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Chronic Morphine Modulates the Contents of the Endocannabinoid, 2-Arachidonoyl Glycerol, in Rat Brain

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Opioids and cannabinoids are among the most widely consumed drugs of abuse in humans and the phenomena of cross-tolerance or mutual potentiation have been demonstrated between the two drugs. Several authors have suggested that both drugs share common links in their molecular mechanisms of action, although this has been a matter of controversy. Furthermore, no data exist on the possible adaptive changes in the contents of arachidonoylethanolamide (anandamide, AEA) and 2-arachidonoylglycerol (2-AG), the two major endogenous ligands for cannabinoid receptors, in morphine-tolerant rats. In the present work, we investigated the alterations in cannabinoid receptor functionality and endocannabinoid levels in rats chronically treated with morphine (5 mg/kg, s.c., twice a day for 5 days). Autoradiographic-binding studies using [^{3}H]CP-55 940 revealed a slight but significant reduction in cannabinoid receptor level in the cerebellum and hippocampus of morphine-tolerant rats, while CP-55 940-stimulated [^{35}S]GTPγS binding showed a strong decrease (40%) in receptor/G protein coupling in the limbic area of these animals. Moreover, in the same brain regions we measured, by isotope-dilution gas chromatography/mass spectrometry, the contents of AEA and 2-AG. Chronic morphine exposure produced a strong reduction in 2-AG contents without changes in AEA levels in several brain regions (ie striatum, cortex, hippocampus, limbic area, and hypothalamus). These findings clearly demonstrate that prolonged activation of opioid receptors could alter the cannabinoid system, in terms of both receptor functionality and endocannabinoid levels, and suggest the involvement of this system, alone or in combination with other mediators, in the phenomenon of morphine tolerance.
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INTRODUCTION

Opiates and cannabinoids are two classes of psychoactive drugs that share a similar pharmacological profile: both induce analgesia, hypothermia, sedation, hypotension, inhibition of intestinal motility and locomotor activity, changes in mood, and depression of the immune function (Manzanares *et al*, 1999; Massi *et al*, 2001). At the cellular level they activate different receptors, which are both coupled to Gi/Go GTP-binding proteins and inhibit adenylyl cyclase activity, block voltage-dependent calcium channels, and activate potassium channels. Receptors for these drugs are co-localized in the same neurons in various brain areas (caudate putamen, hippocampus, and substantia nigra) and might also compete for the same pool of Gi proteins (Childers *et al*, 1992).

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It is now well-established that opiates and cannabinoids exhibit cross-tolerance and/or mutual potentiation for antinociception after chronic treatment (Thorat and Bhargava, 1994; Rowen *et al*, 1998; Cichewicz *et al*, 2001; Rubino *et al*, 1997). The nature of such interactions is still unclear: some studies have reported that chronic exposure to opiates results in alterations of cannabinoid receptor density and/or signal transduction (Rubino *et al*, 1997; Romero *et al*, 1998; Shapira *et al*, 1998; Gonzalez *et al*, 2002). On the other hand, in mice where the 'central' cannabinoid CB_1 receptor was genetically deleted (CB_1 knockout mice), the acute effects of opiates were unaffected (Ledent *et al*, 1999), whereas the reinforcing properties of morphine and the severity of the withdrawal syndrome were strongly reduced (Ledent *et al*, 1999). Moreover the acute administration of SR141716, a selective central cannabinoid CB_1 receptor antagonist, blocked heroin self-administration in rats as well as morphine-induced place preference and morphine self-administration in mice (Navarro *et al*, 2001).

Recently, some authors have suggested that the functional interaction between opioids and cannabinoids could be ascribed to alterations of the output of their endogenous tone. In fact, the induction of the synthesis and/or release of endogenous opioid peptides after exposure to cannabinoids

have been demonstrated (Pugh *et al*, 1996; Welch and Eads, 1999, Valverde *et al*, 2001). By contrast, no data are available on possible alterations of the endocannabinoid system in the CNS after prolonged exposure to morphine. Therefore, we undertook the present study with the aim of investigating, in several brain regions of rats chronically treated with morphine, possible changes of the levels of: (1) cannabinoid receptor-binding sites and G-protein-coupled activity, and (2) anandamide (AEA) and 2-arachidonoylglycerol (2-AG), the two major endogenous ligands of cannabinoid receptors (Di Marzo *et al*, 1998).

METHODS

Animals and Treatment

Male Sprague–Dawley rats (Charles River, Calco, Italy) weighing 125–150 g at the beginning of the experiment were used. The animals were housed three per cage in standard conditions. After about 1 week of habituation, the rats received morphine hydrocloride (SALARS, Como, Italy) twice a day (between 9:00 and 10:00 am and 5:00 and 6:00 pm) for 4.5 days at a dose of 5 mg/kg s.c. Control rats received the same treatment with saline. On days 1, 3, and 5 the analgesic effect of morphine was evaluated by the tail flick test to monitor the development of tolerance. Pain thresholds were evaluated 15, 30, 60, and 120 min after the morning dose. A maximum cutoff time of 15 s was used and the rat was removed from the apparatus if it failed to respond within this interval. The results were expressed as total area under the time–response curve (AUC) over the 120 min. This chronic regimen of morphine administration induced a clear development of tolerance as previously demonstrated (Massi *et al*, 2001). In the acute condition rats received a single morphine injection (5 mg/kg, s.c.) or saline.

Autoradiographic-Binding Studies

For autoradiographic-binding studies rats were killed by decapitation 2 h after the last morphine or saline injection. This interval of time after the last treatment is needed in order to avoid any interference of the residual drug injected *in vivo*. The brains were rapidly removed and frozen into liquid nitrogen. They were stored at −80°C until processing. Sagittal sections of 20 μm were cut on a cryostat and thaw-mounted onto gelatin-coated slides and stored at −80°C until use. Adjacent sections from the same brains were used for the two assays.

[^{3}H]CP-55 940 receptor autoradiography. The slides were brought rapidly to room temperature, then incubated for 2.5 h at 37°C with 10 nM [^{3}H]CP-55 940 (Perkin-Elmer Life Science, Milan, Italy) in binding buffer (50 mM Tris-HCl, pH 7.4, 5% BSA). Adjacent cerebral sections were coincubated in parallel with 10 μM CP-55 940 to assess nonspecific binding. Sections were washed for 1 h at 0°C in 50 mM Tris-HCl pH 7.4, 1% BSA buffer and left in the same conditions for 3 h. Sections were then dipped in 50 mM Tris-HCl buffer (pH 7.4, 5 min) to remove excess BSA, dipped briefly in distilled water (1 min), and dried under a cool air stream. Autoradiograms were generated by exposing the dried sections for 7 days to tritium-sensitive film, Hyperfilm-^{3}H (Amersham Pharmacia Biotech, Milan, Italy).

Agonist-stimulated [^{35}S]GTPγS autoradiography. Cannabinoid-stimulated [^{35}S]GTPγS binding was determined as described previously by Sim *et al* (1996) with slight modifications. Briefly, slides were incubated in assay buffer (50 mM Tris-HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, 0.1% BSA, adenosine deaminase 10 mU/ml, pH 7.4) at 25°C for 10 min and then in 3 mM GDP in assay buffer at 25°C for 15 min. Slides were then transferred into assay buffer containing 3 mM GDP and 0.04 nM [^{35}S]GTPγS (Perkin-Elmer Life Science, Milan, Italy) with (stimulated) or without (basal) 5 μM CP-55,940 and incubated at 25°C for 2 h. Slides were rinsed twice in cold 50 mM Tris buffer and once in deionized water, dried, and exposed to β_{max} film (Amersham Pharmacia Biotech, Milan, Italy) for 48 h.

Image analysis. The image intensity of the autoradiographic film was assessed by measuring the gray levels with an image analysis system consisting of a video camera (Hamamatsu, Photonics K.K., Tokyo, Japan) connected to an Apple MacIntosh II personal computer. The public domain Image 1.47 software was used (National Institute of Health, Bethesda, MD). Each cerebral area was traced with the mouse cursor control and the light transmittance was determined as the gray level. The gray level of densitometric measurements calculated after subtraction of the film background density was established within the linear range, determined by using tritium standards (^{3}H Microscales, Amersham Pharmacia Biotech, Milan, Italy) for receptor-binding studies, and [^{35}S]-labelled standards prepared in laboratory for [^{35}S]GTPγS-binding studies.

Measurement of Endocannabinoid Contents

Dissection procedure. Rats were killed 2 h after the last morphine or saline injection, and the brains were quickly and carefully removed; the following areas, striatum, cortex, hippocampus, limbic area (that contains nucleus accumbens, septum nuclei, and parts of the anterior amygdaloid nuclei) hypothalamus, mesencephalon, and cerebellum were rapidly dissected on ice using the Paxinos and Watson atlas (1986) for morphological orientation, and immediately frozen at –80° to avoid the post-mortem rise in the concentrations of long-chain *N*-acylethanolamines. This starts approximately 30 min after sacrifice, as reported by Schmid *et al* (1995).

Procedure of extraction. Each brain region was extracted when still frozen. The tissue was homogenized in 5 vol of chloroform/methanol/Tris-HCl 50 mM (2 : 1 : 1) containing 1 nmol of d_8-AEA and d_8-2-AG. Deuterated standards were synthesized from d_8 arachidonic acid and ethanolamine or glycerol as described, respectively, in Devane *et al* (1992) and Bisogno *et al* (1997). Homogenates were centrifuged at 13 000 *g* for 16 min (4°C), the aqueous phase plus debris were collected and extracted again twice with 1 vol of chloroform. The organic phases from the three extractions were pooled and the organic solvents evaporated in a

rotating evaporator. Lyophilized samples were then stored frozen at −80°C under nitrogen atmosphere until analyzed.

Analysis of endocannabinoid contents by GC/MS. Lyophilized extracts were resuspended in chloroform/methanol 99 : 1 by vol. The solutions were then purified by open bed chromatography on silica as described in Fontana *et al* (1995). Fractions eluted with chloroform/methanol 9 : 1 by vol. (containing AEA and 2-AG) were collected and the excess solvent evaporated with a rotating evaporator. The former fractions were further fractionated by normal phase high-pressure liquid chromatography (NP-HPLC) carried out using a semipreparative silica column (Spherisorb S5W, Phase Sep, Queensferry, CLWYD, UK) eluted with a 40 min linear gradient from 9 : 1 to 8 : 2 (by vol) of n-hexane/2-propanol (flow rate = 2 ml/min). These elution conditions allow the separation of 1(3)- and 2-acyl-glycerols (retention time of 18 and 20 min, respectively) from *N*-acylethanolamines (retention time = 26–27 min). NP-HPLC fractions from 17 to 22 min and from 24 to 28 min were pooled, the solvent evaporated in a speed-vac, and the components derivatized with 20 µl *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide+1% trimethylchlorosylane for 2 h at room temperature and analyzed by gas chromatography/mass spectrometry (GC-MS) carried out under conditions described previously (Bisogno *et al*, 1997) and allowing the separations of monoacylglycerols or *N*-acylethanolamines with different fatty acid chains. MS detection was carried out in the selected ion monitoring mode using *m/z* values of 427 and 419 (molecular ions for deuterated and undeuterated AEA), 412 and 404 (loss of 15 mass units from deuterated and undeuterated AEA), 530 and 522 (molecular ions for deuterated and undeuterated 2-AG), and 515 and 507 (loss of 15 mass units from deuterated and undeuterated 2-AG). The area ratios between signals of deuterated and undeuterated AEA varied linearly with varying amounts of undeuterated AEA (20 pmol–20 nmol). The same applied to the area ratios between signals of deuterated and undeuterated 2-AG in the 100 pmol–20 nmol interval. AEA and 2-AG levels in unknown samples were therefore calculated on the basis of their area ratios with the internal deuterated standard signal areas. Two GC-MS peaks for both deuterated and undeuterated mono-arachidonoylglycerol were found, corresponding to 2-AG and 1(3)-AG, in agreement with the previous observation that 2-AG undergoes isomerization during the purification procedure (Stella *et al*, 1997). Therefore, the amounts of 2-AG were calculated by adding the amounts of the two isomers. The amounts of endocannabinoids are expressed as pmol or nmol per gram of wet tissue extracted. Further details on the GC/MS technique, including the detection and quantification limits, have been published (De Petrocellis *et al*, 1999).

Statistical analysis

The data presented in the figures are means ± SEM of at least five animals. Statistical analysis of the data was carried out by using the Student's *t*-test and the one-way analysis of variance (ANOVA) followed by Tukey's and Bonferroni's test performed by Prism (GraphPad Software, Inc.).

RESULTS

Behavioral Studies

The acute injection of morphine (5 mg/kg, s.c.) in rats elicited a significant degree of analgesia and as expected, rats chronically treated with morphine (5 mg/kg, s.c., twice a day for 4.5 days) developed tolerance to its analgesic effect (Figure 1).

Autoradiographic-Binding Studies

At 2 h after the last morphine injection, rats were killed, and the brain was quickly removed for autoradiographic studies of [^{3}H]CP-55 940 binding to CB_1 receptors and agonist-stimulated [^{35}S]GTPγS binding. First, we checked the effect of acute morphine on these parameters, and alterations were found neither in CB_1 receptor binding nor in CP-55 940-stimulated [^{35}S]GTPγS binding (data not shown). Figure 2 shows the results of densitometric analysis of rat brain sagittal sections in animals given morphine or saline chronically. Five days exposure of morphine induced a slight (15%) but significant downregulation of cannabinoid receptor in the hippocampus and cerebellum, without any changes in the other regions. Representative autoradiograms of rat brain sagittal sections showing the effect of chronic morphine administration on cannabinoid receptor binding are presented in Figure 2.

Since the cannabinoid receptor is linked to the G protein transduction system, the second step in our work was to check for any alteration in receptor/G protein coupling following chronic morphine administration. We assayed cannabinoid-stimulated [^{35}S]GTPγS binding, with CP-55 940 as the agonist. Basal [^{35}S]GTPγS binding was not affected by chronic morphine in any of the considered cerebral areas (data not shown). By contrast, prolonged exposure to morphine significantly decreased the net binding value only in the limbic area (40%), whereas no significant differences were found between morphine-treated and control rats in the other regions (Figure 3).

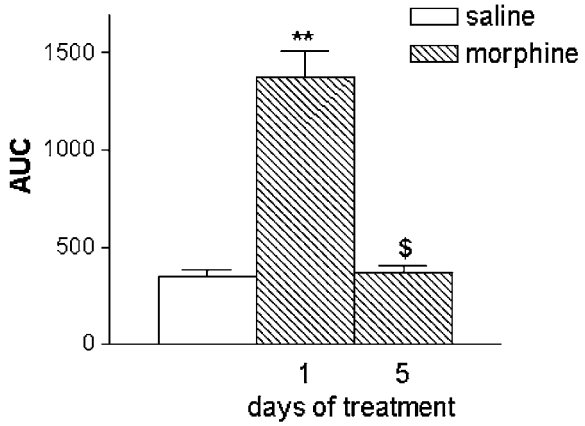

Figure 1 Effect of morphine (5 mg/kg, s.c., twice a day for 4.5 days) on analgesia expressed as area under the analgesic curve (AUC). The data are the means ± SEM of at least five animals. $^{**}p < 0.01$ by one-way ANOVA followed by Tukey's test *vs* saline; $ $p < 0.05$ by one-way ANOVA followed by Tukey's test *vs* morphine 1 day.

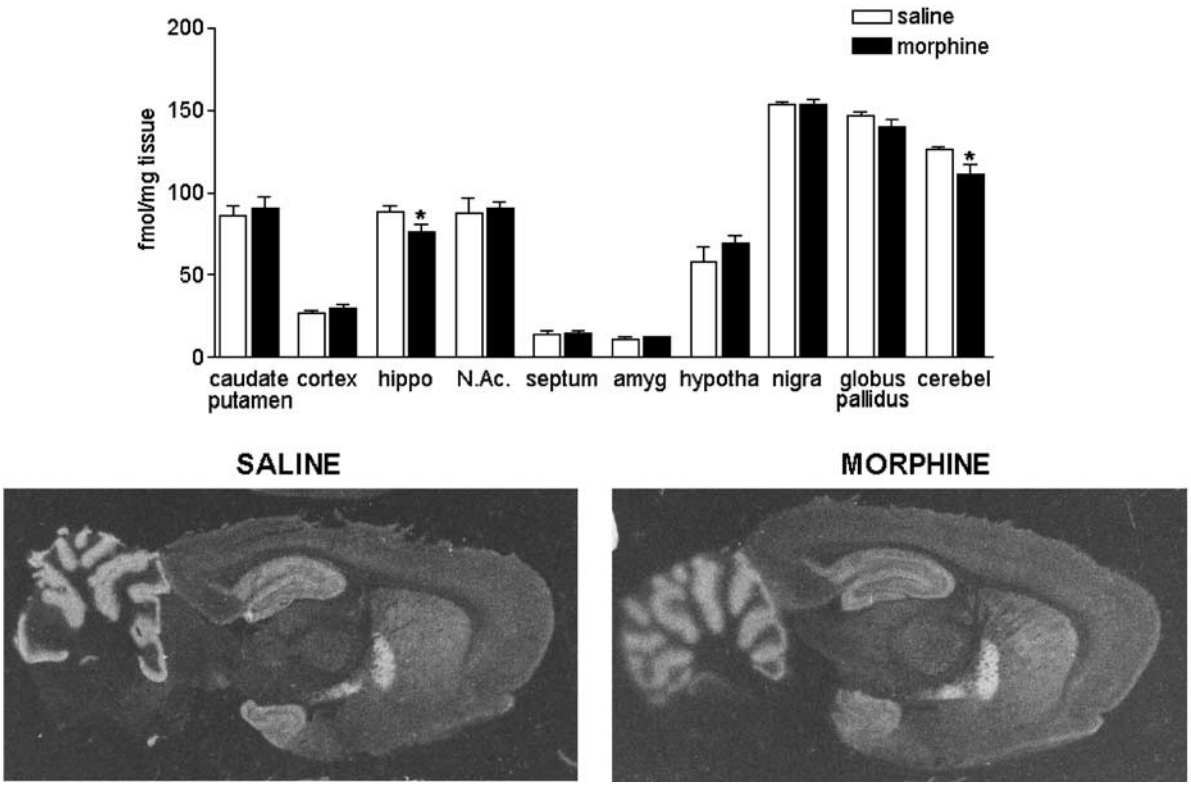

Figure 2 Effect of morphine chronic treatment on CB_1 receptor binding in different rat brain areas. Gray levels obtained with densitometric analysis were transformed into fmol/mg tissue using [^{3}H]standards. Bars indicate the means ± SEM of at least five animals (three sections/animal). * $p<0.05$ by Student's *t*-test *vs* saline. The bottom panel shows autoradiograms of representative sagittal sections of the brain of saline and morphine rats. hippo: hippocampus; N. Ac.: nucleus accumbens; amyg: anterior amygdala; hypothal: hypothalamus; nigra: substantia nigra; cerebel: cerebellum.

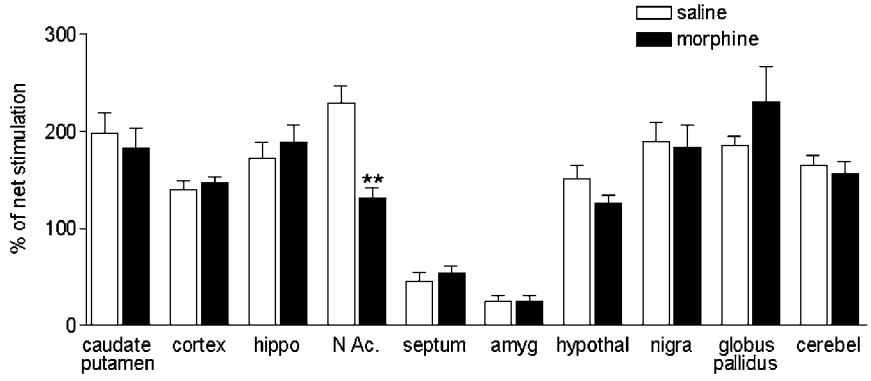

Figure 3 Effect of morphine chronic treatment on net cannabinoid-stimulated [^{35}S]GTPγS binding in different brain regions, determined by subtracting basal [^{35}S]GTPγS binding. Bars indicate the means ± S.E.M. of at least five animals (six sections/animal). ** $p<0.005$ by Student's *t*-test *vs* saline. hippo: hippocampus; N. Ac.: nucleus accumbens; amyg: anterior amygdala; hypothal: hypothalamus; nigra: substantia nigra; cerebel: cerebellum.

Endocannabinoid Contents

The contents of both endocannabinoids, AEA and 2-AG, measured in seven brain regions of control-injected rats, were mostly similar to those previously reported by other authors (Di Marzo *et al*, 2000a; Bisogno *et al*, 1999; Felder *et al*, 1996) in naive and control animals, allowing for the large standard deviations with which these compounds are usually quantified by the GC/MS technique. In a pilot study, acute morphine treatment did not induce any changes of either AEA or 2-AG levels in rat whole brain (data not shown). Therefore, we focused on chronic morphine treatment, which instead produced a dramatic decrease in 2-AG contents in most of the brain regions analyzed, that is, striatum (69%), cortex (62%), hippocampus (42%), limbic area (42%), and hypothalamus (35%), with no significant alterations in the mesencephalon and cerebellum (Figure 4). On the contrary, no statistically significant alterations were found in any of the cerebral regions in the levels of AEA after 5 days of morphine

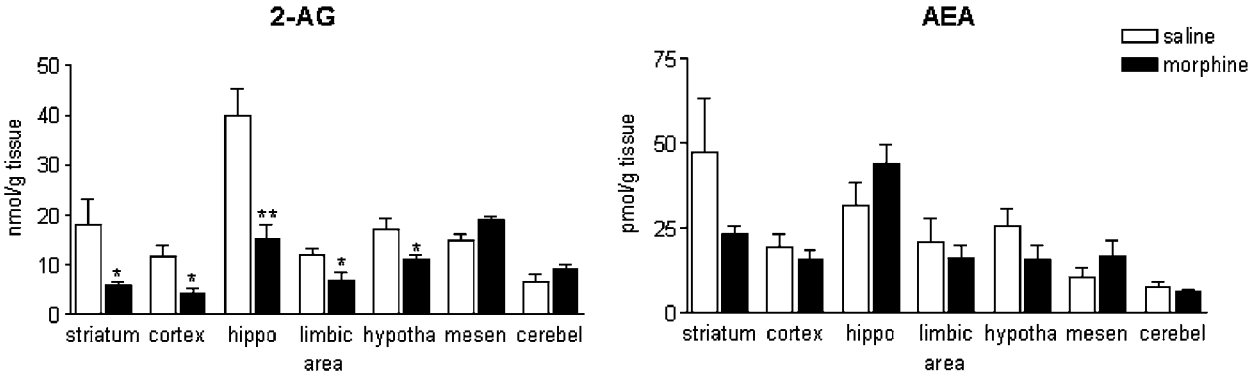

Figure 4 (a) Effect of morphine chronic treatment on 2-AG and anandamide (AEA) contents measured in different rat brain regions. Values are means ± SEM of four determinations/each corresponding to a different pool of animals. *$p<0.05$; **$p<0.005$ by one-way ANOVA followed by Bonferroni's test *vs* saline. hippo: hippocampus; hypothal: hypothalamus; nigra: substantia nigra; cerebel: cerebellum.

treatment, although a trend for a decrease was observed in the striatum (Figure 3). As a control, we measured the amounts of the anandamide congener, *N*-palmitoylethanolamine (PEA), a substance with cannabimimetic actions and little, if any, binding affinity to cannabinoid receptors. We found no differences in its levels between control and treated animals in any of the brain regions under study (data not shown).

DISCUSSION

This study provides new insights into the molecular mechanisms of the crosstalk between opiates and cannabinoids. In morphine-tolerant rats we observed, limited to the hippocampus and cerebellum, a slight but significant decrease in CB_1 receptor binding, which was not accompanied by a corresponding decrease in cannabinoid receptor-induced signal transduction. More importantly, a strong decrease in cannabinoid-induced [^{35}S]GTPγS binding, but not in CB_1 receptor binding, was observed in the limbic area, a region involved in the rewarding and addictive properties of morphine and other substances of abuse. None of these changes was observed in the brain of rats after acute administration of morphine. The region specificity of the changes observed could be related to the high density of CB_1 receptors in these areas, and might correlate with the important functional roles in cognition (hippocampus), control of motor behavior (cerebellum), and reward processes (limbic area), of these regions. Previous studies demonstrated that chronic morphine exposure induces divergent and region-dependent effects on CB_1 receptor expression, binding and signal transduction. In fact, some authors reported no significant changes in CB_1 receptors in mice chronically treated with morphine (Romero *et al*, 1998), while other recent studies carried out in morphine-tolerant rats reported both a decrease (in the cerebellum and hippocampus) and an increase (in the caudate putamen and most of the limbic structures) in different brain regions (Gonzalez *et al*, 2002), or an increase in the caudate putamen only (Rubino *et al*, 1997) of the density of cannabinoid CB_1 receptor protein and/or mRNA. These somehow discrepant data are likely because of the different species, strains, and protocols used to induce tolerance. The protocol used in the present study was milder than that used in previous investigations, but it was nevertheless able to induce tolerance to the analgesic effect of morphine.

Our study presents for the first time data regarding alterations in the contents of endocannabinoids in the brain of morphine-tolerant rats. Chronic treatment with morphine produced a widespread decrease in 2-AG contents without significant changes in AEA levels, therefore suggesting that this treatment influences in a different way the regulatory mechanisms controlling AEA and 2-AG homeostasis in the brain. Again, no change was observed after acute administration of morphine. Thus, the endocannabinoid system, besides being involved in the development of tolerance to Δ^9-THC (Di Marzo *et al*, 2000a), might play an important role also in opiate tolerance (see below). However, it must be emphasized that chronic exposure to cannabinoids and opioids influences endocannabinoid levels in a different way, in terms of both the brain region and the type of endocannabinoid affected. In fact, according to Di Marzo *et al* (2000a), chronic Δ^9-THC induced an increase in AEA contents only in the limbic forebrain, without affecting 2-AG levels, whereas a decrease of both endocannabinoids was found in the striatum. By contrast, in the present study, chronic morphine affected 2-AG levels in five out of the seven regions examined without significantly altering AEA levels, although, like with chronic THC treatment (Di Marzo *et al*, 2000a), a decrease of 2-AG and a trend for a decrease of AEA was observed in the caudate putamen. Interestingly, the regions where chronic morphine decreased 2-AG levels to the highest extent were also those with the highest density of CB_1 receptors, thus potentially leading to a strong impact on endocannabinoid signalling in these regions. In particular:

(1) In the caudate putamen, we observed a significant decrease in the contents of 2-AG without any alterations in CB_1 receptor functionality, which might result in a decreased endocannabinoid transmission. This event, as discussed by Di Marzo *et al* (2000a) for THC-tolerant rats, could explain in part the loss of motor inhibition by morphine observed in rats made

tolerant to this drug. In fact, endocannabinoids in the striatum are thought to depress motor activity, probably through both inhibition of dopamine neurotransmission and enhancement of GABA-mediated effects (Glass *et al*, 1997; Di Marzo *et al*, 1998).

(2) In the cortex the picture was similar to that observed in the caudate putamen, that is, no changes in the receptor functionality with decreased 2-AG levels, thus again potentially resulting in a decreased endocannabinoid tone. In this area, cannabinoids inhibit the release of glutamate, which, at the level of cortico-striatal synapses, causes an impairment of motor coordination. Thus, the observed decrease in 2-AG amounts could at least in part account for the loss of morphine effect on motor coordination in morphine-tolerant rats (Auclair *et al*, 2000).

(3) In the hippocampus, we found a decrease of both 2-AG contents and receptor binding. We found, however, no changes in receptor/G protein coupling, suggesting the presence of CB_1 receptors with increased functionality. This might represent an adaptive process induced in hippocampal neurons to counteract the reduced endocannabinoid levels. In fact, previous data obtained using CB_1 knockout mice showed that: (1) the endocannabinoid system is tonically active in the hippocampus with the function of controlling acetylcholine and glutamate signalling, long-term potentiation, and memory (Bohme *et al*, 2000; Martin *et al*, 2002); (2) the levels/activity of CB_1 receptors and their ligands are tightly and mutually regulated in this region (Di Marzo *et al*, 2000b).

(4) In the hypothalamus, we observed a reduction of 2-AG amounts again with no changes in receptor functionality. As shown by studies using CB_1 knockout mice, an endocannabinoid tone in this brain area modulates the hypothalamic–pituitary axis as well as body temperature, appetite, and food intake (Di Marzo *et al*, 2001; Wenger *et al*, 2001). The decreased endocannabinoid transmission observed in this area could thus account in part for the reduced food intake observed in morphine chronically exposed rats, and for the development, in these animals, of tolerance to the hypothermic effect normally induced by acute morphine.

(5) In the limbic area, the reduction in cannabinoid-stimulated G protein activity was restricted to the nucleus accumbens, while we found a decreased 2-AG content analyzing the whole limbic area (nucleus accumbens, septum, and part of anterior amygdaloid nuclei), thus we cannot link the alteration in endocannabinoid levels to a specific brain regions belonging to the limbic area. Nevertheless, our data represent the first example of the existence of an alteration in limbic forebrain endocannabinoid levels and nucleus accumbens CB1 receptor signalling during chronic morphine. The observed reduction in 2-AG content in this heterogeneous region could play a role in the complex emotional and motivational state observed during chronic morphine exposure, being the septum and anterior amygdala notoriously linked to the inhibition of anxiety-like responses (Adamec and Young, 2000) and the nucleus accumbens a key region in the reward neural circuitry as well as in drug and food craving and in drug addiction.

(6) Finally, the widespread reduced levels of 2-AG found in the brain of morphine-tolerant rats could account for the enhanced susceptibility to neurodegenerative processes observed after chronic morphine exposure (Pearson *et al*, 1975/76; Coomb *et al*, 1985; London *et al*, 1989; Sala *et al*, 1994). In fact, recently it has been demonstrated that 2-AG exerts a neuroprotective action in rats after brain injury (Panikashvili *et al*, 2001), and the reduction in its amounts could in part underlie morphine-induced premature aging (Sala *et al*, 1994).

In conclusion, our results suggest that the widely described interactions between opiates and cannabinoids, as well as some of the behavioral features of animals tolerant to morphine, might be partly because of decreased endocannabinoid levels together with changes in cannabinoid receptor binding and receptor/G protein coupling; the possible functional significance of these changes being dependent on the brain region where they occur. Of the two endocannabinoids most widely studied to date, only the levels of the 2-AG were found here to be significantly decreased in brain regions with the highest density of cannabinoid CB_1 receptors. There are several possible reasons for this different behavior between AEA and 2-AG, which are produced and inactivated by neurons and astrocytes through different biochemical pathways. For example, it is possible that chronic morphine impacts more on the phospholipase C enzymes responsible for the biosynthesis of some 2-AG precursors, rather than on the phospholipase D, which catalyses AEA production (Di Marzo *et al*, 1998). Alternatively, it is possible that prolonged treatment with the drug upregulates only the monoacylglycerol lipase responsible for 2-AG hydrolysis, and not the fatty acid amide hydrolase catalysing anandamide hydrolysis. Importantly, chronic morphine has been previously reported to both enhance phospholipase C activity in rat mesolimbic neurons (Wolf *et al*, 1999) and reduce the formation of phospholipase C products in chick neurons (Mangoura and Dawson, 1991). Clearly, a full characterization of all the proteins that participate in the regulation of endocannabinoid levels must be awaited before addressing this issue in greater detail. At any rate, our finding of selective effects on 2-AG levels might explain in part the recent report that exogenous 2-AG attenuates some of the signs caused by morphine withdrawal in morphine-dependent rats (Yamaguchi *et al*, 2001).

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