLU/VEH, SAL/CRH, SAL/VEH, FLU/CRH, with individual pigs starting at different points in the sequence and a minimum interval of 2 days between consecutive treatments. All experimental procedures were carried out in accordance with the UK Animals Scientific Procedures Act 1986 (Project Licence No. 80/1269).

Behaviour was scored using a 3-min time-sampling procedure similar to that in previous studies [20,21]. Records were made for three categories of behaviour: 'A', activity state; 'B', posture and elimination; and 'C', oronasal activity and vocalisation. In category 'A', an assessment was made as to whether the animal was generally calm (or drowsy), alert (showing low level activity), active (engaged in continuous or high level activity) or agitated (indicated by vigorous activity and obvious emotional distress). These states were not mutually exclusive, e.g. an animal might sometimes be scored as both 'calm' and 'active' in the same 3-min time bin. Under 'B', records were made of standing or lying (non-mutually exclusive), turning around, shaking, urination and defaecation. Similarly, 'C' was concerned with the occurrence of drinking, gagging, nosing, chewing and regular grunting/barking. In addition, ordinal data were collected in category 'C' for the number of bouts of continuous nosing or chewing in each 3-min period. Event totals (maximum of 25 for nominal data, no maximum for ordinal data) were calculated for each 75-min test and used to derive medians and interquartile ranges for each treatment.

The Friedman two-way ANOVA was used to determine whether there were significant differences due to treatment. If this was the case, paired comparisons were made using the Sign test to determine whether FLU modified behaviour in either test (CRH or VEH) condition. Unfortunately, because one animal failed to complete the test series, the results are based on data from five pigs only. This means that the highest level of significance attainable in a two-tailed Sign test ($p < 0.06$) just exceeded the minimally acceptable criterion.

A second experiment was carried out to determine whether the trends apparent in Experiment 1 could be confirmed. To do this, the precision of the behavioural analysis was increased by using 1- instead of 3-min time bins. In addition, because FLU has a half-life of about 60 min [11], and this dose of CRH induces behavioural responses with a latency of about 18 min (Experiment 1),

it was reasoned out that FLU might be more effective when given 15 min after CRH. The animals were also weighed regularly so that the dose of FLU could be better controlled. The opportunity was also taken to test the effect of DZ in CRH-treated pigs.

Six prepubertal Large White boars were prepared with i.c.v. cannulae and i.v. catheters, as in Experiment 1, and housed under the same conditions. However, food availability was restricted to 90 min/day (09:00 to 10:30 hours) to limit growth rate. When tested with AII (420 ng), all animals displayed a drinking response, 39.6 ± 5.2 reinforcements, with a latency of 56 s. The pigs weighed 24.2 ± 0.7 kg (mean ± sem) on arrival and 38.2 ± 0.8 kg in the final week of the investigation.

In weeks 1 and 2, the animals were tested with i.c.v. CRH (75 μg) and i.v. FLU (0.09 mg/kg), made up, as before, in DMSO and SAL (VEH). Half of the group received CRH/FLU, and the remainder CRH/VEH, in week 1, with the treatments reversed in week 2. Two animals were tested consecutively each afternoon, as follows. The i.c.v. injection of CRH was given 15 min before i.v. FLU or VEH; observations commenced immediately after the i.v. injection with behaviour scored at 1-min intervals for 1 h. In addition, blood samples were collected before (0 min) and after (30, 60, 90 min) CRH injection, held on ice, and subsequently centrifuged. The plasma was then stored as frozen aliquots (−30°C) pending radioimmunoassay to determine cortisol concentrations.

In week 3, pigs were tested over a 3-day period with i.c.v. CRH followed 15 min later by i.v. DZ (0.2 mg/kg; Valium injection, Roche), using the above protocol; these tests were carried out separately from those involving FLU to avoid any drug interactions. On the fifth day, blood samples were taken before (0 min) and after (30, 60 and 90 min) i.c.v. (700 μl) and i.v. SAL (SAL/SAL) for the measurement of plasma cortisol concentrations. Finally, in week 4, the pigs were given a higher i.v. dose (0.3 mg/kg) of DZ 15 min before CRH injection, i.e. 30 min prior to the behavioural recording session.

Behaviour was scored in the same way as for Experiment 1, the only difference being that, under category 'C', ordinal data were collected for time (s) spent nosing per 1-min period, rather than for the number of nosing bouts. The Friedman test was used to investigate whether the responses to CRH/VEH, CRH/FLU and CRH/DZ in weeks 1–3 differed and relevant paired comparisons were made using the two-tailed Sign test. Hormonal data were analysed by examining the differences in plasma concentration between the pre-treatment (0 min) value and post-treatment (30, 60 and 90 min) mean, using the two-tailed

Table 1

(A) Activity scores (medians with interquartile ranges in parentheses), derived using a 3-min time-sampling procedure, in pigs ($n = 5$) given i.v. SAL or FLU (3 mg/animal) followed by i.c.v. VEH or CRH (75 μg) and observed for 75 min (Experiment 1)

State[a]	SAL/VEH	FLU/VEH	SAL/CRH	FLU/CRH
Calm ($p < 0.001$)	22 (9/23)	21 (21/24)	4 (1/5)	7 (4/8)
Alert ($p < 0.05$)	8 (6/11)	7 (4/18)	12 (10/15)	17 (12/20)
Active ($p < 0.02$)	0 (0/0)	0 (0/0)	15 (12/16)	4 (3/13)
Agitated (NS)	0 (0/0)	0 (0/0)	0 (0/2)	0 (0/0)

(B) Posture and elimination scores (medians with interquartile ranges in parentheses), derived using a 3-min time-sampling procedure, in pigs ($n = 5$) given i.v. SAL or FLU (3 mg/animal) followed by i.c.v. VEH or CRH (75 μg) and observed for 75 min (Experiment 1)

Event[a]	SAL/VEH	FLU/VEH	SAL/CRH	FLU/CRH
Stand ($p < 0.001$)	6 (2/6)	4 (4/4)	15 (13/16)	15 (14/16)
Lie ($p < 0.01$)	25 (25/25)	25 (24/25)	18 (16/21)	20 (13/24)
Turn ($p < 0.05$)	1 (0/1)	2 (0/2)	3 (2/5)	4 (4/6)
Shake ($p < 0.05$)	4 (1/4)	3 (2/3)	10 (6/11)	7 (6/8)
Urinate (NS)	1 (0/1)	1 (1/1)	1 (1/2)	1 (1/1)
Defaecate (NS)	3 (1/4)	2 (1/3)	4 (3/4)	2 (2/3)

(C) Oro-nasal activity and vocalisation scores (medians with interquartile ranges in parentheses), derived using a 3-min time-sampling procedure, in pigs ($n = 5$) given i.v. SAL or FLU (3 mg/animal) followed by i.c.v. VEH or CRH (75 μg) and observed for 75 min (Experiment 1)

Event[a]	SAL/VEH	FLU/VEH	SAL/CRH	FLU/CRH
Drink (NS)	0 (0/0)	0 (0/0)	1 (0/5)	2 (0/2)
Gag ($p < 0.02$)	0 (0/0)	0 (0/0)	6 (3/6)	3 (1/5)
Nose ($p < 0.01$)	2 (1/3)	1 (0/3)	18 (18/21)	15 (13/18)
Nose bout ($p < 0.01$)	3 (1/13)	2 (0/4)	106 (95/121)	45* (38/105)
Chew ($p < 0.01$)	3 (2/4)	4 (2/4)	20 (20/23)	17* (15/19)
Chew bout ($p < 0.01$)	4 (4/10)	8 (3/11)	83 (67/85)	46 (36/105)
Reg grunt (NS)	0 (0/0)	0 (0/0)	2 (1/9)	0 (0/1)

* $p < 0.06$ vs. SAL/CRH (Sign test, two-tailed).

[a] Probability values indicate significant differences due to treatment (Friedman two-way ANOVA).

paired *t*-test. The overall change in cortisol concentrations was also compared between treated (CRH/VEH, CRH/FLU, CRH/DZ) and control (SAL/SAL) conditions using the paired *t*-test.

2. Results

Findings relating to activity state, posture and elimination, and oro-nasal behaviour and vocalisation in Experiment 1 are expressed as medians with interquartile ranges in Table 1A, B and C, respectively. Table 1A indicates that the animals were relatively inactive after the control (VEH) i.c.v. injection, whereas CRH treatment decreased ($p < 0.001$) the number of instances when they were calm and increased the number of periods in which they were alert ($p < 0.05$) and/or active ($p < 0.02$). However, this dose of CRH (75 μg) did not induce a state of agitation. Moreover, FLU failed to alter activity states in either VEH- or CRH-treated pigs, although the median interval (based on the number of 3-min periods) to the first 'active' record appeared to be greater ($p < 0.06$, Sign test) after FLU/CRH (27 min) than with SAL/CRH (18 min).

Posture and elimination scores are presented in Table 1B. Pigs given i.c.v. VEH lay down for most of the time, whereas CRH treatment increased ($p < 0.001$) the incidence of standing and, correspondingly, decreased ($p < 0.01$) the occurrence of lying. The frequency of turning and shaking was also increased (both $p < 0.05$) by CRH, whereas the incidence of urination and defaecation was unchanged. However, there was no effect on any of these behaviours in CRH- or VEH-treated pigs as a consequence of FLU administration.

Results for oro-nasal activity and vocalisation are indicated in Table 1C. Drinking was unaffected by CRH, whereas gagging (different from yawning) became more frequent ($p < 0.02$). There was also a marked increase in the incidence of nosing and in the number of nosing bouts (both $p < 0.01$). Similarly, CRH intensified chewing ($p < 0.01$) and increased ($p < 0.02$) the number of chewing bouts. However, CRH did not induce a pattern of regular grunting and associated barking. In addition, although most of these behaviours exhibited by VEH- or CRH-treated pigs were unchanged by FLU, there was an indication ($p < 0.06$) that FLU may have reduced the number of nose bouts and the frequency of chewing.

Table 2

(A) Activity scores (medians with interquartile ranges in parentheses), derived using a 1-min time-sampling procedure, in pigs ($n = 6$) given i.c.v. CRH (75 μg) followed by VEH, FLU (0.09 mg/kg) or DZ (0.2 mg/kg), or CRH preceded by DZ (0.3 mg/kg), and observed for 60 min (Experiment 2)

State[a]	CRH/VEH	CRH/FLU	CRH/DZ	DZ/CRH[b]
Calm (NS)	0 (0/0)	0 (0/0)	0 (0/0)	7.5 (6/9)
Alert ($p < 0.01$)	26.5 (22/28)	39.5 (31/45)	50.0 * (47/55)	45.0 (43/56)
Active ($p < 0.01$)	35.0 (31/27)	22/5 (17/35)	8.0 * (6/13)	7.0 (0/19)
Agitated (NS)	0 (0/0)	0 (0/0)	0 (0/0)	0 (0/0)

(B) Posture and elimination scores (medians with interquartile ranges in parentheses), derived using a 1-min time-sampling procedure, in pigs ($n = 6$) given i.c.v. CRH (75 μg) followed by VEH, FLU (0.09 mg/kg) or DZ (0.2 mg/kg), or CRH preceded by DZ (0.3 mg/kg), and observed for 60 min (Experiment 2)

Event[a]	CRH/VEH	CRH/FLU	CRH/DZ	DZ/CRH[b]
Stand ($p < 0.05$)	50.5 (48/55)	48.0 (35/57)	36.5 (29/45)	29.5 (24/44)
Lie (NS)	25.5 (19/33)	26.0 (13/38)	36.5 (23/41)	38.0 (22/49)
Turn ($p < 0.05$)	12.5 (3/16)	7.0 (2/11)	1.5 (0/6)	0.5 (0/1)
Shake (NS)	10.5 (0/22)	11.5 (2/16)	2.0 (1/6)	5.0 (0/13)
Urinate (NS)	2.0 (1/3)	1.0 (0/3)	1.5 (1/3)	1.5 (1/2)
Defaecate (NS)	1.5 (1/3)	1.0 (0/3)	1.5 (0/2)	2.0 (1/3)

(C) Oro-nasal activity and vocalisation scores (medians with interquartile ranges in parentheses), derived using a 1-min time-sampling procedure, in pigs ($n = 6$) given i.c.v. CRH (75 μg) followed by VEH, FLU (0.09 mg/kg) or DZ (0.2 mg/kg), or CRH preceded by DZ (0.3 mg/kg), and observed for 60 min (Experiment 2)

Event[a]	CRH/VEH	CRH/FLU	CRH/DZ	DZ/CRH[b]
Drink (NS)	5.5 (1/8)	5.0 (2/10)	3.0 (0/7)	1.5 (0/8)
Gag ($p < 0.01$)	12.5 (11/18)	14.5 (12/21)	5.5 * (1/7)	1.0 (0/2)
Nose (NS)	54.0 (52/59)	53.5 (46/57)	46.5 (39/50)	32.5 (19/45)
Nosing time (NS)	408 (319/495)	266 (160/360)	241 (181/307)	118 (21/229)
Chew ($p < 0.02$)	51.5 (50/53)	54.5 (52/58)	49.0 * (41/50)	34.0 (18/50)
Chew bout ($p < 0.02$)	132 (117/154)	144 (132/162)	109 (99/133)	81 (22/127)
Reg grunt (NS)	2.5 (0/11)	0.0 (0/1)	1.5 (0/2)	0.0 (0/2)

* $p < 0.03$ vs. CRH/VEH (Sign test, two-tailed).

[a] Probability values indicate significant differences due to treatment (CRH/VEH × CRH/FLU × CRH/DZ; Friedman two-way ANOVA).

[b] Included for comparative purposes only, no statistical analysis.

In summary, Experiment 1 confirmed the expected activational effect of CRH and indicated a possible inhibitory action of FLU on some CRH-dependent responses. However, the power of the statistical analysis was limited by the small number of pigs used. Accordingly, a second experiment was carried out using a protocol designed to enhance the possibility of detecting an inhibitory effect of FLU (see Methods). Moreover, to determine how increased GABA tone might affect CRH-induced behaviour in pigs, the animals were also treated with DZ. Results outlining changes in activity, posture and elimination, and oro-nasal behaviour are given in Table 2A, B and C, respectively. In each case, the statistical analysis compared treatments administered using the same protocol, i.e. CRH/VEH, CRH/FLU and CRH/DZ. The data obtained using a higher dose of DZ and a different protocol (DZ/CRH, week 4) were not subjected to statistical analysis and are merely included for comparative purposes.

Activity records (Table 2A) indicate that there were few occasions (in weeks 1–3) when the animals were calm; this reflects the fact that observations did not commence until 15 min after CRH administration. The occurrence of alert and active episodes differed (both $p < 0.01$) between treatments; this was due to an effect of DZ in reducing ($p < 0.03$) scores for activity and reciprocally increasing ($p < 0.03$) those for alertness (see Methods). However, as in Experiment 1, the degree of agitation induced by this dose of CRH was minimal. The results obtained with the higher dose of DZ in week 4 were similar to those in week 3, although there appeared to be more occasions when the animals were calm.

With respect to posture and elimination (Table 2B), only standing and turning differed ($p < 0.05$) between treatments. These effects were probably due to an inhibitory action of DZ, even though paired comparisons (CRH/DZ vs. CRH/VEH) failed to detect a significant difference. The apparent reduction in the incidence of standing was associated with a tendency for DZ to increase the frequency of lying. Similarly, both turning and shaking seemed to occur least often in the CRH/DZ treatment condition, whereas the incidence of urination and defaecation was similar in each case. The tendency for DZ to reduce the occurrence of standing and turning was reaffirmed by the results obtained in week 4.

Oro-nasal and vocalisation scores are presented in Table 2C. The frequency of drinking, nosing, regular grunting and the time spent nosing did not differ between treatments. However, there were differences in the incidence of gagging and chewing, and in the number of chewing bouts ($p < 0.01$, $p < 0.02$ and $p < 0.02$, respectively). In the case of gagging and chewing, these effects were due to a reduction in activity caused by DZ (both $p < 0.03$). These differences are in line with a general tendency for DZ to reduce nearly all behaviours under category 'C', a trend that was further enhanced by the injection of a higher dose in week 4.

The effects on plasma cortisol concentrations of the experimental treatments used in weeks 1–3, together with the response to control treatment (SAL/SAL), are illustrated in Fig. 1. The results show that there was a small non-significant increment in mean hormone concentrations (9.5 nmol/l) in the SAL/SAL condition and similar significant

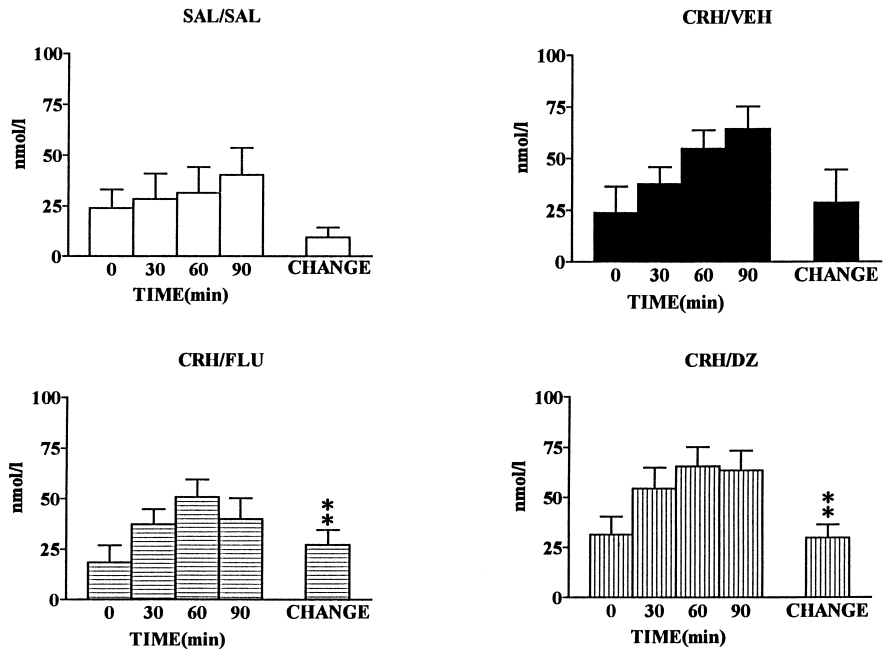

Fig. 1. Plasma cortisol concentrations (nmol/l; mean ± sem) in pigs before (0 min) and after (30–90 min) i.c.v./i.v. treatment with SAL/SAL, CRH/FLU, CRH/DZ ($n = 6$) and CRH/VEH ($n = 5$) in Experiment 2. Injection of CRH (75 μg i.c.v.) induced a significant (** $p < 0.02$, paired t-test) change when the animals were given i.v. FLU (0.09 mg/kg) or DZ (0.2 mg/kg). See text for further details.

increases after CRH/FLU (27.4 nmol/l, $p < 0.02$) and CRH/DZ (29.7 nmol/l, $p < 0.01$). The change induced by CRH/VEH was of the same order (28.7 nmol/l) but failed to achieve significance; this was because blood samples were obtained from only five of the pigs and one animal had a high initial hormone concentration. Additional paired comparisons revealed that the overall changes in plasma cortisol concentrations after CRH/FLU and CRH/DZ were greater ($p < 0.03$ and $p < 0.04$, respectively) than in the SAL/SAL condition, whereas the increase produced by CRH/VEH was not significant, probably for the reason noted above. However, these differences were no longer significant when correction was made for multiple comparisons. Comparison between CRH/VEH and CRH/FLU or CRH/DZ also failed to detect significant differences.

In summary, the results of Experiment 2 failed to confirm the apparent inhibitory effect of FLU observed in Experiment 1. There were only three instances where median scores for particular behaviours were noticeably smaller than for those obtained with CRH/VEH (active, 22.5 vs. 35.0; turn, 7.0 vs. 12.0; nosing time, 266 vs. 408), but none of these effects was significant. By contrast, there was a general reduction in activity attributable to the action of DZ, indicated by significant overall differences due to treatment (alert, active, stand, turn, gag, chew and chew bout) and between CRH/VEH and CRH/DZ (alert, active, gag and chew). However, this dose of CRH did not affect defaecation or vocalisation and neither FLU nor DZ modified its effect on cortisol release. Furthermore, the higher dose of DZ (DZ/CRH treatment) appeared to have a greater anxiolytic effect, but because a different protocol was used this could not be verified statistically.

3. Discussion

In contrast to the anxiolytic action of pharmacological agonists acting at the BZ site on the $GABA_A$ receptor, the existence of an endogenous psychoactive ligand for this receptor remains controversial. Some studies [1,4,7] have examined this hypothesis in rats treated with both CRH and FLU, but none has investigated the behaviour of individual animals in the home environment. However, this approach is appropriate for pigs [20,21], and the present study represents the first attempt to address this issue in a non-rodent species. The results indicate that FLU did not significantly alter the response to CRH whereas DZ was effective in this respect. There were also no effects of FLU or DZ on CRH-induced cortisol release.

Centrally administered porcine CRH (100 μg) produces long-lasting behavioural reactions in swine [20], but the lower dose (75 μg) used here produced a less intense response, characterised by an absence of agitation and little regular grunting/barking. The animals also received a high (clinically effective) i.v. dose of FLU judged to be sufficient to abolish the activity of any endogenous BZ receptor ligands. However, FLU did not activate behaviour in SAL-treated pigs, or enhance the response to CRH. Therefore, the drug did not appear to exhibit BZ inverse agonist properties.

Studies with FLU in man (i.v. doses up to 1 mg; 0.01 mg/kg) have reported anxiolytic effects during emotional stress [15] and anxiogenic responses in panic attack patients, but not in normal subjects [17]. A lower dose (0.56 mg; 0.008 mg/kg) could be discriminated by some individuals after training [24] and a higher dose (3 mg; 0.04 mg/kg) produced dizziness [9]. Therefore, the dose use here (0.09 mg/kg) may have had some effect on arousal. However, the behavioural effects of FLU were not significant, whereas DZ caused a generalised inhibition and significant decreases in activity, gagging and chewing.

These negative findings indicate that FLU has no BZ agonist activity in pigs at this dose level and suggest that any involvement of endogenous betacarbolines in the response to CRH is negligible. In agreement with this interpretation, ODN was found to be devoid of activational effects in pigs [20] and the behavioural response to exogenous betacarbolines was dissimilar to that induced by CRH [21]. In addition, FLU did not alter the behaviour of CRH-treated rats in the elevated plus maze, or during social interaction [7].

In contrast to the above, conflict tests in rats have provided evidence for a modifying effect of FLU on CRH-dependent behaviour. A stimulatory (BZ agonist [2]) effect on appetite can be ruled out as an alternative explanation for these findings because the enforced 15% reduction in body weight would have ensured maximal feeding motivation. However, in one of the reports [1], the anxiogenic effect of CRH was unclear because punished and unpunished response rates were identical; moreover, there was also no effect on CRH-induced locomotion. In the other study [4], punished responding was selectively reduced by CRH and reinstated by i.c.v. FLU or chlordiazepoxide (CDP). Nevertheless, because the same doses (10 μg) of FLU and CDP were used, even though CDP has a much lower potency, the FLU dosage may have been sufficiently high to produce BZ agonist (anxiolytic) effects.

Punished responding for food in swine is increased by DZ (1 mg/kg, i.m.) through a mechanism that is independent of motivational state [3]. Similarly, DZ (0.3 mg/kg, i.v.) reverses conditioned suppression in operant feeding pigs (Baldwin and Ebenezer, unpublished results). In addition, in the present study, DZ treatment (0.2 mg/kg, i.v.) similar to the acute anxiolytic dose in man (0.14 mg/kg [16]) decreased the activational effect of CRH. Together, therefore, these findings confirm that DZ reduces experimental and CRH-induced anxiety in pigs. However, although BZ's act on CRH neurones in man to inhibit pituitary/adrenocortical activation [14] and prevent stress-induced cortisol release [22], no such effect was apparent in this study. This may be due to species differences or because DZ has a low potency in this regard compared to other BZs [14].

In conclusion, the results of this and former studies provide little support for the hypothesis that the anxiogenic action of CRH in swine involves the release of an endogenous BZ inverse agonist. Therefore, any physiological role for the $GABA_A$ receptor in the mediation of anxiety probably depends upon different neuromodulators. In addition, it is likely that other neurotransmitter systems are also involved in the anxiety-inducing effects of CRH. Further work will be needed to characterise these factors.

References

[1] Britton KT, Lee G, Koob GF. Corticotropin releasing factor and amphetamine exaggerate partial agonist properties of benzodiazepine antagonist RO 15-788 in the conflict test. Psychopharmacology 1988;94:306–11.

[2] Cooper SJ, Estall LB. Behavioural pharmacology of food, water and salt intake in relation to drug actions at benzodiazepine receptors. Neurosci Biobehav Rev 1985;9:5–19.

[3] Dantzer R. Anti-punishment effects of diazepam: interaction with shock and food deprivation levels in pigs. Psychopharmacology 1978;58:99–104.

[4] De Boer SF, Katz JL, Valentino RJ. Common mechanisms underlying the proconflict effects of corticotropin-releasing factor, a benzodiazepine inverse agonist and electric foot-shock. J Pharmacol Exp Ther 1992;262:335–42.

[5] Ferrero P, Guidotti A, Conti-Tranconi B, Costa E. A brain octadecaneuropeptide generated by tryptic digestion of DBI (diazepam binding inhibitor) functions as a proconflict ligand of benzodiazepine recognition site. Neuropharmacology 1984;23:1359–62.

[6] File SE, Baldwin HA. Effects of β-carbolines in animal models of anxiety. Brain Res Bull 1987;39:293–9.

[7] File SE, Johnston AL, Baldwin HA. Anxiolytic and anxiogenic drugs. Changes in behavior and endocrine responses. Stress Med 1988;4:221–30.

[8] File SE, Pellow S. Intrinsic actions of the benzodiazepine receptor antagonist RO 15-1788. Psychopharmacology 1986;88:1–11.

[9] Griffiths RR, Evans SM, Guarino JJ, Roache JD, Furman WR, Liebson I, Schwam EM. Intravenous flumazenil following acute and repeated exposure to lorazepam in healthy volunteers: antagonism and precipitated withdrawal. J Pharmacol Exp Ther 1993;265:1163–74.

[10] Guoth J, Olsen DB, Kovacs M, Schally AV. Synthesis, purification and biological evaluation of porcine corticotropin-releasing factor. Life Sci 1987;41:1003–10.

[11] Hobbs WR, Rall TW, Verdoorn TA. Hypnotics and sedatives; ethanol. In: Harman JG, Limbird LE, editors. Goodman and Gilman's the pharmacological basis of therapeutics. 9th ed. New York: McGraw-Hill, 1996. pp. 372–3.

[12] Hunkeler W, Mohler H, Pieri L, Polc P, Bonetti EP, Cumin R, Schaffner R, Haefely W. Selective antagonists of benzodiazepines. Nature 1981;290:514–6.

[13] Johnston RW, van Borrell EH, Anderson LL, Kojic LD, Cunnick JE. Intracerebroventricular injection of corticotropin-releasing hormone in the pig: acute effects on behavior, adrenocorticotropin secretion, and immune suppression. Endocrinology 1994;125:642–8.

[14] Kalogeras KT, Calogero AE, Kuribayiashi IK, Gallucci WT, Kling GP, Chrousos GP, Gold PW. In vitro and in vivo effects of the triazolobenzodiazepine alprozolam on hypothalamic–pituitary adrenal function: pharmacological and clinical implications. J Clin Endocr Metab 1990;70:1462–71.

[15] Kapezinski F, Curran HV, Gray J, Leder M. Flumazenil has an anxiolytic effect in simulated stress. Psychopharmacology 1994;114:187–9.

[16] Monthly Index of Medical Specialities, Haymarket, London.

[17] Nutt DJ, Glue P, Lawson C, Wilson S. Flumazenil provocation of panic attacks. Evidence for altered benzodiazepine receptor sensitivity in panic disorder. Arch Gen Psychiatry 1990;47:917–25.

[18] Parrott RF. Central administration of corticotropin releasing factor in the pig: effects on operant feeding, drinking and plasma cortisol. Physiol Behav 1990;47:519–24.

[19] Parrott RF. Cortisol release in pigs following peripheral and central administration of ovine and human corticotropin-releasing hormone. Acta Endocrinol 1990;123:108–12.

[20] Parrott RF, Vellucci SV, Goode JA. Behavioral and hormonal effects of centrally injected "anxiogenic" neuropeptides in growing pigs. Pharmacol Biochem Behav 2000;65:123–9.

[21] Parrott RF, Vellucci SV, Goode JA. Behavioural effects of beta-carbolines in pigs: anxiety or aversion? Pharmacol Biochem Behav (In Press).

[22] Petraglia F, Bakalakis S, Facchinetti F, Volpe EE, Genazzani AR. Effects of sodium valproate and diazepam on beta-endorphin, beta-lipotropin and cortisol secretion induced by hypoglycemic stress in humans. Neuroendocrinology 1986;44:320–5.

[23] Salak-Johnston JL, McGlone JJ, Whisnant CS, Norman RL, Kraeling RR. Intracerebroventricular porcine corticotropin-releasing hormone and cortisol effects on immune measures and behavior. Physiol Behav 1997;61:15–23.

[24] Smith BJ, Bickel WK. Flumazenil discrimination by humans under a two-response and a novel-response procedure. J Pharmacol Exp Ther 1999;291:1257–68.

[25] Vellucci SV, Webster RA. Is RO 15-1788 a partial agonist at benzodiazepine receptors? Eur J Pharmacol 1983;90:263–8.