

Differential role of the nitric oxide pathway on Δ^9 -THC-induced central nervous system effects in the mouse

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Abstract

This study investigated whether the nitric oxide pathway was involved in the central effects of Δ^9 -tetrahydrocannabinol (Δ^9 -THC), the major psychoactive constituent of *cannabis sativa*. Body temperature, nociception and locomotion were measured in neuronal nitric oxide synthase (nNOS) knock-out (KO) mice and wild-type (WT) controls after intraperitoneal application of Δ^9 -THC. These Δ^9 -THC-induced effects are known to be mediated through the brain-type cannabinoid receptor 1 (CB1). Therefore, *in situ* hybridization (ISH) experiments were performed in the adult murine brain to determine possible changes in CB1 mRNA levels in nNOS-KO, compared with WT mice, and to reveal brain areas where CB1 and nNOS were coexpressed in the same neurons. We found that an intraperitoneal injection of 10 mg/kg Δ^9 -THC led to the same increase in the hot plate latencies in both genotypes, suggesting that Δ^9 -THC-mediated antinociception does not involve nNOS. In contrast, a significant Δ^9 -THC-induced decrease of body temperature and locomotor activity was only observed in WT, but not in nNOS-KO mice. ISH revealed significantly lower levels of CB1 mRNA in the ventromedial hypothalamus (VMH) and the caudate putamen (Cpu) of the nNOS-KO animals, compared with WT mice. Both areas are known to be among the regions involved in cannabinoid-induced thermoregulation and decrease of locomotion. A numerical evaluation of nNOS/CB1 coexpression showed that approximately half of the nNOS-positive cells in the dorsolateral Cpu also express low levels of CB1. ISH of adjacent serial sections with CB1 and nNOS, revealed expression of both transcripts in VMH, suggesting that numerous nNOS-positive cells of VMH coexpress CB1. Our findings indicate that the nitric oxide pathway is involved in some, but not all of the central effects of Δ^9 -THC.

Introduction

Δ^9 -tetrahydrocannabinol (Δ^9 -THC), the major psychoactive constituent of *cannabis sativa*, exhibits a variety of central effects including hypothermia, antinociception and changes of locomotor activity (Pertwee, 1997; Felder & Glass, 1998; Ameri, 1999). Δ^9 -THC is known to act on CB1 and CB2 receptors, two subtypes of G-protein-coupled cannabinoid receptors. CB1 is mainly distributed in the central nervous system and CB2 in the lymphoid organs (Pertwee, 1997). CB1 mediates Δ^9 -THC-induced hotplate analgesia, hypothermia and decrease of locomotor activity. These effects of Δ^9 -THC cannot be induced in CB1 receptor knock-out mice (Ledent *et al.*, 1999; Zimmer *et al.*, 1999), or in animals pretreated with the selective CB1 receptor antagonist SR141716A (Lichtman & Martin, 1997; Welch *et al.*, 1998).

Results of recent studies imply that the cannabinoid system is likely to interact with several neurotransmitter systems such as the GABAergic, dopaminergic, opioidergic and glutamatergic system (Bidaut-Russell & Howlett, 1991; Herkenham *et al.*, 1991; Pertwee &

Wickens, 1991; Glass & Felder, 1997; Hampson *et al.*, 1998; Ameri, 1999; Manzanares *et al.*, 1999; Piomelli *et al.*, 2000). These systems are also strongly linked to the nitric oxide (NO) pathway (Bredt & Snyder, 1994; Herman *et al.*, 1995; East *et al.*, 1996; Liu, 1996; Jayakumar *et al.*, 1999; Contestabile 2000). NO is produced intracellularly by three isoforms of nitric oxide synthase (NOS): the endothelial (eNOS), inducible (iNOS) and neuronal NOS (nNOS) (Garthwaite & Boulton, 1995; Huang & Lo, 1998; Contestabile, 2000) with the latter being the focus of the present investigation. nNOS is a calcium/calmodulin-dependent enzyme which was first found in neurons (Bredt & Snyder, 1994). NO is considered to participate in a variety of physiological and pathological processes such as neuronal plasticity and neurotoxicity (Dawson *et al.*, 1998). In addition, NO is known to be involved in the effects of several centrally acting anaesthetic and analgesic drugs (Ferreira *et al.*, 1991; Johns *et al.*, 1992; Tonner *et al.*, 1997).

Various *in vitro* studies suggest a link between cannabinoid signalling through cannabinoid receptors and the NO pathway. The potent CB1 agonist CP-55,940 is able to decrease the release of NO from endotoxin/cytokine-activated rat microglial cells (Waksman *et al.*, 1999). In addition, several CB1 agonists were shown to inhibit K⁺-induced activation of NOS from primary cerebellar cultures whereas the cannabinoids had no effect on basal NOS activity (Hillard *et al.*, 1999). However, most of the *in vivo* studies analysing

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the interaction of the NO pathway and the cannabinoid system have been inconclusive so far (Thorat & Bhargava, 1994; Spina *et al.*, 1998).

In this study, we used a genetic approach to further characterize the functional link between Δ^9 -THC and the neuronal NO pathway. Nociception, body temperature, and locomotion were measured, both in neuronal nitric oxide synthase (nNOS) knock-out (KO; Huang *et al.*, 1993), and wild-type (WT) control mice after intraperitoneal application of the drug. In addition, we investigated possible changes in CB1 mRNA levels in nNOS-KO, as compared with WT mice, as well as coexpression of CB1 and nNOS using *in situ* hybridization.

Materials and methods

Animals

Homozygous males with a deficiency in the nNOS gene (KO), and wild-type male control mice (WT) were used for the study. Targeted disruption of the nNOS gene is described in Huang *et al.* (1993). The genetic background consisted of a combination of the strains 129/Sv and C57BL/6J, with a predominance of C57BL/6J, as mutants were backcrossed for three generations into C57BL/6J and then intercrossed to obtain experimental animals. As it is known that male nNOS-KO mice show a markedly increased aggressive behaviour (Nelson *et al.*, 1995), all animals were housed individually in the same temperature and humidity-controlled room with a 12 : 12-h light : dark cycle (light from 0700 to 1900), and with access to food and water *ad libitum*. At the time of investigation, the animals were 9–10 weeks-old and had a body weight of 21–26 g. All animals were drug-naïve and were injected only once with Δ^9 -THC or vehicle. All behavioural experiments were performed between 9 : 00 a.m. and 1 : 00 p.m. All behavioural and molecular investigations were evaluated with the investigator blind to treatment and mouse genotype. The experimental protocols were approved by the Ethical Committee on the Use and Care of Animals (Government of Bavaria, Germany).

Drugs and chemicals

Δ^9 -THC (Sigma, Deisenhofen, Germany) was purchased as a 100-mg/mL (w/v) solution in 100% ethanol. Immediately before injection, Δ^9 -THC was diluted 1 : 100 in 45% β -hydroxy-cyclodextrin (RBI/Sigma, Deisenhofen, Germany), and stirred for 10 min at 37 °C. As a vehicle control, 45% β -hydroxy-cyclodextrin containing 1% ethanol was used. All drugs were administered intraperitoneally (i.p.) with an injection volume of 10 mL/kg body weight. For behavioural tests, 10 mice of each genotype received Δ^9 -THC or vehicle. In a pilot study carried out on C57BL/6J mice, using various doses of Δ^9 -THC, 10 mg/kg Δ^9 -THC was found to be the lowest dose leading to clear analgesic and hypothermic effects.

Δ^9 -THC-induced effects *in vivo*

In both genotypes, antinociceptive effects of Δ^9 -THC were measured using a hot-plate analgesia meter (Bachofner, Reutlingen, Germany) at 30, 60 and 90 min after injection of the drug or the vehicle. The plate was heated to 55 ± 0.5 °C and the time taken for mice to first show signs of discomfort (licking or flinching of the paws or jumping on the plate) was recorded. A cut-off time of 60 s was set to prevent tissue damage. Δ^9 -THC-induced hypothermia was determined using an infrared thermometer (C-1600, Linear Laboratories, Fremont, California, USA), which was placed between the forepaws at a distance of exactly 3 cm. Body temperature was recorded immediately before, as well as 60 and 120 min after injection of drug or

vehicle. Spontaneous locomotor activity was assessed by an automated open field system (box size 32 × 32 cm; illumination of 40–60 lux, MOTION, TSE GmbH, Bad Homburg, Germany). Fifteen minutes after injection of Δ^9 -THC or vehicle, the animals were individually open-field tested for 30 min. The cumulative horizontal distance the animals moved within the box was recorded.

In situ hybridization was used to reveal possible differences in CB1 mRNA levels between the two genotypes. Six untreated mice of each genotype, which had the same age and weight as those tested in behavioural experiments, were used for *in situ* hybridization experiments (using a ³⁵S-labelled riboprobe) performed according to Marsicano & Lutz (1999). After hybridization and stringency washes, the slide-mounted sections were apposed to autoradiographic films (Kodak Biomax MR film, Integra Biosciences GmbH, Fernwald, Germany) for 11 h. Developed films were illuminated with a light box and sections were scanned as grey scale images with 256 grey values using a computer-assisted video camera. Mean densities of the regions of interest were measured using Object-Image 1.62 (N. Vischer, University of Amsterdam, the Netherlands) for Macintosh with a value of zero reflecting white, and 255 reflecting black.

For colocalization of CB1 and nNOS, double *in situ* hybridization experiments were performed according to Marsicano & Lutz (1999). The nNOS template was obtained by a reverse transcriptase-polymerase chain reaction (RT-PCR) using the 5' primer 5'-CCT GGT GGA GAT TAA CAT TGC-3' and the 3' primer 5'-CTG GTA CTG CAA CTC CTG ATT-3'. The amplification product was 1197 bp and spanned positions 1985–3182 of Genbank accession number NM008712. The PCR product was cloned in pBluescript KS⁻ (Stratagene, CA, USA) and the clone was confirmed by sequencing. Sense and antisense riboprobes were obtained as described in Marsicano & Lutz (1999), linearizing the plasmid with the restriction enzyme *Bam*HI and *Eco*RI, respectively, and using T3 and T7 RNA polymerase to obtain ³⁵S-labelled riboprobes. CB1 and nNOS sense probes gave no detectable signals.

Statistical analysis

Hot plate analgesia and temperature were first analysed using analysis of variance (ANOVA) with repeated measures, and the genotypes and therapy as between-subject-factors. In case of significant interactions, univariate *F*-tests were performed in order to evaluate significant differences at the respective time points. Univariate analysis of variance was used for statistical evaluation of the open field observations. Results of *in situ* hybridization of the different brain regions were evaluated using multivariate analysis of variance. In all cases, a *P*-value < 0.05 was considered as statistically significant. All results are shown as mean and standard error of the mean (SEM) of the absolute values. As the WT mice showed a lower basal activity in the open field system than the KO mice, Δ^9 -THC-induced changes in locomotor activity were evaluated and expressed as a percentage of the mean activity of the vehicle-treated group for each genotype.

Results

Δ^9 -THC-induced effects *in vivo*

The effect of Δ^9 -THC on nociceptive responses to acute pain were assessed by using the hot plate test, which is considered to involve mainly supraspinal mechanisms of the nociceptive system. As shown in Table 1, hot plate latencies after injection of the vehicle did not differ between the mutant mice and the wild-type controls. Acute intraperitoneal application of 10 mg/kg Δ^9 -THC exerted significant

TABLE 1. Effect of 10 mg/kg Δ^9 -THC (i.p.) on hot plate latencies, body temperature and locomotor activity in nNOS KO and wild-type control mice

	Wild-type mice (WT)		Knock-out mice (KO)	
	Vehicle-group	Δ^9 -THC-group	Vehicle-group	Δ^9 -THC-group
Hot plate latencies (s)				
30 min	15.6 \pm 1.1	22.3 \pm 2.2*	14.1 \pm 0.9	23.5 \pm 1.8*
60 min	16.4 \pm 1.8	30.5 \pm 3.9*	20.6 \pm 1.7	26.6 \pm 2.7*
90 min	19.1 \pm 1.9	25.3 \pm 3.2	19.0 \pm 2.3	28.6 \pm 1.6*
Body temperature ($^{\circ}$ C)				
basal	31.1 \pm 0.2	31.2 \pm 0.2	30.9 \pm 0.1	30.8 \pm 0.2
60 min	31.5 \pm 0.2	30.6 \pm 0.4	31.7 \pm 0.2	31.2 \pm 0.3
120 min	32.0 \pm 0.1	30.0 \pm 0.4*	31.3 \pm 0.2	30.9 \pm 0.3
Open field activity (%)	100 \pm 27	42.5 \pm 16.8*	100 \pm 14.4	82.5 \pm 10.9

Note the similar Δ^9 -THC-induced antinociceptive responses in both genotypes in contrast to the lack of Δ^9 -THC-induced hypothermia, and decrease of locomotor activity in the KO in comparison to the WT animals ($n = 10$ per group). Hot plate test and body temperature data are taken at the indicated time point after injections and are shown as mean \pm SEM; * $P < 0.05$ vs. vehicle. Data of locomotion are expressed as percentage of the moved distance/30 min of the respective vehicle-treated animals; * $P < 0.05$ vs. vehicle.

antinociceptive effects, as measured by an increase of hot plate latencies in both the KO and the WT mice. Significant effects of Δ^9 -THC on latency were observed at all three time points analysed (Univariate F -tests, P -value < 0.05), but there were no differences in the antinociceptive reactions to Δ^9 -THC between the genotypes. (Wilks multivariate test of significance; effect of treatment: $F_{3,34} = 9.505$, $P < 0.0001$; influence of genotype: $F_{3,34} = 0.151$, $P = 0.928$).

Measurement of body temperature (Table 1) also revealed no differences in basal values between the genotypes (Wilks multivariate test of significance; influence of genotype: $F_{2,34} = 0.750$; $P = 0.480$). However, in contrast to the analgesic effects, a significant Δ^9 -THC-induced hypothermia was only observed in the WT mice (Wilks multivariate test of significance; effect of treatment: $F_{2,34} = 5.87$, $P < 0.01$; effect of type \times treatment: $F_{2,34} = 3.407$, $P < 0.05$). Locomotor activity was assessed by open field observations. This test revealed a significantly higher activity of the vehicle-treated KO mice compared to the vehicle-treated WT controls (Univariate test of significance, $P < 0.01$, data not shown). The Δ^9 -THC-induced changes in locomotion were, therefore, evaluated separately in each genotype and expressed as a percentage of the mean distance moved by the respective vehicle-treated group. The results in Table 1 show that injection of 10 mg/kg Δ^9 -THC led to a decrease of movement in both genotypes, but this reduction reached significance only in the WT animals (Univariate test of significance, $P < 0.05$).

Cannabinoid receptor CB1 mRNA expression

Levels of CB1 mRNA were determined using *in situ* hybridization, and included those brain areas which are proposed to be involved in mediating the respective cannabinoid-induced effects (Breivogel & Childers, 1998; Martin & Lichtman, 1998; Ameri, 1999). Periaqueductal grey, dorsal raphe, ventroposterolateral thalamus and amygdala were investigated regarding antinociceptive effects. The median preoptic area and ventromedial hypothalamus were evaluated in respect to hypothermia. Caudate putamen, globus pallidus and substantia nigra were analysed regarding locomotor activity. Statistical analysis revealed a significant interaction between the genotype and the mean density values reflecting CB1 mRNA levels (Wilks multivariate test of significance; effect of genotype: $F_{8,1} = 12371.84$, $P < 0.01$). A subsequent detailed analysis of the particular areas showed that there were significantly lower levels of

TABLE 2. Cannabinoid CB1 receptor mRNA expression

Area	Wild-type mice	Knock-out mice
Periaqueductal grey	63.2 \pm 5.5	63.2 \pm 7.4
Dorsal raphe	38.9 \pm 6.5	38.3 \pm 4.7
Ventroposterolateral thalamus	10.2 \pm 3.2	8.4 \pm 2.4
Amygdala	144.1 \pm 6.7	128 \pm 6
Median preoptic area	107 \pm 10.7	101 \pm 17.8
Ventromedial hypothalamus	151.2 \pm 10.3	114.8 \pm 23.9 *
Caudate putamen	201 \pm 8.9	182 \pm 8.7 *
Globus pallidus	21.3 \pm 3.6	18.0 \pm 1.4
Substantia nigra	13.8 \pm 1.2	15.4 \pm 1.4

Density values of the regions of interest reflecting CB1 mRNA levels in nNOS knock-out (KO) and wild-type (WT) control mice. Evaluation was made on the autoradiographic films. Data are shown as mean \pm SEM; * $P < 0.05$ between the two genotypes.

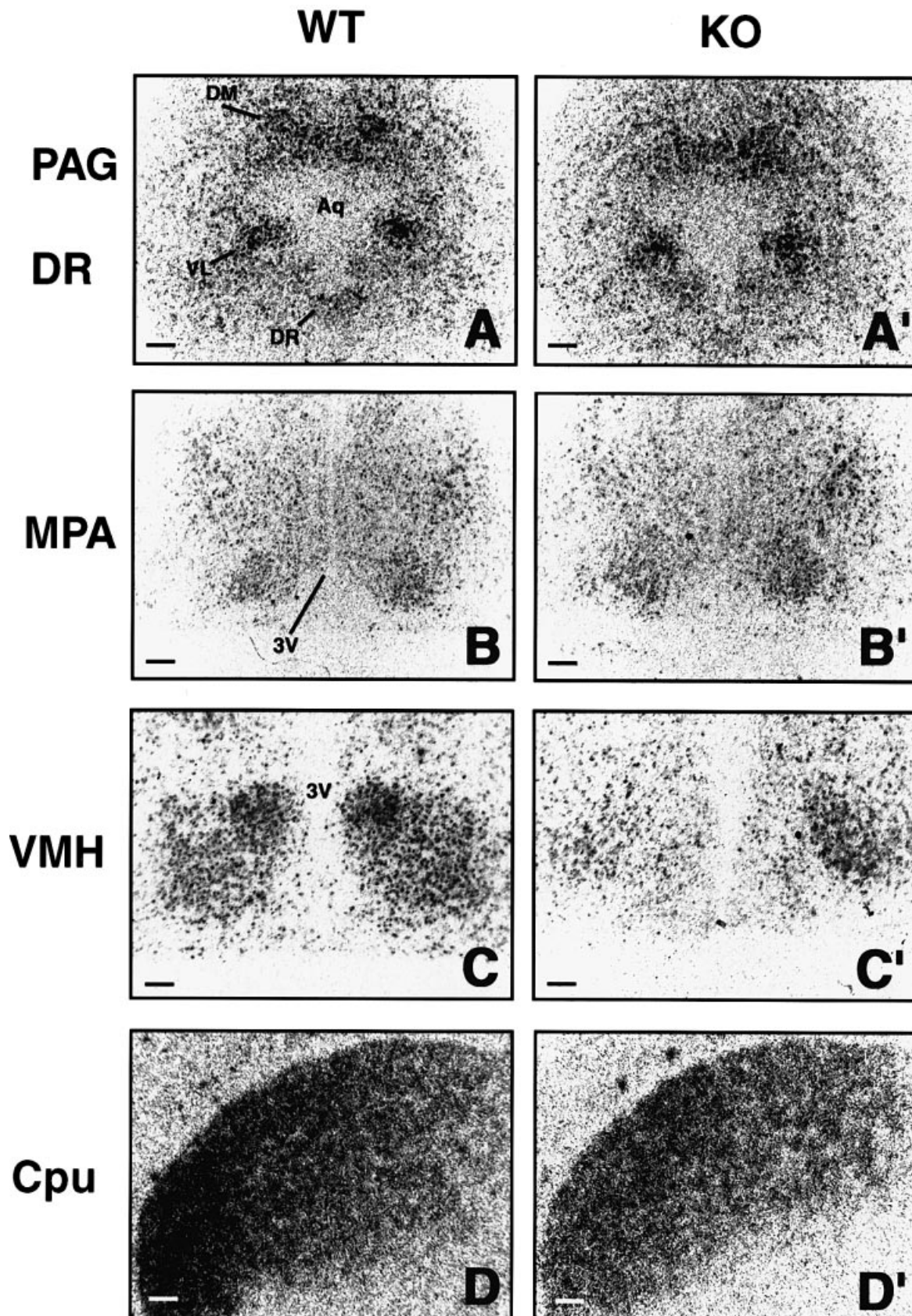
CB1 mRNA in the ventromedial hypothalamus, and the caudate putamen of the KO, in comparison to the WT mice ($P < 0.05$). No significant differences between the two genotypes were observed in either of the other regions (Table 2 and Fig. 1).

Analysis of CB1/nNOS coexpression

A double *in situ* hybridization study was carried out on forebrain tissue of wild-type animals to describe the expression of nNOS and CB1 at a single cell level. The murine forebrain contains cells that express both high and low levels of CB1 mRNA (Matsuda *et al.*, 1993; Pettit *et al.*, 1998; Tsou *et al.*, 1998; Marsicano & Lutz, 1999). While cortical areas possess both high and low CB1-expressing cells, subcortical regions contain mostly low CB1-expressing cells that are usually densely packed. Cortical areas, such as the hippocampus (Fig. 2A), neocortex (Fig. 2B), entorhinal cortex or basolateral amygdala (data not shown) display a very low extent of coexpression of CB1 with nNOS. Only principal cells in CA1 and CA3 regions of the hippocampus (Fig. 2A), and a few cells in the basolateral amygdala (data not shown), express low levels of CB1 together with low levels of nNOS mRNA. Due to the low levels of CB1 expression and, thus, to the diffuse appearance of the signals in subcortical areas, we were not always able to determine single CB1-expressing cells with the same high precision as in the cortical regions. However, in the dorsolateral caudate putamen, one of the two regions showing

altered levels of CB1 mRNA expression in the KO compared to the WT controls, an indicative evaluation of coexpression was possible.

In the striatum, nNOS is present in scattered cells containing generally high levels of mRNA. In comparison, CB1 is expressed at



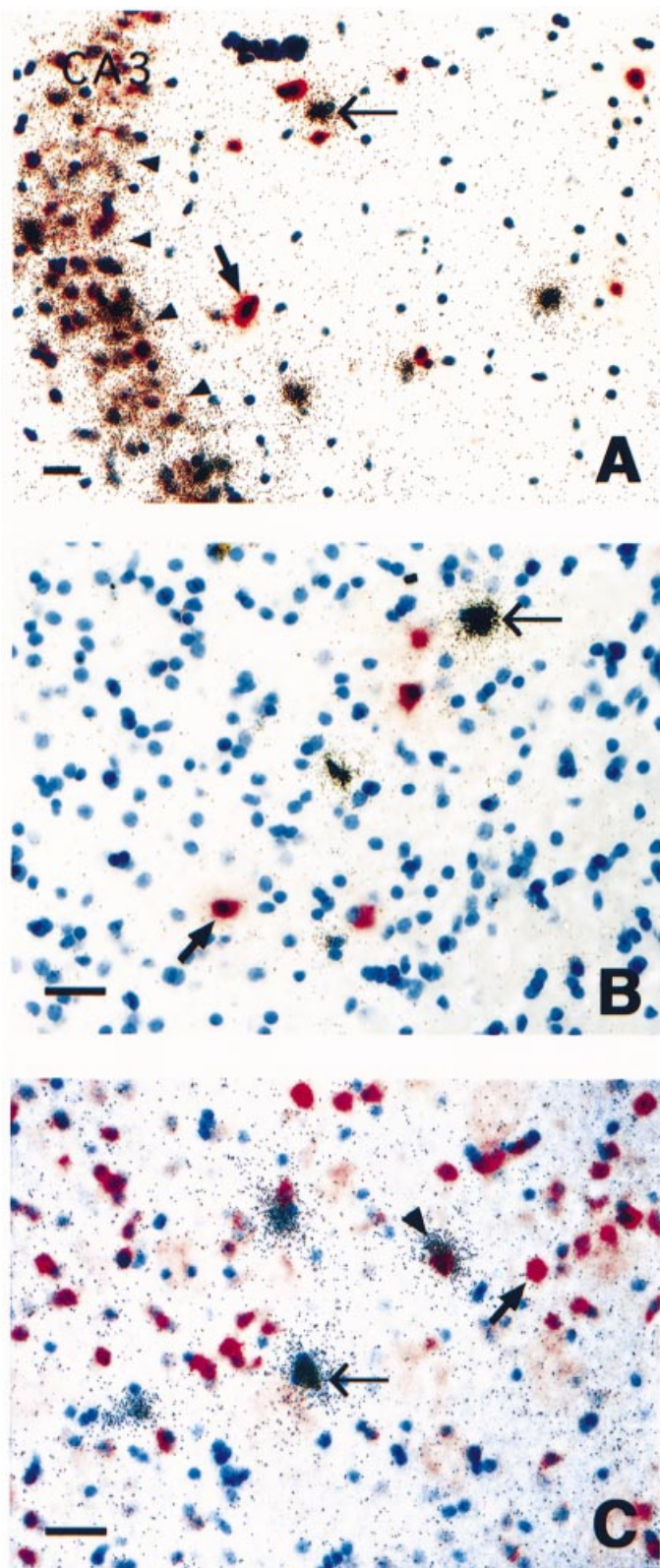


FIG. 1. Reversed dark-field micrograph of coronal sections showing examples of CB1 mRNA levels in different brain areas of nNOS knock-out (KO) in comparison to wild-type (WT) animals. (A and A') Periaqueductal grey and dorsal raphe. (B and B') Median preoptic area. (C and C') Ventromedial hypothalamus. (D and D') Dorsolateral caudate putamen. Note the lower expression of CB1 in VMH and Cpu of nNOS-KO. Abbreviations: 3 V, third ventricle; Aq, aqueductus; Cpu, caudate putamen; DR, dorsal raphe; MPA, median preoptic area; PAG, periaqueductal grey (DM, dorsomedial; VL, ventrolateral); VMH, ventromedial hypothalamus. Scale bars, 100 μ m.

low and uniform levels in the majority of medium-spiny neurons of the dorsolateral caudate putamen (Marsicano & Lutz, 1999). An approximate numerical evaluation of nNOS/CB1 coexpressing cells revealed that about 50% of nNOS-positive cells also express low levels of CB1 mRNA (Fig. 2C). Due to the low levels of both CB1 and nNOS mRNA in the ventromedial hypothalamus, it was not possible to evaluate numerically the coexpression in this area using the double *in situ* technique. However, parallel sections, hybridized either with radioactive riboprobes for CB1 or nNOS, revealed a similar pattern of expression in this brain area (Fig. 3). This observation allowed us to conclude that, in the murine ventromedial hypothalamus, nNOS-expressing cells also contain CB1 mRNA at a rather high percentage.

Discussion

The aim of the present study was to investigate whether the nitric oxide pathway is involved in Δ^9 -THC induced analgesia, thermoregulation and changes of locomotion. The effects of Δ^9 -THC were examined in mice with targeted disruption of the nNOS gene, in comparison to their wild-type controls. Unexpectedly, in the mutant animals, Δ^9 -THC still evoked analgesia, whereas the typical effects on body temperature and locomotion were lacking. The ventromedial hypothalamus and the caudate putamen of the nNOS KO animals showed significantly lower levels of CB1 mRNA. In addition, in these two areas, which are involved in Δ^9 -THC-induced thermoregulation and locomotion, respectively, numerous neurons coexpressed CB1 and nNOS mRNA.

Antinociceptive effects

Δ^9 -THC reduces the Ca^{2+} influx triggered by the activation of NMDA receptors in rat brain slices (Hampson *et al.*, 1998). A blocking effect on glutamatergic transmission provides the most simple explanation for the antinociceptive action of Δ^9 -THC (Zieglgänsberger & Tölle, 1993; Tölle *et al.*, 1996). Current research suggests that the Ca^{2+} influx, following activation of NMDA receptors, triggers the NO production in the postsynaptic neuron (Garthwaite, 1991). An unaltered antinociceptive response to Δ^9 -THC in these two different genotypes is therefore quite unexpected. Several studies indicate that Δ^9 -THC-induced antinociception involves the noradrenergic and the opioidergic system. Both apparently do not play such a prominent role in other central effects of Δ^9 -THC. The antinociceptive effects of intravenously applied Δ^9 -THC are blocked by intrathecally applied

FIG. 2. Bright-field micrograph of coronal sections from a wild-type murine brain showing examples of coexpression of CB1 (red staining) with nNOS (silver grains), as detected by double ISH in wild-type (WT) mice. All sections were counterstained with toluidine blue. (A) CA3 area of the hippocampus, showing coexpression only in the principal cell layer. (B) Double staining for CB1 and nNOS in the neocortex. Almost no coexpression is observed. (C) Dorsal caudate putamen. Note the scattered distribution of nNOS-positive cells and the diffuse staining of CB1. A certain fraction of nNOS-expressing cells also contain CB1 transcripts. Filled arrows, CB1-expressing cells; open arrows, nNOS-expressing cells; arrowheads, cells coexpressing CB1 and nNOS mRNAs. Scale bars, 20 μ m.

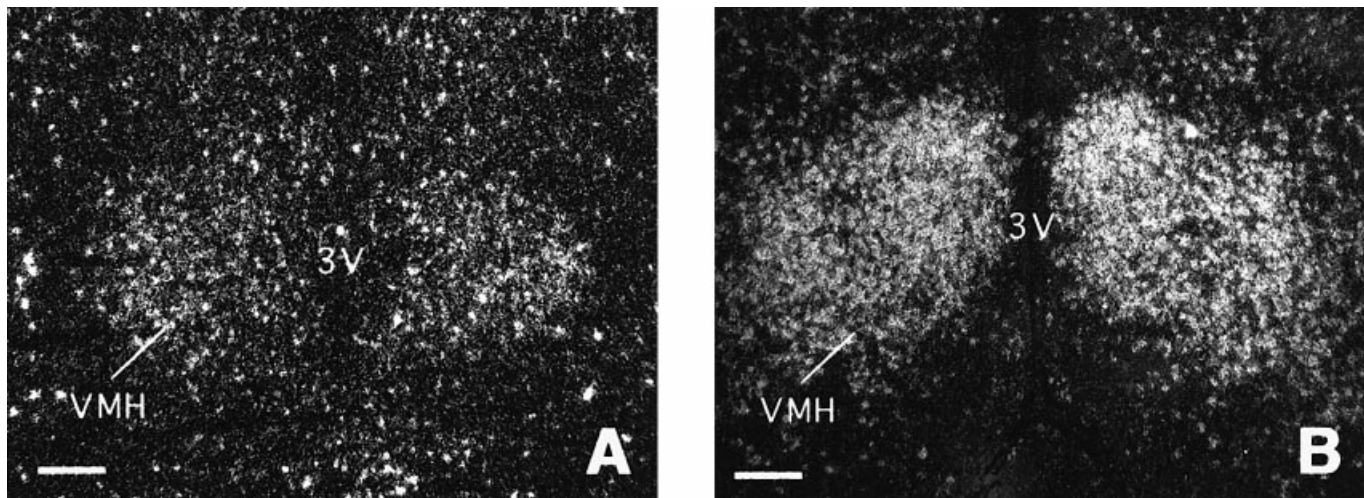


FIG. 3. Dark-field micrograph of parallel coronal sections from a wild-type mouse showing the expression of nNOS (A) and CB1 (B) in the ventromedial hypothalamus. Note the similar distribution pattern of the two transcripts. Abbreviations: 3 V, third ventricle; VMH, ventromedial hypothalamus. Scale bars, 100 μm .

α_2 -noradrenergic antagonists and kappa opioid antagonist. Both failed to antagonize the cataleptic or hypothermic effects of Δ^9 -THC (Lichtman & Martin, 1991; Martin & Lichtman, 1998). Our present results also suggest that Δ^9 -THC-induced antinociception involves transmitter systems different from the glutamatergic NMDA/NO signalling cascade.

Effects on thermoregulation and locomotion

Conflicting results have been reported concerning the role of the NO pathway in thermoregulation and locomotion (Calignano *et al.*, 1993; Gourine, 1995; Sandi *et al.*, 1995; Scammell *et al.*, 1996; Johansson *et al.*, 1997; Simon, 1998). The present study indicates that nNOS is required for Δ^9 -THC-induced decrease of body temperature and locomotor activity. Moreover, we found that CB1 and nNOS are coexpressed in the striatum and the VMH. Recent results show that dopamine D_2 -like receptors, which are also present in the striatum and VMH (Weiner *et al.*, 1991), possess a permissive role in hypothermia induced by Δ^9 -THC (Nava *et al.*, 2000). In the latter report, coadministration of D_2 antagonists completely abolished the hypothermic effects of Δ^9 -THC, suggesting that costimulation of CB1 and D_2 -like receptors is necessary for the hypothermic effects of Δ^9 -THC. CB1 and D_2 receptors are also colocalized in the dorsolateral caudate putamen (H. Hermann, G. Marsicano & B. Lutz, unpublished results), and it was shown that their costimulation increases the level of cAMP in striatal neurons (Glass & Felder, 1997). It remains to be shown whether such an increase in cAMP, which in turn stimulates NO production (Inada *et al.*, 1998), could finally exert the hypothermic and, possibly, also the locomotor effects of Δ^9 -THC. This interpretation of our results, however, appears to be inconsistent with the report of Thorat & Bhargava (1994). These authors found that pretreatment of mice with the NOS inhibitor NG-monomethyl-L-arginine (L-NMMA) neither changed the analgesic nor the hypothermic effects of Δ^9 -THC. An incomplete block of NO production by the pretreatment with L-NMMA could explain this discrepancy. However, it is also feasible to assume that a disruption of the nNOS gene could lead to more profound alterations in signalling pathways that cannot be achieved by a pharmacological treatment with an nNOS inhibitor. In line with this assumption, is our observation that the disruption of the nNOS gene decreased the

CB1 receptor mRNA levels in the striatum and the ventromedial hypothalamus in the mutant mice.

Therefore, the changes in CB1 expression could also explain the decreased effects of Δ^9 -THC in locomotion and thermoregulation in the transgenic animals. Anandamide, the principal endogenous ligand of CB1 (Di Marzo *et al.*, 1998), is rapidly taken up by a high affinity transporter which can be activated by NO (Maccarrone *et al.*, 1998, 2000). A lack of NO may, thus, increase the extracellular concentration of the endogenous CB1 ligand due to a decreased re-uptake. There is evidence that high levels of extracellular cannabinoids downregulate CB1 expression in selected areas such as the caudate putamen and VMH (Zhuang *et al.*, 1998; Corchero *et al.*, 1999; Gonzalez *et al.*, 1999). Such decreased levels of CB1 would explain the reduced responsiveness to Δ^9 -THC observed in the present study.

In conclusion, our behavioural and molecular findings indicate that the NO signalling pathway is involved in Δ^9 -THC-induced hypothermia and locomotion but plays a negligible role in Δ^9 -THC-induced antinociception.

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Abbreviations

3V, third ventricle; Aq, aqueductus; CA3, CA3 field of hippocampus; cAMP, cyclic adenosine-monophosphate; CB1, cannabinoid receptor 1; Cpu, caudate putamen; D2, dopamine receptor 2; DR, dorsal raphe; eNOS, endothelial nitric oxide synthase; GABA, γ -aminobutyric acid; i.p., intraperitoneal; iNOS, inducible nitric oxide synthase; ISH, *in situ* hybridization; KO, knock-out; L-NMMA, NG-monomethyl-L-arginine; MPA, median preoptic area; NMDA, N-methyl-D-aspartate; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; PAG, periaqueductal grey; RT-PCR, reverse transcriptase-polymerase chain

reaction; SEM, standard error of the mean; Δ^9 -THC, Δ^9 -tetrahydrocannabinol; VMH, ventromedial hypothalamus; w/v, weight per volume; WT, wild-type.

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