British Journal of Pharmacology (2002) 136, 550–557
www.nature.com/bjp

Endocannabinoid levels in rat limbic forebrain and hypothalamus in relation to fasting, feeding and satiation: stimulation of eating by 2-arachidonoyl glycerol

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1 Endocannabinoids are implicated in appetite and body weight regulation. In rodents, anandamide stimulates eating by actions at central CB1 receptors, and hypothalamic endocannabinoids may be under the negative control of leptin. However, changes to brain endocannabinoid levels in direct relation to feeding or changing nutritional status have not been investigated.

2 We measured anandamide and 2-arachidonoyl glycerol (2-AG) levels in feeding-associated brain regions of rats, during fasting, feeding of a palatable food, or after satiation. Endocannabinoid levels were compared to those in rats fed *ad libitum*, at a point in their daily cycle when motivation to eat was absent. 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.

3 As 2-AG was most sensitive to variation during feeding, and to leptin regulation in a previous study, we examined the behavioural effects of 2-AG when injected into the nucleus accumbens shell, a limbic forebrain area strongly linked to eating motivation. 2-AG potently, and dose-dependently, stimulated feeding. This effect was attenuated by the CB1 receptor antagonist SR141716.

4 These findings provide the first direct evidence of altered brain levels of endocannabinoids, and of 2-AG in particular, during fasting and feeding. The nature of these effects supports a role for endocannabinoids in the control of appetitive motivation.

British Journal of Pharmacology (2002) **136,** 550–557

Keywords: Cannabinoid; anandamide; 2-arachidonoyl glycerol; nucleus accumbens; appetite; SR141716

Abbreviations: AcbSh, nucleus accumbens shell; 2-AG, 2-arachidonoyl glycerol; GC-MS, gas chromatography-mass spectrometry; LV, lateral ventricle; NP-HPLC; normal phase high pressure liquid chromatography; Δ^9-THC, Δ^9-tetrahydrocannabinol

Introduction

The discovery of cannabinoid receptors within the central nervous system (Devane *et al*., 1988; Matsuda *et al*., 1990), and their endogenous ligands, the endocannabinoids arachidonoyl ethanolamide (anandamide; Devane *et al*., 1992), 2-arachidonoyl glycerol (2-AG; Mechoulam *et al*., 1995, Sugiura *et al*., 1995), and 2-arachidonyl glyceryl ether (noladin ether; Hanus *et al*., 2001) has generated considerable research into their behavioural significance. There is increasing evidence for a role of endocannabinoid systems in the regulation of appetite (Kirkham & Williams, 2001a), with both the exogenous cannabinoid, Δ^9-tetrahydrocannabinol (Δ^9 -THC) and anandamide reported to reliably induce overeating (Foltin *et al*., 1988; Williams & Kirkham, 1998; Williams & Kirkham, 1999; 2002a; Hao *et al*., 2000; Jamshidi & Taylor, 2001; Koch, 2001; Koch & Matthews, 2001). These hyperphagic actions are mediated by central type CB1 cannabinoid receptors (Williams & Kirkham, 1999; 2002a), since they are selectively blocked by the CB1 antagonist, SR141716, but not SR144258, an antagonist of peripherally expressed CB2 receptors (Munro *et al*., 1993; Rinaldi-Carmona *et al*., 1998). By contrast, selective CB1 blockade, alone, suppresses feeding (Arnone *et al*., 1997; Simiand *et al*., 1998; Colombo *et al*., 1998; Kirkham & Williams, 2001b; Le Fur *et al*., 2001), while mice with genetically impaired CB1 receptors eat less than wild-type mice in response to food deprivation (Di Marzo *et al*., 2001).

There are other indications that endocannabinoids are key components of systems that regulate both feeding and body weight. For example, endocannabinoids appear to be crucial to suckling in neonates (Fride *et al*., 2001), and are involved in feeding responses across the phylogenetic scale (De petrocellis *et al*., 1999). In addition, the biosynthesis of hypothalamic anandamide and 2-AG may be subject to control by leptin, a peptide hormone produced by adipocytes that is believed to signal nutritional status to brain regions controlling appetite (Friedman & Halaas, 1998). Thus, leptin administration suppresses hypothalamic endocannabinoid

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levels in normal rats; while genetically obese, chronically hyperphagic rats and mice express elevated, leptin-reversible, hypothalamic anandamide or 2-AG levels (Di Marzo *et al*., 2001).

The ability of blockade, or genetic deletion, of CB1 receptors to suppress eating indicates that tonic endocannabinoid release may be crucial to the normal expression of feeding. However, there have so far been no direct measures of brain endocannabinoid levels tied specifically to the occurrence of feeding, or to motivational factors that influence food intake.

There is evidence that CB1 agonist and antagonist effects on food intake reflect alterations to appetitive or consummatory aspects of reward processes. For example, agonists can energise food-seeking behaviour, advancing the onset of feeding (Kirkham & Williams, 2001a; Williams & Kirkham, 2002b) and increasing the effort an animal will expend to obtain ingesta (Gallate *et al*., 1999). Conversely, CB1 blockade reduces responding for food (Gallate & McGregor, 1999; Freedland *et al*., 2000; 2001). Such data suggest that endocannabinoid activity may be linked to the appetitive phase of feeding, perhaps orienting the organism towards food and increasing the incentive value of food. However, there are some data to support a specific interaction with food palatability (orosensory reward). Thus, in people Δ^9–THC may selectively enhance the consumption of snack foods, while in animals SR141716 is reported to preferentially attenuate the intake of palatable foods (Arnone *et al*., 1997; Simiand *et al*., 1998). Of course, distinct actions of cannabinoids on appetitive and consummatory aspects of ingestion are not necessarily mutually exclusive, and may be indicative of a general activation of reward circuitry that both instigates feeding and enables a greater appreciation of food once eating commences. Indeed, cannabinoid involvement in general reward processes is supported by the ability of SR141716 to reduce rats' sensitivity to rewarding electrical brain stimulation (Deroche-Gamonet *et al*., 2001), and prevent the acquisition of drug- or food-induced place preferences (Chaperon *et al*., 1998).

In the present study we explored feeding-related changes in brain endocannabinoids, and we chose to examine anandamide and 2-AG levels in the hypothalamus and limbic forebrain of rats. As outlined above, there is indirect evidence of hypothalamic endocannabinoids being susceptible to manipulations that affect feeding behaviour, such as the potentially anorectic effects of leptin administration and the hyperphagia displayed by genetically obese animals. The limbic forebrain contains a number of feeding-related areas, including the shell area of the nucleus accumbens (AcbSh). The accumbens shell region is heavily implicated in the generation of emotional arousal and behavioural activation in response to rewarding stimuli, including the stimulation of eating (Kelley, 1999; Ikemoto & Panksepp, 1999; Stratford *et al*., 1998). As a control, we also examined anandamide and 2-AG levels in the cerebellum, a brain area not directly implicated in feeding motivation.

Using a sensitive and specific gas chromatography-mass spectrometric assay (Bisogno *et al*., 1999), we examined regional anandamide and 2-AG levels in animals that were killed: after food deprivation; while engaged in consumption of a palatable food, or after satiating on that food. Endocannabinoid levels in these animals were compared against those from non-deprived rats, killed at a point during their daily feeding cycle when motivation to eat was minimal, and feeding absent. We reasoned that if endocannabinoid activity is key to the appetitive phase of eating motivation, then food deprivation, with its obvious ability to provoke hunger, would maximise our ability to detect relevant changes in anandamide and 2-AG. Alternatively, if endocannabinoid activity contributes more directly to orosensory reward during ingestion, relevant changes should be maximized by allowing animals to avidly consume a highly palatable food. Finally, examining the brains of animals that had eaten to satiety provided an additional control to assess the specific involvement of endocannabinoids at all stages of appetite and feeding.

In addition to those assays, we also examined the acute actions on food intake of 2-AG administration. Although exogenously administered anandamide has been shown to induce hyperphagia in rats and mice (Williams & Kirkham, 1999; Hao *et al*., 2000; Jamshidi & Taylor, 2001), and 2-AG can restore suckling in neonatal mice treated with SR141716 (Fride *et al*., 2001), direct hyperphagic effects of 2-AG have yet to be published. We chose to investigate the effects of 2-AG injected directly into the nucleus accumbens shell. This region expresses CB1 receptors, as well as being associated with the stimulation of feeding and the processing of the incentive and reward value of food, and is consequently a potentially sensitive site for the hyperphagic actions of cannabinoid receptor agonists. To assess the localization of any behavioural effects of 2-AG to the accumbens shell, we also administered the endocannabinoid into the lateral ventricle.

We provide evidence of variation in both anandamide and 2-AG in rat brain according to changes in nutritional status and feeding behaviour, and demonstrate that 2-AG can stimulate eating *via* actions at forebrain CB1 receptors within a key appetite-related nucleus.

Methods

Animals

Adult male Lister hooded rats (Harlan, U.K.) weighing approximately 450 g at the start of testing were housed singly under a reversed 12 : 12 h light/dark cycle (lights off at 1000). Food and water were freely available at all times, except where specified below. All procedures were conducted according to the specifications of the United Kingdom Animals (Scientific Procedures) Act, 1986.

Brain endocannabinoid levels in relation to feeding and deprivation

Animal treatment Groups of 8–10 rats were killed during the dark phase of their daily cycle, following one of four manipulations. Rats with *ad libitum* food access (Group 1) were killed 5 h into the dark phase (at 1500). As nocturnal feeding is concentrated at predictable periods, this time was chosen to coincide with naturally low levels of spontaneous eating. Intake measures ensured that the rats had not eaten within the previous hour. Animals in this group should thus represent an adequate control, with any tonic, appetite-

related endocannabinoid activity minimized. Rats in Group 2 were presented with 40 g of a highly palatable wet mash diet. The mash comprised 200 g Rat and Mouse Expanded Ground diet (Special Diet Services, Witham, U.K.) mixed with 250 ml tap water, stirred until all the water was absorbed and the food acquired a firm consistency. Rats will avidly consume this mixture, easily eating 30 g in an hour (Williams & Kirkham, 1999). The wet mash was presented at 1000 and animals were still engaged in eating when they were removed to the euthanasia chamber (at approx. 1015). Rats in Group 3 were also presented with the palatable diet at 1000, but were allowed to eat to satiety. These animals were killed only after they had stopped eating and begun to display the species-typical satiety sequence (Kirkham & Blundell, 1984). Finally, rats in Group 4 were subjected to severe food restriction for 24 h. The rats were provided with 5 g of their normal maintenance diet (equivalent to approximately 20% of their normal daily intake) at the beginning of the dark cycle at 1000, and were observed to have consumed all that food within 5 h. These animals were killed between 1000–1030 on the following day, without any further access to food.

All animals were killed by rising CO_2 concentration, their brains removed immediately and frozen on dry ice. The hypothalamus and limbic forebrain from animals in each group were rapidly dissected, pooled and frozen in liquid nitrogen. Samples were subsequently sent on dry ice to the Naples laboratory.

Extraction and quantification of endocannabinoids Tissues were homogenized in 5 vol of chloroform/methanol/Tris HCl 50 mM (2:1:1) containing 1 nmol of d_8-anandamide and d_8-2-AG. Deuterated standards were synthesized from d_8 arachidonic acid and ethanolamine or glycerol as described, respectively, in Devane *et al*. (1992) and Bisogno *et al*. (1997). Homogenates were centrifuged at 13000 g for 16 min (4°C), the aqueous phase plus debris were collected and extracted again twice with 1 vol of chloroform. The organic phases from the three extractions were pooled and the organic solvents evaporated in a rotating evaporator. Lyophilized samples were then stored frozen at −80°C under nitrogen atmosphere until analysed.

Analysis of endocannabinoid contents by GC–MS Lyophilized extracts were resuspended in chloroform/methanol 99:1 by volume. The solutions were then purified by open bed chromatography on silica, as described in Fontana *et al*. (1995). Fractions eluted with chloroform/methanol 9:1 by volume (containing anandamide and 2-AG) were collected and the excess solvent evaporated with a rotating evaporator. The former fractions were further fractionated by normal phase high-pressure liquid chromatography (NP-HPLC) carried out using a semi-preparative silica column (Spherisorb S5W, Phase Sep, Queensferry, Clwyd, U.K.) eluted with a 40 min linear gradient from 9:1 to 8:2 (by volume) of n-hexane/2-propanol (flow rate = 2 ml min^{-1}). These elution conditions allow the separation of 1(3)- and 2-acyl-glycerols (retention time of 18 and 20 min, respectively) from anandamide plus other N-acylethanolamines (retention time = 26–27 min). NP-HPLC fractions from 17 to 22 min and from 24 to 28 min were pooled, the solvent evaporated in a speed-vac, and the components derivatized with 20 μl N-methyl-N-trimethylsilyl-trifluoroacetamide +1% trimethyl-chlorosylane for 2 h at room temperature and analysed by gas chromatography/mass spectrometry (GC–MS), carried out under conditions described previously (Bisogno *et al*., 1997) and allowing the separations of monoacylglycerols or N-acylethanolamines with different fatty acid chains. MS detection was carried out in the selected ion monitoring mode using m/z values of 427 and 419 (molecular ions for deuterated and undeuterated anandamide), 412 and 404 (loss of 15 mass units from deuterated and undeuterated anandamide), 530 and 522 (molecular ions for deuterated and undeuterated 2-AG), and 515 and 507 (loss of 15 mass units from deuterated and undeuterated 2-AG). The area ratios between signals of deuterated and undeuterated anandamide varied linearly with varying amounts of undeuterated anandamide (20 pmol-20 nmol). The same applied to the area ratios between signals of deuterated and undeuterated 2-AG in the 100 pmol–20 nmol interval. Anandamide and 2-AG levels in unknown samples were therefore calculated on the basis of their area ratios with the internal deuterated standard signal areas. Two GC–MS peaks for both deuterated and undeuterated mono-arachidonoylglycerol were found, corresponding to 2-AG and 1(3)-AG, in agreement with the previous observation that 2-AG undergoes isomerization during the purification procedure. Therefore, the amounts of 2-AG were calculated by adding the amounts of the two isomers. The amounts of endocannabinoids are expressed as pmol or nmol per gram of wet tissue extracted. Further details on the GC–MS technique, including the detection and quantification limits, have been published (De Petrocellis *et al*., 1999).

Statistical analysis Means ± s.e.mean ($n = 4$) of endocannabinoid amounts in the same area from different feeding groups were compared by one-way ANOVA followed by the Bonferroni test with a threshold of significance of $P < 0.05$.

Intracerebral 2-arachidonoyl glycerol administration and food intake

Surgery After habituation to handling, two groups of rats ($n = 10$ per group) were anaesthetised using ketamine and chlorpromazine (100 mg kg^{-1} and 3 mg kg^{-1}, respectively), after induction of buprenorphine analgesia (0.3 mg kg^{-1}). Rats were then implanted with 26-gauge, stainless steel guide cannulae, using flat-skull stereotaxic technique. One group received bilateral cannulae aimed at the nucleus accumbens shell (AcbSh; coordinates relative to bregma: anteroposterior, +1.2 mm; lateromedial, ±1.0 mm; dorsoventral, −7.0 mm). A second group was implanted with a single cannula aimed at the lateral ventricle (LV; co-ordinates: AP, −1.0 mm; LM +1.6 mm; DV −3.0 mm). Co-ordinates were determined by reference to the atlas of Paxinos & Watson (1986). After fixation to the skull with stainless steel screws and dental cement, the cannulae were sealed with stylets to maintain their patency.

Procedure Beginning 14 days after surgery, rats were familiarized with the drug infusion procedures and test conditions outlined below. To establish stable intakes, sham infusions and 2 h intake tests were performed over 1 week, with intracerebral saline infusion on the final baseline day.

On each test day, 10 min before dark onset, rats were removed from the test cages and given single LV or simultaneous bilateral AcbSh microinfusions of 0.15 M saline vehicle, or 2-AG (0.125, 0.5, 2.0 μg per rat; Alexis Chemical, Nottingham, U.K.). The timing of injections was chosen to coincide with the nocturnal feeding habits of rats, in order to augment endogenous, feeding-related activity of brain cannabinoid systems. Infusions were delivered over 90 s through 33-gauge injectors (projecting 1.0 mm beyond the tip of the guide cannula), using automated microsyringe pumps in a volume of 0.5 μl per side (AcbSh), or 1.0 μl per rat (LV). For bilateral injections, one half of the total dose was administered into each side of the brain. One minute after the completion of each infusion, injectors were removed, the cannulae re-sealed, and the rats placed in their test chambers. Food (chow; PCD Mod C; Special Diet Services, Witham, U.K.) and water intake were subsequently measured after 1 and 2 h, with appropriate correction for food spillage. All rats received all treatments, according to a counter-balanced design, with at least 48 h between successive treatments.

After completion of the initial dose–response series, rats with AcbSh cannulae were re-tested to examine the ability of the CB1 antagonist SR141716 ([N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methylpyrazole-3-carboxamide]; Vernalis Ltd, Wokingham, U.K.) to block the behavioural actions of the most potent 2-AG dose. Thirty minutes before dark onset, rats received a subcutaneous injection of 0.5 mg kg^{-1} SR141716 (dissolved in 10% DMSO solution, in a volume of 1 ml kg^{-1}). Bilateral infusions of saline or 0.5 μg 2-AG were administered 20 min later, and food intake monitored over the subsequent 2 h. The SR141716 dose was chosen from our previous observation of its ability to attenuate the hyperphagic actions of Δ^9-THC and anandamide (Williams & Kirkham, 1999; 2002a).

Histology On completion of testing, rats were killed with CO_2, and decapitated. India ink was injected into each LV cannula, in a volume of 1 μl, prior to decapitation. The heads were placed in a 10% sucrose, 10% formaldehyde phosphate-buffered solution for 4 days. Subsequently, brains were removed, sectioned coronally and stained with cresyl violet. Cannula tracks were determined by visual inspection under a microscope. The presence of ink within the ventricles confirmed LV cannula placement. These inspections revealed that injection sites for two animals from the accumbens group lay outside the AcbSh. Data from these animals were excluded from subsequent data analyses.

Statistical analysis Intake data for each group were analysed using one-way ANOVA for repeated measures. *Post hoc* comparisons were made with Newman–Keuls test for multiple comparisons.

Results

Feeding-related changes in endocannabinoid levels

Analysis of regional anandamide and 2-AG levels in brain tissue revealed distinct changes in response to our experimental manipulations (Figure 1). Under control conditions

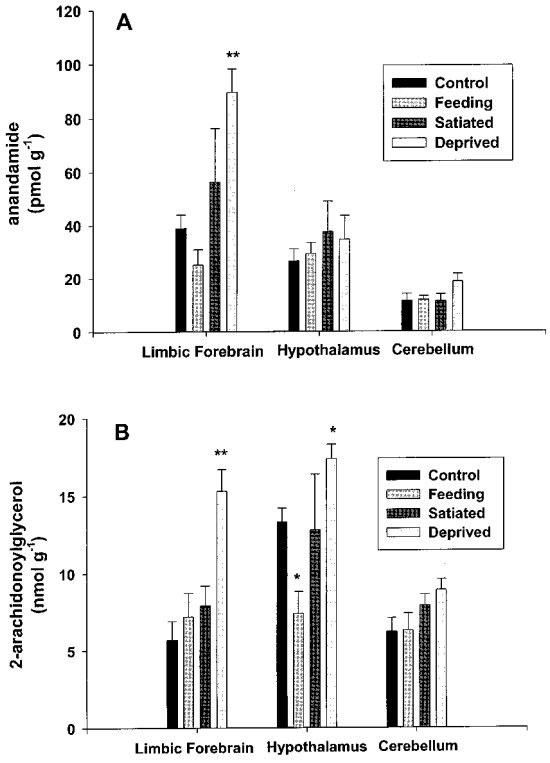

Figure 1 Endocannabinoid levels in the limbic forebrain, hypothalamus and cerebellum of rats in relation to fasting, feeding and satiation. Data for anandamide levels (A), and 2-arachidonoyl glycerol levels (B) are shown, and are expressed as pmol or nmol, respectively, per gramme of wet tissue weight. All values are means ± s.e.mean of $n=4$. * $P<0.05$ and ** $P<0.01$, significantly different from control by ANOVA followed by the Bonferroni test.

(Group 1) anandamide levels were highest within the limbic forebrain, while 2-AG was highest in the hypothalamus. Levels of anandamide in the cerebellum of control rats were lower than in the other tissues, while 2-AG levels were similar to those in the limbic forebrain. Importantly, cerebellar anandamide and 2-AG levels remained constant under all conditions.

The levels of each endocannabinoid were differentially affected by the separate treatments, with distinct regional changes. Considering the limbic forebrain, the most notable changes were induced by the imposition of food deprivation. In food-deprived rats, both anandamide and 2-AG levels displayed significant, 2–3 fold increases respectively ($P<0.01$). The active expression of avid eating in animals consuming a palatable diet had no effect on either anandamide or 2-AG. Similarly, limbic forebrain endocannabinoid levels were unaffected in animals that had been completely satiated after consumption of 30–40 g of the wet mash.

A different, more complex profile of change was apparent in the hypothalamus. In this instance, only 2-AG levels exhibited reliable changes, with the direction dependent on the nutritional status of the animals. Once again, food deprivation significantly increased hypothalamic 2-AG levels ($P<0.05$), although the increase was somewhat smaller ($\sim$30%) than that observed in the limbic forebrain. In contrast to all other measures, in animals that were killed

while still actively feeding, hypothalamic 2-AG levels were significantly reduced ($P<0.05$). When access to the wet mash was uninterrupted, and animals were able to satiate, the feeding-induced reduction of hypothalamic 2-AG was no longer apparent, so that levels were identical to control values.

Stimulation of feeding by 2-arachidonoyl glycerol

Analysis of hourly food intake (Figure 2) revealed that, in our free-feeding rats, bilateral injection of 2-AG into the nucleus accumbens shell produced a clear-cut, short-term stimulatory action on feeding behaviour. Specifically, the endocannabinoid significantly increased the amount eaten during the first hour after administration ($P<0.001$), with particularly marked effects after 0.5 and 2.0 μg. The effect of the lowest dose failed to achieve significance. No significant effects were apparent during the second hour of testing, although there was a slight tendency to compensatory intake reduction at doses that had induced overconsumption during hour 1 (mean hour 2 intakes: vehicle = 1.28 ± 1.43 g; 0.125 μg = 1.05 ± 1.18 g; 0.5 μg = 0.71 ± 0.80 g; 2.0 μg = 0.45 ± 0.51 g). In addition, pretreatment with the selective CB1 antagonist SR141716 significantly attenuated the hyperphagic effects of 0.5 μg 2-AG ($P<0.01$; Figure 2). Administration of the antagonist alone had no reliable effect on the already low baseline food intake (data not shown). In contrast to the effects on food intake, no effects on water intake were detected after 2-AG treatment; animals rarely engaged in drinking during any of the tests. Finally, it should be noted that 2-AG did not appear to introduce any unusual, non-specific behaviour that might account for the increased food intake.

In marked contrast to the hyperphagic effects induced by intra-accumbens 2-AG, infusion of 2-AG into the lateral ventricle failed to exert any obvious effect on food intake during either measurement period. Mean 1-h intakes after lateral ventricular administration of 2-AG were: vehicle = 2.84 ± 0.4 g; 0.125 μg = 2.72 ± 0.57 g; 0.5 μg = 2.05 ± 0.49 g; 2.0 μg = 2.99 ± 0.8 g. Although the baseline intakes for this group were relatively high, it is unlikely that ceiling effects would have obscured 2-AG hyperphagia. Indeed, 1-h intake after the most effective intra-accumbens 2-AG dose (0.5 μg) significantly exceeded the lateral ventricle baseline ($P<0.01$), indicating that the lack of effect of ventricular administration was not due to a failure of detection.

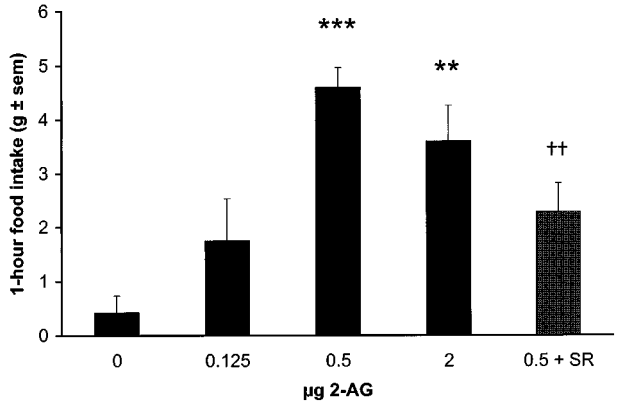

Figure 2 Dose-related increases in food intake over one hour following bilateral accumbens shell 2-arachidonoyl glycerol infusion. All values are mean ± s.e.mean from eight rats. ** $P<0.01$, *** $P<0.0001$, significant difference from vehicle; †† $P<0.01$, significant attenuation of the hyperphagic effects of 0.5 μg 2-AG by pretreatment with the CB1 antagonist SR141716 (0.5 mg kg^{-1}, s.c.).

Discussion

The results of our experiments provide significant support for current hypotheses concerning the putative role of endocannabinoids in appetite and body weight regulation. Specifically, we have provided the first demonstration of alterations to brain levels of anandamide and 2-AG in direct relation to the expression of feeding behaviour and changes in the motivation to eat. We have also shown what we believe to be the first evidence of behavioural activity of 2-AG: specifically we demonstrated that 2-AG can reliably stimulate eating. Moreover, this hyperphagic action appears to have been mediated by cannabinoid receptors within the nucleus accumbens shell, an area with strong associations with appetite stimulation.

As outlined in the introduction, elevated hypothalamic levels of 2-AG have been detected in genetically obese *fa*/*fa* rats and *ob*/*ob* mice, and of both 2-AG and anandamide in the hypothalamus of obese *db*/*db* mice, compared to lean controls (Di Marzo *et al*., 2001). Those animals are either leptin-deficient or express mutated forms of the leptin receptor. Leptin is proposed to be a humoral agent, released by adipocytes, that can signal nutritional status and modulate food intake and energy balance. In normal animals, this integrative function appears to involve the respective up-regulation of anorectic neuropeptides and down-regulation of orexigenic neuropeptides expressed in the hypothalamus (Friedman & Halaas, 1998). In genetically obese animals these actions of leptin are absent or ineffective and animals gain weight; due, in part, to chronic overconsumption (hyperphagia). Given the ability of cannabinoid receptor agonists to stimulate feeding and the elevated endocannabinoid levels observed in genetically obese animals, it is feasible that endocannabinoid systems in the brain constitute an additional component of leptin-sensitive regulatory mechanisms. Consequently, we could expect that, in normal animals, endocannabinoid activity would be provoked by stimuli that actively challenge energy homeostasis.

In our first experiment, we observed that acute food restriction over 24 h provoked significant increases in brain anandamide and 2-AG levels. These effects were most marked in the limbic forebrain, with substantial increases in the levels of both endocannabinoids. Within the hypothalamus, only 2-AG was reliably increased. That the two cannabinoids were differentially regulated probably reflects the high degree of anatomical complexity in the distribution of appetite-related, cannabinoid-sensitive neurons. In particular, the hypothalamus contains a number of zones (e.g., lateral hypothalamic, paraventricular, ventromedial and arcuate nuclei) that have been separately implicated in the stimulation or inhibition of feeding. Although the nature of our tissue samples does not permit any further anatomical resolution, a specific increase in hypothalamic 2-AG is nevertheless consistent with the similarly selective elevation

of this cannabinoid detected in the hypothalamus of *fa*/*fa* rats and *ob*/*ob* mice.

When leptin signalling is defective, animals are effectively in a chronic state of what has been described as perceived starvation (Friedman & Halaas, 1998). Thus, the hyperphagia exhibited by genetically obese rodents arises from a motivational state that mimics the natural hunger generated in our experiment by actual food deprivation. In the present study, deprived rats were killed without any opportunity to eat, so the observed changes in endocannabinoid levels are likely to reflect the specific activation of neural systems involved in the appetitive components of eating motivation, as opposed to those responsible for the active maintenance of eating when food is available. The other significant change in hypothalamic 2-AG that we detected may also support this proposition. Specifically, we found that in non-deprived animals consuming a palatable food and killed while still highly motivated to continue eating, hypothalamic 2-AG levels were actually significantly reduced. By contrast, in animals that ate the same food to satiety, hypothalamic 2-AG levels were restored to levels similar to those in our control animals. The control rats, it will be recalled, were killed at a time when they were replete and their endogenous feeding rhythms naturally suppressed eating. We should also note that cerebellar levels of anandamide and 2-AG remained constant across all conditions, supporting the interpretation that forebrain and hypothalamic changes were due to our specific manipulations, rather than, for example, differences in general activity levels of the animals.

The respective elevation of hypothalamic 2-AG levels with deprivation, and the decline evident during feeding, suggest that, once initiated, eating is no longer dependent on endocannabinoid activity for its maintenance. Indeed, there may be specific mechanisms to suppress 2-AG activity during re-feeding in order to facilitate satiation. Certainly, our observation of reduced hypothalamic 2-AG in animals eating a palatable diet indicates that endocannabinoids are not crucial to food palatability – the orosensory reward that guides the rate, duration and size of meals. Further analyses may reveal that there is a gradual increase in endocannabinoid activity as time elapses after a meal, until the next meal is instigated. The post prandial recovery of 2-AG levels that we observed may be evidence of the first stages in such a process; possibly facilitating opportunistic feeding under less predictable circumstances than those normally facing a laboratory animal. It will be important, in future, to assess the extent to which there are endogenous rhythms in brain endocannabinoids, and the extent to which levels are correlated with the natural, periodic expression of feeding.

A substantial increase in endocannabinoid levels in response to caloric deficit was also apparent within limbic forebrain: in this case for both 2-AG and anandamide. The magnitude of these deprivation-induced increases in this region (and an absence of changes with any other manipulation) suggests activation of circuitry with specific involvement in the generation of appetite and food seeking behaviour. Notably, this region contains the nucleus accumbens. The shell subregion of the accumbens, which contains a relatively high density of CB1 receptors (Herkenham *et al*., 1991; Tsou *et al*., 1998), has particularly strong associations with appetitive processes. Neural activity in the shell is believed to signal incentive salience and to facilitate the generation of motor patterns orienting an animal towards potentially rewarding stimuli, such as food in a hungry animal (Kelley, 1999). Within this region, endocannabinoids may interact with other neurochemical circuits linked to incentive processes, such as the mesolimbic dopamine system. Dopamine release from neurons terminating in the accumbens is considered to be crucial to the appetitive aspects of feeding. Importantly, doses of Δ^9-THC that can induce hyperphagia also promote dopamine release within the accumbens (Tanda *et al*., 1997; Gardner & Vorel, 1998; Ameri, 1999), and significantly stimulate anandamide biosynthesis in the limbic forebrain (Di Marzo *et al*., 2000).

In the light of these associations, and in consideration of 2-AG being the endocannabinoid most sensitive to changes induced by fasting and feeding, or leptin signalling (Di Marzo *et al*., 2001), we examined the effect on food intake of injection of 2-AG into the nucleus accumbens shell. We demonstrated for the first time that administration of 2-AG, like anandamide, can induce significant alterations to feeding behaviour in rats, with substantial short-term increases in food intake being observed during the first hour after administration. This action of 2-AG was significantly attenuated by SR141716, indicating mediation by CB1 receptors. Importantly, the rapidity of onset and magnitude of 2-AG hyperphagia injected into the accumbens shell far outweigh the relatively weak effects of anandamide seen after peripheral or central administration (Williams & Kirkham, 1999; Hao *et al*., 2000; Jamshidi & Taylor, 2001). Moreover, the lack of effect of intraventricular injection of 2-AG suggests that the accumbens shell effects were not due to back-diffusion along the cannulae tracks, and access *via* cerebrospinal fluid to structures remote from the injection site. Of course, it will be necessary to assess the behavioural effects of the cannabinoid in neighbouring sites, such as the nucleus accumbens core, and the relative potency of injection into anterior versus posterior aspects of the shell (Kelley & Swanson, 1998), to fully determine the anatomical specificity of these effects. Nevertheless, the potency of 2-AG strongly suggests that the accumbens shell is a sensitive site of action, and strengthens our hypothesis concerning the possible role of endocannabinoids in appetitive processes.

We have reported elsewhere that peripherally administered Δ^9-THC and anandamide actively provoke satiated animals to approach food and begin feeding (Williams & Kirkham, 2002b). We have interpreted those effects as evidence of an action of cannabinoids to increase the salience or incentive value of food stimuli and induce an appetite for food, analogous to the effects of food deprivation. In line with this proposition, CB1 -/- mice with genetically impaired CB1 receptors are distinguished from their wild-type littermates by a reduced hyperphagic response to fasting (Di Marzo *et al*., 2001), although apparently displaying normal intake levels and body weights under *ad libitum* conditions. The potency of 2-AG within the accumbens shell is also consistent with endocannabinoid involvement in appetite stimulation. As we discussed earlier, there is good evidence from other paradigms for endocannabinoid involvement in incentive/appetitive processes. Thus, Gallate and colleagues found that a CB1 agonist, CP 55940, increased break-points in rats licking for sucrose solutions (i.e., rats would work harder to obtain the sucrose), an effect that was blocked by SR141716 (Gallate & McGregor, 1999).

It is also possible to incorporate the deprivation-induced changes to hypothalamic 2-AG into this scheme. Although different hypothalamic regions differentially influence appetite, one area in particular, the lateral hypothalamus, has been tied to the stimulation of feeding. Moreover, the lateral hypothalamus is anatomically linked to the nucleus accumbens shell (Stratford & Kelley, 1999). Chemical or electrical stimulation of the lateral hypothalamus induces intense feeding activity, apparently by raising the incentive value of food stimuli through the activation of reward circuitry. That lateral hypothalamic reward pathways are sensitive to cannabinoid modulation is supported by the demonstration that Δ^9-THC will facilitate feeding induced by electrical stimulation of that area (Trojniar & Wise, 1991). Interestingly, the threshold level at which rats will respond for rewarding electrical stimulation of the lateral hypothalamus is reduced by food restriction (Carr, 1996), while leptin has the opposite effect (Fulton *et al*., 2000). Recalling the links between leptin and endocannabinoids, such data suggest that leptin may act, through down-regulation of endocannabinoids, to reduce the general incentive value of food and so restrict feeding. Food deprivation and the concomitant suppression of circulating leptin levels would therefore be expected to release endocannabinoids from this control, with increased hypothalamic levels (as in our study), and a consequent increase in the attractiveness of food. Moreover, in spontaneously feeding human volunteers, circulating leptin levels have been shown to increase during intermeal intervals and decline before the onset of a meal (Chapelot *et al*., 2000). It is therefore possible to predict a reciprocal relationship between leptin and hypothalamic endocannabinoids in the regulation of meal patterns. So far, there has been only one study of intra-hypothalamic cannabinoid administration and feeding. Jamshidi & Taylor (2001) reported modest, slow-onset hyperphagia after anandamide administration into the ventromedial hypothalamus, an area more traditionally associated with the inhibition of feeding. There is, therefore, clearly a need for more extensive analyses of the feeding effects of cannabinoids within different hypothalamic regions, and of the corresponding changes to endocannabinoid levels in response to different nutritional manipulations. Again, it will be important to examine whether circadian feeding patterns are linked to elevation of endocannabinoids in feeding-related brain regions.

In conclusion, the present studies have demonstrated clear links between endogenous cannabinoid activity and feeding motivation. They have also shown that the endocannabinoid 2-AG can potently stimulate eating. Overall, these findings are consistent with previous reports and strengthen a role for endocannabinoids in the physiological regulation of appetite and body weight, potentially through actions on brain systems that mediate incentive and reward processes.

This work was supported by a project grant from the U.K. Biotechnology and Biological Sciences Research Council (T.C. Kirkham). The authors are grateful to Dr S. Vickers of Vernalis Ltd, Wokingham, U.K. for the generous gift of SR141716.

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(Received March 13, 2002
Revised April 10, 2002
Accepted April 19, 2002)