

Behavioral pharmacological properties of a novel cannabinoid 1',1'-dithiolane Δ^8 -THC analog, AMG-3

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Newly developed cannabinoids may hold the promise of the development of useful and safe drugs. This study aimed to investigate the behavioral effects of the novel 1',1'-dithiolane Δ^8 -THC analogue AMG-3, a cannabinomimetic molecule with high affinity for CB₁/CB₂ receptors. This analog was chosen for its binding affinity to these receptors, which is higher than that reported for Δ^8 -tetrahydrocannabinol (Δ^8 -THC). Behavioral responses were assessed after the administration of AMG-3 (1, 2, 4, 8 mg/kg, i.p.) in the open field, on the bar test, on the hot plate and in the intracranial self-stimulation procedure. AMG-3 increased the reactivity time on the hot plate in a dose- and time-dependent manner, indicating a long-lasting analgesic effect (at least 24 h). The substance was found dose-dependently to decrease spontaneous motor activity and to induce catalepsy, particularly at the highest dose (8 mg/kg). AMG-3 did not affect the rewarding value of intracranial self-stimulation, except to increase the reward threshold at the highest dose (8 mg/kg). The effects of the highest dose of AMG-3 on spontaneous activity and on the self-stimulation paradigm were completely reversed by pre-treatment with the CB₁ receptor antagonist AM-251. These findings indicate that the administration of AMG-3 to rats elicits a specific

behavioral profile, most probably associated with the activation of CB₁ receptors and without effects indicating abuse potential. *Behavioural Pharmacology* 16:499–510

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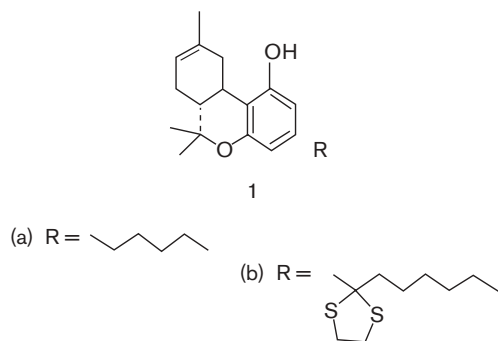
Introduction

Significant contributions made by medicinal chemistry, both in academia and in the pharmaceutical industry, with respect to synthetic cannabinoids have enhanced basic knowledge of the biology of the endocannabinoid system (Piomelli, 2003). A limited number of cannabinoids have already been used as therapeutic agents, while an expanding number of new analogs, currently under preclinical testing, appear to be promising therapeutic interventions for a variety of pathophysiological states (Howlett *et al.*, 2004; Hall *et al.*, 2005). Nevertheless, the development of additional safe and medically useful cannabinoids is still worth pursuing, since the endocannabinoid system seems to be involved in disease states with either central or peripheral pathophysiology (Di Marzo *et al.*, 2004).

The discovery of cannabinoid receptors (Devane *et al.*, 1988; Munro *et al.*, 1993; Pertwee, 1997) provided an impetus for obtaining detailed information regarding the

stereoelectronic requirements of receptor active site(s). The existing literature (Razdan, 1986; Makriyannis and Rapaka, 1990; Khanolkar *et al.*, 2000) recognizes four pharmacophores on the cannabinoid structure that can be associated with cannabinomimetic activity. Among these elements it is now well known that the aliphatic side-chain plays a pivotal role in determining receptor binding affinity and pharmacological activity, as first demonstrated by Adams (1949). Moreover, Papahatjis *et al.* (1998, 2002) pointed out that structural modifications at the benzylic position of the side-chain of the Δ^8 -tetrahydrocannabinol (Δ^8 -THC) core have a profound effect on the affinities of new analogs for both CB₁ and CB₂ cannabinoid receptors. The newly synthesized dithiolane derivative, AMG-3, exhibited greater affinity (at the subnanomolar level), as compared to that of Δ^8 -THC (see Fig. 1), for both CB₁ and CB₂ cannabinoid receptors (Papahatjis *et al.*, 1998, 2002). Although Δ^9 -THC is the most active constituent of cannabis, Papahatjis *et al.* (1998) chose Δ^8 -THC as the reference

Fig. 1



Compounds	CB ₁ (K_i , nmol/l)	CB ₂ (K_i , nmol/l)
(-)- Δ^8 -THC, (a)	47.6	39.3
AMG-3, (b)	0.32	0.52

Chemical structure of Δ^8 -tetrahydrocannabinol (Δ^8 -THC) (a) and AMG-3 (b). Δ^8 -THC has been used as prototype because it has a similar pharmacological profile as Δ^9 -THC and it is chemically more stable. Affinities (K_i) of cannabinoid analogs (a) and (b) for the CB₁ and CB₂ receptors are also given. (From Papahadjis *et al.*, 1998, 2002.)

substance in the binding studies because it shares a similar pharmacological profile with Δ^9 -THC and, in addition, it possesses greater chemical stability. The increased affinity of AMG-3 was attributed to a hydrophobic subsite for both CB₁ and CB₂ at the level of the benzylic side-chain carbon, and suggested the significance of the orientation and conformation of the side-chain in determining cannabinomimetic activity. Compounds with higher affinities for the target site might prove useful as pharmacological tools and therapeutic agents with improved efficacy and safety.

The higher affinity of AMG-3 for CB₁/CB₂ receptors as compared to Δ^8 -THC (Papahadjis *et al.*, 1998) prompted us to examine the behavioral properties of this new cannabinoid, which might reflect its central nervous system action and its potential for abuse. Behaviors most often used to assess cannabinoid activity are antinociception, locomotor inhibition and catalepsy (Fuentes *et al.*, 1999; Pertwee, 2001; Romero *et al.*, 2002). In addition, cannabinoids have addictive liability in humans and a number of attempts have been made, at the preclinical level, to assess their abuse potential using a variety of models, including the intracranial self-stimulation (ICSS) paradigm (Gardner, 2002; Tanda and Goldberg, 2003). The rewarding effects of brain stimulation and of addictive drugs are thought to involve the same reward circuits (Wise, 1980, 1996, 1998) and the ICSS model is well suited for assessing reward-related drug interactions. It is known, for instance, that almost all drugs of abuse increase the rewarding value of self-stimulation, as this is reflected in the decreased threshold for rewarding brain stimulation. Additionally, any aversive effect of a drug

increases the threshold for rewarding brain stimulation (for reviews, see Stellar and Rice, 1989; Wise, 1996, 1998). Therefore, the intracranial self-stimulation paradigm allows clear dissociation and quantification of the reward-potentiating and reward-inhibiting effects of a drug.

The present study aimed to investigate the effects of the novel 1',1'-dithiolane Δ^8 -THC analogue (AMG-3) on analgesia, motor activities and the rewarding properties of ICSS. To this purpose the hot-plate test, the recording of open-field behavioral activity, the bar test and the intracranial self-stimulation paradigm were used.

Methods

Binding studies

AMG-3 was synthesized and characterized by Papahadjis *et al.* (1998). Additional quantities were synthesized for the needs of the current study. Samples of AMG-3 synthesized in the Institute of Organic and Pharmaceutical Chemistry of the Hellenic Research Center were taken and competition studies were performed in cortical membranes according to Khanolkar *et al.* (1996), in order to confirm the high affinity of the ligand in the brain. Prefrontal cortex was removed from rat brains and homogenized in a buffer containing 50 mmol/l Tris-HCl, EDTA (2.5 mmol/l), MgCl₂ (5.0 mmol/l), pH 7.4 (incubation buffer). The mixture was centrifuged three times at 45 000g for 15 min. The final pellet was resuspended in the incubation buffer (2.5 mg wet weight/ml). Membranes (100 µg) were incubated in the presence of [³H]CP55940 (168 Ci/mmol; 0.3 nmol/l, New England Nuclear), different concentrations of AMG-3 and incubation buffer in a final volume of 200 µl for 90 min at 30°C. The reaction was terminated by the addition of 5 ml cold incubation buffer containing bovine serum albumin (BSA; 2.0 mg/ml, wash buffer) and the mixture was filtered over glass fiber/carbon (GF/C) filters that were presoaked in 0.05% polyethyleneimine (PEI). The filters were washed with an additional 2 × 5 ml wash buffer, and added to scintillation vials. Scintillation fluid was added to the filters and the radioactivity was counted in a beta-counter. Specific binding was determined in the presence of WIN212-291 (10 µmol/l).

Behavioral studies

Subjects

Male Sprague-Dawley rats, aged 70–90 days old and weighing approximately 250–300 g at the beginning of experiments, were used. The animals were housed in groups of six or seven in plastic cages (57 × 35 × 20 cm) with food and water freely available, under controlled laboratory conditions, i.e. 12 h light/dark with lights on at 0600 h and constant temperature 21 ± 1°C. All animal experiments were carried out in accordance with the National Institutes of Health *Guide for the Care and Use of*

Laboratory Animals (NIH Publications No. 80-23) revised 1996. All procedures conformed to named local and international guidelines on the ethical use of animals and special care. Efforts were made to use the minimal number of rats necessary for experiments and to reduce their suffering.

Hot-plate test

The rats ($n = 35$) were accustomed to the experimental room for 1 h prior to the experiment. Antinociception was monitored using the hot-plate test (Ugo Basile 7280, Ugo Basile Biological Research Apparatus Comerio, Varese, Italy), which consisted of a Plexiglas frame and an electrically heated surface kept at a constant temperature. In particular, rats were placed in the Plexiglas frame, on the stainless-steel platform, which was set thermostatically at $52 \pm 0.1^\circ\text{C}$. The hot-plate cut-off was 40 s. The antinociceptive response was evaluated by recording the latency (s) to paw licking. Rats were treated with vehicle or AMG-3 (1, 2, 4, 8 mg/kg) and the latency to paw lick was recorded 30 min, 1, 2, 4 and 24 h following vehicle or drug administration. Each rat was treated with a single dose of the drug or vehicle, and the reaction was tested at the time points given above. Group sizes for each dose or vehicle were 6–7 rats.

Catalepsy test

The rats ($n = 34$) were accustomed to the experimental room for 1 h prior to the experiment. Then each rat was tested for catalepsy using the bar test. Briefly, each animal was placed with its front legs over a 10-cm high bar and its hind legs on a stable surface. Catalepsy was evaluated by recording the latency (s) to remove its front legs from the bar. Rats were treated with vehicle or AMG-3 (1, 2, 4, 8 mg/kg) and the descent latency in the bar test was recorded 30 min, 1, 2, 4 and 24 h after vehicle or drug administration. Each rat was treated with a single dose of the drug or vehicle and was tested at the time points given above. Group sizes for each dose or vehicle were 6–7 rats.

Spontaneous motor activity

The behavioral testing was performed between 08.00 and 16.00 h. All animals ($n = 33$; $n = 6$ –7 for each dose or vehicle group) were gently handled before the beginning of the experimental procedure. The rats were initially accustomed to the experimental room for 1 h prior to the experiment. Five groups of rats were then injected intraperitoneally (i.p.) with vehicle or AMG-3 (1, 2, 4, 8 mg/kg) and 10 min later rats were introduced into the testing cage, a transparent plastic open-field cage ($40 \times 40 \times 40$ cm). This time interval appears to be critical for the observation of other behavioral and physiological effects of cannabinoids (see original studies in Chaperon and Thiébot, 1998, for example). In addition, two groups of rats ($n = 6$ and 7) were pre-treated with AM-251 (CB₁ antagonist), 5 min later treated with vehicle or AMG-3

(8 mg/kg), and 10 min after the second injection were introduced into the open-field apparatus. Spontaneous behavior/reaction to novelty was recorded for a 15 min observation period. In brief, an observer (not blind) recorded the behavioral responses using a registration program based on Spruijt and Gispen (1984). The duration of each behavioral response was recorded at the end of the 15-min observation period (Antoniou and Kafetzopoulos, 1996; Antoniou *et al.*, 1998). The following behavioral responses were registered: standing (std), on all four feet, essentially motionless and not sniffing; moving (mov), walking on all four feet; sniffing (sni), not moving but sniffing parts of the walls or floor of the apparatus; rearing (rr), body inclined vertically with hindpaws on the floor of the activity cage and forepaws on the wall of the cage; grooming (grm), washing the face or any other body part with the forepaws; scratching (scr), raising of hindpaw to touch any body part; sniffing-air (sna), rearing in the open-field area of the activity cage.

Self-stimulation procedure

For the brain stimulation experiments, rats were subjected to surgery and following one week, they were allowed to respond to the intracranial self-stimulation (ICSS) procedure.

Animals were anesthetized with ketamine hydrochloride (100 mg/kg, i.m.) and xylazine (10 mg/kg, i.m.). Atropine sulfate (0.6 mg/kg, i.m.) was injected to reduce bronchial secretion. Moveable monopolar stimulating electrodes (Model SME-01, Kinetorods, Ottawa, Ontario, Canada) were lowered into the medial forebrain bundle (MFB) at the level of the lateral hypothalamus (coordinates AP: -2.5 mm from bregma, L: -1.7 mm from the midline, VD: -8.0 from a flat skull), according to Paxinos and Watson (1998). The electrodes consisted of a plastic guiding base and a 0.25 mm diameter moveable stainless-steel wire, which were insulated with Epoxylite except for the conically shaped tip. The anode was an Amphenol pin connected to five miniature skull screws.

One week after surgery, the animals were tested for self-stimulation in an operant chamber made of transparent Plexiglas (25 cm wide, 25 cm deep, 30 cm high). Each bar-press triggered a constant current generator that delivered a 0.4-s train of rectangular cathodal pulses of constant duration (0.1 ms) and intensity (250 μA) and variable frequency (15–100 Hz, i.e. 6–40 pulses/0.4 s). The pulse frequency, i.e. the number of pulses within a train, was progressively increased up to 40/stimulation train until the subject showed vigorous self-stimulation. During the acquisition phase the animals were trained to self-stimulate for at least three consecutive days (1 h daily), using stimulation parameters that maintained near maximal bar-pressing rates. The animals were subsequently trained to self-stimulate using four alternating series of ascending and descending pulse frequencies.

The pulse frequency was varied by steps of approximately 0.1 log units. Each frequency was tested within trials of 60 s in duration, followed by an extinction period of 30 s. At the beginning of each trial, the animals received three trains of priming stimulation at the frequency of the stimulation which was available for that trial. A rate-frequency determination lasted about 45 min. One rate-frequency curve was established daily, for 10–12 days, depending on the period when the self-stimulation indices (i.e. curve shift and threshold measure) were stable.

Drug testing began for each animal when the function relating bar-pressing rate to pulse frequency (the rate-frequency function) was stable for at least three consecutive days. Following the baseline period, each animal was injected with the drug or its vehicle. Each drug or vehicle self-stimulation test consisted of a baseline and a drug rate-frequency function determination (for 45 min each). The animals were tested 10 min after the last injection. This time interval has also been used in self-stimulation studies with other drugs of abuse (see, for example, Maldonado-Irizarry *et al.*, 1994; Ranaldi and Beninger, 1994; Vlachou *et al.*, 2003), and appears to be critical for the observation of other behavioral and physiological effects of cannabinoids (see original studies in, for example, Chaperon and Thiébot, 1998). The sequence of injections for the different drug doses was counterbalanced and a 3-day period was allowed between injections. As we have observed in a previous study (Vlachou *et al.*, 2003), this period is considered sufficient for the behavior of the animals to return to stable, pre-treatment levels without being affected by prior cannabinoid administration, i.e. no carry-over effects of the cannabinoids were detected.

Rats ($n = 14$) received multiple doses of AMG-3 (1, 2, 4 and 8 mg/kg, i.p.) or its vehicle and a treatment of AM-251 (1 mg/kg, i.p.) or its vehicle followed 5 min later by AMG-3 (8 mg/kg, i.p.) or its vehicle, with a minimum of 3 days between consecutive combination drug treatments. The sequence of injections for the different drug doses was counterbalanced.

Data gathered from pre- and post-injection portions of each session were curve fitted and threshold and asymptote estimates were obtained using the Gompertz sigmoid model (Coulombe and Miliaressis, 1987):

$$f(x) = \alpha e^{-e^{b(x_i - x)}}$$

In this equation, α represents the maximum rate (asymptote), whereas X_i (X at inflection) represents the threshold frequency. The latter is the pulse number producing 36.7% of the asymptotic rate, i.e. the rate lying on the fastest-accelerating region of the curve. Parameter b represents an index of the slope whereas e is the base of natural logarithms.

At the end of the experiment, the animals were given a lethal dose of sodium pentothal. The location of the terminal stimulation site was then marked according to the following procedure: a direct anodal current of 0.1 mA and 15 s duration was passed through the electrode tip. The animals were given an intracardiac perfusion of saline 0.9%, which was followed by a 50 ml solution of potassium ferrocyanide (3%), potassium ferricyanide (3%), and trichloroacetic acid (0.5%) in 10% formalin. The brains were then removed and stored in 10% formalin for 3 days, and 2 days in a 30% sucrose solution. Finally, the brains were sliced in a cryostat microtome and the sections containing the electrode tract were mounted on slides and stained with cresyl violet. Only data from the rats in which tracks from the electrode were verified to be located in the MFB were included in this study.

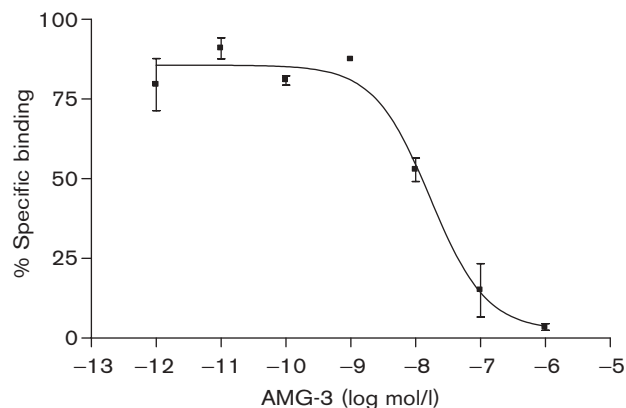
Drugs

AMG-3 (Papahadjis *et al.*, 1998) was dissolved in a vehicle solution that consisted of 15% dimethylsulfoxide, 5% cremophor EL and 80% of 0.9% NaCl; AM-251 (Tocris) was dissolved in a vehicle solution that consisted of 5% dimethylsulfoxide, 5% cremophor EL and 90% of 0.9% NaCl. Both compounds were injected intraperitoneally (i.p.) at a volume of 3 ml/kg of body weight.

Statistical evaluation

Non-parametric tests (Kruskal–Wallis) were performed on all data from hot-plate and catalepsy bar tests. Mann–Whitney tests were used for specific group comparisons. Spearman's Rank correlation coefficient was used for estimation of time-dependent differences in latency of analgesia and catalepsy data.

Fig. 2



Inhibition of specific [^3H]CP55940 binding to rat cortical membranes. [^3H]CP55940 (0.3 nmol/l) binding to cortical membranes (100 μg) was inhibited by increasing concentrations of AMG-3. Specific binding was defined in the presence of WIN212-291 (10 $\mu\text{mol/l}$). Values represent the mean and standard deviation of two experiments utilizing seven concentrations of AMG-3 in duplicate.

A one-way analysis of variance (ANOVA) with treatment as factor was performed on the open-field data, including duration of each behavioral response. LSD multiple-range tests were used for specific group comparisons.

The post-treatment ICSS threshold and asymptote values were expressed as a percentage of pre-drug values. The results were evaluated statistically using two-way

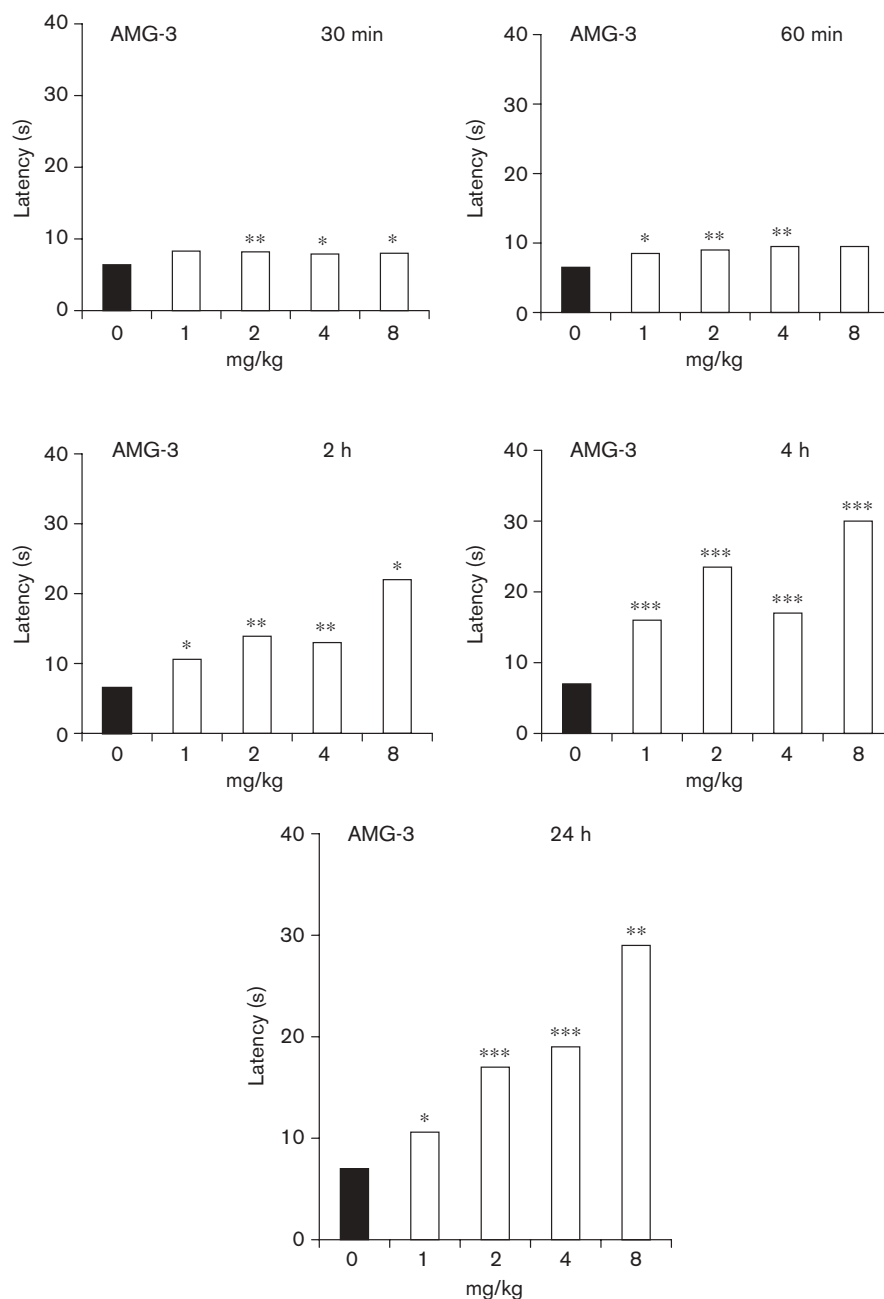
and one-way analysis of ANOVA, followed, whenever appropriate, by LSD test.

Results

Binding studies

The chemical structure of Δ^8 -THC and AMG-3 and their binding characteristics are presented in Fig. 1. The

Fig. 3



Indices of antinociception in rats following vehicle or AMG-3 administration (1, 2, 4, 8 mg/kg, i.p.). Antinociception is expressed by the latency (s) to paw licking in the hot-plate test at various time points: 30 min, 60 min, 2 h, 4 h and 24 h following vehicle or AMG-3 administration. Values are medians. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, AMG-3 versus vehicle.

specific binding of [^3H]CP55940, performed in the current study, represented 68% of the total binding and was inhibited by AMG-3 in a concentration-dependent manner with a K_i value of 5×10^{-9} mol/l (Fig. 2). This K_i value for AMG-3 is higher than previously reported (Papahadjis *et al.*, 1998) and confirms the high affinity of the compound for CB receptors. For the determination of the K_i value, the K_d value of 133×10^{-12} mol/l was employed from the study of

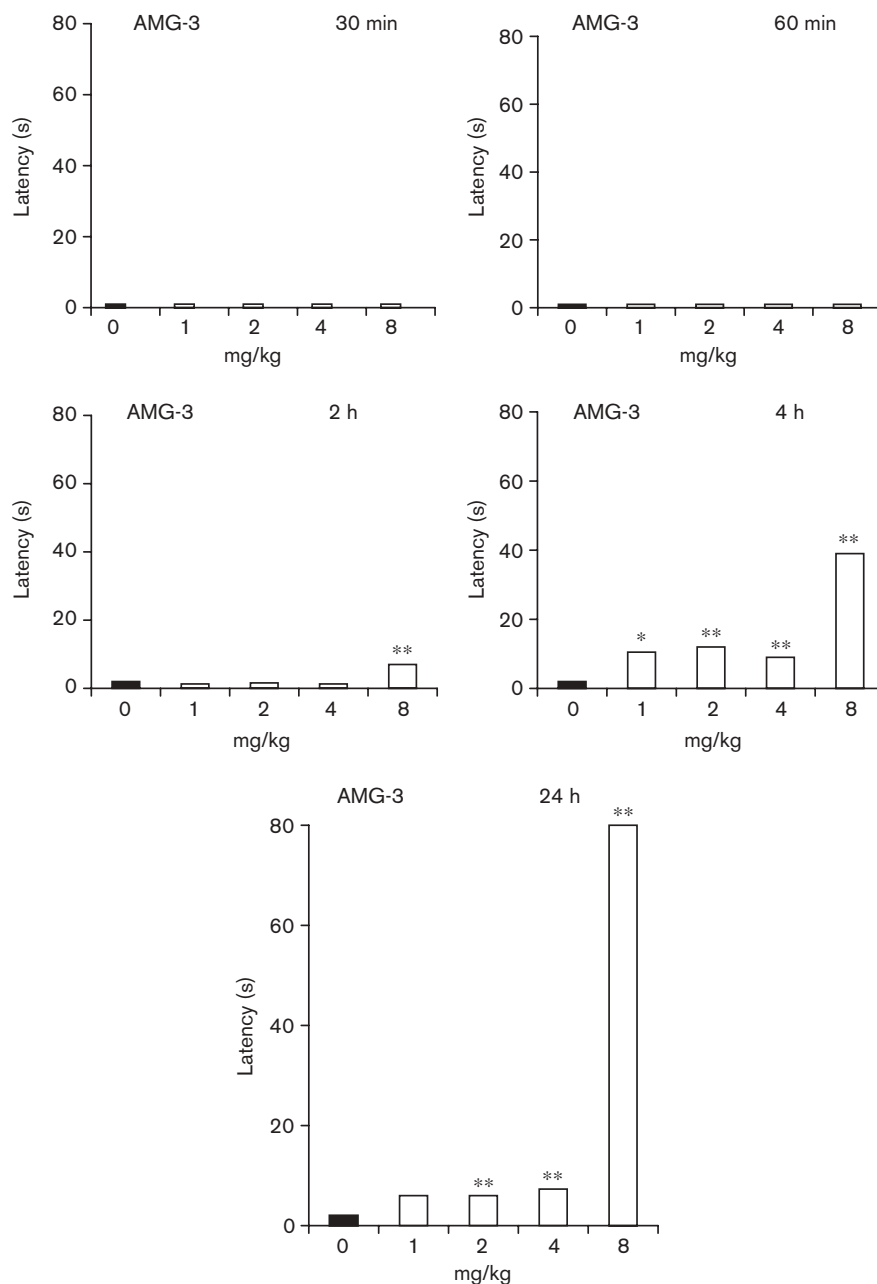
Devane *et al.* (1988) who used the same radioligand and cortical membranes.

Behavioral studies

Hot-plate test

AMG-3 induced analgesic effects in a dose- and time-dependent manner (Fig. 3). Kruskal–Wallis analysis revealed a dose effect on the latency to paw licking at

Fig. 4



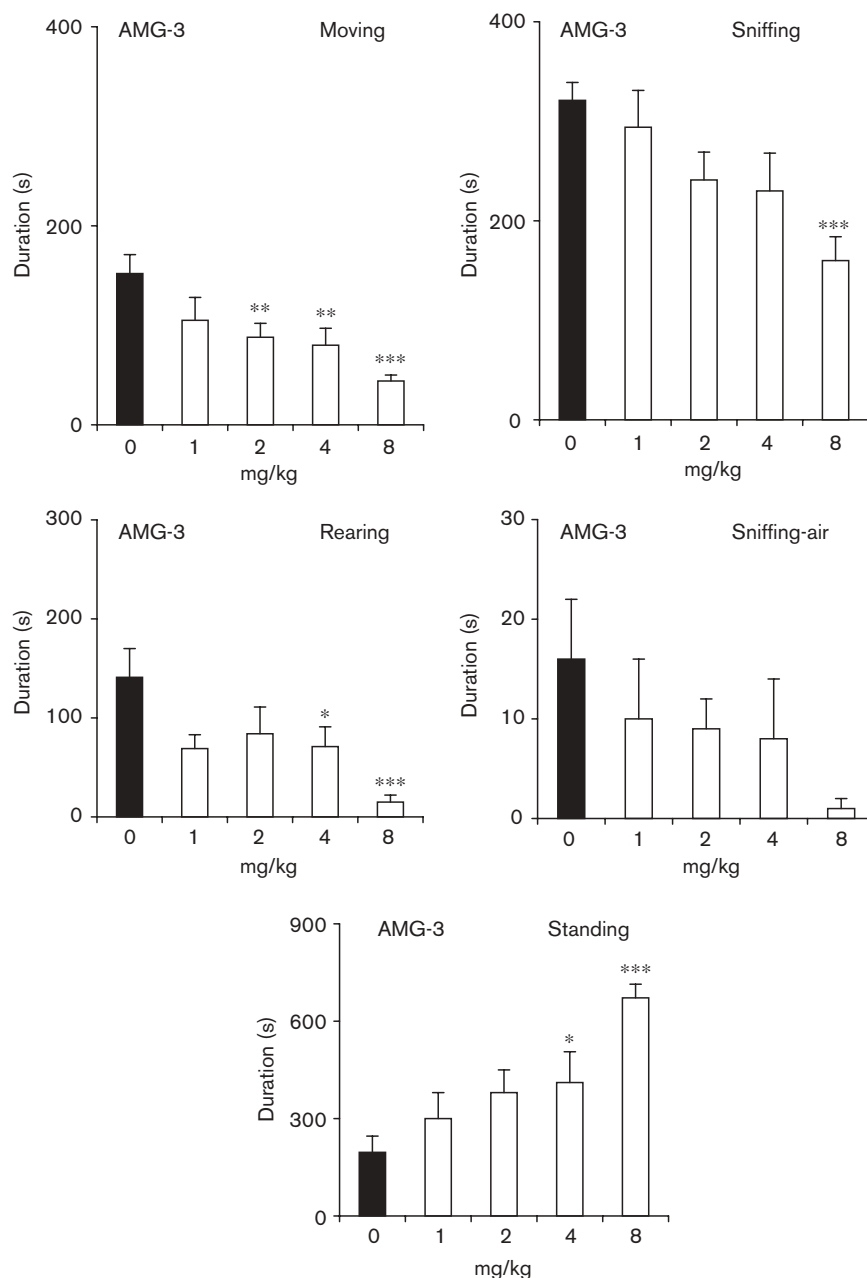
Indices of catalepsy behavior in rats following vehicle or AMG-3 administration (1, 2, 4, 8 mg/kg, i.p.). The latency (s) to remove its front paws from the bar in the bar test (descent latency) reflects catalepsy-like behavior at various time points: 30 min, 60 min, 2 h, 4 h and 24 h following vehicle or AMG-3 administration. Values are medians. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, AMG-3 versus vehicle.

30, 60 min, 2, 4 and 24 h ($\chi^2 = 11.7, 12.4, 14.1, 24.2, 21.4$; $df = 4$; $P < 0.05, P < 0.01, P < 0.01, P < 0.001, P < 0.001$, respectively). Subsequent Mann–Whitney U tests revealed a statistically significant increase in latency at all doses (except the lowest one) at the first 30 min following AMG-3 administration ($z = -3.2, 2.1, 2.1$; $df = 4$; $P < 0.001, P < 0.05, P < 0.05$, respectively) (Fig. 3). Similar results were observed at 60 min ($z = -2, -2.7, 2.8$; $df = 4$; $P < 0.05, P < 0.01, P < 0.01$,

respectively), 2 h ($z = -2.1, -2.7, -2.4, -2.4$; $df = 4$; $P < 0.05, P < 0.01, P < 0.01, P < 0.01$, respectively), 4 h ($z = -3.2, -3.2, -3, -2.9$; $df = 4$; $P < 0.001$, respectively) and 24 h ($z = -2.2, -3.1, -3, -2.7$; $df = 4$; $P < 0.05, P < 0.001, P < 0.001, P < 0.01$, respectively) following drug administration, even at the lowest dose (Fig. 3).

Spearman's correlation coefficient did not reveal any time effect on latency to paw licking following vehicle

Fig. 5



Mean $\pm$ SE of the duration of moving, sniffing, rearing, sniffing-air and standing spontaneous behavior following vehicle or AMG-3 administration (1, 2, 4, 8 mg/kg, i.p.). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, AMG-3 versus vehicle.

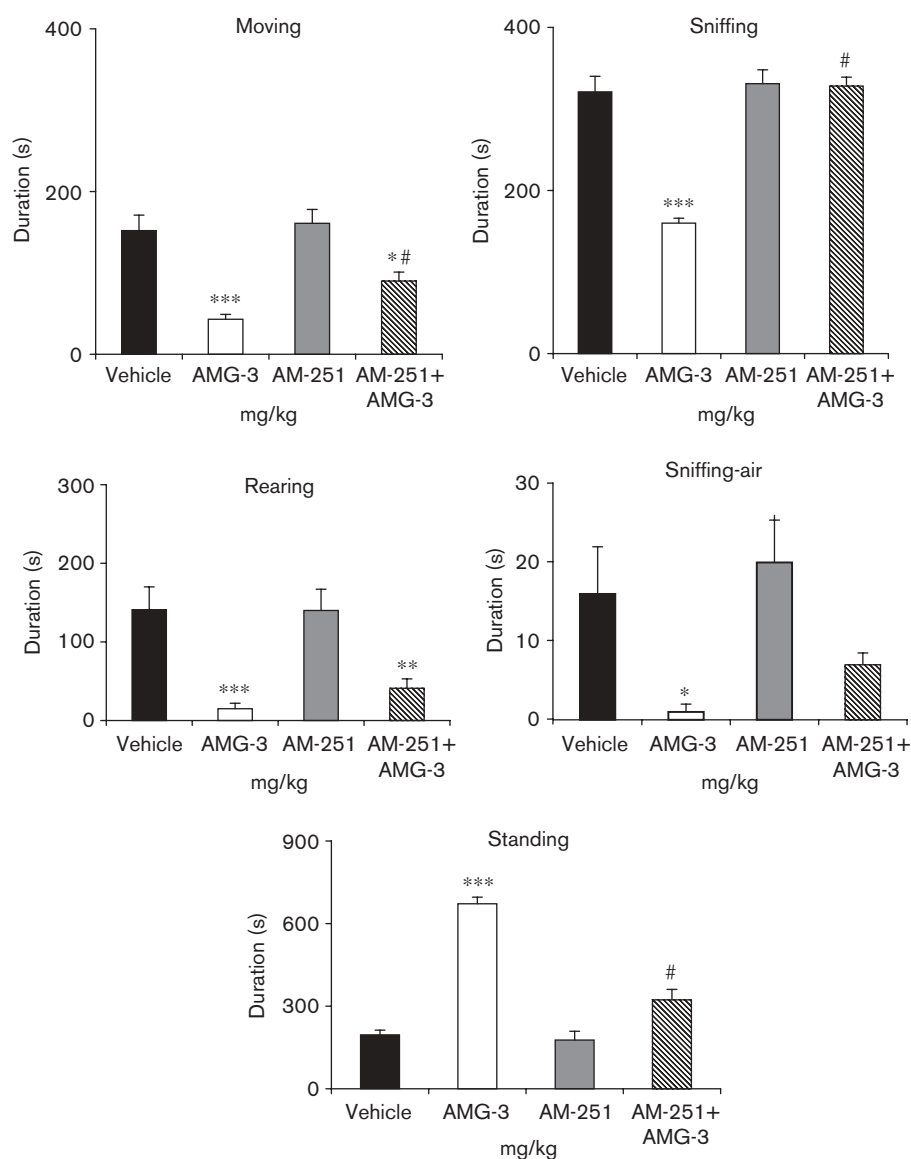
administration. The same analysis revealed a time effect on latency at all doses of AMG-3 ($R = 0.482, 0.768, 0.870, 0.794$; $P < 0.01, P < 0.001, P < 0.001, P < 0.001$ for 1, 2, 4, 8 mg/kg AMG-3, respectively]. In particular, latency to paw licking is positively correlated to different time points of reassessment, showing that the effect of the drug increased with time up to 24 h after administration.

Bar test

Indices of catalepsy at different time intervals and at the higher doses of AMG-3 administration are presented in Fig. 4. Kruskal–Wallis analysis revealed a dose effect on the latency to remove front paws from the bar, especially

at 4 and 24 h ($\chi^2 = 4.1, 15.1$; $df = 4$; $P < 0.05, P < 0.01$) following AMG-3 administration. In particular, Mann–Whitney U tests did not reveal any statistically significant effect on descent latency at 30 min or 60 min following AMG-3 administration. There is an increase in the descent latency at the highest dose ($z = -2.7, P < 0.01$), 2 h following AMG-3 administration (Fig. 4). Moreover, the descent latency was increased in a dose-dependent manner ($z = -1.9, -2.6, -2.1, -2.6$; $P < 0.05, P < 0.01$, respectively), 4 h after the AMG-3 administration (Fig. 4). Finally the descent latency was increased 24 h after the AMG-3 administration, except at the lowest dose ($z = -2.3, -2.7, -2.6$; $P < 0.01$, respectively) (Fig. 4).

Fig. 6



Mean $\pm$ SE of the duration of moving, sniffing, rearing, sniffing-air and standing spontaneous behavior following administration of vehicle, AMG-3 (8 mg/kg, i.p.), AM-251 (1 mg/kg, i.p.) and co-administration of AM-251 (1 mg/kg) plus AMG-3 (8 mg/kg). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, AMG-3 versus vehicle. # $P < 0.05$, AM-251 + AMG-3 versus AMG-3.

Spearman's correlation coefficients did not reveal any time effect on descent latency in the bar test following vehicle administration. The same analysis revealed a time effect on latency at 1, 2, 4 and 8 mg/kg of AMG-3 administration ($R = 0.472, 0.717, 0.842, 0.837$; $P < 0.01, P < 0.001, P < 0.001, P < 0.001$, respectively). In particular, descent latency in the bar test was mainly apparent at the higher doses of AMG-3 2, 4 and 24 h after administration.

Spontaneous activity

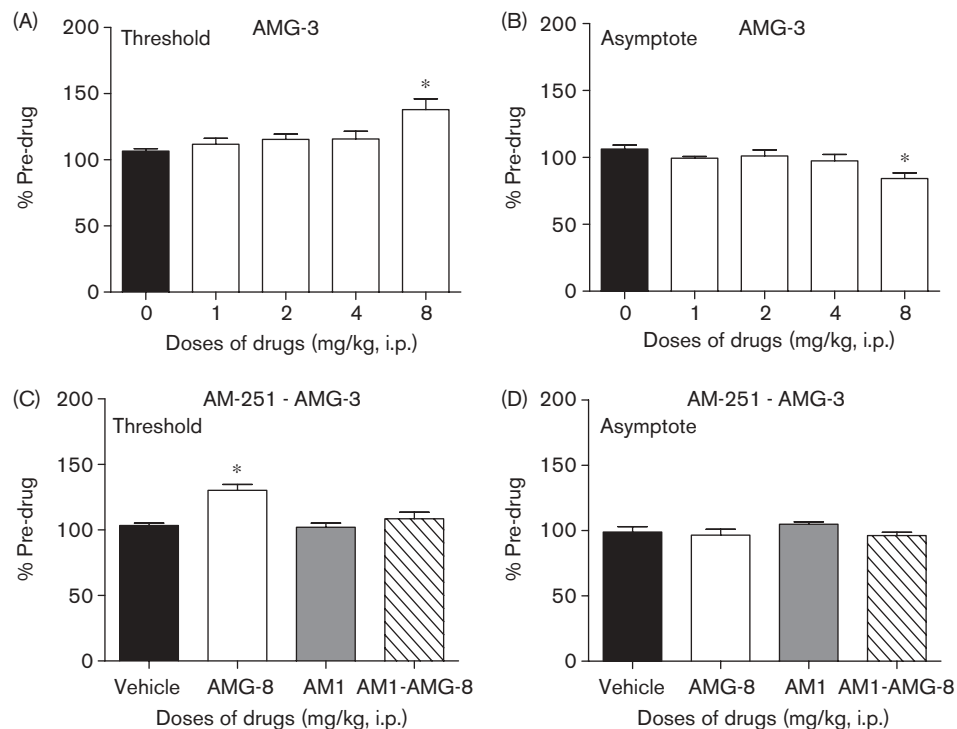
The effects of AMG-3 on various aspects of spontaneous activity are presented in Fig. 5. One-way ANOVA revealed that AMG-3 decreased the duration of moving, sniffing, rearing behavior [$F(4,31) = 6.2, 5.2, 4.2, 4.4, P < 0.001, P < 0.01, P < 0.01, P < 0.01$, respectively]. A decrease in the sniffing-air duration was observed but did not reach statistical significance. On the other hand, standing duration was increased [$F(4,31) = 6.2, P < 0.001$] following AMG-3 administration. Subsequent LSD tests revealed that moving and rearing behavior were decreased in a dose-dependent manner, while sniffing behavior was mainly reduced at the highest dose (Fig. 5). A dose-dependent increase of standing duration was also revealed following AMG-3 administration

(Fig. 5). Separate one-way ANOVAs revealed that pretreatment with AM-251 (cannabinoid antagonist) reversed the decrease in the afore-mentioned behavioral aspects of spontaneous activity induced by AMG-3 at the highest dose. In particular, AM-251 fully reversed the decrease in sniffing duration as well as the increase of standing duration [$F(4,25) = 9.6, 27.5, P < 0.001$, respectively], partially reversed the decrease in moving behavior [$F(4,25) = 10.8, P < 0.001$, respectively] but did not reverse rearing or sniffing-air behavior induced by AMG-3 at the highest dose (Fig. 6).

Self-stimulation procedure

The changes in self-stimulation threshold and asymptotic rate of responding after systemic injection of the CB₁ receptor agonist AMG-3 are presented in Figs 7A and B, respectively. AMG-3 significantly increased self-stimulation thresholds [$F(4,34) = 5.06, P < 0.005$] and decreased the asymptotic rate of responding [$F(4,34) = 4.77, P < 0.005$]. Post-hoc analysis with the LSD test showed that these effects were significant in the group receiving the highest dose of AMG-3 (8 mg/kg). There was a significant increase of the self-stimulation thresholds ($P < 0.001$) and the asymptotic rate of responding ($P < 0.001$), compared with the vehicle group.

Fig. 7



Changes in self-stimulation threshold (A, C) and asymptotic rate (B, D) of responding (expressed as percentage of pre-drug values) following AMG-3 (0, 1, 2, 4, 8 mg/kg, i.p.) and AM-251 (0, 1 mg/kg, i.p.) + AMG-3 (0, 8 mg/kg, i.p.) treatments. Vertical bars represent the standard errors of the mean. AMG8 represents AMG-3, 8 mg/kg; AM1 represents AM-251, 1 mg/kg. *Signifies an intracranial self-stimulation (ICSS) threshold and asymptote value significantly different from the control condition.

Figures 7C and D present the changes in self-stimulation threshold and asymptotic rate of responding after systemic injection of AM-251 or its vehicle and AMG-3 or its vehicle. AMG-3 (8 mg/kg) produced an increase in self-stimulation threshold. Administration of AM-251 (1 mg/kg) significantly blocked this effect [$F(3,27) = 6.972$, $P = 0.014$]. There were no significant differences in the asymptotic rate of responding between the different groups [$F(3,27) = 0.81$, NS].

Discussion

In the present study the behavioral profile of a novel cannabinoid (AMG-3) was examined, using different experimental procedures. Binding studies confirmed that AMG-3 displays an affinity for CB₁ receptors which appears to be superior to that of Δ^8 -THC (Papahadjis *et al.*, 1998). As was mentioned in the Introduction, the high affinity of AMG-3 for CB₁ receptors is attributed to a hydrophobic subsite for CB₁ receptors at the level of the benzylic side-chain carbon. Therefore, the binding data support the significance of the orientation and conformation of the side chain in determining cannabinomimetic activity. This activity was assessed further at the behavioral level.

The behavioral data demonstrated that AMG-3 induced analgesic activity in a dose- and time-dependent manner. Antinociception, as expressed by latency to paw licking in the hot-plate test, was augmented by increasing the dose. Additionally, this effect was more prominent at 4 and 24 h after AMG-3 administration, showing that the drug has a very long duration of action. Antinociceptive action for cannabinoids has been widely described in experimental animals, using a variety of analgesic tests (Fuentes *et al.*, 1999). In particular, analgesic potency, as assessed by the hot-plate test, is one of the most profound effects of THC and several synthetic cannabinoids in most species (Johnson *et al.*, 1981; Dajani *et al.*, 1999; Fuentes *et al.*, 1999). Our data demonstrate a long-lasting analgesic effect of AMG-3 and suggest that this compound might prove therapeutically useful, with interesting pharmacokinetic properties which need to be examined. Also, reassessment of behavioral indices for analgesic effect at additional time points, as well as comparisons with the well-known cannabinomimetics, would add to the overall estimation and clarification of this pharmacological property.

It was also found that AMG-3 decreased spontaneous motor activity, including forward locomotion and vertical activity in a dose-dependent manner. Exploration as indicated by sniffing behavior was also decreased following AMG-3 administration, especially at the highest dose. Interestingly, AMG-3-induced decreases in motor activity, including horizontal and vertical activity as reflected by moving and rearing/sniffing-air, respectively, were more prominent compared to the decrease in exploration

as reflected by sniffing behavior. This differentiation between forward/vertical activity and sniffing behavior possibly indicates that AMG-3-induced effects are related to a discrete behavioral profile, related to decreased motor activity rather than the inhibition of some other aspects of behavior. These findings are in agreement with the major effect of cannabinoid agonists on motor activity (hypoactivity) and they suggest that AMG-3 shares some of the pharmacological properties of other CB₁ agonists (Romero *et al.*, 1996; Ferrari *et al.*, 1999). Nevertheless, cannabinoid agonists induce biphasic, or even triphasic (Sanudo-Pena *et al.*, 2000), effects on motor activity that are both time- and dose-dependent (Davis *et al.*, 1972; Dewey, 1986; Hollister, 1986; McGregor *et al.*, 1996; Poncelet *et al.*, 1999). In particular, an increase in motor activity has been associated with relatively low doses of CB agonists, while higher doses inhibit motor activity and produce catalepsy. The new ligand AMG-3 did induce catalepsy, as shown by increased descent latency in the bar test, but mainly at the highest dose and at longer times after administration. Catalepsy has been considered an active state that may or may not be accompanied by inhibition of movement (Klemm, 1989). Animals rendered cataleptic by CB agonists display distress, mainly manifested by vocalizations and aggressiveness towards handling (Sanudo-Pena *et al.*, 2000). The data of this study showed that the novel cannabinoid induced catalepsy-like behavior at a high dose. Additionally, according to our observations, the rats tested were also distressed during the handling procedure.

It has been reported that cataleptic doses of cannabinoids induce aversion in a conditioned place preference procedure (Sanudo-Pena *et al.*, 2000). In line with these findings, the data of the present study demonstrated that low doses of the CB₁ receptor agonist AMG-3 did not affect the rewarding efficacy of brain stimulation, whereas the highest dose increased the brain stimulation reward thresholds and decreased the response rates. This decrease seems to be independent of cannabinoid agonist-induced motor impairment, since changes in threshold current may discriminate between reward and performance (Liebman, 1983; Miliaressis *et al.*, 1986; Miliaressis and Rompré, 1987; Markou and Koob, 1992). For example, Miliaressis and Rompré have shown that motoric factors that produced profound quantitative changes in the response rate of self-stimulation failed to shift the rate-frequency function to any significant degree (Miliaressis and Rompré, 1987). Moreover, the self-stimulation threshold procedure applied in the present study allowed determining threshold and response rate separately and concurrently in the same self-stimulation session. It is worth noting that a decrease in performance is not always associated with an increase in threshold frequency, and, more importantly, attenuated performance has been observed in association with a

decrease in threshold frequency (Panagis and Spyraiki, 1996). More profound effects might also have been observed if ICSS had been assessed at longer times after AMG-3 administration.

The present results are consistent with previous studies that showed that the synthetic cannabinoid receptor agonists levonantradol, WIN 55,212-2, CP 55,940 and HU-210 either did not affect, or increased, the brain stimulation reward thresholds (Stark and Dews, 1980; Kucharski *et al.*, 1983; Arnold *et al.*, 2001; Vlachou *et al.*, 2003, 2005). However, the present results are not in agreement with data from studies that show that THC increases the rewarding efficacy of self-stimulation (Gardner *et al.*, 1988; Gardner and Vorel, 1998). These discrepant results could be attributed to differences in the pharmacological properties and the dose range of the compounds tested, the methods followed and the strain of the animals used. For example, Lepore *et al.* (1996) reported the most pronounced action of THC in Lewis rats, while they found that the effect was minimal in Sprague–Dawley rats (such as those used in the present study).

The effects of the highest dose of AMG-3 (8 mg/kg) were completely reversed by pre-treatment with the CB₁ receptor antagonist AM-251 at a dose that did not affect the baseline of self-stimulation itself. In particular, the AMG-3-induced decrease in moving and rearing behavior was reversed to some extent, while the respective induced decreases in sniffing, sniffing air and standing behavior were fully reversed by AM-251. This indicates that the inhibitory role of the cannabinoid ligand AMG-3 on spontaneous activity and brain stimulation reward is probably mediated through CB₁ receptor activation.

In summary, the present study clearly shows that the cannabinoid ligand AMG-3 displays significant analgesic properties, decreases motor activity at low doses and induces catalepsy at high doses. AMG-3 does not decrease reward threshold in the intracranial self-stimulation paradigm, but rather has an inhibitory influence on reward mechanisms at the highest dose tested. These effects were reversed by AM-251, a selective CB₁ antagonist. This observation might indicate that the behavioral effects examined are mediated through the CB₁ receptor.

In general, these findings show that AMG-3 produces a characteristic behavioral profile in rats, which, although reminiscent of the stimulation of cannabinoid receptors, does not include a decreased reward threshold in the intracranial self-stimulation paradigm. On the basis of these data, one cannot argue that AMG-3 simply possesses increased potency and efficacy as compared to the classical cannabinomimetics, despite its long-lasting effects on some behavioral tests and its high affinity for

CB receptors. Further studies, including comparisons with additional new cannabinoid ligands of similar structure are under way, with the aim to delineate and establish structure–activity relationships that might prove useful in developing drugs with promising therapeutic value.

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