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REVIEW

Endocannabinoids, feeding and suckling – from our perspective

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In this overview we have summarized some aspects of our published work related to the effects of the endocannabinoid system on appetite and suckling. As noted also by several other groups we have found that anandamide, a major endocannabinoid, enhances appetite in mice. On partial or full food deprivation over 24 h the levels of 2-arachidonoyl glycerol (2-AG), a second major cannabinoid, are initially elevated in mouse brain; however, partial food deprivation over a longer period causes reduction of 2-AG levels. Blocking the endocannabinoid system with a CB1 antagonist on the 1st day after birth leads to inhibition of suckling; later administration also affects suckling, but does not fully block it.
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Introduction

Preparations of the Cannabis plant (marijuana, hashish, ganja, bhang) have been known since times long past, particularly in India, for their enhancement of appetite.[1] However, almost no research was carried out on this aspect of Cannabis action until the identification of the psychoactive constituent, Δ^9-tetrahydrocannabinol (THC).[2] Later, interest in THC as an orexigenic agent rapidly increased when AIDS patients found that their appetite was enhanced and that they lost less weight by smoking marijuana. After animal experiments showed that THC indeed promotes appetite and clinical trials confirmed its activity, this natural product was introduced clinically under the generic name Dronabinol.

With the discovery of the cannabinoid CB_1 receptor and the arachidonic acid derivatives anandamide and 2-arachidonoyl glycerol (2-AG) as endogenous cannabinoids, the field was opened for a thorough investigation of the molecular basis of the action of the cannabinoid system on appetite, weight gain and suckling.

In the present overview, we review and summarize some of the published research carried out in our laboratories in Jerusalem and Naples in the above areas.

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The endocannabinoid system in suckling

Our first foray in this field was the result of a publication in Nature on the presence of anandamide in chocolate.[3] As arachidonic acid derivatives are very rare in plants we looked for anandamide in Israeli-made chocolate and found essentially none. However, we found that oleamide, a sleep producing endocannabinoid-like compound is present, as it is in soybean, hazelnuts, oatmeal and millet.[4] Oleamide is an inhibitor of fatty acid amide hydrolase, the enzyme that breaks down anandamide. Hence, it seems plausible that foods may affect endogenous levels of anandamide and its effects. Surprisingly this line of research has not been pursued, although we reported that oleamide at high doses (200 mg/kg) causes anandamide-like effects, associated with movement, sedation, body temperature and pain. Is it possible that oleamide and *N*-acylethanolamines (NAE), which are present in plants, and which prevent hydrolysis of anandamide, affect some of the subtle effects of endocannabinoids in man?

The possible effects of food on endocannabinoid levels led us to take a look at milk – the only food of newborn mammalians.[5] We first examined the levels of anandamide, 2-AG, oleamide and several NAE's in human as well as bovine and goat milk. While the levels of anandamide were negligible, all milks were found to contain 2-AG (1–9 μ/g of extracted lipids) and considerably higher levels of oleamide,

2-palmitoyl glycerol and 2-linoleoyl glycerol. The presence of 2-AG in milk then led us to examine whether the cannabinoid CB_1 receptor is involved in suckling. The cannabinoid CB_1 receptor antagonist, SR141716A (20 mg/kg), completely inhibited the physical growth of mouse pups and caused death within 1 week, by depriving them of the essential benefits of nursing. This devastating effect of SR141716A was seen after daily injections 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-AG. This is also consistent with our observation that 2-AG is present in mammalian milk from the 1st day after birth ('early' milk or colostrum).[5]

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.

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 and milk bands were observed, 5 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.[5] When THC was co-administered with SR141716A, the detrimental effects on weight gain and feeding were almost completely reversed. Co-administration of cannabidiol, a cannabinoid which does not bind cannabinoid CB_1 receptors, had no influence on SR141716A-induced effects. 2-Arachidonoyl glycerol by itself had no effect on the SR141716A-induced growth stunting. This lack of effect of exogenous 2-AG alone is probably due to the ease of its degradation by enzymatic hydrolysis of its ester bond and to its rapid uptake. However, endogenous 2-AG may be partly protected from hydrolysis and its uptake may be slowed down by monoacyl glycerols. As mentioned above, in all milk samples analyzed by us 2-AG is accompanied by several 2-acyl glycerols. 2-Palmitoyl glycerol and 2-linoleoyl glycerol, which do not bind to cannabinoid CB_1 receptors cause no cannabis-type effects as evaluated in binding assays and in several *in vivo* assays in mice. In all these assays, however, 2-AG activity was significantly enhanced by 2-palmitoyl glycerol and 2-linoleoyl glycerol (an effect named by us 'entourage effect'). If 2-AG is consumed by pups with the milk, the 'entourage' compounds that are present in milk may enhance its suckling activity. Therefore, we evaluated the activity of 2-AG in the presence of the 'entourage' compounds. We found that co-administration of 2-AG (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-AG. However, dose ranges may have to be investigated further in order to find the maximal 'entourage' effect, which appears to be a rather unique mechanism of enhancing biological activity.

The above data strongly suggest that the antisuckling and growth-inhibiting effects of SR141716A, are mediated by blockage of the cannabinoid CB_1 receptors.

The dose of SR141716A needed for a complete inhibition 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 1st week of life in developing pups. Moreover, although lower doses are often found sufficient to block some THC-induced effects, many effects of the antagonist require higher doses.

Zimmer *et al*.[6] have found that cannabinoid CB_1 receptor knockout 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*.[7] 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 Contos *et al*.[8] have described 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 receptor.[9] There is also a chemical relationship between the two families of bioactive compounds. The chemical structures of 2-AG and lysophosphatidic acid (with 2-arachidonoyl as the acyl moiety) only differ by the absence of a phosphate group in 2-AG.

In a later study,[10] we generalized the above findings 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*. We found that (untreated) cannabinoid CB_1 receptor-deficient newborns have almost no milk in their stomachs on day 1 after birth. This observation supports a critical role for the endocannabinoid-CB_1 receptor system in the initiation of milk ingestion by newborn mice. On the other hand, milk intake from day 2 of life by $CB_1^{-/-}$ mice did not resemble that of SR141716A-treated normal pups. 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, but overall weight gain and survival were not affected.

We do not know which compensatory mechanism enables the $CB_1^{-/-}$ pups to start ingesting milk on the 2nd day of life in the absence of cannabinoid CB_1 receptors. As the opioid system plays a regulatory role in milk suckling (see above) and in view of the rich interactions between the cannabinoid and opioid systems, it is possible that the opioid system takes over some of the cannabinoid functions in $CB_1^{-/-}$ mice.

Thus far, two cannabinoid receptors have been identified and cloned, CB_1 and CB_2. The cannabinoid CB_1 receptor is present in neural and in non-neural tissue, whereas the cannabinoid CB_2 receptor is detected mainly in immune cells. Our observations[10] are consistent with the existence of a third cannabinoid receptor, present 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 affects the $CB_1^{-/-}$ pups by blocking this putative 'CB_3' receptor, while an injection of SR141716A in normal mice blocks 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, has been proposed in previous *in vivo* and *in vitro* studies using the same cannabinoid CB_1 receptor knockout mice as in our study.[11] 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 our study, we attempted to encourage $CB_1^{-/-}$ pup growth with WIN55,212-2 or with 2-AG which, as mentioned above, is found in high quantities in milk. 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 by us has different pharmacological properties compared to the 'CB_3' receptor proposed previously.

In conclusion of this portion of our studies, we can state that we have confirmed a critical role for cannabinoid CB receptors for the initiation of milk suckling within the first 24 h of birth. Timing is essential: administration of the cannabinoid CB receptor antagonist on a later day results in a partial effect or no effect at all. We speculate that normally, without experimental interference, endocannabinoids, in particular, 2-AG from the pup's brain which peaks on the 1st day of life, are required to initiate the suckling response/milk ingestion. We further postulate that from day 2, when levels of 2-AG are lower again, endocannabinoids from maternal milk, 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.

Effects of endocannabinoids on appetite

The research on the effect of cannabinoids on feeding and appetite has been summarized in several reviews.[12,13] Of particular relevance to our work is the report by Williams and Kirkham[14] who found that anandamide (0.5, 1, 5, 10 mg/kg) increased food intake in male rats during a 3-h feeding period and that this effect was blocked by the CB_1 antagonist, SR 141716A. In a different protocol (female mice on a 40% diet restriction) we found that doses as low as 0.001 mg/kg enhanced food intake.[15] Anandamide-treated mice consumed 44% more food during 1 week of 2.5 h feeding each day. Doses of 0.7 and 4 mg/day did not cause any significant change. This unexpected behavior has precedence. We have shown that anandamide produces a bi-phasic dose–response in both behavior and neuro-biochemistry.[16] Thus, at low doses, it stimulated ambulation and rearing, as well as gut motility in the open field situation, decreased the rate of immobility on ring standing, and analgesia on a hot plate; it also stimulated aggressive behavior in timid mice and phagocytosis by mouse leukocytes. At high doses, opposite effects of inhibition were observed.

In an early review on the effects of Cannabis, Paton and Pertwee[17] wrote: 'Nor does one readily find another substance so contradictory, capable of taming yet producing aggressiveness, of both enhancing and depressing spontaneous activity, of being anticonvulsant yet generating epileptiform cortical discharges.' Another early example of a cannabinoid biphasic effect is reported by McLaughlin *et al*.[18] They showed that THC, when administered to sheep, initially increased food intake, but later the intake decreased.

Endocannabinoid levels in brain – relevance to appetite

Several groups have looked into the presence and levels of endocannabinoids in the brain and have established excellent methods for analyses of endocannabinoids. Endocannabinoid levels are very sensitive to biochemical and

pathological changes in the brain. Thus, Di Marzo *et al*. have reported[19] the presence of anandamide and 2-AG in two regions of the basal ganglia, the globus pallidus and substantia nigra. In the reserpine-treated rat, an animal model of Parkinson's disease, suppression of locomotion was accompanied by a sevenfold increase in the levels of the 2-AG (up to around 5.0 nmol/g) in the globus pallidus, but not in the other five brain regions analyzed. Defective leptin signalling is also associated with elevated hypothalamic, but not cerebellar, levels of endocannabinoids in obese db/db and ob/ob mice and Zucker rats. Acute leptin treatment of normal rats and ob/ob mice reduces anandamide and 2-AG levels in the hypothalamus. These findings indicate that endocannabinoids in the hypothalamus may tonically activate CB_1 receptors to enhance food intake and thus form part of the neural circuitry regulated by leptin.

In our original publication, in which we described 2-AG as an endogenous cannabinoid, we recorded that this compound is present in about 5.0 nmol/g wet weight of mouse spleen tissue.[20] In a later study, we found that in isolated rat aorta under cholinergic stimulation by carbachol the production of 2-AG was enhanced about fivefold (from 0.41 to 2.0 nmol/g wet weight).[21] We have also shown that, after injury to the mouse brain, 2-AG levels were significantly elevated (in 4 h the level of 2-AG increased on average from 9.18 to 105.4 nmol/g and after 24 h it still remained at a level of 56.0 nmol/g).[22]

The first direct evidence of altered brain levels of endocannabinoids (2-AG particularly) during fasting and feeding was reported by Kirkham *et al*.,[23] who measured anandamide and 2-AG levels in brain regions of rats, during fasting (24 h), feeding of palatable food, or after satiation. Endocannabinoid levels were compared to those in rats fed *ad libitum*. Fasting increased levels of anandamide and 2-AG in the limbic forebrain and, to a lesser extent, of 2-AG in the hypothalamus. By contrast, hypothalamic 2-AG declined as animals ate. No changes were detected in satiated rats. Endocannabinoid levels in the cerebellum, a control region not directly involved in the control of food intake, were unaffected by any manipulation. 2-Arachidonoyl glycerol was sensitive to variations during feeding and this observation supports a role for endocannabinoids in the control of appetitive motivation. It was also demonstrated that 2-AG can reliably stimulate eating.

Our aim was to establish whether a 12-day food restriction regimen in mice affects 2-AG levels in the whole brain, in the hypothalamus, a brain area associated with feeding, and in the hippocampus, a brain area associated with cognitive functions.[24] We assumed that the results of such measurements may be of some relevance to starvation and/or to anorexia nervosa – a psychiatric condition in which the patients impose diet restrictions on themselves resulting not only in reduced food intake but also in multiple endocrine abnormalities. Our results showed that diet restriction over 12 days lowered the levels of 2-AG both in the hippocampus and the hypothalamus, although differences were observed. The amounts of 2-AG in the hypothalamus depended on the severity of the diet restriction, while in the hippocampus the amounts of 2-AG fell to a common level, irrespective of the diet restriction protocol.[24]

Our results differ from those reported from Kirkham *et al*. presumably due to the non-identical experimental conditions.[23] A major difference between the experiments described by Kirkham *et al*. and ours is the food restriction protocol. While Kirkham *et al*. report that the rats used in their experiment were under severe food restriction (20% of their normal daily intake) and were killed after 24 h, the mice used in the experiments described here were on a less severe food restriction (see above) for a considerably longer period (12 days). Thus, Kirkham *et al*. apparently measure the effects of hunger, while we measure presumably the effect of semistarvation and our observations may be relevant to the physiology of starvation and anorexia nervosa. In order to reconcile our results with those of Kirkham *et al*. we analyzed mouse brain after 24 h of full starvation (fasting). Indeed, as in the British report, we found significant enhancement of 2-AG levels, contrary to the decrease of 2-AG levels after 12 days of diet restriction described above. It is well established that endocannabinoids enhance appetite and it is reasonable to expect that over short periods of hunger 2-AG levels will be enhanced, as reported by Kirkham *et al*., and confirmed by us. In our view, the lowering of 2-AG levels after 12 days is compatible with the needs of an animal on starvation. Potentiation of hunger, caused by high levels of endocannabinoids, presumably will be detrimental in these conditions.

We assumed that supplementation of food such as soya containing polyunsaturated fatty acids, in particular linoleate (18:2*n*6) which may be converted into arachidonate, would elevate the 2-AG levels and could counteract the effect of diet restriction. We were surprised to find that such supplementation actually significantly lowered 2-AG levels in non-diet-restricted mice and in the hippocampus in mice on 50% diet restriction. We also assumed that 20% soya oil might cause end product inhibition of 2-AG synthetic enzymes by excess arachidonate and therefore decreased the soya oil level to 5%. Although a strong lowering trend of 2-AG levels in total brain was noted, this effect did not reach statistical significance. Other types of oil also did not cause any significant change in 2-AG levels. Berger *et al*.[25] in a study on piglets also reported that 2-AG levels exhibited a strong trend towards decrease if the milk was supplemented with the long chain unsaturated fatty acids.

If the above observations on diet restriction in mice parallel the human condition, we can expect that diet restriction self-imposed by humans, as in anorexia, may cause lowering of 2-AG levels leading to further reduction of food consumption, thus perpetuating the clinical condition.

The observed lowering of 2-AG levels in mice on prolonged diet restriction may also represent a general coping (psychobehavioral) strategy for intermittent starvation when food is scarce – most wild animal species certainly undergo periods of starvation. As we are all well aware

starvation was also a common occurrence in human populations in former times, or even today, in certain parts of the world.

Possible therapeutic implications

Patients with anorexia nervosa refuse to eat and show decreased concentrations of tyrosine, norepinephrine metabolites in the cerebrospinal fluid and a variety of endocrine abnormalities. Some patients have memory and learning deficits. Two brain areas are prominent in these processes – the hippocampus, which is involved in aspects of cognitive function and the hypothalamus, the center for the regulation of systemic energy balance. In our study, anandamide administration was able to reverse many of the neurochemical and behavioral deficits following semistarvation, even before weight gain. Such properties could have therapeutic potential in the treatment of cachexia associated with cancer and AIDS, the side effects of weight loss and in the maintenance of a reduced body weight and also in the extreme case of anorexia nervosa. However, much further work is required in studying the effects of chronic cannabinoid treatment on these functions in animals at different levels of systemic energy balance.

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