

Differential modulations of striatal tyrosine hydroxylase and dopamine metabolism by cannabinoid agonists as evidence for functional selectivity *in vivo*

Barbara Bosier^a, Giulio G. Muccioli^b, Birgit Mertens^c, Sophie Sarre^c, Yvette Michotte^c,
Didier M. Lambert^d, Emmanuel Hermans^{a,*}

^aNeuropharmacology Group, Institute of Neuroscience, Université catholique de Louvain, 54.10, Av. Hippocrate 54, B-1200 Brussels, Belgium

^bBioanalysis and Pharmacology of Bioactive Lipids Laboratory, Louvain Drug Research Institute, Université catholique de Louvain, B-1200, Brussels, Belgium

^cDepartment of Pharmaceutical Chemistry and Drug Analysis, Center for Neuroscience, Vrije Universiteit Brussel, 1090 Brussels, Belgium

^dPharmaceutical Chemistry and Radiopharmacy Group, Louvain Drug Research Institute, Université catholique de Louvain, B-1200 Brussels, Belgium

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ABSTRACT

It is generally assumed that cannabinoids induce transient modulations of dopamine transmission through indirect regulation of its release. However, we previously described a direct cannabinoid-mediated control of tyrosine hydroxylase (TH) expression, *in vitro*. We herein report on the influence of cannabinoid agonists on the expression of this key enzyme in catecholamine synthesis as well as on the modification of dopamine content in adult rats. As expected for cannabinoid agonists, the exposure to either Δ^9 -THC, HU 210 or CP 55,940 induced both catalepsy and hypolocomotion. Supporting a possible long-lasting control on dopaminergic activity, we noticed a significant HU 210-mediated increase in TH expression in the striatum that was concomitant with an increase in striatal dopamine content. Surprisingly, while a similar trend was reported with Δ^9 -THC, CP 55,940 completely failed to modulate TH expression or dopamine content. Nevertheless, the access of CP 55,940 to brain structures was validated by determinations of drug concentrations in the tissue and by *ex vivo* binding experiments. Furthermore, confirming the central activity of CP 55,940, the analysis of dopamine metabolites revealed a reduction in striatal DOPAC concentrations. Consistent with the involvement of the CB₁ cannabinoid receptor in these different responses, both HU 210- and CP 55,940-mediated effects were prevented by SR 141716A. Therefore, the present data suggest that both HU 210 and CP 55,940 cause a delayed/persistent regulation of the dopamine neurotransmission system. Nevertheless, these commonly used cannabinoid agonists endowed with similar pharmacodynamic properties clearly triggered distinct biochemical responses highlighting the existence of functional selectivity *in vivo*.

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1. Introduction

While G protein coupled receptors (GPCRs) were initially considered as simple molecular switches, the last decade has revealed the complexity and flexibility offered by these membrane

Abbreviations: CP 55,940, (1*R*,3*R*,4*R*)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol; DMSO, dimethylsulfoxide; DOPAC, 3,4-dihydroxyphenylacetic acid; DSE, depolarisation-induced suppression of excitation; DSL, depolarisation-induced suppression of inhibition; GPCR, G protein coupled receptor; HU 210, (6*R*)-*trans*-3-(1,1-dimethylheptyl)-6,7,10,10-tetrahydro-1-hydroxy-6,6-dimethyl-6*H*-dibenzo[*b*,*d*]pyran-9-methanol; SR 141716A, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride; Δ^9 -THC, Δ^9 -tetrahydrocannabinol; TH, tyrosine hydroxylase.

* Corresponding author. Tel.: +32 2 7649339; fax: +32 2 7645460.

E-mail address: emmanuel.hermans@uclouvain.be (E. Hermans).

receptors. Indeed, experimental data, evidencing that a single GPCR has the ability to simultaneously activate multiple G protein subtypes, have accumulated for almost all members of this receptor family. Assuming that these different G protein couplings emerge from various active receptor conformations, this concept supports the possibility of agonist-selective signalling. This concept, also referred to as “agonist trafficking of receptor signalling” (Kenakin, 1995), has been more recently termed functional selectivity (Urban et al., 2007), which is suggestive of the potential selective regulation of functional responses.

With respect to the CB₁ cannabinoid receptor, its interaction with several G protein subtypes is commonly documented (for review see Hudson et al., 2010). This is consistent with accumulating reports showing agonist-selective activations of different G protein subtypes and associated signalling (Bonhaus et al., 1998; Glass and Northup, 1999; Lauckner et al., 2005; Mukhopadhyay and

Howlett, 2005). In a previous study, we demonstrated an opposite regulation of the dopamine synthesising enzyme tyrosine hydroxylase (TH) expression in response to either HU 210, a tricyclic synthetic cannabinoid agonist or CP 55,940, a bicyclic cannabinoid agonist, in a neuroblastoma cell line (Bosier et al., 2007). Because these agonist-selective responses were regulated through different signalling pathways, our data unveiled functional selectivity at the CB₁ cannabinoid receptor (Bosier et al., 2009). However, the question of the physiological consequences of such a complex regulation remains unanswered.

There is evidence for the implication of dopamine in the central actions of cannabinoids in rodents and humans (Fernandez-Ruiz et al., 2010). Indeed, CB₁ cannabinoid receptors are abundantly expressed in basal ganglia (Herkenham et al., 1991), including those regions implicated in the control of reward and motor behaviours which are considerably altered by cannabinoids. On the other hand, endocannabinoids are now emerging as key components in the regulation of dopamine neurotransmission (Maldonado et al., 2006). Thus, by increasing the firing rate of dopaminergic neurons, cannabinoids facilitate dopaminergic transmission in several brain regions including the striatum (Andre et al., 2010; Cheer et al., 2004; Riegel and Lupica, 2004). However, contradictory results have been reported regarding dopamine release in striatum (Cadogan et al., 1997; Malone and Taylor, 1999; O'Neill et al., 2009; Szabo et al., 1999). Indeed, in this structure, as in most of the brain, CB₁ cannabinoid receptors are primarily located on presynaptic GABAergic and glutamatergic nerve terminals where they function together with the endocannabinoids as a retrograde signalling system. Hence, it is generally assumed that modifications of dopaminergic circuits mediated by CB₁ cannabinoid receptors are exerted through the modulation of either inhibitory or excitatory inputs received by the dopaminergic neurons.

Contradictory data have been reported concerning the presence of CB₁ cannabinoid receptors on dopaminergic neurons and it is likely that CB₁ cannabinoid receptors may regulate dopamine transmission through distinct and complementary mechanisms. Cachope et al. (2007) have shown that endocannabinoids-evoked dopamine release occurs through a mechanism that does not involve disinhibition of dopaminergic varicosities. It was also suggested that Δ^9 -tetrahydrocannabinol (Δ^9 -THC) could influence dopamine transmission through modulation of catecholamine uptake (Poddar and Dewey, 1980; Sakurai-Yamashita et al., 1989), although these *in vitro* observations were not supported by *in vivo* studies (Cheer et al., 2004). Finally, it has been reported that either a prenatal exposure to Δ^9 -THC (Bonnin et al., 1994) or a chronic treatment with the synthetic cannabinoid agonist WIN 55,212-2 (Page et al., 2007) induce changes in TH expression and/or activity.

While we previously demonstrated a cannabinoid-mediated transcriptional regulation of TH expression *in vitro*, suggesting that CB₁ cannabinoid receptors may control critical neuronal functions through a delayed and persistent control of dopamine brain levels, no evidence for such a direct and acute regulation has been reported *in vivo*. To strengthen the physiological relevance of our study, and to further investigate the mechanisms of cannabinoid actions in the striatum, we have now examined the regulations of TH expression, dopamine content as well as dopamine metabolism after a single administration of cannabinoid agonists in adult rats. The complex regulation of TH expression that we reported in neuroblastoma cells has revealed functional selectivity at the CB₁ cannabinoid receptor. Therefore, we herein investigated whether this concept could account for agonist-selective responses *in vivo*. Thus, by reporting differential regulations of both TH expression and dopamine metabolism in the rat striatum consecutively to either HU 210 or CP 55,940 administration, this study provides evidence for physiological consequences of functional selectivity.

2. Experimental procedures

2.1. Animals

Male Wistar rats, weighing 275–300 g at the beginning of the experiment, were from Charles River Laboratories (distributed by Iffa-Credo, Lyon, France). Animals were acclimatised in the housing facility from Vrije Universiteit Brussel in a controlled environment (12 h daylight cycle, temperature controlled room) during 1 week before starting the experiments. All experiments were approved by the local ethic committee and housing conditions were as specified by the Belgian Law of 14 November, 1993 on the protection of laboratory animals (LA 1230314).

2.2. Drugs

HU 210 and CP 55,940 were purchased from Tocris Cookson (Bristol, UK) and Δ^9 -THC was from Lipomed (Arllesheim, Switzerland). The CB₁ cannabinoid receptor inverse agonist/antagonist SR 141716A was generously given by Dr. Barth, Sanofi-Synthelabo Research (Montpellier, France). HU 210 and CP 55,940 were prepared as stock solutions in ethanol at 20 mg/ml, stored as aliquots at -80°C and administered i.p. in a volume of 1 ml/kg 1% ethanol, 1% Tween 80 saline solution. Δ^9 -THC was stored in ethanol solution at 100 mg/ml, prepared in 5% ethanol, 2% Tween 80 saline solution and administered i.p. in a volume of 2 ml/kg. SR 141716A was prepared in 4% ethanol, 1% Tween 80 saline solution, and administered i.p. in a volume of 1 ml/kg. In tests involving agonists only, rats were given a single i.p. injection, while for tests combining agonists and the antagonist SR 141716A, the latter was administered 10 min before injection of the agonist.

2.3. Catalepsy and locomotion measurements

Catalepsy and locomotion measures were performed as previously described (Bosier et al., 2010). Briefly, rats were tested for catalepsy by the placement of both forelimbs over a thin metal bar fixed at 10 cm above the ground and timed for the latency to move one or both forelimbs. After catalepsy testing, the motor activities of the animals were recorded in an open field device (60 × 60 cm arena) equipped with a digital video tracking system. The total walking distance was recorded during a 5 min period and scored with the Noldus EthoVision video tracking system (Wageningen, the Netherlands).

2.4. RNA extraction and quantitative real-time PCR

At the indicated time points, animal were sacrificed, the striatum was immediately dissected and stored at -80°C . Total RNA was then extracted using TriPure reagent (Roche Diagnostics, Mannheim, Germany). First strand cDNA was generated from 1 μg total RNA using the iScript cDNA Synthesis kit (Bio-Rad, Nazareth, Belgium) according to the manufacturer's instructions. Real-time PCR amplifications of TH cDNA were carried out using the iCycler IQTM multicolour real-time PCR detection system (Bio-Rad), in a total volume of 25 μl containing 10 ng cDNA template, 0.3 μM of the primers (forward, 5'-AGTCCAATGCTCTGGAGAACT-3'; reverse, 5'-TTCACCTGAGCCGGACTGCT-3') designed to exclude the detection of genomic DNA, and the IQTM SYBR Green Supermix. PCR protocol was conducted using 45 cycles with an annealing temperature of 60°C . The fluorescence was monitored at the end of each elongation step. For quantitative analysis, a relative standard curve was generated using the same amplification conditions, with dilutions of a mix of cDNA templates (from 100 to 0.39 ng). TH mRNA expression was normalised to the relative amplification of GAPDH mRNA. Quantification of mRNA in the samples was performed using the post-run data analysis software provided with the iCycler system.

2.5. Western blot analysis

40 μg of striatum protein extracts diluted in the appropriate amount of 5 × loading buffer (250 mM Tris-HCl, 500 mM dithiothreitol, 10% SDS, 50% glycerol, 0.5% bromophenol blue, pH 6.8) to obtain 1 × buffer were boiled for 5 min before separation on a 10% SDS-polyacrylamide gel. Proteins were then transferred to nitrocellulose membranes for immunodetection. Blots were blocked for 1 h with 5% non fat powdered milk in TTBS (20 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.6) and probed at 4°C overnight in a 1:2500 dilution of rabbit anti-TH (Chemicon, Hampshire, UK) antibody. This was followed by thorough washings in TTBS and 1 h incubation with HRP-conjugated anti-rabbit (1:3000) (Chemicon) secondary antibody. Blots were revealed with Super Signal West Pico system (Pierce, Aalst, Belgium). After antibodies stripping (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7, 60°C , 30 min) the TH expression was normalised by reprobing with an anti-actin antiserum (1:5000) (Abcam, Cambridge, UK). Relative amounts of protein were quantified by scanning densitometry using the software Image Master (Pharmacia Biotech Benelux, Roosendaal, The Netherlands).

2.6. HPLC-MS quantification of exogenous cannabinoids

Rat brains were homogenised in H₂O (5 ml), sonicated in an ice-bath for 5 min after which 2.5 ml of the solution were added to 10 ml of CHCl₃ containing 2 nmol of

CP 55,243 as internal standard. MeOH was then added and the resulting solution extensively mixed to extract the analytes in the organic phase. Phase separation was completed by centrifuging 10 min at 2500 rpm and the organic layer recovered and dried down under nitrogen and mild heating. The residue was solubilised in CHCl_3 and pre-purified by solid phase separation using a silica column. Following CHCl_3 elution, CP 55,940, HU 210 and CP 55,243 were recovered using a 1:1 mixture of ethylacetate-acetone. Note that the complete elution of these derivatives using the above procedure was checked by measuring the recovery of radiolabeled CP 55,940 from an aqueous solution.

The resulting fraction was analysed by HPLC-MS using a LTQ Orbitrap mass spectrometer (ThermoFisher Scientific) coupled to an Accela HPLC system (ThermoFisher Scientific). Analytes separation was achieved using a C-18 Supelguard pre-column and a Supelcosil LC-18 column (3 μM , 4 \times 150 mm) (both from SigmaAldrich). Mobile phase A and B were composed of MeOH–H₂O–ammonium hydroxide 75:25:0.1 (v/v/v) and MeOH–ammonium hydroxide 100:0:1 (v/v), respectively. The gradient (0.5 ml/min) was designed as follows: from 100% A to 100% B in 15 min, followed by 10 min at 100% B and subsequent re-equilibration at 100% A. MS analysis in the negative mode using the Orbitrap mass analyser was performed with an APCI ionisation source. Capillary and APCI vapouriser temperatures were set at 250 °C and 400 °C, respectively. The ratios of CP 55,940 and HU 210 over CP 55,243 were calculated and used to determine the actual amounts of CP 55,940 and HU 210 using a calibration curve built using 0, 1, 2, 5, 10, 15, 50 and 100 pmol of CP 55,940 and HU 210. Finally the data were normalised by tissue sample weight.

2.7. *Ex vivo* binding assay

The penetration of cannabinoid agonists into the striatum was further evaluated by an *ex vivo* binding assay. Rats were killed by decapitation 30 min following i.p. injection of HU 210, CP 55,940 (both at 100 $\mu\text{g}/\text{kg}$) or corresponding vehicle, striatum were rapidly dissected and stored at –80 °C till the binding assay. Tissues samples were homogenised in 50 mM Tris–HCl buffer (50 mM, pH 7.4) containing 3 mM MgCl_2 , 1 mM EDTA and 0.5% bovine serum albumin with a glass-teflon pestle homogeniser. Determination of total [³H]-SR 141716A binding was performed on 20 μl (2 mg tissue) of the total tissue preparation incubated for 60 min at 30 °C with increasing concentration of [³H]-SR 141716A (1–20 nM) (Amersham, Roosendaal, the Netherlands) in a 100 μl final volume of homogenisation buffer. Non specific binding was determined in the presence of 100 μM HU 210. Reactions were then stopped and solutions were vacuum-filtered through 0.5% polyethyleneimine pretreated glass fibre filters. Radioactivity was counted for each filter in 10 ml liquid scintillation using a Pharmacia Wallac1410 β -counter. Specific [³H]-SR 141716A binding was determined by subtracting the non specific binding values from the total binding values. Data were expressed as the specific [³H]-SR 141716A binding expressed in fmol/mg tissue. Each determination was performed in duplicate for at least 3 animals.

2.8. Determination of dopamine and dopamine metabolites contents

The determination of dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) tissue levels was performed as previously described (Izurieta-Sanchez et al., 1998). The striatal tissue was homogenised in 2 ml of anti-oxidant solution 0.05 M HCl, 0.5% $\text{Na}_2\text{S}_2\text{O}_5$ and 0.05% Na_2EDTA . After 20 min centrifugation at 10,000 \times g at 4 °C, the supernatant was diluted 5 times in acetic acid (0.5 M) and 20 μl of sample was injected and analysed directly for dopamine and DOPAC content on a narrow bore (C-18 column: 15 μm , 150 \times 2.1 mm; Alltima, Grace, Lokeren, Belgium) LC system. The mobile phase consisted of 0.1 M sodium acetate trihydrate (Merck, Darmstadt, Germany), 20 mM citric acid monohydrate (Merck), 1 mM 1-octane sulfonic acid (Sigma), 0.1 mM Na_2EDTA (Merck), 1 mM dibutylamine (Sigma) and 3% v/v methanol, adjusted to pH 3.8. All samples were injected via a high precision auto-injector equipped with a cooling system (Kontron, San Diego, CA, USA). The flow rate was set at 0.3 ml/min. The electrochemical detection (Antec, The Netherlands) potential was +700 mV versus the reference electrode (Ag/AgCl). Sensitivity was set at 2 nA full scale. The integration of the chromatograms was performed with the Data Apex Clarity software programme (Antec). Results were expressed as $\mu\text{g}/\text{g}$ wet tissue. The limit of detection of the LC system corresponds to a residual tissue dopamine and DOPAC content of less than 2.5 ng/g wet tissue.

2.9. Data analysis

Unless otherwise stated, data presented in the text and figures were expressed as mean percentages \pm SEM of the corresponding values obtained with vehicle-treated animals. GraphPad PRISM (version 5; San Diego, CA) was used to analyse the data and generate dose–response curves. Statistical analyses were performed by analysis of variance (ANOVA) and the post-hoc analysis was made by Scheffe test.

3. Results

3.1. Cannabinoid-mediated motor effects and striatal regulation of TH expression

Hypolocomotion and catalepsy are typical striatal-controlled processes observed after administration of cannabinoids. As expected, the single administration of either HU 210 (100 $\mu\text{g}/\text{kg}$, i.p.) or CP 55,940 (100 $\mu\text{g}/\text{kg}$, i.p.) rapidly induced a marked and significant reduction of locomotion in rats and a strong cataleptic behaviour (see Bosier et al., 2010). These animals were sacrificed, and TH mRNA or protein contents were examined in the striatum. Administration of HU 210 (100 $\mu\text{g}/\text{kg}$, i.p.) produced a significant increase in TH mRNA level in striatal tissue 6 h after the injection (Fig. 1A). According to our previous report on the regulation of TH (Bosier et al., 2007), the increase in TH mRNA was followed by an up-regulation of TH protein 24 h after the administration (Fig. 1C). To determine whether the effects of HU 210 were mediated through CB₁ cannabinoid receptor activation, the selective antagonist/inverse agonist SR 141716A (1 mg/kg, i.p.) was administered to animals 10 min before HU 210 injections. Using this protocol, SR 141716A alone was without effect, but totally prevented HU 210-mediated responses, suggesting that the increase in either TH mRNA or protein expression elicited by HU 210 is CB₁ cannabinoid receptor-dependent processes (Fig. 1B and D). In addition, administration of the natural partial CB₁ cannabinoid receptor agonist Δ^9 -THC (10 mg/kg, i.p.) was also found to elicit an increase in TH mRNA expression, even though this failed to reach significance (*p* value of 0.051). Nevertheless, at the tested doses, Δ^9 -THC induced the expected profound hypolocomotion (Fig. 2).

Contrasting with this, the potent cannabinoid agonist CP 55,940 failed to elicit changes in either TH mRNA or protein levels in the rat striatum, at all tested times (Fig. 3). Given the pharmacokinetics properties of CP 55,940, likely supporting a shorter tissue half-life of this agonist, we additionally assessed the changes in TH expression 3 h after administration of CP 55,940. Again, CP 55,940 was without influence on striatal TH content, confirming that the CB₁ cannabinoid receptor-mediated control of TH expression is dependent on the agonist used for receptor activation.

3.2. Brain penetration of cannabinoid agonists and CB₁ cannabinoid receptor occupation

To confirm the efficient brain penetration of both cannabinoid agonists, we further analysed HU 210 and CP 55,940 levels in total brain homogenates using HPLC-MS quantification. Thirty min after i.p. administration (100 $\mu\text{g}/\text{kg}$ HU 210, 100 $\mu\text{g}/\text{kg}$ CP 55,940), drug concentrations were higher for CP 55,940 than for HU 210 (Fig. 4A). Confirming an efficient brain penetration for both drugs, these results are consistent with the above mentioned efficient modulation of motor behaviours observed with both agonists.

In addition, to determine whether both drugs reached the CB₁ cannabinoid receptor into the brain and more specifically into the striatum, *ex vivo* binding assays were carried out on striatal homogenates prepared from vehicle, HU 210 or CP 55,940 treated animals, 30 min after i.p. administration. To minimise drug receptor dissociation, care was taken in our assays to rapidly prepare the homogenates, omitting the usual washing steps. In these conditions, the *K_D* value for [³H]-SR 141716A binding on vehicle-treated striatal homogenates was 7.39 ± 3.77 nM, consistent with the reported nanomolar affinity of this radioligand (Govaerts et al., 2004). In samples from HU 210 or CP 55,940 treated animals, the *K_D* values were not statistically different (5.22 ± 2.19 nM and 7.24 ± 2.55 nM respectively) confirming the reliability of our protocol to evaluate receptor occupancy. Focussing on the maximal

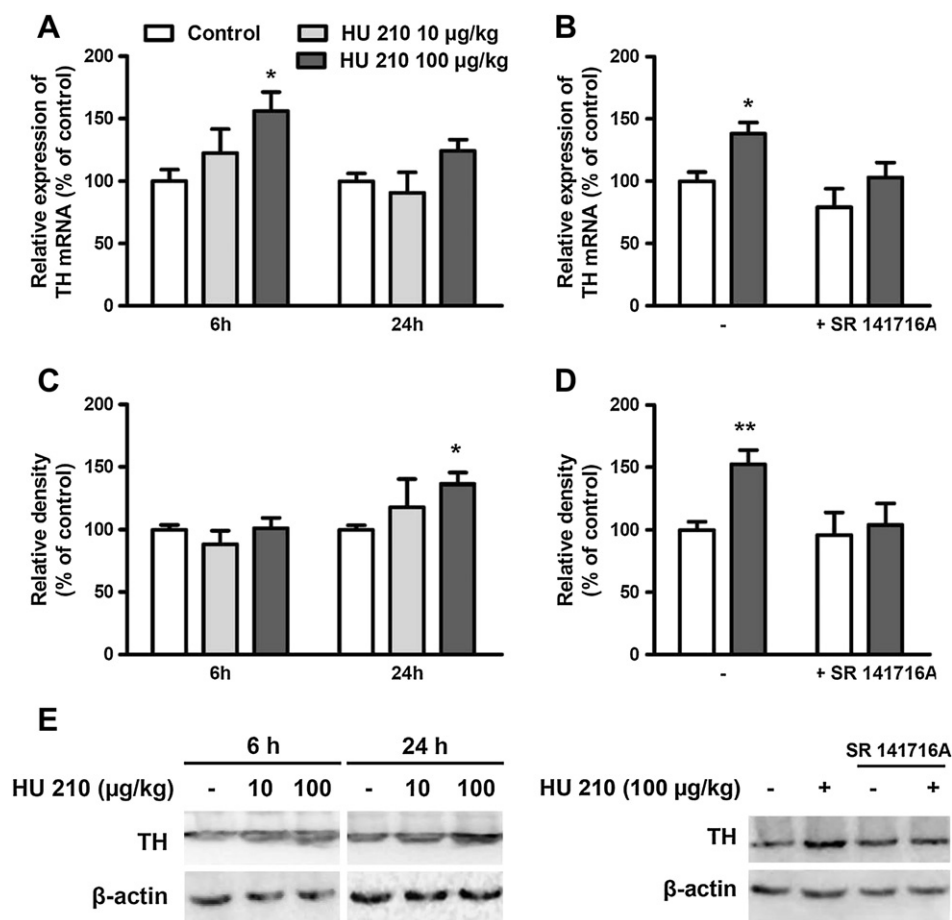


Fig. 1. HU 210-mediated regulation of TH mRNA and protein contents in the rat striatum. mRNA levels for TH (A–B) were determined in striata of adult male rats treated for 6 and 24 h with HU 210 (10 or 100 µg/kg i.p.). The response observed 6 h after the injection was prevented injecting SR 141716A 1 mg/kg 10 min prior to the agonist administration (panel B). Results are given as relative percentage of expression of TH mRNA normalised against GAPDH mRNA expression. Protein levels for TH (C–D) were determined in the striata from the same animals treated for 6 and 24 h with HU 210 (10 or 100 µg/kg i.p.). The 24 h treatment was repeated in animals having received SR 141716A 10 min previously (panel D). The densitometric analysis of the TH protein signals (representative example illustrated in E) was normalised against the measured signals corresponding to actin. Results are expressed in percentage relative to control animals. Data are mean \pm SEM of $n = 4$ –9 animals. ** $p < 0.01$; * $p < 0.05$.

binding values, we observed that i.p. administration of either HU 210 or CP 55,940 (both at 100 µg/kg) significantly reduced the binding of [3 H]-SR 141716A in the striatum (Fig. 4B). Thus analysis of saturation curves revealed B_{max} values of 70.8 ± 10.4 fmol/mg tissue in samples prepared from vehicle injected rats, whereas values of 40.4 ± 5.9 and 45.9 ± 6.5 fmol/mg tissue were measured in samples from HU 210 or CP 55,940 treated rats, respectively. Similar reductions of the maximal [3 H]-SR 141716A binding suggest an equivalent occupancy of CB₁ cannabinoid receptors in the striatum with both drugs. As, CP 55,940 shows a slightly lower affinity for the targeted receptor (Govaerts et al., 2004), this appears thus counterbalanced by its higher brain penetration (Fig. 4A). Note that given the rather low absolute brain concentration of the two drugs reported in Fig. 4A, it is unlikely that the reduction of [3 H]-SR 141716A binding on striatal homogenates prepared from cannabinoid-treated animals results from a contamination of the tissue preparation with residual unbound HU 210 or CP 55,940.

3.3. Cannabinoid-mediated regulation of dopamine and dopamine metabolite contents

To determine whether the HU 210-mediated increase in TH expression was correlated with alterations of dopamine content and metabolism in the striatum, we next assessed total dopamine as

well as DOPAC concentrations in striatal homogenates. Consistent with the up-regulation of TH, administration of HU 210 (100 µg/kg i.p.) produced an elevation of dopamine content in the striatum, an effect which appeared modest after 6 h and became significant 24 h after drug administration (Fig. 5A). When administered 10 min prior to HU 210 injection, SR 141716A totally prevented the dopamine increase, demonstrating the involvement of the CB₁ cannabinoid receptor (Fig. 5B). With respect to dopamine metabolism, an increase in DOPAC concentration was measured after HU 210 administration, which however failed to reach significance. Nevertheless, no significant modulation of DOPAC/dopamine ratio was evidenced (Fig. 5D). Together with the increased dopamine content, these data are suggestive of an elevation of dopamine metabolism in the striatum in these animals.

In contrast to the results obtained with HU 210, the dopamine content in the striatum was not affected by the administration of CP 55,940 (10 and 100 µg/kg, i.p.) (Fig. 6A). However, 6 and 24 h after its administration CP 55,940 significantly reduced DOPAC content in striatum homogenates (Fig. 6B). Consistent with this reduction of dopamine metabolism produced by CP 55,940 we also evidence a decreased DOPAC/dopamine ratio (Fig. 6D). Pre-treatment with the antagonist SR 141716A (1 mg/kg, i.p.) completely blocked this alteration in dopamine metabolism observed 6 h after the injection of CP 55,940 (Fig. 6C and E). This suggests that the effect of CP

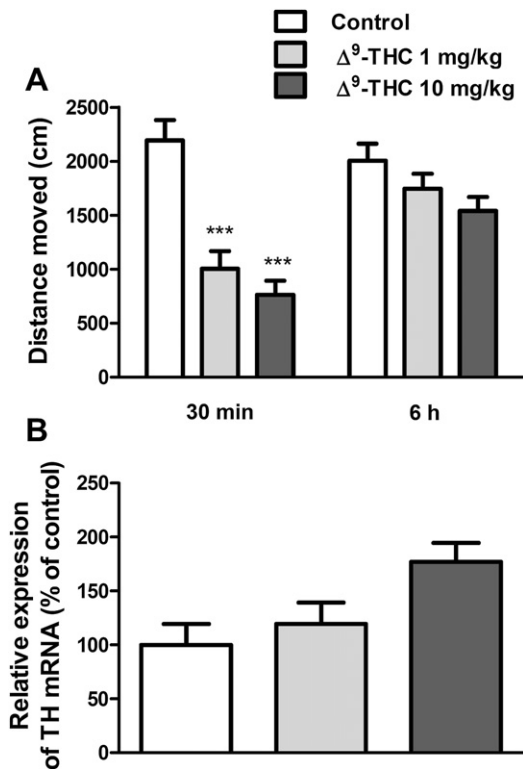


Fig. 2. Δ^9 -THC-mediated hypolocomotion and regulation of TH mRNA. (A) Total distance moved was measured on a 5 min period 30 min after i.p. injection of vehicle or Δ^9 -THC (1 and 10 mg/kg) to adult male Wistar rats. (B) mRNA levels for TH were determined in striata of animals treated for 6 h with Δ^9 -THC (1 and 10 mg/kg). Results are given as relative percentage of expression of TH mRNA normalised against GAPDH mRNA expression. Data points represent the mean values \pm SEM of $n = 5$ animals. *** $p < 0.005$.

55,940 on dopamine turnover in the striatum is mediated by the activation of CB₁ cannabinoid receptors. Of note, treatment with SR 141716A alone did not modulate dopamine and DOPAC contents.

4. Discussion

Several behavioural functions regulated by cannabinoids largely depend on influences on the dopaminergic system. Nevertheless, while it is generally accepted that cannabinoids control neurotransmitter release through rapid and transient inhibition of either excitatory (DSE) or inhibitory (DSI) synaptic transmission (Katona and Freund, 2008), the delayed influence of cannabinoids on the genetic expression of key proteins involved in neurotransmitter metabolism remains poorly examined. Previous studies reporting on the influences of cannabinoids on TH expression and/or activity have focused on the nervous system of foetuses, neonates, or adult rats prenatally exposed to Δ^9 -THC (Bonnin et al., 1994; Suarez et al., 2000). In contrast, little is known regarding the impact of cannabinoid exposure on TH expression in adulthood. At the cellular level, CB₁ cannabinoid receptor-mediated regulation of TH protein was reported in foetal mesencephalic neurons (Hernandez et al., 2000), while *in vivo*, only the chronic administration of WIN 55,212-2 was shown to promote TH protein expression (Page et al., 2007). We previously demonstrated that synthetic cannabinoid agonists regulate TH expression in a neuroblastoma cell line through a transcriptional regulation mechanism (Bosier et al., 2007). Extending these *in vitro* findings, we herein report on a control of TH expression in the striatum consecutive to a single administration of HU 210. Considering both the increase in TH

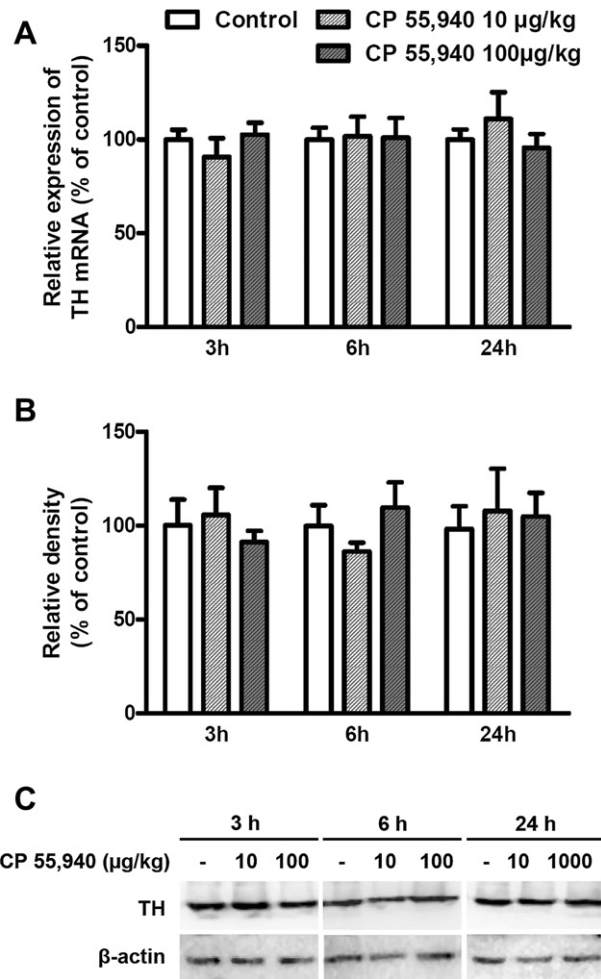


Fig. 3. Lack of CP 55,940-mediated regulation of TH mRNA expression in the rat striatum. TH gene expression (A) was determined total mRNA extract from striata of adult male rats treated for 3, 6 and 24 h with CP 55,940 (10 or 100 µg/kg i.p.). TH mRNA levels are normalised against the corresponding GAPDH and results are given as relative percentage to control animals. TH protein analysis (B) was performed on striatal protein extract from the same animal exposed 3, 6 and 24 h to CP 55,940 (10 or 100 µg/kg i.p.). The densitometric analysis of the TH protein signals (representative example illustrated in C) was normalised against the measured signals corresponding to actin. Results are given as the percentages of expression relative to control animals. Data are mean \pm SEM of $n = 4$ –9 animals.

mRNA and protein expressions, our data suggest that in addition to modifications of neuronal dopamine transmission through the well-established DSI/DSE mechanisms, CB₁ cannabinoid receptors also induce a more delayed and long-lasting control of striatal dopaminergic activity. We indeed validated that the up-regulation of TH expression was correlated with increased striatal dopamine content 24 h after the HU 210 administration.

Increased striatal dopamine content together with enhanced DOPAC concentrations indicates that cannabinoids could promote dopamine turnover. This suggests that newly synthesised dopamine is not only stored in the striatum but also could be released and metabolised. Stereotypy, locomotor hyperactivity and reward behaviour are typical responses to increased striatal levels of dopamine. Enhanced dopamine transmission is also a common feature of several neuropsychiatric diseases. Therefore, cannabinoid-mediated long-lasting increase in dopaminergic activity would likely impact on essential brain functions influencing motor, cognitive and emotional behaviours. As an example it is postulated that striatal hyperdopaminergia predates psychotic symptoms that characterise

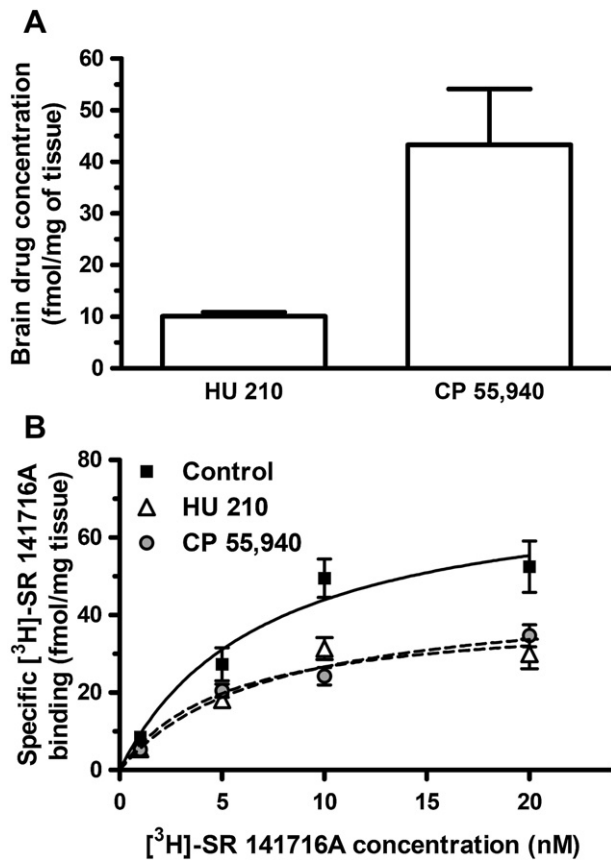


Fig. 4. Brain penetration of HU 210 and CP 55,940. (A) 30 min consecutive to HU 210 or CP 55,940 (both at 100 $\mu\text{g}/\text{kg}$) i.p. injection, animals were sacrificed and brain content in HU 210 and CP 55,940 respectively was measured by HPLC-MS. Results are expressed as the amount of drug (pmol)/g of tissue. Data are means with SEM values from 4 injected animals. (B) Specific binding of [^3H]-SR 141716A was measured on striatum from animal exposed during 30 min to vehicle; HU 210 or CP 55,940 (both at 100 $\mu\text{g}/\text{kg}$ i.p.). Results are expressed as the specific [^3H]-SR 141716A binding (fmol [^3H]-SR 141716A/mg tissue). Results are given as the means with SEM values from 6 animals. Estimated B_{max} values were: 70.840 ± 10.39 , 40.40 ± 5.85 and 45.89 ± 6.37 fmol [^3H]-SR 141716A/mg tissue respectively for vehicle, HU 210 and CP 55,940 treated rats respectively. An overall ANOVA analysis revealed significant effect of the treatment on specific maximal [^3H]-SR 141716A binding ($F(2,15) = 5.621$ $P = 0.0151$). Dunnett's multiple comparison test showed significantly lower [^3H]-SR 141716A binding in tissue homogenates from HU 210 ($P < 0.05$) and CP 55,940 ($P < 0.05$) treated rats.

schizophrenia (Howes et al., 2009). Hence, it is tempting to propose that this mechanism contributes to neuronal dysregulation induced by CB₁ cannabinoid receptor agonists. On the other hand, modifications of TH expression have been reported in several neurodegenerative diseases including Parkinson's disease (Javoy-Agid et al., 1990; Hurley et al., 2003) and Alzheimer's disease (Kastner et al., 1993; Szot et al., 2000). Underlining the potential impact that cannabinoids may have in the treatment of these pathologies, the loss of TH was possibly related to the neurodegenerative process (Kastner et al., 1993).

At variance, CP 55,940 failed to induce any regulation of either TH mRNA or protein levels in the striatum and did not trigger changes in dopamine level in the striatum. Likewise, it was recently reported that TH expression in rat mesolimbic and nigrostriatal pathways was not affected after chronic treatment with this agonist (Higuera-Matas et al., 2010). Of importance, the measurement of brain concentrations of HU 210 and CP 55,940 as well as the *ex vivo* binding experiment evaluating interaction with the receptor (Petitet et al., 1999) validated the brain penetration and CB₁ cannabinoid receptor occupancy for both cannabinoid

ligands after the single i.p. administration. In addition, rapidly after their administration, comparable hypolocomotion and cataleptic responses were observed with both CP 55,940 and HU 210, further confirming the efficiency of CP 55,940 in inducing central effects. Finally, despite the lack of influence on dopamine level, this single CP 55,940 administration provoked a significant reduction in DOPAC content in the striatum. Indeed, as dopamine concentration was not modulated by CP 55,940, the DOPAC/dopamine ratio was reduced, indicating a reduction of dopamine metabolism. Monoamine oxydases (MAO) are enzymes involved in dopamine degradation. Therefore an inhibition of this enzyme could contribute to the reported reduction in DOPAC level. Indeed, several studies have already debated about regulations of MAO activity by diverse cannabinoid agonists (see Fišar, *in press* for review). Even though the CP 55,940-mediated reduction in DOPAC concentrations was antagonised by SR 141716A, we cannot exclude that in this study, this compound also induces an inhibition of MAO through an indirect mechanism requiring CB₁ cannabinoid receptor.

Even though we previously reported that HU 210 displays a longer duration of action in comparison to CP 55,940 (Bosier et al., 2010), a more rapid CP 55,940 pharmacokinetic could not hold clue for the herein reported agonist-selective regulation of TH expression. Indeed, the presented data highlight distinct CB₁ cannabinoid receptor-dependent responses to both compounds at an identical time point (6 h after the injection) while neither TH expression nor dopamine content was affected 3 h after CP 55,940 administration. In addition, the previously reported delayed HU 210-mediated effects were CB₁-independent (Bosier et al., 2010), while in the present study, both HU 210- and CP 55,940-mediated responses were systematically antagonised using SR 141716A. This confirms that the regulation of TH expression as well as the change in dopamine level and/or metabolism, resulted from CB₁ cannabinoid receptor activation. Therefore the present data are in accordance with our study showing that through interaction with the same cannabinoid receptor, these two chemically unrelated reference agonists promoted distinct regulations of TH transcription (Bosier et al., 2007). Indeed, earlier data provided compelling evidence that distinct cannabinoid ligands could selectively regulate different intracellular signalling pathways (Bosier et al., 2008) presumably through induction of agonist-selective conformations (Georgieva et al., 2008) that promote selective coupling with distinct G proteins (Bonhaus et al., 1998; Glass and Northup, 1999; Mukhopadhyay and Howlett, 2005). This concept, referred to as functional selectivity was proposed to contribute to the agonist-selective regulation of TH *in vitro* (Bosier et al., 2009). Likewise, the paradoxical observations of agonist-selective alteration of dopaminergic activity might be explained by a similar agonist-selective regulation of signalling pathways. This hypothesis is further supported by the experiment in which Δ^9 -THC tends to display similar effects to those of HU 210. Indeed, in our previous experiments we reported that Δ^9 -THC shared similar properties with HU 210, both differing from the ones of CP 55,940 (Bosier et al., 2007). Therefore one may speculate that HU 210 and Δ^9 -THC activate common signalling cascades, which are distinct from those activated by CP 55,940.

In the nucleus accumbens and the dorsal striatum, most of TH protein, and the resulting newly synthesised dopamine, are detected in neuron terminals projecting from ventral tegmental area and substantia nigra respectively. However, recent experimental data and speculative considerations are now supporting the existence of mRNAs and translation machinery at synaptic proximity that would allow individual terminals to promptly respond to local stimuli (Giuditta et al., 2008; Schuman et al., 2006). In addition, the presence of intrastriatal dopaminergic neurons has been

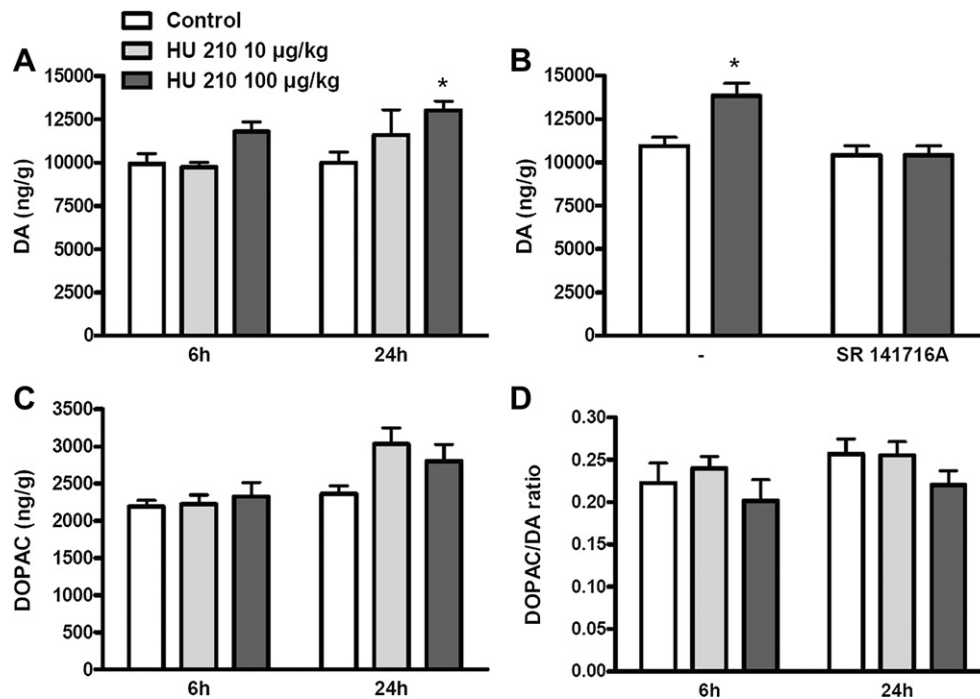


Fig. 5. HU 210-mediated regulation of DA contents in the rat striatum. DA (A–B), DOPAC (C) and DOPAC/DA ratio (D) were determined in striata of adult male rats treated for 6 and 24 h with HU 210 (10 or 100 µg/kg i.p.). In panel B response observed after 24 h was totally prevented when SR 141716A (1 mg/kg) was administered 10 min prior to the HU 210 administration. Data are means with SEM values from $n = 4$ to 9 animals. * $p < 0.05$ relative to control rats injected with vehicle.

documented in both rodents and humans (for review see Huot and Parent, 2007). Even though we measured up to 150 times less TH mRNA in the striatum than in the midbrain structures, we herein evidenced a dose-dependent increase in striatal TH mRNA, 6 h after HU 210 administration which was followed by a TH protein increase 18 h later. Considering both the time-course for this regulation and the concomitant increase in striatal dopamine content, the present findings are in agreement with the concept that the striatum is intrinsically equipped to provide a local source of dopamine which could be pharmacologically manipulated by the cannabinoid system.

Moderate to high densities of CB₁ cannabinoid receptors are observed in regions belonging to the extrapyramidal motor system and the mesolimbic reward system (Herkenham et al., 1991; Hohmann and Herkenham, 2000), including the dorsal and the ventral part of the striatum. Therefore, one may suggest that local cannabinoid receptors could account for a long-term regulation of dopaminergic activity in striatal regions. Supporting the hypothesis of an indirect regulation by cannabinoids, it is generally considered that CB₁ cannabinoid receptors do not colocalise with TH (Herkenham et al., 1991; Julian et al., 2003). In the basal ganglia CB₁ receptors are indeed predominantly located on either GABA or glutamate projections, consequently regulating different neuronal inputs to dopaminergic neurons from the nigrostriatal or the mesolimbic pathways (For review see Fernandez-Ruiz et al., 2010). While the question of a colocalisation between partners from the cannabinoid and the dopamine systems is out of the scope of this study, this should not exclude direct influences of cannabinoids on dopaminergic neurons. Indeed, while still debated in the literature, both functional (Jelsing et al., 2009) and immunohistochemical investigations (Hernandez et al., 2000; Patel and Hillard, 2003; Wenger et al., 2003; Oropeza et al., 2005; Lau and Schloss, 2008) support the colocalisation between CB₁ cannabinoid receptor and TH. These considerations along with our previous studies performed in neuroblastoma cell line support dopaminergic

neurons as putative pharmacological targets of cannabinoids. However, contrasting with this simplified model that only focused on direct modulatory effects, the herein reported increased expression of striatal TH after HU 210 administration likely reflects balanced processes involving both direct and indirect influences of the neuronal network. Indeed, we previously reported on a HU 210-mediated decrease in transcription of TH (Bosier et al., 2007). Besides, we also demonstrated, *in vitro*, that a concomitant stimulation of CB₁ cannabinoid receptors and adenylyl cyclase was sufficient to switch the stimulation to an inhibition of TH transcription (Bosier et al., 2008). Given the observed *in vitro/in vivo* discrepancy, additional endogenous neuronal inputs modulating the constitutive activity of adenylyl cyclase in the striatum have to be considered to explain the stimulatory influence of cannabinoid receptor activation on TH expression. While beyond the scope of this study, a more in depth investigation of these complex regulatory mechanisms would probably lead to a more comprehensive picture of the interactions between cannabinoid and dopamine systems.

Altogether, beside the well-described cannabinoid-mediated transient regulations of neurotransmitter release, the present study suggests that the cannabinoid system participates to a more delayed and persistent regulation of dopamine striatal levels through a CB₁-mediated control of TH gene expression. Modifications of the cannabinoid system take part in a variety of neurological processes or diseases related to dopamine transmission, including modulation of reward behaviour, Parkinson's disease or schizophrenia. This cannabinoid-mediated modification of TH expression may have particular impact on the development or treatment of these disorders. Furthermore, confirming our previous *in vitro* investigations, we now evidence that distinct cannabinoid agonists display different abilities to regulate diverse components of dopamine neurotransmission system *in vivo*. Shedding light on the physiological relevance of functional selectivity, the possibility to selectively manipulate different physiological functions should

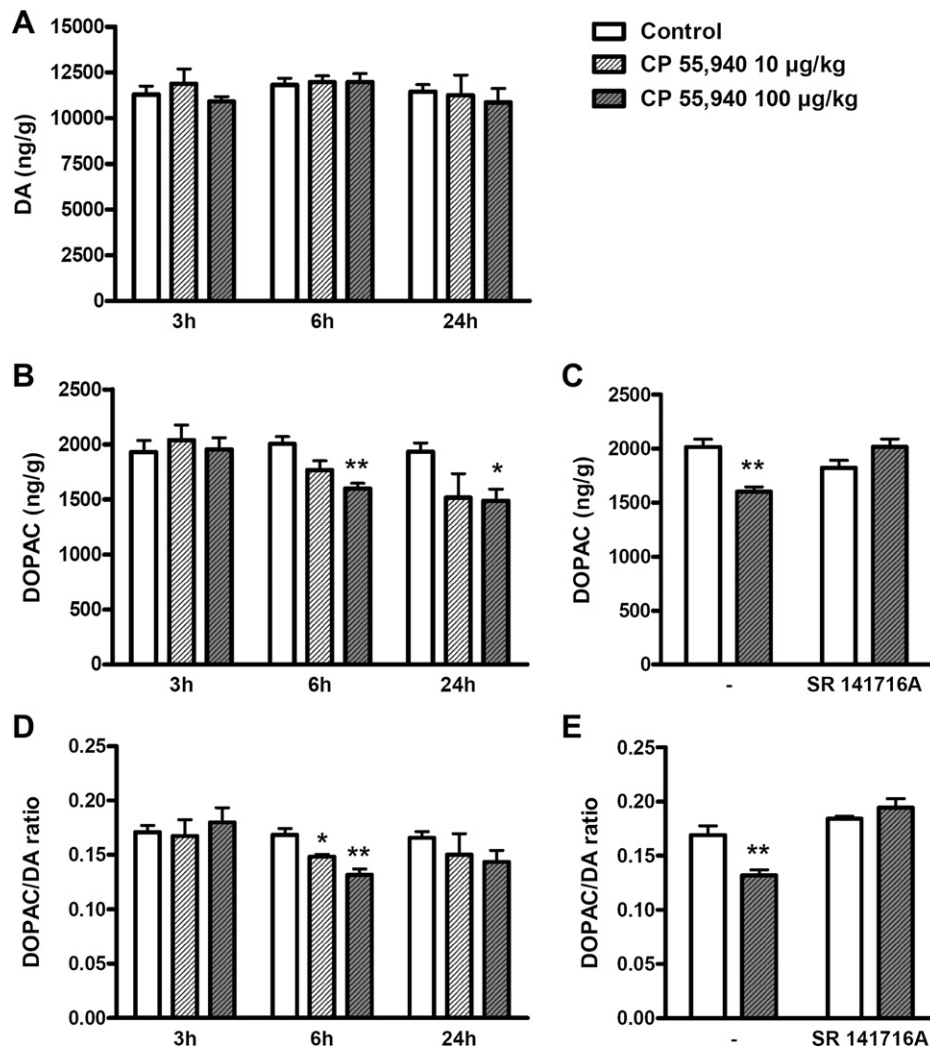


Fig. 6. CP 55,940-mediated regulation of DA metabolism in the rat striatum. DA (A), DOPAC (B–C) and DOPAC/DA ratio (D–E) were determined in striata of adult male rats treated for 3, 6 and 24 h with CP 55,940 (10 or 100 µg/kg i.p.). In panels B and E responses observed after 6 h were suppressed by SR 141716A (1 mg/kg) administered 10 min prior to the agonist administration. Data are means with SEM values from $n = 4$ to 9 animals. ** $p < 0.01$, * $p < 0.05$ relative to control rats injected with vehicle.

receive considerable attention for further development of cannabinoid-related drugs as medicine.

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