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European Journal of Pharmacology 461 (2003) 27–34
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Milk intake and survival in newborn cannabinoid CB_1 receptor knockout mice: evidence for a "CB_3" receptor

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Received 28 October 2002; received in revised form 20 December 2002; accepted 7 January 2003

Abstract

Cannabinoids, whether plant-derived, synthetic or endogenous, have been shown to stimulate appetite in the adult organism. We have reported previously that cannabinoid receptors play a critical role during the early suckling period: The selective cannabinoid CB_1 receptor antagonist *N*-(piperidiny-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (SR141617A) permanently prevented milk ingestion in a dose-dependent manner, when administered to (Sabra, albino) mouse pups, within 1 day of birth. As a consequence, these pups died within the first week of life. We now generalize this finding to a different strain of mice (C57BL/6). Further, we show that cannabinoid CB_1 receptor blockade (20 mg/kg SR141716A) must occur within 24 h after birth as injection of SR141716A into 2- or 5-day-old pups had a much smaller effect or no effect at all, respectively. Cannabinoid CB_1 receptor knockout mice did not ingest milk on the first day of life, similarly to SR141716A-treated normal pups, as measured by the appearance of "milkbands". However, the knockout pups started to display milkbands from day 2 of life. Survival rates of cannabinoid CB_1 receptor knockout mice were affected significantly, but to a lesser extent than normal pups, by the administration of SR141716A. Daily administration of the endocannabinoid 2-arachidonoyl glycerol, or the synthetic agonists (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone (WIN55,212-2, 5 mg/kg) or (−)-*cis*-3-[2-Hydroxy4-(1,1-dimethylheptyl) phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol (CP55,940, 5 or 20 mg/kg) did not promote survival or weight gain in $CB_1^{-/-}$ pups. Our data support previous evidence for a critical role of cannabinoid CB_1 receptors for the initiation of suckling. Further, the present observations support the existence of an unknown cannabinoid receptor, with partial control over milk ingestion in newborns. Our data also suggest that the $CB_1^{-/-}$ neonates possess a compensatory mechanism which helps them overcome the lack of cannabinoid CB_1 receptors.

Keywords: 2-Arachidonoyl glycerol; Endocannabinoid; Cannabinoid receptor; Milk suckling; SR141716A; Cannabidiol

1. Introduction

The discovery of an endogenous cannabinoid system started with the unequivocal proof for a selective cannabinoid receptor for Δ^9-tetrahydocannabinol, the major psychotropic constituent of the *Cannabis sativa* plant (Devane et al., 1992; Matsuda et al., 1990). This receptor, denoted CB_1, is located in the brain, the peripheral nerves and several peripheral organs (Pertwee and Ross, 2002). In 1993, a second, 'peripheral' cannabinoid CB_2 receptor (Munro et al., 1993) was identified in nonneural tissue, mainly in components of the immune system. The series of landmark discoveries continued with the identification of endogenous receptor ligands, which were coined 'endocannabinoids' (Di Marzo and Fontana, 1995; Di Marzo, 1998). Thus far, 3 main 'endocannabinoids' with agonist properties have been identified. They are anandamide, 2-arachidonoyl glycerol and noladin-ether, all fatty acid derivatives: an ethanol amide (Devane et al., 1992), an ester (Mechoulam et al., 1995) and an

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doi:10.1016/S0014-2999(03)01295-0

ether (Hanus et al., 2001), respectively (see Fride, 2002b for review). More recently, an endocannabinoid with partial agonist/antagonist properties has been identified and was denoted "virodhamine" (Porter et al., 2002).

The involvement of tetrahydrocannabinol in feeding was shown decades ago (Abel, 1971; Fride and Sanudo-Pena, 2002); endocannabinoids appear to fulfill a similar role (Williams and Kirkham, 1999; Fride and Sanudo-Pena, 2002; Fride, 2002a). Endocannabinoids have also been detected in milk, 2-arachidonoyl glycerol in at least 100–1000-fold higher concentrations than anandamide (Di Marzo et al., 1998; Fride et al., 2001).

Cannabinoid CB_1 receptors have been detected prenatally, as early as day 11 of gestation in the rat embryo (Buckley et al., 1998) and week 14 of gestation in the human embryo (Biegon and Kerman, 2001). The behavioral response to cannabinoids develops gradually in young mice (Fride and Mechoulam, 1996), which correlates with the postnatal developmental pattern of cannabinoid receptor mRNA and receptor binding over the first weeks of life as observed in some studies (Rodriguez de Fonseca et al., 1993; Belue et al., 1995; McLaughlin et al., 1994). Other findings suggest, however, that the developmental pattern of cannabinoid CB_1 receptors varies between brain regions (Berrendero, et al., 1999; McLaughlin and Abood, 1993), probably due to the transient presence of cannabinoid CB_1 receptors in "atypical" regions during brain development (Fernandez-Ruiz et al., 2000).

Endocannabinoids are also detectable from the fetal period, and similarly to milk, anandamide at much lower (1000-fold) concentrations than 2-arachidonoyl glycerol. Moreover, the developmental pattern differs between the two endocannabinoids. Thus, whereas concentrations of anandamide gradually increase throughout development until adult levels are reached (Berrendero et al., 1999), fetal levels of 2-arachidonoyl glycerol are similar to those in neonatal and in adult brains with a remarkably distinct peak on the first day after birth in rats (Berrendero et al., 1999).

These findings, taken together, suggested that endocannaboinoids and especially 2-arachidonoyl glycerol may have physiological importance in milk suckling by the newborn. Indeed, we have shown recently (Fride et al., 2001) that selective blockade of cannabinoid CB_1 receptors with the antagonist *N*-(piperidiny-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (SR141716A) in 1-day-old mouse pups completely prevented milk intake and caused almost 100% mortality in albino (Sabra) mouse pups. The effect was dose-dependent. Cannabinoid CB_1 receptor selectivity was demonstrated in a number of experimental manipulations, most notably by the almost complete reversal of the SR141716A-induced growth inhibition and mortality by the prototypical cannabinoid CB_1 receptor agonist THC.

Timing was critical for the effects of the antagonist on pup development and survival. Thus, injection of SR141716A on day 2 of life resulted in only 50% mortality and milk ingestion, compared to almost 100% on the first day.

Cannabinoid CB_1 receptor-deficient mice ($CB_1^{-/-}$) display some behavioral abnormalities consistent with an absence of cannabinoid CB_1 receptors (Zimmer, et al., 1999; Ledent et al., 1999). According to our studies, however, where blockade of cannabinoid CB_1 receptors after birth is incompatible with life (Fride et al., 2001), $CB_1^{-/-}$ pups would not be expected to survive beyond the first postnatal week. Yet, in the original paper by Zimmer et al. (1999) in their strain of $CB_1^{-/-}$ mice, increased mortality in the knockout compared to *wild type* (C57BL/6) mice was recorded only from 2 months of age, while no abnormalities in growth, adult body weights and fertility were reported in either strain of cannabinoid CB_1 knockout mice (Ledent et al., 1999; Zimmer et al., 1999).

More recent studies on cannabinoid CB_1 knockout mice have suggested the existence of a third cannabinoid receptor subtype, active in the cannabinoid CB_1 receptor-deficient mice (Breivogel et al., 2001; Di Marzo et al., 2000). According to these studies, this putative cannabinoid "CB_3" receptor is activated by the cannabinoid CB_1 agonist WIN55,212-2 and by the endocannabinoid anandamide, but is not blocked by SR141716A.

In the present study, we further studied the importance of the timing of SR41716A administration and we generalized our previous findings to a different strain of mice (C57BL/6). Moreover, we investigated birth and developmental data and milk intake in cannabinoid CB_1 receptor knockout pups. We also compared the effects of a single injection of SR141716A (on day 1 of life) into $CB_1^{-/-}$ pups with those on wild type pups.

Thus, we report here that SR141716A injected in C57BL/6 mice is equally destructive compared to the albino strain studied previously (Fride et al., 2001) and that injecting SR141716A on postnatal day 5 causes only transient inhibition of milk intake. We also report that SR141716A significantly reduces survival rates and milk intake in the knockout mice, but not as dramatically as in wild type pups.

Finally, in an attempt to "improve" feeding and body weight gain in cannabinoid CB_1 receptor-deficient mice via the putative cannabinoid "CB_3" receptor or another unknown mechanism, the cannabinoid CB_1 agonist (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone (WIN55,212) and the endocannabinoid 2-arachidonoyl glycerol were administered daily to knockout pups for the first 2 weeks of life. The potent cannabinoid CB_1 receptor agonist (−)-*cis*-3-[2-Hydroxy4-(1,1-dimethylheptyl) phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol (CP55,940) was also administered daily at high doses (5 and 20 mg/kg).

The results indicate that none of these treatments improved growth and survival of the $CB_1^{-/-}$ pups.

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 was prepared in our laboratory (Mechoulam et al., 1995). Cremophor EL was purchased from Sigma (St. Louis, MO, USA). WIN55,212-2 and the cannabinoid antagonist SR141716A were received from the US National Institute on Drug Abuse. All drugs were dissolved in a mixture of ethanol, cremophor EL and saline (1:1:18, the vehicle) as described previously (Fride and Mechoulam 1993) and injected at a dose of 20 mg/kg.

2.2. Mice

Four breeding pairs of $CB_1^{-/-}$ knockout mice were kindly donated by Dr. Zimmer, Max Planck Institute, Germany, and bred in our facilities. C57BL/6 mice, the parent strain of the $CB_1^{-/-}$ knockout mice, were purchased from Harlan, Israel. They were either bred in our facilities (one male with 1 or 2 females), or purchased as dams with their litters within 24 h of birth (Day 1). No differences in litter data and pup development were recorded between these two conditions. Females from pairs mated in our facilities were separated from the males when visibly pregnant (day 12–14 of gestation) and housed singly from then onward. Pups were injected on days 1, 2 or 5 with vehicle or with SR141716A. For the daily treatments, pups received daily injections of vehicle, 2-arachidonoyl glycerol, WIN55,212-2 or CP55,940. All injections were performed s.c. in the neck using 30-gauge needles (10 μl/g body weight). 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 4 and 9 litters. The individual group sizes are indicated in the Legends.

2.3. Procedure

Pups were separated each day from their mothers only for the duration of injection, weighing and scoring of the presence of "milkbands" 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 "milkband". The dam was kept in a holding cage in a separate room. Pups were kept at an environmental temperature of 26–28 °C. Injections of SR141716A (20 mg/kg) were performed on a single day only (day 1, 2 or 5). However, pups were examined daily during the first 2 weeks of life. (A dose–response relationship for SR141716A was reported previously; see Fride et al., 2001.) WIN55,212 was injected at a (daily) dose of 5 mg/kg, 2-arachidonoyl glycerol at 20 mg/kg and CP55,940 at a dose of 5 or 20 mg/kg.

2.4. Data analysis

Body weights, survival rates (percent surviving pups/litter) and milkbands (percent pups with milkbands/litter) were analyzed using analyses of variance (ANOVA: 2-way, age $\times$ treatment, or 3-way, age $\times$ treatment $\times$ strain). (Because of the factor design, and large numbers of pups used, preference was given to perform parametric statistics, over chi-square analyses, also for milkbands and survival rates. However, chi-square analyses were also performed for these parameters and yielded similar results). Individual data points were compared by Newman–Keuls post hoc comparisons. T-tests were used to compare knockout vs. wild type litter data. Three-way ANOVA's were performed using SPSS, version 10; the remaining analyses were performed with Graphpad Prism 3.0 software. Data are reported as means ± S.E.M.

3. Results

3.1. Timing of cannabinoid CB_1 receptor blockade in wild type C57BL/6 mice

From Fig. 1, it can be seen that whereas injection of SR141716A (20 mg/kg) within 24 h of birth (day 1) induced almost 100% mortality, injection on day 5 had no effect at all ($F_{treatment} = 46$, $df = 4,130$, $P < 0.001$). (The 50% mortality as shown previously (Fride et al., 2001) is superimposed in Fig. 1.)

From Fig. 2A, it can be seen that the injection on day 5 had an effect on milk intake as visible the next day (day 6). (By day 7, due to thickening of the skin and emerging fur, milkbands could not be scored reliably anymore.) However, since the growth curve was not affected by SR141716A

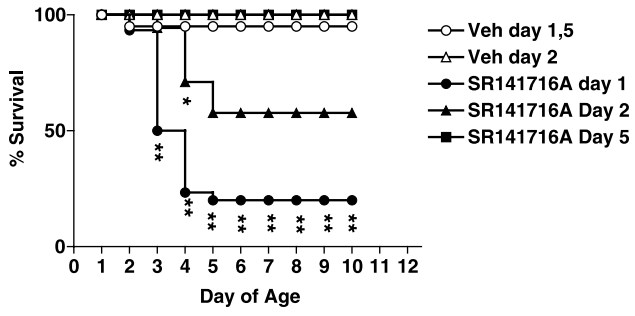

Fig. 1. Survival rates (percent mice/litter) in developing mouse pups. Each litter was divided in control and experimental pups. Pups received a single injection (s.c.) of injection of vehicle (7 litters, open symbols) (ethanol:emulphor:saline = 1:1:18) or SR141716A (5 litters, 20 mg/kg, closed symbols). Daily assessments were made of the number of live pups. $F_{treatment} = 46$ ($df = 4,130$), $P < 0.001$.

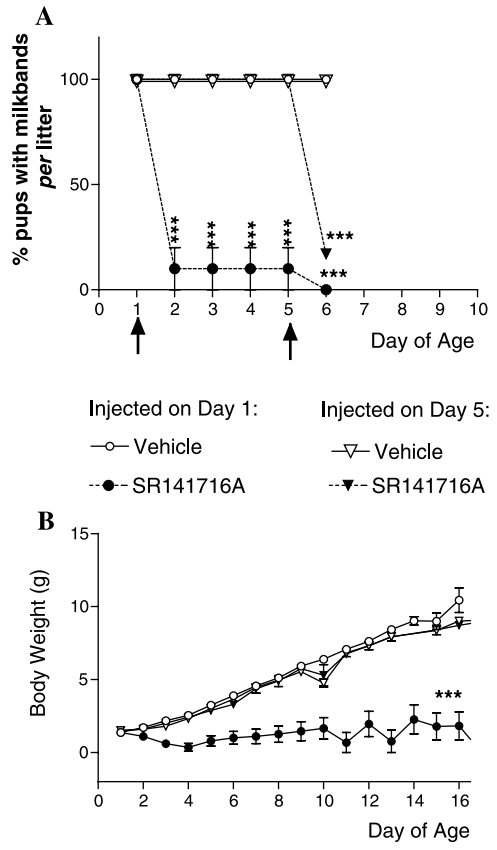

Fig. 2. Milkbands (percent pups with milkbands/litter) (A) and body weights (B) in developing (C57BL/6) mouse pups. Each litter was divided in control and experimental pups. Pups received a single injection of injection of vehicle (ethanol:emulphor:saline = 1:1:18) (open symbols) or of SR141716A (20 mg/kg), either on day 1 (circles) or on day 5 (triangles) (5 litters, 20 mg/kg, closed symbols). Daily assessments were made. (As the skin is not transparent enough for reliable assessments by day 7, the last day of recording milkbands is day 7.) Milkbands: $F_{treatment} = 23$ ($df = 3,26$), $P < 0.001$; Body weights: $F_{treatment} = 261$ ($df = 3,388$), $P < 0.001$. ***$P < 0.001$, SR141716A different from vehicle injected on the same day. (For body weights, this significance level was reached on every day from day 2 onward although, for the sake of clarity, this is indicated only once on the curve.)

when injected on day 5 (Fig. 2B), it is clear that the effect on milkbands (Fig. 2A) was transient. The effect of SR141716A injection on day 1 on the reduction in milkbands ($F_{treatment} = 23$, $df = 3,26$, $P < 0.001$) and weight gain ($F_{treatment} = 261$, $df = 3,388$, $P < 0.001$) was highly significant.

3.2. Litter data and development in wild type and cannabinoid CB_1 receptor knockout mice

Litters from six wild type (C57BL/6) and nine $CB_1^{-/-}$ knockout breeding pairs (Zimmer et al., 1999) were observed over the same period in our animal house. As can be seen from Fig. 3, birth weights of the pups did not differ between the strains. However, significantly fewer knockout pups per litter were born. As we observed frequent cannibalism by the knockout dams as well as a scarcity of milkbands in the knockout pups (see below), we also assessed the number of pups alive by day 4 (Fig. 3). Indeed, the difference in the numbers of living pups between wild type and knockout litters on day 4 ($P < 0.001$) was greater than this difference at birth ($P < 0.05$).

As can be seen from Fig. 4A, survival of (untreated) $CB_1^{-/-}$ knockout pups was lower than that of wild type litters ($F = 20.6$ $df = 1,141$, $P < 0.001$). On the first day after birth, the (untreated) knockout pups had virtually no milk in their stomachs (Fig. 4B) although by day 3, all of them, similarly to wild type pups, had milkbands. Significantly lower body weights were recorded from day 5 of life in the knockout pups (Fig. 4C). This difference was permanent as body weights of knockout mice were still significantly lower than those of wild type mice at 3 months of age (wild type: 29.3 ± 0.76, $CB_1^{-/-}$ 24.0 ± 0.76 g, $t = 4.8$, $df = 15$, $P < 0.001$). Thus, body weights did not recover despite the "catch-up" of the milkbands (Fig. 4B).

3.3. Effects of the cannabinoid CB_1 receptor antagonist on $CB_1^{-/-}$ knockout pup development and milk ingestion

An injection on day 1 of life with SR141716A (20 mg/kg) caused an almost 100% mortality in C57BL/6 wild type pups, consistent with our previous observations in Sabra mice (Fride et al., 2001). Despite the absence of cannabinoid CB_1 receptors, SR141716A also affected the $CB_1^{-/-}$ mice although to a significantly lesser extent ($F_{strain \times treatment} = 131$, $df = 1,193$, $P < 0.001$, see Fig. 5).

The dramatic effect of SR141716A on milkbands in the wild type pups is shown in Fig. 6A: milkbands were absent the day after injection (day 2) and remain absent permanently. On the other hand, SR141716A significantly affected the occurrence of milkbands in knockout pups on the day after injection; yet a gradual increase in milk

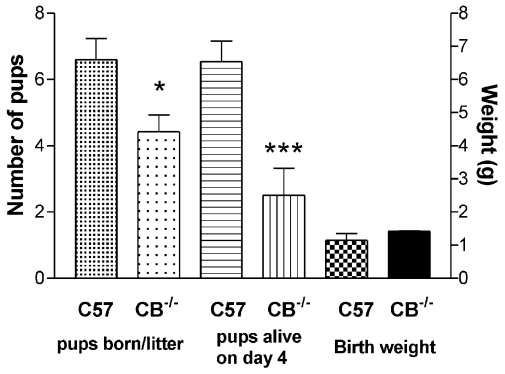

Fig. 3. Litter data in cannabinoid CB_1 receptor knockout mice and wild type (C57BL/6) mice. The number of live pups per litter was recorded on the day of birth as well as on day 4 because frequent cannibalism and a scarcity of milkbands in the knockout pups were observed. Data were derived from 10 wild type and 9 knockout litters. *$P < 0.05$; ***$P < 0.001$, t-tests.

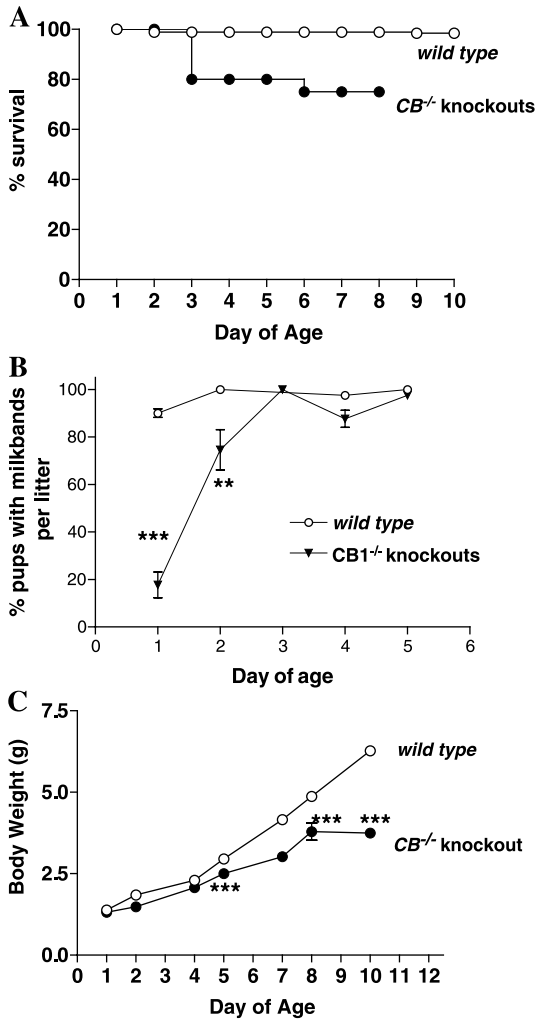

Fig. 4. (A) Developmental data of cannabinoid CB_1 receptor knockout mice. $CB_1^{-/-}$ pups (from 4 litters) displayed about 25% lower survival rates compared to C57BL/6 wild types (from 5 litters) ($F=20.6$, $df=1,141$, $P<0.001$). (B) Milkbands (percent pups/litter) ($F=135$, $df=1,57$, $P<0.001$) and (C) body weights ($F=7.73$ $df=1,89$, $P<0.001$).

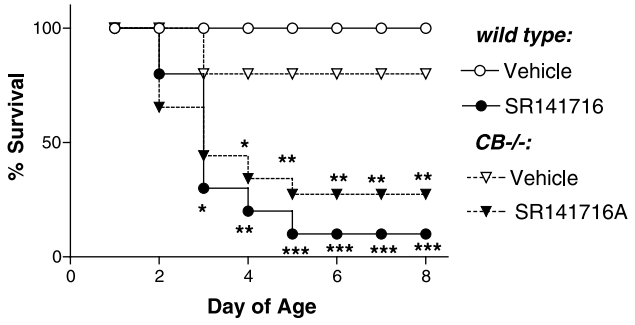

Fig. 5. Effect of SR141716A on the survival of cannabinoid CB_1 receptor knockout pups. On day 1 of life, wild type (4 litters, circles) or $CB_1^{-/-}$ (5 litters, triangles) pups were injected (s.c.) with vehicle (open symbols) (ethanol:emulphor:saline = 1:1:18) or SR141716A (5 litters, 20 mg/kg, closed symbols). Daily assessments were made of the number of live pups. $F_{strain \times treatment}=131$, $df=1,193$, $P<0.001$.

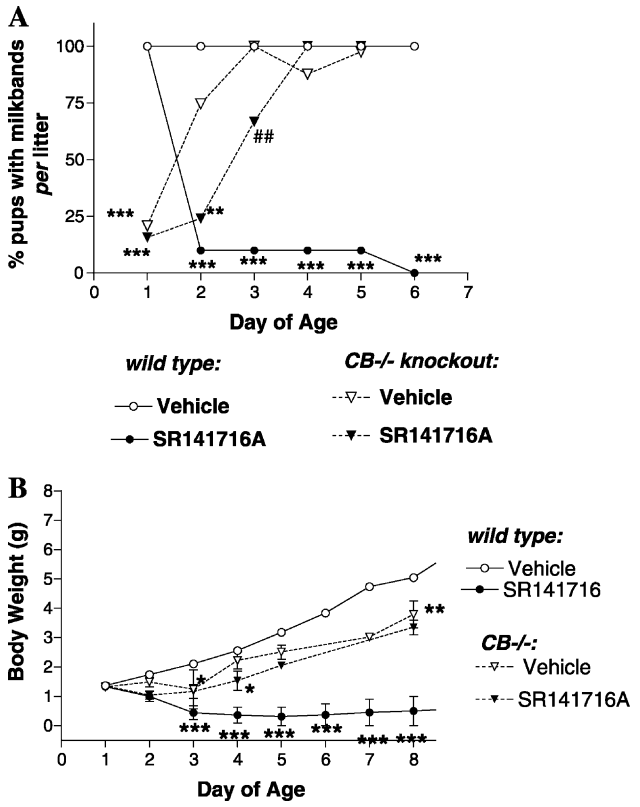

Fig. 6. Effect of SR141716A on body weight and milkbands of cannabinoid CB_1 receptor knockout pups. On day 1 of life, wild type (4 litters, circles) or $CB_1^{-/-}$ (5 litters, triangles) pups were injected (s.c.) with vehicle (open symbols) (ethanol:emulphor:saline = 1:1:18) or SR141716A (5 litters, 20 mg/kg, closed symbols). Daily assessments were made of (A) milkbands ($F_{treat \times strain}=16.5$, $df=1,38$, $P<0.001$; for $CB_1^{-/-}$ only: $F_{treat}=3.57$, $df=1,40$, $P=0.06$; $F_{treat \times strain}=2.74$, $df=4,40$ $P<0.05$) and (B) body weights ($F_{treat \times strain}=131$, $df=1,193$, $P<0.001$).

ingestion was also observed, leading to 100% presence of milkbands by day 4 ($F_{treat \times strain}=16.5$, $df=1,38$; for $CB_1^{-/-}$ only: $F_{treat}=3.57$, $df=1,40$, $P=0.06$; $F_{treat \times strain}=2.74$, $df=4,40$ $P<0.05$).

Body weight gain was virtually absent in SR141716A-treated wild types, consistent with our observations in Sabra mice (Fride et al., 2001). However, weight gain in knockout pups was not significantly affected by the antagonist (Fig. 6B), leading to a significant interactive effect ($F_{treat \times strain}=131$, $df=1,193$, $P<0.001$).

3.4. Effects of cannabinoid CB_1 receptor agonists, CP55,940, WIN55,212-2 and 2-arachidonoyl glycerol on the development of $CB_1^{-/-}$ knockout mice

No enhancement of growth rates or survival of the $CB_1^{-/-}$ knockout mice were recorded after daily injections with any of the CB_1 receptor agonists CP55,940 (5 or 20 mg/kg), WIN55,212-2 (5 mg/kg) or 2-arachidonoyl glycerol (20 mg/kg) (Fig. 7). (The failure of daily injections of tetrahydrocannabinol to enhance body weight gain in normal mice was reported previously, Fride et al., 2001). On the contrary, a

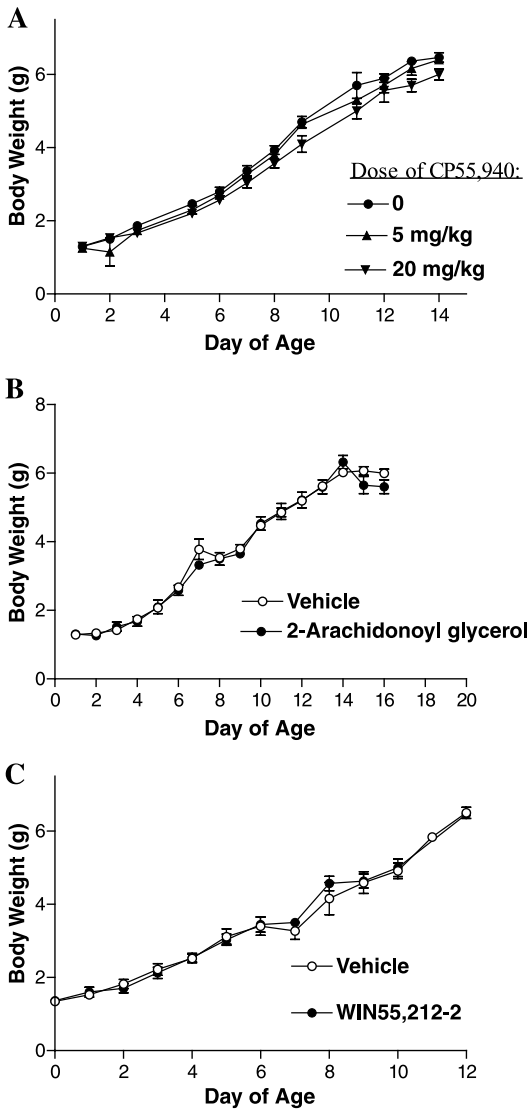

Fig. 7. Effects of daily injections of CP55,940, WIN55,212, or 2-arachidonoyl glycerol on the growth curves of $CB_1^{-/-}$ mouse pups. Developing $CB_1^{-/-}$ knockout mouse pups were injected daily with (A) CP55,940 (5 or 20 mg/kg), (B) WIN55,212-2 (5 mg/kg), and (C) 2-arachidonoyl glycerol (20 mg/kg).

slight, but significant, growth retardation was observed with CP55,940 ($F_{treat} = 14$, $df = 2,74$, $P < 0.001$).

4. Discussion

In the present study, we observed smaller litters and high rates of cannibalism (infanticide) in $CB_1^{-/-}$ knockout mice compared to C57BL/6 wild type mice. We do not know whether the reduced litter sizes at birth were due to the birth of fewer pups or due to maternal cannibalism starting immediately after birth when observers were not consistently present. Thus, although very few births were observed in a number of litters, this phenomenon was not quantified. However, the even lower number of live pups per litter on day 4 of age (see Fig. 3) can be ascribed to maternal cannibalism and possibly other factors such as malnutrition. In their initial study on the $CB_1^{-/-}$ mice, Zimmer et al. (1999) reported increased mortality in adult knockout mice, but no abnormal body weights. Litter data were not reported. No changes in mortality and fertility and birth data were reported regarding the $CB_1^{-/-}$ strain produced by Ledent et al. (1999) using CD1 mice as the wild type.

We have previously reported a devastating effect of the cannabinoid CB_1 receptor antagonist SR141716A when administered to 1-day-old mice in an albino strain (Fride et al., 2001). Thus, the SR141716A-treated pups did not ingest maternal milk (as assessed by "milkbands"), did not gain any weight and, consequently, an almost 100% mortality rate was recorded within the first week of life. In the present study, we generalize this finding to a different mouse strain (C57BL/6) which is the background strain for the cannabinoid CB_1 receptor-deficient mice produced by Zimmer et al. (1999).

We further report in the present study that (untreated) cannabinoid CB_1 receptor-deficient newborns have almost no milk in their stomachs on day 1 after birth (see Fig. 4B). This observation supports a critical role for the endocannabinoid-CB_1 receptor system in the initiation of milk ingestion by newborn mice (Fride et al., 2001).

On the other hand, milk intake from day 2 of life by $CB_1^{-/-}$ mice did not resemble that of SR141716A-treated normal pups (see Fig. 6A). Thus, on day 2 of life, 75% of the $CB_1^{-/-}$ mice displayed milkbands and by day 3, 100% had ingested milk, similarly to controls. Despite the "catch-up" in milk ingestion, however, body weights were significantly lower throughout life. Rather, the delayed onset in suckling observed in the knockout pups resembled the transient effect of cannabinoid CB_1 receptor blockade in normal 5-day-old pups. Thus, when 5-day old C57BL/6 pups were injected with SR141716A, milk ingestion was fully inhibited the next day (see Fig. 2A), but overall weight gain (Fig. 2B) and survival (Fig. 1) were not affected.

We do not know which compensatory mechanism enables the $CB_1^{-/-}$ pups to start ingesting milk on the second day of life in the absence of cannabinoid CB_1 receptors. As the opioid system plays a regulatory role in milk suckling (Petrov et al., 1998) and in view of the rich interactions between the cannabinoid and opioid systems (Maldonado, 2002), it is possible that the opioid system takes over some of the cannabinoid functions in $CB_1^{-/-}$ mice. We are currently investigating potential mechanisms, including motor deficiency or impaired pup-mother contact, which may underlie the failure of SR141716A-treated or untreated $CB_1^{-/-}$ pups to suckle.

Thus far, two cannabinoid receptors have been identified and cloned, CB_1 and CB_2 (Devane et al., 1988; Matsuda et al., 1990; Munro et al., 1993). The cannabinoid CB_1 receptor is present in neural and in nonneural tissue, whereas the cannabinoid CB_2 receptor is exclusively nonneural, detected mainly in immune cells (Pertwee, 1997).

The cannabiniod CB_1 receptor is selectively blocked by SR141716A (Rinaldi-Carmona et al., 1994); SR144528 selectively antagonizes cannabinoid CB_2 (Rinaldi-Carmona et al., 1998).

As we show here, SR141716A had a partial, but significant, detrimental effect on the milk intake and survival of $CB_1^{-/-}$ pups, while weight gain was not affected (see Figs. 5 and 6). These observations are consistent with the existence of a third cannabinoid receptor, which exists in cannabinoid CB_1 receptor-deficient mice, but which, similar to the cannabinoid CB_1 receptor, is blocked by SR141716A. Thus, it is possible that SR141716A affected the $CB_1^{-/-}$ pups by blocking this putative "CB_3" receptor, while an injection of SR141716A in normal mice blocked both cannabinoid CB_1 and "CB_3" receptors. According to this scenario, the "CB_3" receptor is only partially responsible for newborn milk suckling since the cannabinoid CB_1 knockout mice were only partially affected by the antagonist. Alternatively, "CB_3" receptors are only partially blocked by SR14716A, thus explaining the partial effect of SR141716A in the knockout pups.

A third cannabinoid receptor, not blocked by SR141716A, was proposed in previous in vivo (Di Marzo et al., 2000) and in vitro (Breivogel et al., 2001) studies using the same cannabinoid CB_1 receptor knockout mice as in our study. In Breivogel et al.'s (2001) experiments, the cannabinoid CB_1 receptor agonist WIN55,212-2 displayed specific binding to $CB_1^{-/-}$ brain tissue, whereas SR141716A did not specifically affect WIN55,212-2-induced stimulation of [^{35}]GTPγS binding in brains of $CB_1^{-/-}$ mice. In the present study, we attempted to encourage $CB_1^{-/-}$ pup growth with WIN55,212-2 or with 2-arachidonoyl glycerol which is found in high quantities in milk (Fride et al., 2001). We also treated $CB_1^{-/-}$ knockout pups with the potent agonist CP55,940 at two doses (5 and 20 mg/kg). However, none of these "treatments" enhanced body weight gain in the $CB_1^{-/-}$ pups. Therefore, the putative third "CB_3" receptor suggested now has different pharmacological properties compared to the "CB_3" receptor proposed previously (Breivogel et al., 2001; Di Marzo et al., 2000).

In conclusion, we have confirmed a critical role for cannabinoid CB_1 receptors for the initiation of milk suckling within the first 24 h of birth. Timing is essential; administration of the cannabinoid CB_1 receptor antagonist on a later day results in a partial effect (Fride et al., 2001) or no effect at all. We speculate that normally, without experimental interference, endocannabinoids, in particular, 2-arachidonoyl glycerol from the pup's brain which peaks on the first day of life (Berrendero et al., 1999), are required to initiate the suckling response/milk ingestion. We further postulate that from day 2, when levels of 2-arachidonoyl glycerol are lower again (Berrendero et al., 1999), endocannabinoids from maternal milk (Di Marzo, 1998; Fride et al., 2001), maintain the suckling process.

Surprisingly, milk intake and survival were also impaired upon administration of the CB_1 receptor antagonist in CB_1 receptor-deficient pups although not as dramatically as in wild type pups. These results support previous evidence for the existence of additional cannabinoid receptor(s). We suggest two interpretations, both consistent with our finding of a partial effect of SR141716A in the cannabinoid CB1 knockout pups: Either SR141716A partially blocks the putative "CB_3" receptor present on cannabinoid CB_1 knockout mice, or SR141716A is a full antagonist of the "CB_3", but this receptor only partially controls the initial stages of milk ingestion in the newborn mouse.

Acknowledgements

This work was supported by the Danone Research Institute, Israel (to E.F. and H.B.).

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