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Agonist selective modulation of tyrosine hydroxylase expression by cannabinoid ligands in a murine neuroblastoma cell line

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Abstract

Functional interactions between catecholamines and cannabinoid transmission systems could explain the influence of Δ^9-tetrahydrocannabinol on several central activities. Hence, the presence of cannabinoid CB_1 receptors in tyrosine hydroxylase (TH) containing cells has been suggested, providing clue for a direct control of catecholamines synthesis. In the present study, we evidenced the constitutive expression of functional cannabinoid CB_1 receptors in N1E-115 neuroblastoma and reported on the use of this model to examine the influence of diverse cannabinoid ligands on TH expression. Exposure of the cells to the high-affinity agonist HU 210 (5 h) resulted in a significant decrease in TH content (pEC_{50}: 6.40). In contrast, no change was observed after a similar treatment with the structurally unrelated agonist CP 55,940. Besides, the use of a luciferase reporter assay revealed that these two agonists showed opposite influences on TH gene promoter activity. Thus, in cells expressing pTH-luc constructs, inhibition and induction of luciferase activity were respectively observed with HU 210 (pEC_{50}: 8.95) and CP 55,940 (pEC_{50}: 9.09). Pharmacological characterisation revealed that these reciprocal responses were both related to the specific activation of cannabinoid CB_1 receptor, suggesting an agonist-dependent modulation of distinct signalling pathways. While these data points out the possible pharmacological manipulation of TH expression by cannabinoid ligands, such approach should take into account the existence of agonist selective trafficking of cannabinoid CB_1 receptor signalling.

Keywords: agonist selective trafficking of receptor signalling, cannabinoid, catecholamine, luciferase, transcription, tyrosine hydroxylase.

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Compelling evidence indicates that alteration in mammals behaviour induced by cannabis largely depends on the influence of cannabinoid systems on catecholaminergic transmissions. Thus, enhanced noradrenaline release or turnover was demonstrated in the rat prefrontal cortex shortly after administration of Δ^9-tetrahydrocannabinol (Δ^9-THC) or the synthetic agonist WIN 55,212-2 (Jentsch *et al.* 1997; Oropeza *et al.* 2005). In contrast, in human hippocampus, cannabinoid CB_1 (CB_1) receptors were proposed to contribute to the psychotropic effects of cannabis through a negative influence on electrically evoked noradrenaline release (Schlicker *et al.* 1997). More complex is the crosstalk between cannabinoid and dopamine systems as both appear to exert a mutual control on each other. In the nigrostriatal dopamine pathway, both an increase (French *et al.* 1997; Wu and French 2000) and a decrease in dopamine release (Cadogan *et al.* 1997; Steffens *et al.* 2004) were reported. Similarly, contradictory effects were observed in the mesolimbic and mesocortical dopaminergic projections (for review see (van der Stelt and Di Marzo 2003). Finally, convincing data regarding the physiological role of endogenous cannabinoids systems were obtained using antagonists of cannabinoid receptors. Thus, SR 141716A was found to

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Abbreviations used: CHO, Chinese hamster ovary; DMSO, dimethylsulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate-buffered saline; PFK-2, 6-phosphofructo-2-kinase; PKA, protein kinase A; SDS, sodium dodecyl sulfate; TH, tyrosine hydroxylase; Δ^9-THC, Δ^9-tetrahydrocannabinol; TRPV, transient receptor potential vanilloid.

increase hypothalamic and cortical norepinephrine outflow, as well as forebrain and cortical dopamine release (Darmani *et al.* 2003; Tzavara *et al.* 2003).

The inconsistency of these *in vivo* studies probably results from differences in methodologies and/or specificity in different anatomical structures. It also reflects the complex interactions of cannabinoid systems with monoamine transmission and up to now, the molecular and cellular mechanisms remain poorly understood. Hence, the absence of co-expression or co-localisation of CB_1 receptors with tyrosine hydroxylase (TH), the first rate-limiting enzyme for catecholamines synthesis has been suggested (Herkenham *et al.* 1991a; Tsou *et al.* 1998; Julian *et al.* 2003). Widely distributed throughout the brain (Herkenham *et al.* 1991b), CB_1 receptors are mainly restricted on striatoafferent GABAergic neurons in the basal ganglia. Their absence on dopaminergic nigrostiatal cell bodies or terminals (Herkenham *et al.* 1991a) suggests that cannabinoid-mediated regulation of catecholaminergic transmission involves a retrograde signalling process. On the other hand, others reported that TH immunoreactive fibres are distributed in the same region along the CB_1 terminals of striatonigral or striatopallidal neurons (Julian *et al.* 2001). Immunohistochemical investigations demonstrated expression of CB_1 receptors in TH containing cells in parts of mesocorticolimbic system, including the nucleus accumbens, the ventral tegmental area and the striatum and pyriform cortex (Wenger *et al.* 2003). This provides a cellular background for a direct control of cannabinoid system over dopamine synthesis and release. Besides, the endocannabinoid anandamide was shown to activate transient receptor potential vanilloid (TRPV1) (Zygmunt *et al.* 1999), suggesting an additional mechanism for cannabinoid-mediated regulation of dopamine transmission. Indeed, several studies have demonstrated that capsaicin-mediated activation of TRPV1 located on dopaminergic neurons participate in the control of dopamine release (Hajos *et al.* 1986; Marinelli *et al.* 2005).

During the last decade, several groups reported on the influence of hashish extracts, including the psychoactive cannabinoid agonist Δ^9-THC, on the gene expression and/or functional activity of TH. In particular, the studies focused on the consequences of pre-natal exposure to Δ^9-THC, revealing changes in TH mRNA levels (Bonnin *et al.* 1994) with or without modification of TH protein content (Suarez *et al.* 2000) and activity (Bonnin *et al.* 1996). The decrease in TH activity induced by natural phytocannabinoids was mimicked by administration of anandamide, one of the principal endocannabinoids (Romero *et al.* 1995) and of AM404, a synthetic inhibitor of the putative anandamide transporter (Gonzalez *et al.* 1999). These studies indicate that the cannabinoid system participates in the mammalian brain development by modulating TH function.

Tyrosine hydroxylase plays a pivotal role in the synthesis of catecholamines and both its expression and activity are dynamically regulated by a large variety of stimuli. Whilst the molecular mechanisms involved in this regulation remain not fully elucidated, nearly all physiological regulatory mechanisms affecting a cell protein synthesis and function have been documented for TH (for review see Kumer and Vrana 1996). The TH gene promoter was characterised in the late 1980s and since then, the modulation of TH gene transcription by pharmacological and biochemical inducers have been deeply investigated. Of potential interest, intracellular effectors such as protein kinase A (PKA), mitogen-activated protein kinase and intracellular Ca^{2+} which participate in the signalling cascade triggered by CB_1 receptors agonists (Howlett 2005) have been reported to regulate TH gene expression. Therefore, the aim of this study was to examine the influence of cannabinoid agonists on TH expression and gene transcription in a murine neuroblastoma cell line, N1E-115. Indeed, we first demonstrated the presence of cannabinoid receptors in this TH containing cell line which therefore constitutes a valuable cellular model to study this direct regulatory mechanism. In addition, the availability of structurally distinct synthetic cannabinoid agonists allowed to further characterise the possible pharmacological specificity of this regulation.

Materials and methods

Materials

HU 210 and CP 55,940 were purchased from Tocris Cookson (Bristol, UK). CP 47,947 and CP 55,244 were generously provided by Pfizer Inc. (Groton, CT, USA). WIN 55,212-2 and WIN 55,212-3 were from Research Biochemicals International (Boornem, Belgium). Δ^9-THC was obtained from GW Pharmaceuticals (Salisbury, UK). The CB_1 receptor inverse agonist/antagonist SR 141716A, and the CB_2 receptor inverse agonist/antagonist SR 144528 were generously given by Dr Barth and Dr Mossé respectively, Sanofi-Synthélabo Research (Montpellier, France). Forskolin, 3 isobutyl-1-methylxanthine and phorbol 12-myristate 13-acetate (TPA) were purchased from Sigma (Boornem, Belgium). Stock solutions of drugs were prepared in dimethylsulfoxide (DMSO) at 10 μmol/L and stored as aliquots at −80°C. The final DMSO concentration never exceeded 0.1% which had no significant effect on assays.

Cell culture

All cell culture media and supplements were obtained from Invitrogen (Merelbeke, Belgium). Transfected Chinese hamster ovary (CHO) cells stably expressing the human CB_1 or CB_2 receptors (CHO-CB_1 and CHO-CB_2), kindly provided by Euroscreen (Gosselies, Belgium) were maintained using Ham's F-12 medium supplemented with 10% foetal calf serum, 500 μg/mL geneticin G418, 100 UI/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL fungizone/amphotericin B and 2 mmol/L L-glutamine. Mouse neuroblastoma N1E-115 and murine macrophages J774 were grown in Dulbecco's MEM/NUT mix F-12 medium and RPMI 1640 medium respectively, both supplemented with 10% foetal calf serum, 100 UI/mL penicillin, 100 μg/mL streptomycin and 2 mmol/L L-glutamine. At confluence, cells were trypsinised for dilutions.

Cells were cultured at 37°C in an atmosphere of humidified air and 5% CO_2.

cAMP assay

N1E-115 cells were seeded in 96-well plates (10^4 cells/well), 24 h prior cannabinoid treatment. Cells were pre-treated for 30 min with 0.1 mmol/L 3 isobutyl-1-methylxanthine. Then, cannabinoid agonists were added for another 30 min incubation treatment in the presence of 1 μmol/L forskolin. When SR 141716A and SR 144528 were used, cells were pre-treated for 5 min with these inverse agonists/antagonists at 1 μmol/L before the addition of the cannabinoid agonist. cAMP levels were measured using cAMP Biotrak enzyme immunoassay system (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) following the protocol provided by the manufacturer. The cell incubations were performed at 37°C.

RNA extraction and RT-PCR

Total RNA was isolated using TriPure isolation reagent (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. One step RT-PCR for CB_1 and CB_2 receptors were performed on a 1 μg total RNA sample using access RT-PCR system (Promega, Leiden, The Netherlands) according to manufacturer's recommendations. After 45 min at 45°C for reverse transcription and 2 min at 94°C for reverse transcriptase inactivation, 45 cycles of 30 s denaturation at 94°C, 1 min annealing and 2 min 68°C extension were run. Annealing temperatures were 50°C and 58°C for the CB_1 and CB_2 receptors respectively. Appropriate primers used for amplification (Invitrogen) are described in Table 1.

Cell homogenates and western blotting

For CB_1 receptor expression, cells were washed and scrapped in phosphate-buffered saline (PBS) buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 4.3 mmol/L Na_2HPO_4 and 1.47 mmol/L KH_2PO_4, pH 7.4). Pelleted cells were lysed, homogenised in 50 mmol/L Tris–HCl pH 7.4 and centrifuged at 15 000 *g* for 10 min. The resulting pellet (membranes) was again washed twice under the same conditions and re-suspended in the following buffer: 50 mmol/L Tris–HCl, 1 mmol/L phenylmethylsulfonyl fluoride, 20 mmol/L dithiotreitol, 1 mmol/L EDTA, 40 μmol/L leupeptin, 5 μmol/L antipain, 1 μmol/L pepstatin A and 0.5% Triton X-100. For TH protein determinations, cells were plated in six-well culture dishes 24 h prior assay. HU 210 and CP 55,940 were used for 5–72 h treatment. When specified, forskolin and TPA were respectively used at 1 μmol/L and 80 nmol/L. The assay was terminated by aspirating the medium, scrapping and washing cells in PBS buffer. Cells were lysed by sonication in lysis buffer [50 mmol/L HEPES, 50 mmol/L KF, 1 mmol/L Na_3PO_4, 1 mmol/L EDTA, 1 mmol/L EGTA, 0.5% 2-mercaptoethanol, 5 mmol/L β-glycerol phosphate, 5 mmol/L $Na_4P_2O_7$, 1 mmol/L phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Sigma)]. Homogenate preparations were performed at 0–4°C, and protein contents were determined using Bradford method. Cell extracts were then diluted in appropriate amount of 5x loading buffer (250 mmol/L Tris–HCl, 500 mmol/L dithiotreitol, 10% sodium dodecyl sulphate (SDS), 50% glycerol, 0.5% bromophenol blue, pH 6.8) to obtained 1x buffer, boiled for 5 min, separated on a 10% SDS–polyacrylamide gel and transferred to nitrocellulose membranes for immunodetection. Blots were then blocked for 1 h with 5% non-fat powdered milk in TTBS (20 mmol/L Tris, 150 mmol/L NaCl and 0.05% Tween 20, pH 7.6) and probed at 4°C overnight in a 1 : 250 dilution of goat anti-CB_1 receptor (Santa Cruz Biotechnology, Bergheimer, Germany), 1 : 200 rabbit anti-CB_2 (Alpha Diagnostic International, Gentaur Molecular product, Brussels, Belgium) or in a 1 : 2500 dilution of rabbit anti-TH (Chemicon, Hampshire, UK) antibodies. Blots were then washed thoroughly, incubated 1 h with horseradish peroxidase conjugated anti-goat (1 : 5000) (Santa Cruz Biotechnology) or anti-rabbit (1 : 3000) (Chemicon) secondary antibodies respectively, and revealed with super signal west pico system (Pierce, Aalst, Belgium). After antibodies stripping (100 mmol/L 2-mercaptoethanol, 2% SDS and 62.5 mmol/L Tris–HCl, pH 6.7, 60°C, 30 min) the consistency of sample loading was validated by reprobing with an anti-actin antiserum (1 : 5000) (Abcam, Cambrige, UK). Relative amounts of protein were quantified by scanning densitometry using the software Image Master (Pharmacia Biotech Benelux, Roosendaal, The Netherlands).

Table 1 Pairs of PCR primers and amplification products

CB_1 receptor (amplification product: 328 bp)
Forward 5′-AAGCTGCAATCTGTTTGCTCA-3′
Reverse 5′-AGACATCATACACCATGATCG-3′
CB_2 receptor (amplification product: 325 bp)
Forward 5′-AAGTGACCAACGGCTCCAAC-3′
Reverse 5′-GTCATGGTCACACTGCCGAT-3′
TH (amplification product: 100 bp)
Forward 5′-AGTTCTCCCAGGACATTGGACTT-3′
Reverse 5′-ACACAGCCCAAACTCCACAGT-3′
GAPDH (amplification product: 115 bp)
Forward 5′-CCCCCAATGTATCCGTTGTG-3′
Reverse 5′-TAGCCCAGGATGCCCTTTAGT-3′

TH, tyrosine hydroxylase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Quantitative real-time PCR

Quantitative real-time-PCR was performed for quantification of the genetic expression of TH. N1E-115 were grown in six-well culture dishes and treated for 5 h with HU 210, CP 55,940, forskolin or TPA at the indicated concentrations and total RNA was extracted from using TriPure reagent. First strand cDNA was generated from 1 μg total RNA using iScript cDNA Synthesis kit (Bio-Rad, Nazareth, Belgium) according to the manufacturer's instructions. Real-time PCR amplifications were carried out using the iCycler IQ™ multicolour real time PCR detection system (Bio-Rad), in a total volume of 25 μL containing 2 ng cDNA template, 0.3 μmol/L of the primers (Table 1) and the IQ™ SYBR Green Supermix. PCR protocol was conducted as previously described (Vermeiren *et al.* 2006) using 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 same amplification conditions, with dilutions of a mix of cDNA templates (from 20 to 0.078 ng). Each sample was normalised to the relative amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Quantification of mRNA in the samples was performed using the post-run data analysis software provided with the iCycler system.

Plasmid descriptions

Construction of the reporter plasmids pTH-Luc and pTH250-Luc was previously described (Najimi *et al.* 2002). These constructs respectively included 5300 and 250 base pairs of the upstream sequence of the rat TH gene fused to the firefly luciferase gene. pRL 138 obtained from Dr E. Pierreux (UCL, ICP, Brussels, Belgium) was used as an internal control to normalise for transfection variability. pRL 138 was constructed by introduction of a 225 base pairs sequence of the 6-phosphofructo-2-kinase (PFK-2) promoter in the pRL null vector from Promega encoding for renilla luciferase as previously described (Pierreux *et al.* 1998).

Transient transfection and dual luciferase assay

Cells were plated at a density of 10^5 cells/well in 24-well plates. Culture medium was changed after 24 h, then cells were co-transfected with a reporter plasmid and the pRL 138 plasmid at 2 and 0.25 μg/well respectively. Transfection was performed overnight using a calcium phosphate co-precipitation method. After transfection, cells were washed three times by PBS buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 8.1 mmol/L Na_2HPO_4 and 1.47 mmol/L KH_2PO_4, pH 7.4) and fresh medium was added 24 h prior the 5 h treatment with the appropriate amounts of cannabinoid agonists. When indicated, SR 141716A and SR 144528 were added 5 min before applying agonist. To stop the reaction, cells were washed three times with PBS buffer before lysis by addition of 100 μL passive lysis buffer supplied with the Dual Luciferase Reporter Assay System (Promega). Firefly luciferase reporter activity was normalised for the renilla luciferase activity. Respective measurements of light emission were determined according to manufacturer's instructions. Luminescence was detected using a TD20/20 luminometer (Turner design, Sunnyvale, CA, USA).

Data analysis

Data presented in the text and in the figures were expressed as percentages of the corresponding values obtained with cells treated with vehicle alone (DMSO diluted in culture medium). Statistical analysis was performed on the log-transformed value of relative luminescence or on net cAMP level, mRNA amount or immunoblot density, by analysis of variance (ANOVA) with repeated measurements or Student's *t*-test. A *post hoc* analysis was made by the Scheffe test, using the Statistical Package for Social Science (SPSS for Windows; SPSS Inc., Chicago, IL, USA). Unless otherwise stated, data are expressed as mean ± SEM from at least three times performed experiments (triplicate).

Results

Evidence for the expression of functional CB_1 receptors in N1E-115 cells

Preliminary RT-PCR studies revealed the genetic expression the CB_1 receptor in mouse neuroblastoma N1E-115 cells. Thus, using specific primers validated using a cDNA sample from mouse hypothalamus, where abundant expression of the CB_1 receptor is documented, the expected amplification product of 328 bp was obtained when tested on samples from N1E-115 cells (Fig. 1a). The expression of the CB_2 receptor

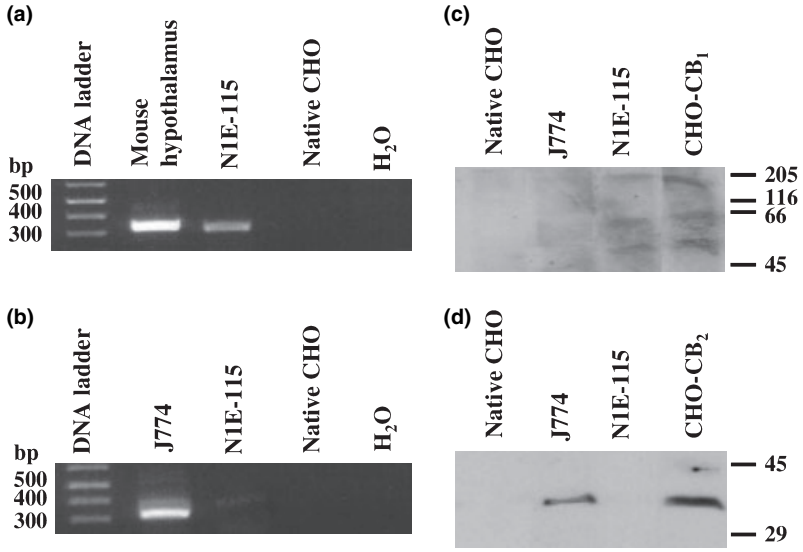

Fig. 1 Expression of CB_1 receptors in N1E-115 cells. (a and b) RNA extracts from N1E-115 cells, mouse hypothalamus, J774 cells or native CHO cells were used in RT-PCR performed with specific primers targeting the mouse CB_1 (panel a) or CB_2 (panel b) receptor cDNA, yielding the predicted products of 328 or 325 bp for CB_1 or CB_2 receptor transcripts respectively. Negative control was performed without any DNA template. (c and d) Western blotting analysis of crude membrane protein extracts from CHO-CB_1, CHO-CB_2, N1E-115, J774 or native CHO cells. Using specific antibodies for the CB_1 receptor (c), specific bands at 53 and 64 kDa corresponding to monomer receptors as well as an additional band at higher molecular weight corresponding to multimer of the receptor were observed in N1E-115 and CHO-CB_1 cell extracts, whereas no signal was observed for J774 and native CHO cells. Immunodetection with specific CB_2 receptor antibodies (d) revealed an expected signal at 39 kDa in extracts from CHO-CB_2 and J774 cells, whereas no specific signal was observed for N1E-115 and native CHO cells.

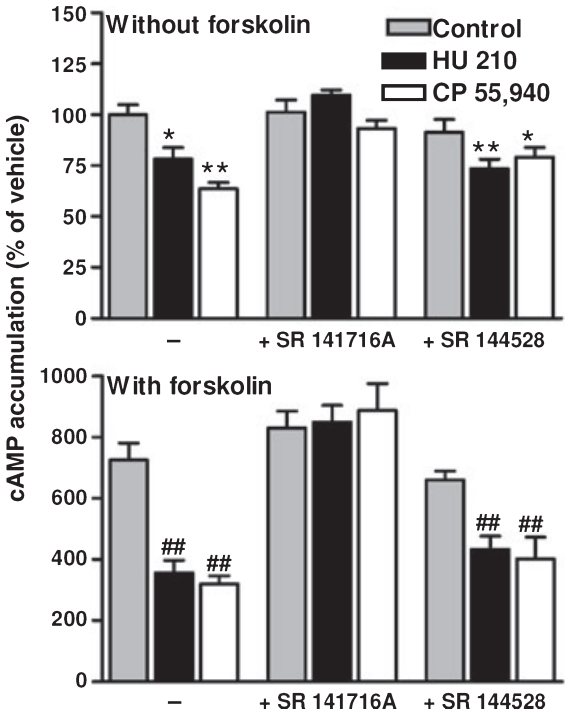

Fig. 2 Effect of CB_1 receptor agonists on cAMP level in N1E-115 cells. Intact N1E-115 cells pre-treated for 30 min with 0.1 mmol/L 3 isobutyl-1-methylxanthine were exposed for 30 min to 0.1 μmol/L HU 210 or 0.1 μmol/L CP 55,940 in the cells exposed (lower panel) or not (upper panel) to 1 μmol/L forskolin. As indicated, the same treatments were repeated in the presence of 1 μmol/L SR 141716A or 1 μmol/L SR 144528. Shown are variations in cAMP accumulation expressed as percentages of control (cell exposed to vehicle only). Data are the mean values with SEM from at least three separate experiments performed in triplicate. $^{**}p < 0.01$; $^{*}p < 0.05$; relative to control; $^{\#\#}p < 0.01$; $^{\#}p < 0.05$ relative to forskolin-induced response. Effect of forskolin was highly significant ($p < 0.01$) compared with control.

was also examined using appropriate primers. While positive amplification (325 bp amplicon) was obtained using a cDNA sample from the murine macrophage cell line J774, no specific PCR signal was detected for N1E-115 cells (Fig. 1b).

The selective expression of the CB_1 receptor in N1E-115 cells was further confirmed after immunodetection of the corresponding protein in crude membrane extracts. Specific CB_1 receptor antibodies specifically recognised two protein bands at 53 and 64 kDa which putatively correspond to the native and glycosylated cannabinoid receptor. Hence, both signals were detected in transfected CHO cells (CHO-CB_1) and were absent in non-transfected cells. Another protein band was also recognised at a higher molecular weight (between 160 and 200 kDa) suggesting the presence of multimeric forms or aggregated CB_1 receptors. Contrasting with the robust signal detected in extracts from the J774 cells, the specific CB_2 receptor antibodies failed to detect any specific protein in samples from N1E-115 cells, confirming the lack of expression of this receptor subtype in this cell line (Fig. 1d).

The functionality of CB_1 receptor in N1E-115 cells was investigated by measuring agonist-mediated reduction of cAMP production. As shown on Fig. 2, both HU 210 (0.1 μmol/L) and CP 55,940 (0.1 μmol/L) significantly decreased the cAMP level. These experiments were repeated on cells in which the basal activation of adenylyl cyclase was artificially enhanced by addition of forskolin (1 μmol/L), causing a robust increase in cAMP levels (up to 700% as compared with basal). In these conditions, the inhibitory effect of the agonists was confirmed as both HU 210 and CP 55,940 caused a noticeable reduction in forskolin-mediated response. Both in naive cells and cells exposed to forkolin, the inhibition of cAMP accumulation by the cannabinoid agonists was prevented by SR 141716A (1 μmol/L), a selective CB_1 inverse agonist/antagonist. In contrast, SR 144528, a selective CB_2 inverse agonist/antagonist had no influence on the functional response to both agonists, confirming the involvement of the CB_1 receptor only. Together, these results clearly demonstrate the expression of functional CB_1 receptors in N1E-115 cells and the absence of the CB_2 receptor subtype.

HU 210 but not CP 55,940 regulates TH protein content in N1E-115 cells

The influence of HU 210 and CP 55,940 on TH protein content was determined by western blotting analysis of total cell protein extracts. N1E-115 cells were treated for different periods of time in the presence of the cannabinoid receptor agonists at various concentrations. In naive cells, the TH antibody recognised a single band at the expected molecular mass (60 kDa). As previously documented (Vyas *et al.* 1990; Rusnak and Gainer 2005), the TH protein content was dramatically increased (up to 200% above basal) when cells were exposed to 1 μmol/L forskolin or to 0.08 μmol/L TPA and for both chemicals, the maximal effect was observed after 5 h of treatment. Exposure of the cells to HU 210 concentration-dependently reduced the level of TH protein (by up to 60%, pEC_{50} of 6.40 ± 0.09) (Figs 3a and b). This effect was transient as maximal decrease was observed between 5 and 24 h of treatment. Thereafter, the response progressively weakened and no effect was observed after 72 h of treatment (Fig. 3c). A similar time-course was observed when measuring the stimulatory effect of forskolin and TPA. Contrasting with the data obtained with HU 210, the exposure of the cells to CP 55,940 in the same conditions (duration and concentrations) did not significantly alter TH protein content. These paradoxical results suggest that the CB_1 receptor-mediated regulation of TH protein expression is dependent on the agonist tested.

Dual responses of CB_1 receptor activation on TH mRNA level in N1E-115

Considering that the regulation of protein expression may result from alteration of gene transcription or mRNA stability,

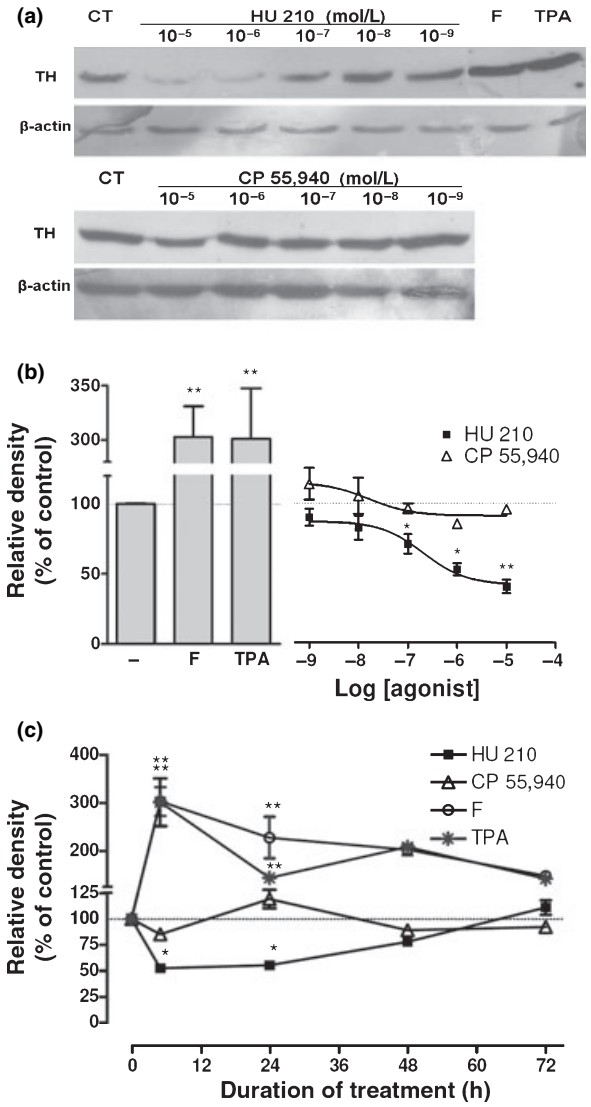

Fig. 3 Influence of HU 210 and CP 55,940 on tyrosine hydroxylase (TH) protein expression in N1E-115 cells. (a and b) Western blotting analysis of TH expression (upper panels) in crude extracts from N1E-115 cells exposed for 5 h to HU 210 or CP 55,940 (1 nmol/L to 10 μmol/L), 1 μmol/L forskolin (F), 0.08 μmol/L phorbol 12-myristate 13-acetate (TPA) or to vehicle (CT). After antibodies stripping, detection of actin on the same blots was examined for data normalisation (lower panels). (c) Influence of exposure duration on the modulation of TH protein expression by 1 μmol/L HU 210, 1 μmol/L CP 55,940, 1 μmol/L forskolin (f) and 0.08 μmol/L TPA. (a) Shows data from typical experiments, while (b) and (c) combine the data from three independent experiments. Autoradiograms were analysed by densitometry, and TH signals were normalised against the signals corresponding to actin. Results expressed in percentages of control cells are mean values with SEM. $^{**}p < 0.01$; $^{*}p < 0.05$; relative to control.

quantitative real-time PCR analysis of TH mRNA levels were carried out. Expression of TH mRNA in N1E-115 cells was examined following 5 h of treatment with 1 nmol/L to

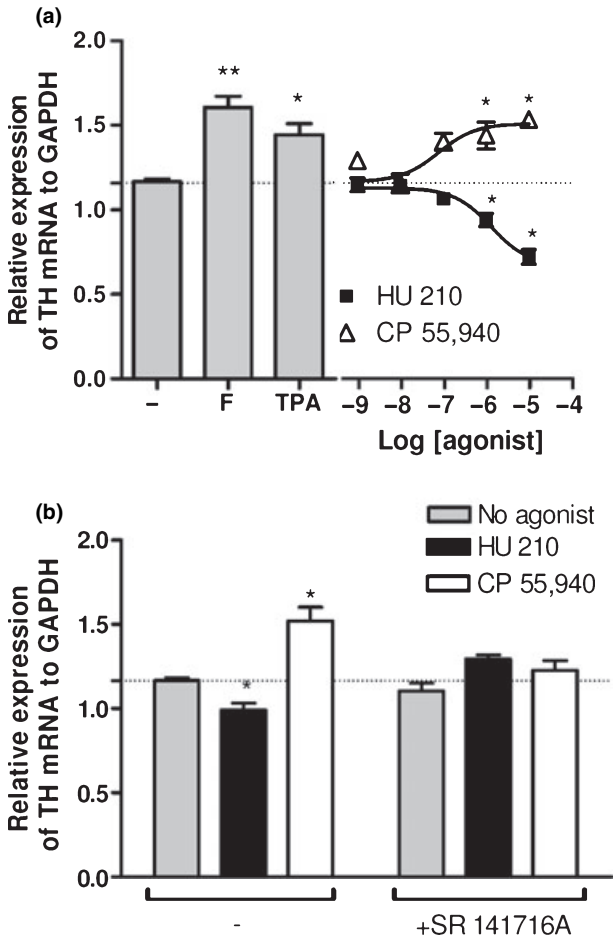

Fig. 4 Regulation of tyrosine hydroxylase (TH) mRNA level by CB_1 receptor. (a) Quantification of TH mRNA relative expression by real-time PCR on cDNA of N1E-115 neuroblastoma cells exposed for 5 h to HU 210 or CP 55,940 (1 nmol/L to 10 μmol/L), 1 μmol/L forskolin (F), 0.08 μmol/L phorbol 12-myristate 13-acetate (TPA) or vehicle (CT). Parameters derived from non-linear analysis of concentration-response curves are indicated in the text. (b) Exposure to HU 210 or CP 55,940 (1 μmol/L) were repeated in the presence of SR 141716A (1 μmol/L). Data shown are relative expression of TH mRNA obtained after normalisation against GAPDH amplification data as internal standard. Values are mean ± SEM of three independent experiments performed in duplicate. $^{*}p < 0.05$; relative to control.

10 μmol/L of the two cannabinoid agonists HU 210 and CP 55,940. As expected, 1 μmol/L forskolin and 0.08 nmol/L TPA produced an increase in TH mRNA level. Figure 4a revealed that the HU 210-mediated reduction in TH protein expression correlates with a reduction of the amount of TH mRNA, as indicated by the dose-dependent reduction of TH mRNA level upon HU 210 treatment (pEC_{50}: 5.88 ± 0.05). In contrast, while no effect was noticed at the protein level, CP 55,940 displayed a dose-dependent increase in the amount of TH mRNA (pEC_{50}: 7.11 ± 0.20) with the effect being significant from 1 μmol/L. In accordance with a CB_1 receptor-mediated mechanism, both responses were totally preven-

ted by SR 141716A (Fig. 4b). These data suggest that the modification of protein expression is due to a cannabinoid-mediated regulation of TH gene expression and that the regulation of TH mRNA level is dependent on the agonist used to activate the receptor.

Transcriptional regulation of TH gene expression

In order to evaluate the possible influence of cannabinoid receptor agonists on the transcriptional activity at the TH promoter, firefly luciferase reporter gene constructs were expressed in N1E-115 cells. Thus, plasmids containing the putatively entire TH promoter (pTH-Luc) extending over 5000 nucleotides of the rat TH gene or a fragment of 250 nucleotides of its 5′-flanking region (pTH250-Luc) cloned upstream of the firefly luciferase reporter gene were transiently transfected in order to indirectly monitor the influence of drugs on TH gene transcription. With both plasmids, transfected cells showed a substantial basal luciferase activity, confirming the constitutive expression of TH by N1E-115 cells and allowing the evaluation of positive or negative pharmacological modulators. Noteworthy, basal luciferase activity appeared sixfold weaker in cells expressing the pTH-Luc construct (not shown). Nevertheless, forskolin 1 μmol/L and TPA 0.08 nmmol/L were found to strongly promote luciferase activity in cells expressing either pTH250-Luc or pTH-Luc (Table 2).

As shown in Fig. 5, HU 210 and CP 55,940 were found to differentially regulate the activity of the TH promoter. In cells expressing pTH-Luc, HU 210 (0.1 μmol/L, 5 h incubation) caused a significant decrease (20%) in luciferase activity, while CP 55,940 (0.1 μmol/L, 5 h incubation)

Table 2 Agonist-dependent dual regulation of TH promoter activity in N1E-115 cells

Constructs:	pTH-Luc	pTH250-Luc
Forskolin	467.3 ± 37.2**	482.9 ± 50.4**
TPA	133.2 ± 3.2**	137.7 ± 7.8**
Δ^9-THC		73.8 ± 8.6*
HU 210	80.3 ± 3.6*	63.6 ± 2.2**
CP 55,940	122.2 ± 9.5*	161.1 ± 8.2**
CP 55,244		156.2 ± 23.5**
CP 47,947		176.7 ± 10.7**
WIN 55,212-2		60.0 ± 7.3**
WIN 55,212-3		102.7 ± 6.1**

Relative luciferase activity measured in transfected N1E-115 cells carrying pTH-Luc and/or pTH250-Luc and treated for 5 h with forskolin (1 μmol/L), TPA (80 nmol/L) or diverse cannabinoid ligands (all at 0.1 μmol/L). Results (mean with SEM from at least three independent experiments performed in triplicate) are given as the percentage of relative luciferase activity (firefly luciferase relative to *Renilla* luciferase activity) compared with untreated cells. $^{**}p < 0.01$; $^{*}p < 0.05$; relative to control. Δ^9-THC, Δ^9-tetrahydrocannabinol; TPA, phorbol 12-myristate 13-acetate; TH, tyrosine hydroxylase.

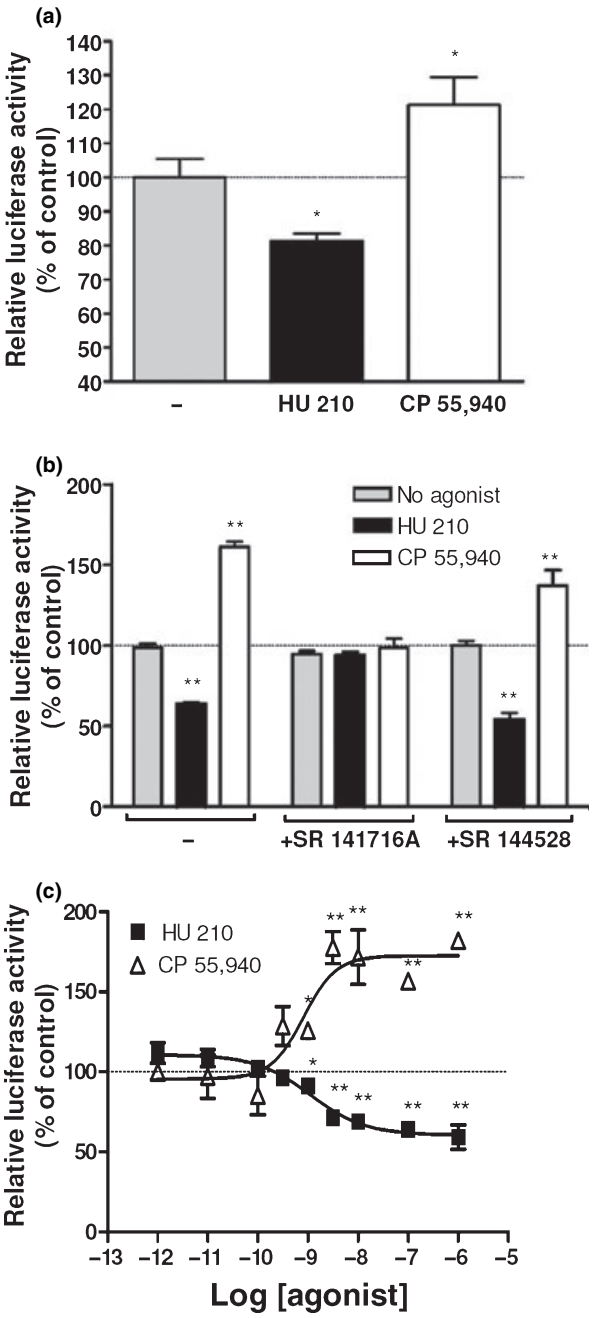

Fig. 5 Regulation of tyrosine hydroxylase (TH) promoter activity in N1E-115 cells using a luciferase-based reporter assay. Luciferase activity measured in transiently transfected N1E-115 cells carrying pTH-Luc (a) and pTH250-Luc (b) constructs. Cells were treated with HU 210, CP 55,940 (both at 0.1 μmol/L) or vehicle. Same treatment was repeated in the presence of SR 141716A or SR 144528 used at 1 μmol/L. (c) Concentration-response modulation of luciferase activity by HU 210 or CP 55,940. Parameters derived from non-linear analysis of concentration-response curves are indicated in the text. Results (mean with SEM from at least three independent experiments performed in triplicate) are given as the percentage of relative luciferase activity (firefly luciferase relative to *Renilla* luciferase activity) compared with untreated cells. $^{**}p < 0.01$; $^{*}p < 0.05$; relative to control.

significantly induced this response (by 22%) (Fig. 5a). Similar results were obtained using the pTH250-Luc reporter construct, although the influence of both agonists appeared more pronounced (36% decrease and 61% increase respectively) (Fig. 5b). Therefore, this second model was used for further pharmacological characterisation of the responses. As shown in Fig. 5c, the effects of HU 210 and CP 55,940 were concentration dependent, and non-linear analysis revealed pEC_{50} values of 8.95 ± 0.17 and 9.09 ± 0.19 respectively, correlating with their nanomolar affinities for the cloned rat CB_1 receptor (Govaerts *et al.* 2004a). Again, the responses to both agonists were totally abolished when cells were simultaneously exposed to SR 141716A, while SR 144528 was without effect, confirming the involvement of CB_1 receptors. In order to rule out any unspecific responses, native CHO were transfected with pTH250-Luc and treated for the same time with either HU 210, CP 55,940 or forskolin. In these cells, basal luciferase activity was 10-fold weaker as compared with that observed in the neuroblastoma cells, consistent with the lack of TH expression in fibroblasts such as CHO cells. Nevertheless, the reporter gene assay was proven effective as indicated by a significant forskolin-induced stimulation of luciferase activity. In contrast, no significant induction was observed after exposure to the cannabinoid ligands. Of importance, the dual response pattern observed in the neuroblastoma cell line was recapitulated in transfected CHO cells expressing both the CB_1 receptor and the gene reporter construct (Fig. 6).

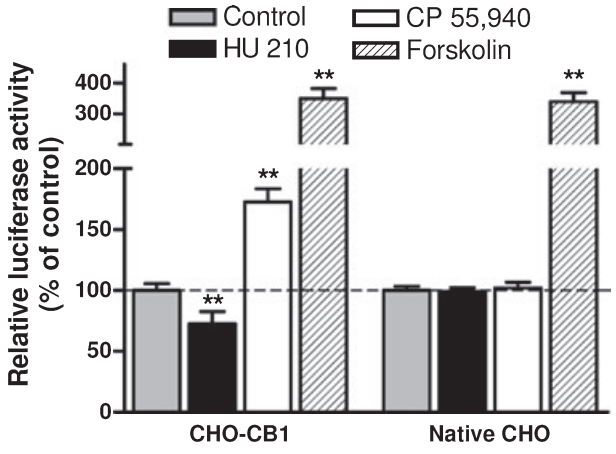

Fig. 6 Regulation of tyrosine hydroxylase (TH) promoter activity in CHO-CB_1 or native CHO cells using a luciferase-based reporter assay. Luciferase activity was measured in CHO-CB_1 or native CHO cells transiently transfected with the pTH250-Luc construct and treated for 5 h with HU 210, CP 55,940 (both at 0.1 μmol/L), forskolin (1 μmol/L) or vehicle. Results (mean with SEM from at least three independent experiments performed in triplicate) are given as the percentage of relative luciferase activity (firefly luciferase relative to *Renilla* luciferase activity) compared with untreated cells. $^{**}p < 0.01$; $^{*}p < 0.05$; relative to control.

Regulation of TH transcription reflects distinct properties of agonists belonging to different chemical families

The contrast between the results obtained with HU 210 and CP 55,940 suggests a dual regulation of TH expression induced by distinct cannabinoid ligands in a cell line that constitutively expresses the CB_1 receptor. To examine the pharmacological specificity of the dual response, N1E-115 transfected with pTH250-Luc were treated with different cannabinoid ligands belonging to distinct chemical families (Table 2). In addition to CP 55,940, other non-classical cannabinoids CP 47,947 and CP 55,244 were assessed for their ability to regulate TH transcription. All these cannabinoid agonists lacking the dibenzopyran ring showed the same influence on TH promoter in inducing luciferase activity (Table 2). This result suggests that, probably depending on particular structural features, different non-classical cannabinoids may induce the same signalisation pathway through the CB_1 receptor, leading to activation of TH transcription. Δ^9-THC, a natural classical cannabinoid and WIN 55,212-2, the most widely studied aminoalkylindole, behave as the synthetic classical cannabinoid HU 210, in mediating a reduction of luciferase activity. In agreement with its reported partial agonist properties (Govaerts *et al.* 2004b), Δ^9-THC was found less effective as compared with the other agonists. Taken together, these data indicate that cannabinoid agonists induce a reduction of TH promoter activity with the exception of non-classical cannabinoid ligands, which promote a stimulation of this promoter.

Discussion

Several studies have reported on the influence of Δ^9-THC on TH activity and TH gene expression (Rodriguez *et al.* 1991; Bonnin *et al.* 1996; Suarez *et al.* 2000). Most of these studies have mainly considered regulatory processes occurring during brain development and have focused on the consequences of pre-natal exposure to Δ^9-THC on neonates, but the mechanisms of such regulation remain unexplored. This question was addressed using N1E-115 neuroblastoma cells, a commonly used model for *in vitro* studies of the regulation of TH expression and activity (Richelson 1973). Indeed, these cells exhibit typical catecholaminergic properties including catecholamines synthesis (Brautigam *et al.* 1982) and elevated TH and acetylcholinesterase activities (Amano *et al.* 1972). In addition, expression of CB_1 receptor was already evidenced by PCR and immunofluorescence microscopy in differentiated N1E-115 cells (Zhou and Song 2001). Herein, the expression of the CB_1 but not the CB_2 receptor was evidenced in non-differentiated N1E-115 cells. Despite the failure to detect cannabinoid receptors in radioligand-binding studies using either [^{3}H]-SR 141716A or [^{3}H]-CP 55,940 (not shown), the robust reduction in cAMP levels in N1E-115 cells exposed to cannabinoid agonists, demonstrates the expression of functional CB_1

receptors coupled to $G_{i/o}$ type G protein. While an influence of cannabinoid agonists on catecholamines release has been previously documented, the expression of cannabinoid receptor on catecholaminergic neurons is still debated in the literature. In this respect, the present study clearly establishes functional responses to CB_1 receptor agonists in a TH-expressing neuroblastoma cell line, modelling the co-expression evidenced in cultured foetal mesencephalic neurons (Hernandez *et al.* 2000).

Cannabinoid-mediated regulation of TH expression

The present study provides the first evidence for a concentration-dependent reduction of TH gene transcription in neuroblastoma cells by HU 210, correlated with a concentration-dependent reduction of TH mRNA level and TH protein. While previous *in vivo* studies have already demonstrated the regulation of TH expression and activity consecutively to pre-natal exposure to Δ^9-THC, few have demonstrated the involvement of CB_1 receptors. However recent studies suggested that this natural cannabinoid agonist also triggers CB_1 and CB_2 independent effects (Bueb *et al.* 2001), raising the question of the pharmacological selectivity. Noteworthy, at cellular level, Hernandez *et al.* have suggested a CB_1-mediated mechanism by reversing the Δ^9-THC-indced increase in TH content and activity with SR 141716A (Hernandez *et al.* 2000). Similarly, the blockade of HU 210-mediated responses by SR 141716A evidenced in the present report, strongly suggests implication of the CB_1 receptor. Furthermore, consistent responses were observed with synthetic analogues belonging to different chemicals families. Thus, WIN 55,212-2, but not the inactive enantiomer WIN 55,212-3, elicited a strong reduction in TH gene transcription. Besides, a weaker response was obtained with Δ^9-THC, in agreement with previous studies demonstrating the partial agonist profile of this natural cannabis extract (Govaerts *et al.* 2004b). Finally, regulation of the TH promoter by cannabinoids was experimentally recapitulated in transfected CHO cells expressing the CB_1 receptor, whereas no significant responses to cannabinoids were obtained in native CHO excluding the involvement of another putative cannabinoid receptor or TRPV1. While these data provide strong evidence for the CB_1 receptor-mediated modulation of TH expression, we cannot exclude the contribution of additional regulatory mechanisms in N1E-115 cells.

Multistep process of TH gene expression regulation

With reference to the broad literature on this topic, several if not all mechanisms of regulations have been reported to participate in the control of TH (for review see Kumer and Vrana 1996). Unexpectedly, we observed marked differences in the potency of HU 210 when examining regulation of TH protein expression, mRNA level or promoter activity. Indeed, these approaches differ in their sensitivity and maximal detection limit. Considering the measures of TH gene promoter activity, attention must be drawn to the need for cell transfection of selected exogenous DNA sequences. Consistent with original experiments describing the 212 first bp of TH promoter as the minimum essential element regulating TH transcription (Cambi *et al.* 1989), similar responses were observed with the pTH-Luc and the pTH250-Luc constructs. However, expression of the full-length promoter construct led to a sixfold lower basal luciferase activity as compared with the 250 bp fragment (not shown) suggesting presence of repressor sequences in the native promoter. While the deletion of presumed repressors in the shorter construct facilitates the characterisation of the pharmacological response, it could also positively influence the potency of the agonists tested. However, such a marked discrepancy in the pharmacological parameters related to HU 210 probably reveals the multifaceted regulation of TH by CB_1 receptors. Thus, one could propose that the regulation operates at the levels of gene transcription, mRNA processing and protein synthesis/stability through distinct signalling pathways which are differentially affected by the CB_1 agonist. Indeed, previous studies reported on the complexity of TH regulation that involves post-transcriptional modulation and/or modification of mRNA or protein stability (Vyas *et al.* 1990; Fossom *et al.* 1992; Alterio *et al.* 2001; Roe *et al.* 2004), at least partly mediated by cAMP-mediated processes.

Agonist-dependent regulation of TH

Beside HU 210, Δ^9-THC and WIN 55,212-2 which mediate inhibition of TH expression, a paradoxal induction of TH gene promoter was observed with CP 55,940 and related compounds belonging to the same family of so-called non-classical cannabinoid agonists. This induction by CP 55,940 was also demonstrated in transfected CHO cells expressing the CB_1 receptor. In N1E-115 cells, this response was correlated with a rise in TH mRNA level although no change in TH protein content was evidenced in the conditions tested. As for HU 210, a marked difference was noticed between the EC_{50} values calculated when studying the influence of CP 55,940 on TH gene transcription activity and on mRNA level. Together with the unexplained lack of regulation of TH protein content, this dissimilar potency of CP 55,940 in different assays raises again the question of the complexity of the regulation of TH at different levels. Indeed, a possible explanation is that in these cells, the signalling pathways potentially activated by CP 55,940 acting at cannabinoid receptors show distinct and possibly reciprocal influences on TH expression.

The existence of distinct responses to HU 210 and CP 55,940 when considering TH regulation contrasts with the similar alteration of cAMP accumulation mediated by both agonists. Hence, HU 210 and CP 55,940 are known to share equivalent pharmacodynamic properties. Although we failed to characterise the interaction of these agonists with CB_1

receptors in N1E-115 cells, our previous binding and functional studies in the model of transfected CHO cells revealed their similar nanomolar affinities and potencies (Govaerts *et al.* 2004a). The influence of cAMP on TH has been extensively documented. Indeed, this second messenger enhances TH transcription rate (Kim *et al.* 1993) and increases TH activity through PKA-mediated phosphorylation (for review see Dunkley *et al.* 2004). In contrast, cAMP accumulation was also associated with reduction of TH enzyme stability and TH mRNA levels (Fossom *et al.* 1992; Gahn and Roskoski 1995). Considering the induction of TH expression by forskolin, the data obtained with HU 210 are compatible with the coupling of the CB_1 receptor with inhibition of adenylyl cyclase. However, our data also suggest that beyond the conventional negative coupling to adenylyl cyclase, the CB_1 receptors shows complex influence on intracellular signalling cascades. Nowadays, there is accumulated evidence for the coupling of a single receptor with multiple G proteins (Hermans 2003), and agonist-directed trafficking of G-protein coupled receptor signalling is now well established (Kenakin 1995). In this view, the CB_1 receptor was shown to relay multiple intracellular signals through efficient coupling to pertussis toxin sensitive $G_{i/o}$-type G proteins but also under certain conditions to G_s-type G proteins (Glass and Felder 1997; Calandra *et al.* 1999; Jarrahian *et al.* 2004) and possibly to $G_{q/11}$-type G protein (Lauckner *et al.* 2005). With respect to the CB_1 receptor, further studies have demonstrated agonist selective signalling discriminating between $G_{i/o}$ subtypes of G proteins (Mukhopadhyay and Howlett 2005). Of interest, CB_1 receptors were shown to independently induce mitogen-activated protein kinase and inhibit PKA activity, providing a possible background for opposite influences on TH expression (Howlett 2005). Although the aim of the present study was not to elucidate the signalling pathways involved in control of TH expression, our results suggest that via an agonist selective trafficking of the receptor, distinct cannabinoid agonists may operate differential control on TH.

Noteworthy, multiplicity of coupling was frequently reported in artificial cell models where recombinant receptors are expressed at very high densities. The present study was conducted in a neuroblastoma cell line in which the CB_1 receptor is constitutively expressed at a putatively low density, as indicated by the lack of detection in radioligand-binding studies. While the physiological significance of the multiplicity of coupling of G-protein coupled receptor remains obscure, a meaningful outcome is that the response to a given agonist would differ according to the tissue/organ where it is examined, because of differences in the expression level of signalling partners (G proteins, effectors, etc.) (Hermans 2003). Worth mentioning previous studies concerning the regulation of TH in the central nervous system consecutive to pre-natal exposure to Δ^9-THC led to inconsistent and sometimes paradoxical results. Indeed, the influence of this cannabis agonist appeared highly dependent on the sex of the animal, the brain area examined and the duration of the treatment and the embryonic development stage (Rodriguez *et al.* 1990;, 1991 Bonnin *et al.* 1996; Suarez *et al.* 2000).

In conclusion, the present study clearly indicates that cannabinoid agonists operate a direct control on TH expression in non-differentiated N1E-115 cells. Confirming the complexity of the regulation of TH expression, these data should help to better understand the relationships between cannabinoid and catecholaminergic transmission systems and open new perspectives in the use of cannabinoid ligands in neuropharmacology. A major observation is that the regulation may differ, depending on the agonist tested despite their specific interaction with the same receptor. As these data were obtained in a model where the CB_1 receptor is constitutively expressed, they constitute a typical and relevant example of the concept of agonist-selective trafficking of receptor signalling and related functional responses.

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