Short-term fasting and prolonged semistarvation have opposite effects on 2-AG levels in mouse brain

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Abstract

2-Arachidonoyl glycerol (2-AG) levels in whole mouse brain and two of its regions—hippocampus and hypothalamus—were determined after diet restriction (between 60 and 40%) lasting 12 days. The diet restriction lowered the level of 2-AG, which in the hypothalamus depended on the severity of the diet restriction, while the level in the hippocampus was not dependent on the diet regimen. As these observations differ from previously published data showing elevation of 2-AG levels in rat brain after 24 h of severe food restriction, we measured 2-AG levels in whole mouse brain after a comparable period of full starvation (fasting). We confirmed the elevation of 2-AG levels. It seems possible that these time-dependent variations of 2-AG levels may be of importance as a general coping strategy by animals during periods of starvation.

2. Introduction

2-Arachidonoyl glycerol (2-AG) is the most abundant endogenous cannabinoid present in the mammalian body. It was first found in canine gut [24] and later in rat brain [36]. While numerous other endocannabinoids, such as arachidonoyl ethanolamide (anandamide) [7], homö-linoleoyl ethanolamide, docosatetraenoyl ethanolamide [12], noładin [13] and virodhamine [27] are now known, they are present in considerably lower amounts.

Both anandamide and 2-AG bind to the CB₁ cannabinoid receptor. While anandamide binds the receptor with moderate affinity and has the characteristics of a partial agonist, 2-AG binds with low affinity, but exhibits full efficacy [35,34].

Stella et al. have reported that the brain levels of 2-AG in the rat are about 4.0 nmol/g brain tissue, although some of it is actually present as 1-AG, an isomerisation product, formed from 2-AG mostly during the extraction (work-up) and analytical procedures [29]. This amount is about 170 times higher than the amount of anandamide found in brain (23 pmol/g). Similar values have been recorded by Sugiura et al. [32], namely 3.25–4.75 nmol/g wet weight of rat brain. They also recorded isomerization to 1-AG (about 30%). 2-AG is one of the most abundant molecular species of monoacylglycerols in the rat brain (44.1% of the total monoacylglycerols). Numerous other 2-mono-acyl glycerol esters are found together with 2-AG. Some of them are known to enhance the activity of 2-AG (a phenomenon named ‘the entourage effect’) [3].

Substantial amounts of 2-AG are also found in the liver...
of rats (1.15 nmol/g tissue), spleen (1.17 nmol/g tissue), lung (0.78 nmol/g tissue) and kidney (0.98 nmol/g tissue), but the levels are considerably lower than that in the brain [21]. While a small amount of 2-AG is generated in a brain homogenate during incubation in the absence of Ca$^{2+}$, the generation of 2-AG is markedly augmented in the presence of Ca$^{2+}$, suggesting that Ca$^{2+}$ plays a key role in regulation of the generation of 2-AG in this tissue [21,33].

More recently the amount of 2-AG (2.0–14.0 nmol/g) was determined by isotope-dilution gas chromatography–mass spectrometry in nine different rat brain regions (brainstem, striatum, hippocampus, medulla, mesencephalon, limbic forebrain, cerebellum, cortex and diencephalon) [6]. The highest amounts of 2-AG was found in the brainstem and striatum.

Sugiura et al. [31] have shown that rapid determination of brain levels of 2-AG is important in order to minimize the effects of post-mortem formation. 2-AG can presumably be formed from diacylglycerols, containing the arachidonoyl moiety at the C-2 position and from (2-arachidonoyl) lysophosphatidic acid, as both types of compounds are known to be present in brain [2,25].

We have shown that 2-AG levels are very significantly enhanced in mouse brain after close head injury and that this effect represents a protective response [26]. Hansen et al. have recorded similar observations with anandamide in rat brains [11]. 2-AG levels are also elevated in ob/ob mice which lack leptin [9]. Berger et al. have shown that 100% of mice which consumed different oils.

2. Materials and methods

2.1. Animals

Young female Sabra mice were assigned at random to different groups of five mice to a cage (each group contained 10 mice). All cages contained wood-chip bedding and were placed in a temperature-controlled room at 22 °C, on a 12-h light/dark cycle (lights on at 07.00 h). The mice had free access to water 24 h a day. The food provided was Purina chow with the following nutritional values: 54.9% carbohydrate, 21.1% protein and 4.7% fat. The food was given at 10.00 h and left for 24 h. The mice were fed a food restriction diet (DR) for 12 days. DR 100% represents control mice, which received a diet of approximately 95 kcal/week per mouse, e.g., 3.6 g/day per mouse as suggested by Ingram et al. [17]. This diet kept stable the weight of the mice. A restricted diet DR 60% represents approximately 57 kcal/week; and DR 40% represents approximately 47.5 kcal/week; and DR 50% represents approximately 38 kcal/week. In some 100% DR experiments the diet was supplemented with 20% soya, or 5% soya, olive or coconut oil.

The dietary supplements were mixed and produced a homogeneous mixture with the food. The mice had not consumed comparable diet before the experiment and there was no significant weight change between the mice on DR 100% which consumed different oils.

The weights of all animals were similar (36.70±0.15 g) at the beginning of each experiment. In each cage there were two containers of food, which facilitated free access. Body weight was measured twice a week during each experiment. Only small, insignificant, variations in body weight between the animals were observed in each set of experiments, which showed that all mice in a group had consumed similar amounts (data not shown).

Mice were decapitated between 10.00 and 12.00 h after 12 days on diet restriction (see above) or 24 h of full starvation (fasing) or after consumption ad libitum for 24 h.

Brains were rapidly removed and the hypothalamus and hippocampus were dissected out.

2.2. Determination of 2-arachidonoylglycerol (2-AG)

Whole brains, and/or parts (hippocampus, hypothalamus) of young female Sabra mice were weighed and placed in glass tubes containing 7 ml chloroform–methanol (2:1) immediately after decapitation, and manually homogenized in a glass homogenizer (Pyrex) for 1–2 min. As an internal standard, 10 μL of 1 mM arachidinin were added. Vacuum filtration was carried out and the filter was washed three times with the organic mixture. The solvents were evaporated to dryness using a rotary evaporator under reduced pressure at 35 °C and 1 ml of absolute ethanol was added to ensure removal of water from the solid residue.
The solution was then evaporated again to dryness, the solid residue was re-dissolved in 2 ml of chloroform and dried out under nitrogen.

Pre-coated TLC plates (20×20 cm, with silica gel 60 and thickness 0.25 mm with fluorescent indicator; Merck) were used to separate 2-AG and arachidinin from other lipids. Synthetic 2-AG and arachidinin were applied alongside the unknown lipid, dissolved in chloroform, to help in their identification in the lipid sample.

The solvent system for developing of the sample was hexane–ether–acetic acid (40:20:7:1, v/v). 2-AG and arachidinin were identified with a visualizing reagent (phosphomolybdic acid). Developed silica gel layer at the height of this two compounds was scrapped, placed into tube and extracted with 10 ml of chloroform–methanol (19:1). After two repeated extractions the fractions were collected into a flask and evaporated to dryness on a rotary evaporator at 35 °C. The solid residue was dissolved in 2 ml of chloroform and dried under a stream of nitrogen.

2.3. GC–MS analysis

For quantitative analysis the samples were analyzed by GC–MS in a Hewlett-Packard G1800A GCD system with a HP-5971 gas chromatograph with electron ionization detector. Ultra low-bleed 5% phenyl capillary column (28-m×0.25-mm (i.d.)×0.25-μm film thickness) based on diphenyl methylsiloxane chemistry (HP-5MS; Agilent Technologies) was used. This column was temperature programmed from 150 to 280 °C at 50 °C/min (inlet, 250 °C; detector, 280 °C; splitless injection/purge time, 1.0 min; initial temperature, 180 °C; initial time, 2.0 min) using selective ion monitoring (SIM). The selected qualifier ions were m/z 103 (100%), 74 (84%) and 129 (50%) for 2-AG and m/z 73 (100%), 57 (57.1%) and 147 (49.6%) for arachidinin. Quantifier ions were m/z 218 for 2-AG and 427 for arachidinin, respectively. 2-AG concentrations from 0.5 to 16 nmol were used for the calibration curve together with 10 nmol arachidinin.

The solid residue, separated by TLC (vide supra), was dissolved in 1 ml of chloroform, dried under nitrogen, transferred with 100 μl chloroform into a small vial and dried again. Ten μl of bis(trimethylsilyl)trifluoroacetamide (BSTFA) were added in order to silylate the free hydroxyl groups. One μl of this solution was injected into the GC–MS. 2-AG and the standard arachidinin were eluted after 9.0 and 10.34 min, respectively. The ratio between 2-AG and the standard was used to calculate the level of 2-AG in the brain.

2.4. Statistical analysis

Programs GraphPad Prism (Version 3.02) and GraphPad InStat (Version 3.05) were used. Statistical analysis was realized as one-way ANOVA (and nonparametric) with Newman–Keuls test (compare all pairs of columns). With confidence interval 95%, results were evaluated as not significant (ns, \( P > 0.05 \)), significant (*\( P = 0.001–0.05 \)), very significant (**\( P = 0.001–0.01 \)) and extremely significant (**\( P < 0.001 \)).

3. Results

3.1. Body weight

All groups had identical body weight at the beginning of each experiment namely 36.7 ± 0.15 g. At the end of each trial mice on 60, 50 and 40% DR had lower weight than the control (100%); the 60% group decreased to an average weight of 30.1 ± 0.5 g (\( P < 0.001 \)); the 50% group to 27.9 ± 0.5 g (\( P < 0.001 \)) and the 40% to 28.9 ± 0.6 g (\( P < 0.001 \)) (Figs. 1 and 2).

3.2. Levels of 2-AG in the mouse brain and its parts

Diet restriction over 12 days led to lower levels of 2-AG in whole mouse brain as well as in hippocampus and hypothalamus compared to 100% diet restricted (DR) control mice (Fig. 3). In the whole brain 2-AG levels decreased from 31.220 ± 0.707 on a 100% diet to 18.690 ± 1.535 nmol/g on a 50% diet restriction. In the hypothalamus a gradual decrease was observed depending on the severity of the diet restriction. Thus, in the hypothalamus of control animals the 2-AG levels were 7.928 ± 0.949 nmol/g brain wet tissue, while those on 60, 50 and 40% DR had levels of 4.973 ± 1.431, 3.604 ± 0.172 and 2.745 ± 0.669 nmol/g brain wet tissue, respectively. The \( P \) values are presented in the legend of Fig. 3. In the hippocampus the picture was different. In the control animals the 2-AG levels were 2.679 ± 0.216 nmol/g. All diet restriction regimens led to lower values (1.338 ± 0.317; 1.990 ± 0.1132; 1.406 ± 0.0962 nmol/g, for the 60, 50 and 40% DR, respectively), but the differences between the regimens were not significant. However the differences between the levels of 2-AG in the 60, 50, 40% DR animals and the 100% DR control group were significant (Fig. 3).

Addition of 20% soya oil to the 100% DR diet significantly lowered the levels of 2-AG in the whole brain, in the hypothalamus and in the hippocampus as compared with the levels in organs of animals which were on 100% diet restriction without oil supplementation. Thus, addition of 20% soya oil to the food of mice decreased the levels of 2-AG from 31.220 ± 0.707 to 14.120 ± 0.995 nmol/g in the whole brain (Fig. 4A), from 2.679 ± 0.2160 to 1.183 ± 0.1713 in the hippocampus (Fig. 4B) and from 9.928 ± 0.994 to 2.925 ± 0.470 in the hypothalamus (Fig. 4C). Addition of 20% soya oil to the diet of mice which were on 50% DR (which by itself causes 2-AG reduction—see Fig. 3) did not cause further lowering of 2-AG in the whole brain and hypothalamus (Fig. 4A,C), but a
significant reduction was observed in the hippocampus (from 1.990±0.1132 to 1.243±0.0612 nmol/g) (Fig. 4B). Contrary to the addition of 20% soya oil, addition of 5% soya oil to the food of mice on 100% DR did not cause significant reduction in 2-AG level in total brain, although a non-significant trend was noted (from 31.220±0.707 to 20.260±2.950). Addition of 5% coconut oil or olive oil to mice on 100% DR caused neither significant changes nor strong trends of change of 2-AG levels in total brain. However, a significant difference between the 2-AG levels in mice supplemented with soya oil (5%) and olive oil (5%) was observed.

As our findings of lowered 2-AG levels after 12 days of diet restriction differ from those of Kirkham et al. [20], measured after 24 h, who found elevation in 2-AG levels, we analysed mouse brain after 24 h of full starvation (fasting with free access to water). We also found significant enhancement of 2-AG levels (59.100±8.478 for the starved animals vs. 28.730±4.825 nmol/g brain for animals on a ad libitum diet) (Fig. 5).

4. Discussion

It is well established that endocannabinoids affect appetite and food consumption. For recent reviews see Berry and Mechoulam [5] and Kirkham and Williams [19]. Williams and Kirkham have reported that anandamide
Fig. 3. Effects of diet restriction over 12 days on 2-AG levels in mouse hypothalamus (A) and hippocampus (B). Diet restriction (60, 50 and 40%) causes dose-dependent, significant lowering of 2-AG levels (compared to 100%) in hypothalamus (A) and none dose-dependent lowering in the hippocampus (B). Data represent the means±S.E.M. from each group (five to 10 mice). The level of 2-AG was determined in each animal and the results represent the average of (5–10) mice from each group. Key: (A) 60 vs. 100% (P<0.05); 50 vs. 100% (P<0.05) and 40 vs. 100% (P<0.01); (B) 60 vs. 100% (P<0.01); 50 vs. 100% (P<0.05) and 40 vs. 100% (P<0.01).

(0.5–10 mg/kg) causes stimulation of feeding in rats, which can be blocked by the CB₁ cannabinoid receptor antagonist SR-141716A [37]. Hao et al. have also observed stimulation by anandamide of food consumption by mice at the considerably lower dose of 1 μg/kg [14]. Kirkham et al. also found that 2-AG potently and dose-dependently stimulated feeding, an effect also attenuated by SR-141716A [20]. Numerous groups have noted that Δ⁹-tetrahydrocannabinol (THC) is a potent stimulator of feeding [5,19]. Indeed THC (generic name dronabinol) is an approved drug for appetite enhancement, particularly in

Fig. 4. Effects of diet restriction and 20% soya oil on 2-AG levels in mouse whole brain (A), hippocampus (B) and hypothalamus (C). Soya oil (20% in food) led to lowering of 2-AG levels in mice on 100% diet restriction (DR) in whole brain (A), hippocampus (B) and hypothalamus (C). Soya oil (20%) in mice on 50% DR led to significant lower levels in the hippocampus (B). Key (only the statistically significant data are presented): (A) 100%+oil vs. 100% (P<0.001); (B) 100%+oil vs. 100% (0.001) and 50%+oil vs. 50% (P<0.01); (C) 100%+oil vs. 100% (P<0.001).
obese db/db and ob/ob mice and Zucker rats. Acute leptin treatment of normal rats and ob/ob mice reduces anandamide and 2-arachidonoyl glycerol levels in the hypothalamus. These findings indicate that endocannabinoids in the hypothalamus may tonically activate CB₁ receptors to enhance food intake and thus form part of the neural circuitry regulated by leptin [9].

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 [24]. In a later study we found that in isolated rat aorta under cholinergic stimulation by carbachol the production of 2-AG was enhanced about 5-fold (from 0.41 to 2.0 nmol/g wet weight) [23]. 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 in average from 9.18 to 105.42 nmol/g and after 24 h it still remained at a level of 56.00 nmol/g) [26].

The first direct evidence of altered brain levels of endocannabinoids (2-AG particularly) during fasting and feeding was reported by Kirkham et al., who measured anandamide and 2-AG levels in brain regions of rats, during fasting (24 h), feeding of palatable food, or after satiation [20]. 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-AG 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. 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 [10,30]. Our results showed that diet restriction over 12 days lowered the levels of 2-AG both in the hypothalamus and the hippocampus, 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.

Our results differ from those reported from Kirkham et al. [20] (see above) presumably due to the non-identical experimental conditions. A major difference between the experiments described by Kirkham et al. and ours is the

AIDS patients, while SR-141716A reduces hunger, caloric intake and body weight in obese men [16]. A better understanding of the role of the endocannabinoids could be of importance in finding novel therapeutic leads in diseases such as anorexia nervosa.

Chronic voluntary and involuntary weight loss may lead to similar changes in autonomic tone [22] and brain neurotransmitters [1,15,18,28]. These may lead to endocrine and neurobehavioral changes similar to those found in anorexia nervosa. We have studied other models such as enhanced activity and enforced separation, which also cause similar changes [22,1,15,18,28]. Thus DR is an attempt to model anorexia nervosa although it is yet not sufficiently established that the two conditions are fully comparable.

Several groups have looked into the presence and levels of endocannabinoids in the brain and have established excellent methods for analyses of endocannabinoids. Some of the methods described, and the data reported so far, are presented in Section 1.

Endocannabinoid levels are very sensitive to biochemical and pathological changes in the brain. Thus, Di Marzo et al. have reported the presence of anandamide and 2-AG in two regions of the basal ganglia, the globus pallidus and substantia nigra [8]. In the reserpine-treated rat, an animal model of Parkinson’s disease, suppression of locomotion was accompanied by a 7-fold 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

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**Fig. 5.** Effect of ad libitum and starvation over 24 h on total brain levels of 2-AG. Key (only the statistically significant data are presented): ad libitum versus 24 h fast (P < 0.05).
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 (Fig. 5), contrary to the decrease of 2-AG levels after 12 days of diet restriction described above. It is well established that endocannabinoids enhance appetite (for reviews, see Refs. [5,19]) 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 now 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 (18:2n−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 (Fig. 4) in the 100% DR mice and in the hippocampus in mice on 50% DR mice. 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% in mice on 100% DR. 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. [4], in their study on piglets detected that 2AG levels exhibited a strong trend towards decrease (although the level of significance was only $P=0.1$) if the milk was supplemented with the long chain PUFAs.

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

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