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Research report

Chronic treatment with a synthetic cannabinoid CP-55,940 alters G-protein expression in the rat central nervous system

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Abstract

Prolonged exposure of rats to the synthetic cannabinoid receptor ligand, CP-55,940 (0.4 mg/kg, i.p. for 11 days), induced tolerance to analgesia, to the reduction in spontaneous locomotor activity and the incidence of splayed hind limbs. One hour after the last injection on day 11, the rats were killed and in situ hybridization was used to investigate the effect of treatment on G-protein α-subunit expression throughout the brain. Chronic cannabinoid exposure markedly reduced $G\alpha_s$, $G\alpha_i$ and $G\alpha_o$ mRNA levels. The message for the α_s-subunit was decreased in all the brain areas containing the basal autoradiographic signal; the decrease ranging from 25% in the thalamus to 45% in the mesencephalon. Also the basal $G\alpha_i$ expression was reduced in tolerant rats showing the greatest decrease in the forebrain (63%) in the cerebellum (58%) and in the mesencephalon (38%). The reduction in $G\alpha_o$ expression (25%) was more localized, being present only in the rostral portion of the brain (cortex, striatum and olfactory area). The alterations in α-subunits gene expression were not followed by any change in the amount of proteins. Our results indicate that, besides the receptor modification, alteration to the G-protein expression could be a molecular event associated with the development of cannabinoid tolerance.

Keywords: Cannabinoid tolerance; Rat central nervous system; G-protein level; G-protein expression

1. Introduction

The first direct evidence of a cannabinoid receptor came from Howlett's laboratory [3] where it was demonstrated that radiolabeled CP-55,940 (a synthetic cannabinoid compound) bound to brain membranes in a highly specific and selective manner, features characteristic of a receptor. Behaviorally, active cannabinoids, including Δ9-THC, presented high affinity for this site, lending credence to its being the cannabinoid receptor. It has now been established that there is an excellent correlation between the pharmacological potencies of cannabinoids in several behavioral tests and their affinity for this binding site [1].

Subsequently, Matsuda et al. [10] isolated a clone from a rat library that had a high degree of homology with G-protein-coupled receptors. The discovery that the distribution of mRNA of the clone paralleled that of cannabinoid receptors, reported by Herkermann et al. [6], led them to conclude that they had cloned the cannabinoid receptor. All evidence points to the brain cannabinoid receptor being associated to G-proteins and negatively coupled to adenylyl cyclase and N-type calcium channels [7,9,15]. Thus, the best characterized cannabinoid effects are receptor-mediated, including the reduction in spontaneous motor activity, catalepsy and antinociception. Tolerance to these effects has been reported in many animal studies and they appear to result, to same extent, from agonist-induced down-regulation [12,16].

Recently, using in situ hybridization, we demonstrated that levels of mRNA coding for the cannabinoid receptor decreased in specific brain areas of rats chronically treated with CP-55,940, indicating that prolonged occupancy of the cannabinoid receptor primes a sequence of intracellular events resulting in alteration of its expression [17].

Starting from these data and considering that the cannabinoid receptor is associated with the second-messenger system through G-protein, the study examined whether $G\alpha$-proteins synthesis is altered in the rat central nervous system as a result of prolonged CP-55,940 treatment. We employed the in situ hybridization technique to

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map the spatial expression of α mRNA transcript throughout the rat brain and to verify any changes induced by the treatment. We also carried out semi-quantitative immunoblotting analysis of the proteins in the brain regions that showed altered expression, with a view to correlating the changes in the messages with changes in the total amounts of proteins.

2. Materials and methods

2.1. Animals and treatment

Male Sprague–Dawley rats (Charles River, Calco, Italy) weighing 175–200 g at the beginning of the experiment were used. The animals were housed three per cage in standard conditions. Rats received CP-55,940 (0.4 mg/kg in 20% ethanol), vehicle (ethanol 20%) or saline, intraperitoneally (i.p.) once daily for 11 days. CP-55,940 was a generous gift from Dr. D. Casilli, Pfizer Italiana S.p.A., Rome, Italy.

2.2. Behavioral studies

The analgesic effect of CP-55,940 was assessed using the tail-flick test according to D'Amour and Smith [2]. Pain thresholds were measured twice before drug administration and again 30, 60, 90, 120, 240 and 360 min after the injection on days 1, 5 and 10. To prevent tissue damage, a maximum cut-off of 15 s was used and the rat was removed from the apparatus if it failed to respond within this interval. The results for each time were expressed as latency in seconds and the total area under the time response curve (AUC) was calculated as long as the analgesic effect lasted. At each time considered, the incidence of splayed hind limbs was quantitated as a percentage.

Thirty minutes after drug injection on days 1, 5 and 10, animals were submitted to a locomotor activity test. The apparatus, a square Plexiglas arena ($43 \times 43 \times 12$ cm), painted black and elevated 65 cm above the floor, was placed in the center of an acoustically insulated room, with a surface area of 6.25 m^2 and lit by fluorescent bulbs. All experiments were recorded by a TV-video system, and a Delta System computer (Addonics) was used for image analysis and pattern recognition, using software provided by Biomedica Mangoni (Pisa, Italy). Locomotor activity was recorded for 5 min and quantitated as the overall distance travelled (cm).

2.3. Quantitative in situ hybridization

On days 5 and 11, 1 h after the last injection, the rats were killed by decapitation and their brains rapidly removed and frozen in liquid nitrogen. Coronal sections (12 μm) were cut at -18°C and processed for in situ hybridization histochemistry as previously described [12]. Briefly, sections were hybridized with ^{35}S-labeled oligonucleotide probes (3×10^5 d.p.m. per section), washed, air dried and exposed to X-ray film (βmax, Amersham) for 5–8 days. The intensity of the hybridization signal was assessed by measuring the gray levels of the autoradiographic films with an image-analysis system consisting of a video camera (Hamamatsu, Japan) connected to an Apple Macintosh II personal computer. The public domain Image 1.47 software was used (NIH, Bethesda, MD, USA). The gray level measurements were made within the linear range as determined using radioactive ^{35}S-standards prepared in the laboratory.

The probes (all supplied by Du Pont, Milan, Italy) consisted of a 39-mer oligonucleotide directed against bases 580–618 of the rat $G\alpha_s$ mRNA sequence; a 39-mer directed against bases 384–422 of the rat $G\alpha_o$ mRNA sequence; a 39-mer directed against 514–552 of the rat $G\alpha_i$ mRNA sequence. Northern blot analysis of RNA from rat cerebral cortex and hippocampus showed that each probe identified specific mRNA species with a size corresponding to that previously reported when full-length or restriction fragment cDNA probes were used [8,13].

2.4. Immunoblot analysis

Brains were removed rapidly from decapitated rats and the various areas were obtained by gross dissection according to the atlas of Paxinos and Watson [14], and homogenized by sonication in 4 vols. (w/v) of Laemmli's sample buffer containing 20 mM dithiothreitol. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was done on a discontinuous Laemmli's SDS–PAGE (12.5%) system. The proteins were then transferred onto nitrocellulose paper with a semi-dry apparatus (Trans-blot, Biorad) by applying constant voltage (15 V for 30 min). After blocking overnight in a blocking solution containing 5% (w/v) dried skimmed milk, 0.2% (v/v) Tween 20, 0.02% (w/v) sodium azide in Tris-HCl 20 mM, pH 7.5, 150 mM NaCl (TBS), and repeated washing with TBS, the blots were incubated for 2 h with polyclonal antisera raised against the C-terminal decapeptides of $G\alpha_s$ and $G\alpha_o$ (1/500 v/v dilution in blocking solution). After repeated washing with TBS, the immunoblots were incubated for 1 h with goat anti-rabbit immunoglobulins coupled to alkaline phosphatase (1/600 v/v dilution in blocking solution). Finally, the blots were washed as above, dried and the color reaction was developed with 5-bromo-4-chloro-3-indolyl phosphatase and nitro blue tetrazolium as substrate. Relative intensities were quantified using the Image software described above.

2.5. Statistical analysis

Data are the mean $\pm$ S.E.M. of at least six animals; For analgesia and hypomotility the significances were calcu-

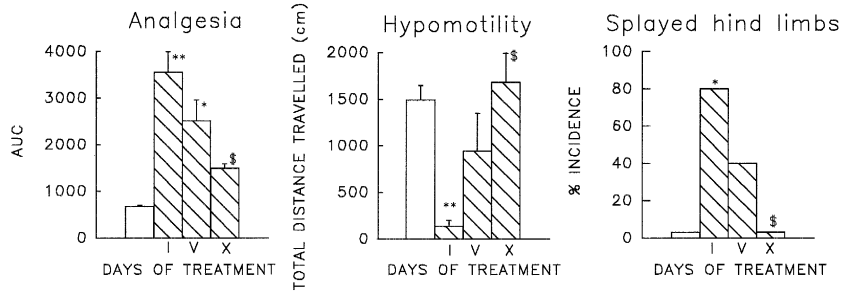

Fig. 1. Effect of chronic treatment with CP-55,940 (0.4 mg/kg, i.p., for 11 days) on analgesia (expressed as area under the analgesic curve, AUC), reduction of spontaneous locomotor activity (expressed as total distance travelled) and splayed hind limbs (expressed as % incidence at 30 min). The data are the mean ± S.E.M. of at least six animals. * $P < 0.05$; ** $P < 0.01$ vs. vehicle; $^{\$}$ $P < 0.01$ vs. 1 day (Dunnett's test); for splayed hind limbs * $P < 0.05$ vs. vehicle; $^{\$}$ $P < 0.01$ vs. 1 day (Fisher's exact test).

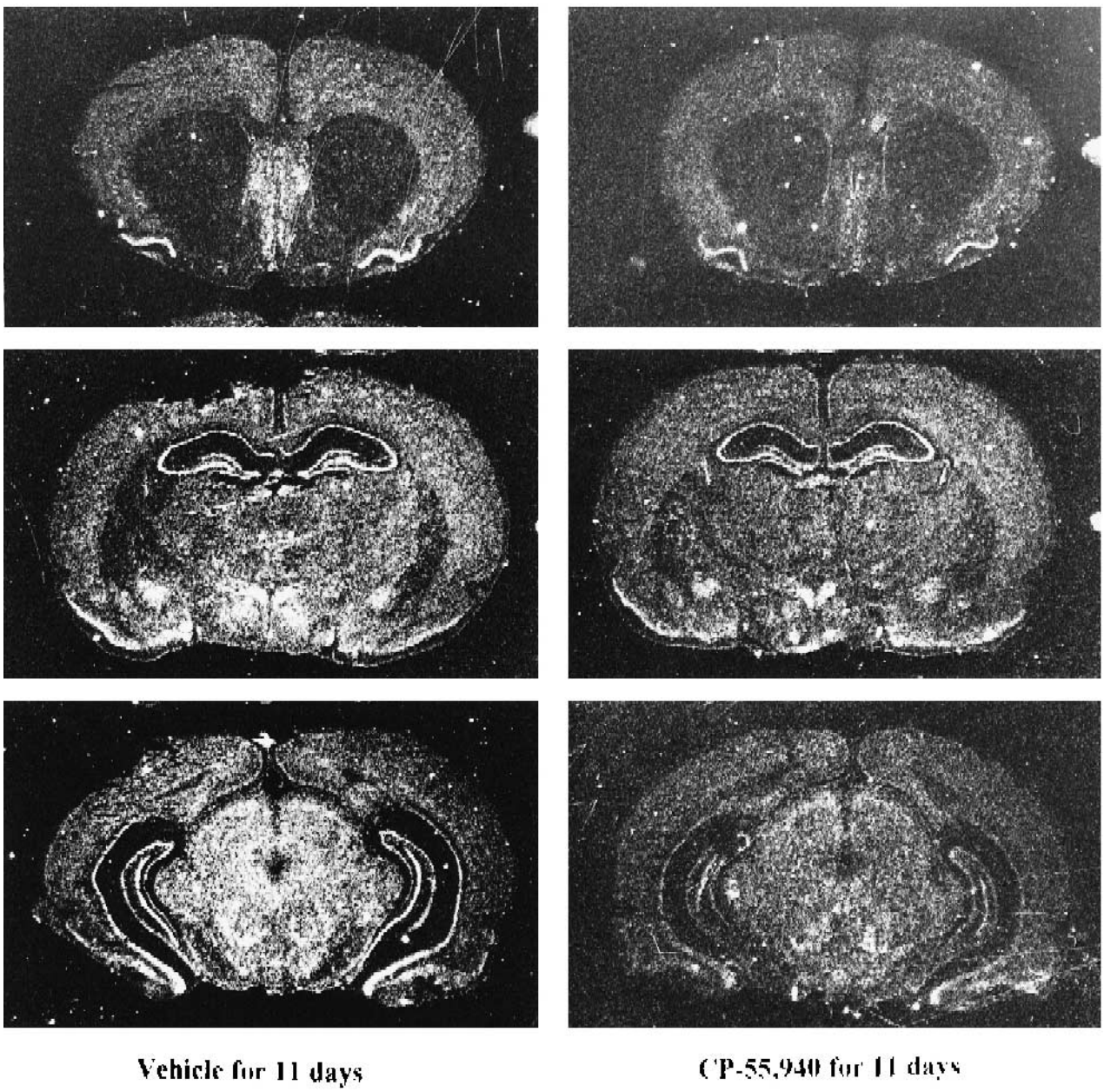

Fig. 2. Autoradiograms of representative rat brain coronal sections showing the effect of chronic CP-55,940 on $G\alpha_s$ mRNA.

lated with one-way analysis of variance followed by Dunnett's procedure; SHL data were analyzed by Fisher's exact test. For biochemical studies the significances were calculated using Student's *t*-test.

3. Results

3.1. Behavioral studies

The effects of repeated CP-55,940 injections on analgesia, reduction of spontaneous locomotor activity and splayed hind limbs are reported in Fig. 1. The first injection of CP-55,940 (0.4 mg/kg, i.p.) produced a significant degree of analgesia, hypomotility and splayed hind limbs. All these effects disappeared during treatment and within 10 days, a high degree of tolerance developed. Tolerance appeared to be complete for hypomotility and splayed hind limbs, which gave the same values as controls, whereas analgesia was reduced only by about 60%. During the entire observation period, vehicle alone never induced any behavioral effects different from controls (data not shown).

3.2. Biochemical studies

Our recent investigations have indicated that chronic treatment with CP-55,940 lowers cannabinoid receptor mRNA levels in the caudate-putamen and in the cerebral cortex [17]. Since the association of this receptor with second-messenger systems is mediated through G-proteins, we checked whether chronic CP-55,940 also affected the expression and level of G-proteins. Considering the behavioral results, we have measured the Gα-protein mRNAs after 5 and 11 days of treatment. One hour after the last injection on day 5, the rats were killed and in situ hybridization was done to see whether the levels of Gα_s, Gα_i and Gα_o mRNA were affected. First of all, we checked whether vehicle per se affected the basal expression of the three considered α-subunits: chronic treatment with ethanol 20% i.p. did not alter α_s, α_o or α_i gene expression in any of the cerebral regions containing the message (data not shown). Table 1 shows that 5 days after the chronic treatment with CP-55,940 some alterations appeared in α-subunit expression. Particularly the abundance of α_s mRNA was significantly decreased in the cerebral cortex, while the slight alterations we observed in the other considered brain regions did not reach the statistical significance. Concerning α_o and α_i, 5 days CP-55,940 treatment did not alter the abundance of their messages, the decrease being around 10%.

Table 1
Effect of 5 days of treatment with CP-55,940 on α_s, α_o and α_i mRNAs in the rat central nervous system

Cerebral areas	α_s (% vs. controls)	α_o (% vs. controls)	α_i (% vs. controls)
Cerebral cortex	20 ↓ [a]	15 ↓	=
Olfactory area		=	
Striatum		10 ↓	
Thalamus	18 ↓		=
Hypothalamus	12 ↓		=
Mesencephalon	10 ↓		=

=, alterations lesser than 10% vs. controls; ↓, decrease vs. controls.
[a] $P < 0.05$ vs. controls.

In contrast on day 11, when behavioral tolerance has been developed, the level of α-subunits expression was significantly altered. Fig. 2 shows the autoradiograms of rat brain coronal sections, presenting the α_s mRNA level in vehicle and treated animals. Chronic cannabinoid exposure caused a marked reduction in Gα_s expression in all brain areas containing the message, the decrease ranging from 25% in the thalamus to 45% in the mesencephalon. The immunoreactivity level of α_s protein did not show any real change, although sometimes we observed some slight alteration, not reaching statistical significance (see Fig. 5).

Concerning Gα_i mRNA that had a very similar regional distribution to Gα_s, even if generally less abundant than α_s, chronic CP-55,940 treatment decreased α_i expression among all the brain areas containing the basal message. Specifically the greatest intensity of reduction was localized in the cerebral regions in which the cannabinoid receptor was present (i.e., forebrain, 63% of reduction; cerebellum, 58%; and mesencephalon, 38%) (Fig. 3). Again, the changes in α_i expression were not coupled to analog changes in protein level (see Fig. 5).

Chronic treatment with CP-55,940 produced a more localized change in the Gα_o-subunit expression. In fact, among all the cerebral areas containing the α_o message, only the rostral portion showed a significant reduction in α_o mRNA. Representative coronal sections of these re-

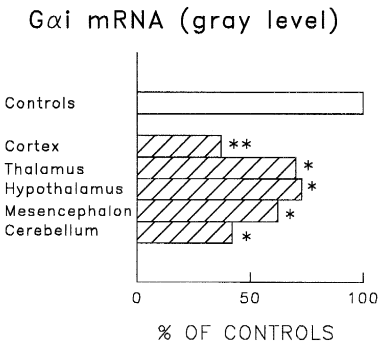

Fig. 3. Quantitative assessment of the Gα_i mRNA hybridization signal measured in tolerant and control rats. The data are mean ± S.E.M. of at least six animals. ** $P < 0.01$; * $P < 0.05$ vs. controls (Student's *t*-test).

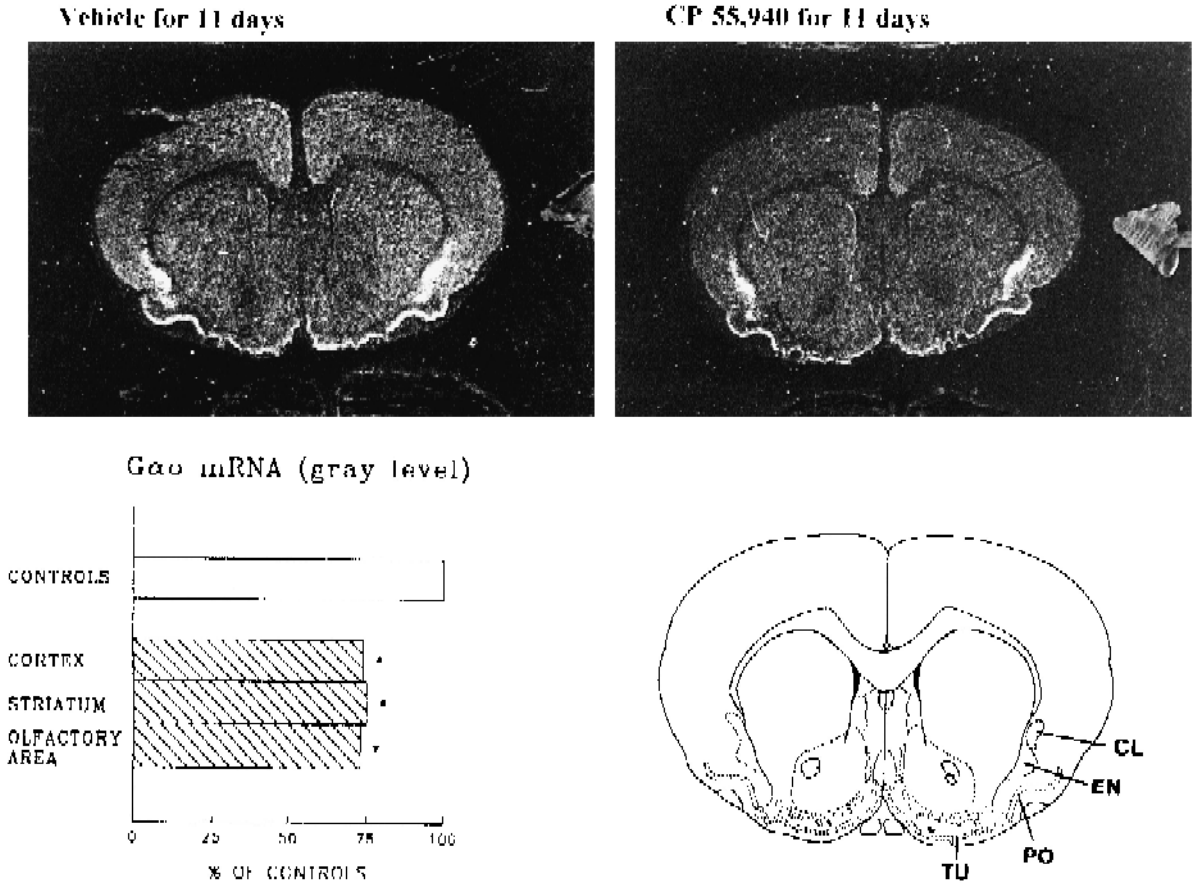

Fig. 4. Autoradiograms of representative coronal sections of the rostral portion of rat brain showing the effect of chronic CP-55,940 on $G\alpha_o$ mRNA. Camera lucida drawing from the atlas of Paxinos and Watson is shown for reference. EN, endopiriform nucleus; CL, claustrum; PO, piriform cortex; TU, olfactory tubercle. The bottom left-hand panel shows the quantitation of $G\alpha_o$ mRNA in the cerebral areas presented in the autoradiograms. The data are the mean ± S.E.M. of at least six animals. * $P < 0.05$ vs. controls (Student's *t*-test).

gions are presented in Fig. 4, showing that the autoradiographic signal was lower in CP-55,940 than in vehicle-treated animals. The decrease amounted to 26% in the cortex, 25% in the caudate-putamen and 27% in the olfactory area (Fig. 4). As with other α-subunit proteins, these changes in α_o message were not followed by equivalent changes in the amount of protein in the same areas (Fig. 5).

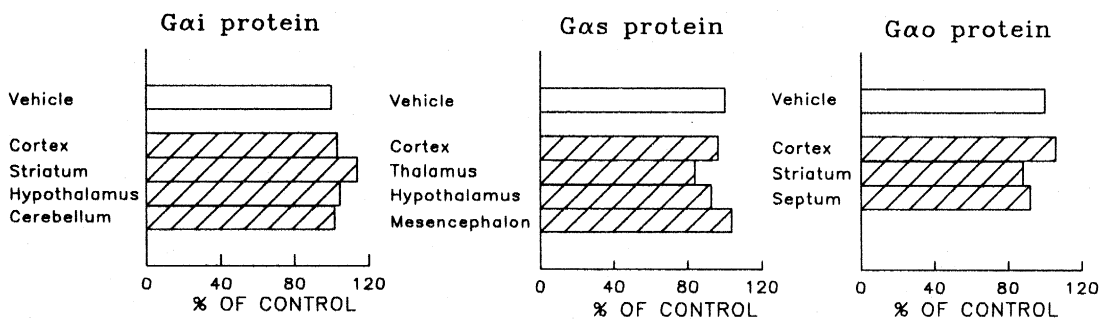

Fig. 5. Effect of chronic CP-55,940 on α_i-, α_s- and α_o-subunit immunoreactivity in the main cerebral areas presenting altered gene expression. The data are the mean ± S.E.M. of at least six animals.

4. Discussion

The current study confirms the finding of tolerance to the motor and analgesic effects of CP-55,940 in rats [4,12] and extends it to another behavioral manifestation, the incidence of splayed hind limbs. The development of tolerance followed different time courses, and full tolerance appeared for hypomotility and splayed hind limbs, whereas analgesia was only reduced by 60%.

The main finding of this study is that long-term in vivo exposure to the synthetic cannabinoid compound CP-55,940 changed the contents of the mRNA coding for G-protein α-subunits, following the development of cannabinoid tolerance. Concerning the time course of biochemical and behavioral parameters, it appears that the modifications in Gα-subunits expression are significant only after 11 days of treatment, when a complete behavioral tolerance has been developed. In fact, early time of cannabinoid exposure (5 days) did not trigger significant modification in α mRNA levels. Thus it could be suggested that only long-term exposure to CP-55,940 leads to adaptations in G-protein α-subunit expression in whole animals. In fact, the expression of α_s, α_i and α_o messages was significantly reduced in the brain of 11-day-treated rats. Specifically, the grain density associated with α_s and α_i mRNA was significantly reduced in almost all brain areas containing the basal message whereas alteration of the α_o signal showed striking regional specificity, being diminished only in some areas of the anterior forebrain (cortex, striatum and olfactory area). Therefore, chronic treatment with CP-55,940 had different effects on G-protein α-subunit expression.

The effect on the α_s- and α_i-subunit appeared to be generalized to all the brain areas containing the message, including regions with no cannabinoid receptors. Thus we can interpret these widespread alterations in their mRNA levels as the result not only of changes at the cannabinoid receptor level, but also of cross-regulation between cannabinoid receptors and other neurotransmitter systems which ends by causing an alteration in the α_s/α_i signal within cerebral regions that are not strictly related to the presence of the cannabinoid receptor itself. Moreover, a further explanation of this effect may be found – as suggested by Felder and coworkers for cannabinoid stimulation of arachidonic acid release [5] – in the ability of cannabinoid agonists to produce receptor-independent effects. CP-55,940, with its high lipophilicity, may penetrate the cell membrane and interfere with intracellular pathways able to modify gene expression. The use of a receptor-inactive stereoisomer or enantiomer of CP-55,940 could be helpful to better discriminate the receptor-independent vs. receptor-dependent mechanism.

In contrast, chronic CP-55,940 exposure had quite a different effect on Gα_o mRNA: among all brain areas containing the basal message, we observed a localized decrease only in the anterior portion of the brain. In these areas, we demonstrated a strong reduction in cannabinoid receptor mRNA levels [17], and since one of the intracellular consequences of cannabinoid receptor activation is inhibition of voltage-gated calcium channels through a pertussis toxin-sensitive G-protein belonging to the G_o/G_i family, we suggest that the alteration in Gα_o could be linked to permanent activation of cannabinoid receptors inducing adaptive changes both in the receptor and in α_o-subunit expression.

Another important observation arising from our data is that in spite of the alteration in gene expression, the levels of α-proteins were not significantly affected by the chronic cannabinoid treatment. There are plenty of published reports of no correlation between the effects on mRNA and on the protein, and no clear explanation has yet been offered. The neuronal level of a protein is always based on a balance between several factors: biosynthesis of the protein in terms of transcription of DNA or mRNA, translation of mRNA, packaging, transport and degradation of the biologically active protein produced. In our hands, the alteration in Gα mRNA might represent a destabilization of the message not affecting protein amount during the time of the experiment. Moreover immunoblotting data represent only the total amount of protein not providing any information about its functional activity. Thus, we can hypothesize that the constant level of protein we measured could include G-protein possessing altered biological activity. Finally, changes in Gα-subunit levels could occur at a later time than mRNA alterations.

We have previously shown [13,18,19] that changes in the Gα_o and Gα_s expression and level occur in rat brain upon the development of morphine tolerance; alteration in G-protein levels in specific brain regions was also reported after chronic morphine or cocaine treatment by Nestler's group [11,20]. Anyway, the alterations in G-protein system observed by the various authors are quite different depending on the drug, the protocol used for chronic treatment and the biochemical assay performed (immunoblotting, ADP-ribosylation and in situ hybridization). Comparing cannabinoids with opioids and cocaine, the most striking difference is the regional selectivity of the observed effect. In fact, whereas opioids and cocaine regulate G-protein expression/level into selective central nervous system areas (i.e., hypothalamus, olfactory system and locus coeruleus), at least for α_s and α_i, CP-55,940 effect appears to be generalized to several brain areas, suggesting that chronic exposure induced a global response into the CNS. However, taken together, all these findings suggest that adaptations in G-protein expression and/or synthesis could represent part of the biochemical mechanism underlying drug addiction.

Overall, the data of the present work suggest that two sets of cellular events contribute to the maintenance of cellular homeostasis during cannabinoid tolerance: proximal events taking place in the cannabinoid receptor transduction machinery (receptor amount and expression

down-regulation) and more distal events through the balance of other signal transduction systems, that affect the G-protein expression.

In conclusion, we found that the expression of G-protein α-subunit mRNAs in rat brain was significantly altered by in vivo chronic CP-55,940. These findings emphasize the need to map the precise intracellular pathways activated by chronic cannabinoids during the development of tolerance and to investigate the associated molecular events.

Acknowledgements

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