Available online at www.sciencedirect.com
ScienceDirect
Pharmacology, Biochemistry and Behavior 86 (2007) 334–345
PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR
www.elsevier.com/locate/pharmbiochembeh

Enhancement of endocannabinoid signalling during adolescence: Modulation of impulsivity and long-term consequences on metabolic brain parameters in early maternally deprived rats

Eva María Marco [a,c], Walter Adriani [a], Rossella Canese [b], Franca Podo [b], Maria Paz Viveros [c], Giovanni Laviola [a,*]

[a] Unit of Behavioural Neurosciences, Department of Cell Biology and Neurosciences, Istituto Superiore di Sanità, Roma, Italy
[b] Unit of Molecular and Cellular Imaging, Department of Cell Biology and Neurosciences, Istituto Superiore di Sanità, Roma, Italy
[c] Depto. de Fisiología (Fisiología Animal II), Facultad de Biología, Universidad Complutense de Madrid, Spain

Received 21 March 2006; received in revised form 3 October 2006; accepted 4 October 2006
Available online 13 November 2006

Abstract

Pharmacological modulation of the endocannabinoid system is a novel but poorly explored field for potential therapy. Early maternal deprivation represents an animal model for specific aspects of neuropsychiatric disorders. This study explored whether a pharmacological manipulation of the endocannabinoid system at adolescence may restore altered phenotypes resulting from early maternal deprivation. Wistar male rats, maternally deprived for 24 h on postnatal day (PND) 9, were administered the fatty-acid amide hydrolase (FAAH) inhibitor URB597 (0, 0.1 or 0.5 mg/kg/day) for six days during adolescence (PND 31–43), while tested in the intolerance-to-delay task. Deprived (DEP) adolescent rats showed a trend for higher impulsivity levels and an increased locomotor response to novelty when compared to non-deprived (NDEP) controls. The low dose of URB597 effectively decreased impulsive behaviour specifically in DEP subjects. Moreover, long-term metabolic brain changes, induced by drug treatment during adolescence, were detected in DEP animals using proton magnetic resonance spectroscopy (^{1}H MRS). Significant changes were only found within the hippocampus: *N*-acetyl-aspartate and total creatine were up-regulated by the low dose; glutamate and glutamate plus glutamine were conversely down-regulated by the higher dose. In summary, administration of URB597 during adolescence increased self-control behaviour and produced enduring brain biochemical modifications, in a model for neuropsychiatric disorders.

Keywords: Maternal deprivation; Adolescence; Cannabinoid drugs; Fatty-acid amide hydrolase (FAAH); Intolerance-to-delay; URB597; Rat; Magnetic resonance spectroscopy (MRS)

1. Introduction

The existence of an endocannabinoid system is nowadays well established. Endogenous ligands, known as endocannabinoids, activate metabotropic receptors coupled to Gi/o proteins, mainly CB_1 and CB_2, with the former being densely expressed within the central nervous system (Herkenham et al., 1990; Di Marzo et al., 2002). Deactivation of the endocannabinoid signalling in the brain requires two cooperative mechanisms: carrier-mediated transport into cells and intracellular hydrolysis by a fatty-acid amide hydrolase (FAAH) (Cravatt et al., 1996). The endocannabinoid system has been involved in the modulation of diverse physiological processes such as control of motor activity, analgesia, memory and cognitive functions and modulation of emotions (Fride, 2005; Piomelli, 2003; Ramos et al., 2005). In recent years the endocannabinoid system has become an appealing objective for the research of novel therapeutic drugs. A number of animal studies and clinical trials indicate that cannabinoids may have clinical application in a wide range of pathological states (Croxford, 2003; Jonsson et al., 2006; Williamson and Evans, 2000). Cannabinoids may be suitable in the management of anxiety disorders, particularity, in the treatment of diseases associated with inappropriate

* Corresponding author. Tel.: +39 06 4990 2105; fax: +39 06 495 7821.
E-mail address: laviola@iss.it (G. Laviola).

0091-3057/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.pbb.2006.10.006

retention of aversive memories such as post-traumatic stress disorders (Haller et al., 2004; Marsicano et al., 2002), and have also been proposed as an efficient tool for the treatment of depression and stress-related disorders (Viveros et al., 2005b; Witkin et al., 2005). The endocannabinoid system may represent a therapeutic target for other neuropsychiatric disorders: indeed, a dysregulation of the endocannabinoid system has been implicated in neurocognitive disorders such as schizophrenia (Leweke et al., 1999; Schneider et al., 1998). More recently, modulation of the cannabinoid system has been proposed as a possible therapeutic approach, to improve some of the behavioural anomalies seen in the attention deficit/hyperactivity disorder (ADHD) (Adriani and Laviola, 2004).

Putatively, the development of compounds that indirectly enhance the endocannabinoid signalling might represent a more convenient therapeutic tool, achieving greater clinical efficacy with fewer side effects. In this sense, both inhibitors of metabolism or transport of endocannabinoids have been developed and successfully tested on animal models of anxiety and depression (Hill and Gorzalka, 2004; Kathuria et al., 2003). URB597 is a potent and selective inhibitor of FAAH, the enzyme responsible for the degradation of the endogenous cannabinoid anandamide, i.e. arachidonyl–ethanolamide (AEA). URB597 increases the levels of fatty-acid ethanolamides, and magnifies responses to anandamide, without exerting the typical signs of CB_1 receptor activation, namely catalepsy, hypothermia and hyperphagia (Fegley et al., 2005; Kathuria et al., 2003). The notion of a localised increase in endocannabinoid levels raises an obvious possibility for real usage of these compounds (Fowler et al., 2005). CB_1 cannabinoid receptors are present from the first days after birth but reach adult levels only after the first weeks of life (Belue et al., 1995; McLaughlin and Abood, 1993; Rodriguez de Fonseca et al., 1993). During the perinatal period, CB_1 receptors exhibit an atypical transient distribution that seems to suggest a specific involvement of the endocannabinoid system in brain development (Fride, 2004; Ramos et al., 2002). Inhibition of the hydrolysis of anandamide by URB597 produces anxiolytic-like effects not only in adult rats, but also in the isolation-induced ultrasonic emission test in rat pups (Kathuria et al., 2003). Thus, the endocannabinoid system might be involved in the control of emotional states since early developmental stages.

The periadolescent period represents a crucial phase in development, characterised by enhanced neurobehavioural plasticity and specific neurobiological and behavioural features, such as novelty seeking, impulsivity and risk taking (Laviola et al., 1999, 2003; Spear, 2000; Adriani and Laviola, 2004). During this period, maturation and rearrangement of major neurotransmitter pathways, including the endocannabinoid system, are still developing (Rodriguez de Fonseca et al., 1993). There is relatively scarce information about a possible role of the endocannabinoid system on self-control behaviours. Impulsivity, one of main features of ADHD syndrome, is defined as a reduced ability to tolerate a delay of reward. It can be studied in animal models by providing a choice between a large and delayed *vs*. a smaller and immediate reinforcement (Evenden and Ryan, 1999). As the length of the delay increases, animals progressively shift their preference from the large and delayed towards the smaller and immediate reward. In humans, acute Δ^9-tetrahydrocannabinol (THC) has been reported to increase certain forms of impulsive behaviour, while not affecting others (McDonald et al., 2003). However, recent findings on animal models support the notion for an involvement of the endocannabinoid system, particulary CB_1 receptors, in the modulation of impulsive/self-control behaviour. Indeed, a subpopulation of adolescent spontaneously hypertensive rats (SHR), showing an elevated basal level of impulsive behaviour, were also characterised by a reduction of CB_1 cannabinoid receptor density in the prefrontal cortex (Adriani and Laviola, 2004). Moreover, acute administration of the cannabinoid agonist WIN 55,212 was effective in modulating levels of self-control behaviour in SHR adolescent animals, a putative model of ADHD (Adriani and Laviola, 2004). Therefore, as impulsive choice can be a feature of diverse neuropsychiatric disorders, manipulation of the endocannabinoid system might represent a novel and useful therapeutic tool.

Stressful events early in life are reported to be more prevalent among patients with an adult life psychiatric disorder. Neonatal aversive experiences, such as rat pups being reared in neglect-like environments, lead to long-term alterations in the neurobiology, physiology and emotional behaviour of adult animals, resembling depressive and schizophrenic symptomatology (Ellenbroek et al., 1998; Pryce et al., 2005). Early maternal deprivation is considered as an animal model of early life stress. A single 24 h period of maternal deprivation leads to an enhanced sensitivity of the hypothalamus–pituitary–adrenal axis to stressors (Levine et al., 1991), to a disruption of prepulse inhibition of the acoustic startle response (Ellenbroek et al., 1998), to an increased behavioural response to amphetamines (Zimmerberg and Shartrand, 1992), and to a retardation in normal development, especially of the dopaminergic system (Ellenbroek et al., 2005).

In the present study we aimed to explore whether a pharmacological manipulation of the endocannabinoid system at adolescence may render a useful therapeutic tool for altered phenotypes resulting from early maternal deprivation (see e.g. Macrì and Laviola, 2004). We therefore deprived rat pups from dams for 24 h early in life, postnatal day (PND) 9, and tested them for impulsivity at adolescence (PND 31–43) when a subchronic treatment with URB597 was also administered. Considering typical cannabinoid effects on locomotion, and its potential interference with performance on the impulsivity task, levels of locomotor activity in adolescent rats were also assessed both in drug-free state and upon a cannabinoid challenge.

Long-term consequences of URB597 administration were also assessed within the maternally deprived group. From the point of view of cannabinoid effects, the hippocampus (HIP) has been pointed out as an important target mediating cannabinoid-induced behavioural effects (Jiang et al., 2005). Moreover, this brain region is heavily connected to a number of other structures known to play a role in the delayed gratification choice task (Cheung and Cardinal, 2005), like the dorsal/ventral striatum (STR) (Cardinal et al., 2004) or the nucleus accumbens (NAcc), whose dysfunction has been suggested to be a key element in the

neuropathology of impulsivity (Cardinal et al., 2001). In order to assess possible long-lasting changes at adulthood, produced by increased brain endocannabinoid levels during adolescence, we used non-invasive in vivo proton magnetic resonance spectroscopy (^{1}H MRS) approaches to analyse basal level metabolites in three brain regions (HIP, STR and NAcc).

2. Material and methods

2.1. Animals and rearing conditions

Experimental animals were the male offspring of time pregnant Wistar rats (Harlan, Italy). Animals were housed in an air-conditioned room (temperature 21±1 °C, relative humidity 60±10%) in a standard 12-h light–dark cycle (lights on at 7.00 a.m.), with free access to water and food (Mucedola, Italy).

Although only male subjects were used in the present experiments, litters were culled to 6 males and 2 females, when possible, on the day after birth (birth being postnatal day 0, PND 0). Females were left to avoid potential carry-over effects of rearing rats in a sexual segregation condition (Cirulli and Laviola, 2000). A total of eight litters were used, and randomly assigned to one of the two neonatal conditions (non-deprived, NDEP *vs*. deprived, DEP). At weaning (PND 21) male pups were housed in pairs of sibling subjects, originating from the same litter. The pair of siblings housed in the same cage was assigned to the same dose of drug treatment, whereas siblings pairs housed in separate cages were assigned to receive one of the three levels of pharmacological treatment during the adolescent period (for literature see Adriani and Laviola, 2004; Laviola et al., 2003; Spear, 2000).

2.2. Maternal deprivation protocol

Half of the litters were deprived (DEP) from their mother for 24 h as described by Ellenbroek (Ellenbroek et al., 1998). In brief, on PND 9 the dam was removed, pups were weighted, and the entire offspring then remained undisturbed in the home cage. During the 24 h deprivation period, pups were housed in an adjacent room, under the same temperature and lighting conditions, to disallow communication with their mothers by use of ultrasound vocalizations (Hofer et al., 1994). No food or water was available during this period. On PND 10 pups were weighted again, and dams were then replaced in their corresponding cages. Pups from the control litters (NDEP) were submitted to a similar manipulation: dams were briefly removed both on PND 9 and 10, to allow weighing of the offspring, but dams were immediately replaced in their home cages. Thus, the NDEP offspring remained undisturbed with their mothers in the animal room. Pups were henceforth left undisturbed except for changing the sawdust once until weaning.

2.3. Impulsivity: apparatus and schedule

At adolescence (from 31 to 43 days old) all the animals were tested for levels of behavioural impulsivity (Evenden and Ryan, 1999). We employed a variant of the classical intolerance-to-delay protocol, which has been developed and validated in our lab (Adriani et al., 2004). Two days before the experimental schedule started, animals were habituated to the special rewarding pellets (45-mg precision pellets: BioServ, Frenchtown, USA), and were food-restricted to increase motivation to work for food delivery. Animals were placed daily in computer-controlled operant chambers (Coulbourn Instruments, Allentown, USA), provided with two nose-poking holes, a chamber light, a feeder device, a magazine where food (the 45-mg pellets) was dropped, and a magazine light. Nose-poking in either hole was detected by a photocell and was recorded by a computer, which controlled food delivery. After a 20-min session, animals were returned to their home cages, where they were given standard rat chow (approx. 6 g. per rat), to keep animals at 80–85% of their free-feeding weight. Once the two-week impulsivity schedule was completed, animals were given free access to food.

During the "training" phase (6 consecutive days), nose-poking in one hole (H1) resulted in the delivery of one pellet, whereas nose-poking in the other hole (H5) resulted in the delivery of five pellets. After nose-poking and before food delivery, the chamber light was turned on for 1 s. After food delivery, the magazine light was turned on for 20 s, during which further nose-poking was recorded but had no consequences (time out). On the last day of the "training" phase, a delay (7.5 s) was inserted between H5 nose-poking and delivery of the five pellets. The chamber light was turned on during the length of this delay. Any additional nose-poking, taking place during this delay interval, was recorded but had no consequences. During the "test" phase (6 consecutive days), the delay between H5 nose-poking and delivery of the five pellets was fixed for each daily session and was increased progressively every day (15, 30, 45, 60, 75 and 90 s).

2.4. Pharmacological treatment

URB597 (Alexis USA, San Diego, CA, USA) was dissolved in Tween-80 (5%), polyethylene glycol (PEG) (5%) and saline (90%) and administered i.p. at a volume of 2 ml/kg body weight. Doses of 0.1 mg/kg and 0.5 mg/kg of URB597 were selected based on a pilot study and on available literature about the behavioural effects of this compound (Gobbi et al., 2005; Kathuria et al., 2003; Patel and Hillard, 2006). All animals were injected once daily for 6 days with either vehicle or URB597 (0.1 or 0.5 mg/kg i.p.). This drug treatment was administered only during the "test" phase of the impulsivity schedule, 30 min before the daily testing session.

2.5. Locomotor activity: apparatus and schedule

The apparatus consisted of opaque-Plexiglas compartments (70×30×35 cm) provided with eight infrared photobeams, placed on the wall a few centimeters from the floor. Beam interruptions were recorded by a computer provided with custom-made software. "Activity rate" was obtained automatically and expressed as number of beam interruptions during

5-min intervals. Locomotor activity was measured on a single session at least 10 days after finishing impulsivity testing. Undrugged animals were individually placed on the arena, and locomotor activity was registered for 30 min. Subsequently, animals were administered with the same drug dose they received at adolescence, and activity was registered for an additional hour in order to evaluate the acute effects of URB597 on locomotion. Locomotor activity data were expressed as averaged activity rate per pair of sibling rats coming from the same cage. The floor of the apparatus was cleaned after each rat was tested, and the test was carried out under dim illumination.

2.6. Magnetic resonance imaging and spectroscopy

At adulthood (>PND 70), the maternally deprived animals underwent MRI/MRS analyses, with the aim to measure any long-term modifications in biochemical parameters in relevant brain areas. Experiments were performed on a VARIAN Inova MRI/MRS system operating at 4.7 T, equipped with actively shielded gradient coils (maximum gradient strength 120 mT/m; rise time<150 μs), with a volume coil as transmitter and a surface coil constructed with a stereotaxic rat head holder as receiver (RAPID Biomedical, Germany). Animals were anaesthetised with isofluran 1.5–2.5% in O_2 flow (1 l/min).

Scout multi-slice T_1-weighted gradient-echo axial MR images (repetition time TR=134.3 ms, echo time TE=5.3 ms, number of scans NS=2, slice thickness 1 mm, FOV=30×30 mm^2, matrix 128×128) were acquired in *xy* planes to control rat head positioning. Sagittal multi-slice T_2-weighted spin-echo images were acquired (TR/TE=3000/70 ms, slice thickness 1 mm, NS=2, FOV=30×30 mm^2, matrix 128×256). In order to cover the whole brain, 15 contiguous slices were acquired. Single voxel localised ^{1}H MR spectra (PRESS, TR/TE=4000/23 ms, NS=256) were collected from different brain areas: HIP (33.6 μl), STR (58.8 μl) and NAcc (21 μl) as shown in Fig. 1a. Field homogeneity was optimised by manual shimming up to signal line widths ranging between 6 and 11 Hz for water signal. In the metabolite spectra, the water signal was efficiently suppressed by three CHESS pulses. The averages were sufficient to obtain good sensitivity in 17 min.

The region between 0.2 and 4.0 ppm of in vivo MR spectra was processed by using LCModel 6.1 fitting program (Provencher, 1993). The LCModel analysis calculates the best fits to the experimental spectrum as a linear combination of solution spectra of brain metabolites. Solution spectra of 18 brain metabolite and simulated spectra of macromolecules and lipids were included in the basis set. The estimated standard deviations are given by LCModel as a percentage of the estimated concentration. A percent standard deviation (%SD)<20% has been used in the present study as a rough criterion for estimates of acceptable reliability. Means over a group of LCModel analyses of spectra deriving from the same region reduced the uncertainties. The unsuppressed water signal acquired from the same VOI was used as internal reference for metabolite quantification (Keevil et al., 1998). For signals arising from overlapping resonances, the fitting program calculates the best fit of both the overall resonance and the separate contributions.

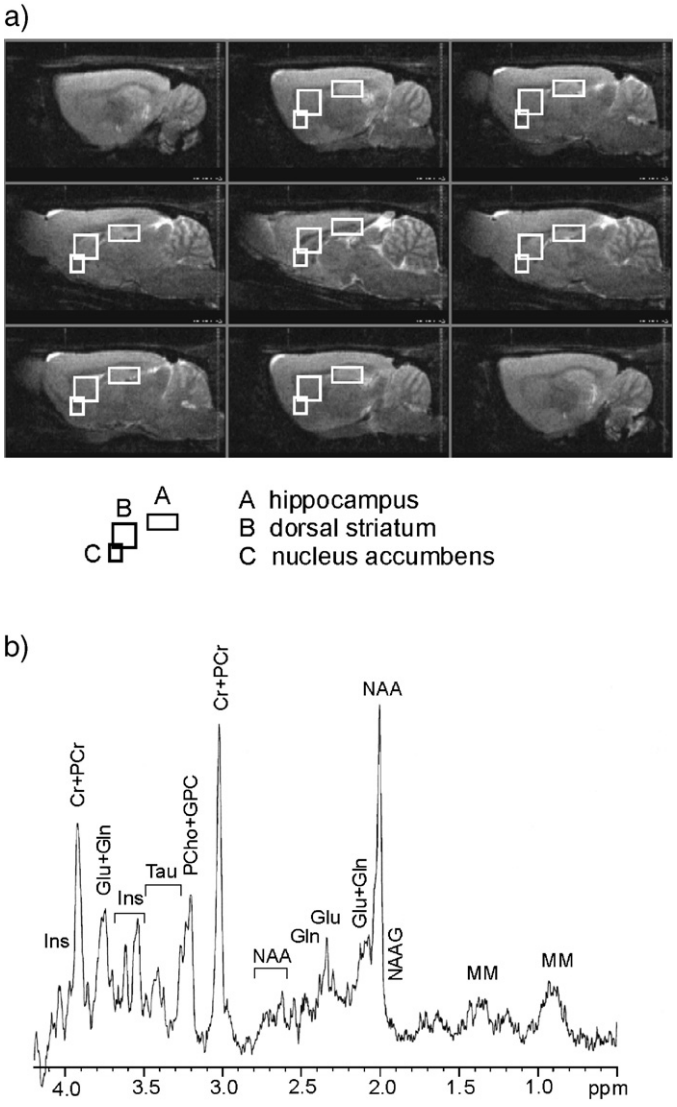

Fig. 1. a) Example of *in vivo* sagittal T_2-weighted spin-echo images of rat brain (TR/TE=3000/70 ms, slice thickness 1 mm, NS=2, FOV=30×30 mm^2, matrix 128×256). Voxels localised on HIP, STR and NAcc are indicated by the white rectangles. b) Example of *in vivo* ^{1}H MR spectrum acquired from hippocampus (PRESS, TR/TE=4000/23 ms, VOI=33.6 μl, NS=256). Principal brain metabolite levels measured within these volumes are reported in Table 3.

2.7. Design and data analysis

All data were analyzed by a *split-plot* ANOVA where the litter was the blocking factor. Neonatal condition was a between-litter factor (NDEP *vs.* DEP), whereas drug (URB597 dose: 0, 0.1, 0.5 mg/kg/day) and all the other variables were within-litter factors. Separate analyses were performed when allowed. Post-hoc multiple comparisons within a significant interaction were performed using the Tukey HSD test. A complete scheme of the experimental design is outlined in Fig. 2.

2.7.1. Body weight gain

Body weight of rats was measured regularly all along the experimental procedure. Body weight gain on each day (D) was expressed as percent increase relative to the reference day (R):

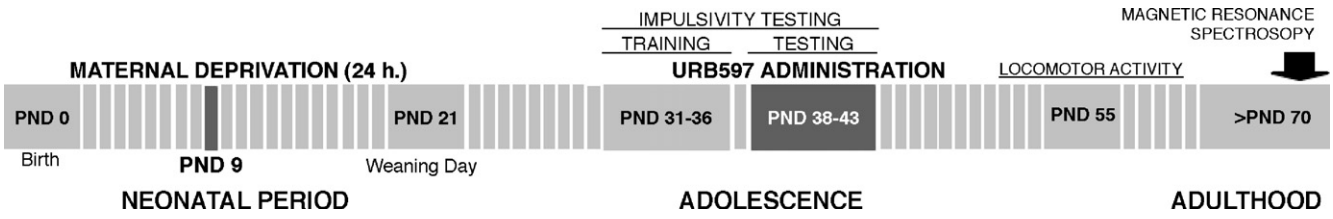

Fig. 2. Outline of the experimental design.

$\%\Delta BW = (BW_D - BW_R)/BW_R * 100$. For the maternal deprivation effect, PND 9 was considered as the reference day. Since subjects were not individually identified until weaning, body weight increment during infancy was calculated for each litter (average of 6 sibling males). At adolescence, because of the experimental schedule, animals were slightly food restricted. Therefore, the day before starting the schedule (PND 31) was considered as the reference day during this period. Body weight was then registered until adulthood, and the reference day was PND 45, when animals were provided with free access to food.

2.7.2. Impulsivity test

Three parameters were considered: 1) the percentage of large-reward preference (H5/(H5+H1)*100); 2) the slope of the preference–delay curve, calculated using Microsoft Excel "slope" function, with the large-reward preference as *Y*-axis data and log(delay+1) as *X*-axis data, (Evenden and Ryan, 1999); and 3) the amount of non-reinforced responding (i.e. nose-poking during the timeout or the length of the delay, when it was without scheduled consequence). The design included a delay factor. Since some of the animals were not sensitive to the procedure, data were screened for individual failure to perform the operant task correctly. Indeed, those animals showing either large-reward preference lower than 50% at the end of the training phase, or large-reward preference at delay=90 higher than that shown at delay=0, were excluded. Across groups, the quantity of excluded animals did not vary considerably (n=0/2 per group).

2.7.3. MRI/MRS

Statistical analysis was performed by using a set of randomised block ANOVAs. Only the maternally deprived group was assessed with MRS. Each of the metabolite levels within each of the brain regions were analyzed separately. In the Results section, we report only those metabolites whose URB597-induced alterations were statistically significant ($p<0.05$) when compared to the vehicle-exposed group.

3. Results

3.1. Body weight gain

During infancy, as expected, body weight increased as animals were growing up [age: $F(1,6)=442.2$, $p<0.001$], and maternal deprivation induced a moderate but significant decrement on body weight gain on male rats [neonatal condition: $F(1,6)=6.56$, $p<0.05$]. Decreased body weight gain was only evident immediately after the deprivation protocol [$F(1,6)=908.9$, $p<0.001$], but did not remain significant at weaning age [$F(1,6)=0.988$, NS] (Table 1). During adolescence, age-related body weight increment [age: $F(4,56)=449.7$, $p<0.001$] was accompanied by an increase in body weight gain as a consequence of cannabinoid administration [age×drug: $F(8,112)=2.61$, $p<0.05$]. Both doses of URB597 increased body weight gain to a similar extent during the whole cannabinoid treatment (Fig. 3). At adulthood, no long-term consequences of either early maternal deprivation or adolescent cannabinoid treatment were evident (data not shown), ANOVA only rendering a significant overall effect of age [$F(5,70)=2680$, $p<0.001$].

3.2. Effects of URB597 on impulsivity during adolescence

3.2.1. Choice between reinforcers

As expected, after the training period, animals developed a significant preference for the H5 hole, delivering the large reinforcer. This preference progressively shifted towards the H1 hole (delivering the smaller and immediate reinforcer) when the length of the delay was increased, as demonstrated by a significant effect for the delay factor [$F(7,98)=22.84$ $p<0.001$]. ANOVA also revealed significant interactions between drug and delay [$F(14,196)=2.12$ $p<0.05$], and between the three main factors [neonatal condition×drug×delay, $F(14,196)=1.78$ $p<0.05$]. Post-hoc comparisons revealed no significant drug effects within NDEP animals. Conversely, at the delay of 75 s, DEP animals treated with the low dose of URB597 displayed higher preference for the large and delayed reward, when compared to vehicle-injected ones. Therefore, within DEP group, levels of impulsivity were reduced upon administration of the low dose of URB597. In order to further clarify this scenario, separate analyses were performed for NDEP and DEP adolescent male rats. Both ANOVAs rendered the expected significant effect of the delay factor [NDEP: $F(7,49)=10.36$; DEP: $F(7,56)=16.92$, $p<0.0001$], as well as a significant interaction between drug and delay [NDEP: $F(14,98)=2.06$,

Table 1
Maternal deprivation effect on mean (±SEM) body weight (BW) gain, expressed as percent increase (%) from the day prior to the deprivation (PND 9)

	Neonatal condition	
	NDEP	DEP
PND 10	10.18±0.36	−3.77±0.29***
PND 21	92.66±5.19	84.30±6.61
PND 28	226.04±10.75	223.69±9.31

***$p<0.001$ *vs.* the NDEP control group at the same age.
As subjects were not individually identified until weaning, the BW increment was calculated for each litter ($n=3/4$ per neonatal condition).

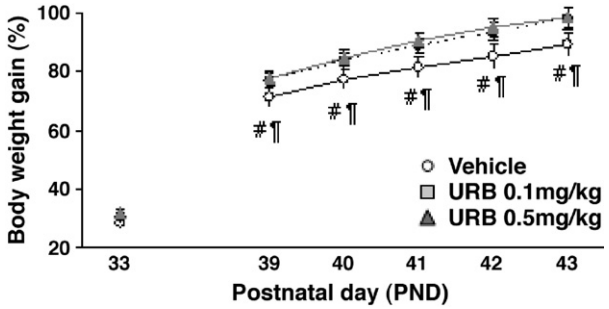

Fig. 3. Mean (±SEM) body weight (BW) gain during adolescent URB597 administration, expressed as percent increase (%) from the day before starting the schedule (PND 31). In the absence of significant differences, the two neonatal conditions were pooled ($n=15$ per group). $^{\#}p<0.05$ *vs.* animals treated with the low dose, ¶ $p<0.05$ *vs.* animals treated with the high dose.

$p<0.05$; DEP: $F(14,112)=2.38$, $p<0.01$]. Post-hoc comparisons again revealed no significant drug effects within NDEP animals. Conversely, within the DEP group, impulsivity-reducing properties of the low dose of URB597 became significant when the delay was fixed at 30 and 75 s (Fig. 4).

Further analyses, performed within each delay value, rendered a significant interaction between neonatal condition and drug [$F(2,28)=4.09$, $p<0.05$] at delay=90 s. Once again, the low dose of URB597 tended to increase self-control behaviour exclusively within DEP animals. Furthermore, in this occasion vehicle-injected DEP animals showed a marked tendency for higher impulsivity levels, compared to the corresponding NDEP controls.

3.2.2. Slope of the preference–delay curve

ANOVA did not render any significant interaction between neonatal condition and drug [$F(2,28)=1.44$, NS]. However, multiple comparisons performed within this interaction seemed to confirm the observations achieved by the analysis of the percent choice between reinforcers (Table 2). Modulation of the endocannabinoid system had no effects on NDEP controls, whereas the low dose of URB597 (0.1 mg/kg) showed a tendency to reduce the absolute value of the slope in DEP animals.

Table 2
Slope of the preference–delay curve

Condition	Treatment	Mean (SEM)
NDEP	Vehicle	−11.42±5.12
	URB (0.1 mg/kg)	−13.85±2.19
	URB (0.5 mg/kg)	−18.67±3.71
DEP	Vehicle	−17.42±2.33
	URB (0.1 mg/kg)	−7.23±2.70
	URB (0.5 mg/kg)	−17.53±5.23

Adolescent male rats, neonatally exposed to a single episode of maternal deprivation (24 h on PND 9), were tested in the intolerance-to-delay paradigm. Animals were administered with URB597, 30 min before the daily session, during the testing phase of the task ($n=7/8$ per group).

3.2.3. Non-reinforced responding

The nose-poking in either holes during the course of the timeout period or the delay interval had no scheduled consequences, and such a "non-reinforced" response was considered an index of motor impulsivity. As expected, a significant effect of delay was found [$F(7,98)=76.81$, $p<0.001$], as well as a significant effect of neonatal condition [$F(1,14)=5.73$, $p<0.05$] and a significant interaction between neonatal condition and delay [$F(7,98)=3.04$, $p<0.01$]. DEP adolescent rats showed a higher frequency of non-reinforced nose-poking in either holes, such difference reaching statistical significance at the higher delays, 60 and 75 s (Fig. 5). Cannabinoid administration had no significant effects on this parameter (data not shown). The expression of non-reinforced nose-poking behaviour during the length of the delay, when such a response

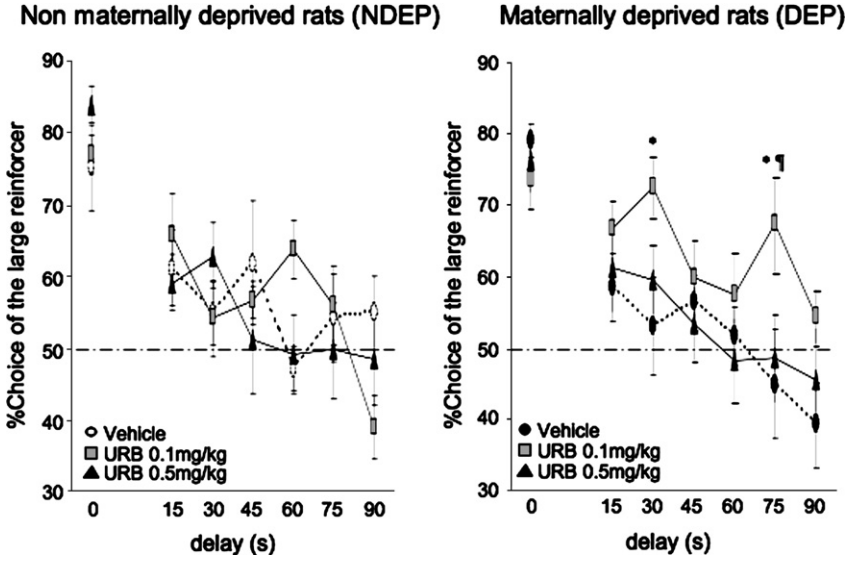

Fig. 4. Impulsivity test: mean (±SEM) choice (%) of the large and delayed reinforcer shown by adolescent rats during the impulsivity task. URB597 administration occurred 30 min before the daily session. The line indicates the 50% indifferent-choice value ($n=7/8$ per group). Within DEP animals: $^{*}p<0.05$ *vs.* vehicle-injected animals, ¶ $p<0.05$ *vs.* animals treated with the high dose.

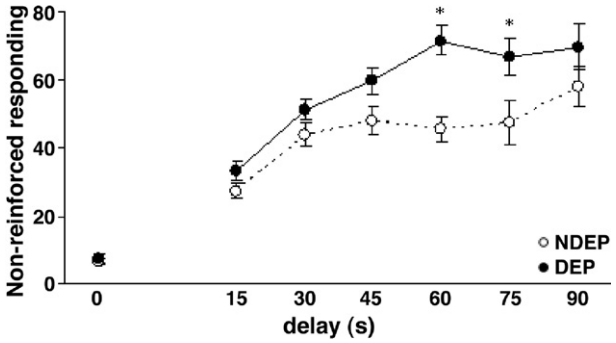

Fig. 5. Impulsivity test: mean (±SEM) frequency of non-reinforced responding at either holes (i.e., nose-poking when it was without any consequence) shown by adolescent rats during the impulsivity task. URB597 administration occurred 30 min before the daily session. In the absence of significant differences, animals with different levels of drug administration were pooled (n=21/24 per group). *p<0.05 *vs.* NDEP adolescent males.

is not rewarded nor punished, is considered an index of restlessness (Adriani and Laviola, 2003).

3.3. Locomotor activity

Spontaneous locomotor activity performed by drug-free animals rendered a significant overall effect of time [F(5,30)= 52.18, p<0.001], levels of locomotor activity showed a clear habituation profile after the first half of the session. The finding of a significant interaction between neonatal condition and time [F(5,30)=2.95, p<0.05] indicated that DEP animals exhibited a higher rate of activity in response to novelty compared to NDEP (Fig. 6). Accordingly, after acute drug administration, a main effect of time [F(11,88)=23.53, p<0.001] and a significant interaction between neonatal condition and time [F(11,88)=3.06, p<0.005] were found. Acute cannabinoid administration did not alter locomotor activity, suggesting that data from the intolerance-to-delay task were not biased by cannabinoid-induced effects on locomotor activity. Differences on locomotion as a long-term consequence of the neonatal adverse experience became more evident when analysing the performance of animals injected with vehicle during adolescence in a separate analysis. Similarly, a significant effect of time was found [baseline: F(5,30)=21.89; post-drug: F(11,88)= 5.99, p<0.001], as well as an interesting significant effect of neonatal condition [baseline: F(1,6)=9.26, post-drug: F(1,8)= 7.81, p<0.05]. Early maternal deprivation caused an increase on locomotor activity in response to novelty that was only evident on animals with a previous history of vehicle administration.

3.4. MRS-detected metabolic changes in different brain areas

The use of a stereotaxic holder together with the well contrasted and spatially resolved T_2-weighted images of rat brain enabled precise and reproducible positioning of the VOIs in the HIP, STR and NAcc brain areas. An example of in vivo spectrum acquired from HIP is shown in Fig. 1b, to represent the spectral quality achieved in this study. The high quality spectra allowed reliable quantification (%SD<20%) not only for the commonly observed *N*-acetyl-aspartate, total creatine and total choline resonances, but also for glutamine, glutamate, taurine and inositol in all the investigated brain regions. The quantitative results of these analyses are summarised in Table 3. The levels of metabolites are given in arbitrary units, relative to the unsuppressed water signal. Long TR and short TE parameters were selected in our pulse sequences, in order to minimize the error due to any potential carry-over influence of adolescent drug treatment on the relaxation times, and therefore to be able attribute any change in signal intensities to actual changes of metabolite levels.

In the STR and NAcc areas, no significant changes were found for any of the observed metabolites at any drug dose used. Statistically reliable changes were conversely evident in the HIP. An inverted U shaped profile was evident for *N*-acetyl-aspartate [F(2,14)=6.21, p<0.05], and total creatine [F(2,14)= 4.89, p<0.05]. Multiple comparisons revealed that these two metabolic parameters were increased as a specific consequence of adolescent exposure to the low URB597 dose, when compared to controls (ps<0.05). Indeed, the high-dose and control values did not differ significantly. Conversely, a significant dose-dependent reduction was found in glutamate, when considered alone (Glu), [F(2,14)=23.0, p<0.001], or together with glutamine (Glu+Gln), [F(2,14)=16.4, p<0.001]. Multiple comparisons revealed that high-dose values were considerably lower when compared to vehicle-injected controls

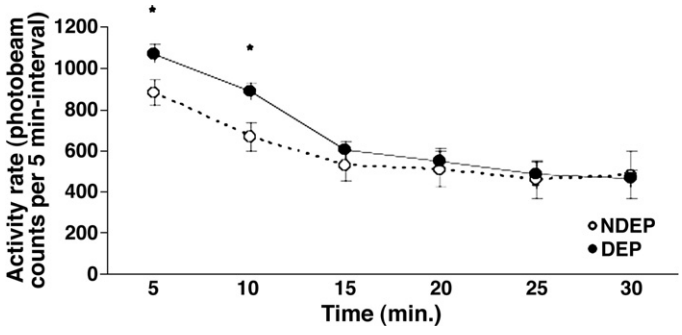

Fig. 6. Mean (±SEM) activity rate (number of photobeams interruptions per each 5-min interval) during the first thirty minutes of locomotor activity measurement in drug-free state. In the absence of significant differences, animal pairs with different levels of drug-history were pooled (n=9/12 per group). *p<0.05 *vs.* NDEP adolescent males.

Table 3
Levels of metabolites in the HIP of adult rats ($n=8$) which underwent a single episode of maternal deprivation (24 h on PND 9) and were administered with URB597 (0, 0.1, 0.5 mg/kg/day for 6 days) during adolescence (PND 38 to 43)

Parameter	Vehicle	URB 0.1	URB 0.5
N-acetyl-aspartate	1.70±0.04	1.81±0.05*	1.62±0.06
Total choline	0.271±0.01	0.272±0.01	0.247±0.01
Total creatine	1.71±0.03	1.85±0.06*	1.69±0.05
Glx (Glu+Gln)	2.78±0.06	2.65±0.08	2.29±0.06***
Glutamine (Gln)	0.91±0.05	0.87±0.06	0.75±0.04
Glutamate (Glu)	1.87±0.04	1.77±0.04	1.55±0.05***
Taurine	1.18±0.03	1.25±0.05	1.15±0.05
Inositols	1.22±0.06	1.23±0.08	1.26±0.06

*$p<0.05$ and ***$p<0.001$ *vs.* control rats, injected with vehicle during adolescence.
Levels of the metabolites were referred to the unsuppressed water signal, and are given in arbitrary relative units.

($ps<0.001$), with low-dose values being at intermediate levels. No differences were evident for all other metabolites in the HIP.

4. Discussion

Present investigations indicate that an early adverse experience during development and pharmacological manipulation of the endocannabinoid system during adolescence may influence the behavioural performance in opposite ways. Moreover, the latter treatment also produced enduring metabolic effects in relevant brain areas. A single prolonged (24 h) episode of maternal deprivation at PND 9 affected the behavioural profile shown at adolescence. Indeed, DEP animals exhibited a slight increase of intolerance to delay, as suggested by reduced preference for the large and delayed food reward at the highest delay. Furthermore, DEP rats displayed elevated levels of nose-poking behaviour when adolescent, and remarkably during the length of the delay, when such a response was not rewarded nor punished. The sustained expression of a given behaviour when it is no longer reinforced might be considered as an index of restlessness (Adriani and Laviola, 2003). Likewise, neonatal deprivation from the dam increased spontaneous locomotor activity in adolescent males, when exposed to a novel environment. Taken together, these findings suggest that adolescent males, submitted to an aversive event in early life, were prone to impulsive-like behaviour, including the forms of basal restlessness (i.e. motor impulsivity, inability to wait) and of increased reactivity to a novel environment. This kind of modified behavioural phenotype might reflect an altered neurodevelopmental pattern, as a consequence of the maternal deprivation episode. It may be worth mentioning that effects of maternal deprivation may not be clearly apparent until adulthood. In this direction, Ellenbroek et al. (1998) showed that disruptions on prepulse inhibition of the acoustic startle were evident in adult rats, but not at adolescence.

Uppermost, the observed behavioural and long-lasting metabolic effects cannot be solely attributed to an URB597-induced effect on AEA, the endogenous cannabinoid. Indeed, URB597 was recently shown to increase *N*-oleyl-ethanolamide (OEA) and *N*-palmitoyl-ethanolamine (PEA) (Lambert and Di Marzo, 1999; Kathuria et al., 2003; Gobbi et al., 2005), and a role for these non-cannabinoid fatty-acid ethanolamides cannot be ruled out. However, OEA has been mostly associated with the peripheral regulation of feeding and body weight (Fu et al., 2003), whilst PEA is mainly believed to modulate inflammation and pain (Calignano et al., 1998; LoVerme et al., 2005). Hence, AEA is suggested to play a primary role in the present findings.

Maternal deprivation induced a moderate but significant decrement on body weight gain on male rats that was only evident immediately after the deprivation protocol (for contrasting results, see Ellenbroek et al., 2005). Blockade of anandamide hydrolysis by URB597 administration at adolescence increased body weight gain exclusively during drug administration in food-restricted animals. The role of the endocannabinoid system in the regulation of appetite and lipid metabolism has been extensively studied (Di Marzo and Matias, 2005; Fride et al., 2005; Pagotto et al., 2006). Administration of exogenous and endogenous cannabinoids leads to robust increases in food intake and can promote body weight gain (Vickers and Kennett, 2005), however, acute URB597 administration was not found to increase food intake in free-feeding rats (Kathuria et al., 2003). Recent results suggests that endogenous cannabinoids, acting at CB_1 receptors, may be involved in maintaining food intake in mice submitted to a brief food-deprivation period (Di Marzo et al., 2001; Ravinet-Trillou et al., 2004). In our experimental conditions, where the operant-behaviour protocol may have changed food intake patterns, subchronic URB597 administration temporarily increased body weight gain in slightly food-deprived adolescent subjects. Since cannabinoid effects on ingestive behaviour may drastically depend on experimental conditions, it is not possible to fully account for mechanisms underlying the observed effect.

Regarding impulsive behaviour, enhancement of endocannabinoid signalling was only effective on DEP adolescent males. Within this experimental group, solely the low dose of URB597 succeeded in reducing impulsive behaviour, without affecting basal locomotor status. Present results show that 1) only DEP rats were sensitive to pharmacological blockade of FAAH, and 2) only a low dose of URB597 was effective, in the short-term, as an effective modulatory tool for increasing self-control behaviour. Previous results from adolescent mice support the current findings, since an increased susceptibility to the behavioural effects of a cannabinoid challenge was found after a similar protocol of early maternal deprivation (Macrì and Laviola, 2004). Present effects of URB597 can be compared with previous work from our group: a single acute injection of the cannabinoid agonist, WIN 55,212 (2 mg/kg s.c.) was found to reduce the impulsive profile of adolescent SHR individuals, whereas it was completely unable to modulate self-control in control Wistar–Kyoto (WKY) rats (Adriani and Laviola, 2004). Apparently, a pharmacological stimulation of the endocannabinoid system may be unable to modify behaviour of 'normal' animals (WKY and NDEP control rats). Conversely, manipulation of the endocannabinoid system became in both cases an effective pharmacological tool, positively influencing self-control behaviour in animals that showed a tendency to impulsive-like profiles ('impulsive' SHRs subjects, present

DEP rats). It seems therefore plausible that (in)direct pharmacological stimulation might be proposed as a therapeutic approach, in order to obtain recovery from some dysfunction within brain endocannabinoid systems. Indeed, a reduced density of CB_1 receptors within the prefrontal cortex may be associated with elevated levels of impulsivity (Adriani and Laviola, 2004). Therefore, repeated administration of URB597 may be useful to produce a sustained elevation of endocannabinoid levels (Kathuria et al., 2003; Gobbi et al., 2005), and consequently an increased activation of CB_1 receptors. The consequences of repeated cannabinoid exposure in the juvenile period on both expression and activity of CB_1 cannabinoid receptors remains unknown, and hence further experiments become of critical interest.

In view of the important role played by the serotonergic system in impulsive behaviour, the observed actions of the low dose of URB597 might be due to interactions between endocannabinoid and serotonergic systems. On one hand, reduced central serotonin (5-HT) activity has been associated with impulsive-choice behaviour (Linnoila et al., 1983; Soubrié, 1986), and on the other, repeated injections with URB597, at the same dose used in the present experiment, influenced serotonergic activity. Specifically, URB597 was able to enhance 5-HT release in dorsal raphe nucleus terminal fields of anaesthetised rats, and 5-HT outflow in the hippocampus, assessed by in vivo microdialysis in awake rats, however it did not increase 5-HT outflow in the prefrontal cortex and did not produce desensitization of 5-HT_{1A} auto-receptors (Gobbi et al., 2005). Thus, it can be hypothesised that the reduction in impulsive behaviour, achieved after administration of the low dose of URB597, might be associated with similar effects on serotonergic activity due to the prolonged blockade of endocannabinoid deactivation. On the other hand, it has been recently suggested that a chronic cannabinoid treatment may up-regulate 5-HT_{2A} receptor activity while concurrently down-regulating 5-HT_{1A} receptor activity (Hill et al., 2006). 5-HT_{2A} receptors have been involved in impulsive behaviour, with agonists enhancing impulsivity and antagonists inducing the opposite effect (Bjork et al., 2002). Thus, an up-regulation of this serotonergic receptor subtype would support higher impulsivity levels (Adriani et al., 2006). However, this discrepancy may be reconciled, since Hill et al. (2006) chronically administered a very high dose of the potent direct cannabinoid agonist, HU-210 (100 μg/kg for 12 days). As strongly supported by previous findings, cannabinoids exert biphasic effects on diverse neurobehavioural responses (Chaperon and Thiebot, 1999; Genn et al., 2004; Marco et al., 2004; Martín-Calderón et al., 1998; McGregor et al., 1996; Sulcova et al., 1998), so that a down-regulation of 5-HT_{2A} receptors may be hypothesized following treatment with the low URB597 dose. Although a linear, dose-dependent profile has been suggested for this compound (Kathuria et al., 2003; Patel and Hillard, 2006), to the best of our knowledge no behavioural studies so far have used a higher dose than the one presently used. Accordingly, it would not be surprising to find such a bidirectional profile on other behaviours or even at different neurobiological levels. Albeit the higher URB597 dose might probably increase AEA levels, in the present experiment effects on impulsive-related parameters were surprisingly absent. However, at the higher dose URB597 may display reduced selectivity for FAAH relative to other serine hydrolase enzymes. Alternatively, the higher dose might have caused a prolonged over-activation of endocannabinoid receptors, possibly leading to an altered functional status within other diverse neurotransmitter systems, thus masking "pure" AEA effects on impulsivity. Indeed, the higher URB597 dose produced adverse long-term modifications that persisted until adulthood (e.g. within the glutamatergic system, as shown in the present study by in vivo MRS).

The use of the intolerance-to-delay protocol may carry some disadvantages when analysing cannabinoid compounds. This paradigm involves choice between reinforcers that differ in both magnitude and delay. Therefore, impulsive choice might be influenced by altered sensitivity to reinforcer magnitude (i.e. perceived rewarding properties), inappropiate perception of time (i.e. delay), or both (Ho et al., 1999). Cannabinoid agonists have been shown to alter time perception in humans (McDonald et al., 2003) and in rats (Crystal et al., 2003), and to enhance an aversive motivational state (Arguello and Jentsch, 2004). Necessarily, present results should be interpreted carefully. Nevertheless, the dose of URB597 that was effective in the intolerance-to-delay task showed no effects in the conditioned place preference test (Gobbi et al., 2005), thus ruling out a potential bias due to altered sensitivity to reinforcement.

As modulation of the endocannabinoid system was only effective on maternally deprived animals, solely this group was submitted to MRS for evaluation of brain metabolic patterns at adulthood. Long-term consequences of adolescent URB597 administration at a low dose were evident in the hippocampus. Both *N*-acetyl-aspartate and total creatine exhibited a similar inverted U shaped profile. On the one hand, *N*-acetyl-aspartate levels appear to be a sensitive marker of neuronal number and viability (Bruhn et al., 1989; Henriksen et al., 1992) while, on the other, creatine can be used as a reliable marker of cellular integrity (Imamura and Proton, 2003). Creatine and its transporter have been reported to be of interest in modern models of depression, since the latter is almost exclusively expressed in two cerebral regions of serotonergic pathways, hippocampus and raphe nuclei (Schloss et al., 1994), importantly involved in depressive-like phenotypes (Sartorius et al., 2003). Lately, cannabinoid-induced neurogenesis in adult hippocampus has been reported, and, moreover, this cannabinoid-induced neurogenesis has been related to diverse behavioural effects such as anti-anxiety and anti-depressant-like actions (Jiang et al., 2005). Some previous work supports a role of the cannabinoid system on hippocampal neurogenesis, which was profoundly suppressed in CB_1 knock-out mice (Jin et al., 2004). It can consequently be speculated that metabolic changes, namely increased *N*-acetyl-aspartate and total creatine, observed within the hippocampus following adolescent exposure to a low dose of URB597, may reflect increased neurogenesis and hence underlie the beneficial outcome reported for behavioural self-control. Hippocampus appeared to be selectively affected, compared to the other forebrain areas. Such finding is consistent

with existing literature, pointing to hippocampal serotonergic levels as playing a crucial role in the performance of impulsive-choice tests (Liu et al., 2004).

Despite the lack of behavioural effects of the higher dose of URB597 on impulsivity, long-lasting effects on metabolite brain contents were found. A significant reduction in glutamate levels, when considered alone and together with glutamine, were achieved in adult rats receiving the higher dose of URB597 during the adolescence period. Notwithstanding a modest decline on glutamatergic activity was related to the lower dose of the cannabinoid compound. URB597 is known to increase the levels of endocannabinoids, which act on presynaptic CB_1 receptors serving as retrograde messengers particularly in the hippocampus (Alger, 2002; Hájos and Freund, 2002). Here, CB_1 receptors are selectively expressed on a particular subset of GABAergic inhibitory interneurons, so that endocannabinoids may indirectly increase glutamatergic signalling. CB_1-mediated modulation of glutamatergic signalling may account for present findings where a decrease on hippocampal glutamate levels was found as a long-term consequence of adolescent exposure.

Hypoglutamatergy has been proposed as one of the primary causes of schizophrenia (Jentsch and Roth, 1999; Olney et al., 1999). In addition, chronic cannabis consumption has been connected with mental disturbances and neuropsychiatric disorders, suggesting that exposure to cannabis derivatives at a premature age might be associated with an increased risk of schizophrenia, depression, and anxiety (Arseneault et al., 2004; Hollister, 1986; Patton et al., 2002; O'Shea et al., 2004; Schneider and Koch, 2003; Viveros et al., 2005a). Furthermore, the endocannabinoid system has been recently involved in both positive and negative symptoms of schizophrenia (Haller et al., 2005). Taken together, it can be speculated that the decreased hippocampal glutamate levels, achieved after adolescent exposure to the higher dose of URB597, might exert detrimental effects that could contribute to psychophysiological modifications, possibly related with schizophrenia or other neuropsychiatric disorders. Metabolic changes due to early maternal deprivation cannot be excluded, nor differential effects of URB597 during adolescence within DEP rats.

In conclusion, a single prolonged episode of maternal deprivation rendered long-lasting behavioural modifications, such as a trend for impulsive-like behaviour, inability to wait, and increased locomotor activity in response to novelty. This modified behavioural phenotype might reflect an altered neurodevelopmental pattern as a consequence of maternal deprivation episode, which appears to be susceptible to positive modulation via the endocannabinoid system. Indeed, within DEP animals, pharmacological blockade of FAAH at adolescence decreased impulsive behaviour. These findings suggest a putative therapeutic approach for the treatment of some aspects of neurodevelopmental disorders, particularly neurobehavioural alterations resulting from neglect parenting. Present findings also highlight a critical role to the magnitude of the pharmacological intervention upon endocannabinoid signalling. Beside beneficial behavioural and neurochemical effects of adolescent administration of FAAH inhibitors at low doses, an excessive activation of the endocannabinoid signalling (due to exaggerated blockade of anandamide deactivation) may exert long-lasting detrimental neurochemical effects, contributing to an increased risk for suffering diverse psychophysiological modifications.

Acknowledgements

EM Marco received a pre-doctoral fellowships from the Ministerio de Educación y Ciencia, Spain. Work supported by the NIH-ISS Collaboration Project, and by Dipartimento Nazionale Antidroga, Pres. Cons. Min., Italy (grants to GL). We wish to thank Giovanni Dominici for expert technical help and Massimo Giannini for top-quality maintenance of the MR equipment.

References

Adriani W, Laviola G. Elevated levels of impulsivity and reduced place conditioning with D-amphetamine: two behavioral features of adolescence in mice. Behav Neurosci 2003;117:695–703.

Adriani W, Laviola G. Windows of vulnerability to psychopathology and therapeutic strategy in the adolescent rodent model. Behav Pharmacol 2004;15:341–52.

Adriani W, Rea M, Baviera M, Invernizzi W, Carli M, Ghirardi O, et al. Acetyl-L-carnitine reduces impulsive behaviour in adolescent rats. Psychopharmacology 2004;176:296–304.

Adriani W, Giannakopoulou D, Bokulic Z, Jernej B, Alleva E, Laviola G. Response to novelty, social and self-control behaviors, in rats exposed to neonatal anoxia: modulatory effects of an enriched environment. Psychopharmacology 2006;184:155–65.

Alger BE. Retrograde signaling in the regulation of synaptic transmission: focus on endocannabinoids. Prog Neurobiol 2002;68:247–86.

Arguello PA, Jentsch JD. Cannabinoid CB1 receptor-mediated impairment of visuospatial attention in the rat. Psychopharmacology 2004;177:141–50.

Arseneault L, Cannon M, Witton J, Murray RM. Causal association between cannabis and psychosis: examination of the evidence. Br J Psychiatry 2004;184:110–7.

Belue RC, Howlett AC, Westlake TM, Hutchings DE. The ontogeny of cannabinoid receptors in the brain of postnatal and aging rats. Neurotoxicol Teratol 1995;17:25–30.

Bjork JM, Moeller FG, Dougherty DM, Swann AC, Machado MA, Hanis CL. Serotonin 2a receptor T102C polymorphism and impaired impulse control. Am J Med Genet 2002;114:336–9.

Bruhn H, Frahm J, Gyngell ML, Merboldt KD, Hanicke W, Sauter R. Cerebral metabolism in man after acute stroke: new observations using localized proton NMR spectroscopy. Magn Reson Med 1989;9:126–31.

Calignano A, La Rana G, Giuffrida A, Piomelli D. Control of pain initiation by endogenous cannabinoids. Nature 1998;394:277–81.

Cardinal RN, Pennicott DR, Sugathapala CL, Robbins TW, Everitt BJ. Impulsive choice induced in rats by lesions of the nucleus accumbens core. Science 2001;292:2499–501.

Cardinal RN, Winstanley CA, Robbins TW, Everitt BJ. Limbic corticostriatal systems and delayed reinforcement. Ann NY Acad Sci 2004;1021:33–50.

Chaperon F, Thiebot MH. Behavioral effects of cannabinoid agents in animals. Crit Rev Neurobiol 1999;13:243–81.

Cheung TH, Cardinal RN. Hippocampal lesions facilitate instrumental learning with delayed reinforcement but induce impulsive choice in rats. BMC Neurosci 2005;6:36.

Cirulli F, Laviola G. Paradoxical effects of D-amphetamine in infant and adolescent mice: role of ender and environmental risk factors. Neurosci Biobehav Rev 2000;24:73–84.

Cravatt BF, Giang DK, Mayfield SP, Boger DL, Lerner RA, Gilula NB. Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. Nature 1996;384:83–7.

Croxford JL. Therapeutic potential of cannabinoids in CNS disease. CNS Drugs 2003;17:179–202.
Crystal JD, Maxwell KW, Hohmann AG. Cannabinoid modulation of sensitivity to time. Behav Brain Res 2003;144:57–66.
Di Marzo V, Matias I. Endocannabinoid control of food intake and energy balance. Nat Neurosci 2005;8:585–9.
Di Marzo V, Goparaju SK, Wang L, Liu J, Batkai S, Jarai Z, et al. Leptin-regulated endocannabinoids are involved in maintaining food intake. Nature 2001;410:822–5.
Di Marzo V, De Petrocellis L, Fezza F, Ligresti A, Bisogno T. Anandamide receptors. Prostaglandins Leukot Essent Fatty Acids 2002;66:377–91.
Ellenbroek BA, van den Kroonenberg PT, Cools AR. The effects of an early stressful life event on sensorimotor gating in adult rats. Schizophr Res 1998;30:251–60.
Ellenbroek BA, Derks N, Park HJ. Early maternal deprivation retards neurodevelopment in Wistar rats. Stress 2005;8:247–57.
Evenden JL, Ryan CN. The pharmacology of impulsive behaviour in rats VI: the effects of ethanol and selective serotonergic drugs on response choice with varying delays of reinforcement. Psychopharmacology 1999;146:413–21.
Fegley D, Gaetani S, Duranti A, Tontini A, Mor M, Tarzia G, et al. Characterization of the fatty acid amide hydrolase inhibitor cyclohexyl carbamic acid 3′-carbamoyl-biphenyl-3-yl ester (URB597): effects on anandamide and oleoylethanolamide deactivation. J Pharmacol Exp Ther 2005;313:352–8.
Fowler CJ, Holt S, Nilsson O, Jonsson KO, Tiger G, Jacobsson SO. The endocannabinoid signaling system: pharmacological and therapeutic aspects. Pharmacol Biochem Behav 2005;81:248–62.
Fride E. The endocannabinoid-CB receptor system: importance for development and in pediatric disease. Neuro Endocrinol Lett 2004;25:24–30.
Fride E. Endocannabinoids in the central nervous system: from neuronal networks to behavior. Curr Drug Targets CNS Neurol Disord 2005;4:633–42.
Fride E, Bregman T, Kirkham TC. Endocannabinoids and food intake: newborn suckling and appetite regulation in adulthood. Exp Biol Med 2005;230: 225–34.
Fu J, Gaetani S, Oveisi F, Lo Verme J, Serrano A, Rodriguez De Fonseca F, et al. Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-alpha. Nature 2003;425:90–3.
Genn RF, Tucci S, Marco EM, Viveros MP, File SE. Unconditioned and conditioned anxiogenic effects of the cannabinoid receptor agonist CP 55,940 in the social interaction test. Pharmacol Biochem Behav 2004;77: 567–73.
Gobbi G, Bambico FR, Mangieri R, Bortolato M, Campolongo P, Solinas M, et al. Antidepressant-like activity and modulation of brain monoaminergic transmission by blockade of anandamide hydrolysis. Proc Natl Acad Sci U S A 2005;102:18620–5.
Hájos N, Freund TF. Distinct cannabinoid sensitive receptors regulate hippocampal excitation and inhibition. Chem Phys Lipids 2002;121:73–82.
Haller J, Varga B, Ledent C, Freund TF. CB1 cannabinoid receptors mediate anxiolytic effects: convergent genetic and pharmacological evidence with CB1-specific agents. Behav Pharmacol 2004;15:299–304.
Haller J, Szirmai M, Varga B, Ledent C, Freund TF. Cannabinoid CB1 receptor dependent effects of the NMDA antagonist phencyclidine in the social withdrawal model of schizophrenia. Behav Pharmacol 2005;16:415–22.
Henriksen O, Gideon P, Sperling B, Olsen TS, Jorgensen HS, Arlien-Soborg P. Cerebral lactate production and blood flow in acute stroke. J Magn Reson Imaging 1992;2:511–7.
Herkenham M, Lynn AB, Little MD, Johnson MR, Melvin LS, de Costa BR, et al. Cannabinoid receptor localization in brain. Proc Natl Acad Sci U S A 1990;87:1932–6.
Hill MN, Gorzalka BB. Enhancement of anxiety-like responsiveness to the cannabinoid CB(1) receptor agonist HU-210 following chronic stress. Eur J Pharmacol 2004;499:291–5.
Hill MN, Sun JC, Tse MT, Gorzalka BB. Altered responsiveness of serotonin receptor subtypes following long-term cannabinoid treatment. Int J Neuropsychopharmacol 2006;9:277–86.
Ho MY, Mobini S, Chiang TJ, Bradshaw CM, Szabadi E. Theory and method in the quantitative analysis of "impulsive choice" behaviour: implications for psychopharmacology. Psycho-pharmacology 1999;146:362–72.
Hofer MA, Brunelli SA, Shair HN. Potentiation of isolation-induced vocalization by brief exposure of rat pups to maternal cues. Dev Psychobiol 1994;27:503–17.
Hollister LE. Health aspects of cannabis. Pharmacol Rev 1986;38:1–20.
Imamura K, Proton MR. spectroscopy of the brain with a focus on chemical issues. Magn Reson Med Sci 2003;2:117–32.
Jentsch JD, Roth RH. The neuropsychopharmacology of phencyclidine: from NMDA receptor hypofunction to the dopamine hypothesis of schizophrenia. Neuropsychopharmacology 1999;20:201–25.
Jiang W, Zhang Y, Xiao L, Van Cleemput J, Ji SP, Bai G, et al. Cannabinoids promote embryonic and adult hippocampus neurogenesis and produce anxiolytic- and antidepressant-like effects. J Clin Invest 2005;115:3104–16.
Jin K, Xie L, Kim SH, Parmentier-Batteur S, Sun Y, Mao XO, et al. Defective adult neurogenesis in CB1 cannabinoid receptor knockout mice. Mol Pharmacol 2004;66:204–8.
Jonsson KO, Holt S, Fowler CJ. The endocannabinoid system: current pharmacological research and therapeutic possibilities. Basic Clin Pharmacol Toxicol 2006;98:124–34.
Kathuria S, Gaetani S, Fegley D, Valino F, Duranti A, Tontini A, et al. Modulation of anxiety through blockade of anandamide hydrolysis. Nat Med 2003;9:76–81.
Keevil SF, Barbiroli B, Brooks JC, Cady EB, Canese R, Carlier P, et al. Absolute metabolite quantification by in vivo NMR spectroscopy: II. A multicentre trial of protocols for in vivo localised proton studies of human brain. Magn Reson Imaging 1998;16:1093–106.
Lambert DM, Di Marzo V. The palmitoylethanolamide and oleamide enigmas: are these two fatty acid amides cannabimimetic? Curr Med Chem 1999;6:757–73.
Laviola G, Adriani W, Terranova ML, Gerra G. Psychobiological risk factors for vulnerability to psychostimulants in human adolescents and animal models. Neurosci Biobehav Rev 1999;23:993–1010.
Laviola G, Macrì S, Morley-Fletcher S, Adriani W. Risk-taking behavior in adolescent mice: Psycho-biological determinants and early epigenetic influence. Neurosci Biobehav Rev 2003;27:19–31.
Levine S, Huchton DM, Wiener SG, Rosenfeld P. Time course of the effect of maternal deprivation on the hypothalamic–pituitary–adrenal axis in the infant rat. Dev Psychobiol 1991;24:547–58.
Leweke FM, Giuffrida A, Wurster U, Emrich HM, Piomelli D. Elevated endogenous cannabinoids in schizophrenia. Neuroreport 1999;10:1665–9.
Linnoila M, Virkkunen M, Schenin M, Nutila A, Rimon R, Goodwin FK. Low cerebrospinal fluid 5-hydroxyindolacetic acid concentrations differentiate impulsive from non-impulsive violent behaviour. Life Sci 1983;33:2609–14.
Liu YP, Wilkinson LS, Robbins TW. Effects of acute and chronic buspirone on impulsive choice and efflux of 5-HT and dopamine in hippocampus, nucleus accumbens and prefrontal cortex. Psychopharmacology 2004;173:175–85.
LoVerme J, La Rana G, Russo R, Calignano A, Piomelli D. The search for the palmitoylethanolamide receptor. Life Sci 2005;77:1685–98.
Macrì S, Laviola G. Single episode of maternal deprivation and adult depressive profile in mice: interaction with cannabinoid exposure during adolescence. Behav Brain Res 2004;154:231–8.
Marco EM, Perez-Alvarez L, Borcel E, Rubio M, Guaza C, Ambrosio E, et al. Involvement of 5-HT1A receptors in behavioural effects of the cannabinoid receptor agonist CP 55,940 in male rats. Behav Pharmacol 2004;15:21–7.
Marsicano G, Wotjak CT, Azad SC, Bisogno T, Rammes G, Cascio MG, et al. The endogenous cannabinoid system controls extinction of aversive memories. Nature 2002;418:530–4.
Martín-Calderón JL, Muñoz RM, Villanua MA, Del Arco I, Moreno JL, Rodriguez De Fonseca F, et al. Characterization of the acute endocrine actions of (−)-11-hydroxy-Δ8-tetrahydrocannabinol-dimethylheptyl (HU-210), a potent synthetic cannabinoid in rats. Eur J Pharmacol 1998;344:77–86.
McDonald J, Schleifer L, Richards JB, de Wit H. Effects of THC on behavioral measures of impulsivity in humans. Neuropsychopharmacology 2003;28: 1356–65.
McGregor IS, Issakidis CN, Prior G. Aversive effects of the synthetic cannabinoid CP 55,940 in rats. Pharmacol Biochem Behav 1996;53:657–64.
McLaughlin CR, Abood ME. Developmental expression of cannabinoid receptor mRNA. Brain Res Dev Brain Res 1993;76:75–8.
Olney JW, Newcomer JW, Farber NB. NMDA receptor hypofunction model of schizophrenia. J Psychiatr Res 1999;33:523–33.

O'Shea M, Singh ME, McGregor IS, Mallet PE. Chronic cannabinoid exposure produces lasting memory impairment and increased anxiety in adolescent but not adult rats. J Psychopharmacol 2004;18:502–8.

Pagotto U, Marsicano G, Cota D, Lutz B, Pasquali R. The emerging role of the endocannabinoid system in endocrine regulation and energy balance. Endocr Rev 2006;27:73–100.

Patel S, Hillard CJ. Pharmacological evaluation of cannabinoid receptor ligands in a mouse model of anxiety: further evidence for an anxiolytic role for endogenous cannabinoid signaling. J Pharmacol Exp Ther 2006;304–11.

Patton GC, Coffey C, Carlin JB, Degenhardt L, Lynskey M, Hall W. Cannabis use and mental health in young people: cohort study. BMJ 2002;325: 1195–8.

Piomelli D. The molecular logic of endocannabinoid signalling. Nat Rev Neurosci 2003;4:873–84.

Provencher SW. Estimation of metabolite concentrations from localized in vivo proton NMR spectra. Magn Reson Med 1993;30:672–9.

Pryce CR, Ruedi-Bettschen D, Dettling AC, Weston A, Russig H, Ferger B, et al. Long-term effects of early-life environmental manipulations in rodents and primates: Potential animal models in depression research. Neurosci Biobehav Rev 2005;29:649–74.

Ramos JA, De Miguel R, Cebeira M, Hernández M, Fernández-Ruiz J. Exposure to cannabinoids in the development of endogenous cannabinoid system. Neurotox Res 2002;4:363–72.

Ramos JA, Gonzalez S, Sagredo O, Gomez-Ruiz M, Fernandez-Ruiz J. Therapeutic potential of the endocannabinoid system in the brain. Mini Rev Med Chem 2005;5:609–17.

Ravinet Trillou C, Delgorge C, Menet C, Arnone M, Soubrie P. CB1 cannabinoid receptor knockout in mice leads to leanness, resistance to diet-induced obesity and enhanced leptin sensitivity. Int J Obes Relat Metab Disord 2004;28:640–8.

Rodríguez De Fonseca F, Ramos JA, Bonin A, Fernández-Ruiz JJ. Presence of cannabinoid binding sites in the brain from early postnatal ages. Neuroreport 1993;4:135–8.

Sartorius A, Vollmayr B, Neumann-Haefelin C, Ende G, Hoehn M, Henn FA. Specific creatine rise in learned helplessness induced by electroconvulsive shock treatment. Neuroreport 2003;14:2199–201.

Schloss P, Mayser W, Betz H. The putative rat choline transporter CHOT-1 transports creatine and is highly expressed in neural and muscle-rich tissues. Biochem Biophys Res Commun 1994;198:637–45.

Schneider M, Koch M. Chronic pubertal, but not adult chronic cannabinoid treatment impairs sensorimotor gating, recognition memory, and the performance in a progressive ratio task in adult rats. Neuropsychopharmacology 2003;28:1760–9.

Schneider U, Leweke FM, Mueller-Vahl KR, Emrich HM. Cannabinoid/anandamide system and schizophrenia: is there evidence for association? Pharmacopsychiatry 1998;31:110–3.

Soubrié P. Reconciling the role of central serotonin neurones in human and animal behaviour. Behav Brain Sci 1986;9:319–64.

Spear LP. The adolescent brain and age-related behavioral manifestations. Neurosci Biobehav Rev 2000;24:417–63.

Sulcova E, Mechoulam R, Fride E. Biphasic effects of anandamide. Pharmacol Biochem Behav 1998;59:347–53.

Vickers SP, Kennett GA. Cannabinoids and the regulation of ingestive behaviour. Curr Drug Targets 2005;6:215–23.

Viveros MP, Llorente R, Moreno E, Marco EM. Behavioural and neuroendocrine effects of cannabinoids in critical developmental periods. Behav Pharmacol 2005a;16:353–62.

Viveros MP, Marco EM, File SE. Endocannabinoid system and stress and anxiety responses. Pharmacol Biochem Behav 2005b;81:331–42.

Williamson EM, Evans FJ. Cannabinoids in clinical practice. Drugs 2000;60:1303–14.

Witkin JM, Tzavara ET, Nomikos GG. A role for cannabinoid CB1 receptors in mood and anxiety disorders. Behav Pharmacol 2005;16:315–31.

Zimmerberg B, Shartrand AM. Temperature-dependent effects of maternal separation on growth, activity, and amphetamine sensitivity in the rat. Dev Psychobiol 1992;25:213–26.