

Corticotropin-Releasing Hormone (CRH) mRNA Expression in Rat Central Amygdala in Cannabinoid Tolerance and Withdrawal: Evidence for an Allostatic Shift?

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Chronic treatment with cannabinoid agonists leads to tolerance. One possible mechanism for this is receptor internalization, but tolerance has also been reported with compounds that only cause internalization to a low degree. Furthermore, cannabinoid antagonist administration precipitates a characteristic withdrawal syndrome in tolerant subjects, accompanied by neuronal activation and enhanced release of corticotropin-releasing hormone (CRH) in the central amygdala. The underlying molecular mechanisms are unknown. We examined the role of cannabinoid tolerance and withdrawal for the expression of the cannabinoid 1 (CB1) receptor and of CRH in rats. Tolerance was first established functionally. An acute dose (100 µg/kg) of the CB1 agonist HU-210 suppressed locomotor activity, and had an anxiogenic-like effect on the elevated plus-maze. Both effects were absent following daily treatment with the same agonist or a lower (40 µg/kg) dose for 14 days. Next, withdrawal was reliably precipitated by a single dose (3 mg/kg) of the CB1 antagonist SR141716A in rats treated subchronically with 14-day HU-210. Using *in situ* hybridization, a robust suppression of CB1 mRNA expression was found in the caudate-putamen, indicating a downregulation of CB1 expression levels as one mechanism for tolerance to the locomotor suppressant effects of HU-210. The CRH transcript was upregulated in the central amygdala in precipitated withdrawal compared to nonwithdrawn tolerant subjects, suggesting that increased gene expression contributes to the previously reported CRH release in withdrawal. Most importantly, this increase occurred from a suppressed level in tolerant subjects, and behavioral signs of withdrawal, presumably mediated by CRH, were seen at the CRH expression that had only returned to normal nontolerant levels. This suggests the possibility of an allostatic shift, as previously proposed on theoretical grounds. The expression of CRH-R1, CRH-R2α, NPY, and its Y1 receptor mRNA was analyzed in search of neural substrates for the allostatic shift observed, but did not seem to contribute to the dysregulated state.

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INTRODUCTION

Cannabis sativa preparations are among the most abused drugs in the world. Their major psychoactive constituent, Δ^9 -tetrahydrocannabinol (THC), produces a wide range of psychoactive effects through the activation of central cannabinoid CB1. CB1 receptors are widely distributed throughout the rat and human CNS, with a high expression in the extrapyramidal motor system (Herkenham *et al*, 1990). Withdrawal effects upon abstinence from marijuana have been reported but are typically mild, presumably due to the long half-life of cannabinoids that distributes the emergence of abstinence symptoms over a prolonged period of time. A more distinct withdrawal syndrome can be

precipitated using an antagonist challenge in animals treated chronically with cannabinoids, and the CB1 antagonist SR141716A has been used successfully to achieve this objective (Rinaldi-Carmona *et al*, 1994).

A component of negative affect, that is, depressed mood and/or increased anxiety is clinically observed in cannabinoid withdrawal (Smith, 2002). The corticotropin-releasing hormone (CRH) has an established role in stress and negative affect (Koob and Heinrichs, 1999), and is therefore an obvious candidate for fully or partially mediating these phenomena. CRH has been linked to aversive states associated with alcohol, cocaine, and opiate withdrawal (Koob, 1999), and has also been suggested to mediate anxiogenic effects of brain cannabinoid receptor agonists (Rodriguez *et al*, 1996, 1997). Of particular interest is the observation that a single dose of a cannabinoid agonist was associated with suppressed CRH release from the central nucleus of the amygdala, while the opposite was observed during precipitated withdrawal from subchronic treatment with the same ligand (Rodriguez *et al*, 1997). This indicates the possibility that cannabinoid and CRH-ergic signaling

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might be organized as opposing processes, which in turn could provide a substrate for allostatic regulation proposed as a central mechanism in the development of dependence (Koob and Le Moal, 2001).

Here, we therefore investigated the role of central cannabinoid receptors and of CRH in cannabinoid dependence and withdrawal, with particular regard for the allostatic shift hypothesis. For this purpose, we analyzed the behavioral and neurochemical changes in the cannabinoid and CRH systems after acute and subchronic administration of HU-210, a potent cannabinoid agonist (Ottani and Giuliani, 2001), and after precipitated withdrawal. Finally, in view of the changes in the neuropeptide Y (NPY) system in the prefrontal cortex of human abusers of marijuana (Caberlotto and Hurd, 2000), we analyzed the possible changes in NPY and its Y1-receptor mRNA expression in cannabinoid tolerance and withdrawal.

MATERIALS AND METHODS

Subjects

Male Wistar rats ($n = 80$, Charles River, Hamburg, Germany) were housed in groups of four/cage, in an environmentally controlled room with a standard light/dark cycle (light on 0700 light off 1900). Animals had free access to food and water. All procedures were conducted in accordance with permits obtained from the Stockholm South Animal Ethics Committee (permit S85-89/98).

Compounds

HU-210 (Tocris, Great Britain) was used as the selective cannabinoid agonist, and SR141716A (SANOFI Recherche, Paris, France) was used as the antagonist. Both drugs were diluted in a vehicle solution of saline, propylene glycol, and Tween 80 (90:5:5).

Experiment 1

In the first experiment, the induction of functional tolerance was examined using subchronic administration of HU-210. Four groups were injected i.p. daily for 14 days. The groups were as follows:

1. vehicle on days 1–14 ($n = 10$),
2. vehicle on days 1–13 and a single injection of 100 $\mu\text{g/kg}$ HU-210 on day 14 ($n = 10$),
3. 40 $\mu\text{g/kg}$ HU-210 daily ($n = 10$),
4. 100 $\mu\text{g/kg}$ HU-210 daily ($n = 10$).

The development of functional tolerance was examined by behavioral analysis as described below. At 1 h following the last injection, subjects were assessed for elevated plus-maze behavior, after which they were immediately transferred to locomotor activity monitoring boxes. Subjects were killed after additional 23 h via decapitation for *in situ* studies of CB1, NPY, and NPY Y1 receptor mRNA expression.

Experiment 2

In the second experiment, precipitated withdrawal was studied using subchronic HU-210 as an agonist, followed by

SR141716A. Four groups were injected i.p. daily for 14 days, followed by an additional acute injection on the last day, 1 h following the injections of the agonist, or its placebo. The groups were as follows:

1. vehicle daily, vehicle acutely ($n = 10$),
2. 100 $\mu\text{g/kg}$ HU-210 daily, vehicle acutely ($n = 10$),
3. vehicle daily, SR141716A 3 mg/kg acutely ($n = 10$),
4. 100 $\mu\text{g/kg}$ HU-210 daily, SR141716A 3 mg/kg acutely ($n = 10$).

Withdrawal scoring (see below) was initiated immediately after the last injection. After 1 h, subjects were killed via decapitation for *in situ* studies of CRH and NPY expression.

In situ Hybridization

Brains were quickly removed and frozen by immersion in dry-ice cooled 2-methylbutane and stored at -70°C until use.

The NPY riboprobe was made from a 508 bp cDNA of the entire NPY sequence (Hänze *et al*, 1991) subcloned into a pGEM4 vector. The NPY Y1 receptor riboprobe consisted of a 245 bp cDNA fragment of the Y1 receptor corresponding to the fourth and fifth transmembrane domains, subcloned into a Bluescript II SK-vector. The CB1 receptor riboprobe was generated by PCR and corresponded to nucleotides 1232–1272 of the patent sequence (NM_012784). The CRH riboprobe corresponded to nucleotides 588–746 and was also generated by PCR, based on the patent sequence (NM_031019) and subcloned in a pCRII-TOPO vector. For CRH-R1 and R2 α , a 500 bp N-terminal region also containing TM1, TM2, and a portion of TM3 (Chalmers *et al*, 1995) was amplified by PCR from rat brain RNA using primers specific for the respective isoform, and cloned into the pCR2.1-TOPO vector.

Prior to transcription, plasmids were linearized with the appropriate restriction enzymes for generating the antisense and sense riboprobes. RNA probes complementary to the coding sequences were transcribed from the linearized plasmid template with 150 μCi α -[^{35}S]UTP (Dupont NEN, Boston, USA). T3, T7, or SP6 RNA polymerase was used for generating the antisense and sense probes. Transcription occurred in the presence of 10 mM dithiothreitol, 0.5 mM each of ATP, GTP, CTP, and 1 μg linearized plasmid template in a $1 \times$ transcription buffer for 60 min at 37°C . The labeled probes were then separated from unincorporated nucleotides using spin columns (Pharmacia Biotech, Stockholm, Sweden).

In situ hybridization was carried out as previously described (Caberlotto *et al*, 1997). Briefly, brain sections were fixed in 4% paraformaldehyde/ $1 \times$ phosphate-buffered saline for 10 min, rinsed twice in $1 \times$ PBS, and treated with 0.25% acetic anhydride/0.1 M triethanolamine/0.9% sodium chloride for 5 min. The sections were then rinsed in $2 \times$ SSC, dehydrated in a graded series of ethanol (70, 80, 95, 100%), and delipidated with chloroform. They were allowed to air dry before being used or were frozen at -70°C until use. All aqueous solutions were pretreated with 0.1% diethylpyrocarbonate before use. The hybridization buffer consisted of 0.5 mg/ml sheared ss DNA, 250 $\mu\text{g/ml}$ yeast tRNA, $1 \times$ Denhardt's solution, 10% dextran sulfate, $4 \times$ SSC, and 50% formamide. Before hybridization, the

labeled probe was added to the hybridization cocktail at a concentration of 20×10^3 cpm/ μ l. A volume of 0.15 ml of this hybridization mixture was applied to the sections. The sections were coverslipped to prevent evaporation and the hybridization was carried out in a humidified chamber overnight at 55°C. Incubation was followed by washing in a graded series of SSC ($2 \times$, $1 \times$, $0.5 \times$, $0.5 \times$ /50% formamide, $0.1 \times$) containing 1 mM DTT, all at room temperature except for the $0.1 \times$ SSC (53°C), and dehydration was carried out with graded ethanol solutions containing 300 mM ammonium acetate. The slides were then air dried and exposed to Kodak Biomax Films (Amersham) along with 14 C standards for a period of 3–7 days (CB1, CRH, NPY, and NPY-Y1 receptor *in situ*), or exposed to Fuji BAS-5000 Phosphorimager plates (CRH-R1 and R2 α receptor *in situ*).

Film autoradiograms were scanned using a ScanMaker III (Microtek, USA) at a resolution of 400 dpi. The light transmittance values were measured from the digitalized images with a PC-based image analysis software system (IMAGE, Wayne Rasband, NIMH). Phosphorimager-generated digital images were analyzed using AIS Image Analysis Software (Imaging Research Inc., St Catharines, Ontario, Canada). The regions of interest were defined by anatomical landmarks according to the Paxinos brain atlas (Paxinos and Watson, 1986). Based on the known radioactivity in the 14 C standards, image values were converted to nCi/g using a Rodbard calibration curve. Each specific brain region was selected by individually tracing the structures on the screen with a cursor.

Locomotion

The exploratory locomotor activity was determined in sound-attenuated chambers containing locomotor activity cages ($380 \times 200 \times 160$ mm) equipped with infrared beam detection (Med Associates, St Albans, VT, USA). The interbeam distance was 8.5 cm horizontally and 6.5 cm vertically, and activity was recorded for 30 min in intervals of 10 min.

Plus-maze

Plus-maze testing was essentially as originally described (Pellow *et al*, 1985, 1986). The apparatus was made of black plastic, and consisted of two open arms measuring 50×10 cm, and two arms of the same size surrounded by a 40 cm high end and side-walls. The arms were connected by a central area measuring 10×10 cm, and the maze was 50 cm above the floor during testing, which was undertaken under dim red light. Behavior was scored by a trained observer unaware of treatment conditions. The subjects were placed in a novel environment (an empty locomotor chamber) for 5 min prior to testing; this has been shown to increase the exploratory activity and improve the reliability of subsequent plus-maze testing (Pellow *et al*, 1985). At the beginning of a session, the rat was placed in the central area of the maze, facing one of the open arms. The number of entries made into open and closed arms and the time spent in open and closed arms were recorded over a 5-min session. An arm entry was defined as all four paws into an arm. The percentage of time spent and entries into the open

arm, as well as the total number of entries were compared using one-way ANOVAs.

Withdrawal Scoring

Animals were placed in single Macrolon cages (dimensions: $20 \times 38 \times 16$ height) after 1 h habituation in the experimental room. The experimenter was always blind to the treatment conditions. The withdrawal behaviors initially assessed were scratching, rearing, wet dog shake, rubbing, grooming, head shakes (turning or twisting head side to side), penile erection, and paw tremors (lateral rapid and episodic movements of the paws). The withdrawal signs such as scratching and rubbing were scored according to the following scale: 0 = no signs; 1 = intermittent or occasional signs; 2 = continuous signs. Signs such as rearing, wet dog shake, head shakes, penile erection, and paw tremors were counted. Grooming was scored according to the following scale (similar to that previously described in Molloy and Waddington, 1984): 0 = no grooming present; 1 = grooming of any form; 2 = intense grooming, a characteristic pattern of grooming of the face with the forepaws followed by vigorous grooming of the hind flank with the snout. A withdrawal score (for each withdrawal sign) was determined every 15 min for a total period of 60 min by direct observation during 1-min periods for each rat.

Statistical Analysis

Expression analysis. Data were analyzed separately for each region by one-way ANOVA, because variances were homogeneous within, but not between regions. Due to the multiple regions examined, the ANOVA-computed probabilities for a treatment effect in each region were corrected for multiplicity of testing using the Holm sequentially rejective Bonferroni method (Holm, 1979). In regions within which an overall treatment effect was established by this procedure, individual comparisons between treatment groups within that region were computed using Duncan's *post hoc* test.

Locomotion. Data were analyzed via two-way repeated measures ANOVA (treatment $\times$ time) and followed, when appropriate, by Duncan's *post hoc* test.

Withdrawal signs. Raw data for the individual parameters were not normally distributed, but summary scores constructed from the four most sensitive items (grooming, scratching, paw tremor, wet dog shakes) fulfilled the criteria of normal distribution and homogeneity of variance. The summary scores were therefore analyzed using a standard two-way ANOVA with respect to time and treatment, followed, when appropriate by Duncan's *post hoc* test.

RESULTS

Induction of Functional Tolerance by HU-210—Locomotion (Figure 1)

A highly significant overall effect of treatment on locomotor activity was found (horizontal activity: $F_{3,35} = 4.35$; $p = 0.01$; vertical activity: $F_{3,35} = 8.69$; $p = 0.0002$; Figure 1). *Post hoc*

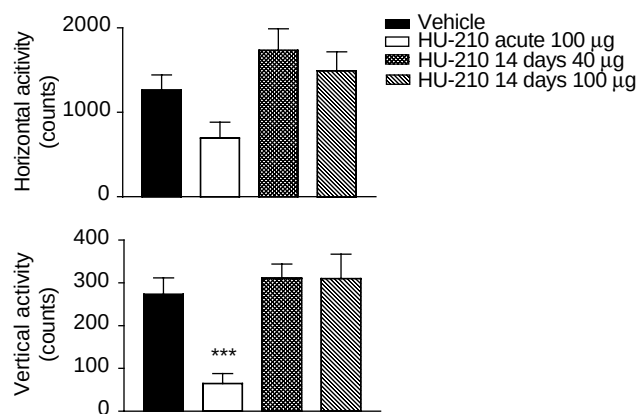


Figure 1 Induction of tolerance to locomotor suppressant actions by subchronic treatment with the selective cannabinoid agonist HU-210. Horizontal (upper panel) and vertical (lower panel) exploratory locomotor activity (mean ± SEM) is shown for subjects treated with HU-210 (40 or 100 µg/kg) or vehicle daily for 14 days, or an acute injection of the higher of the HU-210 doses. Acute HU-210 treatment profoundly suppressed the vertical parameters of exploration ($p = 0.0001$), an effect that was entirely absent following repeated treatment with the same, or the lower dose. For detailed statistical analysis, see the Results section.

analysis revealed that this was due to acute administration of 100 µg/kg of the agonist HU-210, which induced a suppression of horizontal activity vs subchronic treatment ($p = 0.01$ vs 14 day 100 µg/kg; $p = 0.002$ vs 14 day 40 µg/kg). Furthermore, acute treatment induced a profound reduction in vertical activity ($p < 0.001$) vs each of the three other treatments. A qualitative observation indicated that this was due to motor incoordination/ataxia. In contrast, no effect or trend for an effect was observed vs vehicle-treated controls in the group receiving either the same or lower (40 µg/kg) dose daily for 14 days.

Induction of Functional Tolerance by HU-210—Plus-Maze Behavior (Table 1)

On the elevated plus-maze, a significant overall treatment effect was found in one of the two anxiety-related parameters of open-arm exploration (% open entries; overall effect $F_{3,33} = 2.9$; $p = 0.04$). *Post hoc* analysis revealed a difference between rats treated acutely with the cannabinoid agonist HU-210 and controls ($p = 0.04$) or rats treated chronically (40 µg/kg, $p = 0.02$; 100 µg/kg, $p = 0.04$). Furthermore, a significant effect on the index of activity, total number of entries, was found ($F_{3,33} = 15.4$; $p < 0.0001$). Here, *post hoc* analysis revealed a difference between rats treated acutely with the cannabinoids agonist and controls ($p < 0.000056$) or rats treated chronically (40 µg/kg, $p < 0.0001$; 100 µg/kg, $p < 0.000065$), but no difference between any of the chronic groups and vehicle.

Precipitation of Functional Withdrawal by SR141716A (Figure 2)

Summary scores (grooming, scratching, paw tremor, and wet dog shakes) showed a highly significant treatment effect ($F_{3,35} = 27.357$, $p < 0.00001$). *Post hoc* analysis revealed significant differences at 30 and 45 min between rats in which 14 days HU-210 treatment was followed by

Table 1 Induction of Functional Tolerance with Regard to Elevated Plus-Maze Behavior by Subchronic Treatment with the Selective Cannabinoid Agonist HU-210

	Vehicle	HU-210 (40 µg) daily for 14 days	HU-210 (100 µg) daily for 14 days	HU-210 (100 µg) acute
% Open time	33.7 ± 13.1	53.3 ± 8.7	44.7 ± 4.2	33.7 ± 13.1
% Open entries	49.6 ± 5.6	47.5 ± 6.4	50.1 ± 2.7	26.9 ± 9.4*
Total entries	12.5 ± 0.7	10.2 ± 0.8	12.2 ± 1.6	3.1 ± 1***

Data (mean ± SEM) are shown for subjects treated with HU-210 (40 or 100 µg/kg) or vehicle daily for 14 days, or an acute injection of the higher of the HU-210 doses. Acute HU-210 treatment suppressed one of the anxiety-related indices (% open entries; $p = 0.04$) but not the other, and also suppressed the activity index (total entries; $p < 0.0001$); both these effects were entirely absent following repeated treatment with the same HU-210 dose. For detailed statistical analysis, see Results.

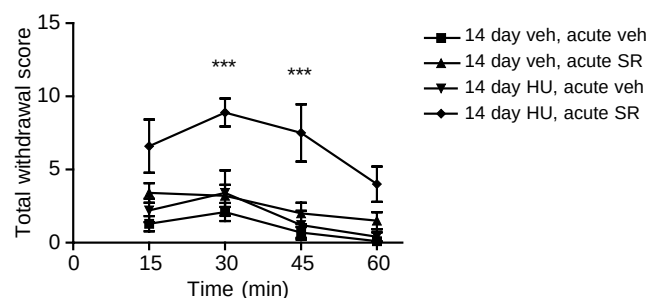


Figure 2 Precipitation of a marked withdrawal syndrome by acute injection of the cannabinoid antagonist SR141716A ('SR'; 3 mg/kg) in subjects treated daily for 14 days with the cannabinoid agonist HU-210 ('HU'; 100 µg/kg), but not in subjects either pretreated with vehicle ('veh'), or pretreated with HU-210 and challenged with vehicle. The total withdrawal scores were the sum of scores for grooming, scratching, paw tremor, and wet dog shakes. There was a highly significant overall treatment effect ($p < 0.00001$), and on *post hoc* analysis the group in which antagonist challenge followed 14 days of agonist treatment differed from each of the other treatment groups ($p < 0.001$), which in turn did not differ between each other at any time point (for detailed statistics, see Results).

SR141716A challenge, and all other treatments ($p < 0.001$ for all comparisons). The other groups did not differ between each other at any time point.

Regulation of CB1 Receptor mRNA by Acute Agonist Treatment and Tolerance (Figure 3)

The distribution of CB1 mRNA-expressing cells was in line with that previously described (Herkenham et al, 1990), with a strong expression in the striatum, cortex, hippocampus, and amygdala. Following agonist treatment, the cannabinoid CB1 receptor mRNA expression was downregulated in the caudate/putamen (overall effect $F_{3,18} = 7.75$; Holm-Bonferroni corrected $p = 0.0017$). *Post hoc* analysis showed downregulation to be present both in chronically (40 µg/kg, $p = 0.0008$; 100 µg/kg, $p = 0.01$) and acutely treated animals ($p = 0.0009$) vs controls. In the other brain regions examined, cingulate cortex, CA region, dentate gyrus, central, and basolateral amygdala, no differences were detected.

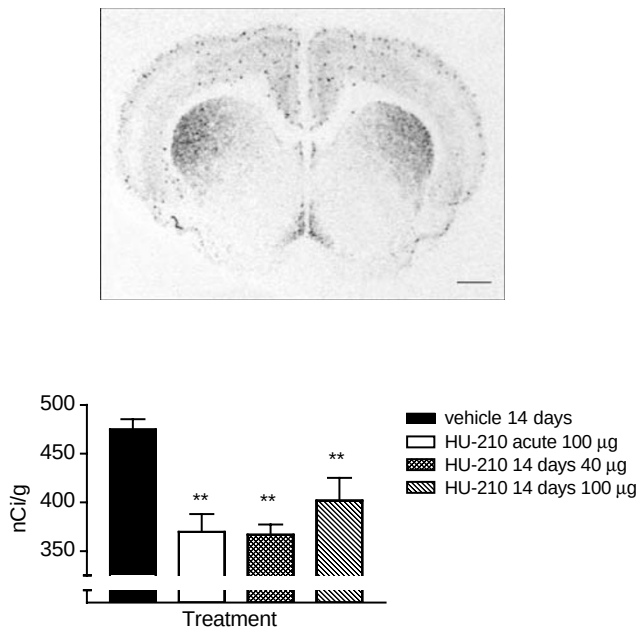


Figure 3 Downregulation of CB1 receptor mRNA in the dorsolateral caudate-putamen by acute as well as 14-day daily treatment with the cannabinoid agonist HU-210. Upper panel: distribution of the CB1 mRNA expression signal in the brain of a typical vehicle-treated control. Scale bar = 1 mm. Lower panel: quantification of mRNA expression levels (see Materials and methods) revealing a significant difference between each of the treatment groups receiving HU-210 and vehicle-treated controls (** $p < 0.01$ for all; for detailed statistics, see Results).

CRH mRNA Expression in Subchronic Cannabinoid Agonist Treatment and Precipitated Withdrawal (Figure 4)

The pattern of expression of CRH mRNA was consistent with the previous description of CRH mRNA-expressing cells in the rat brain (reviewed in Koob, 1999), with hybridization signals mainly found in the hippocampus, hypothalamus, and central amygdala. A significant overall treatment effect was found within the amygdala ($F_{3,27} = 3.92$; Holm-Bonferroni corrected $p = 0.019$). *Post hoc* analysis revealed a downregulation in the central amygdala of chronically HU-210-treated rats compared to vehicle controls ($p = 0.003$). In contrast, in rats treated chronically with HU-210 and subsequently with the antagonist SR141716A to precipitate withdrawal, CRH mRNA expression in central amygdala returned to normal, that is, it was not different from vehicle control rats, but was significantly higher than in chronically HU-210-treated rats ($p = 0.026$). As a control, the antagonist SR141716A alone, when not preceded by subchronic HU-210 treatment, did not affect the CRH mRNA expression.

Regulation of CRH-R1 and CRH-R2 α Receptor Expression (Table 2)

These parameters were analyzed to examine whether the regulation of CRH receptor expression could account for the apparent setpoint shift of the CRH system observed (cf Discussion). The distribution of the receptor expression was consistent with that published previously (Chalmers et al,

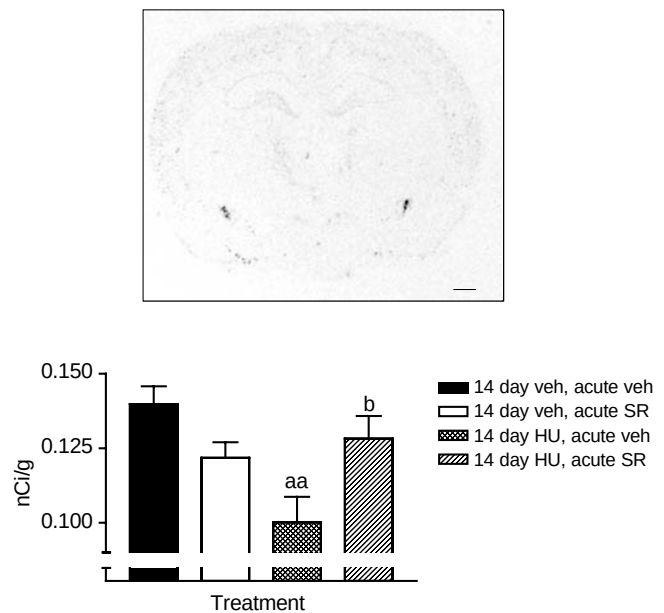


Figure 4 Regulation of CRH mRNA in the central amygdala by tolerance induced through daily treatment with 100 µg/kg of the cannabinoid agonist HU-210, and by withdrawal precipitated through subsequent administration of the antagonist SR141716A. Upper panel: distribution of CRH mRNA expression signal in the brain of a typical vehicle-treated control. Scale bar = 1 mm. Lower panel: quantification of CRH mRNA expression levels (see Materials and methods) demonstrated a significant decrease in the CRH transcript in the group treated for 14 days with HU-210, followed by vehicle, in comparison with the vehicle-vehicle control group ('aa'; $p = 0.003$); precipitation of withdrawal by the administration of SR141716A in HU-210-pretreated subjects led to a significant increase of CRH expression vs the nonprecipitated group ('b'; $p = 0.026$), and returned this group to a level that no longer differed from vehicle-vehicle controls. For detailed statistics, see Results.

1995). An overall treatment effect was found within the following regions:

The hippocampal CA1 region ($F_{3,12} = 22.6$, Holm-Bonferroni corrected $p = 0.000124$), where *post hoc* analysis indicated that both chronic agonist treatment (HU-210; $p = 0.026$), acute antagonist administration (SR141716A; $p = 0.0018$), and to an even higher degree, the combination of the two ($p = 0.000076$) decreased CRH-R1 expression.

1. The hippocampal CA3 region ($F_{3,13} = 5.11$, Holm-Bonferroni corrected $p = 0.015$), where *post hoc* analysis indicated that the precipitated withdrawal group had a lower CRH-R1 expression than any of the other groups ($p = 0.004$ – 0.027), while the other groups did not differ from each other.
2. The hippocampal CA4 region ($F_{3,12} = 6.90$, Holm-Bonferroni corrected $p = 0.012$), where *post hoc* analysis showed that precipitated withdrawal decreased ($p = 0.032$) CRH-R1 expression.
3. The frontoparietal cortex ($F_{3,15} = 7.3$, Holm-Bonferroni corrected $p = 0.009$), where *post hoc* analysis indicated that the precipitated withdrawal group was significantly lower than the vehicle only control group ($p = 0.0012$).

A trend level overall treatment effect (uncorrected $p = 0.056$) with a similar profile, that is with a decrease in the precipitated withdrawal group, was also seen in the basolateral amygdala. In contrast, no treatment effect was

Table 2 CRF-R1 mRNA Levels after Subchronic Treatment with the Cannabinoid Agonist and Precipitated Withdrawal (nCi/g, mean $\pm$ SEM)

Treatment	cing ctx	frontal ctx	fp ctx	MeA	BLA	CA1	CA3	CA4
Veh-veh	19.7 $\pm$ 1.3 (n = 5)	19.4 $\pm$ 1.0 (n = 5)	24.0 $\pm$ 0.6 (n = 5)	21.9 $\pm$ 1.3 (n = 5)	26.6 $\pm$ 0.5 (n = 5)	12.0 $\pm$ 0.6 (n = 5)	16.4 $\pm$ 2.1 (n = 5)	17.2 $\pm$ 0.9 (n = 5)
Veh-SR	18.3 $\pm$ 0.9 (n = 5)	17.3 $\pm$ 1.3 (n = 5)	21.5 $\pm$ 1.1 (n = 5)	18.5 $\pm$ 2.0 (n = 5)	22.9 $\pm$ 0.5 (n = 5)	9.1 $\pm$ 0.4 (n = 4)**	16.3 $\pm$ 0.8 (n = 4)	16.1 $\pm$ 0.4 (n = 4)
HU-veh	19.2 $\pm$ 0.7 (n = 4)	16.7 $\pm$ 0.6 (n = 4)	23.2 $\pm$ 0.9 (n = 4)	21.4 $\pm$ 3.9 (n = 3)	24.4 $\pm$ 1.0 (n = 3)	10.2 $\pm$ 0.4 (n = 3)*	18.8 $\pm$ 2.1 (n = 3)	21.4 $\pm$ 3.0 (n = 3)
HU+SR	17.6 $\pm$ 0.5 (n = 5)	18.1 $\pm$ 0.8 (n = 5)	18.6 $\pm$ 1.0 (n = 5)**	18.1 $\pm$ 1.9 (n = 5)	21.8 $\pm$ 1.9 (n = 5)	6.7 $\pm$ 0.3 (n = 4)***	10.5 $\pm$ 0.8 (n = 4)**	12.4 $\pm$ 0.9 (n = 4)*

Significant overall treatment effect was found in the hippocampal CA1, 3, and 4 regions, and the frontoparietal cortex, but not in the other regions examined. A similar pattern emerged in the regions where a treatment effect was present, with the precipitated withdrawal group consistently showing lower CRH-R1 expression than vehicle-treated controls (*** p < 0.001; ** p < 0.01; * p < 0.05; regions analyzed: frontal ctx—frontal cortex; cing ctx—cingulate cortex area 1 and 2; fp ctx—frontoparietal cortex; MeA—medial amygdaloid nucleus; BLA—basolateral amygdaloid nucleus; CA1, 3, and 4—dorsal hippocampal subregions Cornu Ammonis areas CA1–CA4). For detailed statistical analysis, see Results.

found in the frontal cortex, cingulate cortex, medial amygdala, and basolateral amygdala (Table 2). Furthermore, CRH-R2 receptor expression was not significantly affected in any of the regions examined (medial amygdala; posterior amygdaloid nucleus; hippocampal CA1, 3 and 4 areas, dentate gyrus; data not shown).

NPY and Y1 Receptor mRNA

NPY and Y1 receptor mRNAs distribution pattern was consistent with that previously described (Morris, 1989; Larsen *et al*, 1993). No differences in the expression of the NPY system was detected in any region analyzed: cingulate cortex, caudate/putamen, accumbens, bed nucleus stria terminalis, CA region, dentate gyrus, and medial amygdala. The NPY Y1 receptor mRNA expression was also not altered in the cingulate cortex, caudate/putamen, CA region, dentate gyrus, medial amygdala, and arcuate nucleus of rats treated both acutely and chronically with the cannabinoid antagonist HU-210.

DISCUSSION

The acute administration of HU-210, a potent cannabinoid agonist, at a dose of 100 μ g/kg markedly suppressed both the horizontal and vertical exploratory locomotor activity. Following 14 days of daily treatment with the same dose of HU-210, both parameters of exploratory activity returned to normal, indicative of full tolerance. A similar pattern was seen on the elevated plus-maze, where acute treatment with HU-210 suppressed both parameters of open-arm exploration, and thus produced an anxiogenic-like effect, while chronic treatment was ineffective in this respect. Some caution is warranted in the interpretation of the plus-maze data, since only one of the two anxiety-related parameters of this model was significantly affected, and a suppression of the total number entries was observed, which is an intrinsic activity index of the plus-maze. However, the anxiety-related parameters of the elevated plus-maze are relatively activity-independent (Pellow *et al*, 1985). In summary, the behavioral data provide consistent evidence for a high

degree of tolerance developing over the course of 14 days HU-210 treatment with regard to locomotor activity, and suggestive evidence for tolerance to anxiogenic-like effects of CB1-receptor stimulation. This is in agreement with an extensive literature demonstrating the development of tolerance after repetitive administration of cannabinoids both in humans (McMillan *et al*, 1972; Solowij *et al*, 1995) and animals (Carlini *et al*, 1970; Pertwee *et al*, 1993; Rubino *et al*, 1997).

Presumably, as a correlate of functional tolerance, the expression of the cannabinoid CB1 receptor transcript was markedly downregulated in the caudate/putamen following agonist treatment. This was evident already after a single agonist administration, as well as after chronic treatment. It is unsurprising that the suppression of CB1 expression preceded the appearance of functional tolerance. A down-regulated expression was observed at the mRNA level, while the functional output reflects the availability of coupled receptor protein, which is affected by altered transcription only following a temporal lag. Our results are in line with previous reports of a decreased cannabinoid receptor protein and gene expression in the striatum after acute and chronic treatment with the cannabinoid agonists Δ^9 THC and CP-55,940 (Rubino *et al*, 1994; Corchero *et al*, 1999). As a possible mechanism for tolerance to cannabinoid agonists, it has previously been reported that rapid internalization of the CB1 receptor occurs upon agonist administration, which can be reversed after acute treatment, but not after long-term exposure. However, this is unlikely to be the only cause for the development of tolerance, since Δ^9 THC only caused internalization to a low degree (Hsieh *et al*, 1999). Our present data indicate that a downregulated CB1 gene expression contributes to the development of tolerance to cannabinoids.

The downregulation of CB1 receptors found in the caudate-putamen is an obvious candidate mechanism for the observed development of tolerance in motor behavior, in agreement with the reported ability of the antagonist SR141716A in reversing the hypoactivity induced by the agonist (Arevalo *et al*, 2001). Although less pronounced, tolerance was also seen in the elevated plus-maze, where the acute injection of HU-210 was somewhat anxiogenic, but

where behavior returned to normal after the 14-day treatment. An acute anxiogenic effect of HU-210 is in agreement with the findings of anxiogenic effects in humans after acute exposure to marijuana (Zuardi *et al.*, 1982). These observations are also supported by animal studies, in which cannabinoid agonists have been reported to increase the neophobic response in the holeboard test, and enhance emotional reactivity in the dark-light emergence test (Navarro *et al.*, 1993; Hernandez-Tristan *et al.*, 2000). The anatomical site(s) and neurochemical systems mediating the anxiogenic effects of acute cannabinoid agonist treatment and the tolerance to this effect are presently not clear.

In contrast, the aversive effects of precipitated cannabinoid withdrawal following the development of tolerance are better understood. It has been shown that, within the amygdala, precipitated withdrawal induces neuronal activation and enhanced release of CRH (Rodriguez *et al.*, 1997), a neuropeptide that has well established anxiogenic-like effects, mediated within this structure (Koob and Heinrichs, 1999). Our finding of differential CRH expression regulation within the central amygdala in the chronic tolerant state *vs* precipitated withdrawal is therefore of particular interest. Similar to a previous study (Rodriguez *et al.*, 1997), our behavioral data demonstrated a clear withdrawal syndrome upon the administration of SR141716A to rats previously treated with HU-210 for 14 days. Upon this precipitation of withdrawal, CRH expression was upregulated in the central amygdala compared to agonist-only-treated subjects, again in agreement with the enhanced CRH release previously shown in this structure under the same conditions (Rodriguez *et al.*, 1997). This in itself is of interest, since it provides a mechanism that could contribute to an increased availability and release of CRH. CRH is involved in withdrawal responses to numerous drugs, and is likely to contribute to the behavioral withdrawal syndrome observed here (Koob, 1999).

An important observation, in our opinion, is that the increase of CRH expression upon precipitation of withdrawal was only a relative one. It occurred from a significantly downregulated level in the tolerant subjects, and in fact, precipitated withdrawal only increased CRH transcript levels back to those of untreated control subjects. This suggests the presence of an allostatic setpoint shift during the development of cannabinoid tolerance, as proposed previously on theoretical grounds (Koob and Le Moal, 2001). In this conceptualization, an initial anxiogenic stimulus of the cannabinoid agonist would activate counter-regulatory processes, attempting to return the system to equilibrium despite the presence of the drug, at the expense of demanding adaptive changes, within the same or other signaling systems. The rapid removal of agonist action in precipitated withdrawal would uncover this dysregulation, and would account for abnormal behavior in the presence of normal CRH activity.

We examined two candidate systems that could account for the allostatic shift observed. According to a previously postulated conceptualization (Koob and Bloom, 1988), the first of these is a candidate for a 'within-system', while the second is for a 'between-system' adaptation. Thus, for the former, an upregulated expression of CRH receptors could lead to maintained CRH function, despite down-regulated peptide expression and release, in turn producing

exaggerated CRH effects upon normalized peptide synthesis. However, our expression analysis does not support this mechanism. In fact, 14 days of agonist treatment did not affect CRH receptor expression in a consistent manner, and where an effect was seen, a somewhat downregulated CRH-R1 expression was found. Of note, this is not likely to reflect the insensitivity of our methodology, which was capable of demonstrating a consistent downregulation of CRH-R1 expression following precipitated withdrawal. This finding is likely to represent a response to increased CRH release and receptor stimulation known to exist under these conditions (Rodriguez *et al.*, 1997). Secondly, an opposing-process organization between the CRH system and NPY has been proposed to exist within the amygdala (Heilig *et al.*, 1994). Also, NPY has been shown to attenuate, for example, naloxone-precipitate morphine withdrawal (Woldbye *et al.*, 1997), while a potential role of NPY in cannabinoid dependence was suggested by the reduction of NPY Y1 receptor expression in the prefrontal cortex of marijuana abusers (Caberlotto and Hurd, 1999). Thus, altered expression of NPY or its receptors could constitute a 'between-systems' adaptation leading to the allostatic shift observed. However, the development of functional tolerance in the present study was not accompanied by changes in the NPY and Y1 receptor mRNA expression. The neuroadaptive processes leading to the observed allostatic shift therefore remain to be established.

In summary, here we report behavioral tolerance following prolonged cannabinoid agonist treatment, accompanied by the downregulated expression of cannabinoid CB1 receptors in the caudate-putamen, and of the CRH transcript in the central amygdala. The suppression of amygdala CRH expression is reversed, but only back to normal upon precipitation of withdrawal; however, under these conditions, a normalization of CRH expression is now accompanied by markedly dysregulated behavior. These findings provide empirical evidence for an allostatic shift, previously suggested mainly on theoretical grounds (Koob and Le Moal, 2001). The system(s) mediating this mechanism remain to be discovered.

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