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Perinatal Exposure to Δ^9-Tetrahydrocannabinol Alters Heroin-Induced Place Conditioning and Fos-Immunoreactivity

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In the present study, the effects of perinatal exposure to Δ^9-tetrahydrocannabinol (THC) on heroin-induced place conditioning and Fos-immunoreactivity (Fos-IR) were examined. Male albino Wistar rats ($N = 104$) were pretreated with vehicle ($n = 52$) or 5 mg/kg THC ($n = 52$) from postnatal days 4 through 14. At approximately 8 weeks of age, 72 rats were divided into six equal groups ($n = 12$ per group) and injected subcutaneously (s.c.) with vehicle, 0.5, or 2.0 mg/kg heroin and tested in an unbiased two-compartment place conditioning task. In vehicle-pretreated rats, 2.0 mg/kg but not 0.5 mg/kg heroin produced a significant place preference. Perinatal THC exposure significantly enhanced the rewarding properties of both doses of heroin. In the second experiment, 32 rats were divided into four equal groups ($n = 8$ per group) and injected with vehicle or 0.5 mg/kg heroin s.c. and perfused 2-h later. Fos-IR was examined in several brain regions directly or indirectly involved in reward. Acute administration of heroin in vehicle pretreated rats increased Fos-IR in the central, medial, and dorsomedial caudate putamen (CPu), nucleus accumbens (NAC, core and shell regions), lateral septum, islands of Calleja-major (ICjM), bed nucleus of the stria terminalis (BNST), central nucleus of the amygdala (CEA), dorsolateral and dorsomedial periaqueductal gray (PAG), ventral tegmental area (VTA), Edinger–Westphal nucleus (EW). Perinatal THC exposure significantly increased heroin-induced Fos-IR in the dorsomedial CPu. Conversely, perinatal THC exposure reduced heroin-induced Fos-IR in the NAC (shell), BNST, CEA, dorsolateral and lateral PAG, VTA, and EW. The present study demonstrates an increase in the rewarding properties of heroin following exposure to THC at an early age and provides new evidence regarding possible neural correlates underlying this behavioral alteration.

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INTRODUCTION

Cannabis is among the most widely abused illicit drugs in the world (Hall and Solowij, 1998). A recent survey suggests that at least 2.8% of pregnant women take illicit drugs during pregnancy, with cannabis being the most common among these (Ebrahim and Gfroerer, 2003). Prenatal marijuana exposure negatively impacts the foetus as evidenced by reduced birth weight and length (Fried *et al*, 1999; Zuckerman *et al*, 1989) and increased tremor and prolonged startle in response to spontaneous and mild stimulation (Fried, 1982; Fried and Makin, 1987). In addition to negative effects on fetal development, the use of cocaine, heroin, tobacco, and alcohol is generally higher in cannabis users compared to nonusers (Hall *et al*, 1991; Miller *et al*, 1990), suggesting that cannabis may enhance the vulnerability to other drugs of abuse. However, these studies are correlational in nature, and thus cannot establish a causal relationship between cannabis use and subsequent transition to other drugs. Thus, there is a need for laboratory studies using animal models of addiction to provide noncorrelational evidence for the possible gateway effect of cannabis.

Animal studies have shown that perinatal cannabinoid exposure affects the ontogeny of the central nervous system and alters the functioning of various neurotransmitter systems (for a review, see Fernandez-Ruiz *et al*, 2000, 1999) leading to several behavioral changes (Navarro *et al*, 1996, 1994). Among the systems altered by perinatal cannabinoid exposure is the opioid system (Thornton *et al*, 1998). Exposure to the cannabis extract Δ^9-tetrahydrocannabinol (THC) at an early age leads to long-lasting alterations in the brain resulting in characteristic behavioral changes during adulthood (Fernandez-Ruiz *et al*, 2000, 1999). These changes include an enhancement of the rewarding properties of drugs of abuse such as opiates. For example, THC pretreatment cross-sensitizes to heroin (Lamarque *et al*, 2001), increases morphine self-administration (Vela *et al*, 1998), and leads to higher levels of μ-opioid receptor binding (Vela *et al*, 1998). Furthermore, THC pretreatment leads to changes in opioid peptide mRNA levels in drug

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addiction-related regions of the brain (Corchero *et al*, 1998).

It should be noted that most studies discussed so far have administered cannabinoids to pregnant rats and examined their pups at foetal, early postnatal, and/or adult ages (Vela *et al*, 1998). However, very few animal studies have administered THC during the early neonatal stages of development. In terms of synaptic development, the neonatal period is considered important, as this period (ie postnatal day (PND) 5–21) in rats corresponds to the third trimester of gestation in humans; that is, these are both critical periods of neuronal development in which the majority of synapses are formed (Meyer and Kunkle, 1999). Hence, this is the period when THC administration is likely to produce long-lasting effects on the central nervous system. Thus, the first aim of the present study was to determine whether there are differences in the strength of heroin-induced conditioned place preferences (CPP) in adult rats with or without prior neonatal exposure to THC. The CPP paradigm is a task typically used to evaluate the rewarding properties and determine the incentive motivational value of addictive substances (Mucha *et al*, 1982; Tzschentke, 1998).

Secondly, this study examined whether long-term exposure to cannabinoids leads to changes in heroin-induced Fos-immunoreactivity (Fos-IR). Fos-IR as a dependent measure represents a useful marker for neuronal activation following drug administration (Arnold *et al*, 1998). Long-term exposure to addictive drugs leads to altered responses to other drugs of abuse such as cocaine and MDMA (ecstasy) (Erdtmann-Vourliotis *et al*, 2000). Given that an interaction exists between the cannabinoid and opioid systems, it is possible that pre-exposure to a cannabinoid receptor agonist may lead to permanent changes in opioid-induced gene expression. This hypothesis is supported by our previous work where we have shown that long-term exposure to the cannabinoid receptor agonist CP 55, 940 during adolescent and adult ages alters subsequent morphine-induced Fos-IR (Singh *et al*, 2002).

The first experiment reported here sought to examine the effects of chronic THC exposure on heroin-induced CPP (Hand *et al*, 1989). It was hypothesized that heroin-induced CPP would be significantly enhanced in rats pre-exposed to THC compared to non-THC pre-exposed rats. The second experiment sought to examine the effects of chronic THC exposure on heroin-induced Fos-IR in brain regions directly or indirectly involved in reward. Examination of any alterations in heroin-induced Fos-IR in THC pretreated rats compared to non-THC pretreated rats was expected to provide insights into the mechanisms underlying the functional interactions between these neurotransmitter systems as a result of long-term cannabinoid exposure.

METHODS

Subjects

Wistar rats bred in our animal facility and weighing 6–8 g on PND 2 were randomly redistributed into litters of 10 pups and placed with foster mothers who had recently birthed. Each foster mother had eight male and two female pups. The remaining pups were culled. The foster mother and pups were housed in opaque plastic cages on a 12 : 12 h reverse light–dark cycle (lights off at 0800). Rats had free access to standard lab chow (Barastoc, Ridley AgriProducts, Australia) and tap water at all times during the experiments. Experimental testing was conducted during the dark cycle between 0830 and 1600. Animals were treated in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1985) and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. This study was approved by the University of New England Animal Ethics Committee.

Drugs

THC (AGAL, Pymble, NSW, Australia), available as a 2.0 mg THC/ml ethanol solution was first mixed with a few drops of Tween-80 (polyoxyethylene sorbitan monooleate, ICN Biomedicals). The suspension was stirred continuously under a stream of nitrogen gas until all ethanol was evaporated. Physiological saline was then added and the solution was stirred until the Tween-80/THC suspension was well dispersed. The final vehicle solution contained 15 μl Tween-80 per 2 ml saline. THC was administered intraperitoneally (i.p.) at a dose of 5.0 mg/kg in a volume of 5 ml/kg.

Heroin (API/AMED, Australia) was dissolved in a vehicle containing 0.05 μl acetic acid (Sigma-Aldrich, Australia) in 2 ml of physiological saline (0.9%). Heroin was administered subcutaneously (s.c.) at a dose of either 0.5 or 2.0 mg/kg in a volume of 1 ml/kg. Vehicle conditions comprised heroin vehicle (acetic acid in saline) and THC vehicle (Tween-80 in saline).

Procedure

Upon weaning litters were divided into two groups: vehicle control and THC. Neonatal rat pups were injected with either vehicle ($n = 52$) or THC ($n = 52$) from PND 4 through PND 14. Rats were weaned at 21–23 days of age, after which males were retained and the females culled. Of the 104 rats, 72 were used for Experiment 1 (place conditioning) and 32 were used for Experiment 2 (immunohistochemistry). Both experiments were conducted when rats were approximately 8 weeks old.

Experiment 1: Effects of THC Pre-Exposure on Heroin Place Conditioning

Apparatus. The experiment was conducted in eight identical dimly lit (13 lux) rectangular Perspex place-conditioning boxes (28 cm long × 23 cm wide × 30 cm high), divided into two compartments connected by an aluminum tunnel (10 cm long × 14 cm wide × 10 cm high). The compartment walls were constructed of clear Perspex and aluminum. Two types of galvanized wire mesh floors were used: painted white diamond-shaped (1.5 cm^2) and unpainted square-shaped (1 cm^2). Metal dropping pans were placed below the galvanized wire mesh floors. Aluminum guillotine doors were slid into place at both ends of the tunnels to restrict access when needed.

Both compartments in each of the place conditioning boxes were visually and tactually different. In half the boxes, the left compartment had walls with vertical black and red stripes, white diamond-shaped floors, and dropping pans containing wood shavings. The right compartments had horizontal white and black striped walls, unpainted square-shaped floors, and dropping pans without wood shaving. This arrangement was reversed for the other half of the boxes such that the left compartment now had walls with horizontal white and black stripes, unpainted square-shaped floors, and dropping pans without wood shavings. The right compartments had walls with vertical black and red stripes, white diamond-shaped floors, and dropping pans containing wood shavings.

A computer-controlled passive infrared detector (Quantum passive infrared motion sensors, NESS Security Products, Australia, Part No. 890-087-2) was mounted to the ceiling of each box to quantify locomotor activity using custom designed software. The locomotor activity recorded by the passive infrared detector included ambulation, rearing, grooming, and small horizontal movements of the head and neck. A 10 μF capacitor located on the sensor's printed circuit board was replaced with a 0.1 μF capacitor serving to alter the sensor alarm period from 5 s to approximately 50 ms. Activity chambers were located inside sound attenuating boxes (80 cm long × 36 cm wide × 65 cm high) and fitted with a fan, to provide ventilation and masking noise.

Procedure. Rats ($n = 72$) were handled for 1 min per day for 6 consecutive days before starting experimental testing. Next, rats were divided into six equal groups ($n = 12$ per group) as follows: perinatal vehicle/heroin vehicle (VEH → VEH), perinatal vehicle/0.5 mg/kg heroin (VEH → 0.5HER), perinatal vehicle/2.0 mg/kg heroin (VEH → 2.0HER), perinatal THC/heroin vehicle (THC → VEH), perinatal THC/0.5 mg/kg heroin (THC → 0.5HER), and perinatal THC/2.0 mg/kg heroin (THC → 2.0HER). It is important to note that all rats were already pre-exposed to vehicle or THC; hence, only vehicle or heroin injections were given at this stage of the experiment. Each animal was tested once per day for 8 consecutive days at approximately the same time of day. The experiment was conducted in three phases: preconditioning (day 1), conditioning (days 2–7), and test (day 8).

The preconditioning phase consisted of one 20-min undrugged session during which each rat was placed in one of the two compartments and given free access to the entire box with the guillotine doors removed. This compartment (start box) was counterbalanced across rats throughout the experiment. The conditioning phase consisted of six 30-min sessions, one per day. Doors restricted the animals to one compartment for the duration of each conditioning session. Drug injections were given on even-numbered days, and vehicle injections were administered on odd-numbered days. During the conditioning phase, rats were injected with vehicle, 0.5, or 2.0 mg/kg heroin, then placed in the test apparatus 5 min later. After the conditioning phase, each animal received a 20-min test session, identical to the preconditioning session. The amount of time spent in each compartment was recorded during the preconditioning and test sessions. The total time spent in motion was recorded during the conditioning phase.

Statistical analysis. Place conditioning was indexed by calculating the difference in time (s) spent on the drug-paired side from the preconditioning to the test sessions. One-way analysis of variance (ANOVA) was used to compare groups. Where a significant ANOVA main effect was found, groups were compared using planned unpaired *t*-tests with Bonferroni adjustments for multiple comparisons. The seven contrasts of interest ($\alpha = 0.05$) were: (1) VEH → VEH *vs* VEH → 0.5HER, (2) VEH → VEH *vs* VEH → 2.0HER, (3) VEH → VEH *vs* THC → VEH, (4) THC → VEH *vs* THC → 0.5HER, (5) THC → VEH *vs* THC → 2.0HER, (6) VEH → 0.5HER *vs* THC → 0.5HER, and (7) VEH → 2.0HER *vs* THC → 2.0HER.

Locomotor activity data were analyzed using a day by drug treatment ANOVA with repeated measures on the second factor. Where significant ANOVA main effects were found, groups were compared using planned unpaired *t*-tests with Bonferroni adjustments for multiple comparisons. The seven contrasts of interest were similar to those listed above. Where a significant interaction was found, a separate one-way ANOVA was conducted for group at each level of day.

Experiment 2: Effects of THC Pre-Exposure on Heroin-Induced Fos-IR

Procedure. Rats ($n = 32$) were randomly assigned to one of four equal treatment groups ($n = 8$) according to a standard 2 × 2 design. The groups were: perinatal vehicle/heroin vehicle (VEH → VEH), perinatal THC/heroin vehicle (THC → VEH), perinatal vehicle/heroin (VEH → HER), and perinatal THC/heroin (THC → HER). It is important to note that all rats were already pre-exposed to vehicle or THC; hence, only vehicle for heroin or heroin (0.5 mg/kg) injections were given at this stage of the experiment.

The experiment was conducted in eight dimly lit rectangular Perspex locomotor activity chambers (28 cm long × 23 cm wide × 30 cm high) placed inside sound attenuating boxes. The locomotor activity chambers were constructed with aluminum plate walls and galvanized wire mesh floors (1 cm^2). Locomotor activity (time spent in motion) was recorded using the same type of motion sensors used in Experiment 1. All rats were handled for 1 min per day for 6 consecutive days before starting experimental testing. Next, all rats were habituated to the testing apparatus, injection, and handling procedures for 4 consecutive days in order to minimize novelty-induced Fos-IR. On each of the habituation days, rats were injected s.c. with the vehicle for heroin and 5 min later were placed into the locomotor activity boxes for 120 min. Treatments were staggered at 10-min intervals to allow sufficient time between consecutive perfusions on the test day. Each home cage contained at least one rat from each treatment group. The procedure for the test day was similar to the habituation days with the exception of drug treatments and perfusions. On the test day, rats were injected with vehicle or 0.5 mg/kg heroin (s.c.) and placed into the

locomotor activity boxes for 120 min and were then perfused.

Immunohistochemistry. Rats were deeply anaesthetized with 120 mg/kg sodium pentobarbital (i.p.) and perfused transcardially with 100 ml phosphate-buffered saline (PBS, 0.1 M, pH 7.2) followed by 150 ml 4% paraformaldehyde in PBS. Brains were then extracted and placed in 4% paraformaldehyde at 4°C for 24 h, then transferred to 15% sucrose in a phosphate buffer solution (PB, 0.1 M, pH 7.2) at 4°C for 24 h, and finally placed in 30% sucrose in PB at 4°C for 48 h. Whole brains were then sectioned coronally at 40 μm using a cryostat and sections were collected in PB.

Free-floating sections were given two consecutive 30-min washes in PB, a 30-min wash in 0.9% hydrogen peroxide in 50% ethanol, and a 30-min wash in 3% normal horse serum in PB. Sections were then incubated for 72 h at 4°C in the primary *c-Fos* antibody (s52, Santa Cruz Biotechnology, Santa Cruz, CA; rabbit polyclonal, specific for the amino-acid terminus of *c-Fos* p62, non-crossreactive with FosB, Fra-1, or Fra-2) diluted 1 : 2000 in phosphate-buffered horse serum (PBH, 0.1% bovine serum albumin, 0.2% Triton X-100, 2% normal horse serum in PB). Next, sections were washed for 30 min in PB and incubated for 60 min in biotynilated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) diluted 1 : 500 in PBH. They were then washed in PB for 30 min, and subsequently incubated for 60 min in extrAvidin-horseradish peroxidase (Sigma-Aldrich, Castle Hill, NSW) diluted 1 : 1000 in PBH. After three further 30 min washes in PB, horseradish peroxidase activity was visualized with the nickel diaminobenzidine and glucose oxidase reaction with nickel enhancement as described by Shu *et al* (1988). The reaction was terminated 10 min later by washing in PB. Sections were mounted onto gelatine-coated slides, dehydrated, Histolene-cleared, and cover-slipped.

A total of 15 brain regions or subregions were examined using an Olympus CH-2 light microscope set at $\times 200$ magnification. The atlas of Paxinos and Watson (1998) was used to identify brain regions shown in Figure 1. A 10×10 square graticule was positioned over each structure and the

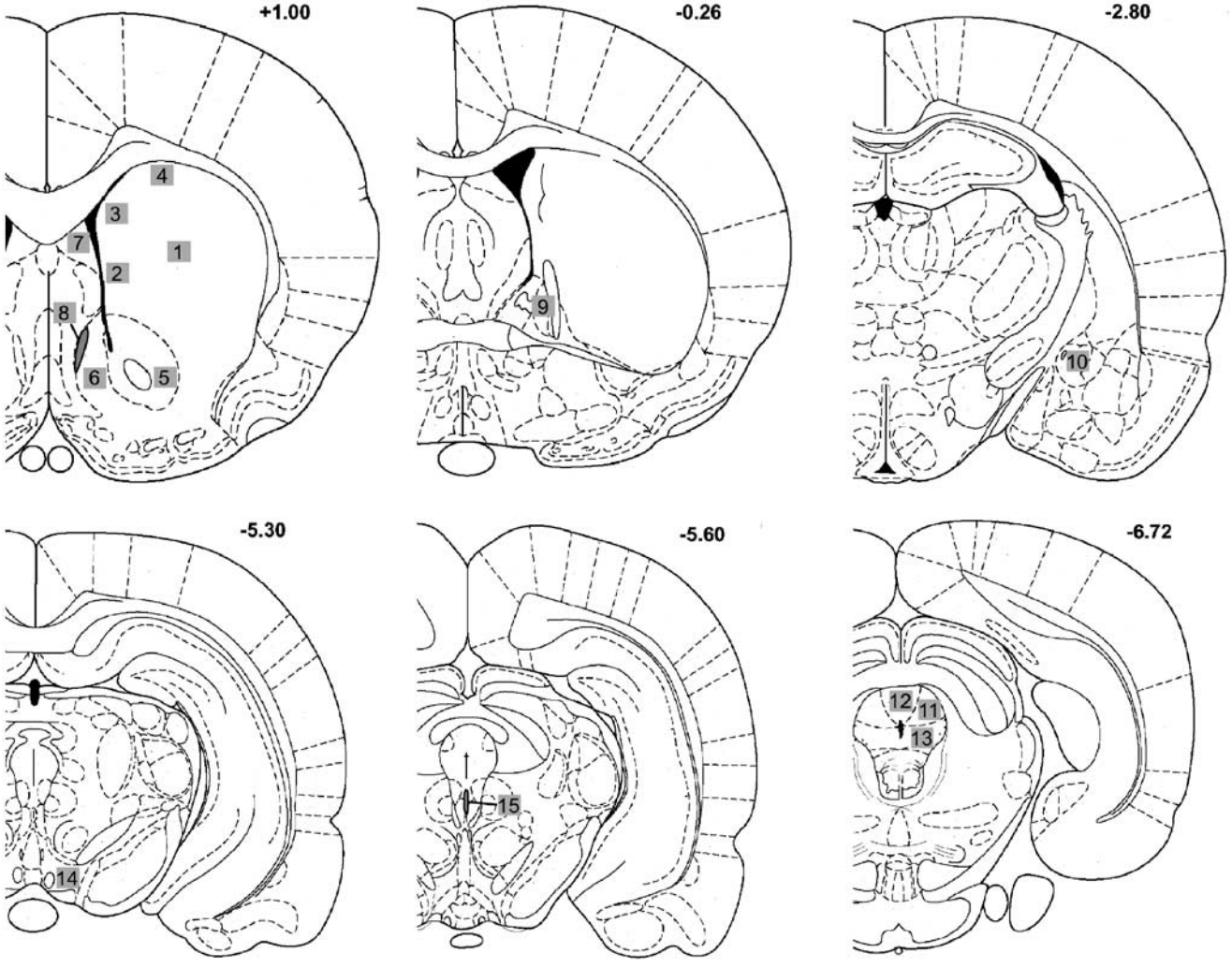

Figure 1 Schematic diagrams of coronal sections of the rat brain (Paxinos and Watson, 1998). The number of Fos-positive nuclei were counted within the areas numbered and shaded in gray. The areas shown correspond in scale to the exact areas counted. The numbers indicated correspond to the brain regions listed in Table 1.

number of labelled cells within the graticule, which covered a $500 \times 500\,\mu m$ area, was counted manually. Where the structure was larger than the graticule, a standardized region within the structure was counted. Only round and oval cells that were completely black were counted. As manual counting can be subject to experimenter bias, microscope slides were labelled such that the person counting the labelled cells did not know which group the rats belonged to.

Images of Fos-IR cells were captured using a Micropublisher FireWire camera (QImaging, Burnaby BC, Canada) attached to an Olympus CH-2 microscope. Images were acquired using a PowerMac computer running Adobe Photoshop Elements version 2.0. Postproduction image processing and layout was conducted using Deneba Canvas 9.0, and included reduction of color to grayscale, and standardization of brightness and contrast.

Statistical analysis. Locomotor activity data were grouped into four 30-min bins and were analyzed using a split plot dose by time ANOVA with repeated measures on the second factor. Group differences in locomotor data were also compared by conducting a one-way ANOVA at each 30 min. Where significant main effects were found, Tukey *post-hoc* tests were used to compare groups.

Group comparisons between the numbers of labelled cells were made using one-way ANOVAs. Planned contrasts with Bonferroni corrected differences were used to compare groups where significant ANOVA main effects were observed. The five contrasts of interest ($\alpha = 0.05$) were: (1) VEH→VEH *vs* THC→VEH, (2) VEH→VEH *vs* VEH→HER, (3) VEH→VEH *vs* THC→HER, (4) THC→HER *vs* THC→VEH, and (5) THC→HER *vs* VEH→HER. A two-way ANOVA was also conducted for each brain region to determine whether the combined effect of the drugs was significantly higher or lower than would be expected by the sum of the individual drug effects. The first factor was the presence or absence of THC and the second was the presence or absence of heroin. As the individual main effects provided no relevant information, only the interaction term was reported.

All ANOVAs and planned contrasts were conducted using SPSS 11.0.2 for Mac OS X. Where the homogeneity of variance assumption was not met, randomization tests of scores were conducted using NPFact version 1.0.

RESULTS

Experiment 1: Effects of THC Pre-Exposure on Heroin Place Conditioning

The CPP data are shown in Table 1 and Figure 2. As expected, perinatal vehicle pretreated animals that received an acute dose of heroin showed an increase in the preference for the drug-paired side. Furthermore, perinatal THC pretreated rats that received an acute dose of heroin also showed a preference for the drug-paired compared to the vehicle-paired side. Interestingly, perinatal THC pretreatment significantly enhanced heroin-induced place preference compared to the heroin-induced place preference observed in perinatal vehicle pretreated rats. The one-way ANOVA comparing groups was significant ($F(5,71) = 10.24$, $P < 0.001$). Pairwise comparison showed that the VEH→VEH group was significantly different from VEH→2.0HER but not from VEH→0.5HER or THC→VEH. THC→VEH was significantly different from THC→0.5HER and THC→2.0HER. Further, VEH→0.5HER and VEH→2.0HER were significantly different from THC→0.5HER and THC→2.0HER, respectively (Figure 2).

Exposure to heroin during the conditioning phase produced a biphasic effect on locomotor activity with 0.5 mg/kg heroin stimulating while 2.0 mg/kg heroin inhibiting locomotion (Figure 3). The ANOVA revealed a significant main effect of drug treatment ($F(5,66) = 21.26$, $P < 0.001$) and a significant main effect of conditioning day ($F(5,330) = 23.72$, $P < 0.001$). Further, the drug treatment by day interaction was also significant ($F(25,330) = 23.53$, $P < 0.001$). Pairwise comparisons revealed that the VEH→VEH group differed significantly from the VEH→0.5HER and VEH→2.0HER groups. The THC→VEH group was significantly different from THC→0.5HER and THC→2.0HER. Furthermore, THC→0.5HER was significantly different from VEH→0.5HER. One-way ANOVA comparing all groups during each conditioning day revealed a nonsignificant effect at days 1, 3, and 5 (vehicle conditioning days). One-way ANOVAs were significant for day 2 ($F(5,71) = 28.77$, $P < 0.001$), day 4 ($F(5,71) = 35.90$, $P < 0.001$), and day 6 ($F(5,71) = 36.84$, $P < 0.001$) (drug conditioning days). Pairwise comparisons revealed that VEH→VEH was significantly different from VEH→2.0HER on days 2, 4, and 6. VEH→VEH was significantly different from VEH→0.5HER on day 6 only. Further, THC→VEH was significantly different from THC→0.5HER and THC→2.0HER on days 2, 4, and 6.

Experiment 2: Effects of THC Pre-Exposure on Heroin-Induced Fos-IR

The split plot ANOVA examining the effects of heroin on locomotor activity in rats with or without perinatal pre-exposure to THC yielded a significant main effect of dose (Figure 4) ($F(3,24) = 7.12$, $P < 0.001$), main effect of time ($F(3,72) = 3.24$, $P < 0.05$), and dose by time interaction ($F(9,72) = 4.50$, $P < 0.001$). One-way ANOVAs with Tukey *post hoc* tests revealed that heroin stimulated locomotor activity relative to control at the 60- and 90-min but not at the 30- and 120-min measurement intervals. Perinatal THC pre-exposure did not affect locomotor activity, and did not affect heroin-induced locomotion.

The number of labelled cells for the 15 brain regions examined is presented in Table 2. In addition, representative photomicrographs of labelled cells from the nucleus accumbens (NAS, core) and the central nucleus of the amygdala (CEA) are presented in Figures 5 and 6, respectively. Fos-IR in vehicle/vehicle-treated rats was low in all regions in accordance with the typical low levels of such expression found in well-habituated control animals. The ANOVA comparing groups was significant in 11 regions examined as follows: Caudate putamen (CPu, central) ($F(3,28) = 5.64$, $P < 0.01$), CPu (medial) ($F(3,28) = 3.80$, $P < 0.05$), CPu (dorsomedial) ($F(3,28) = 41.84$, $P < 0.001$), NAS (core) ($F(3,28) = 22.95$, $P < 0.001$), NAS (shell) ($F(3,28) = 5.89$, $P < 0.01$), islands of Calleja (ICjM) ($F(3,28) = 7.00$, $P < 0.001$), bed nucleus of stria terminalis (BNST)

Table 1 Time (sec ± SEM) Spent in the Vehicle- and Drug-Paired Sides during Preconditioning and Test Sessions

	Preconditioning session		Test session	
Treatment	**Vehicle side**	**Drug side**	**Vehicle side**	**Drug side**
VEH → VEH	539.63 ± 38.96	572.24 ± 38.58	611.94 ± 61.37	501.93 ± 61.72
VEH → 0.5HER	545.70 ± 34.07	552.22 ± 31.38	520.86 ± 54.68	592.77 ± 53.81
VEH → 2.0HER	517.21 ± 27.85	591.82 ± 29.25	358.37 ± 25.53	735.32 ± 36.05
THC → VEH	522.18 ± 30.89	585.43 ± 32.93	556.55 ± 44.55	527.52 ± 48.08
THC → 0.5HER	516.35 ± 36.65	569.18 ± 24.50	372.83 ± 26.69	797.78 ± 14.75
THC → 2.0HER	589.09 ± 31.96	511.07 ± 27.83	313.42 ± 17.16	769.91 ± 15.67

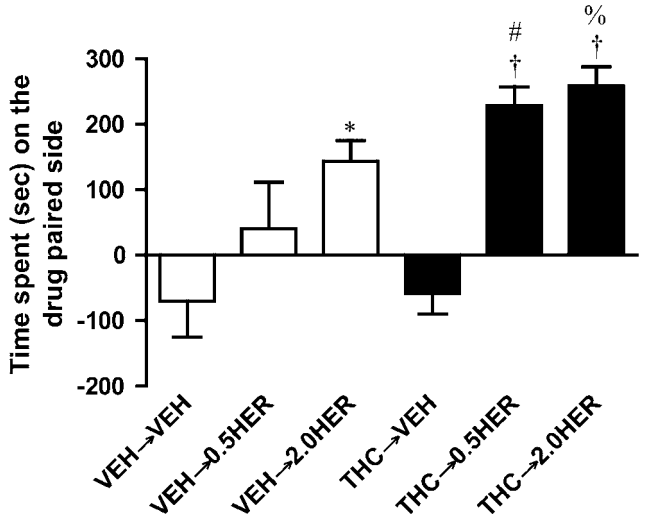

Figure 2 Effect of heroin on the establishment of CPP in rats with or without perinatal exposure to THC. The bars represent the difference between the time spent on the drug-paired side from the preconditioning to the test session (mean ± SEM). *VEH* = vehicle; *THC* = dose of THC in mg/kg; *Her* = dose of heroin in mg/kg. **P* < 0.05, significantly different from VEH → VEH; †*P* < 0.05, significantly different from THC → VEH; #*P* < 0.05, significantly different from VEH → 0.5HER; %*P* < 0.05, significantly different from VEH → 2.0HER. Note that all rats were perinatally exposed to vehicle or THC; only vehicle or different doses of heroin was administered at this stage of the experiment.

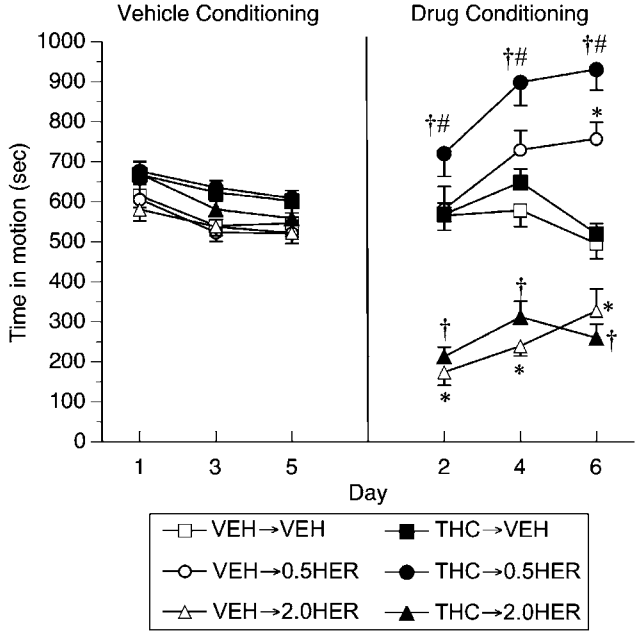

Figure 3 Effect of heroin on locomotor activity (mean ± SEM number of seconds spent in motion) during the conditioning phase in Experiment 1. Abbreviations as in Figure 1. **P* < 0.05, significantly different from VEH → VEH, †*P* < 0.05, significantly different from THC → VEH, #*P* < 0.05, significantly different from VEH → 0.5HER. Note that all rats were perinatally exposed to vehicle or THC; only vehicle or heroin was administered at this stage of the experiment.

($F(3,28) = 3.65$, $P < 0.05$), central nucleus of amygdala (CEA) ($F(3,28) = 3.73$, $P < 0.05$), periaquaductal gray (PAG, dorsolateral) ($F(3,28) = 5.14$, $P < 0.01$), ventral tegmental area (VTA) ($F(3,28) = 3.23$, $P < 0.05$), and Edinger–Westphal nucleus (EW) ($F(3,28) = 3.48$, $P < 0.05$).

A significant effect of perinatal THC pretreatment on Fos-IR was observed in nine of the 15 regions counted (Table 2). The planned contrasts ($\alpha = 0.05$) comparing THC → VEH to VEH → VEH revealed that perinatal THC pre-exposure increased Fos-IR in the NAS (core and shell regions), ICjM, BNST, CEA, dorsolateral and lateral PAG, VTA, and EW.

A significant effect of acute heroin administration on Fos-IR was observed in 13 of the 15 regions examined (Table 2). The planned contrasts ($\alpha = 0.05$) comparing VEH → HER to VEH → VEH revealed that heroin increased Fos-IR in the central, medial, and dorsomedial CPu, NAS (core and shell regions), lateral septum (LS), ICjM, BNST, CEA, dorsolateral and dorsomedial PAG, VTA, and EW.

Planned contrasts ($\alpha = 0.05$) comparing THC → HER to THC → VEH and VEH → HER revealed that THC → HER-induced Fos-IR was significantly higher than THC → VEH in the central and dorsomedial CPu, NAS (core), and ICjM. THC → HER-induced Fos-IR was significantly lower than THC → VEH in the EW. THC → HER was significantly higher than VEH → HER in the dorsomedial CPu, and NAC (core). THC → HER was significantly lower than VEH → HER in the NAC (shell), dorsolateral PAG, and VTA. THC and heroin produced an interactive effect on Fos-IR in eight of the 15 structures counted, including the dorsomedial CPu ($F(3,28) = 11.23$, $P < 0.001$), NAC (shell)

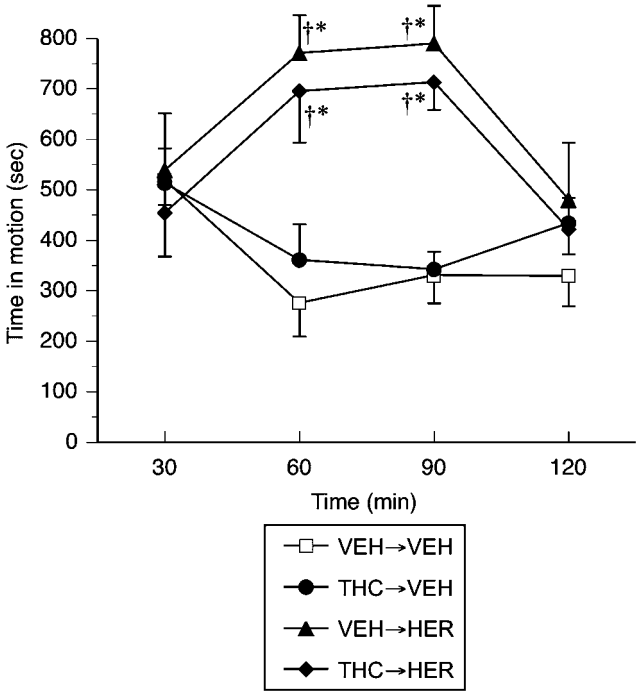

Figure 4 Locomotor activity (mean ± SEM number of seconds spent in motion) for rats receiving VEH → VEH, THC → VEH, VEH → HER, or THC → HER. *$P < 0.05$, significantly different from VEH → VEH, †$P < 0.05$, significantly different from THC → VEH. *VEH* = vehicle, *HER* = 0.5 mg/kg heroin, *THC* = 5 mg/kg THC. Note that all rats were perinatally exposed to vehicle or THC; only vehicle or heroin was given at this stage of the experiment.

(F(3,28) = 11.09, $P < 0.01$), BNST (F(3,28) = 5.31, $P < 0.01$), CEA (F(3,28) = 5.31, $P < 0.01$), dorsolateral PAG (F(3,28) = 8.15, $P < 0.01$), lateral PAG (F(3,28) = 6.11, $P < 0.05$), VTA (F(3,28) = 9.38, $P < 0.01$), and EW (F(3,28) = 9.10, $P < 0.01$). Higher levels of Fos-IR than expected by the sum of the individual drug effects were observed in the dorsomedial CPu. Lower levels of Fos-IR than were expected by the sum of the individual drug effects were observed in the NAS (shell), BNST, CEA, dorsolateral and lateral PAG, VTA, and EW.

DISCUSSION

The results of the present study can be summarized as follows: (1) perinatal THC exposure increased the rewarding effect of heroin in the place conditioning paradigm; (2) consistent with previous studies, heroin significantly elevated Fos-IR in a number of brain regions; (3) perinatal THC exposure increased basal Fos-IR during adulthood in several brain regions known to directly or indirectly mediate reward; (4) perinatal treatment with THC significantly altered heroin-induced Fos-IR during adulthood in several brain regions.

Experiment 1 showed that perinatal exposure to THC enhances the rewarding effects of heroin in adulthood. To our knowledge, this is the first report that perinatal exposure to THC modulates heroin-induced place conditioning. The observation that heroin-treated rats showed a significant increase in the preference for the drug-paired

Table 2 Mean Number (± SEM) of Fos-Immunoreactive Cells

Region	Bregma	VEH → VEH	THC → VEH	VEH → HER	THC → HER
Frontal regions					
1. Caudate-putamen, central	+1.00	0.5 ± 0.3	1.9 ± 0.7	4.8 ± 1.4*	4.4 ± 0.6*,†
2. Caudate-putamen, medial	+1.00	5.0 ± 2.4	11.1 ± 3.3	15.5 ± 3.3*	17.9 ± 2.5*
3. Caudate-putamen, dorsomedial	+1.00	4.5 ± 0.8	8.7 ± 2.2	14.1 ± 1.2*	29.9 ± 2.2$^{*,\#,\dagger,b}$
4. Caudate-putamen, dorsal	+1.00	0.6 ± 0.5	1.9 ± 0.6	5.4 ± 2.5	1.6 ± 0.3
5. Nucleus accumbens, core	+1.00	1.1 ± 0.5	5.3 ± 1.1*	9.0 ± 1.9*	17.8 ± 1.9$^{*,\#,\dagger}$
6. Nucleus accumbens, shell	+1.00	2.6 ± 0.9	9.1 ± 1.9*	17.4 ± 3.9*	6.9 ± 2.4$^{\#,a}$
7. Lateral septum	+1.00	3.6 ± 1.3	7.0 ± 2.1	12.4 ± 3.8*	7.9 ± 1.6
8. Islands of Calleja, major	+1.00	1.8 ± 0.7	9.1 ± 1.8*	24.1 ± 4.8*	24.8 ± 6.9*,†
9. BNST lateral division, dorsal	−0.26	3.3 ± 1.0	8.4 ± 1.5*	22.1 ± 8.1*	7.5 ± 2.2^{a}
Amygdala					
10. Amygdala, central nucleus	−2.80	2.4 ± 0.9	9.5 ± 1.6*	18.3 ± 5.1*	9.9 ± 4.0^{a}
Periaqueductal gray					
11. Dorsolateral	−6.72	1.5 ± 0.5	6.9 ± 1.8*	18.4 ± 6.0*	5.5 ± 1.1$^{*,\#,a}$
12. Dorsomedial	−6.72	2.1 ± 1.1	3.9 ± 1.2	9.8 ± 3.3*	5.5 ± 2.0
13. Lateral	−6.72	2.4 ± 0.8	6.9 ± 1.8*	8.0 ± 2.8	3.9 ± 0.9^{a}
Other					
14. Ventral tegmental area	−6.04	0.8 ± 0.4	4.4 ± 1.7*	3.6 ± 0.9*	1.0 ± 0.4$^{\#,a}$
15. Edinger–Westphal nucleus	−5.60	1.5 ± 0.7	6.4 ± 1.5*	6.6 ± 1.7*	3.6 ± 1.1^{†,a}

*$P < 0.05$ *vs* VEH → VEH ; †$P < 0.05$ *vs* THC → VEH; #$P < 0.05$ *vs* VEH → HER using planned contrasts assuming unequal variances.
[a]Significantly lower than would be expected by the sum of individual drug effects.
[b]Significantly higher than would be expected by the sum of the individual drug effects.

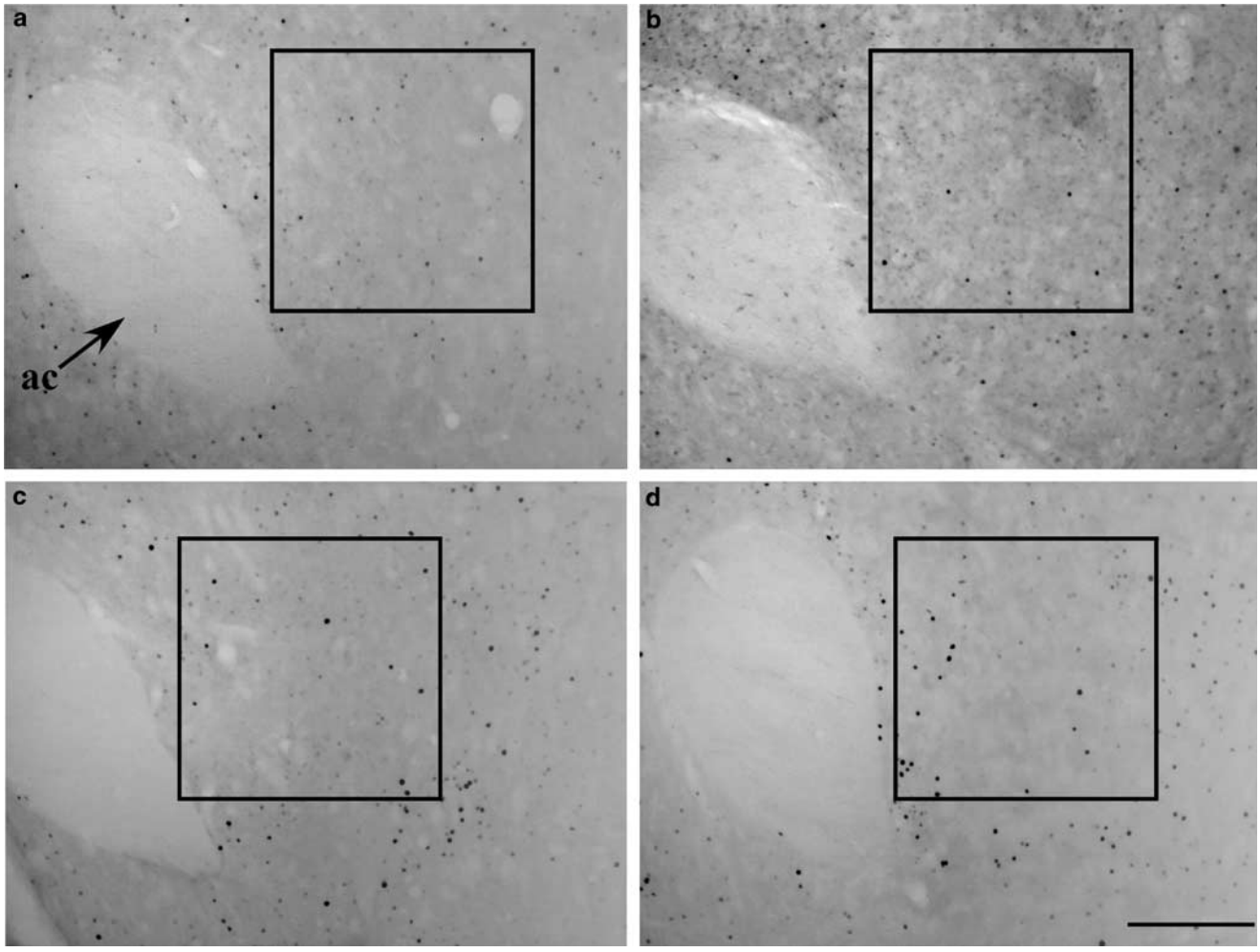

Figure 5 Photomicrograph showing Fos-labelled neurons in the nucleus accumbens (core) in representative sections from rats treated with VEH → VEH (a), THC → VEH (b), VEH → HER (c), or THC → HER (d). Scale bar = 250 μm; ac = anterior commissure. The square indicates the region in which labelled cells were quantified.

side compared to control rats is in good agreement with previous findings (Cole *et al*, 2003; Hand *et al*, 1989). Importantly, Experiment 1 demonstrates that perinatal exposure to THC may produce changes in the rewarding efficacy of heroin. Specifically, although the low dose of heroin (0.5 mg/kg) did not produce place conditioning when given to drug naïve rats, this dose induced a significant place preference in rats exposed perinatally to THC (Figure 2). In addition, the place preference produced by the high dose of heroin (2.0 mg/kg) was significantly potentiated by perinatal THC exposure (Figure 2).

The results of Experiment 1 complement previous studies showing that maternal THC exposure enhances morphine self-administration (Vela *et al*, 1998) and place conditioning (Rubio *et al*, 1998) during adulthood. More recently, our group has reported that CP 55, 940 exposure in adulthood enhances self-administration of morphine (Norwood *et al*, 2003). However, Solinas *et al* (2004) recently reported that THC exposure in adulthood increases the frequency of heroin taking without increasing its reinforcing efficacy. One explanation for this seeming contrast between the present study and the study by Solinas *et al* may be related to differences in the paradigms used to measure heroin reward. It is also possible that the age at which rats are exposed to cannabinoids plays a critical role in modulating the reinforcing efficacy of opiates.

It is noteworthy that in Experiment 1 heroin produced a biphasic effect on locomotion when given to rats with perinatal vehicle exposure, with the low dose (0.5 mg/kg) inducing hyperactivity, and the high dose (2.0 mg/kg) inducing hypoactivity (Figure 3). Importantly, locomotor hyperactivity produced by 0.5 mg/kg heroin was significantly potentiated by perinatal THC exposure, demonstrating locomotor cross-sensitization between the cannabinoid and opioid systems. These results suggest that perinatal THC exposure increases the incentive-motivational properties of heroin, which may have important implications in understanding cannabinoid involvement in opiate addiction.

It is not clear why the high dose of heroin (2.0 mg/kg) did not produce locomotor cross-sensitization to THC (Figure 3). However, opiate drugs produce biphasic effects on locomotor activity with small doses inducing hyperactivity and high doses inducing sedation (Browne and

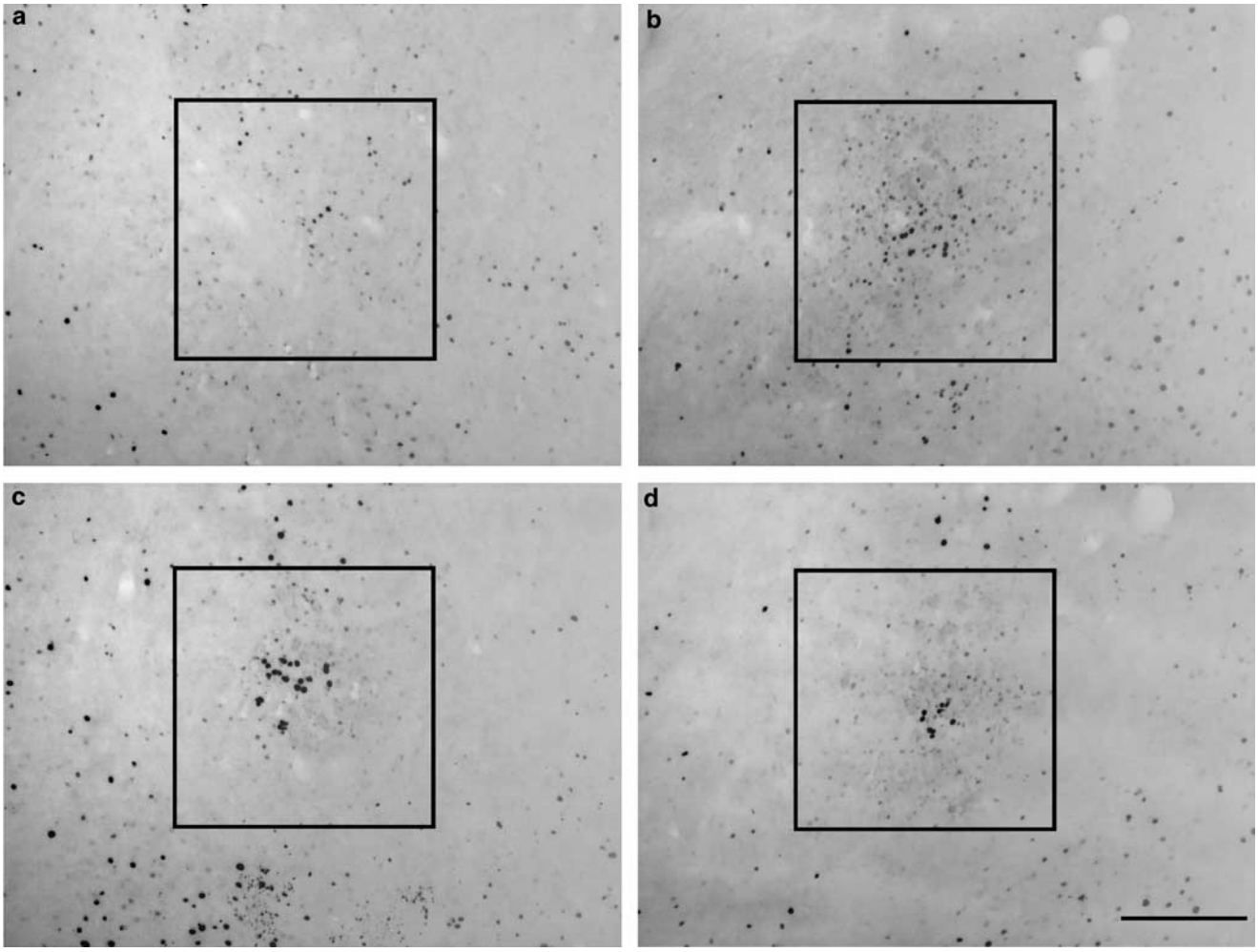

Figure 6 Photomicrograph showing Fos-labelled neurons in the central nucleus of the amygdala (CEA) in representative sections from rats treated with VEH→VEH (a), THC→VEH (b), VEH→HER (c), or THC→HER (d). Scale bar = 250 μm. The square indicates the region in which labelled cells were quantified.

Segal, 1980; Calenco-Choukroun *et al*, 1991; Meyer *et al*, 1995). It is therefore possible that sedation caused by the 2.0 mg/kg dose of heroin masked any cross-sensitizing effects of THC. This hypothesis is supported by our previous work showing that a high dose of an opiate can induce locomotor stimulation (Norwood *et al*, 2003), but this effect only becomes apparent following a long delay after drug administration, presumably because most of the drug has by then been metabolized.

Experiment 2 sought to examine whether perinatal exposure to THC affects heroin-induced Fos-IR in adulthood. Results revealed that Fos-IR differed according to the rat's drug history. There are several findings worth noting in this regard. First, heroin administration to drug naïve rats induced significant Fos-IR in the central, medial, and dorsomedial CPu, NAS (core and shell regions), LS, ICjM, BNST, CEA, dorsolateral and dorsomedial PAG, VTA, and EW. The increase in Fos-IR in the CPu and NAS (shell) are in good agreement with a recent study (D'Este *et al*, 2002). To the best of our knowledge, the increase in acute heroin-induced Fos-IR in the NAS (core), ICjM, BNST, CEA, PAG, VTA, and EW are novel findings. It should however be noted that previous studies using the μ-opioid receptor agonist morphine have found similar results to those obtained in the present study using heroin (Grabus *et al*, 2004; Warty *et al*, 2002).

Second, acute heroin administration to adult rats following perinatal THC exposure increased Fos-IR in the dorsomedial region of the CPu. Anatomical data suggest that the dorsomedial CPu receives strong inputs from the NAC (core) (Brown *et al*, 1998) and these regions are involved in mediating the stereotypic activity related to drug sensitization (Cadoni *et al*, 2001; D'Este *et al*, 2002). These regions also contain DA terminals and denervation of DA terminal fields reduces locomotion (Fink and Smith, 1980). The change in heroin-induced Fos-IR by perinatal THC exposure may therefore represent an important neural correlate for the interactive effects of perinatal THC exposure on heroin-induced hyperactivity observed during adulthood in Experiment 1 (Figure 3).

Third, Experiment 2 showed that heroin-induced Fos-IR was significantly reduced in rats perinatally exposed to THC in several regions including the NAS (shell), BNST, CEA, PAG, VTA, and the EW. Heroin increased Fos-IR in the

NAS (shell) and VTA that was significantly reduced in rats perinatally treated with THC. This finding can be linked to previous studies showing reduced heroin-induced Fos-IR in the NAS (shell) in heroin sensitized rats (D'Este *et al*, 2002), as well as rats sensitized to psychostimulants (Meng *et al*, 1998; Turgeon *et al*, 1997). These studies have shown that as behavioral activation increases, heroin-induced Fos-IR decreases. Thus, the findings of the current study fit in with the hypothesis that as heroin-induced place preference increases in THC pre-exposed rats, the induction of Fos-IR in reward-related neural correlates such as the NAS (shell) and the VTA decreases.

As noted above, heroin Fos-IR was reduced in THC-exposed rats in the lateral BNST and the CEA. The lateral BNST, as part of the extended amygdala, projects to the CEA (Davis, 1998) and shares common morphology, connectivity, and function (such as stress-induced reinstatement of cocaine-seeking behavior) (Alheid and Heimer, 1988; Erb *et al*, 2001; Leri *et al*, 2002). In non-human primates and rodents, the BNST and CEA have strong inputs to NAS (Koob, 1992) and VTA dopaminergic neurons (Fudge and Haber, 2001; Krettek and Price, 1978), and stimulating these inputs can increase NAS (Koob, 1992) and VTA (Aston-Jones *et al*, 1999; Georges and Aston-Jones, 2001, 2002) dopamine (DA) release. These studies suggest that the BNST and CEA may act as an interface for the NAS-VTA reward pathway. Taken together, neonatal THC exposure may inhibit activation of DA neurons throughout the reward circuitry leading to an increase in the rewarding properties of addictive substances as a means to compensate for this deficit. Indeed, perinatal THC has been shown to alter DA sensitivity (García-Gil *et al*, 1996). This hypothesis agrees well with the increase in the rewarding properties of heroin in THC-pretreated rats observed in Experiment 1.

A significant decrease in heroin-induced Fos-IR was also observed in the EW of rats perinatally exposed to THC. Evidence suggests a role for the EW in mediating reward. For example, the EW is involved in alcohol self-administration induced by morphine (Vacca *et al*, 2002). This effect is possibly mediated via stimulation of DA transmission in the EW (Bachtell *et al*, 2003), and this region receives dopaminergic projections from the VTA (Kamenetsky *et al*, 1997). Additionally, CB_1 receptors are localized on dopaminergic neurons (Wenger *et al*, 2003) suggesting an endocannabinoid modulation of the DA reward mechanism within the EW. Taken together, these data invite the hypothesis that THC pre-exposure may inhibit DA neurons in the EW leading to a compensatory increase in the rewarding properties of heroin. Future studies could explore this hypothesis further.

A surprising finding in Experiment 2 was the widespread stimulation of basal Fos-IR in adult rats perinatally exposed to THC. This increase was observed in the NAC (core and shell regions), ICjM, BNST, CEA, dorsolateral and lateral PAG, VTA, and EW. It is important to note that there was a long delay lasting several weeks between drug exposure and perfusion. It is not clear at present why Fos-IR was observed so long after cessation of drug exposure; however, these results provide evidence that brain function continues to be altered weeks after cannabinoid exposure has ceased. This long-lasting change in basal Fos-IR by THC should, however, be considered with caution as it may simply reflect a conditioned drug effect. That is, the increase in basal Fos-IR by THC may be the result of the development of a conditioned response to one or more drug-related cues (eg handling, injection, weighing). The administration of vehicle to these rats after a long period of abstinence from THC may therefore have induced a motivational state previously associated with THC administration. Animals were perfused 2 h following vehicle administration, and after being placed in the locomotor activity chambers. Therefore, Fos production associated with a conditioned state would have had sufficient time to develop. In support of this hypothesis, a related experiment recently conducted in our laboratory (unpublished) also examined the residual effects of chronic cannabinoid exposure on basal Fos-IR, but because we were not interested in the acute effects of a second drug on Fos-IR, locomotor activity was not measured, and animals were not injected with vehicle prior to perfusion. Under these conditions we did not observe any residual cannabinoid-induced changes in basal Fos-IR.

It is interesting to compare the heroin-induced locomotor activity observed in the THC→0.5HER group in the CPP experiment (Experiment 1, Figure 3) to a similar group (THC→HER) in the Fos-IR study (Experiment 2, Figure 4). In the CPP experiment the THC→0.5HER group showed significantly increasing locomotion with repeated heroin injections compared to the THC→VEH group. By comparison, the locomotion observed with a single injection of heroin in the THC→HER group was not significantly higher than the THC→VEH group. These results suggest that the repeated exposure to heroin may be needed to develop the locomotor cross-sensitization observed in the CPP experiment.

However, there is a discrepancy between the heroin locomotor activity data from the first drugged conditioning sessions in Experiment 1 and the locomotor data during the first 30 min of locomotor activity measurement in Experiment 2. In Experiment 1, locomotor activity was significantly higher in the THC→0.5HER group relative to the THC→VEH group on the first day of heroin administration (Figure 3, Day 2). In Experiment 2, no significant differences were observed between the THC→VEH and THC→HER groups during the first 30 min of locomotor activity measurement (Figure 4). Although we can only speculate as to the reasons for this discrepancy, it is possible that the potentiation of THC-induced locomotion by 0.5 mg/kg heroin differs in the two experiments as a result of differing amounts of contextual habituation prior to drug exposure. In Experiment 1, rats received a single 20-min habituation session; however, in Experiment 2 rats received four 120-min habituation sessions prior to drug exposure. The additional exposure to contextual cues in a nondrugged state in Experiment 2 may therefore have slowed or masked the development of locomotor cross-sensitization.

In summary, the present study demonstrates that the rewarding properties of heroin are enhanced by perinatal THC pre-exposure and provides important new clues regarding the potential neural correlates underlying this long-lasting behavioral alteration. It should, however, be noted that the present results alone are not sufficient to determine causal relationships between neural effects and long-lasting behavioral changes. Rather, the present study

serves only to identify candidate sites that may be responsible for THC's 'gateway' effects. It remains the goal of future studies to test the hypotheses generated here. It is also important to note that the evidence for the 'gateway' hypothesis using animal models of drug addiction obtained here and elsewhere (Norwood *et al*, 2003; Pontieri *et al*, 2001a, b) has relied on the use of rather high doses of THC and other cannabinoids. Caution is therefore advised when extending the results from the present study to human drug abuse.

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REFERENCES

Alheid GF, Heimer L (1988). New perspectives in basal forebrain organization of special relevance for neuropsychiatric disorders: the striatopallidal, amygdaloid, and corticopetal components of substantia innominata. *Neuroscience* **27**: 1939.

Arnold JC, Topple AN, Hunt GE, McGregor IS (1998). Effects of pre-exposure and co-administration of the cannabinoid receptor agonist CP 55940 on behavioural sensitization to cocaine. *Eur J Pharmacol* **354**: 9–16.

Aston-Jones G, Delfs JM, Druhan J, Zhu Y (1999). The bed nucleus of the stria terminalis. A target site for noradrenergic actions in opiate withdrawal. *Ann NY Acad Sci* **877**: 486–498.

Bachtell RK, Weitemier AZ, Galvan-Rosas A, Tsivkovskaia NO, Risinger FO, Phillips TJ *et al* (2003). The Edinger–Westphal-lateral septum urocortin pathway and its relationship to alcohol consumption. *J Neurosci* **23**: 2477–2487.

Brown LL, Smith DM, Goldbloom LM (1998). Organizing principles of cortical integration in the rat neostriatum: corticostriate map of the body surface is an ordered lattice of curved laminae and radial points. *J Comp Neurol* **392**: 468–488.

Browne RG, Segal DS (1980). Behavioral activating effects of opiates and opioid peptides. *Biol Psychiatry* **15**: 77–86.

Cadoni C, Pisanu A, Solinas M, Acquas E, Di Chiara G (2001). Behavioural sensitization after repeated exposure to Δ^9-tetrahydrocannabinol and cross-sensitization with morphine. *Psychopharmacology (Berl)* **158**: 259–266.

Calenco-Choukroun G, Dauge V, Gacel G, Feger J, Roques BP (1991). Opioid delta agonists and endogenous enkephalins induce different emotional reactivity than mu agonists after injection in the rat ventral tegmental area. *Psychopharmacology (Berl)* **103**: 493–502.

Cole JC, Sumnall HR, O'Shea E, Marsden CA (2003). Effects of MDMA exposure on the conditioned place preference produced by other drugs of abuse. *Psychopharmacology (Berl)* **166**: 383–390.

Corchero J, García L, Manzanares J, Fernández-Ruiz JJ, Fuentes JA, Ramos JA (1998). Perinatal Δ^9-tetrahydrocannabinol exposure reduces proenkephalin gene expression in the caudate-putamen of adult female rats. *Life Sci* **63**: 843–850.

Davis M (1998). Are different parts of the extended amygdala involved in fear *versus* anxiety? *Biol Psychiatry* **44**: 1239–1247.

D'Este L, Scontrini A, Casini A, Pontieri FE, Renda TG (2002). Heroin sensitization as mapped by c-Fos immunoreactivity in the rat striatum. *Brain Res* **933**: 144–149.

Ebrahim SH, Gfroerer J (2003). Pregnancy-related substance use in the United States during 1996–1998. *Obstet Gynecol* **101**: 374–379.

Erb S, Salmaso N, Rodaros D, Stewart J (2001). A role for the CRF-containing pathway from central nucleus of the amygdala to bed nucleus of the stria terminalis in the stress-induced reinstatement of cocaine seeking in rats. *Psychopharmacology (Berl)* **158**: 360–365.

Erdtmann-Vourliotis M, Mayer P, Riechert U, Hollt V (2000). Prior experience of morphine application alters the c-Fos response to MDMA ('ecstasy') and cocaine in the rat striatum. *Mol Brain Res* **77**: 55–64.

Fernandez-Ruiz JJ, Berrendero F, Hernandez ML, Ramos JA (2000). The endogenous cannabinoid system and brain development. *Trends Neurosci* **23**: 14–20.

Fernandez-Ruiz JJ, Berrendero F, Hernandez ML, Romero J, Ramos JA (1999). Role of endocannabinoids in brain development. *Life Sci* **65**: 725–736.

Fink JS, Smith GP (1980). Mesolimbicocortical dopamine terminal fields are necessary for normal locomotor and investigatory exploration in rats. *Brain Res* **199**: 359–384.

Fried PA (1982). Marihuana use by pregnant women and effects on offspring: An update. *Neurobehav Toxicol Teratol* **4**: 451–454.

Fried PA, Makin JE (1987). Neonatal behavioural correlates of prenatal exposure to marihuana, cigarettes and alcohol in a low risk population. *Neurotoxicol Teratol* **9**: 1–7.

Fried PA, Watkinson B, Gray R (1999). Growth from birth to early adolescence in offspring prenatally exposed to cigarettes and marijuana. *Neurotoxicol Teratol* **21**: 513–525.

Fudge JL, Haber SN (2001). Bed nucleus of the stria terminalis and extended amygdala inputs to dopamine subpopulations in primates. *Neuroscience* **104**: 807–827.

García-Gil L, de Miguel R, Ramos JA, Fernández-Ruiz JJ (1996). Perinatal Δ^9-tetrahydrocannabinol exposure in rats modifies the responsiveness of midbrain dopaminergic neurons in adulthood to a variety of challenges with dopaminergic drugs. *Drug Alcohol Depend* **42**: 155–166.

Georges F, Aston-Jones G (2001). Potent regulation of midbrain dopamine neurons by the bed nucleus of the stria terminalis. *J Neurosci* **21**: RC160 (1–6).

Georges F, Aston-Jones G (2002). Activation of ventral tegmental area cells by the bed nucleus of the stria terminalis: a novel excitatory amino acid input to midbrain dopamine neurons. *J Neurosci* **22**: 5173–5187.

Grabus SD, Glowa JR, Riley AL (2004). Morphine- and cocaine-induced c-Fos levels in Lewis and Fischer rat strains. *Brain Res* **998**: 20–28.

Hall W, Solowij N (1998). Adverse effects of cannabis. *Lancet* **352**: 1611–1616.

Hall WD, Carless JM, Homel PJ, Flaherty BJ, Reilly CJ (1991). The characteristics of cocaine users among young adults in Sydney. *Med J Aust* **155**: 11–14.

Hand TH, Stinus L, Le Moal M (1989). Differential mechanisms in the acquisition and expression of heroin-induced place preference. *Psychopharmacology (Berl)* **98**: 61–67.

Kamenetsky S, Rabinowitz R, Urca G, Korczyn AD (1997). Neuroanatomical aspects of mydriatic action of morphine in rats. *J Ocul Pharmacol Ther* **13**: 405–413.

Koob GF (1992). Drugs of abuse: anatomy, pharmacology and function of reward pathways. *Trends Pharmacol Sci* **13**: 177–184.

Krettek JE, Price JL (1978). Amygdaloid projections to subcortical structures within the basal forebrain and brainstem in the rat and cat. *J Comp Neurol* **178**: 225–254.

Lamarque S, Taghzouti K, Simon H (2001). Chronic treatment with Δ^9-tetrahydrocannabinol enhances the locomotor response to amphetamine and heroin. Implications for vulnerability to drug addiction. *Neuropharmacology* **41**: 118–129.

Leri F, Flores J, Rodaros D, Stewart J (2002). Blockade of stress-induced but not cocaine-induced reinstatement by infusion of noradrenergic antagonists into the bed nucleus of the stria terminalis or the central nucleus of the amygdala. *J Neurosci* **22**: 5713–5718.

Meng ZH, Feldpaush DL, Merchant KM (1998). Clozapine and haloperidol block the induction of behavioral sensitization to amphetamine and associated genomic responses in rats. *Mol Brain Res* **61**: 39–50.

Meyer JS, Kunkle R (1999). Behavioral responses to a D_1 dopamine agonist in weanling rats treated neonatally with cocaine and Δ^9-tetrahydrocannabinol. *Neurotoxicol Teratol* **21**: 375–380.

Meyer ME, McLaurin BI, Meyer ME (1995). DALDA (H-Tyr-D-Arg-Phe-Lys-NH2), a potent μ-opioid peptide agonist, affects various patterns of locomotor activities. *Pharmacol Biochem Behav* **51**: 149–151.

Miller NS, Klahr AL, Gold MS, Sweeney K, Cocores JA, Sweeney DR (1990). Cannabis diagnosis of patients receiving treatment for cocaine dependence. *J Subst Abuse* **2**: 107–111.

Mucha RF, van der Kooy D, O'Shaughnessy M, Bucenieks P (1982). Drug reinforcement studied by the use of place conditioning in rat. *Brain Res* **243**: 91–105.

Navarro M, de Miguel R, de Fonseca FR, Ramos JA, Fernandez-Ruiz JJ (1996). Perinatal cannabinoid exposure modifies the sociosexual approach behavior and the mesolimbic dopaminergic activity of adult male rats. *Behav Brain Res* **75**: 91–98.

Navarro M, Rodriguez de Fonseca F, Hernandez ML, Ramos JA, Fernandez-Ruiz JJ (1994). Motor behavior and nigrostriatal dopaminergic activity in adult rats perinatally exposed to cannabinoids. *Pharmacol Biochem Behav* **47**: 47–58.

Norwood CS, Cornish JL, Mallet PE, McGregor IS (2003). Pre-exposure to the cannabinoid receptor agonist CP 55940 enhances morphine behavioral sensitization and alters morphine self-administration in Lewis rats. *Eur J Pharmacol* **465**: 105–114.

Paxinos G, Watson C (1998). *The Rat Brain in Stereotaxic Coordinates*. Academic Press: New York.

Pontieri FE, Monnazzi P, Scontrini A, Buttarelli FR, Patacchioli FR (2001a). Behavioral sensitization to heroin by cannabinoid pretreatment in the rat. *Eur J Pharmacol* **421**: R1–R3.

Pontieri FE, Monnazzi P, Scontrini A, Buttarelli FR, Patacchioli FR (2001b). Behavioral sensitization to WIN55212-2 in rats pretreated with heroin. *Brain Res* **898**: 178–180.

Rubio P, de Fonseca FR, Martin-Calderon JL, Del Arco I, Bartolome S, Villanua MA *et al* (1998). Maternal exposure to low doses of Δ^9-tetrahydrocannabinol facilitates morphine-induced place conditioning in adult male offspring. *Pharmacol Biochem Behav* **61**: 229–238.

Shu SY, Ju G, Fan LZ (1988). The glucose oxidase-DAB-nickel method in peroxidase histochemistry of the nervous system. *Neurosci Lett* **85**: 169–171.

Singh ME, Warty NA, McGregor IS, Mallet PE (2002). Repeated exposure to a cannabinoid receptor agonist alters subsequent basal and morphine-induced Fos immunoreactivity. 32st Annual Meeting of the Society for Neuroscience, Orlando, FL, USA.

Solinas M, Panlilio LV, Goldberg SR (2004). Exposure to Δ^9-tetrahydrocannabinol (THC) increases subsequent heroin taking but not heroin's reinforcing efficacy: a self-administration study in rats. *Neuropsychopharmacology* **29**: 1301–1311.

Thornton SR, Compton DR, Smith FL (1998). Ontogeny of μ opioid agonist anti-nociception in postnatal rats. *Dev Brain Res* **105**: 269–276.

Turgeon SM, Pollack AE, Fink JS (1997). Enhanced CREB phosphorylation and changes in c-Fos and FRA expression in striatum accompany amphetamine sensitization. *Brain Res* **749**: 120–126.

Tzschentke TM (1998). Measuring reward with the conditioned place preference paradigm: a comprehensive review of drug effects, recent progress and new issues. *Prog Neurobiol* **56**: 613–672.

Vacca G, Serra S, Brunetti G, Carai MAM, Gessa GL, Colombo G (2002). Boosting effect of morphine on alcohol drinking is suppressed not only by naloxone but also by the cannabinoid CB_1 receptor antagonist, SR 141716. *Eur J Pharmacol* **445**: 55–59.

Vela G, Martin S, Garcia-Gil L, Crespo JA, Ruiz-Gayo M, Fernandez-Ruiz JJ *et al* (1998). Maternal exposure to Δ^9-tetrahydrocannabinol facilitates morphine self-administration behavior and changes regional binding to central μ opioid receptors in adult offspring female rats. *Brain Res* **807**: 101–109.

Warty NA, Singh ME, McGregor IS, Mallet PE (2002). Cannabinoid receptor antagonist modulatioin of morphine-induced Fos expression in feeding-related brain regions. 32nd Annual Meeting of the Society for Neuroscience, Orlando, FL, USA.

Wenger T, Moldrich G, Furst S (2003). Neuromorphological background of cannabis addiction. *Brain Res Bull* **61**: 125–128.

Zuckerman B, Frank DA, Hingson R, Amaro H, Levenson SM, Kayne H *et al* (1989). Effects of maternal marijuana and cocaine use on fetal growth. *N Engl J Med* **320**: 762–768.