Prenatal Cannabis Exposure Increases Heroin Seeking with Allostatic Changes in Limbic Enkephalin Systems in Adulthood

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Background: *Prenatal cannabis exposure is a growing concern with little known about the long-term consequences on behavior and neural systems relevant for reward and emotional processing.*

Methods: *We used an animal model to study the effects of prenatal exposure to Δ^9-tetrahydrocannabinol (THC) on heroin self-administration behavior and opioid neural systems in adult males (postnatal day 62). Rats were exposed to THC (.15 mg/kg) or vehicle from gestational day 5 to postnatal day 2.*

Results: *Both pretreatment groups showed similar heroin intake, but THC-exposed rats exhibited shorter latency to the first active lever press, responded more for low heroin doses, and had higher heroin-seeking during mild stress and drug extinction. THC exposure reduced preproenkephalin (PENK) mRNA expression in the nucleus accumbens during early development, but was elevated in adulthood; no adult striatal changes on preprodynorphin mRNA or PENK in caudate-putamen. PENK mRNA was also increased in the central and medial amygdala in adult THC-exposed animals. THC animals had reduced heroin-induced locomotor activity and nucleus accumbens μ opioid receptor coupling.*

Conclusions: *This study demonstrates enduring effects of prenatal THC exposure into adulthood that is evident on heroin-seeking behavior during extinction and allostatic changes in mesocorticolimbic PENK systems relevant to drug motivation/reward and stress response.*

Key Words: Cannabinoid receptor, CB_1, extended amygdala, G protein-coupling, locomotor activity, opioid neuropeptide, rimonabant

Exposure to Cannabis sativa preparations (hashish, marijuana) during prenatal development is a major health problem with harmful effects including higher rates of fetal distress, growth retardation and adverse neurodevelopmental outcome (Day et al 1994; Fried 1995; Hurd et al 2005). It has been estimated that, depending on age and ethnicity, 7–17% of women used marijuana during pregnancy (SAMHSA 2002). Despite these concerns, long-term neural effects of prenatal exposure to cannabinoids are still poorly understood. CB_1 receptors, which mediate the neural actions of cannabinoids, emerge early in the brain during fetal development (Berrendero et al 1998; Romero et al 1997) and are involved in neurodevelopmental events such as synaptogenesis, proliferation and migration of neuronal cells (Fernandez-Ruiz et al 2000). Cannabinoids can be transferred from the mother to the offspring via placental blood during gestation (Hutchings et al 1989) and through maternal milk during lactation (Jakubovic and McGeer 1977). As such, cannabis can interfere with the ontogeny of neurodevelopment that can potentially have long-term impact on neural function and behavior.

Epidemiological and clinical studies indicate that in utero marijuana exposure is associated with impulsive behavior, cognitive impairment and psychiatric disorders (e.g., schizophrenia, depression and anxiety) in later life (Arseneault et al 2002; Fried and Watkinson 2001; Patton et al 2002). The potential role of cannabis to increase the risk of consumption of highly addictive substances has also been suggested (Kandel 2003; Porath and Fried 2005). Therefore, it can be hypothesized that long-lasting neurobiological changes in neuronal systems linked with limbic function important for reward and emotional regulation might be affected by in utero marijuana exposure.

Of the neuronal populations associated with limbic function, the endogenous opioid system shares many neuroanatomical and neurochemical characteristics with the cannabinoid system (Corchero et al 2004; Vigano et al 2005). The evidence for a tight cannabinoid-opioid interaction is now overwhelming in regard to the modulation of behavioral responses linked with drug reinforcement/reward processes and relapse (Cossu et al 2001; De Vries et al 2003; Fattore et al 2004; Ledent et al 1999). There are three families of endogenous opioid peptides—enkephalin, dynorphin, endorphin—derived from preproenkephalin (PENK), preprodynorphin and proopiomelanocortin genes (Herz 1998). Enkephalin and endorphin peptides mediate their actions primarily through μ and δ receptors and are reinforcing, whereas dynorphins mediate their actions via the κ receptor which is associated with aversion and dysphoria (Herz 1998). Cannabinoids have been documented to stimulate the release of enkephalin in the nucleus accumbens (NAc; Valverde et al 2001), a key brain area of the mesocorticolimbic reward pathway, as well as β-endorphin in the ventral tegmental area (Solinas et al 2004), the origin of dopamine mesocorticolimbic circuits. Of these opioid neuropeptides, PENK is more widely expressed throughout the mesocorticolimbic system (NAc, amygdala, prefrontal cortex) and more highly implicated in modulating hedonic homeostasis (Kelley et al 2002; Skoubis et al 2005).

Human studies of fetal mid-gestational subjects have recently revealed significant impairment of PENK (Wang et al, in press) and mesolimbic-related dopamine (Wang et al 2004) genes in association with in utero marijuana exposure. The question remains as to whether these effects are enduring and can influence adult behavior relevant to addiction disorders. Previous rat studies have documented that perinatal cannabis exposure alters opioid gene expression (Corchero et al 1998) and receptor binding (Vela et al 1998) as well as morphine self-administration behavior

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(Gonzalez et al 2003; Vela et al 1998) in adult female offspring. However, those studies examined the effects of cannabis throughout gestation and the entire lactation period until the pups were young adolescents, approximately 24 days old. Cannabis was also administered to the dams orally, which has a different pharmacokinetic property as compared to smoking. In order to examine the impact of early cannabis exposure more consistent with the human fetal studies, the present study investigated drug exposure limited to the prenatal period that corresponds to mid-gestation in humans and used an intravenous route of administration that better mimics the pharmacokinetics of marijuana smoking in pregnant women (Grotenhermen 2003). To address issues as to whether neural alterations in the human fetus ascribed to maternal marijuana use does directly relate to cannabis and not to other drug exposure or even to by-products of smoking, our experimental rat study specifically examined prenatal exposure to Δ^9-tetrahydrocannabinol (THC; the major psychoactive component of cannabis) during prenatal development. The results demonstrate that, similar to the human findings, THC significantly affects PENK mRNA expression during the prenatal period, and that alterations of the opioid system are long-lasting into adulthood with enhanced vulnerability to opiate-seeking behavior.

Methods and Materials

All animal experiments were conducted in accordance with the guidelines of the European Community and The Swedish National Board for Laboratory Animals, under protocols approved by the Ethical Committee of Northern Stockholm.

Behavioral Experiments

Subjects. Long Evans female rats (Charles River, Germany) weighing 200–250 g (approximately 6–7 weeks old) were housed under 12 h reversed light/dark cycle (light on 11:00 pm) with controlled room temperature (±21°C), humidity (40%); water and food were available ad libitum. After 1 week, a permanent intravenous catheter (Brian Fromant, Cambrige, UK) was surgically implanted and secured to the right jugular vein under deep anesthesia with Isoflurane (Forene, Apoteket AB, Sweden). After surgery, rats received daily intravenous (i.v.) injections of Heparin 10U + ampicillin (50 mg/kg; Doctacillin, Apoteket AB, Sweden) for 3 days to prevent infection and subcutaneous (s.c.) injection of Caprofen (.5 mg Rimadyl vet, Apoteket AB, Sweden) as post-surgery pain management. From the fourth day, the catheters were flushed with .1 ml of saline containing 30U heparin. Once recovered from surgery (one week), females were allowed to mate with a male. Only females with the presence of a vaginal plug were accepted as pregnant and used in the subsequent drug treatment phase. Pregnant females received daily intravenous injections of THC (.15 mg/kg) or vehicle (.3% Tween 80-sterile saline solution) from gestational day 5 to postnatal day (PND) 2. This corresponds to the mid-gestation (≈ week 20) development stage in humans (Bayer et al 1993), the time period examined in our human fetal studies. An intravenous route of administration was used in the animal model since it more closely mimics the pharmacokinetics of cannabis smoking, the normal route used in pregnant women (Grotenhermen 2003). The dose of THC is an extrapolation from current estimates of low cannabis cigarettes (about 16 mg of THC), correcting for differences in route of administration and body weight (Grotenhermen 2003). During the course of the drug treatment, gestational parameters were recorded, such as maternal weight gain, gestational length and fetal weights. On PND 2, pups from both groups were cross-fostered and culled 8 to 10 from vehicle mothers; brains were taken from other sets of animals at this time period. On PND 21, male offspring were weaned from their mothers and housed 4/cage until 55 days of age when surgery was carried out in one set of animals. THC- and vehicle-exposed males at PND 55 were surgically implanted with an intravenous catheter in the right jugular vein and treated as reported above. All subjects were housed individually following surgery and given at least 7 days of recovery before behavioral experiments (outlined below). Brains were taken from another group of rats for post-mortem studies at PND 62 when the behavioral studies were initiated in the other animals.

Drugs. THC (10 mg/ml in ethanol solution; Sigma-Aldrich, Apoteket AB, Sweden) was evaporated under nitrogen gas, dissolved in .3% Tween 80 and diluted with .9% NaCl to the concentrations of 1 mg/ml. For intravenous self-administration training, heroin-HCl (Apoteket, AB, Sweden) was dissolved in .9% sterile saline solution and filtered through .2 μm syringe filters prior to use. For reinstatement tests (i.e., drug priming), heroin-HCl was dissolved in .9% sterile saline solution and injected subcutaneously (s.c.) at a volume of 2 ml/kg. SR 141716A [N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride] (Rimonabant; Sanofi-Aventis) was dissolved in .3% Tween 80 and diluted in sterile saline solution and administered intraperitoneal (i.p.) at a volume of 2 ml/kg.

Apparatus. Heroin self-administration studies were carried out in operant chambers (29.5 × 32.5 × 23.5 cm) equipped with two retractable levers and infrared locomotor sensors (Med Associates Inc., Vermont, U.S.); each enclosed in sound-attenuating chambers. Depression of one lever (defined as active) resulted in an intravenous drug injection, while depression on the other lever (defined as inactive) had no programmed consequence but was always recorded. A single active lever press resulted in a 5s drug infusion (85 μl); concurrently a white light cue above this lever was turned on for 5s. A 10s timeout period was then introduced, during which the white cue light was turned off and further presses on this lever had no additional consequence, but were recorded as well as the presses on the inactive lever. Each self-administration session started with the extension of the two levers. A red house light was illuminated throughout the sessions. Assessment of the self-administration schedule and data collection were controlled by Med Associates PC software.

Self-administration Training. Animals were allowed to self-administer heroin (15 μg/kg/inf) under a fixed-ratio 1 schedule of reinforcement and lever presses as operandum in 3 h daily sessions. The self-administration sessions were conducted daily during the dark cycle (11:00–18:00). To ensure patency, catheters were flushed daily before and after each IVSA session with a sterile saline solution containing heparin (30U). After 6 days, the dose of heroin was increased to 30 μg/kg/inf and acquisition sessions were carried out until stable baseline was reached. Baseline responding was considered stable when the number of responses did not differ more than 15% for 3 consecutive days. During heroin self-administration training, food supply was restricted (20 g/day). Once stable responding was reached the rats were again given unlimited access to food.

Dose Response and Mild Stress Test. After the acquisition phase, a between session dose-response test was conducted. The training dose of heroin (30 μg/kg) was substituted by either higher (60 and 100 μg/kg) or lower (7.5 and 15 μg/kg) doses: each dose was self-administered for 3 consecutive days and the order of presentation was randomly determined. After the dose-

response experiment was performed, rats were maintained on 30 μg/kg for 5 additional days before determining the consequence of exposure to a mild stress (24 hours food deprivation).

Extinction and Reinstatement Test. An extinction phase started following the procedures described above. Responding for heroin was extinguished in daily sessions by replacing the heroin solution in the syringe with physiological saline. Since not all catheters were patent for the entire extinction phase, saline infusion was only given during the first week of extinction. Subsequently, animals were connected to the liquid swivel and an empty syringe was connected to the infusion tubing to seal the system, but was not placed in the syringe pump. All other parameters were left unchanged. Drug-reinforced behavior was considered extinguished when responding on the active lever had decreased by at least 85% for 3 consecutive days. After extinction criteria were reached, each rat was given one of the following priming injections to test its effect on reinstating heroin-seeking behavior: saline, heroin (.25 mg/kg, s.c.; 10 min before the session) and CB_1 antagonist, Rimonabant (3 mg/kg, i.p.; 30 min before the session and 20 before heroin priming) + heroin combination. The dose of heroin was chosen due to its known effect to reinstate heroin-seeking behavior following long-term extinction (De Vries et al 2003; Leri and Stewart 2001). The dose of Rimonabant was selected due to its ability to attenuate heroin seeking (De Vries et al 2003; Ellgren et al, in press).

Post-mortem Studies. Tissue sections preparation. THC- and vehicle-exposed rats on PND 2 (1h after the last maternal THC injection) and PND 62 were anaesthetized in a CO_2 chamber and decapitated. Brains were quickly removed, frozen in isopentane (≈ −30°C) for 1 min, and stored at −80°C. Coronal sections (20-μm thick) were cut through the striatum and amygdala in a refrigerated cryostat (−15°C; Frigocut 2800E, Leica Instruments, Nobloch, Germany) and mounted onto Superfrost Plus slides (Brain Research Laboratories, Newton, Massachusetts). The sections were stored at −30°C until processed as described below.

In Situ Hybridization Histochemistry (ISHH). PENK and preprodynorphin mRNA expression levels were studied by ISHH. The PENK riboprobe was complementary to a 333 kb fragment of the rat PENK cDNA (bp291–624 GenBank accession number Y07503). The preprodynorphin riboprobe was complementary to a 534 kb fragment of the rat preprodynorphin cDNA (bp349–883; accession number NM_019374). The RNA probes were transcribed in the presence of [^{35}S]uridine5′-[α-thio]triphosphate (specific activity 1000–1500 Ci/mmol; New England Nuclear, Boston, Massachusetts). The ISHH procedure was similar to published protocols (Hurd et al 2001). Briefly, the labeled probe was applied to the brain sections in a concentration of 2×10^3 cpm/mm^2 of the coverslip area. Two adjacent sections from each subject were studied. Hybridization was carried out overnight at 55°C in a humidified chamber. After hybridization, the slides were apposed to β-max Hyperfilm (Amersham, Bucks, United Kingdom) along with ^{14}C-standards (American Radiolabelled Chemicals, St Louis, MO, USA): 1–4 days for PENK; 6 days for preprodynorphin.

Agonist-stimulated [^{35}S]GTPγS Autoradiography. Agonist-stimulated [^{35}S]GTPγS binding was carried out according to established protocols (Sim et al 1995) with 2 mM GDP, .04 nM [^{35}S]GTPγS (specificy activity 1099 Ci/mmol; Amershan Biosciences) and appropriate agonists. μ opioid receptor (μOR) agonist stimulation was carried out using 3 μM DAMGO ([D-Ala^2,*N*-Me-Phe^4, Gly^5-ol]-enkephalin; Sigma-Aldrich, Missouri). CB_1 receptor agonist stimulation was carried out using 10 μM WIN 55,212-2 (R-(+)-(2,3-dihydro-5-methyl-3-[(4-morpholinyl) methyl]pyrol[1,2,3-de]-1,4-benzoxazinyl) (1-naphthalenyl)methanonemesylate; Sigma-Aldrich). Non-specific binding was determined in the absence of the agonist and in the presence of 10 μM unlabeled GTPγS. Brain sections were exposed to Hyperfilm for 24 hours with [^{14}C]-standards.

Data Analyses. Films images were digitalized into the 256 levels of optical density with a Microtek scanner (SM III, Microtek Europe, Rotterdam, The Netherlands) and quantitation of film autoradiograms was carried out using computer-assisted optical densitometry (NIH Image, version 1.58, Wayne Rasband, NIMH). The mRNA expression level was estimated within the NAc (+1.60 mm from Bregma), caudate-putamen (+1.60 mm), amygdala (−2.30 mm), ventral tegmental area (−5.80 mm), and substantia nigra (−5.80 mm) in accordance with the Paxinos and Watson Rat Atlas (Paxinos and Watson 1997). Values obtained from duplicate brain sections for each subject were averaged. For ISHH experiments, the values were expressed as dpm/mg of tissue by reference to the co-exposed standard. For receptor autoradiography, specific binding was calculated by subtracting non-specific binding from total binding in the specific area of interest. The percent agonist-induced stimulation from the [^{35}S]GTPγS autoradiography experiments was calculated as [stimulated-basal]/basal × 100.

Statistical Analysis. For behavioral studies, data were analyzed using a two-way ANOVA for repeated measures followed, when appropriate, by planned comparison tests with Bonferroni correction for multiple comparisons. Mann-Whitney non-parametric test was used for group size less than five. The significance level was set at $P < .05$ and trends considered for $P < .10$. For molecular and receptor activation studies, statistical comparison between vehicle- and THC-pretreated animals was performed by one-way ANOVA.

Results

There was no significant group difference in the % weight gain by the pregnant females (vehicle 45.4 ± 1; THC 47.3 ± 3 g), gestational length (vehicle 21 ± .38; THC 20.8 ± .66 days), pup length at PND 2 (vehicle 6.98 ± .12; THC 7 ±.13 cm) and pup weight (vehicle 6.83 ±.18; THC 6.91 ±.16 g). There was also no significant difference ($P = .565$) in weight between animals pretreated with THC (317 ± 11 g) or vehicle- (330 ± 16 g) at the start of the heroin self-administration on PND 62.

Heroin Self-administration

Acquisition. Two THC-exposed and 1 vehicle rat did not meet the acquisition criteria and were excluded from the study as well as 3 rats that developed blocked catheters. Six animals/group remained.

The acquisition data of heroin self-administration is presented in Figure 1. At the lowest dose of heroin tested (15 μg/kg/inf), no reliable heroin self-administration was observed; both groups of animals did not discriminate between the active and the inactive lever (Figure 1A). When the dose of heroin was increased to 30 μg/kg/inf both groups of animals achieved a reliable acquisition pattern of heroin intake (Figure 1B). There was a significant effect of active lever presses during self-administration sessions ($F_{(12,108)} = 19.87$, $P < .000001$). The number of inactive lever presses during the self-administration acquisition sessions (30 μg/kg/inf) was similar between vehicle and THC-exposed rats, which indicate that all rats maintained a good discrimination between the active and inactive lever presses. The number of stable active lever presses in the THC- and vehicle-exposed rats did not differ statistically (last day of acquisition: 26.7 ± 2.7 vs. 23.8 ± 1.96, respectively; $F_{(1,9)} = .1641$, $P = .69$) (Figure 1B).

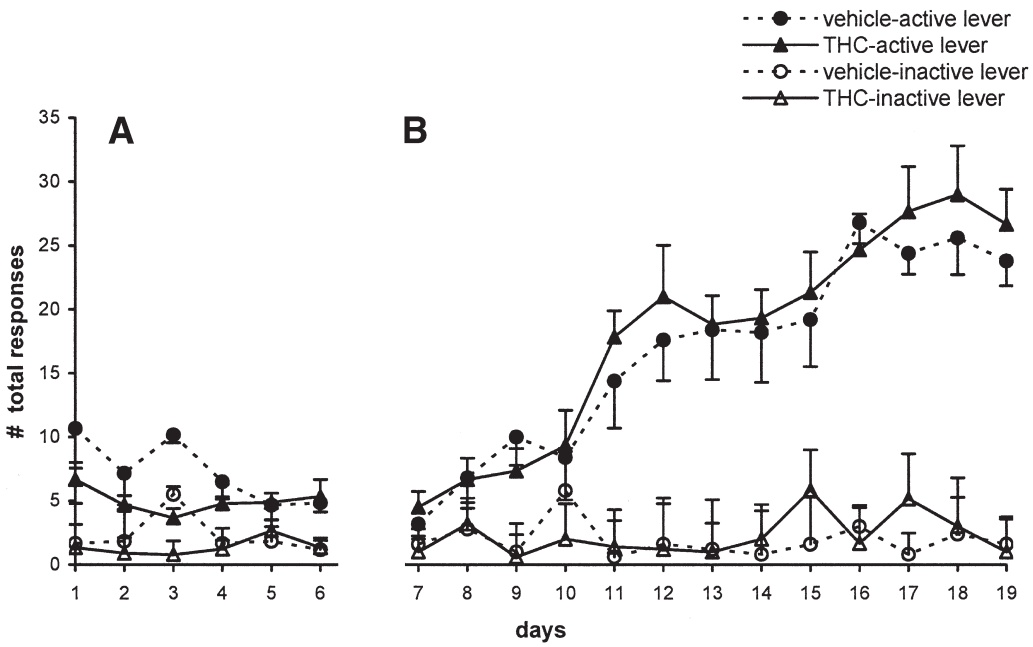

Figure 1. Acquisition of heroin self-administration at **(A)** 15 μg/kg/infusion and **(B)** 30 μg/kg/infusion under a fixed-ratio 1 schedule of reinforcement in adult rats (beginning postnatal day 62) with perinatal exposure (gestation day 5 to postnatal day 2) to vehicle or THC. Values are expressed as mean ± SEM of number of total responses on both active and inactive lever; 6 animals per group.

However, examination of the mean latency to the first active lever press on the last day of acquisition, when all animals maintained stable intake, showed a significant difference in the onset of responding ($P < .05$) between the pretreated groups. THC offsprings displayed a shorter latency (3.4-fold) to the first active response (34 ± .84s) than the vehicle group (115 ± .91s; Figure 2).

Dose-response. Figure 3 shows the total active responses for the between dose-response curve. There was a dose-dependent decrease in responding with increasing heroin dose. There was a main effect of dose ($F_{(4,36)} = 55.79$; $P < .0001$) and a dose × treatment interaction ($F_{(4,36)} = 2.70$; $P < .05$). THC-exposed rats exhibited a higher rate of responding to the lower doses, but statistically significant increase was found only at the dose of 15 μg/kg/inf ($P < .02$) compared to the vehicle animals.

Stress Test. The total responses following an acute mild stress are presented in figure 4. ANOVA revealed a main effect of stress ($F_{(1,8)} = 11.27$; $P < .05$) and stress × treatment interaction ($F_{(1,8)} = 9.24$; $P < .02$). Interestingly, prenatal THC-exposed offspring increased the percentage of active lever presses (26%) at a level significantly higher than the vehicle group. In contrast, the mild stress had no effect on rate of responding as compared to the previous baseline level in vehicle-exposed animals (31 ± 5.9 vs. 29 ± 4.1).

Extinction and Reinstatement. Figure 5 shows the results of the heroin-seeking behavior observed during the extinction

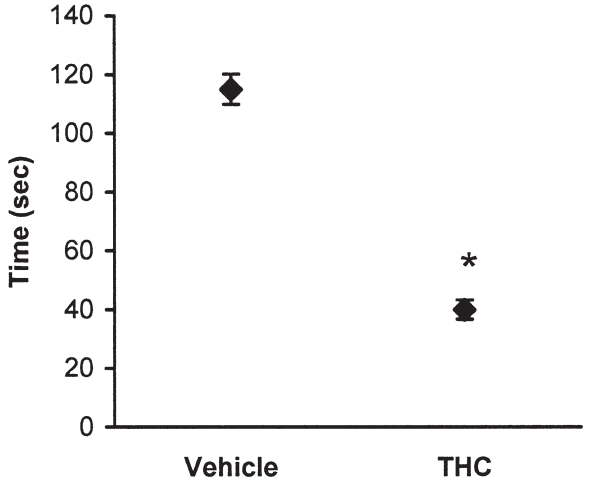

Figure 2. Mean latency to the first active lever press during the last day of acquisition (day 13) of heroin self-administration (30 μg/kg/infusion) in adult rats perinatally exposed to vehicle or THC. Values are expressed as the mean ± SEM. *, $P < .05$ as compared to the vehicle control.

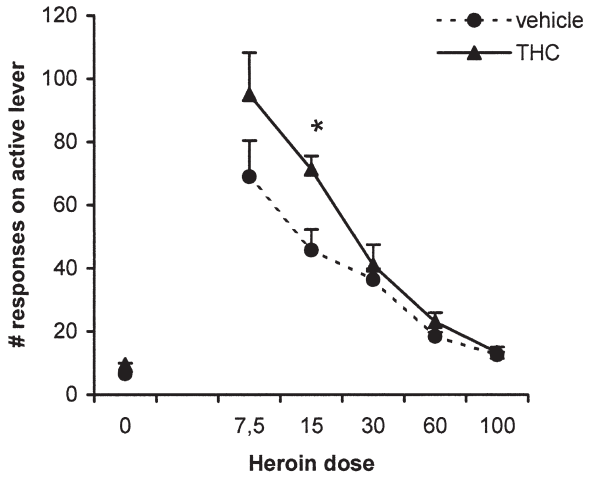

Figure 3. Heroin self-administration behavior (active lever presses) dose-response curve in adult rats with perinatal vehicle or THC exposure. Data refer to the last self-administration session of a given dose. Values are expressed as mean ± SEM for 6 animals per group. *, $P < .05$ as compared to vehicle control.

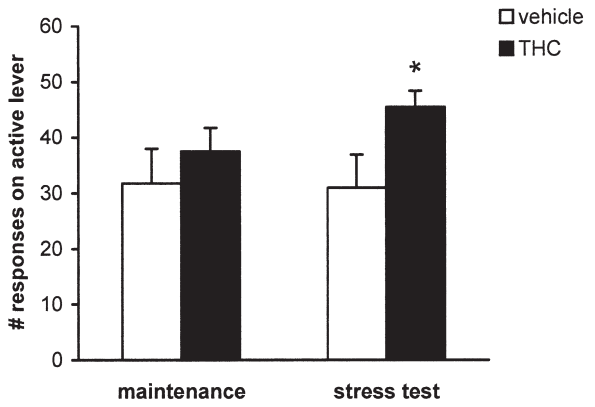

Figure 4. Effect of mild stress (1 day food deprivation) in adult rats with perinatal vehicle or THC exposure. Each bar represents the mean ± SEM of the number of active lever presses for 5 animals per group. *, $P < .02$ as compared to vehicle control.

and reinstatement phases. ANOVA revealed an overall main effect of phase ($F_{(4,32)} = 25.00$; $P < .00001$) and treatment ($F_{(1,8)} = 9.46$; $P < .02$). Responding on the heroin-paired lever during the first day of extinction differed significantly between the two groups (vehicle 35.6 ± 5.6; THC 58.8 ± 5.4; $P < .02$). THC and vehicle animals took 21 days to achieve extinction of the heroin self-administration behavior. Although both group of animals decreased responding similarly over the extinction phase, THC-exposed rats continued to show significantly higher active responses as compared to the vehicle group (vehicle 6.6 ± .4; THC 9.6 ± .05; $P < .01$) even on the last day of extinction. Responding on the inactive lever did not differ significantly between the pretreated groups either during the first or last days of extinction, which indicates that the noted differences between the groups were specific to heroin-seeking.

Following extinction, no difference was observed in the mean number of active lever responses between the extinction baseline and reinstatement session when animals received saline priming (Figure 5). However, a non-contingent, non-reinforced priming injection of heroin (.25 mg/kg, s.c.) triggered relapse to heroin-seeking behavior, selectively reinstating active responding in both groups of animals. The vehicle group reinstated responding at a similar level compared to the pre-extinction baseline (33 ± 4.02 vs. 39.6 ± 3.3); $P = .55$), whereas THC-exposed rats showed a trend for a higher level of responding compared to the pre-extinction baseline ($P = .07$), but there was variability in the response between the rats.

Notably, pretreatment with the selective CB_1 receptor antagonist rimonabant (3 mg/kg, i.p.) completely prevented heroin-induced reinstatement of drug-seeking behavior in both groups of animals (vehicle, $P = .005$; THC, $P = .0005$ vs. heroin prime session). The mean responding rate during reinstatement did not differ from that observed during the previous extinction in both THC-(12.4 ± .45 vs. 9.6 ± .05) and vehicle-exposed rats (6.6 ± .39 vs. 8.6 ± .6).

Locomotor Activity

Forward locomotor activity recorded during heroin self-administration (infrared sensors in 8 operant chambers; 4/group) revealed reduced activity in THC-exposed rats during the acquisition ($P = .029$) and maintenance ($P = .021$) phases (Figure 6). No significant difference in locomotor activity was evident during the drug-free extinction phase ($P = .686$) demonstrating that the behavioral differences noted in the other self-administration conditions were in response to heroin intake.

CB_1 and μ Opioid Receptor G-protein Coupling

Agonist-stimulated [^{35}S]GTPγS Autoradiography. The effects of prenatal THC exposure was studied for μ- and CB_1-activated G-protein coupling. Basal [^{35}S]GTPγS binding was similar in vehicle- and THC-exposed animals suggesting that basal G-protein activity was unaltered in the brain regions

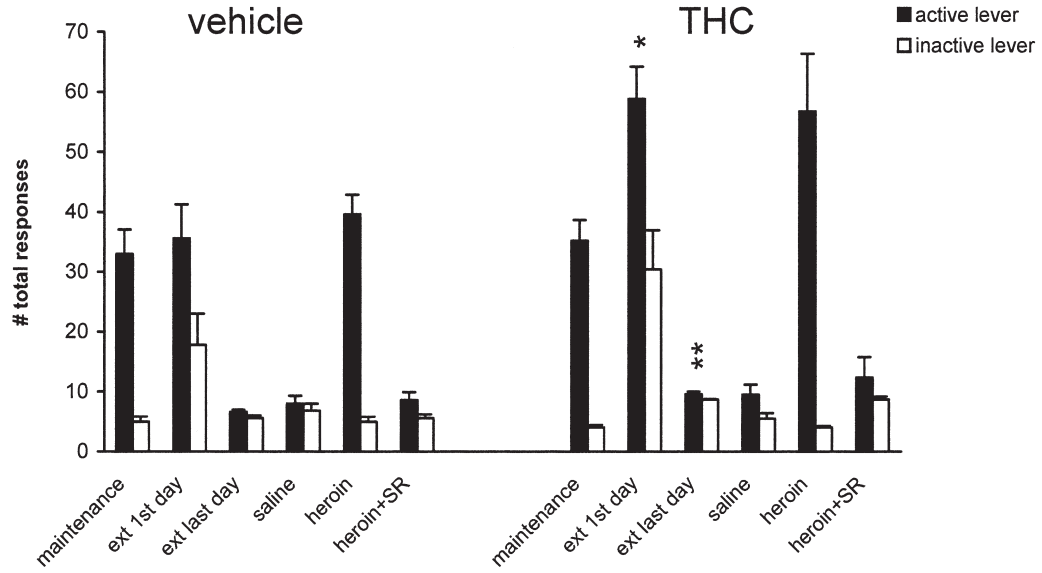

Figure 5. Effect of acute primings on the reinstatement of heroin-seeking behavior following heroin extinction in adult rats with perinatal THC or vehicle exposure. Each bar represents the mean ± SEM of active and inactive lever press over the last 3 days of heroin self administration (maintenance), during drug-free extinction (ext) and during the reinstatement sessions with heroin prime (0.25 mg/kg, s.c.) and SR 141716A (rimonabant; 3 mg/kg, i.p.) administration. $N = 5$ animals per group. *, $P < .02$; **, $P < .01$ as compared to vehicle control.

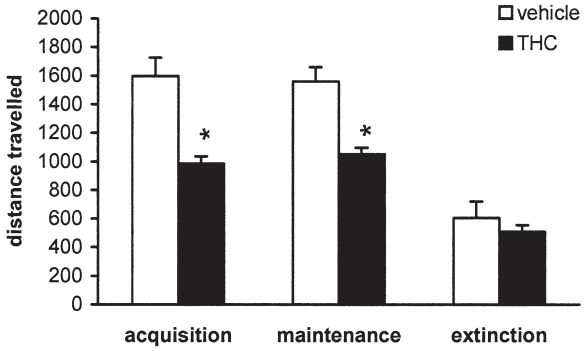

Figure 6. Locomotor activity measured during acquisition, maintenance and extinction (drug-free) phases of the self-administration paradigm in adult rats with perinatal vehicle or THC exposure. Values are expressed as mean ± SEM for 4 animals per group.

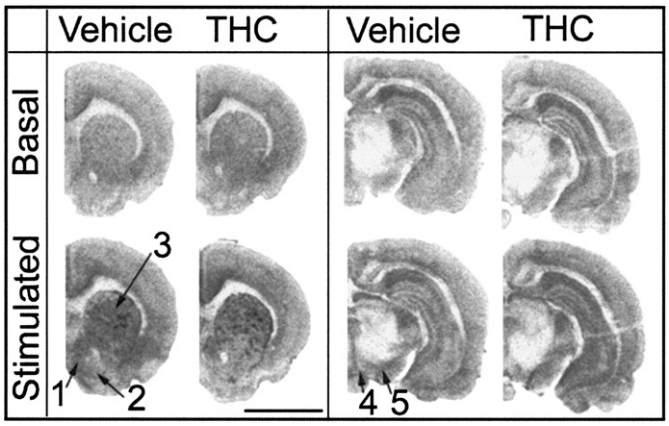

Figure 7. DAMGO-stimulated [^{35}S]GTPγS binding autoradiography at the level of the striatum and brainstem of perinatal THC- and vehicle-pretreated adult animals. Basal and DAMGO-stimulated [^{35}S]GTPγS binding are presented in the upper and lower panels, respectively. 1, nucleus accumbens (NAc) shell; 2, NAc core; 3, caudate-putamen; 4, ventral tegmental area; 5, substantia nigra. Note reduction of DAMGO-stimulated [^{35}S]GTPγS binding in the NAc shell and increase in the substantia nigra of THC-pretreated animals. Scale bar = .05 cm.

studied (Table 1). The prenatal THC exposure was only associated with a trend increase in WIN 55,212-2 [^{35}S]GTPγS binding in the NAc core ($F_{(1,8)}$ = 3.95; P = .08). No statistical group differences were found in the caudate-putamen, NAc shell, substantia nigra or ventral tegmental area (Table 1).

In adult animals, THC prenatal exposure was significantly associated with decreased DAMGO-stimulated [^{35}S]GTPγS binding in the medial shell of NAc ($F_{(1,6)}$ = 7.5; $P < .05$; Figure 7). In contrast, a significant increase was found in the substantia nigra ($F_{(1,7)}$ = 27.83; $P < .002$) of the THC-exposed rats. No statistical differences were detected in the caudate-putamen, NAc core or ventral tegmental area between the groups (Table 1).

Opioid Peptide mRNA Expression

PENK. Figure 8 shows striatal and amygdala subregions measured for PENK mRNA levels in adult rats. Prenatal THC exposure significantly increased PENK mRNA in the NAc core ($F_{(1,8)}$ = 85.46; $P < .0001$) and NAc medial shell ($F_{(1,8)}$ = 9.28; $P < .02$) as well as the central ($F_{(1,8)}$ = 12.4; $P < .008$) and medial ($F_{(1,6)}$ = 31.36; $P < .002$) amygdala nuclei of THC-exposed rats at PND 62 (Figure 9). No significant differences were observed in the caudate-putamen ($F_{(1,8)}$ = 2.17; P = .19). Examination of the striatum of the PND 2 rats showed significantly decreased PENK transcript levels in the NAc of THC pups ($F_{(1,9)}$ = 5.475; P = .04) and a trend in the caudate-putamen ($F_{(1,9)}$ = 4.34; P = .07; Figure 9). Amygdala levels were not well matched in the PND 2 animals which prevented reliable measurements of PENK in this region.

Preprodynorphin. The preprodynorphin mRNA levels were also studied in the caudate-putamen and NAc of adult rats. As shown in Table 2, the prenatal THC exposure did not affect the preprodynorphin gene expression in these regions.

Discussion

The present study provides evidence of enduring behavioral and neural impairments related to the opioid reward/stress limbic system in adult rats exposed prenatally to THC. Hyperactivity of the mesocorticolimbic enkephalinergic system was identified in adulthood that appeared a counteractive response to blunted PENK gene expression during the repeated THC exposure in early development.

The most direct experimental animal model to assess reward and reinforcement effects of drugs of abuse is the self-adminis-

Table 1. Effect of THC or Vehicle Perinatal Exposure (Gestation Day 5 to Postnatal Day 2) on Basal As Well As μ- and CB_1-Stimulated [^{35}S]GTPγS Binding in Adult Rat Brain (Postnatal Day 62)

	Basal		% Stimulation	
	Vehicle	THC	Vehicle	THC
DAMGO stimulated [^{35}S]GTPγS binding (μCi/g)				
Caudate-putamen	69.7 ± 7.5	78.2 ± 2.6	150.6 ± 15.6	155.5 ± 9.4
Nucleus accumbens core	78.5 ± 11.1	70.9 ± 10	52.9 ± 10.6	78.4 ± 10.8
Nucleus accumbens shell	70.4 ± 10.2	86.2 ± 7.3	133.3 ± 7.2	94.4 ± 12.2*
Substantia nigra	86.4 ± 11.1	93.6 ± 7.6	43.3 ± 5.4	77.4 ± 3.9**
Ventral tegmental area	49.4 ± 7	41.9 ± 6.9	88 ± 18.2	118.4 ± 15.6
WIN55,212-2 stimulated [^{35}S]GTPγS binding (μCi/g)				
Caudate-putamen	111.2 ± 11.2	114.4 ± 1.6	83.4 ± 3.3	99.9 ± 10.6
Nucleus accumbens core	123.8 ± 15.9	128.2 ± 6.4	29 ± 6.1	71.7 ± 20.5
Nucleus accumbens shell	95.7 ± 6.9	110.8 ± 13.9	53.3 ± 6.9	54.2 ± 8.5
Substantia nigra	166.6 ± 13.6	214.9 ± 16.7	240.9 ± 52.7	207.41 ± 33.3
Ventral tegmental area	89.6 ± 8.1	113.6 ± 9.1	62.5 ± 6.3	70.9 ± 2.9

Values are the mean ± SEM.
*, $p < .05$; **, $p < .01$; as compared to the vehicle control.

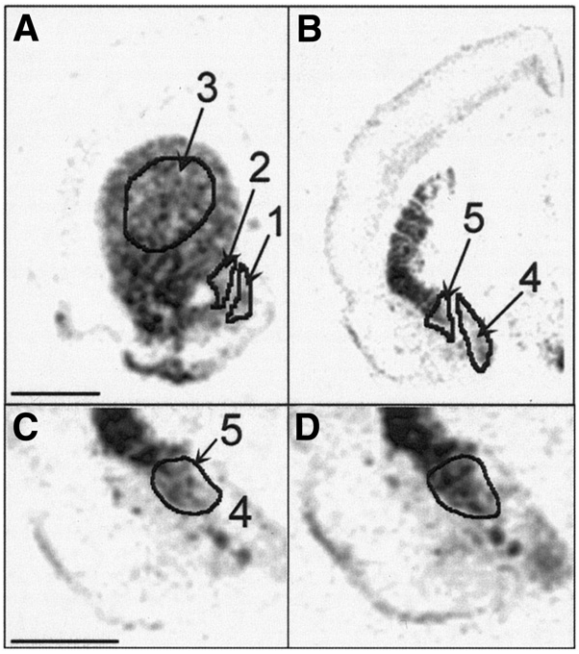

Figure 8. Preproenkephalin (PENK) hybridization signal in the rostral striatum **(A)** and amygdala **(B, C, D)** of adult rats (postnatal day 62). Panels **A** and **B** show the general distribution pattern of PENK in vehicle-treated control animals. Note increased PENK mRNA expression in the central amygdala of THC-exposed rats **(D)** as compared to vehicle controls **(C)**. Longer film exposure, which enhanced the hybridization signals, was carried out for measurements of the medial amygdala. Outlined regions represent the areas measured: 1, NAc shell; 2, NAc core; 3, caudate-putamen; 4, medial amygdala; 5, central amygdala. Scale bar in panels **A** and **B** = .2 cm. Scale bar in panels **C** and **D** = .1 cm.

tration paradigm, which reliably mimics the intake behavioral patterns seen in humans for most addictive drugs. A fixed ratio-1 schedule of self-administration showed that prenatal THC exposure did not lead to enhanced heroin acquisition or intake in adulthood. However, a number of specific behavioral findings point to significant opiate sensitivity as a consequence of early cannabinoid exposure given that THC adult animals: (i) exhibited a shorter latency to the first active lever press during the stable phase of drug acquisition; (ii) responded more to lower doses of heroin in the dose-response curve; (iii) had increased rates of responding following exposure to a mild stress; and (iv) showed higher levels of heroin-seeking during drug extinction as compared to the vehicle group. These findings would appear to suggest long-term vulnerability in the motivation to self-administer heroin rather than just altered sensitivity to the drug's reinforcing effects. Vertical upward shift of the dose-response curve that was evident with the lower doses of heroin is, however, usually hypothesized to reflect both a decrease in the rewarding effect of heroin (as reflected by increased consumption) and increased motivation for the drug (as evidenced by increased peak of the dose-response curve; (Ahmed and Koob 2005). Prenatal THC-exposed animals did not exhibit increased heroin intake except, for example, extinction conditions, when they maintained higher heroin-seeking behavior than control animals.

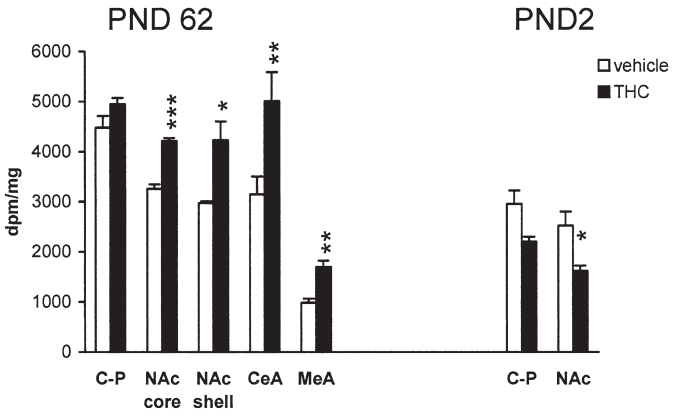

Figure 9. PENK mRNA expression levels in the various brain areas of adult postnatal day 62 (left panel) and post-natal day 2 (right panel) rats with perinatal THC or vehicle exposure. C-P, caudate-putamen; CeA, central amygdala; MeA, medial amygdala. Values are expressed as mean ± SEM (dpm/mg) for 5 animals per group. *, $P < .05$; **, $P < .01$; ***, $P < .001$ as compared to the vehicle control.

Table 2. Preprodynorphin mRNA Expression (dpm/mg) in the Striatum of Adult Rats (Postnatal Day 62) with Perinatal Exposure (Gestation Day 5 to Postnatal Day 2) to THC or Vehicle

	Vehicle	THC
Caudate-putamen	541 ± 3.08	549.5 ± 5.5
Nucleus accumbens core	1326 ± 35	1280 ± 33
Nucleus accumbens shell	1513 ± 27	1472 ± 34

Values are expressed as a mean ± SEM.

Relapse behavior induced after approximately 21 days of drug extinction by an acute priming dose of heroin did not differ significantly between THC and control animals, though THC offspring showed a trend for higher heroin-seeking. Moreover, the blockade of heroin-seeking behavior by the CB_1 receptor antagonist Rimonabant (SR 141716A) was similar in both pretreatment groups. It cannot, however, be excluded that the doses used might not have been low enough to dissociate the differential sensitivity between the THC- and vehicle-pretreated groups. Nevertheless, these results strengthen previous studies and add further support for the cross interaction between opioid and cannabinoid systems in the modulation of central mechanism underlying relapse (De Vries et al 2003; Fattore et al 2003; Navarro et al 2001).

In addition to long-term effects of THC on adult heroin-seeking behavior, there was a profound disruption of the PENK mesocorticolimbic system even after approximately 60 days following THC perinatal exposure. These time periods would be comparable to human subjects studied as young adults following prenatal cannabis exposure into the mid-gestational stage of development. The selective alteration of the PENK gene in mesocorticolimbic neural populations is intriguing since enkephalin is well known to modulate hedonic state (Kelley 2004; Skoubis et al 2005). It has been speculated that Enkephalin is critical for appetitive, but not consummatory, reward behaviors (Hayward et al 2002; Kelley 2004). Moreover, the nucleus accumbens and amygdala are highly involved in emotional regulation, reward, goal-directed behavior, and motivation (Cardinal et al 2002; Kelley 2004; Koob 1999). No PENK mRNA changes were evident in the dorsal striatum that is most associated with sensorimotor function (Nakano et al 2000). Preprodynorphin mRNA, which is also abundantly expressed in the striatum and known to be involved in the long-term regulation of other drugs of abuse, such as psychostimulants (Svensson and Hurd 1998) and opiates (Tjon et al 1997), was not altered in

THC-exposed offspring. PENK and preprodynorphin are co-expressed in subpopulations of NAc neurons (Curran and Watson 1995), so the lack of alterations on the preprodynorphin mRNA emphasize the PENK-selective opioid involvement in the long-term effects of THC in the current model. It should be noted that some studies have been published regarding perinatal cannabinoid exposure with discrepant observations on opioid peptide mRNA and receptor binding (Corchero et al 1998; Perez-Rosado et al 2000; Vela et al 1998). These discrepancies are most likely due to differences in, for example, sex of the animal, as well as the time course and route of drug administration. The current study is the first to use a route of administration that most mimics the human pharmacokinetic profile of marijuana smoking in pregnant women and is limited predominantly to the prenatal period.

The preferential limbic association of early THC administration is not unique to exposure during the prenatal stage of development. We have previously observed enhanced PENK mRNA expression, though restricted to the NAc shell of the striatum (amygdala was not studied), in adult animals exposed to THC during adolescent development (Ellgren et al, in press). Interestingly, in the adolescent model, the relative enhanced PENK expression in the NAc shell was maintained in THC animals even after heroin self-administration that reduced the striatal opioid transcription. We hypothesized that increased NAc PENK mRNA reflected an allostatic compensatory response during the drug-free period to reduced transcription during the active exposure to THC. This was predicted since THC, acting on CB_1 receptor which are $G_{i/o}$ protein-coupled receptors (Howlett et al 2002), should negatively regulate PENK transcript. Indeed, the current findings revealed a decrease of the PENK mRNA expression immediately after THC exposure during early development.

Disturbance of PENK in the medial and central amygdala has been predominantly associated with stress and anxiety (Drolet et al 2001; Kang et al 2000) since these nuclei are critical components of the extended amygdala. Subtle, but significant, increased heroin seeking was evident in THC-exposed animals following a mild food deprivation stress. The choice of food deprivation as a mild stress model could be questioned since the cannabinoid system is involved in food related behavior (Di Marzo and Matias 2005). The fact that the groups did not differ in weight either at birth or at the start of the self-administration experiment as young adults supports that the increase in heroin intake following food deprivation is not related to an alteration of feeding behavior. Drug abstinence, particularly during its early phase, is a very stressful event for drug-dependent subjects. Thus, the marked increase of heroin-seeking in THC-pretreated animals, especially during the first day of heroin extinction, could reflect a behavioral response to stress which intensifies the motivation for drug use. Early cannabis exposure has been shown to predispose individuals to anxiety-related behavior in later life (Patton et al 2002). Increased PENK as that present in the THC-exposed animals is, however, normally associated with a reduced anxiety response (Kang et al 2000). Paradoxically, acute and chronic elevation of corticosterone that facilitate potentiation and termination of stress response, respectively, both elevate PENK in various brain regions (Ahima et al 1992). Systematic evaluation of the glucocorticoid system is necessary with the current prenatal THC model to fully understand the potential role of the stress system in contributing to the PENK mesocorticolimbic mRNA levels. It also has to be determined whether the elevated PENK mRNA state reflects compensation for tonically reduced enkephalin peptide levels.

Long-term alteration of prenatal THC on the adult NAc opioid system was also apparent for the μOR coupling. THC-exposed offspring had significantly decreased μOR function in the NAc shell, a key brain region in reward processing (Cardinal et al 2002; Kelley 2004), which would suggest decreased opioid reward. Possible evidence of reward deficit in the THC animals was the apparent vertical shift of the dose-response curve. The NAc also strongly modulates locomotor function and THC animals had decreased locomotion in response to heroin intake. Reduced opiate-induced locomotor activity and reward is normally characteristic of animals lacking μORs (Chefer et al 2003; Contarino et al 2002).

Cannabinoid CB_1 and μOR exhibit overlapping neuroanatomical distribution, are members of the G-protein coupled family of receptors and modulate similar transduction systems (Corchero et al 2004; Vigano et al 2005). Despite the significant alteration of the μOR coupling, the prenatal exposure to THC was not associated with persistent changes in WIN 55,212-2-stimulated [^{35}S]GTPγS binding in adulthood. Indeed, the only alteration detected was a trend for increased cannabinoid-stimulated G-protein coupling in the NAc core. The lack of significant effect on CB_1 receptor function with the long drug-free period after repeated cannabinoid treatment is consistent with the literature showing apparent normalization of desensitized CB_1 receptors over time (Garcia-Gil et al 1999).

It is important to note that there was also evidence of non-limbic opioid alterations in adult animals with prenatal THC exposure. Increased μOR signaling observed in the substantia nigra would predict elevated dopamine levels in output regions, for example, the dorsal striatum, since stimulation of opioid receptors reduce inhibitory GABA-mediated regulation of dopamine cell firing (Johnson and North 1992). Acute and chronic effects of cannabinoids in adulthood on striatal dopamine release have been documented (Malone and Taylor 1999; Tanda et al 1997), but studies of the in vivo dopamine system following protracted THC withdrawal has yet to be evaluated.

In conclusion, the results of this study would suggest that adult subjects exposed prenatally to THC do not show an enhanced risk for heroin intake under normal conditions. However, the neural mesocorticolimbic systems linked to reward and stress are significantly altered and adult THC offspring have enhanced heroin-seeking during extinction. The PENK mesocorticolimbic system appears to be a significant allostatic marker that could underlie such long-term behavioral vulnerability.

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