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Effects of direct periaqueductal grey administration of a cannabinoid receptor agonist on nociceptive and aversive responses in rats

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Abstract

The analgesic potential of cannabinoids may be hampered by their ability to produce aversive emotion when administered systemically. We investigated the hypothesis that the midbrain periaqueductal grey (PAG) is a common substrate mediating the anti-nociceptive and potential aversive effects of cannabinoids. The rat formalin test was used to model nociceptive behaviour. Intra-PAG microinjection of the excitatory amino acid D,L-homocysteic acid (DLH) was used to induce an aversive, panic-like reaction characteristic of the defensive "fight or flight" response. Administration of the cannabinoid receptor agonist HU210 (5 μg/rat) into the dorsal PAG significantly reduced the second phase of formalin-evoked nociceptive behaviour, an effect which was blocked by co-administration of the CB_1 receptor antagonist SR141716A (50 μg/rat). This anti-nociceptive effect was accompanied by an HU210-induced attenuation of the formalin-evoked increase in Fos protein expression in the caudal lateral PAG. Intra-dorsal PAG administration of HU210 (0.1, 1 or 5 μg/rat) significantly reduced the aversive DLH-induced explosive locomotor response. The anti-nociceptive effect of HU210 is likely to result from activation of the descending inhibitory pain pathway. Mechanisms mediating the anti-aversive effects of cannabinoids in the PAG remain to be elucidated. These data implicate a role for the PAG in both cannabinoid-mediated anti-nociceptive and anti-aversive responses.

Keywords: Cannabinoid receptor; Pain; Periaqueductal grey; Aversion; Formalin test; Panic; c-Fos

1. Introduction

The analgesic potential of cannabis and cannabinoids has long been recognised (Pertwee, 2001). However, cannabinoids induce aversive emotion and precipitate anxiety or panic attacks in about 22% of individuals (Thomas, 1996). A greater understanding of the mechanisms underlying cannabinoid-induced analgesia and aversion is needed if analgesic drugs without this adverse side-effect profile are to be developed.

Systemic administration of cannabinoids is anti-nociceptive in the tail-flick test (Lichtman and Martin, 1991) and in the formalin model of tonic, persistent pain (Jaggar et al., 1998). The finding that the anti-nociceptive effects of systemically administered cannabinoids are reduced following disruption of the descending inhibitory control pathway (Hohmann et al., 1999b) supports studies demonstrating that supraspinal sites of action play a crucial role in the analgesia mediated by systemically administered cannabinoids (Lichtman and Martin, 1991).

Cannabinoid agonists are aversive in conditioned place preference, taste avoidance and defensive withdrawal paradigms (Parker and Gillies, 1995; McGregor et al., 1996a; De Fonseca et al., 1996; Sanudo-Pena et al., 1997; Hutcheson et al., 1998; Cheer et al., 2000a). However, there is also some evidence for anti-aversive effects of cannabinoids in rat pup ultrasonic vocalisation (McGregor et al., 1996b) and light–dark box (Berrendero and Maldonado, 2002) paradigms.

The midbrain periaqueductal grey (PAG) mediates

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behavioural responses to both aversive and nociceptive stimuli (for review, see Bandler and Keay, 1996). The PAG is an important component of the descending inhibitory pain control pathway (Wall and Melzack, 1999). PAG stimulation results in profound analgesia (Levine et al., 1991) which may occur with or without aversive side-effects (Hosobuchi, 1983). Indeed Besson et al. (1991) have suggested that anti-nociception mediated by the dorsal PAG may be a consequence of aversion. The defensive "fight or flight" response can be induced in animals following chemical stimulation of the PAG with excitatory amino acids such as D,L-homocysteic acid (DLH) (Bandler, 1982; Beckett et al., 1992).

CB_1 receptors are present in moderate density and distributed heterogeneously in the PAG (Herkenham et al., 1991; Tsou et al., 1998). Intra-PAG microinjection of cannabinoid agonists is anti-nociceptive in the rat tail-flick test (Lichtman et al., 1996). To date, the role of cannabinoid receptors in the PAG in modulation of tonic, persistent pain behaviour or aversion has not been reported.

The aim of this study was to investigate the effect of intra-dorsal PAG administration of the potent non-selective cannabinoid agonist HU210 on formalin-evoked nociceptive behaviour, PAG Fos protein expression and DLH-induced aversive behaviour in rats. We demonstrate for the first time that the PAG is a common anatomical substrate mediating both cannabinoid-induced anti-nociceptive and anti-aversive responses. Some of this work has been published in abstract form (Finn et al., 2001, 2002).

2. Methods

2.1. Animals

Adult male Sprague-Dawley rats (250–300 g, Charles River, UK) were used for these experiments. One group of rats was used for the study of formalin-evoked behaviour and Fos expression, another group was used for determination of CB_1 receptor involvement in the effects observed and a separate group was used for the DLH-induced aversion study. Rats were group housed in cages of five prior to surgery. Food and water were available ad libitum and rats were housed on a 12 h light–dark cycle. All experiments were carried out in the light period according to the UK Home Office Animals (Scientific Procedures) Act 1986.

2.2. 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. A non-steroidal anti-inflammatory agent, carprofen (4.5 mg kg^{-1}, s.c., Rimadyl, Pfizer, UK), was administered before the surgery for post-operative 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 once daily.

2.3. Microinjections

Drugs or vehicle were microinjected manually into the dorsal PAG (as defined by Bandler and Keay, 1996) in a volume of 250 nl or 500 nl over 60 s using an injector and Hamilton syringe. These injection volumes were selected on the basis of a large number of studies which have investigated the effects of direct microinjection of cannabinoids into discrete brain regions in models of nociception including the tail-flick and formalin tests (Martin et al., 1998, 1999; Lichtman et al., 1996; Lichtman and Martin, 1997; Monhemius et al., 2001). 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) and an attached 1 m long polystyrene tube (0.75 mm OD, 0.28 mm ID) to allow injection of the drug when the rat was placed in the arena.

2.4. Effect of HU210 on formalin-induced nociceptive behaviour

Each rat received HU210 (0.1, 1, 5 μg/250 nl) or dimethyl sulphoxide (DMSO, 60%) microinjection into the dorsal PAG followed 10 min later by formalin (50 μl, 2.5% formaldehyde in 0.9% NaCl) or saline (0.9%) injection into the sub-plantar region of the right hind paw. Intra-plantar injections were carried out under brief isoflurane anaesthesia to minimise stress to the animals. Behaviour was tracked for 10 min post-drug and 60 min post-formalin in a transparent perspex box (30 × 30 × 30 cm) and scored using Ethovision software by a trained experimenter, blind to the treatments. The maximum time taken to recover from anaesthesia, as judged by regain of the righting reflex, was 2.5 min, and this initial period of the formalin trial was removed from the analysis. The video image was obtained from a camera positioned under the perspex box enabling clear examination of paw-directed behaviour throughout the experiment. In addition to rearing, grooming and distance moved, pain behaviours were scored according to the weighted composite pain scoring technique (CPS-$WST_{0,1,2}$) described by Watson et al. (1997). According to this method, pain behaviours were categorised as time

spent raising the paw above the floor without contact with any other surface (C1) and holding, licking, biting, shaking and flinching (C2) to obtain a composite pain score (CPS = (C1 + 2×C2)/(total duration of analysis period)). Post-formalin oedema was assessed by measuring the difference in paw diameter of the left and right hind paws with Vernier callipers.

2.5. Immediate early gene c-fos immunohistochemistry

The translation of the immediate early gene *c-fos* in the brain reaches a peak at 90–120 min post stimulus (Harris, 1998). Therefore, 90 min after formalin injection, rats were perfused transcardially with saline (120 ml) followed by paraformaldehyde (PFA, 4% w/v in 0.1 M sodium phosphate buffer) under sodium pentobarbitone (100 mg/kg, ip) anaesthesia. Fos immunohistochemistry was carried out according to the method of Leslie et al. (1993) and using the Vectastain ABC sheep IgG Elite Kit (Vector Laboratories, UK). Briefly, brains were removed and post-fixed overnight at room temperature in PFA. Six coronal sections (100 μm thickness), each 500 μm apart, were taken through the PAG using a Vibratome (Campden Instruments, UK). Each brain section was placed individually into immunobuffer (120 mM NaCl, 5 mM KCl, 9 mM Na_2HPO_4, 15 mM NaH_2PO_4, 1 mM merthiolate containing 0.3% w/v Triton X-100). Endogenous peroxidase activity was removed with hydrogen peroxide (H_2O_2, 30 min, 0.03% v/v in immunobuffer), prior to sequential incubation in rabbit serum (blocking buffer, 2% v/v in immunobuffer) for 15 min. After removal of blocking buffer, sections were sequentially incubated in affinity purified polyclonal sheep antiserum raised to the Fos protein (1:2000 in blocking buffer, 48–60 h, Sigma-Genosys, UK) followed by incubation in biotinylated secondary rabbit anti-sheep antibody (1:200 in blocking buffer, 24 h). Control sections were incubated in blocking buffer minus Fos antiserum. Sections were incubated in avidin/biotin/horseradish peroxidase conjugate (3 h), followed by incubation in 3,3′-diaminobenzidine (DAB, 0.3% w/v,10 min) in phosphate buffer (0.1 M, pH 7.4). To reveal sites of Fos-like immunoreactivity (FLI, black/dark brown nuclear stain), H_2O_2 (0.1% v/v) solution was added to the DAB solution and washed off with Tris–HCl (50 mM, pH 7.4) once sections had turned a beige/brown colour. All sections were further washed (×2) in Tris–HCl to ensure the removal of all DAB solution, mounted onto glass microscope slides, and allowed to dry overnight. The sections were then dehydrated in absolute alcohol, and cleared using Citroclear (xylene substitute), and coverslipped with Styrolite mounting medium. The number of Fos-like immunoreactive (FLI) cells present in the sub-divisions of the PAG (as defined by Bandler and Keay, 1996; Paxinos and Watson, 1997) was counted by an observer blind to the treatment groups using a light microscope (Zeiss, West Germany) and Openlab imaging software (Improvision, UK).

2.6. Effect of HU210 and SR141716A, alone or in combination, on formalin-induced nociceptive behaviour

A separate group of rats was used to investigate the involvement of CB_1 receptors in mediating the anti-nociceptive effects of direct intra-dorsal PAG administration of HU210. Rats had a guide cannula surgically implanted above the dorsal PAG as described above. Four experimental groups were studied: vehicle (100% DMSO); HU210 (5 μg/rat); HU210 (5 μg/rat) + SR141716A (50 μg/rat); SR141716A (50 μg/rat). The dose of SR141716A (50 μg/rat), the vehicle (100% DMSO) and the injection volume (500 nl) were identical to that used by Martin et al. (1998), where it was demonstrated that co-administration of this dose of SR141716A into the rostral ventromedial medulla antagonised HU210-induced anti-nociception in the rat tail-flick test. Drugs or vehicle were microinjected manually into the dorsal PAG (as described above) 10 min prior to intraplantar formalin injection (50 μl, 2.5% formaldehyde in 0.9% NaCl) into the right hind paw (under brief isoflurane anaesthesia). The maximum time taken to recover from anaesthesia, as judged by regain of the righting reflex, was 3 min, and this initial period of the formalin trial was removed from the analysis. Behaviour was tracked for 10 min post-drug and 60 min post-formalin and scoring was as described earlier.

2.7. Effect of HU210 on DLH-induced aversive behaviour

Each rat received two intra-dorsal PAG microinjections. (1) HU210 (0.1, 1, 5 μg/rat) or vehicle (60% DMSO) and after 10 min, (2) DLH (5 nmol/rat) or saline (0.9% NaCl). The methodology was identical to that used in previous studies in our laboratory (Beckett et al., 1992). Behaviour was tracked for 10 min after the first, and for 5 min after the second microinjection in a circular open arena (75 cm diameter, 70 cm high) using the video image from a camera fixed vertically above the arena and Ethovision software (Noldus, Netherlands). A trained experimenter, unaware of the treatments, scored the rearing, grooming and jumping behaviour by computer while the distance moved and velocity were calculated automatically by Ethovision software.

2.8. Histology

The site of injection was confirmed prior to data analysis. Pontamine sky blue dye (250 or 500 nl) was microinjected through the guide cannula following all behavioural experiments. Coronal brain sections (100

μm) with the blue dye mark were background stained with Neutral Red and mounted on glass slides for the precise location of the site of microinjection using a light microscope. Eighty-eight percent of the injectors were successfully positioned in the dorsal PAG with the remaining 12% positioned either outside the PAG, in the aqueduct or in the lateral PAG. Results of experiments in which the cannula was positioned in the dorsal PAG were included in the analysis (Fig. 1). Data from experiments where the cannula was misplaced in the lateral PAG were analysed separately to verify specificity of the site of action in the dorsal PAG. No distinction was made between dorsomedial and dorsolateral PAG.

2.9. Drugs

Drug doses and concentrations of vehicle were selected on the basis of previous studies in the literature (Beckett et al., 1992; Martin et al., 1998, 1999; Lichtman et al., 1996; Lichtman and Martin, 1997; Monhemius et al., 2001). HU210 (Tocris) was dissolved in DMSO and diluted with artificial cerebrospinal fluid (1.5 mM NaH_2PO_4/Na_2HPO_4, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 147 mM NaCl, 3 mM KCl; pH = 7.4) to obtain a 5 μg/250 nl solution in 60% DMSO. This solution was further diluted with 60% DMSO to obtain 1 μg/250 nl and 0.1 μg/250 nl solutions. DLH (Sigma) was dissolved in saline (0.9% NaCl) to obtain a 5 nmol/250 nl solution. In the second formalin experiment, HU210 and SR141716A were dissolved either alone or in combination in 100% DMSO to give final concentrations of 5 and 50 μg/500 nl, respectively.

2.10. Data analysis

All data are presented as means ± SEM. The data from all the experiments were analysed by one- or two-way ANOVA followed by Fisher's PLSD post-hoc test. Comparisons between saline- and formalin-treated controls (Table 2) were made using Student's unpaired, two-tailed *t*-test. A *P* value less than or equal to 0.05 was considered significant.

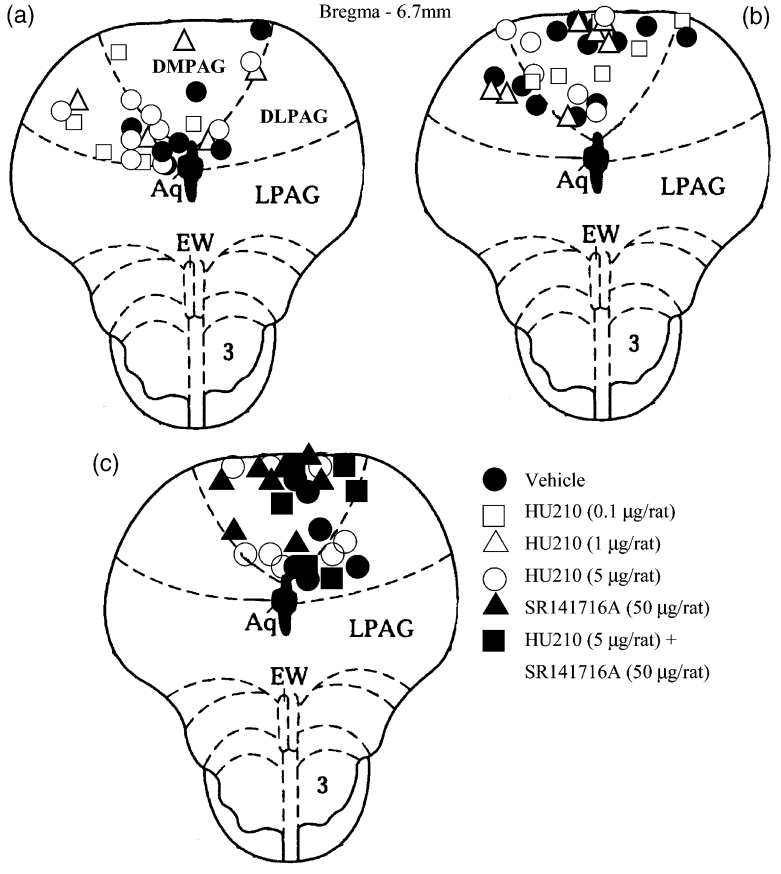

Fig. 1. Histological localisation of injection sites within the PAG. Each rat received (a) microinjection of vehicle or HU210 (0.1, 1, 5 μg/rat) into the dorsal PAG prior to formalin injection; (b) microinjection of vehicle or HU210 (0.1, 1, 5 μg/rat) into the dorsal PAG prior to intra-PAG DLH microinjection; (c) microinjection of vehicle, HU210 (5 μg/rat), SR141716A (50 μg/rat) or HU210 + SR141716A into the dorsal PAG prior to formalin injection or Pontamine sky blue dye was microinjected prior to brain removal. DMPAG, dorsal-medial PAG; DLPAG, dorsal-lateral PAG; LPAG, lateral PAG; Aq, aqueduct; EW, Edinger Westphal Nucleus; 3, oculomotor nucleus. PAG outline is from the atlas of Paxinos and Watson (1997). Some injector positions overlapped and thus may not be clearly differentiated in this figure.

Table 1
The effect of intra-dorsal PAG administration of HU210 (0.1, 1 and 5 μg/250 nl) on the behaviour of rats during the 10 min pre-formalin trial

	Vehicle	HU210 (0.1)	HU210 (1)	HU210 (5)
Distance (cm)	1570 ± 113	1628 ± 117	1622 ± 130	1624 ± 141
Number of rears	26 ± 4	28 ± 5	33 ± 7	21 ± 2
Grooming (s)	70.4 ± 10.1	98.4 ± 29.3	20.6 ± 4.8*	59.2 ± 16.1

Values expressed as means ± SEM (n = 5–11).
* $P < 0.05$ compared with vehicle-injected controls.

Table 2
The effect of formalin injection (50 μl, 2.5%, s.c.) into the right hind paw on the behaviour and paw diameter of rats during the 60 min post-formalin trial

	Distance (cm)	Δ Paw diameter (cm)	Number of rears	Grooming (s)
Saline (4)	2480 ± 430	0.017 ± 0.01	13 ± 2	210.5 ± 59.8
Formalin (7)	2813 ± 240	0.12 ± 0.007***	3 ± 1*	128.9 ± 25.8

Values expressed as means ± SEM (numbers in brackets).
*** $P < 0.001$ compared with saline-injected controls.
* $P < 0.05$ compared with saline-injected controls.

3. Results

3.1. Effect of HU210 on the behaviour of rats prior to formalin injection

One-way ANOVA revealed that intra-dorsal PAG administration of HU210 (0.1, 1 or 5 μg) had no significant effect on the distance moved ($F_{3,26}$ = 0.051; P = 0.98) or rearing ($F_{3,26}$ = 1.25; P = 0.31) in rats during the 10 min pre-formalin trial, compared with vehicle-treated controls (Table 1). There was a significant effect of treatment on duration of grooming behaviour ($F_{3,26}$ = 2.92; P = 0.05) which was significantly reduced by 1 μg HU210 ($P < 0.05$), but not by 0.1 or 5 μg HU210, compared with vehicle-treated controls (Table 1).

3.2. Effect of formalin injection on rat behaviour

Formalin injection into the right hind paw produced robust licking, flinching, shaking and elevation of the injected paw. The early phase (2.5–7.5 min) and late phase (12.5–57.5 min) marked two distinct phases of nociceptive behaviour (measured as CPS) following formalin injection in vehicle-treated control rats (Fig. 2). Saline-injected animals did not exhibit nociceptive behaviour (data not shown).

The difference between the paw diameter of the left and right hind paw of formalin-injected rats was significantly greater than saline-injected controls ($P < 0.001$; Table 2). Formalin-injected rats also exhibited a significant decrease in rearing behaviour ($P < 0.01$) compared with saline controls (Table 2). Formalin injection had no significant effect on grooming or on the distance moved over the 60 min trial (Table 2).

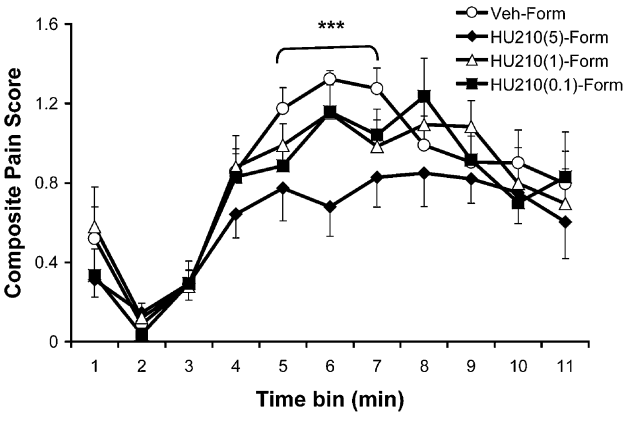

Fig. 2. Effects of intra-dorsal PAG administration of the cannabinoid agonist HU210 (0.1, 1, 5 μg/rat) on the composite pain score of formalin-evoked nociceptive behaviour in rats. Data are means ± SEM (n = 5–9). ***$P < 0.001$ comparing HU210 (5 μg) with vehicle-injected controls 22.5–37.5 min post-formalin. Veh (Vehicle); Form (Formalin).

3.3. Effect of HU210 on formalin-evoked nociceptive behaviour

The first phase of formalin-evoked nociceptive behaviour was not significantly affected by intra-PAG administration of HU210 ($F_{4,25}$ = 2.153; P = 0.1). However, the second phase of formalin-evoked behaviour was significantly reduced by intra-dorsal PAG administration of HU210 ($F_{4,25}$ = 17.25; P = 0.0001). Fisher's PLSD post-

hoc test revealed that HU210 (5 μg) significantly ($P < 0.001$) reduced nociceptive behaviour at the peak (22.5–37.5 min post-formalin) of the late phase, compared with vehicle-treated rats receiving formalin (Fig. 2). Lower doses of HU210 (0.1 μg: $P = 0.14$, 1 μg: $P = 0.16$) did not alter formalin-evoked responses compared with vehicle-treated rats. To confirm the local site of action of HU210 administered into the dorsal PAG, comparison was made with injection of HU210 into a different column of the PAG. Injection of HU210 (5 μg/250 nl, $n = 5$) into the lateral column of the PAG had no significant effect on formalin-evoked nociceptive behaviour, compared with rats receiving vehicle injection ($n = 3$) into this sub-division of the PAG (data not shown). These data confirm that the anti-nociceptive effects of HU210 administered into the dorsal PAG are mediated by a local site of action.

Intra-dorsal PAG administration of HU210 did not significantly affect rearing ($F_{4,25} = 2.818$; $P = 0.5$) or grooming ($F_{4,25} = 0.25$; $P = 0.91$) during the second phase of formalin-evoked nociceptive behaviour. Intra-dorsal PAG administration of HU210 had no significant effect on the increase in paw diameter observed in formalin-injected rats (data not shown).

3.4. Intra-dorsal PAG administration of SR141716A blocks the inhibitory effect of HU210 on formalin-evoked nociceptive behaviour

In a second experiment, the receptor mediating the inhibitory effects of HU210 on formalin-evoked nociceptive behaviour was investigated with the selective CB_1 receptor antagonist SR141716A. The first phase of formalin-evoked nociceptive behaviour was not significantly affected by intra-dorsal PAG administration of HU210 or SR141716A ($F_{3,26} = 0.405$; $P = 0.75$; data not shown). One-way ANOVA revealed a significant effect of treatment during the second phase of formalin-evoked nociceptive behaviour (8–23 min post-formalin; $F_{3,26} = 3.313$; $P = 0.036$). Fisher's PLSD post-hoc test revealed that intra-dorsal PAG administration of HU210 (5 μg) significantly ($P < 0.05$) reduced formalin-evoked nociceptive behaviour compared with vehicle-treated rats receiving formalin (Fig. 3). This anti-nociceptive effect of HU210 was significantly ($P < 0.05$) blocked by co-administration of the CB_1 receptor antagonist SR141716A (50 μg; Fig. 3). Intra-dorsal PAG administration of SR141716A alone did not significantly affect formalin-evoked nociceptive behaviour compared with vehicle-treated rats receiving formalin.

3.5. Effect of HU210 on formalin-evoked Fos expression in the PAG

Intra-plantar injection of formalin resulted in increased Fos expression in the caudal (−8.3 relative to bregma) lateral PAG but not in other PAG sub-regions (Table 3). One-way ANOVA revealed a significant ($F_{2,18} = 4.71$; $P = 0.022$) effect of treatment on the number of FLI cells in the caudal lateral PAG. Post-hoc analysis revealed that the number of FLI cells was significantly increased ($P < 0.01$) in formalin-injected animals compared with saline-injected controls. Intra-dorsal PAG administration of HU210 (5 μg/250 nl) significantly attenuated ($P < 0.05$) the formalin-evoked increase in Fos expression in the caudal lateral PAG (Table 3).

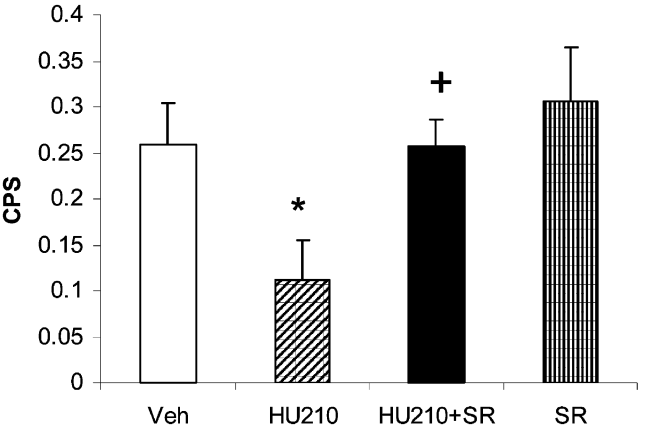

Fig. 3. Effects of intra-dorsal PAG administration of the cannabinoid agonist HU210 (5 μg/rat) and the CB_1 receptor antagonist SR141716A (50 μg/rat) on the composite pain score of formalin-evoked nociceptive behaviour in rats 8–23 min post-formalin. Data are means ± SEM ($n = 7$–8). $^{*}P < 0.05$ for HU210 vs. Veh; $^{+}P < 0.05$ for HU210 vs. HU210 + SR. Veh (Vehicle); SR (SR141716A).

3.6. Effect of HU210 on DLH-induced aversive behaviour

Intra-dorsal PAG administration of HU210 had no significant effect on the velocity of movement ($F_{3,14} = 0.682$; $P = 0.58$) or on the distance moved ($F_{3,14} = 0.704$; $P = 0.57$) by rats which received vehicle injection into the PAG (Fig. 4). Following intra-PAG DLH administration, 78% of rats exhibited explosive running and jumping behaviour. DLH administration resulted in a significant increase in the distance moved ($F_{1,9} = 10.9$; $P = 0.001$) and the maximum velocity of movement ($F_{1,9} = 11.85$; $P = 0.001$) of rats over the 5 min post-DLH trial, compared with vehicle-treated controls (Fig. 5). DLH-induced explosive behaviour was defined by an early phase in which there was a rapid, transient increase in locomotor activity followed by a later phase characterised by less intense locomotor activity. DLH data have been divided into two 2.5 min time bins to represent these two components of DLH-induced behaviour. One-way ANOVA revealed a significant effect of HU210 on the maximum velocity of movement of rats 0–2.5 min post-DLH ($F_{3,31} = 3.5$; P

Table 3
Effect of intra-dorsal PAG administration of the cannabinoid agonist HU210 (5 μg/250 nl) on the number of Fos-like immunoreactive cells throughout the PAG 90 min post-formalin

	Veh-Sal	Veh-Form	HU210 (5 μg)-Form
Rostral PAG (bregma −5.8 mm)			
DMPAG	13 ± 5.9	7.5 ± 2	3.5 ± 0.5
DLPAG	30.5 ± 17.8	13.5 ± 4	9.5 ± 9.5
LPAG	10 ± 4.1	11.5 ± 3.1	12 ± 3
Intermediate PAG (bregma −7.3 mm)			
DMPAG	2.3 ± 1.1	4 ± 1.1	56 ± 50.5
DLPAG	17 ± 10.9	15 ± 3.1	40.7 ± 33.8
LPAG	16.6 ± 7.2	21.5 ± 6.3	43.7 ± 32.7
Caudal PAG (bregma −8.3 mm)			
DMPAG	4.6 ± 1.7	2.7 ± 0.7	3.8 ± 0.7
LPAG	9.6 ± 3.9	21.2 ± 1.6**	8.3 ± 2.7*
VLPAG	19.6 ± 10.7	30.6 ± 6.3	18.3 ± 6.4

Data are expressed as means ± SEM (n = 3–6). DMPAG (dorsal-medial PAG); DLPAG (dorsal-lateral PAG); LPAG (lateral PAG); VLPAG (ventral-lateral PAG).
** $P < 0.01$ vs. Veh-Sal in the caudal LPAG.
* $P < 0.05$ vs. Veh-Form in the caudal LPAG.

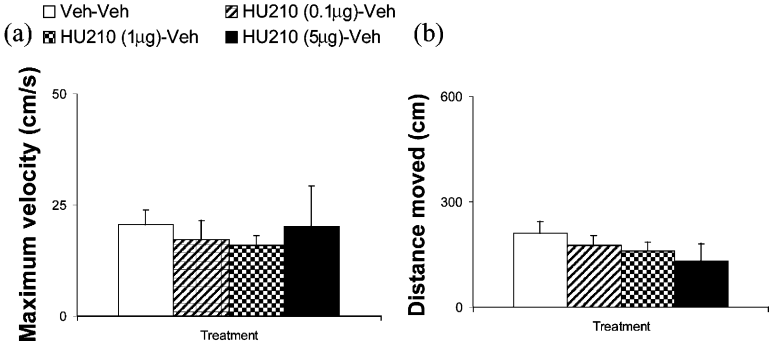

Fig. 4. Effect of intra-dorsal PAG administration of HU210 (0.1, 1, 5 μg in 250 nl 60% DMSO) on (a) maximum velocity and (b) distance moved 0–5 min post-vehicle (0.9% NaCl). Data are expressed as means ± SEM (n = 3–7).

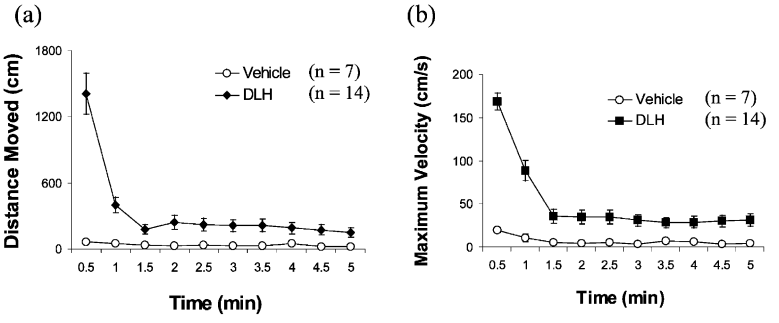

Fig. 5. Effect of intra-PAG administration of DLH on (a) distance moved and (b) maximum velocity of movement of rats. Data are expressed as means ± SEM.

= 0.03). Post-hoc analysis revealed that HU210 (1 and 5 μg/250 nl) significantly reduced ($P < 0.05$) the maximum velocity during the first 2.5 min post-DLH compared with vehicle-treated controls (Fig. 6a). There was no significant effect of treatment on the distance moved during the first 2.5 min post-DLH ($F_{3,31}$ = 1.273; P = 0.3) (Fig. 6b). The increase in maximum velocity of movement of rats 2.5–5 min post-DLH was

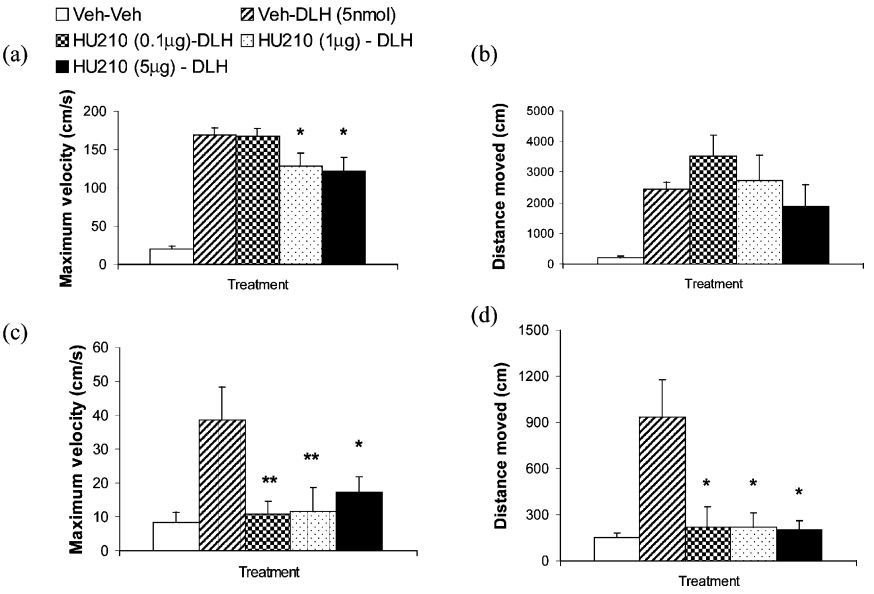

Fig. 6. Effect of intra-dorsal PAG administration of HU210 (0.1, 1, 5 μg in 250 nl 60% DMSO) on (a) maximum velocity and (b) distance moved 0–2.5 min post-DLH (5 nmol in 0.9% NaCl) and on (c) maximum velocity and (d) distance moved 2.5–5 min post-DLH. Data are expressed as means ± SEM (n = 6–14). $^{**}P < 0.01$, $^{*}P < 0.05$ compared with Veh-DLH group.

significantly attenuated by HU210 as revealed by one-way ANOVA ($F_{3,31}$ = 4.4; P = 0.01). Following post-hoc analysis, it was found that the maximum velocity was significantly reduced by all three doses of HU210 compared with vehicle controls (Fig. 6c). The increase in distance moved, 2.5–5 min post-DLH administration, was also significantly attenuated ($F_{3,31}$ = 3.733; P = 0.021) by all three doses of HU210 (Fig. 6d).

HU210 did not significantly affect grooming, rearing, jumping or freezing behaviour post-DLH administration, compared with vehicle-treated controls (data not shown).

4. Discussion

This study investigated the role of the midbrain PAG as a common substrate in cannabinoid-mediated anti-nociception and aversion. Here, we demonstrate that intra-dorsal PAG administration of the potent cannabinoid agonist HU210 reduces the second phase of formalin-evoked nociceptive behaviour in rats. Microinjection of HU210 into the lateral PAG did not alter formalin-evoked nociceptive behaviour confirming the local site of action of HU210 injected into the dorsal PAG. Our data corroborate previous studies in rats demonstrating that systemic administration of cannabinoids reduces the second phase of formalin-evoked nociceptive behaviour (Jaggar et al., 1998). Systemically administered cannabinoids may produce anti-nociceptive effects at either peripheral or central cannabinoid receptors, including at the level of the PAG. Hohmann and colleagues have shown that spinal transection attenuates the anti-nociceptive effects of systemically administered cannabinoids, providing evidence for a supraspinal site of action of cannabinoids (Hohmann et al., 1999b). Our data are in agreement with studies demonstrating that administration of the cannabinoid agonist CP 55,940 into the ventrolateral PAG (Lichtman et al., 1996) and of WIN 55,212-2 into the dorsal PAG (Martin et al., 1999) is anti-nociceptive in the rat tail-flick test.

The receptor mediating the effects of intra-dorsal PAG administration of HU210 on formalin-evoked nociceptive behaviour was investigated with the selective CB_1 receptor antagonist SR141716A. The anti-nociceptive effect of HU210 was fully blocked by co-administration of SR141716A (50 μg/rat) into the dorsal PAG. These data demonstrating the role of the CB_1 receptor in mediating the anti-nociceptive effects of HU210 administered into the dorsal PAG, corroborate previous work demonstrating the contribution of the CB_1 receptor to electrical stimulation-evoked PAG-mediated analgesia (Walker et al., 1999). In addition, our data are in agreement with studies demonstrating that the anti-nociceptive effects of cannabinoid agonists administered i.c.v. (Lichtman and Martin, 1997; Welch et al., 1998) directly into the rostral ventromedial medulla (Martin et al., 1998) or into the *nucleus reticularis gigantocellularis pars alpha* (Monhemius et al., 2001) are mediated by CB_1 receptors. Our data indicate that stimulation of CB_1 receptors in the dorsal PAG is sufficient to achieve an anti-nociceptive effect in the rat formalin test.

Fos expression is a useful indicator of neuronal activation in specific brain regions following stressful or painful stimuli (for review, see Harris, 1998). The anti-nociceptive effects observed following HU210 adminis-

tration were also accompanied by an attenuation of the formalin-evoked increase in Fos expression in the caudal lateral PAG. This is the first report of the effect of a centrally administered cannabinoid on brain Fos expression in the formalin pain model. This finding supports studies demonstrating that cannabinoids decrease formalin-evoked Fos expression in the spinal cord (Hohmann et al., 1999a) and suggests that cannabinoids may act at all levels in the CNS to suppress nociceptive transmission following a noxious stimulus. The ability of intra-dorsal PAG administration of HU210 to reduce formalin-evoked Fos expression in the caudal lateral PAG provides further evidence for a functional relationship between the columns of the PAG (Jansen et al., 1998).

The second part of this study investigated the role of the PAG in cannabinoid-mediated modulation of aversive behaviour. Specifically, we show for the first time that intra-dorsal PAG administration of HU210 attenuates aversive defence behaviour evoked by microinjection of the excitatory amino acid DLH into the dorsal PAG of rats. Our data support previous studies demonstrating that systemic administration of the cannabinoid agonist CP 55,940 inhibits rat pup ultrasonic vocalisations albeit at high doses, an effect indicative of anti-aversive activity (McGregor et al., 1996b). Administration of a low dose of Δ^9-tetrahydrocannabinol resulted in an anxiolytic effect in the rat light–dark box paradigm (Berrendero and Maldonado, 2002). Furthermore, rewarding properties of cannabinoids have been demonstrated using self-administration and intra-cranial self-stimulation paradigms (for review, see Maldonado, 2002). These effects may result from cannabinoid-induced activation of the mesolimbic dopaminergic system (Cheer et al., 2000b). Despite this evidence for anti-aversive effects of cannabinoids, there is a substantial body of evidence suggesting that systemic administration of cannabinoid agonists is aversive in conditioned place preference paradigms (Parker and Gillies, 1995; McGregor et al., 1996a; Sanudo-Pena et al., 1997; Cheer et al., 2000a) and in the elevated plus-maze (Onaivi et al., 1995). The behavioural effects of exogenously administered cannabinoid agonists will undoubtedly depend on the drug and dose administered, the route of administration and the aversive model under investigation. Our data demonstrate that the PAG does not mediate pro-aversive effects of HU210 at a dose which is anti-nociceptive. This finding substantiates earlier work demonstrating that the central nucleus of the amygdala mediates pro-aversive effects of Δ^9-THC (Onaivi et al., 1995).

There may be common mechanisms underlying the effects of cannabinoids on aversion and nociception at the level of the PAG. It is likely that the anti-nociceptive effect of HU210 observed results from activation of the descending inhibitory pain pathway, of which the PAG is an important component (Wall and Melzack, 1999). Neurones project from the PAG to the rostral ventromedial medulla which, in turn, projects extensively to the dorsal horn of the spinal cord. Agonist binding to CB_1 receptors generally leads to an inhibition of neuronal firing and neurotransmitter release (Vaughan et al., 2000; Ferraro et al., 2001). Cannabinoids have been shown to act via CB_1 receptors to inhibit GABAergic synaptic transmission in the PAG (Vaughan et al., 2000), cerebral cortex (Ferraro et al., 2001) and hippocampus (Hajos et al., 2000). It is possible that HU210 binding to CB_1 receptors located on inhibitory GABA interneurones in the PAG leads to disinhibition of output neurones and a corresponding activation of the descending inhibitory pain pathway. Such a mechanism of action would resemble the anti-nociceptive effect mediated by opioid receptors following direct administration of morphine into the PAG (Kishimoto et al., 2001). The mechanism by which intra-PAG administration of HU210 attenuated DLH-induced aversive behaviour may also involve modulation of endogenous GABAergic tone. Evidence suggests that increasing serotonin in the PAG is anti-aversive (Schutz et al., 1985). Stimulation of CB_1 receptors located on GABA interneurones may result in increased 5-HT release via disinhibition of 5-HT neurones and a subsequent attenuation of the aversive behavioural response. The role of such a gain control system involving GABA interneurones in the pathogenesis of panic behaviour has previously been suggested (Lovick, 2000). In addition, there is evidence that 5-HT release in the PAG is under the control of GABA as intra-PAG or i.c.v. microinjection of the GABA antagonist bicuculine increases 5-HT release in this region (Maione et al., 1998; Zhang et al., 2000).

In conclusion, intra-dorsal PAG administration of the potent cannabinoid agonist HU210 is anti-nociceptive in the formalin pain test, an effect mediated by CB_1 receptors. Furthermore, intra-dorsal PAG administration of HU210 is anti-aversive in the DLH-induced aversion model. These behavioural data, together with analysis of Fos expression, highlight the importance of the dorsal PAG as a common substrate mediating anti-nociceptive and anti-aversive responses following cannabinoid receptor activation.

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