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Research report

The distribution of cannabinoid-induced Fos expression in rat brain: differences between the Lewis and Wistar strain

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Abstract

Previous studies have suggested that cannabis-like drugs produce mainly aversive and anxiogenic effects in Wistar strain rats, but rewarding effects in Lewis strain rats. In the present study we compared Fos expression, body temperature effects and behavioral effects elicited by the cannabinoid CB_1 receptor agonist CP 55,940 in Lewis and Wistar rats. Both a moderate (50 μg/kg) and a high (250 μg/kg) dose level were used. The 250 μg/kg dose caused locomotor suppression, hypothermia and catalepsy in both strains, but with a significantly greater effect in Wistar rats. The 50 μg/kg dose provoked moderate hypothermia and locomotor suppression but in Wistar rats only. CP 55,940 caused significant Fos immunoreactivity in 24 out of 33 brain regions examined. The most dense expression was seen in the paraventricular nucleus of the hypothalamus, the islands of Calleja, the lateral septum (ventral), the central nucleus of the amygdala, the bed nucleus of the stria terminalis (lateral division) and the ventrolateral periaqueductal gray. Despite having a similar distribution of CP 55,940-induced Fos expression, Lewis rats showed less overall Fos expression than Wistars in nearly every brain region counted. This held equally true for anxiety-related brain structures (e.g. central nucleus of the amygdala, periaqueductal gray and the paraventricular nucleus of the hypothalamus) and reward-related sites (nucleus accumbens and pedunculopontine tegmental nucleus). In a further experiment, Wistar rats and Lewis rats did not differ in the amount of Fos immunoreactivity produced by cocaine (15 mg/kg). These results indicate that Lewis rats are less sensitive to the behavioral, physiological and neural effects of cannabinoids. The exact mechanism underlying this subsensitivity requires further investigation. Crown copyright © 2001 Published by Elsevier Science B.V. All rights reserved.

Theme: Neural basis of behaviour

Topic: Drugs of abuse: opioids and others

Keywords: Cannabinoid; *c-fos*; Strain difference; Lewis; Wistar; CP 55,940

1. Introduction

Cannabis is the most widely used illicit drug in the world and appears to have addictive potential in a subset of users [32]. The notion that certain individuals may be predisposed to cannabis dependence is supported by data suggesting that genetic factors partly determine whether cannabis is experienced as euphoric or dysphoric in humans [46]. Also reflecting this genetic determination is that major strain differences have been found in the reinforcing effects of cannabinoids in rats [28,29].

Lewis strain rats appear to find the main psychoactive constituent of marijuana, Δ^9-tetrahydrocannabinol (Δ^9-THC), rewarding as assessed by the intracranial self-stimulation paradigm [44]. In contrast, other rat strains such as the Wistar strain appear to find cannabinoids aversive and anxiogenic [52,70]. These observations invite

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the hypothesis that in other rat strains, the effects of cannabinoids on neural substrates mediating anxiety may outweigh their effects on neural substrates underlying reward. In Lewis rats, however, the opposite may be true.

The central nucleus of the amygdala (CEA) appears to be a critical brain region for cannabinoid-induced anxiety. Thus, administration of Δ^9-THC directly into the CEA had an anxiogenic effect on mice on the elevated plus-maze [59]. Extrahypothalamic corticotropin releasing hormone (CRH) receptors such as those found in the CEA, also appear to be important in cannabinoid-induced anxiety [70]. If Lewis rats are less susceptible to cannabinoid-induced anxiety then cannabinoids may have less of an impact on the CEA in these rats. This was one hypothesis tested in the present study in which a range of brain structures were assessed for Fos immunoreactivity following cannabinoid administration in Lewis and Wistar rats.

Previous studies examining the effects of Δ^9-THC and its synthetic analogues on Fos immunoreactivity have shown a consistent distribution of Fos expression following various cannabinoid receptor agonists. Fos expression is typically seen in the caudate-putamen (CPU) and nucleus accumbens (NAS) [50,54,55,66,69], the CEA [50,61,69], the paraventricular nucleus of the hypothalamus (PVN) [50,69], the bed nucleus of the stria terminalis (BNST) [61,69], the ventral tegmental area (VTA) [69], the lateral septum (LS) [50] and the frontoparietal and cingulate cortices [48,66]. However many studies have only examined a relatively small number of brain structures.

In light of the limited anatomical detail provided by these studies, a more comprehensive description of Fos immunoreactivity throughout the brain was sought in the current study. Here we assessed the effects of the synthetic cannabinoid receptor agonist CP 55,940 on Fos immunoreactivity in a total of 33 brain regions. These included a range of reward and anxiety-related areas such as the CEA, the medial prefrontal cortex (MPC), the islands of Calleja (ICjM), the periaqueductal grey (PAG) and the pedunculopontine tegmental nucleus (PPTg).

For comparison purposes, the effects of cocaine on Fos immunoreactivity were also examined in the present investigation. The distribution of cocaine-induced Fos immunoreactivity has some commonalities with that produced by acute exposure to a cannabinoid receptor agonist with Fos immunoreactivity commonly seen in the cortex, the CPU, the NAS, the septum, the ICjM, and the amygdala [33].

In terms of behavior, Lewis rats are more sensitive to the rewarding effects of cocaine as assessed by self-administration and conditioned place preference models when compared to Fischer 344 rats [41,42]. In addition, Lewis rats are more sensitive to acutely administered cocaine where they show greater locomotor activity than Fischer 344 rats [13]. Thus, assessing the effects of cocaine on Fos immunoreactivity in Lewis and Wistar rats provides a useful comparison to the effects of CP 55,940.

2. Materials and methods

2.1. Animals

The subjects were 19 inbred Lewis rats and 19 inbred Wistar rats matched for age (75–90 days old). Wistar rats weighed between 370 and 550 g and Lewis rats weighed between 350 and 430 g at the time of testing. Rats were housed in large plastic tubs in groups of eight and maintained under a 12 h:12 h reversed light/dark cycle (lights off at 09:00 h) with food and water available ad libitum. All experiments were conducted during the dark cycle.

2.2. Drugs

CP 55,940 (Tocris, Bristol, UK) was prepared as described previously [7] and administered intraperitoneally (i.p.) 30 min prior to behavioral testing. Two doses were used in this study, 50 and 250 μg/kg. The 250 μg/kg dose is a relatively high dose that resembles the potency of doses used in previous studies assessing the effects of other cannabinoid receptor agonists on Fos immunoreactivity in the rat brain such as Δ^9-THC (between 3.2 and 15 mg/kg) [48,50,54,55,66], HU-210 (100 μg/kg) [69] and anandamide (20 mg/kg) [50]. The problem with such doses is that they are much higher than typical human recreational doses and cause profound behavioral deficits such as catalepsy in rodents. Thus, the current study also employed a lower dose of CP 55,940 (50 μg/kg) that is more relevant to doses that humans use recreationally [52].

Cocaine hydrochloride (Australian Pharmaceutical Industries, Sydney, Australia) was dissolved in 0.9% saline and injected at a dose of 15 mg/kg in a volume of 1 ml/kg immediately before behavioral testing. This dose was chosen based on previous studies that have assessed the effects of cocaine on Fos immunoreactivity [33].

2.3. Behavioral testing

Rats from each strain were extensively handled for a week prior to the start of the experiment and were randomly assigned to experimental groups. In Experiment 1, CP 55,940 was the drug administered while in Experiment 2 it was cocaine. Exactly the same procedures were used in Experiment 1 as in Experiment 2.

Two habituation days were given immediately prior to the test day in order to minimise any Fos immunoreactivity due to novelty of testing, handling or injection procedures. On these habituation days the rats were subjected to

exactly the same procedures to those used on the test day except that they were given only saline injections.

On the test day individual rats were removed from their home cage and brought through to the test room in a small, enclosed rectangular tub filled with wood shavings. In Experiment 1 all rats were given a single i.p. injection of either 0, 50 or 250 μg/kg CP 55,940 ($n=5$ per group). In Experiment 2, rats were given a single i.p. injection of 15 mg/kg of cocaine ($n=4$ per group).

Thirty minutes following injection with either CP 55,940 or cocaine, rats were subjected to a series of tests that consisted of measuring catalepsy, axillary temperature and locomotor activity. First rats were tested for catalepsy using a bar test [84]. The forepaws of the rat were placed on a horizontal metal bar (25 cm×0.5 cm in diameter) that was held 10 cm above the bench using a retort stand. The experimenter used a stopwatch to measure the time that it took the rat to move both paws on to the bench. Each rat was given a maximum of 2 min to do this. This procedure was repeated twice and the average of these measures formed the basis for statistical comparison.

Rats were then tested for their axillary (underarm) temperature using a digital thermometer and thermocouple probe (Yellow Spring Instruments, BAT-12). The probe was placed under the rats arm for 30 s before a reading was taken. Measuring axillary temperature is thought to be considerably less stressful than taking rectal temperature yet gives a reliable index of body temperature [51].

The rats were then immediately placed in one of eight identical chambers [30 cm (L)×25.5 cm (W)×50 cm (H)] for 30 min for measurement of locomotor activity. The side walls and roof of the chambers were aluminum, while the front wall and rear wall were made of clear perspex. The cage floors consisted of 16 metal bars (0.5 cm diameter, spaced 1 cm apart) connected to a high impedance amplifier. When the rat moved on the grid so that contact or breaking of contact between any four bars and the other 12 occurred, an activity count was recorded by a Macintosh™ computer running WorkbenchMac™ data acquisition software [52]. Each cage was encased in a wooden sound attenuation chamber that was equipped with a fan that provided masking noise during testing.

Immediately after the locomotor activity test, axillary temperature was again taken. Rats were then placed in individual plastic tubs in an adjacent darkened holding room for a further 60 min. On the two habituation days the rats were then returned to their group housing in the colony room. On the test day, the rats were removed to a laboratory where they were given an overdose of pentobarbitone (120 mg/kg, i.p.) and perfused, prior to immunohistochemistry.

2.4. Immunohistochemistry

The immunohistochemical technique was performed as described previously [21,37,77]. Rats were perfused transcardially with 100 ml of 0.1 M phosphate-buffered saline (PBS) followed by 250 ml of 4% paraformaldehyde in PBS (pH 7.3). The brains were removed and placed in paraformaldehyde overnight at 4°C and stored in cold 30% sucrose for 72 h. The brains were then placed on microtome stages, frozen to −17°C and sliced at 40 μm with slices collected in PBS.

Free floating sections were incubated for 30 min in 1% hydrogen peroxide in PBS and then for 30 min in 3% normal horse serum in PBS. They were incubated in the primary c-Fos antibody (Santa Cruz Biotechnology, Santa Cruz, CA; rabbit polyclonal, specific for the amino terminus of c-Fos p62, non cross-reactive with FosB, Fra-1 or Fra-2) diluted 1:2000 in phosphate buffered horse serum (0.1% bovine serum albumin, 0.2% Triton X-100, 2% normal horse serum in PBS) for 72 h at 4°C. Sections were washed for 30 min in PBS at room temperature and then incubated for 1 h in biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA; diluted 1:500) in phosphate buffered horse serum. They were then washed in PBS for a further 30 min and then incubated for 2 h in ExtrAvidin-horseradish peroxidase (Sigma, St Louis, MO; diluted 1:1000 in phosphate buffered horse serum). After three 30-min washes in PBS, horseradish peroxidase activity was visualised with the nickel diaminobenzidine and glucose oxidase reaction. This reaction was terminated after approximately 10 min by washing in PBS. The sections were then mounted onto slides, dehydrated, xylene cleared and coverslipped.

2.5. Counting of labeled cells

The nuclei of Fos immunoreactive cells following administration of CP 55,940 (0, 50 and 250 μg/kg) were quantified in 33 different brain regions or subregions with reference to the rat brain atlas of Paxinos and Watson [62]. As described previously [21,37] quantification was performed manually by an observer who was blind to group assignment. Counts, for the most part, were made within a graticule (0.5 mm×0.5 mm) which equated to 0.25 mm^2 when viewed under the 20× objective. The placement of the graticule in each location is presented in Fig. 1. Regions in which the graticule was not used to count cells were the ICjM, septohippocampal nucleus, suprachiasmatic nucleus (SCh), supraoptic nucleus of the hypothalamus (SO) and PPTg. In these cases the area was smaller than the area of the graticule. Thus, cells in these areas were counted within the regions defined in Paxinos and Watson [62]. A neuron was classified as Fos-positive when the nucleus appeared round or oval, completely filled, and dark brown or black in colour. Digitized images were produced by a Polaroid DMC Ie camera attached to a Olympus BX40 light microscope as previously described [21].

Fos immunoreactivity following administration of 15 mg/kg of cocaine was quantified in eight regions using the same procedure as in Experiment 1. These regions were

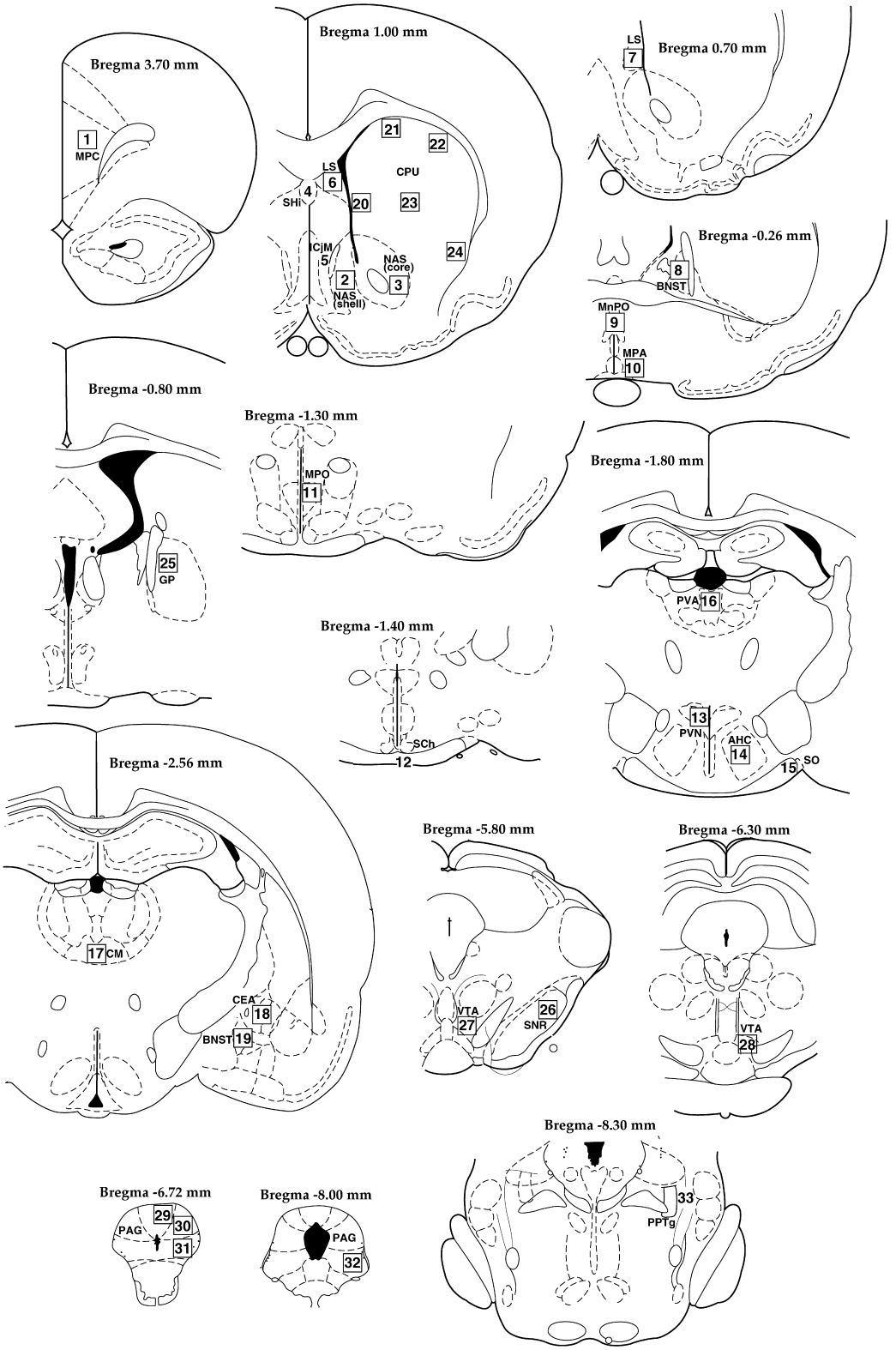

Fig. 1. Schematic diagrams of coronal sections of the rat brain [62]. The number of Fos positive nuclei were counted within the areas numbered and shaded in gray. The areas shown correspond in scale to the exact areas counted. The numbered areas correspond to the brain regions listed in Tables 1 and 2.

the: MPC, NAS (shell and core), median preoptic nucleus (MnPO), PVN, paraventricular nucleus of the thalamus (PV), CEA and dorsolateral PAG. These areas were chosen because they include areas where cocaine-induced Fos immunoreactivity has been previously observed [33].

2.6. Statistical analysis

In Experiment 1, data for Fos immunoreactivity, locomotor activity, catalepsy and axillary temperature were analysed by two-way analysis of variance (ANOVA). The two factors were strain (Lewis and Wistar) and dose of CP 55,940 (vehicle, 50 and 250 μg/kg of CP 55,940). A significance level of $P<0.05$ was chosen for all statistical comparisons.

To examine whether 50 μg/kg of CP 55,940 affected Fos immunoreactivity, locomotor activity, catalepsy and axillary temperature in its own right, an individual contrast compared the vehicle to 50 μg/kg of CP 55,940 conditions for the two strains combined.

To assess strain differences in baseline Fos immunoreactivity the data for vehicle-treated Lewis and Wistar rats were compared using a one-way ANOVA.

The effects of CP 55,940 on body temperature and behavioral data (locomotor activity and catalepsy) were also assessed within strains. Separate one-way ANOVA for Lewis and Wistar rats were conducted followed by Dunnett's post-hoc tests to assess the effects of various CP 55,940 doses within a strain. Data analysed for axillary temperature were an average of the first and second body temperature measures taken before and after a 30-min locomotor activity test. To assess baseline differences in any of these measures, vehicle-treated Lewis and Wistar rat data were compared using a one-way ANOVA.

In Experiment 2, data for Fos immunoreactivity and locomotor activity were analysed using a two-way ANOVA, with the two factors being strain (Lewis and Wistar) and dose (vehicle and 15 mg/kg of cocaine).

Separate one-way ANOVA for Lewis and Wistar rats were conducted to assess the effects of cocaine on locomotor activity within a strain.

3. Results

3.1. Experiment 1. The effects of CP 55,940 on catalepsy, body temperature, locomotor activity and Fos immunoreactivity in Lewis and Wistar rats

3.1.1. Catalepsy

The data from the catalepsy test are shown in Fig. 2. No significant difference was observed between vehicle-treated Lewis and Wistar rats in baseline catalepsy scores [$F(1,8)=2.77$, $P=0.13$].

Wistar rats showed a greater cataleptic response to 250 μg/kg of CP 55,940 than Lewis rats (Fig. 2). Two-way ANOVA revealed a significant overall effect of strain [$F(1,24)=6.75$, $P<0.05$] and dose [$F(1,24)=35.62$, $P<0.001$] and a significant strain by dose interaction [$F(1,24)=5.27$, $P<0.05$].

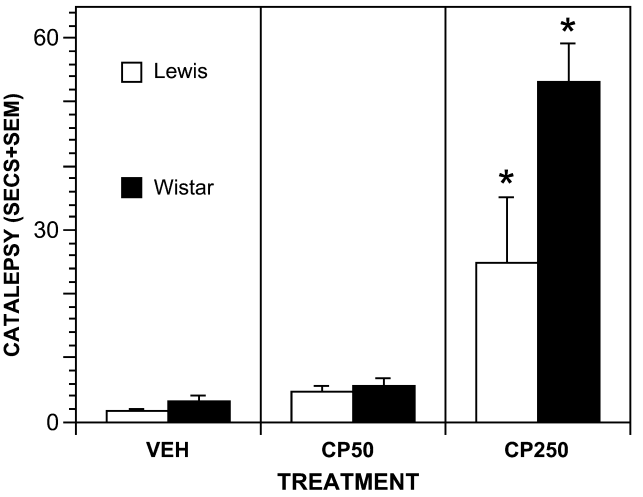

Fig. 2. The effect of CP 55,940 (VEH=0 μg/kg, CP50=50 μg/kg and CP250=250 μg/kg) on catalepsy in Lewis and Wistar rats ($n=5$ per group). *Significant effect of CP 55,940 group compared to vehicle group within that strain ($P<0.05$).

An individual comparison between all rats given vehicle and all rats given 50 μg/kg of CP 55,940 showed no significant cataleptic effect of this low dose ($F<1$). Within strain analysis using post-hoc tests showed that only 250 μg/kg of CP 55,940 promoted catalepsy in Lewis or Wistar strains (Fig. 2) ($P<0.05$).

3.1.2. Body temperature

The body temperature data are shown in Fig. 3. Lewis rats treated with vehicle showed significantly lower axillary temperature than Wistar rats treated with vehicle [$F(1,8)=8.05$, $P<0.05$].

Two-way ANOVA revealed no effect of strain ($F<1$), but a significant effect of dose [$F(2,24)=110.11$, $P<0.001$] and a significant strain by dose interaction [$F(2,24)=5.08$, $P<0.05$]. This significant strain by dose interaction reflects higher body temperature in Wistar rats relative to Lewis rats following vehicle treatment but lower body temperature in Wistar rats following CP 55,940 treatment (Fig. 3). This suggests a greater hypothermic response to CP 55,940 in Wistar rats than in Lewis rats.

Comparison between all rats given vehicle and all rats given 50 μg/kg CP 55,940 showed a significant hypothermic effect of the low dose [$F(1,24)=9.65$, $P<0.01$]. Within strains, this effect of the low dose was only significant in Wistar rats ($P<0.05$) (Fig. 3).

3.1.3. Locomotor activity

The locomotor activity data are shown in Fig. 4. Lewis rats treated with vehicle showed significantly lower

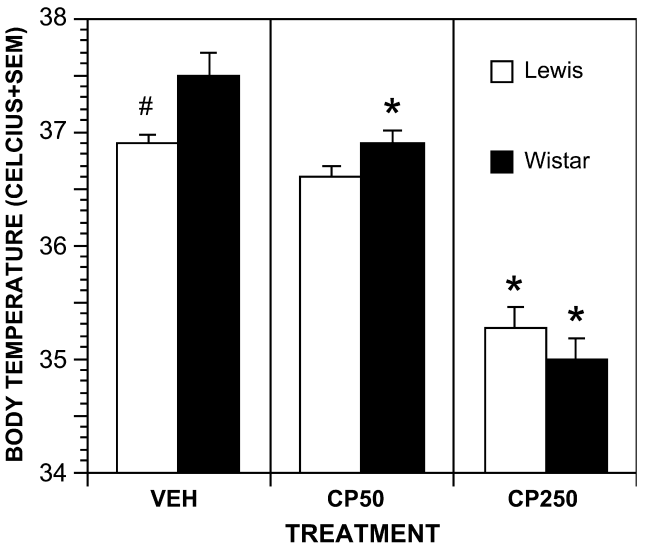

Fig. 3. The effect of CP 55,940 (VEH=0 μg/kg, CP50=50 μg/kg and CP250=250 μg/kg) on axillary temperature in Lewis and Wistar rats (n=5 per group). *Significant effect of CP 55,940 group compared to vehicle group within that strain ($P<0.05$). #Significant difference between vehicle-treated Lewis and Wistar rats ($P<0.05$).

locomotor activity than Wistar rats treated with vehicle [$F(1,8)=18.32$, $P<0.01$] (Fig. 4).

Wistar rats, but not Lewis rats, showed a dose-dependent reduction in locomotor activity with CP 55,940. Two-way ANOVA revealed a significant overall effect of strain [$F(1,24)=18.72$, $P<0.001$], of dose [$F(2,24)=22.06$, $P<0.001$] and a significant strain by dose interaction [$F(2,24)=14.05$, $P<0.001$].

The 50 μg/kg dose of CP 55,940 caused a significant overall reduction in locomotor activity [$F(1,24)=21.52$, $P<0.001$], but within strains this effect was only significant in Wistar rats ($P<0.05$) (Fig. 4).

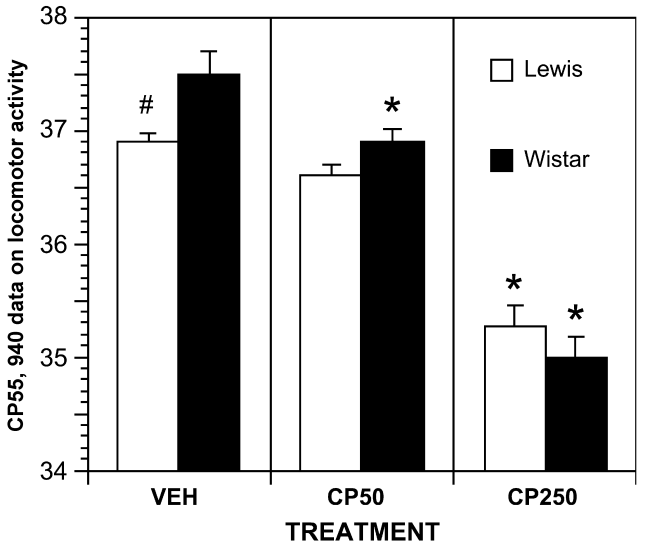

Fig. 4. The effect of CP 55,940 (VEH=0 μg/kg, CP50=50 μg/kg and CP250=250 μg/kg) on locomotor activity in Lewis and Wistar rats (n=5 per group). *Significant effect of CP 55,940 group compared to vehicle group within that strain ($P<0.05$). ##Significant difference between vehicle-treated Lewis and Wistar rats ($P<0.01$).

3.1.4. Regional Fos immunoreactivity

Fos counts for the 33 brain regions counted are shown in Table 1. Photomicrographs showing Fos immunoreactivity in Lewis and Wistar rats for the NAS and ICjM, PVN and CEA are presented in Figs. 5, 6 and 7, respectively.

Vehicle-treated Lewis rats showed significantly less baseline Fos immunoreactivity than Wistar rats in six brain structures: the MPC, the PV, the central medial thalamic nucleus (CM), the intra-amygdaloid nucleus of the BNST, the dorsolateral PAG and the ventrolateral PAG (Table 1).

Independent of any strain differences, CP 55,940 (taking the data for 50 and 250 μg/kg doses together) significantly increased Fos immunoreactivity in 24 of the 33 brain regions counted (Table 1).

The 50 μg/kg dose of CP 55,940 alone significantly increased Fos immunoreactivity in 10 brain regions including the shell of the NAS, the intra-amygdaloid and dorsal area of the lateral division of the BNST, the PVN and the CEA (Table 1).

Throughout the brain, significant strain differences were observed in CP 55,940-induced Fos immunoreactivity. A significant drug by strain interaction effect was seen in 16 brain regions, reflecting greater drug-induced Fos immunoreactivity in Wistar rats (Table 1). In the forebrain this was observed in the NAS (shell and core), the ICjM, the ventral LS, the CEA and the BNST (intra-amygdaloid and dorsal area of the lateral division). Within the hypothalamus, greater CP 55,940-induced Fos immunoreactivity was observed in Wistar rats in the MnPO, the SCh and the PVN. In the basal ganglia more Fos-labeled cells were observed in the dorsal CPU and globus pallidus (GP) of Wistar rats administered CP 55,940 than Lewis rats. In addition, clear strain differences in CP 55,940-induced Fos immunoreactivity were observed in parts of the midbrain, including the VTA, the dorsolateral and ventrolateral PAG and the PPTg.

There was no differential effect of CP 55,940 on Fos immunoreactivity in Lewis and Wistar rats in several brain regions (Table 1). These areas include the dorsolateral and central CPU, the anterior hypothalamic area (AH), the medial preoptic nucleus (MPO), the SO, the PV, the anterior VTA and the lateral region of the PAG.

3.2. Experiment 2. The effects of cocaine on locomotor activity and Fos immunoreactivity in Lewis and Wistar rats

3.2.1. Catalepsy

All rats administered 15 mg/kg cocaine immediately removed their forepaws from the bar to explore the bench below. Thus, cocaine had no cataleptic effects on either strain of rat.

Table 1
The number of Fos-labeled cells in various brain regions of Wistar and Lewis rats following vehicle (VEH) or CP 55,940 (50 or 250 μg/kg; $n=5$ per group)

Region	Bregma	Wistar rats			Lewis rats			Stats
		VEH	CP50	CP250	VEH	CP50	CP250	
Frontal regions								
1. Medial prefrontal cortex	+3.7	93±23	79±37	146±26	24±15	29±9	45±18	a, e
2. Nucleus accumbens shell	+1.0	9±3	15±3	39±8	3±1	11±3	14±0.8	a, b, c, d
3. Nucleus accumbens core	+1.0	4±1	1±0.7	8±3	0.2±0.2	0.6±0.4	0.2±0.2	a, b, c
4. Septohippocampal nucleus	+1.0	0.2±0.2	0.2±0.2	0.5±0.5	0±0	0±0	2±1	
5. Islands of Calleja, major	+1.0	0.6±0.6	3±2	104±15	0.5±0.3	10±4	36±16	a, b, c, d
6. Lateral septum, dorsal	+1.0	16±3	10±1	15±5	5±4	3±0.7	6±3	a
7. Lateral septum, ventral	+0.7	63±12	104±31	217±9	48±8	68±16	88±14	a, b, c
8. BNST lat div, dorsal	−0.26	7±3	68±2	94±13	2±0.6	8±0.8	13±3	a, b, c, d
Hypothalamus/preoptic area								
9. Median preoptic nucleus	−0.26	5±1	12±3	28±5	2±0.8	5±2	9±2	a, b, c
10. Medial preoptic area	−0.26	21±6	23±3	24±12	13±4	23±3	32±8	
11. Medial preoptic nucleus	−1.3	32±7	55±7	82±9	29±5	44±6	57±8	a, b, d
12. Suprachiasmatic nucleus	−1.4	25±7	29±9	89±40	16±4	20±3	16±6	a, b, c
13. Paraventricular nucleus	−1.8	14±5	83±16	185±30	18±3	31±6	63±8	a, b, c, d
14. Anterior hypothalamic area	−1.8	19±8	48±9	44±8	22±5	25±6	31±6	b
15. Supraoptic nucleus	−1.8	2±0.6	10±4	7±2	0.4±0.2	3±2	1±0.6	a, b
Thalamus								
16. Paraventricular nucleus, anterior	−1.8	55±5	100±14	113±11	16±1	52±8	79±11	a, b, d, e
17. Central medial nucleus	−2.56	16±6	13±6	14±3	0.4±0.2	7±3	7±2	a, e
Amygdala								
18. Central nucleus	−2.56	3±1	77±17	82±10	0.6±0.6	29±9	25±4	a, b, c, d
19. BNST intra-amygdaloid	−2.56	3±1	8±3	28±28	0.2±0.2	7±1	4±0.9	a, b, c, d, e
Striatum								
20. Caudate/putamen, medial	+1.0	8±2	6±3	8.5±4	1±0.4	0.2±0.2	6±2	a
21. Caudate/putamen, dorsal	+1.0	3±2	7±2	20±4	0±0	0.8±0.6	3±1	a, b, c
22. Caudate/putamen, dorsolateral	+1.0	2±0.8	3±1	8.5±3	0.2±0.2	0.2±0.2	3±2	a, b
23. Caudate/putamen, central	+1.0	0.4±0.2	1±0.4	13±6	0±0	0±0	5±3	b
24. Caudate/putamen, ventral	+1.0	1±0.8	0.2±0.2	1±0.3	0±0	0.8±0.6	0±0	
25. Globus pallidus	−0.8	0.4±0.4	1±0.5	15±3	0.2±0.2	0±0	3±1	a, b, c
Midbrain								
26. Substantia nigra, reticulata	−5.8	0±0	0±0	0±0	0±0	0.2±0.4	0±0	
27. Ventral tegmental area	−5.8	2±1	2±0.6	5±0.8	0.2±0.2	2±0.6	2±0.5	a, b
28. Ventral tegmental area	−6.3	0.4±0.4	4±0.9	8±0.7	2±0.7	5±2	8±1	b, d
Periaqueductal gray								
29. Dorsomedial	−6.72	16±4	19±5	27±7	10±2	8±2	9±2	a
30. Dorsolateral	−6.72	16±3	17±5	41±6	8±2	12±4	15±2	a, b, c, e
31. Lateral	−6.72	12±3	17±5	28±8	7±2	11±3	16±4	b
32. Ventrolateral	−8.00	22±3	18±3	67±18	4±2	10±9	27±17	a, b, c, e
Other brainstem areas								
33. Pedunculopontine tegmental nucleus	−8.3	2±0.6	3±0.8	12±1	1±0.5	5±2	5±0.5	a, b, c, d

a, a significant effect of strain; b, a significant effect of drug; c, a significant strain by drug interaction; d, a significant overall difference between VEH and CP50 conditions; e, a significant difference between vehicle-treated Lewis and Wistar rats.

3.2.2. Body temperature

The rats given 15 mg/kg of cocaine were hyperactive and difficult to handle. This made reliable measurements of axillary temperature difficult. Thus, data for body temperature were not recorded in Experiment 2.

3.2.3. Locomotor activity

The effects of cocaine on locomotor activity in Lewis and Wistar rats are shown in Fig. 8. The 15 mg/kg dose of cocaine significantly increased locomotor activity in both strains. Two-factor ANOVA revealed a significant effect of

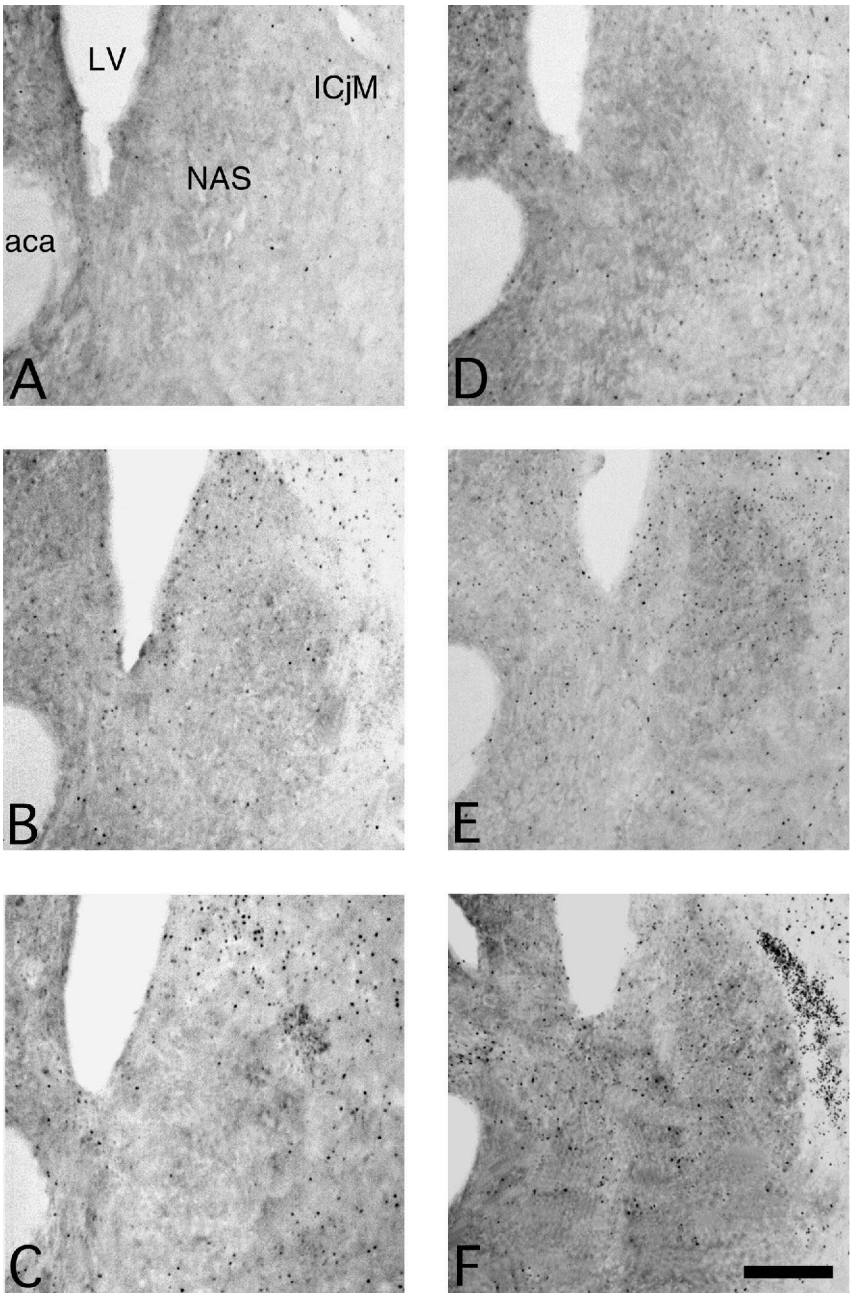

Fig. 5. Fos-labeled neurons within the shell of the nucleus accumbens (NAS) and the islands of Calleja, major islands (ICjM) in representative sections from Lewis and Wistar rats treated with CP 55,940 (0, 50 and 250 μg/kg). A, B and C: Lewis rats treated with 0, 50 and 250 μg/kg of CP 55,940, respectively. D, E and F: Wistar rats treated with 0, 50 and 250 μg/kg of CP 55,940, respectively. The lateral ventricle (LV) and the anterior region of the anterior commissure (aca) are also indicated. Scale bar=250 μm.

cocaine [$F(1,14)=33.88$, $P<0.0001$] but no significant differences between strains [$F(1,14)=4.14$, $P=0.061$]. Lewis rats showed a trend towards increased hyperactivity to 15 mg/kg of cocaine when compared to Wistar rats. However, this may be largely attributable to Lewis rats having lower baseline locomotor activity than Wistar rats (Fig. 8). The strain by dose interaction fell just short of statistical significance [$F(1,14)=4.25$, $P=0.058$].

Within strains, one-way ANOVA revealed that cocaine significantly increased locomotor activity in both Lewis [$F(1,7)=23.72$, $P<0.01$] and Wistar rats [$F(1,7)=10.23$, $P<0.05$] (Fig. 8).

3.2.4. Regional Fos immunoreactivity

Specific counts for eight regions of interest are shown in Table 2. Significant overall effects of drug in a two-way

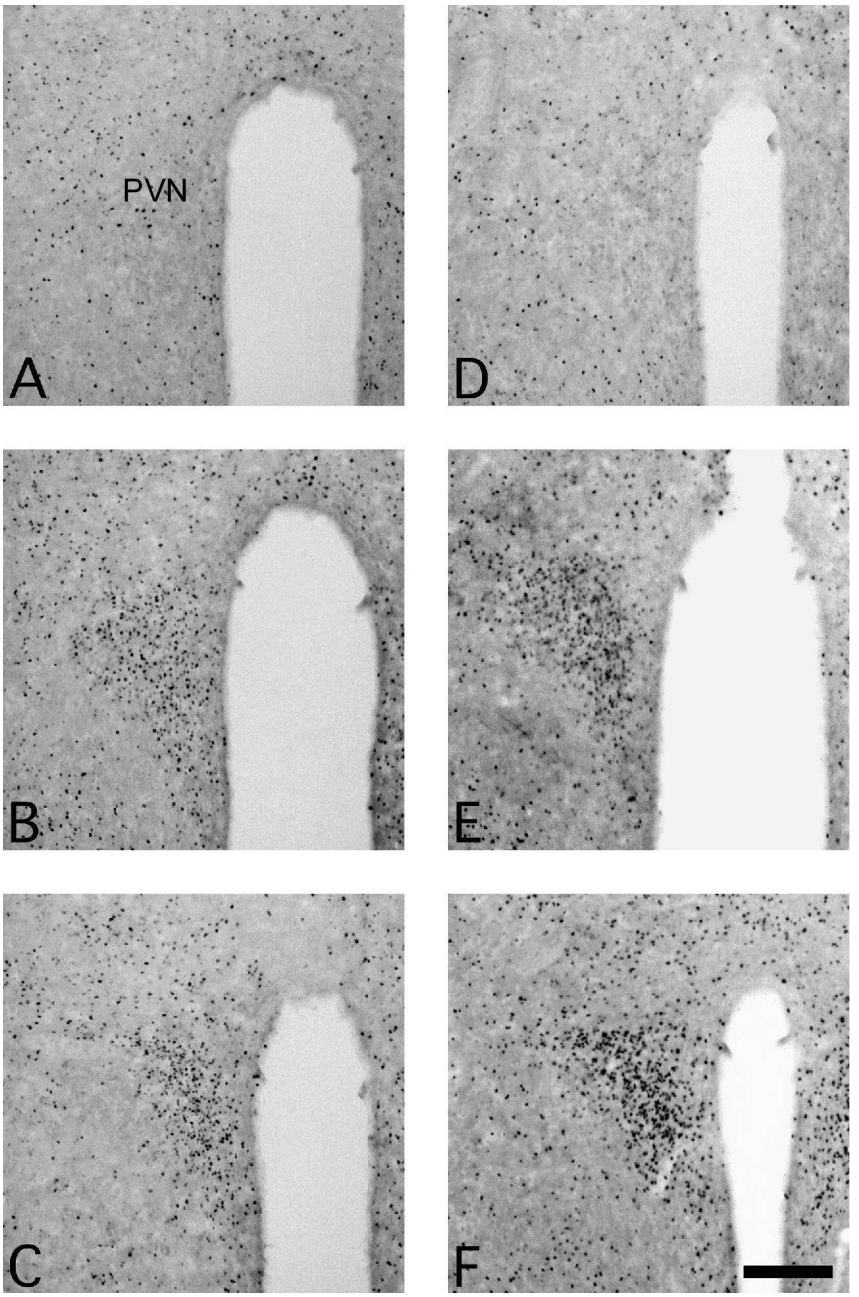

Fig. 6. Fos-labeled neurons within the paraventricular nucleus (PVN) of the hypothalamus in Lewis and Wistar rats treated with CP 55,940 (0, 50 and 250 μg/kg). A, B and C: Lewis rats treated with 0, 50 and 250 μg/kg of CP 55,940, respectively. D, E and F: Wistar rats treated with 0, 50 and 250 μg/kg of CP 55,940, respectively. Scale bar=250 μm.

ANOVA indicated that cocaine increased Fos immunoreactivity across both strains in seven out of eight brain regions of interest. There was also a significant overall effect of strain in three of the eight regions of interest (MPC, PV and dorsolateral PAG) suggesting that Wistar rats expressed more Fos in these three regions regardless of drug treatment. A significant strain by dose interaction effect was obtained in only one region, the MPC, an effect that appeared to be largely due to a strain difference in the response to vehicle rather than cocaine. Thus, there appeared to be minimal, if any, strain difference in the overall Fos response to cocaine.

4. Discussion

The most striking findings of the present study are that: (1) CP 55,940 induces Fos immunoreactivity in a diverse array of specific brain regions, (2) a relatively low dose of CP 55,940 can induce significant Fos immunoreactivity in

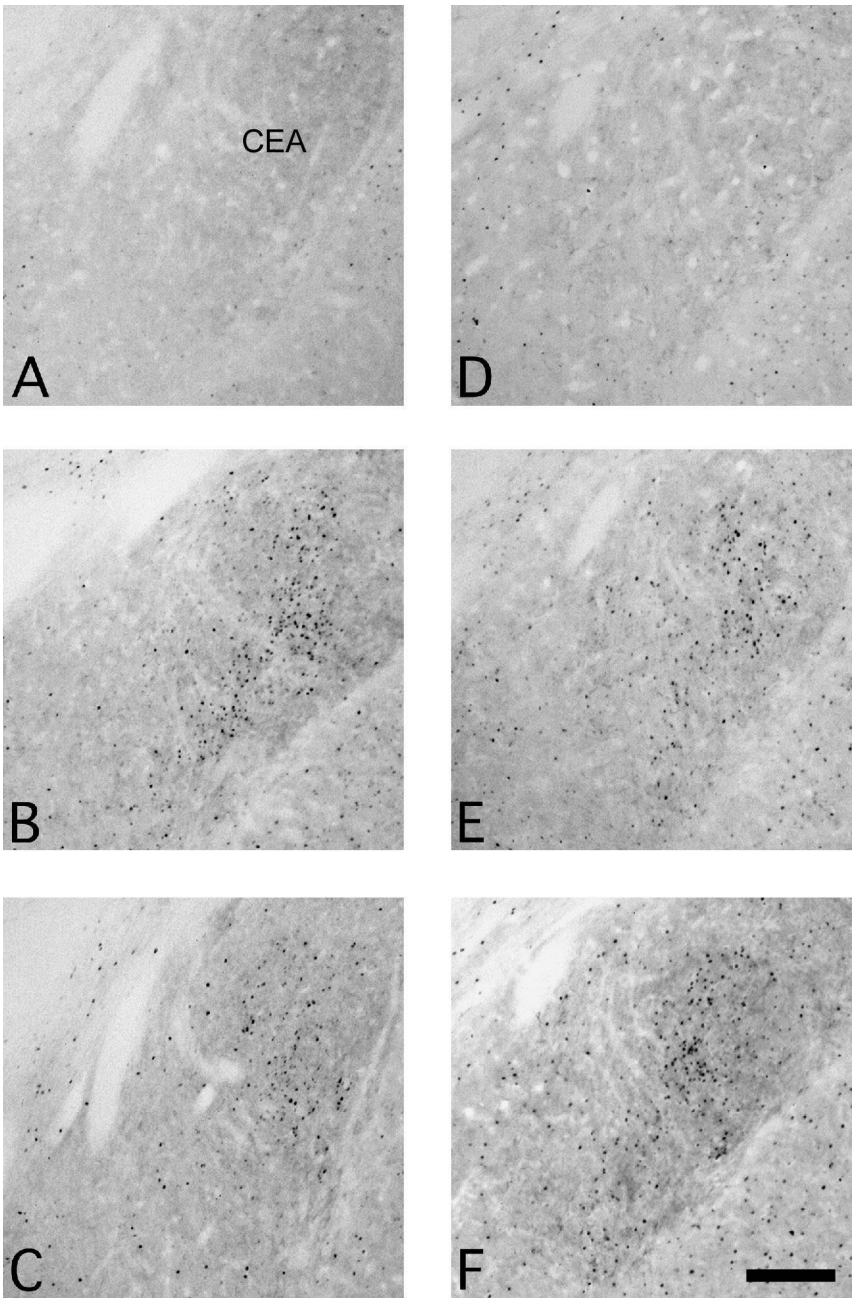

Fig. 7. Fos-labeled neurons within the central nucleus of the amygdala (CEA) for Lewis and Wistar rats treated with CP 55,940 (0, 50 and 250 μg/kg). A, B and C: Lewis rats treated with 0, 50 and 250 μg/kg of CP 55,940, respectively. D, E and F: Wistar rats treated with 0, 50 and 250 μg/kg of CP 55,940, respectively. Scale bar=250 μm.

rat brain, and (3) CP 55,940 produces significantly more Fos-labeled cells in Wistar rats than Lewis rats. This differential effect of CP 55,940 between strains on Fos immunoreactivity appears distinct from the effects of cocaine, where no strain differences were observed.

Confirming previous reports, CP 55,940 promoted catalepsy, hypothermia and an inhibition of locomotor activity [23,51,52,64,73]. Convergent with the data on Fos immunoreactivity, CP 55,940 had more modest effects on these behavioral and physiological measures in Lewis rats than Wistar rats. This suggests that the smaller neural effect in Lewis rats (as indexed by Fos immunoreactivity) is related to a smaller functional effect of the drug (as indexed by behavior and body temperature effects). In contrast, there were no significant differences between Lewis and Wistar rats in either cocaine-induced locomotor activity or cocaine-induced Fos immunoreactivity.

The strain differences in the locomotor suppressant effects of CP 55,940 are somewhat ambiguous because of the significantly lower baseline (i.e. drug-free) locomotor

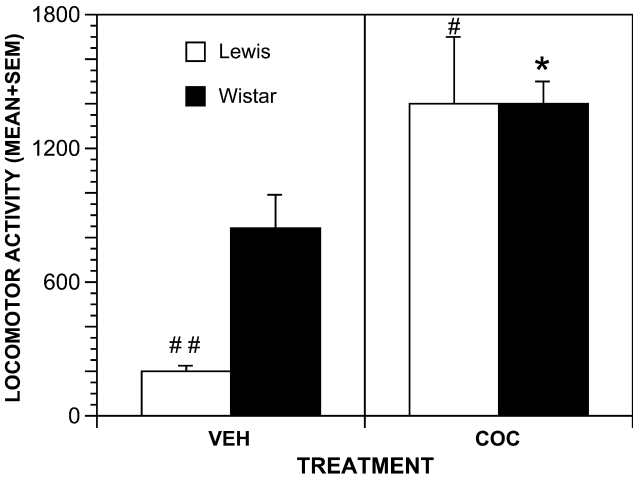

Fig. 8. The effects of cocaine (VEH=0 μg/kg, COC=15 mg/kg) on locomotor activity in Lewis and Wistar rats (n=4 per group). *Indicates a significant effect of cocaine compared to vehicle group within that strain ($P<0.05$). #Significant effect of cocaine compared to vehicle group within that strain ($P<0.01$). ##Significant difference between vehicle-treated Lewis and Wistar rats ($P<0.01$).

activity observed in Lewis rats. That is, as drug-free Lewis rats showed very little locomotion, there was not much opportunity for Lewis rats treated with CP 55,940 to exhibit cannabinoid-induced locomotor depression. However, the observation of clear strain differences in the cataleptic effects of 250 μg/kg of CP 55,940 provides independent confirmation that this drug has differential motoric effects in Lewis and Wistar rats.

The highest CP 55,940 dose administered here (250 μg/kg) is similar in magnitude to the doses of Δ^9-THC, anandamide and other synthetic analogues used in previous studies [50,54,55,66,69]. Such doses produce profound catalepsy and disruption of motor activity that is rarely, if ever, seen in human cannabis users. The low 50 μg/kg dose of CP 55,940 used here is more similar to the self-administered Δ^9-THC doses of humans who are smoking cannabis [52]. This dose does not grossly disrupt rodent behavior with Wistar rats treated with this dose showing only slightly depressed locomotor activity and a mild hypothermia but no signs of catalepsy (Fig. 2).

The 50 μg/kg dose significantly increased Fos immunoreactivity in 10 out of the 33 regions examined. Generally, at any given site, the Fos immunoreactivity with 50 μg/kg of CP 55,940 was far less than that seen with 250 μg/kg. An exception to this was in the CEA where equivalent Fos immunoreactivity was seen with the high and low doses of CP 55,940 in Wistar rats (Table 1 and Fig. 7).

In several brain regions, significant increases in Fos immunoreactivity were seen with 250 but not 50 μg/kg of CP 55,940. Regions only significantly affected by the higher dose included the core of the NAS, dorsal and dorsolateral CPU, ventral LS, MPO, SCh, GP, and the lateral, ventrolateral and dorsolateral PAG. Thus, much regional cannabinoid-induced Fos immunoreactivity may require very large doses that exceed the equivalent doses used recreationally by humans. For example, Glass and Dragunow [31] showed that 2.5 mg/kg of CP 55,940 could only induce sparse amounts of Fos protein in the striatum. This dose is 50 times larger than the dose of CP 55,940 (50 μg/kg) used in the current study and its relevance to human cannabis use is clearly questionable.

The current investigation shows cannabinoid-induced Fos immunoreactivity in a wide variety of brain regions. The distribution of immunoreactivity is consistent with

Table 2
The number of Fos-labeled cells in various brain regions of Lewis and Wistar rats following administration of 15 mg/kg of cocaine (COC15) (n=4 per group)

Region	Bregma	Wistar rats		Lewis rats		Stats
		VEH[a]	COC15	VEH[a]	COC15	
Frontal regions						
1. Medial prefrontal cortex	+3.7	93±23	83±9	24±15	81±3	a, c
2. Nucleus accumbens shell	+1.0	9±3	37±4	3±1	48±10	b
3. Nucleus accumbens core	+1.0	4±1	51±7	0.2±0.2	42±7	b
Hypothalamus/preoptic area						
9. Median preoptic nucleus	−0.26	5±1	20±6	2±0.8	16±2	b
13. Paraventicular nucleus	−1.8	14±5	76±5	18±3	90±9	b
Thalamus						
16. Paraventricular nucleus	−1.8	55±5	107±9	16±1	91±8	a, b
Amygdala						
18. Central nucleus	−2.56	3±1	36±7	0.6±0.6	43±3	b
Periaqueductal gray						
30. Dorsolateral	−6.72	16±3	38±3	8±2	32±2	a, b

a, significant effect of strain; b, significant effect of drug, and c, a significant strain by drug interaction.
[a] Vehicle (VEH) data from Experiment 1 were used for the purposes of statistical comparison.

previous results. For example, previous studies have typically shown cannabinoid-induced Fos immunoreactivity in the CPU [31,54,55], NAS [50,54,55,66,69], PVN [50,61,69], PV [61], CEA [50,61,69], LS [50], and the BNST [61,69]. Other studies have documented Fos immunoreactivity in the PAG and MPO [61], the VTA [69], the SO and the SCh [61,69]. To the best of our knowledge the present findings of Fos immunoreactivity in the ICjM, AH, GP and the PPTg are novel.

As in previous studies, Fos immunoreactivity was seen here in many areas containing only modest densities of CB_1 receptors. These areas include the NAS, VTA, LS, BNST, CEA, PVN and the PAG [35,65,82]. Conversely, CP 55,940 did not affect Fos immunoreactivity in the substantia nigra (SN), an area that contains many CB_1 receptors. Furthermore, the increase in Fos immunoreactivity observed in the GP, another area rich in CB_1 receptors, was relatively sparse and restricted to the medial portion of this structure.

The dissociation between Fos immunoreactivity and CB_1 receptor density may be due to the CB_1 receptor's negative link with adenylate cyclase and the inhibitory consequences this has on cAMP production. When cAMP production is inhibited, Fos immunoreactivity is more likely to be suppressed than promoted [36]. On the other hand, the presence of high levels of Fos immunoreactivity in areas with low CB_1 receptor density presumably reflects transynaptic activation of non-CB_1 receptor containing neurons, or the presynaptic modulation of neurotransmitter release by CB_1 receptors affecting Fos immunoreactivity postsynaptically. For example, in a previous study, Δ^9-THC-induced Fos immunoreactivity in the dorsomedial striatum and the NAS was blocked by the D_1 dopamine receptor antagonist SCH 23390 [55]. This presumably reflects an action of Δ^9-THC on dopamine release in the NAS, with increased dopamine levels caused by Δ^9-THC leading to increased Fos immunoreactivity.

Consistent with its hypothermic effect, CP 55,940 significantly increased Fos immunoreactivity in preoptic and hypothalamic regions thought to be involved in thermoregulation. These areas include the MnPO, MPO and AH [75,76,81,87]. Interestingly, previous studies have shown that injection of Δ^9-THC into the preoptic area of the anterior hypothalamus caused significant reductions in body temperature [25,63]. In addition, exposure to a hot environment increases Fos immunoreactivity in the MnPO [75]. The ICjM might also be involved in the hypothermic effect of cannabinoids. The current study showed clear strain differences in the hypothermic effects of CP 55,940 that correlated with strain differences observed in Fos immunoreactivity in the ICjM. A recent study has shown that intracranial administration of D_3 receptor agonists into the ICjM causes hypothermia, an effect which was potentiated by D_1 receptor agonists [8].

The influence of cannabinoids on locomotor activity is thought to reflect presynaptic modulation of excitatory and inhibitory inputs to the basal ganglia [2]. Dense CB_1 receptor distribution is seen in the GP and the SN, and moderate CB_1 receptor distribution occurs in the CPU [35,65,82]. Most CB_1 receptors in the GP and the SN are localised presynaptically on striatal afferents [34,47,83]. The locomotor depressant effects of cannabinoids seem to involve modulation of GABA, dopamine and glutamate in these brain areas [2].

It is noteworthy that only sparse Fos immunoreactivity was observed in a small and localised area of the GP of Wistar rats administered the highest dose of CP 55,940. Furthermore, no CP 55,940-induced Fos immunoreactivity was observed in the SN in the present study.

CP 55,940 promoted little Fos expression in the CPU, with only sparse Fos immunoreactivity observed in its central, dorsal and dorsolateral regions. This agrees with previous research which has shown that administration of Δ^9-THC promotes some Fos immunoreactivity in the CPU [48,55,66]. Interestingly, a strain difference was observed in CP 55,940-induced Fos immunoreactivity in the dorsal CPU, which is consistent with the strain differences observed in catalepsy promoted by CP 55,940. The view that cannabinoid-induced catalepsy involves the CPU is supported by a previous study showing that administration of CP 55,940 directly into this region produces catalepsy in rats [45].

Another important brain site for cannabinoid effects on locomotor activity may be the core of the NAS. Direct infusion of Δ^9-THC into the NAS promoted catalepsy in mice [59]. In the current investigation, the lesser effects of CP 55,940 on locomotor activity and catalepsy in Lewis rats relative to Wistar rats correlated with the differential pattern of Fos immunoreactivity observed in the core and shell of the NAS between these strains.

CP 55,940 increased Fos immunoreactivity in a range of brain structures known to be involved in fear, anxiety and defensive behavior [21]. These include the CEA, BNST and the PAG. The pattern of Fos immunoreactivity in these areas, particularly in Wistar rats, may highlight a neural circuit that underpins an integrated fear or anxiety response to cannabinoid administration. The divergent effects on Fos immunoreactivity across strains are consistent with the effects of CP 55,940 on Lewis and Wistar rats in a variety of different models of anxiety [5]. In these models, Lewis rats show markedly less anxiety-like behaviors than Wistar rats when administered CP 55,940 (10–75 μg/kg).

When Δ^9-THC is directly infused into the CEA it causes mice to spend less time exploring the open arms of an elevated plus maze [59]. The CEA may therefore underpin an integrated anxiety response seen with the administration of cannabinoids. The CEA gives rise to an important output pathway that modulates many aspects of unconditioned and conditioned fear (e.g. bradycardia, freezing, hypoalgesia and activation of the HPA axis) [72,80]. It provides inputs and outputs to the BNST and output to the PVN (involved in stress and the HPA) and the PAG

(involved in defensive behavior, cardiovascular function and pain transmission) [1,80]. Interestingly, in the current investigation all of the above areas (CEA, BNST and PVN) showed marked CP 55,940-induced Fos immunoreactivity, with significantly more Fos-labeled cells counted in Wistar rats in comparison to Lewis rats.

An extraordinary strain difference in CP 55,940-induced Fos immunoreactivity was observed in the lateral division of the dorsal BNST (Table 1). Notably, both CP 55,940 doses promoted seven times as much Fos-labeled cells in Wistar rats in comparison to Lewis rats in this brain region. The BNST may be a brain region that is crucially involved in cannabinoid-induced anxiety. As part of the extended amygdala, the BNST in thought of as a rostral extension of the CEA [1]. The BNST has a very similar structure to the CEA and contains similar transmitters, cell morphology and has similar efferent connections [1,80] and a similar involvement in fear-related behaviors [19].

The differential behavioral and emotional effects of CP 55,940 in Lewis and Wistar rats could also be attributable to differences in cannabinoid-induced activation of the HPA axis. The HPA axis of Lewis rats is hyporesponsive to stressful stimuli and releases less basal and stress-induced corticosterone [12,68]. This appears to be a consequence of a deficiency in the ability of the hypothalamus to synthesise and secrete CRH in Lewis rats [78,79]. This may also relate to the relative lack of cannabinoid-induced Fos immunoreactivity in the PVN of Lewis rats compared to Wistar rats seen in the present study. The PVN produces CRH, a precursor to corticosterone release [3]. In addition, the effects of CRH on Fos immunoreactivity shows commonalities with the current results with increased Fos-labeled cells observed in PVN, CEA, LS, BNST, NAS and the CPU [4]. Furthermore, administration of the selective CRH antagonist, D-Phe CRF_{12-41}, attenuated the anxiogenic effects of the cannabinoid receptor agonist HU-210 in a defensive-withdrawal model [70].

The PAG is an important brain structure involved in cannabinoid-induced antinociception, catalepsy and hypothermia [45]. Lichtman et al. [45] showed that microinjections of CP 55,940 into the ventrolateral PAG produced all of the above effects. In addition, this study showed that microinjection of CP 55,940 into the dorsolateral PAG had no such effects. Further, the PAG has been implicated in neural circuitry involved in defensive behavior [14,15,21]. Prior studies indicate that the ventrolateral PAG is involved in freezing and immobility, whereas the dorsolateral PAG is involved in the control of flight behaviors [9,16,17,20,40].

The current investigation showed that Lewis rats showed less CP 55,940-induced Fos immunoreactivity than Wistar rats in the ventrolateral and dorsolateral PAG. This is also consistent with Lewis rats being less susceptible to CP 55,940-induced anxiety-related behaviors than Wistar rats [5]. Further, the strain differences observed in the effects of CP 55,940 on Fos immunoreactivity in the ventrolateral PAG are consistent with Lewis rats showing less CP 55,940-induced catalepsy and hypothermia. It is also conceivable that Lewis rats may display lesser antinociceptive effects of CP 55,940 than Wistar rats.

Interestingly, Lewis rats showed less CP 55,940-induced Fos immunoreactivity in reward-related regions as well as those involved in anxiety. In both strains of rat, CP 55,940 promoted Fos immunoreactivity in the shell of the NAS, but the effect was much larger in Wistar rats. This finding contradicts the notion that Lewis rats may be uniquely sensitive to the rewarding effects of cannabinoids via a greater sensitivity to cannabinoid actions on the mesolimbic dopamine system [18,44]. However it might be noted that this theory is somewhat controversial. Thus, Parker and colleagues found a conditioned place aversion to Δ^9-THC in Lewis rats — an effect that suggests an aversive action of cannabinoids in this strain [60]. In addition, we have recently shown that CP 55,940 fails to lower thresholds for rewarding brain stimulation in Lewis rats [6]. This finding is again inconsistent with the idea that Lewis rats are more susceptible to cannabinoid-induced reward.

An increasing body of evidence suggests that increased dopaminergic transmission in the NAS is produced by stressful or anxiogenic stimuli [39,74]. Interestingly, the CEA projects to the shell of the NAS via the lateral BNST [1]. The ambiguity over whether the NAS is involved in the aversive and rewarding effects of cannabinoids raises an issue which may be important to the understanding of the appetitive effects of cannabinoids. This issue involves working out the exact relationship between reward and anxiety, and the implication this relationship may have for appetitive behavior. For example, while cocaine has clearly rewarding effects in humans, it also has aversive effects [10,85]. Cocaine is also at once rewarding and anxiogenic in rats [11,22,49,71,88]. In addition, as shown in the current investigation, cocaine promotes Fos immunoreactivity in similar areas to CP 55,940, including areas implicated in both reward and anxiety, such as the shell and core of the NAS, the CEA, the PVN and the PAG.

For cocaine, the rewarding and anxiogenic effects have been dissociated in an elegant study [22]. Rats avidly self-administering cocaine over days in a runway task showed more retreats than controls from the goal box. As the rats retreated only a short distance and continued to avidly self-administer cocaine, this observation was proposed to reflect a conflict situation for the rat between positive and negative aspects of cocaine's effects. In addition, the anxiolytic compound diazepam, dose-dependently reduced this retreat behavior. Thus, for cocaine it appears the conflict between its aversive and rewarding effects has implications for cocaine self-administration. Future studies will hopefully address the relationship between aversion and reward and what implication this has for appetitive behavior relevant to cannabinoids.

CP 55,940 appeared to have modest and largely equiva-

lent effects on Fos immunoreactivity in the VTA of Lewis and Wistar rats. If anything, CP 55,940 had less of an effect on Fos immunoreactivity in the VTA of Lewis rats than Wistar rats consistent with the distinct effects that CP 55,940 had on Fos immunoreactivity in the shell of the NAS. One proposed mechanism which might mediate cannabinoid-induced dopamine release in the NAS is disinhibition of dopamine cell firing in the VTA via inhibition of GABA interneurons [2]. This theory is based on the finding that cannabinoids increase the spontaneous firing of neurons within the VTA [26,27,30].

Cannabinoid-induced Fos immunoreactivity in the ICjM has not been previously reported. It is interesting to speculate upon the role of the ICjM in the functional effects of cannabinoids. A role for the ICjM in reinforcement appears plausible based on the anatomical proximity and neurochemical similarity of the ICjM and the NAS. The ICjM lies adjacent to the shell of the NAS, and like the shell of the NAS contains dopamine D_1 and D_3 receptors [62,67]. Thus, it is possible that functional interactions may occur between the ICjM and the shell of the NAS. Interestingly, many other recreationally used drugs promote Fos immunoreactivity in the ICjM such as MDMA and cocaine [77,89]. Future studies might usefully explore whether the ICjM has a functional role in the rewarding effects of drugs such as cannabis.

The present study shows for the first time that a cannabinoid increases Fos immunoreactivity in the PPTg. This area has been implicated in a wide range of functions including reinforcement, reward, motor behavior, control of sleep–wake cycles, antinociception and cognitive processing [38,86]. Interestingly, cannabinoids affect all of these functions [2,24,28,29,53]. It is unlikely that the effects of CP 55,940 on Fos immunoreactivity in the PPTg are mediated via direct activation of CB_1 receptors as there appear to be none in this area [35,65,82].

The PPTg has extensive connectivity with the basal ganglia [38]. Specifically, afferent and efferent connections between the PPTg and areas rich in CB_1 receptors, such as the GP and the SN, have been described [38]. Recently, converging evidence has strongly implicated the PPTg as part of the neural circuit underlying reward [86]. Lesions of the PPTg reduced heroin self-administration [58], responding for rewarding brain stimulation [43] and the development of conditioned place preferences to opiates, stimulants or food [56,57]. The role of the PPTg in cannabinoid-induced reward is clearly an interesting topic for further study.

Finally, it should be noted that the mechanism underlying the strain differences in cannabinoid-induced behavior and Fos expression reported in the present study are not entirely clear. One possibility is that Lewis rats have fewer central CB_1 receptors than Wistar rats and may thus be subsensitive to cannabinoid effects. Alternatively, strain differences may exist in the affinity of CP 55,940 for the CB_1 receptor. Another hypothesis is that Lewis rats may have less G proteins in a pool utilised by CB_1 receptors. Evidence shows that Lewis rats have less G proteins in the NAS in comparison to Fischer 344 rats [12]. Another possibility is that pharmacokinetic properties of CP 55,940 differ between these two strains, with Lewis rats perhaps able to metabolise CP 55,940 more efficiently than Wistar rats. Future studies will hopefully be able to give a definitive answer to these hypotheses.

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