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Critical role of the endogenous cannabinoid system in mouse pup suckling and growth

Ester Fride [a,b], Yoav Ginzburg [a], Aviva Breuer [a], Tiziana Bisogno [c], Vincenzo Di Marzo [c], Raphael Mechoulam [a,*]

[a] *Department of Medicinal Chemistry and Natural Products, Faculty of Medicine, Hebrew University, Ein Kerem Campus, Jerusalem 91120, Israel*
[b] *Department of Behavioral Sciences, College of Judea and Samaria, Ariel 44837, Israel*
[c] *Istituto per la Chimica di Molecole di Interesse Biologico, Consiglio Nazionale delle Ricerche, Via Toiano 6, 80072 Felice, Naples, Italy*

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Abstract

Δ^9-Tetrahydrocannabinol, the active principle in marijuana, is a cannabinoid receptor agonist. Both the crude drug and Δ^9-tetrahydrocannabinol have been used as appetite promoters. The endogenous cannabinoid, arachidonoyl ethanolamide (anandamide), likewise a cannabinoid receptor agonist, has been shown to have the same effect. In contrast, the cannabinoid CB_1 receptor antagonist *N*-(piperidin-1-yl)-5(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1-*H*-pyrazole-3-carboxamide (SR141716A) reduces food intake. Here, we report that administration of SR141716A to newly born mouse pups (either a single administration on postnatal day 1, or daily for a week as of postnatal day 2) had a devastating effect on milk ingestion and growth. The first 24 h after birth appeared the most critical for the growth stunting effect of SR141716A. Death followed within 4–8 days. Co-administration of Δ^9-tetrahydrocannabinol almost fully reversed the effect of the antagonist in the week-long regimen. Co-administration of 2-arachidonoyl glycerol, an endocannabinoid, with 2-palmitoyl glycerol and 2-linoleoyl glycerol, which enhance 2-arachidonoyl glycerol potency, resulted in a significant delay in mortality rates caused by the antagonist. We conclude that the endocannabinoid system plays a vital role in milk suckling, and hence in growth and development during the early stages of mouse life. © 2001 Published by Elsevier Science B.V.

Keywords: Tetrahydrocannabinol; 2-Arachidonoyl glycerol; SR141716A; Cannabinoid CB_1 receptor; Milk; Anandamide

1. Introduction

Cannabis and its major psychotropic constituent Δ^9-tetrahydrocannabinol (Gaoni and Mechoulam, 1964), as well as the endocannabinoid arachidonoylethanolamide (anandamide) (Devane et al., 1992), which are cannabinoid receptor agonists, enhance appetite (Mechoulam et al., 1998; Williams et al., 1998; Williams and Kirkham, 1999). Indeed, Δ^9-tetrahydrocannabinol is used clinically for this purpose, particularly in acquired immunodeficiency syndrome (AIDS) patients (Mechoulam et al., 1998). The cannabinoid CB_1 receptor antagonist *N*-(piperidin-1-yl)-5(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1-*H*-pyrazole-3-carboxamide (SR141716A) (Rinaldi-Carmona et al., 1994; Nakamura-Palacios et al., 1999) has been reported to inhibit the intake of palatable food (Arnone et al., 1997; Colombo et al., 1998; Simiand et al., 1998). Endocannabinoids are present in milk, with 2-arachidonoyl glycerol (Mechoulam et al., 1995; Sugiura et al., 1995) found in human milk in higher concentration than anandamide (Di Marzo et al., 1998). Moreover, the observation that the levels of 2-arachidonoyl glycerol, but not of anandamide, in rodent pup brain peak immediately after birth (Berrendero et al., 1999) may indicate a role of 2-arachidonoyl glycerol in pup development.

Evidence for a functional endogenous cannabinoid system was recently obtained for the primitive invertebrate, *Hydra vulgaris*, in which a role in the feeding response was indicated (De Petrocellis et al., 1999). If such a role is found to exist in the mammalian organism, it will point to a very ancient history of the endocannabinoid system in the regulation of feeding.

Very low doses (0.001 mg/kg) of systemically administered anandamide, enhance food intake as well as cognitive function in diet restricted adult mice (Hao et al., 2000).

* Corresponding author. Tel.: +972-2-675-8634; fax: +972-2-641-0740.
E-mail address: Mechou@cc.huji.ac.il (R. Mechoulam).

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We have shown that 2-arachidonoyl glycerol when administered orally, albeit in high doses, is active in the mouse 'tetrad' of tests which provides a gross measure of central cannabimimetic activity (Di Marzo et al., 1998). These findings indicate that 2-arachidonoyl glycerol in milk may, in part at least, reach the central nervous system.

With this background in mind we assumed that the endocannabinoid system in mice may play a role in milk suckling and pup development.

2. Materials and methods

2.1. Materials

Δ^9-Tetrahydrocannabinol was synthesized by acid cyclization of natural, crystalline cannabidiol, isolated from hashish, according to Gaoni and Mechoulam (1971). 2-Arachidonoyl glycerol, 2-palmitoyl glycerol and 2-linoleoyl glycerol were prepared as described by Jensen and Pitas (1976). Anandamide was prepared as described by Devane et al. (1992). Oleamide, as well as deuterated anandamide and 2-arachidonoyl glycerol, were purchased from Cayman Chemical (Ann Arbor, MI, USA). Cremophor EL was purchased from Sigma (St. Louis, MO, USA). The cannabinoid antagonist, SR141716A, was received from the US National Institute on Drug Abuse.

2.2. Analysis of milk

2-Arachidonoyl glycerol, anandamide, oleoylamide (oleamide) and 2-acyl-glycerols were extracted from milk with one volume of chloroform/methanol (2:1 by volume, containing 1 nmol each of [$^2H_8$]anandamide and [$^2H_8$]2-arachidonoyl glycerol) and then twice with one volume of chloroform. The organic phases were lyophilized and then purified by silica column chromatography and normal phase high pressure liquid chromatography (HPLC). The HPLC fractions, with the retention time of anandamide, mono-acylglycerols and oleamide, were trimethyl-silylated and analyzed by isotope dilution gas chromatography-electron impact mass spectrometry as described previously (De Petrocellis et al., 1999).

2.3. Drug administration to pups

Mouse (Sabra strain, Harlan, Israel) dams with their litters arrived in the animal house within 24 h after birth (day 1). The litters were culled to 10 pups. For the daily treatments, pups were injected from day 2 through day 8 after birth. For one-time treatments, pups were injected on day 1 or day 2 only. All injections were administered subcutaneously (s.c.) using 30-gauge needles. For experiments comparing agonists only, each pup received a single injection in the neck. For experiments requiring two injections, in agonist–antagonist experiments, the injections were made, one in the neck and one in the flank (the order neck–flank was alternated). Drugs were injected (10 μl/g body weight) in a mixture of ethanol/cremophor EL/saline (1:1:18) as described previously (Fride and Mechoulam, 1993). SR141716A was injected in doses of 5–20 mg/kg, while Δ^9-tetrahydrocannabinol and 2-arachidonoyl glycerol were injected at doses of 20 mg/kg. Mixtures of 1 mg/kg 2-arachidonoyl glycerol, 10 mg/kg 2-palmitoyl glycerol and 20 mg/kg 2-linoleoyl glycerol were injected, in order to mimic as much as practically feasible, the relative concentrations of 2-arachidonoyl glycerol and acylglycerols ('entourage' constituents, see below) noted previously in spleen and brain (Ben-Shabat et al., 1998). In order to minimize 'litter effects' (Fride and Weinstock, 1984), the various treatments were administered to the pups within each litter. In addition, in all experiments each treatment was administered to between 2

Table 1
Endocannabinoids and oleamide in milk

Milk	2-AG[a]	2-PG[b]	2-LG[c]	Oleamide	Anandamide
Bovine early (1–24 h)	1.0 ± 0.3	415 ± 189	55 ± 13	24.2 ± 15.6	0.0042 ± 0.006
Bovine mature	2.4 ± 1.0	613 ± 202	131 ± 8.9	8.5 ± 6.1	UD
Bovine pasteurized	1.8 ± 0.3	ND	ND	0.4 ± 0.03	0.094 ± 0.006
Bovine UHT	1.0 ± 0.3	ND	ND	UD	0.031 ± 0.004
Human early	6.4 ± 1.9	3498 ± 1038	339 ± 160	4.0 ± 1.0	UD
Human mature	8.7 ± 2.8	2001 ± 644	19 ± 6.2	1.5 ± 0.3	0.0015 ± 0.003
Goat mature	8.3 ± 3.3	1050 ± 417	165 ± 65	34.5 ± 17.5	0.009 ± 0.004

All data are in μg/g extracted lipids (lipid concentration in milk is about 36 g/l) and are means ± S.E.M. of at least three separate determinations. Data for 2-arachidonoyl glycerol, oleamide and anandamide were obtained by grouping previously published results (Di Marzo et al., 1998) with data from new analyses of milk. Note that data on 2-arachidonoyl glycerol, 2-palmitoyl glycerol and 2-linoleoyl glycerol (2-LG) also include 1- arachidonoyl glycerol, 1-palmitoyl glycerol and 1-linoleoyl glycerol, respectively. ND, not determined; UD, under the detection limit; UHT, ultra-high temperature-treated, long life milk. Other monoacyl glycerols were also detected but are not listed in this table.

[a] 2-Arachidonoyl glycerol.

[b] 2-Palmitoyl glycerol.

[c] 2-Linoleoyl glycerol.

and 6 l. The individual group sizes are indicated in the Legends.

2.3.1. Procedure

Pups were separated each day from their mothers for 1 h, during which they were injected, weighed, observed for ear flap detachment, an early postnatal developmental landmark (Fride and Weinstock, 1984), and for the presence of milk bands in their stomachs. As the stomach area in mouse pups is transparent, due to lack of hair and the thinness of the skin, the amount of milk consumed can be observed as a "milk band". Pups were kept at an environmental temperature of 28–30°C. After the separation period litters were reunited for 2.5 h, after which they were briefly separated again for reassessment of body weight and of the developmental parameters.

In the single-dose experiments, this procedure was performed on a single day only (days 1 or 2) and the pups were examined several times during the first week of life. In the daily dose experiment, the procedure was repeated on days 2–8.

2.4. Maternal behavior

Interactions between dams and pups and nest building were recorded based on procedures described by Laviola et al. (1990).

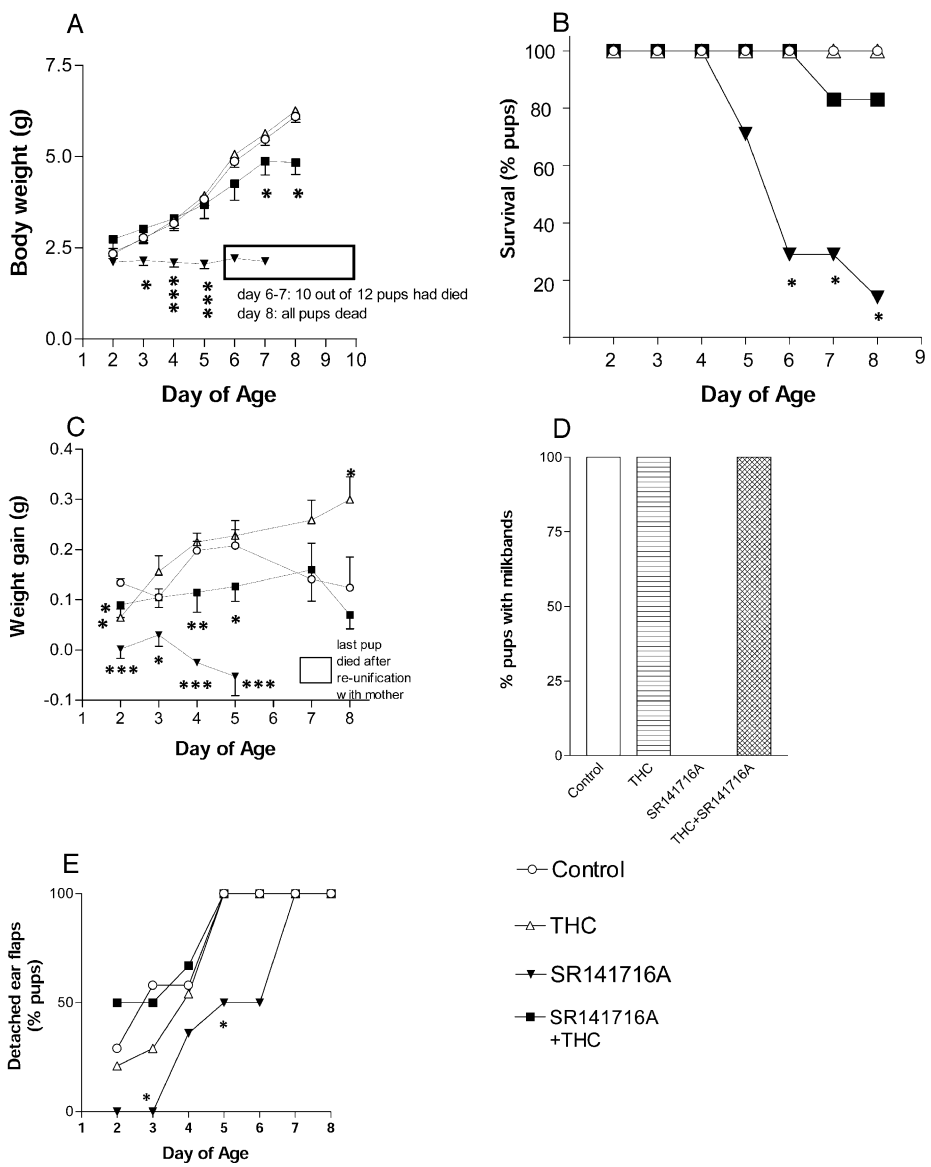

Fig. 1. Week-long administration of antagonist. Mouse pups (Sabra strain) were given two injections (s.c.) each day between days 2 and 8 of life: vehicle (2 ×) (ethanol/cremophor EL/saline = 1:1:18, open circles, $n = 10$), or Δ^9-tetrahydrocannabinol (20 mg/kg) and vehicle (open triangles, $n = 12$), or SR141716A (20 mg/kg) and vehicle (closed triangles, $n = 12$), or Δ^9-tetrahydrocannabinol and SR141716A (closed squares, $n = 12$). Daily assessments were made of body weights (A); survival rates (B); weight gain upon feeding (C); milkbands in stomachs (D); and ear flap detachment (E); $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, different from controls.

2.5. Data analysis

Body weights were analyzed using an analysis of variance and Newman–Keuls post-hoc comparisons. The remaining data were analyzed using χ^2 analyses or Fisher's exact probability tests (Prism 3.0, Graphpad, CA). Data are reported as means ± S.E.M.

3. Results

3.1. Presence of fatty acid derivatives in milk

We analyzed milk from various sources (see Table 1). The concentration of anandamide, oleamide, 2-arachidonoyl glycerol, 2-palmitoyl glycerol and 2-linoleoyl glycerol was determined. While the concentration of anandamide was low, the rest of the constituents tested were found to be present in various concentrations with 2-palmitoyl glycerol and 2-linoleoyl glycerol showing the highest levels (see Table 1).

3.2. Effect of antagonist administered daily between day 2 and day 8

In a typical experiment, administration of the antagonist, SR141716A (20 mg/kg) (Fig. 1A), caused the death of all pups by day 8. The pups stopped putting on weight after the first injection ($F = 7.2$, $df = 2,23$, $P < 0.0001$, Fig. 1A). This observation was repeated four times. The pups apparently stopped suckling as shown by the absence of added weight after a suckling period (Fig. 1C) and by the complete absence of "milk bands" (Fig. 1D). Daily administration of lower doses (15 and 10 mg/kg) had no effect. When Δ^9-tetrahydrocannabinol was administered together with the antagonist, the effect of the antagonist was almost completely reversed and the pups put on weight, closely similar to that of the control animals (Fig. 1A). However, on some days these mice sucked less than the controls (Fig. 1C). Mortality was similar to that of the controls (Fig. 1B). Finally, in the surviving pups, ear flap detachment, was significantly delayed by SR141716A. Co-administration of Δ^9-tetrahydrocannabinol abolished the delay (Fig. 1E). Compared to the controls, pups receiving Δ^9-tetrahydrocannabinol alone had a slight increase in weight gain following a suckling period, reaching significance by day 8 (Fig. 1C). Administration of 2-arachidonoyl glycerol (20 mg/kg) to pups ($n = 15$) did not block the effect of the antagonist.

3.3. Effect of antagonist administered once only on the first or second day of life

Since the previous experiment had suggested that a major effect already occurs on the first day of life, we decided to inject the antagonist only once, on day 1 or on day 2 of life. Thus, a single injection of SR141716A (20 mg/kg) within 24 h after birth, caused devastating effects on pup weight gain ($F = 36.0$, $df = 1,20$, $P < 0.0001$, Fig. 2A) and survival (Fig. 2B), comparable to those recorded in the week-long regimen. Total mortality was observed within 4 days after birth. When SR141716A was injected on day 2 only, half the mortality effect was obtained (Fig. 2B) and the surviving pups gained the same weight as the controls (Fig. 2A). A lower dose (10 mg/kg) had a partial (about 50%) effect on mortality, ear flap development and milk bands and no effect on weight gain. A dose of 5 mg/kg had no effect on any of the parameters studied (Fig. 3).

When Δ^9-tetrahydrocannabinol (20 mg/kg) was co-administered with SR141716A on day 1 in the single dose regimen, we observed that about half the pups survived, while the weight gain in the survivors was similar to that of the controls. We have previously shown that 2-palmitoyl glycerol and 2-linoleoyl glycerol, which do not bind to

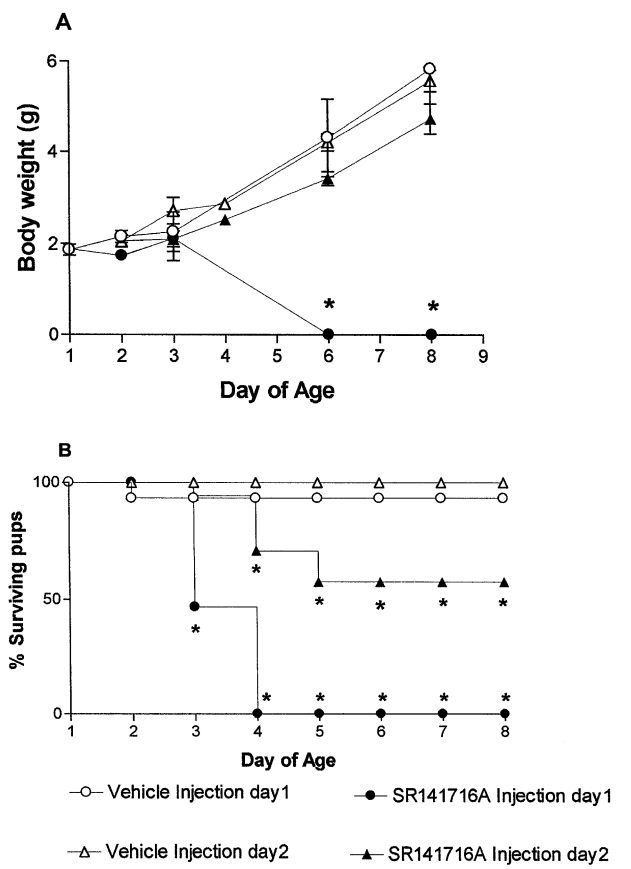

Fig. 2. Single administration of antagonist. Mouse pups (Sabra strain) were given a single injection (s.c.) on day 1 (circles) or on day 2 (triangles) of life. Controls were given vehicle (ethanol/cremophor EL/saline = 1:1:18, open symbols, $n = 15$), or SR141716A (20 mg/kg, closed symbols, $n = 15$). Periodic assessment of body weights (A); and daily assessment of survival rates (B); were made. * $P < 0.001$, different from controls.

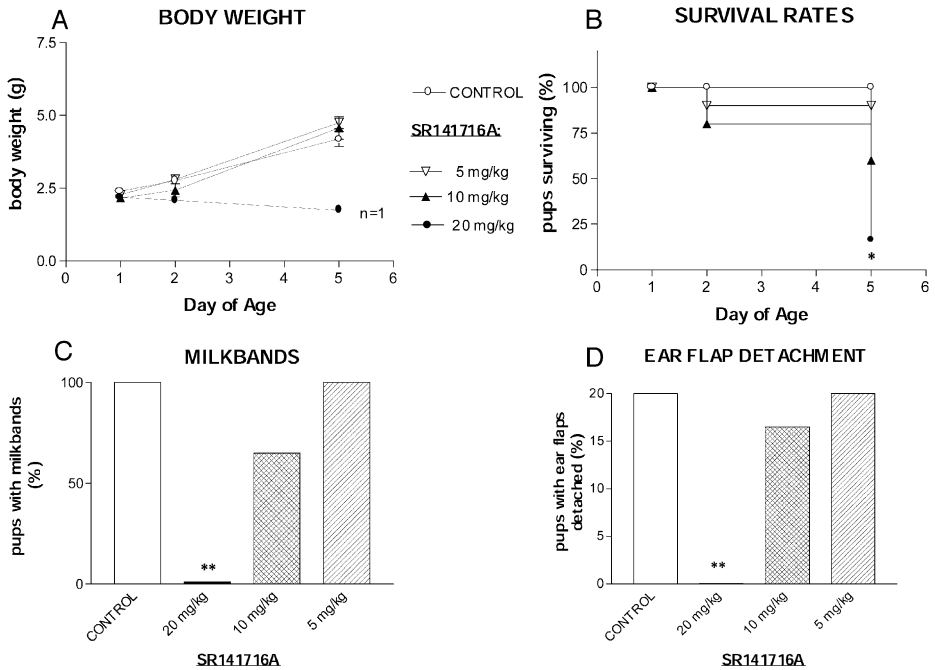

Fig. 3. Comparison of antagonist dose effects. Mouse pups (Sabra strain) were given a single injection (s.c.) on day 1 of life. Vehicle (ethanol/cremophor EL/saline = 1:1:18, open circles, $n = 5$) or SR141716A (5, 10 or 20 mg/kg, $n = 9$, 11, 6 respectively) was administered. Body weights (A); survival rates (B); milk bands (C); and earflap detachment (D); were assessed on postnatal days 1, 2, and 5. $^{*}P < 0.05$, $^{**}P < 0.01$ different from controls.

the cannabinoid receptors, enhance 2-arachidonoyl glycerol activity by blocking its hydrolysis and its uptake (Ben-Shabat et al., 1998). Since the first-day injection of SR141716A was at least as efficient as the multiple-dose regimen (compare Fig. 1B with Fig. 2B), we decided to assess the counteractive effect of 2-arachidonoyl glycerol (with its 'entourage') in the first-day injection regimen. Thus, we co-administered 2-arachidonoyl glycerol (1 mg/kg), 2-palmitoyl glycerol (10 mg/kg) and 2-linoleoyl glycerol (20 mg/kg) with the antagonist (20 mg/kg), on day 1. This regimen resulted in a significant delay in mortality rates as expressed in a significantly lower mortality on day 2 and an approximately overall one-third decrease in mortality compared to the treatment effect of SR141716A alone (Fig. 4B). A partial improvement in weight gain was also observed ($F_{\mathrm{SR141716A}} = 41.0$ $df = 1,53$, $P < 0.0001$, $F_{\mathrm{interaction}} = 3.6$, $df = 1,53$ $P = 0.07$, Fig. 4A). Cannabidiol, a non-psychoactive cannabinoid, which does not bind to cannabinoid CB_1 receptors, did not reverse SR141716A induced mortality, or lack of weight gain ($F = 0.5$ $df = 1,14$, NS, Fig. 5).

3.4. Maternal behavior

Administration of the antagonist had no observable detrimental effect on maternal behavior. Rather, there was a significant increase in "licking" of the pups (1.9 ± 0.00 vs. 1.0 ± 0.1 times in controls, $P < 0.05$) and in the amount of time spent in nursing the antagonist-treated pups (5.9 ± 0.4 vs. 4.4 ± 0.5 min controls, $P < 0.05$). Hence, the dams were not repulsed by the antagonist-treated pups.

4. Discussion

The effects described here show that the cannabinoid CB1 receptor antagonist, SR141716A, completely inhibits physical growth of mouse pups and causes death within 1 week, by depriving them of the essential benefits of nursing. This devastating effect of SR141716A was seen after daily injections of 20 mg/kg between days 2 and 8 of life. At least as dramatic an effect was also seen after a single injection of SR141716A, but only when administered within the first 24 h after birth (day 1). Administration on day 2 only, resulted in 50% mortality. Thus, the first 24 h of life seem to be most critical for the putative endocannabinoid-induced growth-promoting effect, which is compatible with the peak levels of 2-arachidonoyl glycerol found on this day (Berrendero et al., 1999). This is also consistent with the observation that 2-arachidonoyl glycerol is present in mammalian milk from the first day after birth ('early' milk or colostrum) (Table 1).

Apparently, pup mortality was due to impairment of suckling. Thus, from day 1 of treatment, no weight was gained. This effect was also evident from the absence of milk in the stomachs of the SR141716A-treated pups on each day of life. The surviving pups also showed a significant delay in the rate of ear flap detachment (a marker of early development in rodent pups, see Fride and Weinstock, 1984).

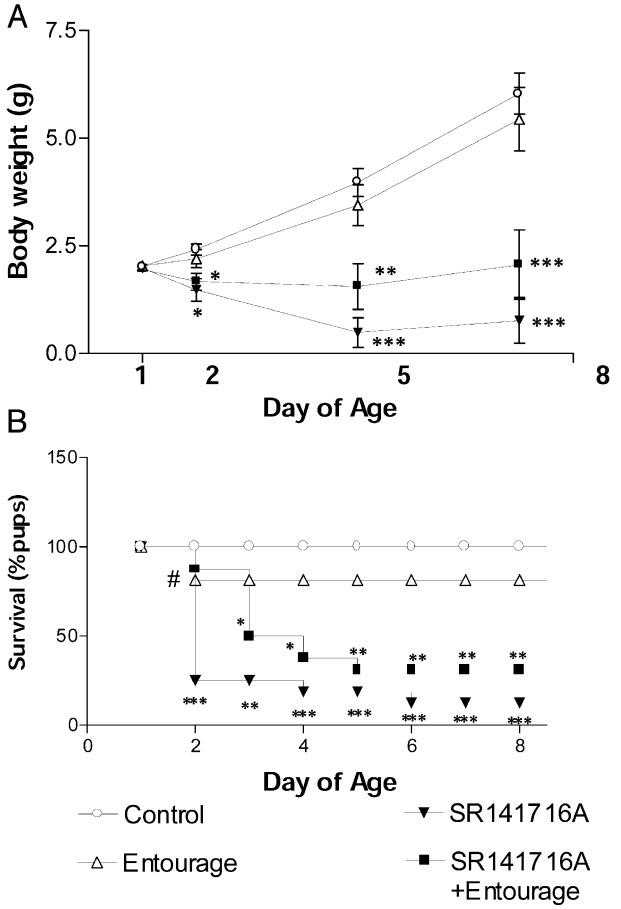

Fig. 4. Reduction of antagonist effects by 2-arachidonoyl glycerol and 'entourage' compounds. Mouse pups (Sabra strain) were given a single injection (s.c.) on day 1 of life. Vehicle (ethanol/cremophor EL/saline = 1:1:18, open circles, $n = 14$); 'entourage' (a mixture of 1 mg/kg 2-arachidonoyl glycerol, 10 mg/kg 2-palmitoyl glycerol and 20 mg/kg 2-linoleoyl glycerol, open triangles, $n = 15$); SR141716A (20 mg/kg, closed triangles, $n = 14$) or 'entourage' and SR141716A (20 mg/kg, closed squares, $n = 15$) was administered. Body weights (A); were assessed on postnatal days 1, 2, 4 and 7. Survival rates (B); were assessed daily. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, different from controls.

A dose–response relationship was noted. Thus, when half the dose (10 mg/kg) of SR 141716A was administered on day 1 in the single day regimen, effects of about 50% magnitude on mortality, milk bands and ear flap detachment, were observed. Five mg/kg had almost no effect.

Several experiments were performed in order to investigate whether the effects of SR141716A were mediated by cannabinoid CB_1 receptors. First, when Δ^9-tetrahydrocannabinol was co-administered with SR141716A, the detrimental effects on weight gain and feeding were almost completely reversed. Second, co-administration of cannabidiol, a cannabinoid which does not bind cannabinoid CB_1 receptors, had no influence on SR141716A-induced effects. The endogenous cannabinoid CB_1 receptor agonist, 2-arachidonoyl glycerol, by itself had no effect on the SR141716A-induced growth stunting. This lack of effect of exogenous 2-arachidonoyl glycerol alone is probably due to its facile degradation by enzymatic hydrolysis

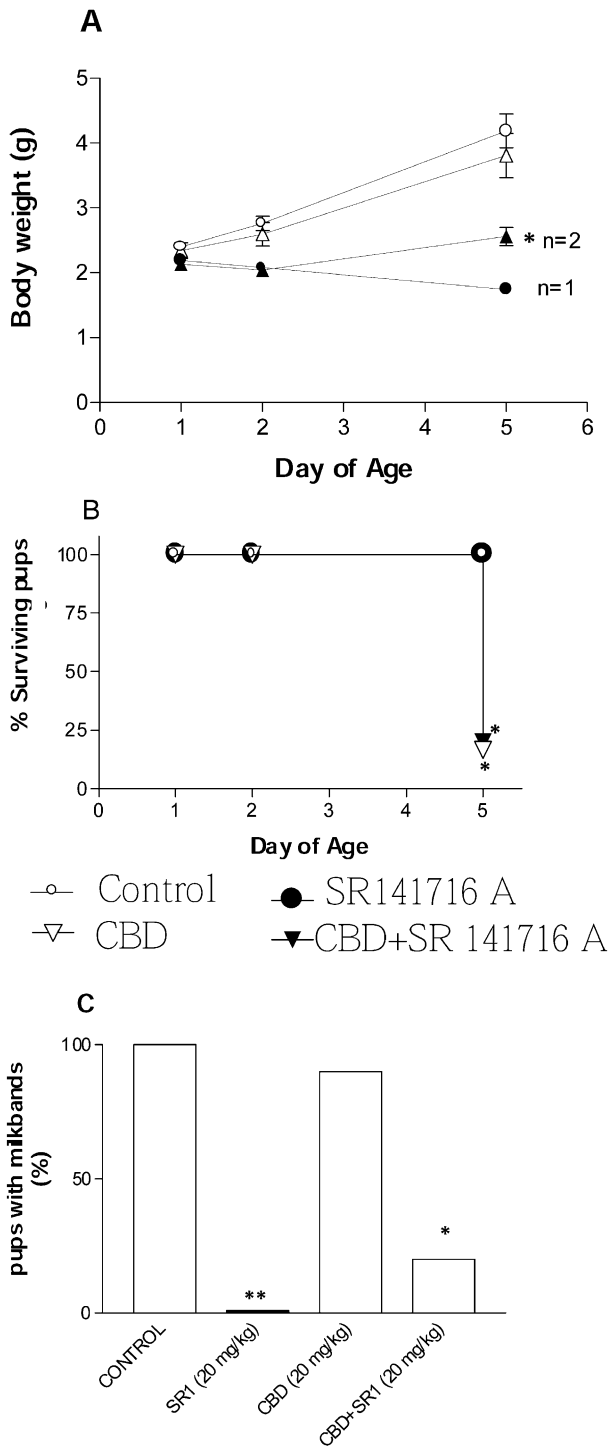

Fig. 5. Administration of cannabidiol. Mouse pups (Sabra strain) were given two injections (s.c.) on day 1 of life: two injections of vehicle (ethanol/cremophor EL/saline, 1:1:18, open circles, $n = 5$) or vehicle and SR141716A (SR1) (20 mg/kg, open triangles, $n = 6$) or vehicle and cannabidiol (CBD) (20 mg/kg closed circles, $n = 10$) or cannabidiol (20 mg/kg) and SR141716A (20 mg/kg closed triangles, $n = 9$). Body weights (A), survival rates (B) and milkbands (C) were assessed on postnatal days 1, 2 and 5. $^{*}P < 0.05$, $^{**}P < 0.01$ different from controls.

of its ester bond and to its rapid uptake. However, endogenous 2-arachidonoyl glycerol may be partly protected from hydrolysis and its uptake may be slowed down by monoacyl-glycerols (Ben-Shabat et al., 1998). 2-Arachidonoyl glycerol was indeed accompanied by several 2-acyl glycerols in all milk samples analyzed by us. 2-Palmitoyl glycerol and 2-linoleoyl glycerol do not bind to cannabinoid CB_1 receptors and cause no cannabis-type effects as evaluated in binding assays, or from lack of inhibition of adenylylcyclase and in several in vivo assays in mice (Ben-Shabat et al., 1998). In all these assays however, 2-arachidonoyl glycerol activity was significantly enhanced by 2-palmitoyl glycerol and 2-linoleoyl glycerol ('entourage effect'). If 2-arachidonoyl glycerol, with suckling-stimulating activity, is consumed by pups with the milk, the 'entourage' compounds that are present in milk (Table 1) may enhance this activity. Therefore, we evaluated the activity of 2-arachidonoyl glycerol in the presence of the 'entourage' compounds. We found that co-administration of 2-arachidonoyl glycerol (1 mg/kg), 2-palmitoyl glycerol (10 mg/kg) and 2-linoleoyl glycerol (20 mg/kg) with the antagonist (20 mg/kg), on day 1, resulted in a significant delay in mortality rates and an approximately overall one-third decrease in mortality compared to the treatment effect of SR141716A alone. A partial improvement in weight gain was also observed. Thus, the 'entourage' effect may enhance the putative growth promoting effects of 2-arachidonoyl glycerol. However, dose ranges may have to be investigated further in order to find the maximal 'entourage' effect.

The above data strongly suggest that the anti-suckling and growth-inhibiting effects of SR141716A, are mediated by a block of the cannabinoid CB_1 receptors.

The dose of SR141716A needed for a complete block of food ingestion and survival is relatively high (20 mg/kg). However, this observation is compatible with the low responsiveness to cannabinoid ligands during the first week of life in developing pups (Fride and Mechoulam, 1993; Fride and Sanudo-Pena, 2000). Moreover, although lower doses are often found sufficient to block some Δ^9-tetrahydrocannabinol-induced effects (Rinaldi-Carmona et al., 1994), many effects of the antagonist require higher doses (between 20 and 100 mg/kg) (see, for example, Adams et al., 1998; Compton et al., 1996; Smith et al., 2000; Lichtman and Martin, 1997; review by Nakamura-Palacios et al., 1999).

Zimmer et al. (1999) have found that cannabinoid CB_1 receptor knock-out mice survive the initial stages of life, which obviously involve suckling. However, increased mortality was noted in such mice. Presumably other mechanisms compensate for the lack of cannabinoid CB_1 receptor-based suckling. Petrov et al. (1998) have reported that endogenous opioids are involved in early suckling and it is possible that this, or other systems assume a more prominent role in CB_1 knockout mice. One such system may involve the receptor gene for lysophosphatidic acid since, in a recent publication, Contos et al. (2000) describe a defective suckling response in neonatal mice with a targeted deletion of this gene. This response may be related to the observation that the lysophosphatidic acid receptors have a sequence homology of nearly 30% with the cannabinoid receptors (Moolenaar, 2000). There is also a chemical relationship between the two families of bioactive compounds. The chemical structures of 2-arachidonoyl glycerol and lysophosphatidic acid (with 2-arachidonoyl as the acyl moiety) only differ by the absence of a phosphate group in 2-arachidonoyl glycerol and a related lysophosphatidic acid (with 1-arachidonoyl as the acyl moiety) has been detected in rat brain (Sugiura et al., 1999).

We conclude that the endocannabinoid system plays a vital role in milk suckling, and hence in growth and development during the early stages of mouse life.

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