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ORIGINAL INVESTIGATION

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Cannabinoids modulate ultrasound-induced aversive responses in rats

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Abstract *Rationale:* Mechanisms and brain substrates mediating cannabinoid-induced modulation of behaviour towards aversive stimuli are poorly understood. *Objectives:* To investigate the effects of systemic and intra-dorsal periaqueductal grey (PAG) administration of the cannabinoid receptor agonist HU210 on behaviour and plasma corticosterone levels in rats exposed to ultrasound and determine the contribution of CB_1 receptors. *Methods:* In experiment 1, rats received vehicle or CB_1 receptor antagonist SR141716A (3 mg/kg, IP) 30 min prior to a second injection of vehicle or HU210 (5, 20 or 80 μg/kg, IP). In experiment 2, rats received intra-dorsal PAG vehicle or SR141716A (30 μg/rat) 10 min prior to intra-dorsal PAG vehicle or HU210 (5 μg/rat). Following injections, rats were exposed to an aversive 20 kHz ultrasonic tone for 3 min. Behaviour, including hyperlocomotor activity and freezing, was monitored during and post-ultrasound. Plasma corticosterone levels 10 min post-ultrasound were measured. *Results:* Ultrasound induced explosive running and freezing behaviour. Systemic administration of HU210 attenuated the expression of ultrasound-induced hyperlocomotor activity and increased freezing. The HU210-induced attenuation of hyperlocomotor activity was blocked by SR141716A. Intra-PAG administration of HU210 reduced the expression of ultrasound-induced hyperlocomotor activity, an effect not blocked by SR141716A. Systemic and intra-PAG administration of HU210 increased plasma corticosterone levels, an effect not blocked by SR141716A. *Conclusions:* The cannabinoid receptor agonist HU210 modulates behaviour towards an aversive ultrasound stimulus in rats, an effect accompanied by increased HPA axis activity. These effects may be mediated, at least in part, by the dorsal PAG but cannot be explained solely by an action at CB_1 receptors.

Keywords Cannabinoid receptor · Aversion · Periaqueductal grey · Ultrasound · HPA axis · Panic

Introduction

Basic preclinical and clinical research has shown that cannabinoid compounds demonstrate clear potential as therapeutic agents for the treatment of a wide range of conditions including pain, inflammation, eating disorders, neurodegenerative disorders and glaucoma. However, cannabinoids induce aversive emotion and precipitate anxiety or panic attacks in about 20% of cannabis users (Annis and Smart 1973; Hollister 1986; Thomas 1996). A greater understanding of the mechanisms underlying cannabinoid-induced aversion will aid the development of drugs without this side-effect profile and may shed light on the therapeutic potential of cannabinoids for the treatment of anxiety or panic disorder.

The aversive effects of cannabinoid receptor agonists have been reported in rats and mice using models of conditioned place and taste aversion (Parker and Gillies 1995; McGregor et al. 1996b; Sanudo-Pena et al. 1997; Hutcheson et al. 1998; Cheer et al. 2000a), the elevated plus-maze (Onaivi et al. 1990), defensive withdrawal test (Rodríguez de Fonseca et al. 1996), and the light-dark emergence test (Navarro et al. 1993). Cannabinoids also elevate plasma levels of the stress hormone corticosterone (Weidenfeld et al. 1994; Rodríguez de Fonseca et al. 1996; Martin-Calderon et al. 1998; Manzanares et al. 1999). In contrast, there is evidence for anti-aversive effects of cannabinoids in rat pup ultrasonic vocalization (McGregor et al. 1996a) and light-dark box (Berrendero and Maldonado 2002) paradigms. Furthermore, the rewarding properties of cannabinoids have been demonstrated using self-administration and intracranial self-

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stimulation paradigms (for review, see Maldonado and Rodríguez de Fonseca 2002). These effects may result from cannabinoid-induced activation of the mesolimbic dopaminergic system (French 1997; Cheer et al. 2000b).

Thus, preclinical studies in the literature relating to cannabinoids and aversion are somewhat ambiguous, with evidence for both aversive and anti-aversive effects following systemic cannabinoid administration. Few studies have investigated the brain regions mediating these effects. The CB_1 receptor is distributed heterogeneously throughout brain regions such as the amygdala, hypothalamus and periaqueductal grey (PAG) (Herkenham et al. 1990; Tsou et al. 1998), all of which play an important role in mediating behavioural responses to aversive stimuli. Onaivi et al. (1995) have demonstrated that intra-amygdaloid administration of the cannabinoid agonist Δ^9-tetrahydrocannabinol(Δ^9-THC) is aversive in the mouse elevated plus-maze test. Increased expression of the immediate early gene *c-fos* in stress-related brain nuclei, including the amygdala and PAG, has been demonstrated following acute systemic administration of the cannabinoid agonist HU210 in rats (Rodríguez de Fonseca et al. 1997). We have demonstrated that intra-dorsal PAG administration of the cannabinoid agonist HU210 attenuates hyperlocomotor behaviour induced by chemical stimulation of the PAG (Finn et al. 2003b). The present study focused on the role of the PAG in mediating the behavioural and hypothalamo-pituitary-adrenal (HPA) axis response to the aversive stimulus of ultrasound exposure.

Rats emit ultrasonic "distress" calls in the range of 18–27 kHz in stressful situations such as exposure to a predator, attack by another rat or on exposure to painful stimuli (Sales 1972; van der Poel et al. 1989; Blanchard et al. 1991). Exposure of rats to artificially generated ultrasound at a frequency of 20 kHz induces a defensive response (Brudzynski and Chiu 1995) characterized by bouts of explosive hyperlocomotor activity and freezing behaviour (Beckett et al. 1996; Commissaris et al. 2000; Neophytou et al. 2000; Finn et al. 2003a). The ultrasound-induced aversive response is very similar to that produced by direct electrical or chemical stimulation of the PAG with the advantage that it is a more ethologically relevant, non-invasive stimulus. In addition, ultrasound exposure results in increased expression of *c-fos* in the PAG (Beckett et al. 1997). The ultrasound-induced response in Lister-Hooded rats is intensity- and duration-dependent (Commissaris et al. 2000). Lower intensities of ultrasound (<81 dB) induce freezing and occasional running, while higher intensities (91–101 dB) result in running, jumping and occasionally convulsions. Explosive running and freezing are characteristic of panic-like defence behaviour in rats and occur in response to exposure to a predator (Blanchard et al. 1986; Blanchard and Blanchard 1989) or to cat odour (Blanchard et al. 2001) or chemical/electrical stimulation of the periaqueductal grey (Hilton and Redfern 1986; Beckett et al. 1992). Presence or perception of threat also reduces non-defensive behaviours such as rearing and grooming (Blanchard et al. 1991). The ultrasound-induced defence response in rats is considered to be analogous to panic in humans (Deakin and Graeff 1991), and both responses share several fundamental components including the cardiovascular, analgesic and behavioural motivational aspects.

The first aim of the present study was to investigate the effect of systemic administration of the potent, non-selective cannabinoid receptor agonist HU210 on the behavioural and corticosterone response to the aversive stimulus of ultrasound exposure in rats. A second series of experiments, using intra-dorsal PAG administration of HU210, examined the role of the PAG in mediating cannabinoid-induced modulation of behavioural and HPA axis responses to ultrasound. The selective CB_1 receptor antagonist SR141716A was used to investigate the specific involvement of the CB_1 receptor subtype in the effects observed.

Materials and methods

Animals

Male Lister-Hooded rats (250–300 g, Charles River, UK) were used in these studies. Food and water were available *ad libitum*. Rats were group housed (four or five rats per cage) for a minimum of 4 days prior to experiments. A 12-h light-dark cycle was maintained (lights on at 0700 hours) and all experiments were carried out in the light period between 0930 and 1600 hours in accordance with UK Home Office guidelines for experiments on animals under Project Licence 40/1955.

Experiment 1(a): effect of systemically administered HU210 and SR141716A on behavioural and corticosterone responses of rats exposed to ultrasound

Rats were randomly divided into ten experimental groups. The groups were: vehicle-vehicle-home cage (n=4), vehicle-vehicle-no ultrasound (n=11), vehicle-vehicle-ultrasound (n=20), vehicle-HU210 (5 μg/kg, IP)-ultrasound (n=7), vehicle-HU210 (20 μg/kg, IP)-ultrasound (n=8), vehicle-HU210 (80 μg/kg, IP)-ultrasound (n=7), SR141716A- HU210 (5 μg/kg, IP)-ultrasound (n=7), SR141716A-HU210 (20 μg/kg, IP)-ultrasound (n=7), SR141716A-HU210 (80 μg/kg, IP)-ultrasound (n=7) and SR141716A-vehicle-ultrasound (n=7).

Rats were injected with the cannabinoid CB_1 receptor antagonist SR141716A (3 mg/kg, IP) or vehicle (ethanol:cremophor:saline, 1:1:18, 1 ml/kg, IP) and 30 min later with the potent non-selective cannabinoid receptor agonist HU210 (5, 20 or 80 μg/kg, IP) or vehicle (1 ml/kg, IP). The doses of HU210 and SR141716A were chosen on the basis of published studies demonstrating their effectiveness in models of aversion and on HPA axis activity (Navarro et al. 1997b; Martin-Calderon et al. 1998; Cheer et al. 2000a; Romero et al. 2002). After a further 30 min, rats were placed in a circular open-field arena (75 cm diameter, 60 cm high). Home cage controls (veh-veh-HC) were not placed in the open-field arena and were not exposed to ultrasound. Another group of controls were placed in the open-field arena but were not exposed to ultrasound (veh-veh-no US). The methodology used was similar to that described previously by Beckett et al. (1996). Two minutes after placement in the arena, ultrasound (continuous square wave, 20 kHz, 70–80 dB) was delivered for 3 min, using a multifunction signal generator (Jupiter 500, Black Star, UK) and Piezo Horn Tweeter (Pro-Sound, China) mounted at a height of 20 cm on the wall of the arena. The signal frequency and intensity was monitored using a digital oscilloscope (Nicolet 310, France). The rats were left

in the arena for a further 10 min post-ultrasound exposure. Rat behaviour was monitored and tracked pre-, during, and for 5 min post-ultrasound using a camera mounted vertically above the arena and Ethovision software (Noldus, Netherlands). The duration of freezing, frequency of explosive running episodes, frequency of rearing and duration of grooming were manually scored, using Ethovision software, by a trained experimenter blind to the treatment groups, while the distance moved and velocity of movement were automatically measured. An explosive running episode was defined as hyperlocomotor activity during which the velocity was greater than or equal to 42 cm/s. This cut-off represented an 8-fold increase in velocity relative to baseline activity in the absence of ultrasound (5.6±0.5 cm/s) and was chosen following correlation of velocity graphs for each individual rat with animal behaviour recorded on video tape. Freezing was defined as the cessation of all visible movement except that necessary for respiration. The number of faecal boli produced by each rat was also counted at the end of the trial. Rats were killed by stunning and decapitation 10 min following termination of ultrasound (or 45 min post-injection). Home cage and open-field control groups were killed at an identical time-point. Trunk blood was collected into heparinized tubes on ice. Blood was centrifuged at 1600 *g* for 10 min and plasma was removed and stored at −20°C prior to radioimmunoassay for corticosterone levels.

Experiment 1(b): effect of systemically administered HU210 on rat locomotor activity in the open-field arena

Rats were randomly divided into three experimental groups. The groups were: vehicle-vehicle-no ultrasound (n=11), vehicle-HU210 (5 μg/kg, IP)-no ultrasound (n=6), vehicle-HU210 (20 μg/kg, IP)-no ultrasound (n=5).

In a similar protocol to that described above, rats were injected with vehicle (ethanol:cremophor:saline, 1:1:18, 1 ml/kg, IP) and 30 min later with the potent non-selective cannabinoid receptor agonist HU210 (5 or 20 μg/kg, IP) or vehicle (1 ml/kg, IP). After a further 30 min, rats were placed in the same circular open-field arena described above and behaviour was monitored for 10 min. Locomotor activity (total distance moved) was assessed automatically by Ethovision tracking software and freezing behaviour was scored manually by an observer blind to the treatments. Effects of the highest dose of HU210 (80 μg/kg, IP) on locomotor activity (distance moved) were assessed from data collected during the 2 min pre-ultrasound period.

Experiment 2: effect of intra-PAG administration of HU210 and SR141716A on behavioural and corticosterone responses of rats exposed to ultrasound

Cannula implantation

A stainless steel guide cannula (18 mm, 23 G) was stereotaxically implanted above the midbrain dorsal PAG (AP −6.7, ML +1.7, DV −3.6, Paxinos and Watson 1997) at an angle of 20°, to avoid the superior sagittal sinus, under isoflurane (1.5–2%) in nitrous oxide (1.5 l/min)-oxygen(0.5 l/min) anaesthetic gas mixture. A stylet made from stainless steel tubing (18 mm, 31 G) was inserted into the guide cannula to prevent blockage by debris. The non-steroidal anti-inflammatory agent, carprofen (4.5 mg/kg, SC, Rimadyl, Pfizer, UK), was administered before the surgery for postoperative analgesia. Following cannula implantation, the rats were housed singly. At least 6 days were allowed for recovery post-surgery. During this recovery period the rats were handled and their body weight and general health monitored daily.

Microinjections

Drugs were microinjected manually into the dorsal periaqueductal grey (as defined by Bandler and Keay 1996) in a volume of 250 nl over 20 s using an injector and Hamilton syringe. The injector comprised a stainless steel tube (31 G) with a collar (23 G) sizing it to 20 mm (2 mm longer than the guide cannula) attached to 50 cm of polythene tubing (0.75 mm OD, 0.28 mm ID) to minimize handling and enable injections to be carried out while rats were in the home cage.

Experimental procedure

There were five groups of rats: vehicle-vehicle-no ultrasound (n=5), vehicle-vehicle-ultrasound (n=7), vehicle-HU210-ultrasound (n=6), SR141716A-HU210-ultrasound (n=6), SR141716A-vehicle-ultrasound (n=4). Each rat received two intra-dorsal PAG microinjections prior to exposure to 20 kHz square wave ultrasound (70–80 dB): (1) SR141716A (30 μg/rat or vehicle (100% DMSO) and 10 min later, (2) HU210 (5 μg/rat) or vehicle. The dose of HU210 was chosen on the basis of previous studies in our laboratory (Finn et al. 2003b) and others (Martin et al. 1998) demonstrating its effectiveness in models of pain and aversion following intracerebral administration, and the dose of SR141716A was chosen on the basis of studies demonstrating its ability to block agonist-induced behavioural effects following intracerebral administration (Welch et al. 1998). Ten minutes after the second intra-dorsal PAG microinjection, the rats were placed in a circular open arena (75 cm diameter, 60 cm high). The procedure for delivering ultrasound and assessing rat behaviour was identical to that described above. Drug effects on locomotor activity (total distance moved) and on freezing behaviour were assessed from data collected during the 2 min pre-ultrasound period. Rats were killed by stunning and decapitation 10 min following termination of 3 min ultrasonic tone, and trunk blood plasma was collected as described for corticosterone radioimmunoassay.

Histology

The site of injection was confirmed prior to data analysis. Pontamine sky blue dye (250 nl) was microinjected through the guide cannula following the behavioural experiments. Coronal brain sections (100 μm) with the blue dye mark were background stained with Neutral Red and mounted on glass slides for precise location of the site of microinjection using a light microscope. Seventy-six percent of the injectors were successfully positioned in the dorsal PAG with 24% positioned either outside the PAG, in the aqueduct or in the lateral or ventrolateral PAG. Only the results of experiments in which the cannula was positioned in the dorsal PAG were included in the analysis (Fig. 1). No distinction was made between dorsomedial and dorsolateral PAG.

Corticosterone radioimmunoassay

Total corticosterone was measured directly in plasma (diluted 1 in 10 in 0.9% saline) using a corticosterone radioimmunoassay kit (IDS, UK). The tracer was [^{125}I]corticosterone with a specific activity of 6.7 kBq/ml. The sensitivity of the assay was 0.39 ng/ml.

Drug preparation

In experiment 1, HU210 (6a*R*-*trans*-3-[1,1-dimethylheptyl]-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6*H*-dibenzo[b,d]pyran-9-methanol, Tocris Cookson Ltd, UK) was diluted with ethanol (100%, Sigma). Surfactant (Cremophor, Sigma) and saline (0.9% NaCl) were then added to produce a final concentration of 5, 20 or 80 μg/ml of HU210 in ethanol-cremophor-saline (1:1:18) vehicle. Similarly, a 3 mg/ml emulsion of SR141716A (N-[piperidin-1-yl]-5-([4-chlorophenyl]-1-[2,4-dichlorophenyl]-4-methyl-1-H-pyrazole-3-carboxamide], NIMH, USA) was obtained by addition of ethanol (100%), followed by addition of cremophor and saline in the ratio of 1:1:18 (ethanol:cremophor:saline). All the drug solutions were

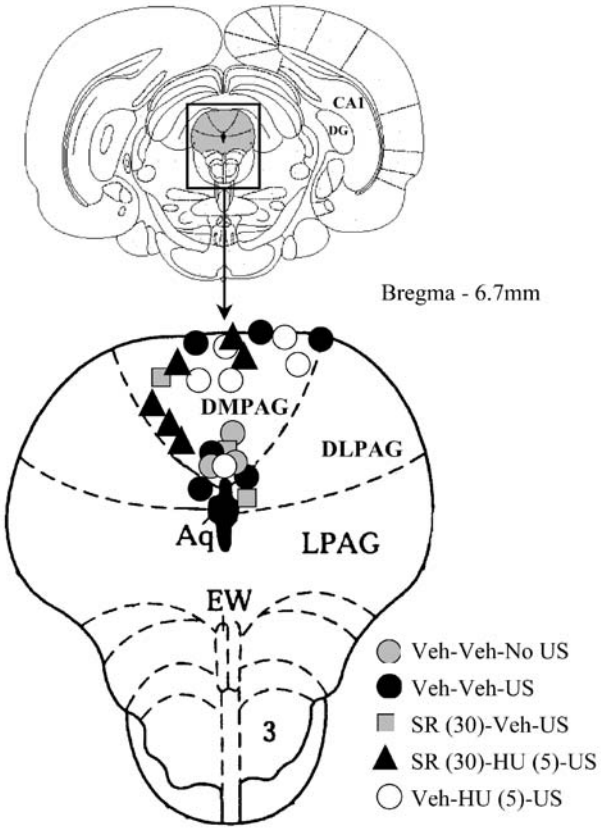

Fig. 1 Schematic showing position of injectors marked by microinjection of pontamine sky blue dye in Lister-hooded rats after assessment of ultrasound-induced behaviour. *CA1* CA1 of hippocampus; *DG* dentate gyrus; *DMPAG* dorsomedial PAG; *DLPAG* dorsolateral PAG; *LPAG* lateral PAG; *Aq* aqueduct; *EW* Edinger Westphal nucleus; *3* oculomotor nucleus; *Veh* vehicle; *HU(5)* HU210, 5 μg/rat; *SR(30)* SR141716A, 30 μg/rat; *US* ultrasound. Brain outline is from the atlas of Paxinos and Watson (1997). Some injector positions overlapped and thus may not be clearly differentiated in this figure

freshly prepared on experimental days and stored on ice and in darkness during the experiments. Drugs or vehicle were administered IP in a volume of 1 ml/kg. In experiment 2, HU210 and SR141716A were dissolved in dimethyl sulphoxide (DMSO) to obtain 5 μg/250 nl and 30 μg/250 nl solutions, respectively. Drugs or DMSO vehicle were microinjected intra-PAG in a volume of 250 nl.

Data analysis

Behavioural data from two time bins: (a) 3 min during ultrasound and (b) 5 min post-ultrasound were analysed using either Student's unpaired two-tailed *t*-test (for analysis of the effects of ultrasound in vehicle-treated rats) or one-way ANOVA followed by Fisher's PLSD post-hoc test (for analysis of drug treatment effects in ultrasound-exposed and naive rats). Corticosterone data were also analysed using one-way ANOVA followed by Fisher's PLSD. The chi-squared test was used to analyse the effects of drug treatment on the percentage of animals exhibiting explosive running behaviour. Data are expressed as means±SEM. *P*-values ≤ 0.05 were considered significant.

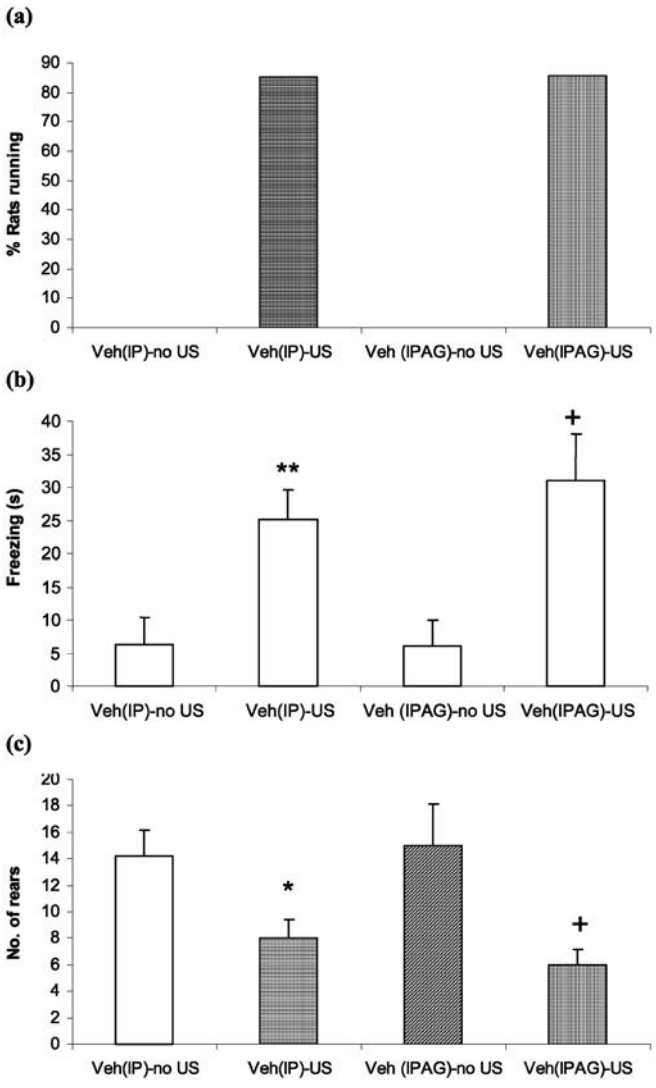

Fig. 2 Behavioural effects of ultrasound exposure in Lister-hooded rats administered vehicle either systemically (*IP*) or intra-PAG (*IPAG*). **a** Percentage of rats which showed explosive running, **b** duration of freezing and **c** frequency of rearing during exposure to ultrasound. Data are means±SEM (n=5–20). $^{**}P<0.01$, $^{*}P<0.05$ versus Veh(IP)-no US and $^{+}P<0.05$ versus Veh(IPAG)-no US (Student's unpaired *t*-test). *Veh* Vehicle; *US* ultrasound

Results

Effects of ultrasound exposure on rat behaviour

During the 3-min ultrasound exposure period, ultrasound induced one or more episodes of explosive running in 85% of rats administered vehicle IP, and in 86% of rats administered vehicle into the dorsal PAG. Thus, surgical insertion of intra-PAG cannulae did not affect the ability of rats to respond to the aversive ultrasound stimulus. Rats not exposed to ultrasound did not exhibit explosive hyperlocomotor activity (Fig. 2a). A significant increase in the duration of freezing behaviour and a significant reduction in rearing also occurred in rats exposed to

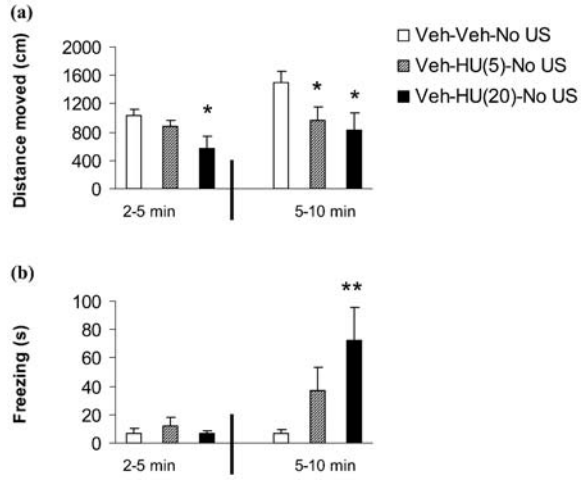

Fig. 3 The effect of HU210 (5 or 20 μg/kg, IP) on **a** locomotor activity (distance moved) and **b** freezing behaviour of rats in an open-field arena. Data are means±SEM (n=5–11). $^{**}P<0.01$, $^{*}P<0.05$ comparing Veh-HU(20)-No US with Veh-Veh-No US and $^{\dagger}P<0.05$ comparing Veh-HU(5)-No US with Veh-Veh-No US (ANOVA, Fisher's PLSD post-hoc test). *Veh* vehicle; *HU* HU210; *US* ultrasound

ultrasound, compared with rats not exposed to ultrasound (Fig. 2b,c; $P<0.01$ or $P<0.05$ Student's unpaired *t*-test). Grooming was unaffected during ultrasound exposure. In the 5-min post-ultrasound period, rats did not exhibit episodes of explosive hyperlocomotor activity. Rearing, grooming and freezing were not significantly altered during the post-ultrasound period compared with rats not exposed to ultrasound.

Effect of HU210 on locomotor activity and freezing behaviour of rats not exposed to ultrasound in the open-field arena

One-way ANOVA on data collected 2–5 min following placement of rats in the open-field arena (i.e. equivalent to the ultrasound exposure period) revealed a significant effect of treatment on distance moved [$F(2,19)=3.79$; $P=0.041$] but not on freezing [$F(2,19)=0.36$; $P=0.7$]. A low dose of HU210 (5 μg/kg) had no significant effect on distance moved over the 2- to 5-min period. However, a higher dose of HU210 (20 μg/kg) significantly reduced the distance moved over this period, compared with vehicle-treated controls ($P<0.05$; Fig. 3a). One-way ANOVA on data collected 5–10 min following placement of rats in the open-field arena (i.e. equivalent to the post-ultrasound period) revealed a significant effect of treatment on distance moved [$F(2,19)=4.3$; $P=0.03$] and on freezing [$F(2,19)=7.73$; $P=0.004$]. Both doses of HU210 significantly reduced the distance moved during the 5- to 10-min period, compared with vehicle-treated controls ($P<0.05$, Fig. 3a). Only the higher dose of HU210 (20 μg/kg) significantly increased freezing behaviour during the 5- to 10-min period, compared with vehicle-treated controls ($P<0.01$, Fig. 3b). The highest dose of HU210 (80 μg/kg, IP) significantly reduced the distance moved and increased freezing during the 2-min pre-ultrasound trial compared with vehicle-injected controls (distance moved: 463.3±108.9 cm versus 913.7±55.8 cm, $P<0.05$; freezing: 29±10.2 s versus 1.2±0.7 s, $P<0.001$).

Intra-PAG administration of HU210 (5 μg/rat) and SR141716A (30 μg/rat) alone, or in combination, had no significant effect on the distance moved or on freezing (data not shown), compared with vehicle-injected controls in the 2-min pre-ultrasound exposure period.

Effect of HU210 and SR141716A on rat behaviour during ultrasound exposure

In the 3-min period during exposure to ultrasound, one-way ANOVA revealed a significant effect of systemic treatment on the duration of freezing [$F(7,62)=7.04$, $P=0.0001$], frequency of rearing [$F(7,62)=5.72$, $P=0.0001$], duration of grooming [$F(7,62)=2.94$, $P=0.01$] and maximum velocity [$F(7,62)=2.22$, $P=0.045$]. Chi-squared analysis revealed a significant effect of treatment on the percentage of rats exhibiting explosive running behaviour (chi-squared value=18.97, $df=7$; $P=0.008$). HU210 (20 μg and 80 μg/kg, IP) significantly reduced the proportion of rats showing ultrasound-induced explosive running to 38% ($P<0.01$) and 14% ($P<0.001$), respectively, compared with vehicle-treated rats exposed to ultrasound (85%) (Fig. 4a). HU210 (5 μg/kg, IP) had no significant effect on the percentage of rats exhibiting explosive running behaviour. HU210 (80 μg/kg, IP) significantly reduced the maximum velocity of movement compared with vehicle-treated rats exposed to ultrasound (47±12 cm/s versus 98±7 cm/s; $P<0.01$). HU210 (5 and 20 μg/kg, IP) had no significant effect on maximum velocity of rats exposed to ultrasound (data not shown). HU210 (5, 20 and 80 μg/kg, IP) significantly increased freezing, reduced rearing, but had no effect on grooming during the 3-min exposure to ultrasound, compared with vehicle-treated rats exposed to ultrasound (Fig. 4a, Table 1). Systemic administration of SR141716A (3 mg/kg, IP) blocked the effect of HU210 (20 μg and 80 μg/kg, IP) on explosive running during the 3-min exposure to ultrasound (Figure 4a). The significant increase in freezing and decrease in rearing induced by HU210 (5, 20 and 80 μg/kg, IP) were not blocked by SR141716A (3 mg/kg, IP) pretreatment (Fig. 4a, Table 1). SR141716A administered alone had no significant effect on the percentage of rats exhibiting explosive running. SR141716A significantly increased grooming, reduced rearing and had no effect on freezing during the 3-min ultrasound exposure, compared with vehicle-treated rats exposed to ultrasound (Fig. 4a, Table 1).

In the 3-min period during exposure to ultrasound, intra-dorsal PAG administration of HU210 (5 μg/rat) reduced the number of rats showing ultrasound-induced explosive running to 50%, compared with vehicle-injected rats (86%) (Fig. 4b). This effect did not reach

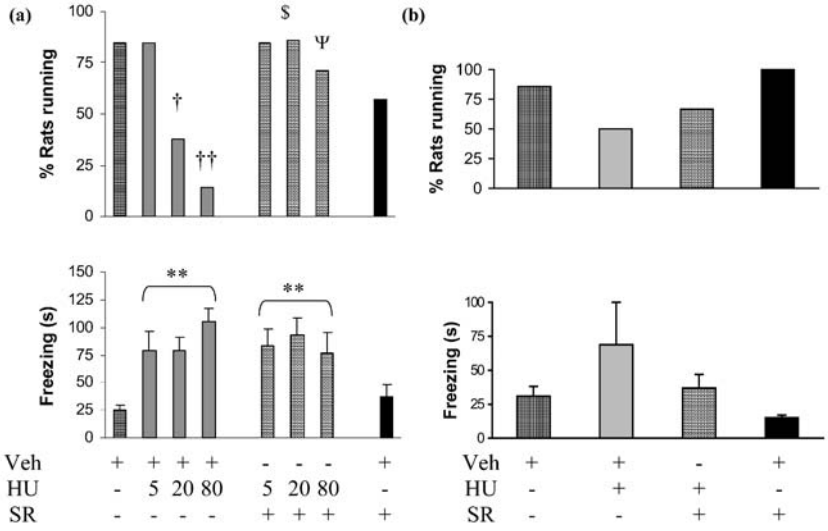

Fig. 4 Behavioural effects of **a** systemically administered HU210 (5, 20 or 80 μg/kg, IP) and SR141716A (3 mg/kg, IP) or **b** intra-dorsal PAG HU210 (5 μg/rat, IPAG) and SR141716A (30 μg/rat, IPAG) during exposure to ultrasound in Lister-hooded rats. Data are means±SEM (**a** n=7–20 and **b** n=4–7). † P<0.01, †† P<0.001 versus vehicle-treated rats exposed to ultrasound; $^{\$}$ P=0.05 versus HU(20) and $^{\Psi}$ P <0.05 versus HU (80) (chi-squared test).** P<0.01 versus vehicle-treated rats exposed to ultrasound (ANOVA, Fisher's PLSD post-hoc test). *Veh* vehicle; *HU* HU210; *SR* SR141716A

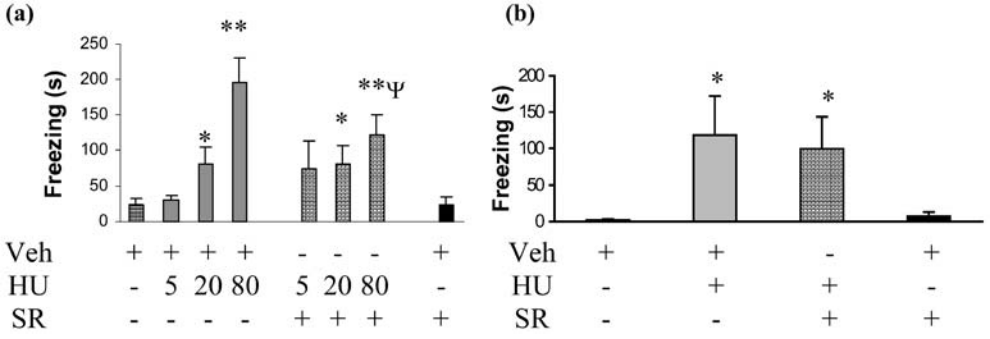

Fig. 5 Behavioural effects of **a** systemically administered HU210 (5, 20 or 80 μg/kg, IP) and SR141716A (3 mg/kg, IP) or **b** intra-dorsal PAG HU210 (5 μg/rat, IPAG) and SR141716A (30 μg/rat, IPAG) after exposure to ultrasound in Lister-hooded rats. Data are means±SEM (**a** n=7–20 and **b** n=4–7). ** P<0.01, * P<0.05 versus vehicle-treated rats and $^{\Psi}$ P<0.05 versus HU (80) (ANOVA, Fisher's PLSD post-hoc test). *Veh* vehicle; *HU* HU210; *SR* SR141716A

statistical significance when analysed with the chi-squared test. This trend towards a reduction in hyperlocomotor activity following HU210 was only partially reversed by SR141716A. Intra-dorsal PAG administration of HU210 or SR141716A, alone or in combination, had no significant effect on freezing (Fig. 4b) or on other measured parameters (data not shown).

Effect of HU210 and SR141716A on rat behaviour following ultrasound exposure

In the period after ultrasound exposure, one-way ANOVA revealed a significant effect of systemic treatment on the duration of freezing [F(7,62)=6.95, P=0.0001], the frequency of rearing [F(7,62)=6.18, P=0.0001], the number of faecal pellets produced [F(7,53)=5.55, P=0.0001] and on the duration of grooming [F(7,62)=2.23, P=0.04]. Post-hoc analysis revealed that systemic administration of HU210 (20 and 80 μg/kg, IP) significantly increased freezing (Fig. 5a) and reduced rearing and defecation (Table 1) when compared with vehicle-treated rats exposed to ultrasound in the 5 min post-ultrasound exposure period. Low dose HU210 (5 μg/kg, IP) had no significant effect on these parameters. SR141716A significantly blocked the increase in freezing induced by HU210 (80 μg/kg, IP), but not HU210 (20 μg/kg, IP), in the 5-min post-ultrasound exposure period (Fig. 5a). Systemic administration of SR141716A did not attenuate the HU210-induced reduction in rearing or defecation. Systemic administration of SR141716A alone did not significantly affect freezing, rearing, grooming or defecation, compared with vehicle-treated rats exposed to ultrasound (Fig. 5a, Table 1).

Table 1 Effects of systemic administration of HU210 (5, 20 or 80 μg/kg, IP) and SR141716A (3 mg/kg, IP) on rearing frequency, grooming duration and defecation during, and after, exposure to ultrasound in rats. *Veh* vehicle; *US* ultrasound; *SR* SR141716A (3 mg/kg, IP); *HU(5)* HU210 (5 μg/kg, IP); *HU(20)* HU210 (20 μg/kg, IP); *HU(80)* HU210 (80 μg/kg, IP). Values represent means±SEM (n=7–20)

		Veh-Veh-US	Veh-HU(5)-US	Veh-HU(20-US	Veh-HU(80)-US	SR-HU(5)-US	SR-HU(20)-US	SR-HU(80)-US	SR-Veh-US
During	No. of rears	8±1	1±1**	2±0.9**	1±0.6**	0.7±0.4**	2±0.7**	2±0.8**	3±1
	Grooming (s)	7±2	8±6	5±2	1±0.6	19±13	10±8	9±3	42±17**
After	No. of rears	20±2	14±2	9±3**	3±3**	10±3**	8±2.1**	3±1**	17±2
	Grooming (s)	25±6	12±6	10±6	2±2	52±19*	35±14.8	26±12.0	36±9.9
	No. of faecal pellets	4±0.3	2±0.9	04±0.4**	0.6±0.6**	4±0.7	1.6±1*	1.2±0.7**	2.8±0.5

** P<0.01, +P<0.05 versus vehicle-vehicle-ultrasound controls. (ANOVA, Fisher's PLSD post-hoc test)

Fig. 6 a Effects of placement in an open-field arena and exposure to ultrasound on plasma corticosterone levels in rats administered vehicle, HU210 (20 or 80 μg/kg, IP) and/or SR141716A (3 mg/kg, IP). **b** Effects of intra-dorsal PAG HU210 (5 μg/rat, IPAG) and SR141716A (30 μg/rat, IPAG) on plasma corticosterone levels of rats exposed to ultrasound. Data are means±SEM (**a** n=5–13; **b** n=5–7). ^{++}P<0.01 versus home cage controls (*veh-veh-HC*) $^{***}P$<0.001, $^{**}P$<0.01, $^{*}P$<0.05 versus veh-veh-US (ANOVA, Fisher's PLSD post-hoc test). *Veh* vehicle; *HC* home cage; *US* ultrasound; *HU* HU210; *SR* SR141716A

One-way ANOVA revealed a significant effect of intra-PAG treatment on the duration of freezing in the post-ultrasound exposure period [F(3,19)=5.156, P=0.0089]. Post-hoc analysis showed that intra-PAG administration of HU210 (5 μg/rat) significantly increased freezing behaviour (P<0.01), compared with vehicle-injected rats, in the 5-min post-ultrasound exposure period (Fig. 5b). This HU210-induced increase in freezing post-ultrasound exposure was not reversed by intra-PAG administration of SR141716A. Neither HU210, nor SR141716A, alone, or in combination, significantly altered any of the other measured parameters (data not shown).

Effect of HU210 and SR141716A on plasma corticosterone levels in rats exposed to ultrasound

Placement of rats in the open-field arena, with or without ultrasound, significantly altered plasma corticosterone levels compared with home cage controls [F(2,20)=8.274, P=0.002]. Post-hoc analysis revealed that plasma corticosterone levels were significantly elevated (P<0.01) in rats 15 min following placement in the novel arena compared with home cage controls (Fig. 6a). Plasma corticosterone levels in the group exposed to ultrasound were also significantly elevated compared with home cage controls (P<0.01); however, plasma corticosterone levels 10 min post-ultrasound exposure were not significantly different to levels in the open-field group not exposed to ultrasound (Fig. 6a).

One-way ANOVA revealed a significant effect of systemic drug treatment on plasma corticosterone levels of rats exposed to ultrasound [$F(5,42)=5.281$, $P=0.0007$]. Systemic administration of HU210 significantly increased plasma corticosterone levels at both the 20 μg/kg ($P<0.01$) and 80 μg/kg ($P<0.001$) doses compared with vehicle-treated rats exposed to ultrasound (Fig. 6a). The HU210-induced increase in plasma corticosterone levels occurred in a dose-related manner, but was not antagonized by pretreatment with systemically administered SR141716A. Systemic administration of SR141716A alone also significantly increased plasma corticosterone levels compared with vehicle-treated rats exposed to ultrasound ($P<0.05$, Fig. 6a). In a separate experiment, a low dose of HU210 (5 μg/kg) had no significant effect on plasma corticosterone levels in rats exposed to ultrasound (108.5±11 ng/ml) compared with vehicle-treated controls exposed to ultrasound (100.6±6.4 ng/ml).

In rats with intra-PAG cannulae, exposure to ultrasound had no significant effect on plasma corticosterone levels measured 10 min post-stimulus (Fig. 6b). One-way ANOVA revealed a significant effect of intra-PAG drug treatment on plasma corticosterone levels of rats exposed to ultrasound [$F(4,23)=3.431$, $P=0.024$]. Intra-dorsal PAG administration of HU210 (5 μg/rat) significantly increased plasma corticosterone levels compared with vehicle-treated rats exposed to ultrasound (Fig. 6b). The HU210-induced increase in plasma corticosterone levels was not antagonized by pretreatment with SR141716A (30 μg/rat). Intra-dorsal PAG administration of SR141716A alone had no significant effect on plasma corticosterone levels compared with vehicle-treated controls ($P<0.05$, Fig. 6b).

Discussion

Systemic administration of the potent, non-selective cannabinoid agonist, HU210, increased ultrasound-induced freezing and reduced the expression of ultrasound-induced explosive hyperlocomotor activity in rats. Importantly, enhancement of freezing was observed following a dose of HU210 (5 μg/kg) that was devoid of overt effects on locomotor activity. The HU210-induced increase in freezing behaviour was not sensitive to SR141716A, suggesting that this effect is not mediated by CB_1 receptors. In contrast, the HU210-induced attenuation of explosive hyperlocomotor activity was only observed at higher doses and was blocked by pre-administration of the selective CB_1 receptor antagonist SR141716A. These data indicate that substrates underlying hyperlocomotor activity associated with ultrasound exposure are modulated by CB_1 receptors. Direct intra-dorsal PAG administration of HU210 (5 μg/rat) tended to reduce the expression of ultrasound-induced explosive hyperlocomotor activity in rats; however, significance was not reached.

The type of defensive behaviour displayed by rats depends on the imminence/intensity of the threat. Thus, as an aversive stimulus increases, the behavioural response of rats increases along a graded scale that may progress from freezing through to explosive hyperlocomotor activity (Blanchard et al. 1986). In the present study, a low dose of HU210 (5 μg/kg, IP) increased ultrasound-induced freezing, with no effect on the expression of explosive hyperlocomotor activity. Importantly, the low dose of HU210 was devoid of any overt effects on locomotor activity. These data suggest that a low dose of cannabinoid agonist modulates behaviour towards an aversive ultrasound stimulus by specifically enhancing the expression of behaviour (i.e. freezing) observed at the lower end of the aversive continuum. Higher doses of HU210 (20 and 80 μg/kg, IP) targeted both ends of the aversive continuum, increasing freezing and attenuating ultrasound-induced explosive hyperlocomotor behaviour. However, it is necessary to exercise caution when interpreting these data, since these higher doses of HU210 reduced locomotor activity in animals not exposed to ultrasound, indicating a possible sedative effect. Nevertheless, the finding that pretreatment with a cannabinoid agonist reduces the ability of the ultrasound stimulus to evoke an explosive aversive episode is interesting, and suggests that cannabinoids may exert an inhibitory effect on the neuronal pathways mediating ultrasound-induced hyperlocomotor activity. The effect of HU210 on hyperlocomotor activity appears to be CB_1-mediated, since it was blocked by SR141716A. In contrast, the HU210-induced increase in freezing was not antagonized by SR141716A, suggesting that this effect may be mediated by a novel cannabinoid receptor subtype.

Studies on the effects of systemically administered cannabinoids and aversion are somewhat mixed with evidence for both aversive and anti-aversive effects. Systemic administration of the cannabinoid receptor agonist CP55, 940, inhibits rat pup ultrasonic vocalizations, an effect indicative of anti-aversive activity (McGregor et al. 1996a). Administration of a low dose of Δ^9-THC is anxiolytic in the rat light-dark box paradigm (Berrendero and Maldonado 2002). In addition, rewarding properties of cannabinoids have been demonstrated (for review, see Maldonado 2002). Nevertheless, there is a substantial body of evidence suggesting that systemic administration of high doses of cannabinoid agonists may be aversive (see Introduction). Differential effects of low and high doses of HU210 on behaviour towards the aversive ultrasound stimulus were also observed in the present study, indicating that aversive behavioural effects of exogenously administered cannabinoids are likely to be dose- and context-dependent. It is likely, however, that the neural substrates and circuitry mediating the unconditioned panic-like response induced by ultrasound are different to those involved in conditioned or conflict-orientated paradigms.

Behavioural effects of systemic administration of cannabinoids are also dependent on their site, or sites, of action in the brain. Microinjection of Δ^9-THC directly into the central nucleus of the amygdala is anxiogenic in

mice tested on the elevated plus-maze (Onaivi et al. 1995). The midbrain PAG also plays an important role in mediating behavioural responses to aversive stimuli (Bandler et al. 1991; Beckett et al. 1992; Bandler and Keay 1996). There is, however, a paucity of studies investigating the role of the PAG in cannabinoid-mediated modulation of behaviour towards aversive stimuli. We observed that intra-dorsal PAG administration of HU210 tended to reduce ultrasound-induced explosive hyperlocomotor activity and increase freezing behaviour, a profile identical to, albeit less pronounced than, that observed following systemic administration of HU210. While the effects of intra-PAG HU210 on freezing during ultrasound exposure appeared to be sensitive to SR141716A, the effects on explosive hyperlocomotor activity and freezing post-ultrasound were not. Thus, while the modulatory effects of HU210 on behaviour towards the aversive ultrasound stimulus may be mediated, at least in part, by the dorsal PAG, these effects cannot be explained solely by an action at CB_1 receptors in this midbrain region.

The results of the present study are in agreement with previous findings in our laboratory demonstrating that intra-dorsal PAG administration of the same dose of HU210 (5 μg/rat) attenuates explosive running behaviour evoked by intra-PAG administration of the excitatory amino acid D,L-homocysteic acid (Finn et al. 2003b). The mechanism by which intra-PAG administration of HU210 produces these anti-aversive effects remains to be elucidated. The role of a gain control system involving GABA interneurones in the pathogenesis of panic has previously been suggested (Lovick 2000). 5-HT release in the PAG is under the control of GABA and intra-PAG or ICV microinjection of the GABA antagonist bicuculine increases 5-HT release in this region (Maione et al. 1998; Zhang et al. 2000). Evidence suggests that increasing serotonin in the PAG is anti-aversive (Schutz et al. 1985; Graeff 1988), an effect which may be mediated by postsynaptic $5\text{-}HT_{1A}$ receptors (Beckett et al. 1992). Cannabinoids act via CB_1 receptors to inhibit GABAergic synaptic transmission in rat PAG neurons in vitro (Vaughan et al. 2000). Stimulation of CB_1 receptors located on PAG GABAergic interneurones may, therefore, result in increased 5-HT release via disinhibition of 5-HT neurones and a subsequent attenuation of the behavioural response induced by the aversive stimulus.

Our results are the first report of the effects of ultrasound exposure on corticosterone levels, and indicate that exposure to 3 min ultrasound (20 kHz, 70–80 dB) does not have any significant effect on plasma corticosterone levels measured 10 min following termination of the ultrasonic tone. This time-point was chosen since exposure of rats to white noise results in increased plasma corticosterone levels which peak at 10 min post-stress (Windle et al. 2001). A preliminary study in our laboratory also demonstrated that corticosterone levels were not significantly altered at an earlier time-point (5 min post-ultrasound). Importantly, exposure of rats to the novel open-field arena was sufficient to induce a robust increase in plasma corticosterone levels. Thus, potential novel environment-induced masking of ultrasound-induced changes in corticosterone levels is an important experimental consideration.

Systemic administration of high (20 and 80 μg/kg), but not low (5 μg/kg), doses of HU210 potentiated the plasma corticosterone response to novel-arena stress. Thus, effects of these higher doses of HU210 on behaviour towards the aversive stimulus were accompanied by activation of the HPA axis. However, the increase in freezing observed with the lowest dose of HU210 was not accompanied by increased levels of plasma corticosterone. Our results corroborate previous studies demonstrating that acute systemic administration of HU210 results in a dose-dependent increase in both plasma corticosterone and adrenocorticotropic hormone levels in rats (Rodríguez de Fonseca et al. 1995, 1996; Martin-Calderon et al. 1998). To date, no study has attempted to block the HU210-induced activation of the HPA axis with SR141716A however the effects of CP55, 940 in young rats were attenuated by this CB_1 receptor antagonist (Romero et al. 2002). In the present study, pretreatment with the same dose of SR141716A (3 mg/kg, IP) did not block the HU210-induced increase in plasma corticosterone levels. In fact, IP administration of SR141716A in rats exposed to ultrasound resulted in increased levels of plasma corticosterone. Interestingly, in non-stressed rats, this dose of SR141716A has no effect on HPA axis activity (Navarro et al. 1997a), suggesting that the behavioural context has an important bearing on HPA axis responsivity to cannabinoids and may influence endocannabinoid system tone.

In the present study, we observed a stimulatory effect of intra-PAG HU210 on plasma corticosterone, suggesting that the effects of HU210 on corticosterone release are not mediated solely by a peripheral site of action. This finding is in agreement with previous studies that have shown that ICV administration of Δ^9-THC to rats increases plasma levels of adrenocorticotropic hormone and corticosterone (Manzanares et al. 1999). Intra-dorsal PAG administration of SR141716A (30 μg/rat) failed to antagonize the stimulatory effect of intra-PAG HU210 on corticosterone levels and induced a small but non-significant increase in corticosterone levels when administered alone into the PAG. This result is in keeping with the findings of Manzanares et al. (1999), who propose that the agonist-like effects of centrally administered SR141716A indicate that endogenous cannabinoids tonically inhibit the release of corticosterone. Alternatively, this activity of SR141716A may be part of an uncharacterized action of this compound not mediated by known cannabinoid receptors.

The present study is the first report of the effect of cannabinoids on behavioural responses towards an unconditioned, aversive ultrasound stimulus. HU210 increased ultrasound-induced freezing and attenuated ultrasound-induced hyperlocomotor activity, suggesting complex modulation of the aversive continuum by cannabinoids in this model. The modulation of behav-

ioural responses to the aversive ultrasound stimulus by the higher doses of HU210 was accompanied by activation of the HPA axis as indexed by an increase in plasma corticosterone levels. The behavioural and neuroendocrine effects of HU210 in this model appear to be mediated, at least in part, at the level of the PAG though not solely by CB_1 receptors. The present study demonstrates that the rat model of ultrasound-induced aversion is a suitable model for assessing cannabinoid-mediated modulation of unconditioned-aversion and complements other behavioural models designed to identify novel therapeutic targets for the treatment of panic and/or anxiety.

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