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Concomitant activation of adenylyl cyclase suppresses the opposite influences of CB_1 cannabinoid receptor agonists on tyrosine hydroxylase expression

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ABSTRACT

The CB_1 cannabinoid receptor shows complex interactions with intracellular signalling partners, and responses to cannabinoid ligands are likely to be influenced by concomitant inputs modifying the overall tone of signalling cascades. This appears even more relevant as we previously evidenced opposite regulations of tyrosine hydroxylase (TH) expression by the two common cannabinoid agonists HU 210 and CP 55,940. Therefore, we studied the consequences of manipulating adenylyl cyclase activity with forskolin on the regulation of TH gene transcription in neuroblastoma cells (N1E-115). Reporter gene experiments performed with the luciferase sequence cloned under the control of modified fragments of the TH gene promoter revealed that the AP-1 consensus sequence is essential for cannabinoid-mediated regulation of TH expression. Consistently, inhibition of PKC totally blocked the responses mediated by both HU 210 and CP 55,940. In addition, forskolin which boosts adenylyl cyclase activity remarkably modified the responses to the cannabinoid agonists. Thus, in these conditions, both agonists efficiently reduced TH gene promoter activity, a response requiring functional PKA/CRE-dependent signallings. Finally, the modulations of the promoter were inhibited in pertussis toxin treated cells, suggesting that responses to both agonists are mediated through $G_{i/o}$-dependent mechanisms. Emphasising on the importance of functional selectivity at GPCRs, these data demonstrate that the concomitant activation of adenylyl cyclase by forskolin strongly influences the biochemical responses triggered by distinct cannabinoid agonists. Together our results suggest that the physiological modulation of TH expression by cannabinoid agonists in dopaminergic neurons would be influenced by additional endogenous inputs.

1. Introduction

Changes in the activity of catecholamine-synthesizing enzymes are of major importance in the regulation of several central neurotransmitters such as dopamine, epinephrine and norepinephrine. Accordingly, the regulation of tyrosine hydroxylase (TH) gene transcription is recognised as a physiological mechanism involved in the control of several behavioural, cognitive and emotional activities. Previous analysis of the regulatory sequences present within the TH

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gene proximal promoter have evidenced the role of consensus binding sites for cAMP responsive element (CRE) and for activator protein 1 (AP-1) in the constitutive transcription of TH gene and its modulation observed with several ligands of G protein-coupled receptors (GPCR) [1–5]. Consistently, the CRE binding protein, primarily regulated via cAMP/PKA-dependent phosphorylation, has been recognised as a major transcription factor involved in the control of TH gene transcription [6–8]. Also, several chemicals causing increased production of cAMP have been shown to induce TH gene expression [9,10]. Likewise, the regulation of TH expression after PKC activation is largely documented, and the role of c-Fos and c-Jun, which assemble into transcription factors acting at AP-1 binding site has been demonstrated [11,12].

Previous studies, including the recent demonstration of a cannabinoid-mediated regulation of TH mRNA level in discrete brain areas [13], suggest that the cannabinoid system participate in the control of mammalian brain development through modulation of TH functions [14]. Consistently, we recently reported on the regulation of TH gene transcription in N1E-115 neuroblastoma cells through the activation of the CB_1 cannabinoid (CB_1) receptor. Even though this receptor mainly couples to $G_{i/o}$-type G proteins to inhibit adenylyl cyclase, an agonist-dependent profile of regulation was observed, with HU 210 reducing and CP 55,940 increasing TH gene transcription, supporting the arising concept of functional selectivity [15]. This concept proposes that distinct agonists can differentially orientate the functional coupling of a given GPCR with unrelated signalling cascades [16]. Nevertheless the molecular mechanisms involved in these agonist-selective responses have not been elucidated so far.

In the majority of studies concerning signalling associated with $G_{i/o}$ coupled receptors, the experimental detection of functional responses to agonists requires an artificial induction of adenylyl cyclase activity. However, when considering the complexity of signalling pathways associated with a single GPCR subtype, it is commonly hypothesised that alterations in the distribution of G proteins or modifications of basal effector activities should qualitatively and quantitatively influence the signals triggered by a given agonist. Therefore, we herein examined the consequence of forskolin on the cannabinoid agonists-mediated regulation of TH expression using a reporter gene assay. In addition, as we recently showed that distinct cannabinoid agonists differentially modulate transcriptional activity at CRE or AP-1 binding sites [17], the study of such functional selectivity was extended to the TH gene promoter in which these sites were specifically mutated.

2. Materials and methods

2.1. Chemicals

HU 210, CP 55,940 and the different kinase inhibitors were purchased from Tocris Cookson (Bristol, UK) while forskolin, phorbol 12-myristate 13-acetate (PMA) and pertussis toxin (PTx) were from Sigma (Boornem, Belgium). Stock solutions of drugs were prepared in dimethylsulfoxide (DMSO) at 0.01 M and stored as aliquots at −80 °C. The final DMSO concentration never exceeded 0.1% which had no significant effect on assays.

2.2. Cell culture

All cell culture media and supplements were obtained from Invitrogen (Merelbeke, Belgium). Mouse neuroblastoma N1E-115 were grown in Dulbecco's MEM/NUT mix F-12 medium supplemented with 10% fetal calf serum, 100 UI/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine. Cells were cultured at 37 °C in an atmosphere of humidified air and 5% CO_2.

2.3. Plasmids

Construction of the reporter plasmid pTH250-Luc was previously described [5]. This construct includes 250 base pairs of the upstream sequence of the rat TH gene fused to the firefly luciferase cDNA sequence. Site directed mutagenesis by overlap extension was carried out to generate specific disrupting mutations in either the CRE or AP-1 binding motives present in the proximal TH gene promoter. For each mutation, two DNA fragments having overlapping ends were produced by a mutagene polymerase chain reaction (PCR) amplification using pTH250-Luc as template and the pairs of complementary oligonucleotide primers indicated in Table 1, containing appropriate nucleotide substitutions. Then these fragments were

Table 1 – Oligonucleotides used as primers in the PCR-based mutagenesis.

Primers	Sequence	Orientation
Mutagenic AP-1 primer	cgggctgaggg**cgcgcc**agaggcaggtg gtgattca	Forward
Mutagenic AP-1 primer	caccgtcctct**ggcgcg**ccctcagcccg	Reverse
Mutagenic CRE primer	cccagaggggctt**gcgcgcc**agcctggccttta tgacgtca	Forward
Mutagenic CRE primer	taaaggccaggct**ggcgcgc**aagcccctctggg	Reverse
Recombinant primer	agatccagttcgatgtaacc	Forward
Recombinant primer	ttatgcagttgctctccagc	Reverse
AP-1mt selection primer	ctcgggctgagggcgcg	Forward
CREmt selection primer	gacccagaggggcttgcg	Forward

The consensus sequences are underlined (bold characters indicate mutated nucleotides). The non-mutated sequences are indicated underneath. Mutagene PCR amplification for AP-1 binding site was performed using forward mutagenic AP-1 primer with the reverse recombinant primer and reverse mutagenic AP-1 primer with the forward recombinant primer. Mutagene PCR amplification for CRE consensus binding site was performed using forward mutagenic CRE primer with the reverse recombinant primer and reverse mutagenic CRE primer with the forward recombinant primer. Selection primers were designed to selectively amplify fragment from mutated AP-1 and CRE constructs when used with the recombinant reverse primers.

recombined by PCR with the recombinant primers (Table 1). The resulting mutated fragments were substituted in pTH250-Luc. Plasmid constructions carrying the appropriate mutations were selected by PCR with primers specifically designed to hybridize at the site of mutation (see selection primer in Table 1) and finally validated by DNA sequencing. pRL 138 construct obtained from Dr. E. Pierreux (UCL, ICP, Brussels, Belgium) was used as an internal control to normalise for transfection variability. pRL 138 derives from the pRL null vector from Promega (Leiden, The Netherlands) encoding for Renilla luciferase and carries a 225 base pairs sequence of the PFK-2 promoter [18].

2.4. 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, and then cells were co-transfected with a reporter plasmid and the pRL 138 plasmid at 2 μg/well 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 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.47 mM KH_2PO_4, pH 7.4) and fresh medium was added 24 h prior the 5 h treatment with the appropriate quantities of cannabinoid agonists. For the treatment in the presence of forskolin, this activator was added simultaneously to the agonist. When indicated, cells were treated overnight with PTx (100 ng/mL) and when required, protein kinase inhibitors were added at the indicated concentrations, 1 h prior to the agonist treatment. 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, Leiden, The Netherlands). Firefly luciferase reporter activity was normalised for the Renilla luciferase activity. Respective measurement of light emission was determined according to manufacturer's instructions. Luminescence was detected by TD20/20 luminometer (Turner design, Sunnyvale, CA, USA).

2.5. Data analysis

Unless otherwise stated, data presented in the text and in the figures were expressed as mean percentages ± SEM of the corresponding values obtained with cells treated with vehicle alone (DMSO diluted in culture medium). Agonist potencies (pEC_{50} values) were determined from at least three independent experiments, by non-linear regression analysis performed using Graph Pad prism software (San Diego, CA). Statistical analysis was performed on the log-transformed values of relative luminescence by analysis of variance (ANOVA) with repeated measures or Student *t* test. A post hoc analysis was made by the Scheffe test, using the software SPSS. Mean percentages ± SEM were obtained from at least three independent experiments, all performed in triplicate.

3. Results

3.1. Constitutive activity of TH gene promoter

The respective roles of CRE and AP-1 consensus binding sites present within the proximal region of the TH gene promoter were investigated using a luciferase reporter gene assay. Three different plasmids containing either the wild-type 250-nucleotide promoter fragment of the TH gene (pTH250-Luc) or the corresponding sequence with mutations within the CRE (pTH250-CREmt-Luc) or the AP-1 (pTH250-AP-1mt-Luc) binding sites were constructed (Fig. 1A). Consistent with the constitutive expression of TH in N1E-115 cells, a substantial luciferase activity was detected after transient transfection of each of these plasmids. As summarised in Table 2, the basal activity of these promoters was weakly reduced with the PKA inhibitors, but not with the PKC inhibitors, suggesting that a constitutive activity of PKA in these cells positively influences the TH gene promoter. Accordingly, the mutations within the CRE consensus site slightly attenuated the activity of the TH gene promoter. Similarly, reduced activity was also observed in cells carrying pTH250-AP-1mt-Luc, suggesting that both CRE and AP-1 consensus sequences support the constitutive expression of TH (Fig. 1B).

The roles of CRE and AP-1 consensus sites in the modulation of TH gene promoter activity were further

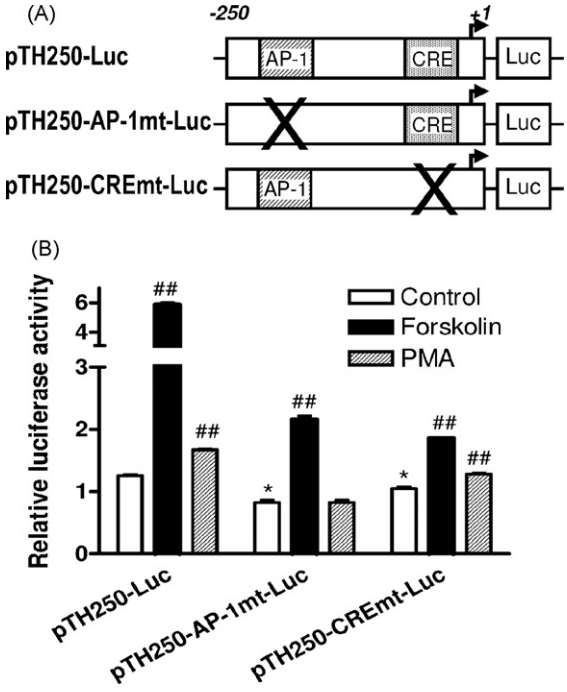

Fig. 1 – Role of CRE and AP-1 consensus sequences in the modulation of basal and stimulated transcription of the TH gene promoter. Panel A illustrates the three constructs of the proximal region of the TH gene promoter fused to the firefly luciferase coding sequence highlighting the mutations disrupting the activities of CRE and AP-1 consensus sequences. Panel B shows the transcriptional activity obtained with these constructs in transfected N1E-115 cells maintained in control conditions or exposed for 5 h to 1 μM forskolin or 0.08 μM PMA. Results (mean values with SEM from three independent experiments performed in triplicate) are given as firefly luciferase activity relative to Renilla luciferase activity. $^{*}p < 0.05$, relative to basal activity of pTH250-Luc construct; $^{\#\#}p < 0.01$, $^{\#}p < 0.05$, relative to basal activity of each individual construct.

Table 2 – Characterisation of the signalling pathways involved in the control of basal and forskolin-induced TH gene promoter activity.

Treatments	pTH250-Luc		pTH250-AP-1mt-Luc		pTH250-CREmt-Luc	
	Control	Forskolin	Control	Forskolin	Control	Forskolin
No inhibitor	100.0 ± 5.9	467.3 ± 37.2**	100 ± 2.89	263.36 ± 15.45**	100 ± 1.25	177.21 ± 29.21**
PKA inhibitors						
H 89	82.57 ± 4.39*	245.42 ± 24.58** 298.56 ± 27.33##	85.48 ± 1.9*	122.88 ± 4.03** 143.7 5 ± 4.71##	105.35 ± 6.33	184.32 ± 4.47** 174.97 ± 4.24##
KT 5720	83.01 ± 0.44**	307.38 ± 7.51** 370.4 1 ± 10.17##				
PKC inhibitors						
GF 109203X	94.08 ± 3.59	320.14 ± 13.91** 351.51 ± 19.69##	92.15 ± 2.7	283.18 ± 11.75** 307.30 ± 13.6##	95.26 ± 0.79	152.36 ± 1.11** 159.94 ± 1.17##
Chelerythrine	96.38 ± 3.05	500.32 ± 9.89** 519.11 ± 19.37##				

Transfected N1E-115 cells carrying pTH250-Luc, pTH250-AP-1mt-Luc or pTH250-CREmt-Luc constructs were pre-treated for 1 h in the absence or in the presence of 10 μM H 89, 5 μM KT 5720,0.25 μM GF 109203X or 10 μM chelerythrine. Cells were the exposed for 5 h to vehicle or 1 μM forskolin and thereafter, luciferase activity was measured. Results (mean values with SEM from at least three independent experiments performed in triplicate) are expressed as the percentages of relative luciferase activity (firefly luciferase relative to Renilla luciferase) compared to untreated cells. Numbers indicated in italic correspond to the same data expressed as percentage of activity when testing the inhibitor alone. $^{**}p < 0.01$, $^{*}p < 0.05$ relative to control; $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ relative to the cells treated with the inhibitor only.

examined using forskolin or PMA. These inducers of either adenylyl cyclase or PKC significantly enhanced the luciferase activity in cells expressing pTH250-Luc. As expected, the response induced by PMA was totally abolished in pTH250-AP-1mt-Luc transfected cells. In contrast, a significant response to forskolin was conserved in these cells, although its relative amplitude was lower, suggesting a role of the AP-1 consensus sequence in the induction of TH gene transcription by forskolin.

Consistent with the integrity and functionality of the AP-1 binding site in the pTH250-CREmt-Luc construct, PMA stimulated gene reporter activity to the same extent as observed with the non-mutated construct. Confirming the indirect activation of CRE binding protein by cAMP, the mutation of the CRE consensus sequence dramatically decreased the response to forskolin. Yet the activity was not totally suppressed, suggesting that forskolin may also act through PKA/CRE-independent signalling pathways. Accordingly, the induction triggered by forskolin observed when testing the non-mutated promoter was reduced using either PKA inhibitors or GF 109203X as a PKC inhibitor. Noteworthy, chelerythrine, another PKC inhibitor failed to inhibit forskolin-mediated TH expression, raising questions regarding differences in the selectivity of these two inhibitors. Also, in cell expressing pTH250-AP-1mt-Luc construct, forskolin-induced transcription was strongly attenuated by H 89 or KT 5720 (PKA inhibitors) while in cells expressing pTH250-CREmt-Luc, either GF 109203X or chelerythrine caused a significant decline of forskolin-induced promoter activity (Table 2).

3.2. AP-1 consensus site is required for the reciprocal regulation of TH gene expression by agonists of the CB_1 receptor

As previously reported, HU 210 and CP 55,940 were found to oppositely regulate the activity of the TH gene promoter (Fig. 2A) [15]. Thus, in cell carrying the non-mutated TH gene promoter construct, HU 210 dose-dependently reduced luciferase activity while CP 55,940 remarkably induced this response (pEC_{50} values of 8.95 ± 0.17 and 9.09 ± 0.19 respectively). As shown in Fig. 2B, the mutation of the AP-1 consensus binding site almost totally abolished the responses to both agonists (Fig. 2B) and modest reductions of luciferase activity were only observed with extremely high concentrations of both ligands. In contrast, the reciprocal regulation of the TH gene promoter induced by HU 210 and CP 55,940 were preserved when tested on cells carrying pTH250-CREmt-Luc (Fig. 2C). Yet, with this construct lacking the CRE consensus binding site, both agonists showed reduced potencies and efficacies compared to those observed with the non-mutated construct. Non-linear analysis revealed pEC_{50} values of 7.38 ± 0.18 and 6.41 ± 0.29 for HU 210 and CP 55,940 respectively. Considering the involvement of AP-1 and CRE consensus sequences in the regulation of the TH gene promoter, the influence of specific kinase inhibitors was examined on the responses induced by the cannabinoid agonists. Because of reported off-target inhibitions caused by several kinase inhibitors, both H 89 (10 μM) and KT 5720 (5 μM) were used to inhibit PKA activity and GF 109203X (0.25 μM) and chelerythrine (10 μM) were selected as PKC inhibitors. Inhibition of either PKA or PKC was sufficient to totally suppress the HU 210-mediated reduction of pTH250-Luc activity (Fig. 3A). In contrast, the stimulatory response induced by CP 55,940 was not influenced by PKA inhibition and efficient impairment was only observed using the PKC inhibitors. When the same experiments were conducted with pTH250-CREmt-Luc transfected cells, total inhibition of HU 210 mediated decrease in luciferase activity was obtained using the PKC inhibitors but not with the PKA inhibitors (Fig. 3B). Again, efficient blockade of CP 55,940-mediated stimulation of pTH250-CREmt-Luc-dependent luciferase activity was only obtained with the PKC inhibitors. As CB_1 receptor has not been commonly reported to

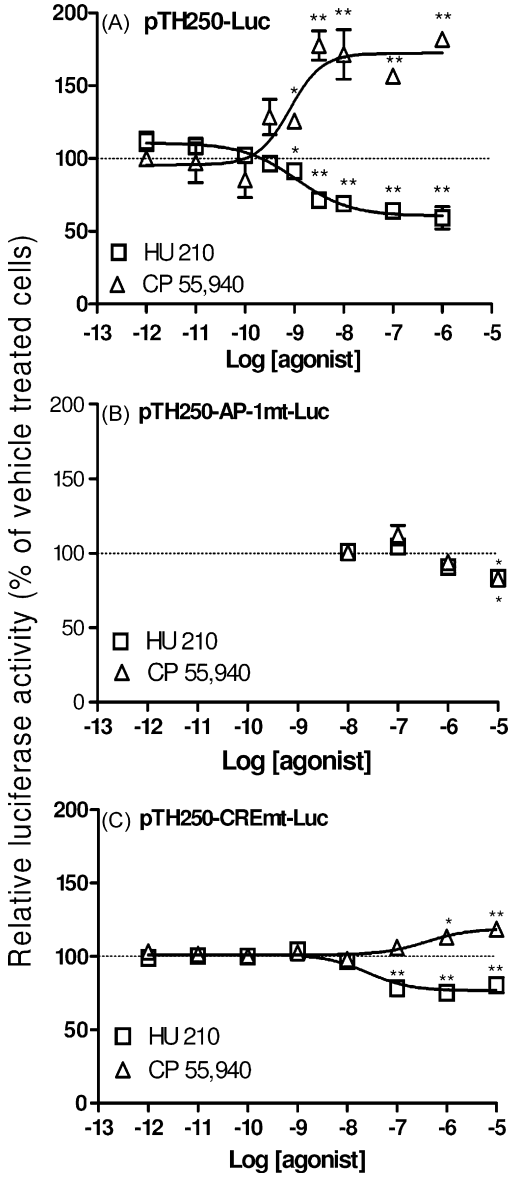

Fig. 2 – Role of CRE and AP-1 consensus sequences in the regulation of TH gene promoter activity by cannabinoid agonists. Luciferase activity was measured in transfected N1E-115 cells carrying pTH250-Luc (A), pTH250-AP-1mt-Luc (B) or pTH250-CREmt-Luc (C) maintained in control conditions or exposed for 5 h to with HU 210 or CP 55,940 (1 pM to 10 μM). Results (mean values with SEM from three independent experiments performed in triplicate) are given as the percentages of relative luciferase activity (firefly luciferase relative to Renilla luciferase) compared with untreated cells. $^{**}p < 0.01$, $^{*}p < 0.05$, relative to control.

regulate PKC-dependent pathways, we also tested Ro-31-6045 (0.25 μM), known as a negative control for GF 109203X in PKC inhibition. As expected, basal luciferase activity was not modified using Ro-31-6045 (107.7 ± 4.6% relative to control) and responses observed for both cannabinoid ligands were similar (67.5 ± 5.6% and 139.5 ± 2.7% relative to cells treated with Ro-31-6045 only, for HU 210 and CP 55,940 respectively).

3.3. *Responses to cannabinoid agonists are altered by forskolin*

As cannabinoid receptors are predominantly coupled to $G_{i/o}$-type G proteins, luciferase reporter gene assays were repeated in cell exposed to forskolin, in order to positively boost the activity of adenylyl cyclase. First, the influence of increasing concentrations of forskolin on the TH gene promoter was characterised. Using the plasmid constructs containing the intact or mutated promoter, forskolin was found to concentration-dependently increase luciferase activity, and non-linear regression analyses revealed pEC_{50} values of 6.16 ± 0.09, 6.19 ± 0.07, 6.25 ± 0.15 for pTH250-Luc, pTH250-AP-1mt-Luc, pTH250-CREmt-Luc transfected cells, respectively (Fig. 4). Avoiding saturation of the reporter system, cells were stimulated with 1 μM forskolin when used in combination with cannabinoid agonists. Contrasting with the reciprocal responses observed in the absence of forskolin, both HU 210 and CP 55,940 dose-dependently decreased luciferase activity in the presence of this co-stimulant (Fig. 5A). Determined pEC_{50} values of 8.81 ± 0.22 and 8.92 ± 0.23 correlated with the respective nanomolar affinities of HU 210 and CP 55,940 for the CB_1 receptor. Similarly, inhibition of luciferase activity by both cannabinoid agonists was observed in cells expressing pTH250-AP-1mt-Luc (pEC_{50} values: 8.76 ± 0.37 and 8.51 ± 0.18 respectively) (Fig. 5B). Finally, when tested using the promoter mutated within the CRE consensus sequence, the co-stimulation with forskolin appeared to totally abolish the positive and negative regulations respectively mediated by CP 55,940 and HU 210 (Fig. 5C).

The signalling pathways contributing to the modulation of the TH gene promoter by the CB_1 receptor agonists in the presence of forskolin were further analysed using PKA and PKC inhibitors. Indeed, this additional study was mainly focused on pTH250-Luc and the pTH250-AP-1mt-Luc constructs, as the presence of the CRE consensus binding site was essential to observe cannabinoid-mediated responses in the presence of forskolin. In these conditions, the blockade of cannabinoid-dependent regulation of TH gene promoter activity was only obtained using H 89 or KT 5720 (Fig. 6A). Thus contrasting with the severe influence of PKC inhibitors on cannabinoid-mediated responses in the absence of forskolin, both GF 109203X and chelerythrine failed to modify either HU 210- or CP 55,940-dependent inhibition of luciferase activity driven by pTH250-Luc (Fig. 6A). Similar results were obtained in cells transfected with the pTH250-AP-1mt-Luc construct, providing convincing evidence for the involvement of PKA-dependent pathways in the cannabinoid-mediated regulation of the TH gene promoter (Fig. 6B).

3.4. *The dual control of TH gene expression by cannabinoids requires $G_{i/o}$-type G proteins*

The putative role of $G_{i/o}$-type G proteins in the cannabinoid-mediated regulation of TH gene expression was examined by neutralizing their activity through overnight exposure to PTx. In the absence of forskolin, basal luciferase activity controlled by the non-mutated promoter appeared substantially increased in PTx treated cells, indicative of a constitutive $G_{i/o}$-protein dependent inhibition of TH gene transcription. As shown in Fig. 7A, in PTx treated cells, the response to HU 210

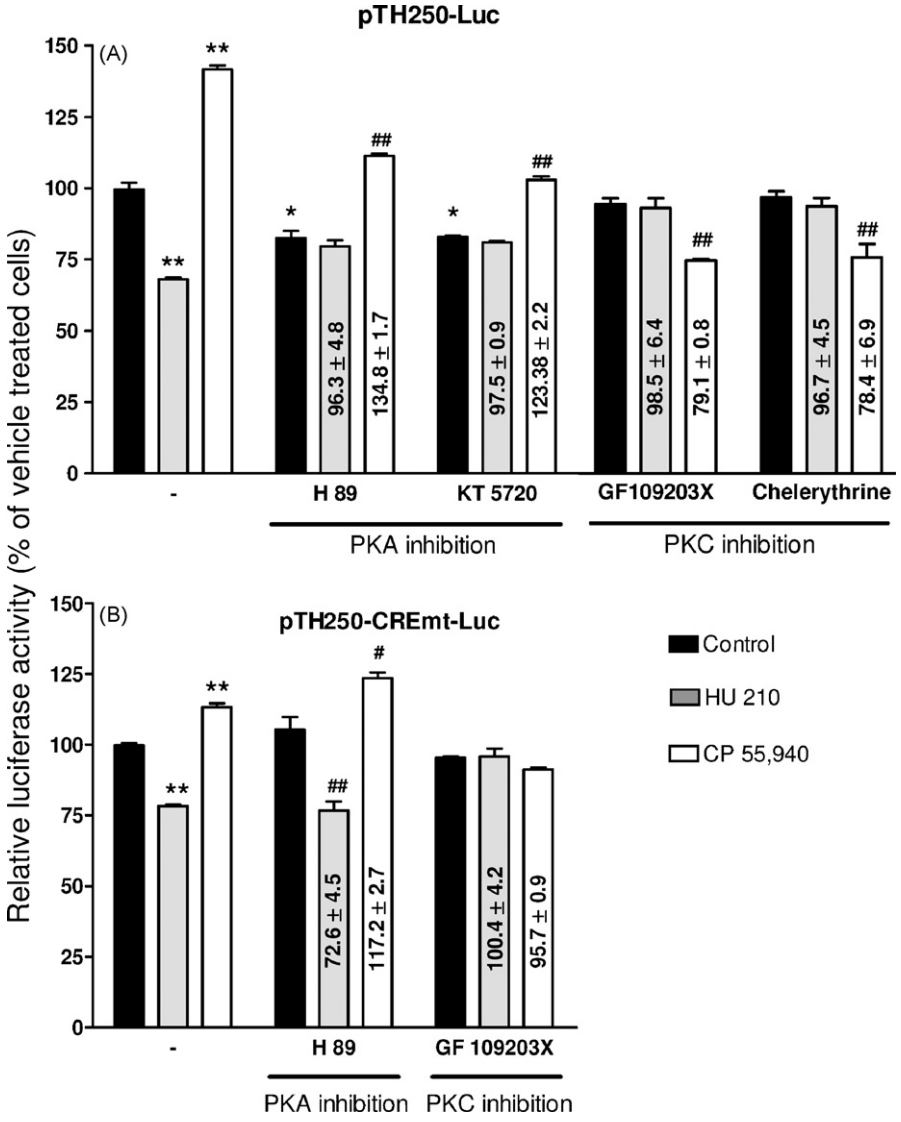

Fig. 3 – Characterisation of signalling pathways involved in regulation of TH gene promoter activity by cannabinoid agonists. Luciferase activity was measured in transfected N1E-115 cells carrying pTH250-Luc (A), or pTH250-CREmt-Luc (C) pretreated for 1 h with vehicle or with kinase inhibitors (10 μM H 89, 5 μM KT 5720, 0.25 μM GF109203X or 10 μM chelerythrine) and subsequently exposed for 5 h to the cannabinoid agonists HU 210 or CP 55,940 (both used at 100 nM in A and 1 μM in B). Results (mean values with SEM from three independent experiments performed in triplicate) are given as the percentages of relative luciferase activity (firefly luciferase relative to Renilla luciferase) compared to untreated cells. Numbers indicated above each bar correspond to the same data expressed as percentage of activity when testing the inhibitor alone. $^{**}p < 0.01$, $^{*}p < 0.05$, relative to control; $^{\#\#}p < 0.01$, $^{\#}p < 0.05$, relative to cells treated with the inhibitor only.

was switched from an inhibition to a dose-dependent induction of TH gene promoter activity (pEC_{50} value of 7.02 ± 0.04). Even though the stimulatory activity of CP 55,940 was conserved in these conditions, non-linear regression analysis revealed a reduced potency for this agonist (pEC_{50} value of 7.27 ± 0.05 vs. 8.92 ± 0.23 in cells non-treated with PTx) suggesting that the signalling pathway involved in this response was also modified.

Even more surprising, in the presence of forskolin, pretreatment of pTH250-Luc transfected cells with PTx reversed the responses to both HU 210 and CP 55,940 (Fig. 7B). Thus, both HU 210 and CP 55,940 induced a dose-dependent increase in luciferase activity. Calculated pEC_{50} values were reduced as compared to the values obtained in basal conditions and were similar to those obtained without forskolin (pEC_{50} of 7.09 ± 0.03 and 7.34 ± 0.04 for HU 210 and CP 55,940 respectively). Together these data suggest that the regulation of TH gene promoter activity by these cannabinoid agonists depends on the coupling of the CB_1 receptor with PTx-sensitive and PTx-insensitive G proteins.

4. Discussion

Emerging evidence indicates that alterations in the intracellular environment such as redistribution of G proteins or

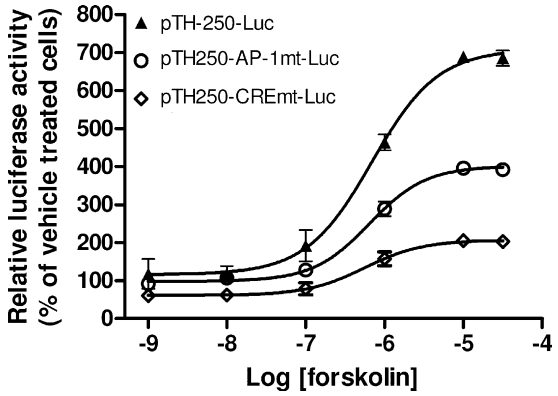

Fig. 4 – Modulation of TH gene promoter constructs by forskolin Luciferase activity was measured in transfected N1E-115 cells carrying pTH250-Luc, pTH250-AP-1mt-Luc or pTH250-CREmt-Luc exposed for 5 h to forskolin (1 nM to 31.6 μM). Results (mean values with SEM from three independent experiments performed in triplicate) are expressed as the percentages of relative luciferase activity (firefly luciferase relative to Renilla luciferase) compared with vehicle treated cells.

modification in basal effector activities can influence signalling cascades associated with GPCR activation. Considering the regulatory crosstalks between cannabinoid and dopaminergic systems [19], this question was addressed in N1E-115 neuroblastoma where constitutive expression of both CB_1 cannabinoid receptor and TH is demonstrated. Using a specific luciferase based reporter assay, our study was focused on the regulation of the activity of the TH gene promoter. We recently reported that HU 210 and CP 55,940, generally used as reference cannabinoid agonists, operate reciprocal influences on the expression of TH through distinct regulations at the transcriptional level [15]. Considering the role of the *cis*-enhancer elements AP-1 and CRE present in the TH gene promoter [20], the responses to these cannabinoids were examined in cells transfected with reporter gene constructs with appropriate mutations in these regulatory sequences. We first observed that basal luciferase activity was significantly reduced when tested on either AP-1 or CRE mutated TH gene promoter constructs, confirming that the constitutive expression of TH is supported by cell signallings acting through both AP-1 and CRE in N1E-115 cells. In addition, our data showed that disrupting the AP-1 consensus binding site impaired both HU 210-mediated reduction and CP 55,940-mediated induction of TH gene promoter activity, revealing that this region of the promoter specifically drives the agonist-selective responses. Paradoxically, our previous experiments revealed that CP 55,940 does not regulate transcriptional activity of a minimal essential promoter constituted by AP-1 consensus repeats [17]. Noteworthy, cross-talks between the AP-1 binding site and adjacent regulatory sequences of the TH gene promoter have been demonstrated [21,22]. At variance with the key role of this AP-1 consensus binding site, our data indicate that the CRE consensus region operates a modulatory influence on the dual responses evoked by the CB_1 receptor ligands. Indeed, the disrupting mutation of this sequence strongly reduced the

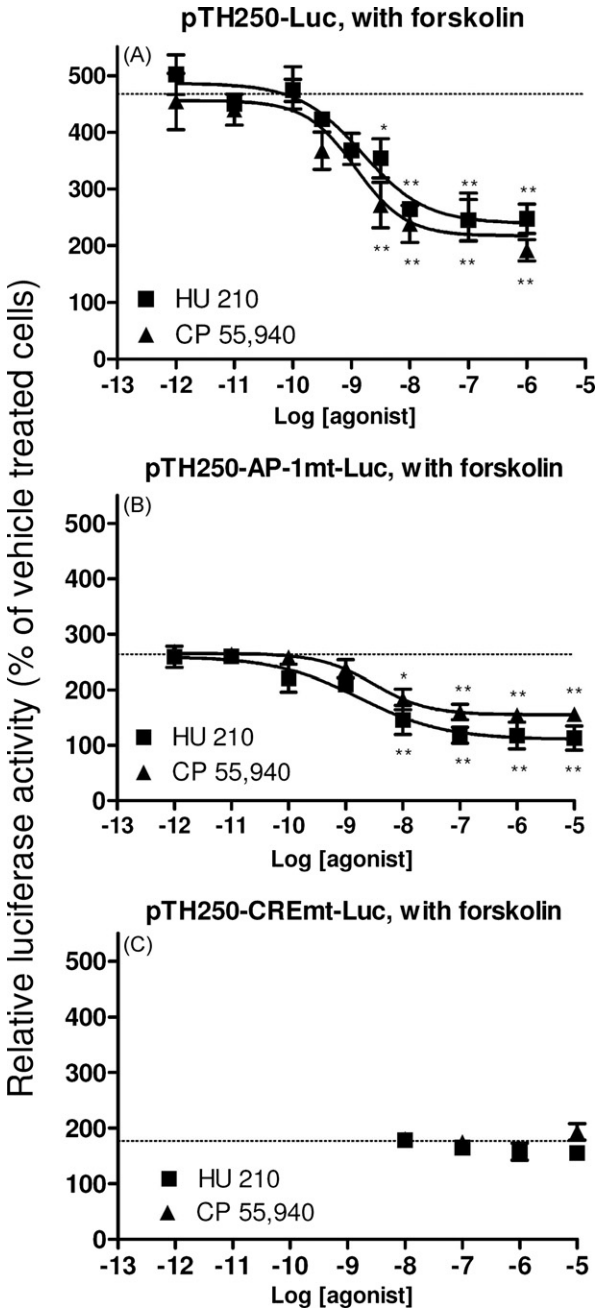

Fig. 5 – Influence of forskolin on the regulation of TH gene promoter activity by cannabinoid agonists. Modulation of luciferase activity by HU 210 and CP 55,940 was measured in transfected N1E-115 cells carrying pTH250-Luc (A), pTH250-AP-1mt-Luc (B) or pTH250-CREmt-Luc (C) concomitantly exposed to forskolin (1 μM) Dotted lines indicate luciferase activity in cells exposed to forskolin only. Results (mean values with SEM from three independent experiments performed in triplicate) are given as the percentages of relative luciferase activity (firefly luciferase relative to Renilla luciferase) compared with control cells. $^{**}p < 0.01$, $^{*}p < 0.05$, relative to forskolin treated cells.

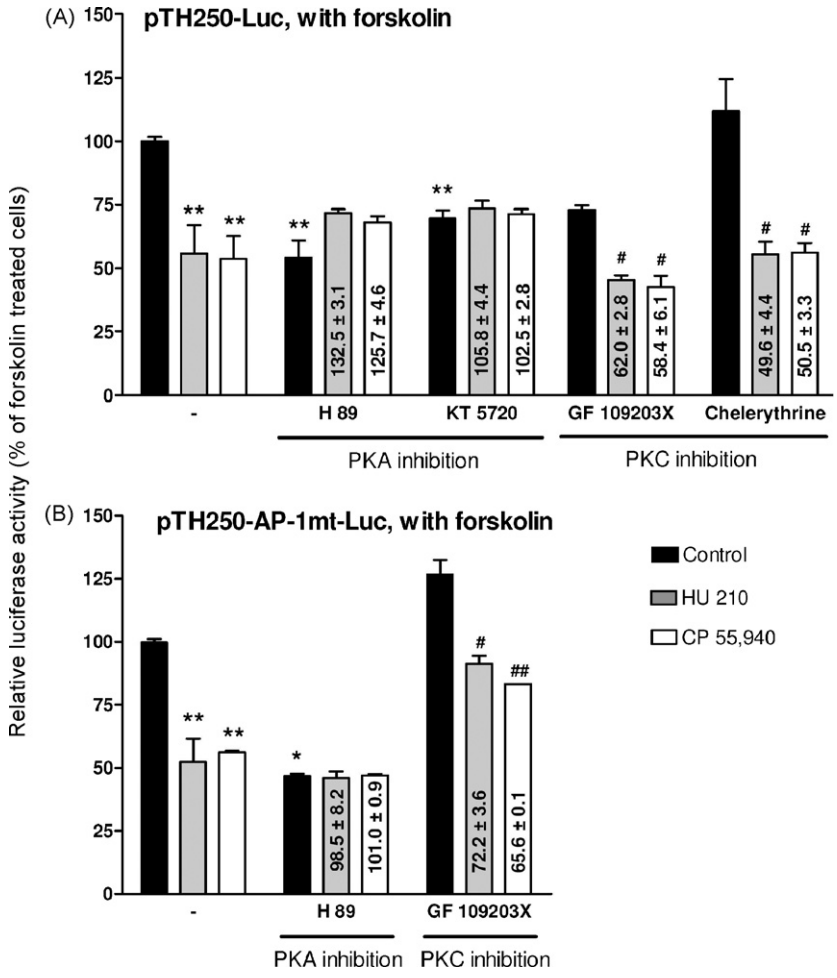

Fig. 6 – Characterisation of signalling pathways involved in regulation of TH gene promoter activity by cannabinoid agonists in the presence of forskolin. The influence of kinase inhibitors (10 μM H 89, 5 μM KT 5720, 0.25 μM GF109203X or 10 μM chelerythrine) on luciferase activity concomitantly modulated by forskolin (1 μM) and by HU 210 or CP 55,940 (both at 100 nM in A and 1 μM B) was examined on transfected N1E-115 carrying pTH250-Luc (A) or pTH250 AP-1mt-Luc (B). Results (mean values with SEM from at least three independent experiments performed in triplicate) are given as the percentages of relative luciferase activity (firefly luciferase relative to Renilla luciferase) compared to forskolin treated cells. Numbers indicated above each bar correspond to the same data expressed as percentage of activity when testing the inhibitor alone. $^{**}p < 0.01$, $^{*}p < 0.05$, relative to control; $^{\#\#}p < 0.01$, $^{\#}p < 0.05$, relative to cells treated with the inhibitor only.

efficacy and potency of HU 210 and CP 55,940 without totally suppressing the respective reduction or induction of reporter gene activity. Even though high concentrations of agonists were required to detect significant regulation of pTH-250-CREmt-Luc, the specificity of the responses should not be questioned since full inhibition was obtained with the selective CB_1 receptor inverse agonist/antagonist SR 141716A (not shown). These alterations of drug potencies reveal the complexity in the molecular mechanisms involved in the finely tuned control of TH gene transcription achieved through the CB_1 receptor. The existence of such intricate mechanisms in the regulation of a limiting enzyme in dopamine synthesis highlights the critical function of the cannabinoid system in the dynamic control of dopaminergic transmission. In addition, consistent with the major role of the transcription factor AP-1 in the regulation of cell life and death [23], cannabinoid receptors have been shown to participate in cell transformation [24] and differentiation [25] through regulation of AP-1 activity. However, considering the dual influence exerted by cannabinoid ligands on AP-1-dependent transcription, one could hypothesise that the differential regulation by distinct agonists may explain the reported multifaceted influences of cannabinoids on the regulation of cell growth, proliferation or death [26,27].

As summarized in Fig. 8, such complexity was confirmed by the results obtained with kinase inhibitors. Several chemically unrelated blockers were used in order to validate the specificity and efficiency of biochemical inhibitions, and their concentrations were chosen on the basis of previous studies [28–32]. Thus, HU 210-mediated reduction in TH expression was totally prevented using either PKA or PKC inhibitors, confirming the simultaneous participation of both PKA and PKC pathways in the emergence of HU 210-directed responses. Whether these two effectors participate in the same of to

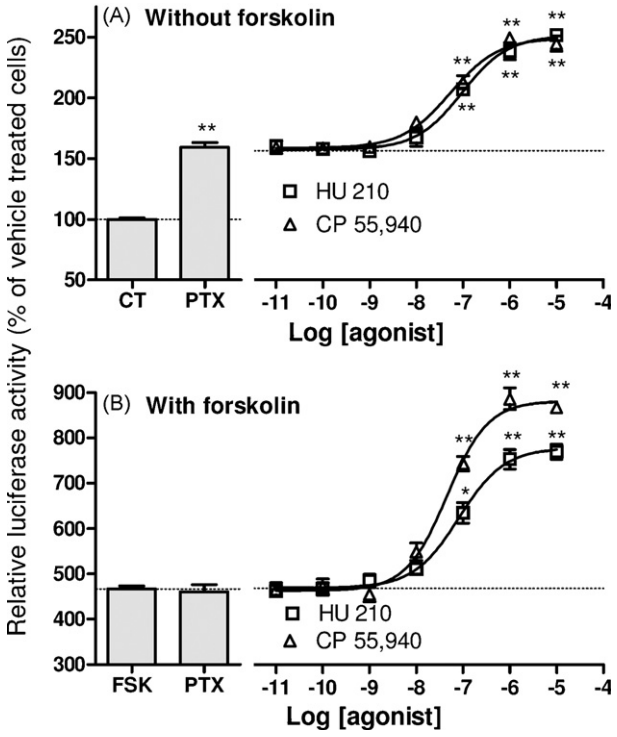

Fig. 7 – Involvement of PTx sensitive G proteins in the modulation of TH gene promoter activity by cannabinoid agonists and forskolin Luciferase activity was measured in transfected N1E-115 cells carrying pTH250-Luc treated overnight with PTx (100 ng/mL). Cells were exposed HU 210 or CP 55,940 (0.01 nM to 10 μM), in the absence (A) or in the presence (B) of 1 μM forskolin. Shown are mean values with SEM from at least three separate experiments performed in triplicate and are given as the percentage of relative luciferase activity (firefly luciferase relative to Renilla luciferase) compared with control. $^{**}p < 0.01$, $^{*}p < 0.05$, relative to control (cells treated with PTx).

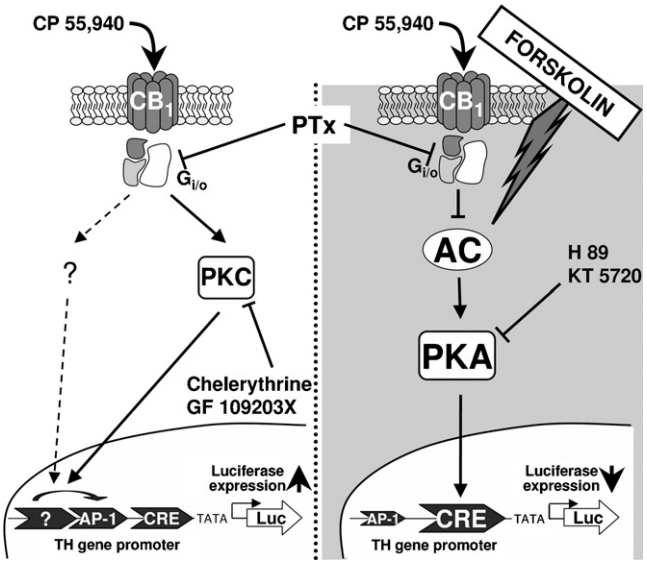

Fig. 8 – Influence of forskolin on the complex signallings involved in the regulation of TH expression by CP 55,940. In the absence of fosrkolin (left), the data obtained with selective biochemical inhibitors suggest that CP 55,940 increases the transcription of TH through $G_{i/o}$- and PKC-dependent regulation of the AP-1 consensus binding site. In addition, the use of mutated constructs of the TH gene promoter revealed that CP 55,940 concomitantly operates a weak negative influence on the expression of TH. On the other hand (right) in cells exposed to forskolin in order to mimic an enhanced constitutive tone of adenylyl cyclase (AC)/PKA activity, CP 55,940 decreases TH gene expression through $G_{i/o}$-dependent inhibition of the AC/PKA/CRE pathway, as confirmed by the use of appropriate inhibitors.

distinct signalling cascades has not been addressed in this study. However the possibility to dissociate the influences of PKA and PKC on pTH250-CREmt-Luc-mediated luciferase activity with selective inhibitors supports the existence of two parallel signalling pathways. A working hypothesis is that PKC constitutes the principal signalling pathway supporting HU 210-mediated reduction of TH expression whereas PKA activity solely amplifies the response. This corroborates our previous findings indicating that HU 210 decreases AP-1-dependent transcriptional activity through regulation of a PKC dependent pathway [17]. In contrast, inhibition of CP 55,940-mediated induction of TH gene expression was only achieved when blocking PKC. Hence, in the presence of the PKC inhibitors, the response to CP 55,940 was switched to a decrease in luciferase activity, suggesting that several and opposite signalling cascades are concomitantly regulated by this ligand. Yet, this CP 55,940-mediated decrease in luciferase activity was not observed when testing PKC inhibitors in cells expressing pTH250-CREmt-Luc suggesting the contribution of a PKA-independent inhibition of CRE element activity.

Consistent with the concept of functional selectivity, our data confirm that distinct cannabinoid ligands selectively regulate different intracellular signalling pathways, presumably through multiple agonist-selective receptor conformations. However, in models where CB_1 receptor is constitutively expressed and in the absence of any co-stimulant, functional couplings to $G_{i/o}$ [33,34], G_s [35] and $G_{q/11}$ [36] type G proteins have been reported. Despite this multiplicity of couplings, both HU 210-and CP 55,940-mediated regulation of TH gene promoter activity appeared sensitive to PTx. Furthermore, we previously reported that in N1E-115 cells, both ligands reduced accumulation of cAMP, confirming that the putative functional selectivity concerns subtypes of $G_{i/o}$-type G proteins. Accordingly, agonist-selective conformations [37] and multifaceted couplings [38,39] with distinct $G_{i/o}$-protein subtypes were recently demonstrated for the CB_1 receptor. On the other hand, it is noteworthy that in several models, PTx treatment unmasked an alternative coupling of the CB_1 receptor with G_s-type G proteins [40–42]. According with this, in PTx treated cells, distinct changes in luciferase activity were observed with either HU 210 or CP 55,940 indicating that beside $G_{i/o}$-type G proteins, some PTx-insensitive G proteins such as G_s proteins are also regulated by the CB_1 receptor.

Paradoxically, while both AP-1 and CRE consensus elements were demonstrated to support the constitutive expression of TH, only the PKA inhibitors appeared to efficiently reduce the basal activity at pTH250-Luc. Likewise, a robust increase in luciferase activity was observed in PTx treated cells, which should be assigned to the suppression of constitutive $G_{i/o}$-mediated inhibition of PKA activity. Also convincing is the observation that a basal tone driven by the CRE consensus sequence is required to enhance cannabinoid agonist-mediated responses. Together, these data highlight the regulatory role of PKA activity in the control of TH expression and suggest that any concomitant stimulus that modulates cellular cAMP levels could influence the regulations driven by CB_1 receptor ligands. Contrasting with the reciprocal regulations induced by HU 210 and CP 55,940, both ligands were shown to concentration-dependently reduce TH gene expression in cells exposed to forskolin. Indeed, it is now accepted that the functional properties exhibited by a given GPCR ligand may differ between models or in different experimental conditions. In particular, ligands termed protean agonists are thought to elicit agonist or inverse agonist properties depending on the constitutive activity of the receptor [43]. Thus, by manipulating the basal tone of adenylyl cyclase activity with forskolin, Yao et al. have recently described AM1241 as a protean agonist at CB_2 receptor [44]. Similarly, our data identify opposite modulations of TH expression by CP 55,940 when examined in the presence or absence of forskolin, suggesting that concomitant activation of cellular effectors strongly influence the response mediated by this functionally selective agonist. In this context, marked regional differences in brain dopamine metabolism have been reported [45,46] possibly underlying the modulation of basal cell tone by different neuronal inputs.

The demonstration that the tone of PKA activity influences the functional properties of HU 210 and CP 55,940 primarily refers to use of forskolin which is recognised as a strong activator of adenylyl cyclase. However, as forskolin interacts with a variety of membrane proteins [47–50], one cannot exclude that alteration of other effectors may also modify CB_1-receptor mediated responses. The reduction of forskolin-induced TH gene expression by both the mutation of AP-1 consensus binding sequence and PKC inhibition is consistent with this hypothesis. Besides, selectivity of adenylyl cyclase isoenzymes regulated by either forskolin [51] or CB_1 receptor [52,53] should also be considered when investigating cannabinoid-mediated regulation of cAMP accumulation. Thus, distinct $G\alpha_i$ subunits contribute to the reduction of adenylyl cyclase activity after either forskolin or $G\alpha_s$ activation, and depending on which GPCR has been activated [54,55]. Rather convincing, Majumdar et al. reported that 8-OH-DPAT, a 5-HT_{1A} receptor agonist, failed to reduce cAMP accumulation produced by forskolin whereas efficient inhibition was achieved when adenylyl cyclase activation was induced with an agonist acting on a G_s coupled receptor [56]. Similarly, it was demonstrated that inhibition of type III adenylyl cyclase driven by CB_1 receptor only take place when forskolin is used as inducer [52], suggesting that the concomitant stimulation of the cells with forskolin may differentially influence the regulation of $G_{i/o}$-type G protein by HU 210 and CP 55,940. Nevertheless, these mechanisms appear not sufficient to explain the distinct regulations by the CB_1 receptor agonists observed in the presence or in the absence of forskolin. Indeed, using the same model, we previously measured reduction of cAMP accumulation consecutively to HU 210 or CP 55,940 exposure, either in the absence or in the presence of forskolin, suggesting that in both conditions, the prevailing effect of both agonists on cAMP/PKA signalling pathway is an inhibition.

In conclusion, this study reinforces our previous findings that through selective interaction with $G_{i/o}$-type G protein subtypes, unrelated cannabinoid agonists differentially influence intracellular signalling cascades leading to radically divergent cellular responses. The manipulation of intracellular signalling using forskolin or PTx revealed further complexity of downstream responses as these appear to largely depend on basal effector activity. This observation is particularly relevant for cannabinoid receptors as well as for other G_i coupled receptor as the pharmacological profile of interacting ligands is frequently studied in conditions where cAMP-dependent signalling pathways are artificially boosted. In addition, these findings raise questions regarding the overall responses that should be expected from ligands interacting with CB_1 receptor in the central nervous system. Indeed, it is commonly recognised that each individual neuron develops thousands contacts with neighbour cells, leading to complex regulation of their activity. Further in vivo studies are required to examine how the modulation of TH gene expression by exogenous cannabinoid ligands would be influenced by additional endogenous inputs in dopaminergic neurons.

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