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ORIGINAL INVESTIGATION

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Effects of the cannabinoid receptor antagonist SR 141716, alone and in combination with dexfenfluramine or naloxone, on food intake in rats

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Abstract *Rationale:* Recent studies in animals have implicated endogenous cannabinoids in the regulation of palatable food intake, but it is not yet clear to what extent pharmacological agents acting on this system may have sustained actions and applicability to different feeding protocols. *Objectives:* In the present study, we examine the effects of the cannabinoid CB1 receptor antagonist SR 141716 on food intake of rats, and its behavioral specificity. We examine whether tolerance develops to the anorectic actions of SR 141716, and whether it has either additive or synergistic actions with dexfenfluramine or naloxone. *Methods:* Undeprived rats were trained to eat a daily sweet milk dessert and on test days were administered single or combination drugs and intakes were recorded. In other studies, rats were deprived for 24 h of either food or water and intakes recorded after drug administration at the end of this time. In one study, rats were fed ad libitum chow with SR 141716 added. *Results:* SR 141716 (1–3 mg/kg) suppressed both palatable food intake in undeprived rats and food, but not water, intake after deprivation. Using an isobolographic analysis, SR 141716 had an additive anorectic effect with dexfenfluramine. In contrast, SR 141716 in combination with naloxone had a significantly supra-additive anorectic action. SR 141716 was also effective orally and no tolerance to its anorectic effect developed over 3 days. *Conclusions:* These data show that SR 141716 is an effective anorectic agent using both palatable foods and bland chow, and is selective because water intake was unaffected. SR 141716 is also effective orally and has an effect sustained for at least several days. There appears to be a synergistic interaction between opioid and cannabinoid systems in the regulation of feeding, whereas the combination of a serotonin releasing agent and the CB1 antagonist is additive.

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Introduction

Peripheral administration of Δ^9-tetrahydrocannabinol (THC), a major active constituent of marijuana (*Cannabis sativa*), has been associated with compulsive or excessive overeating in humans and animals (Brown et al. 1977; Hollister 1970; Mattes et al. 1994; Williams et al. 1998). Peripheral administration of anandamine, an endogenous cannabinoid, also promoted feeding in satiated rats (Williams and Kirkham 1999) although the maximal effect was considerably smaller than that observed with THC using an identical protocol (Williams et al. 1998). These actions appear to be mediated through the G-protein coupled CB1 receptor, found in many brain regions as well as peripherally (Herkenham et al. 1991). Spontaneous food intake was similar in wild-type and CB1-receptor knockout ($CB1^{-/-}$) mice, but in a short-term test of intake after food deprivation $CB1^{-/-}$ ate only ca 50% of wild-type control (Di Marzo et al. 2001). These data suggest that endogenous cannabinoids contribute to the regulation of food intake, at least under some conditions.

The selective CB1 receptor antagonist SR 141716 (Rinaldi-Carmona et al. 1994) provides a tool to investigate this proposed role of endogenous cannabinoids in the central nervous system. In rats, mice, or marmosets, peripheral administration of SR 141716 inhibits food intake (Arnone et al. 1997; Colombo et al. 1998; Di Marzo et al. 2001; Kirkham and Williams 2001; Simiand et al. 1998) and blocks the orexigenic effect of anandamine (Williams and Kirkham 1999). Some, but not all, of these cited studies have suggested that SR 141716 selectively inhibits intake of carbohydrate or sweets.

For CB1 antagonists to be useful anorectic drugs in clinical practice, their effect would have to be sustained over days and apply to more than just sweet food. Colombo et al. (1998) gave SR 141716 daily to rats and

found a suppression of intake for about 6 days and concluded that tolerance developed. However, their protocol of a single daily injection of a relatively high dose is not optimal for producing sustained effects, and so we have re-examined this issue. Further, it may prove useful to combine CB1 antagonists with agents acting on other neurotransmitter systems that have been implicated in food intake, and we examine interactions with serotonin (5-HT) and opioid systems.

Material and methods

Animals

Adult Sprague-Dawley rats of both sexes were either purchased from Harlan Laboratories or were bred in our vivarium from that stock. They received 5001 rodent pellets (Purina Mills) and tap water ad libitum except as noted. During experiments, they were housed individually in stainless steel mesh cages (25×19×19 cm) suspended over absorbent paper pan liners. The vivarium was illuminated by fluorescent lights from 0600 to1800 hours and was maintained at 23±2°C. Animal use was approved by the IACUC and in accordance with the Guide for the Care and Use of Laboratory Animals (1996).

Experiment 1: behavioral specificity, anorectic dose, and time-response of SR 141716

This experiment comprised four studies. In the first, six retired breeder female rats (ca 8 months of age) were acutely deprived of food for 24 h on two occasions, 4 days apart. After the deprivation period, they were injected with either the vehicle (50% aqueous propylene glycol, 1 ml/kg i.p.) or SR 141716 (1 mg/kg) and weighed chow pellets were presented inside the cage 15 min later. Intakes were recorded 1 h later and were corrected for spillage collected on clean paper under the cages. Half of the rats were injected with vehicle on the first day and with SR 141716 on the second, while the other half received the reverse order of presentation. These tests were conducted at about 1400 hours. Intakes from the two sessions were combined and analyzed by paired *t*-test. Deprivation was used in this study only to provide a comparison with the second study to assess behavioral specificity.

The same rats were used in the second study performed 1 week later with a similar test design but for water intake. The rats were deprived of water for 24 h (with food present) and, after drug or vehicle injections, 1 h water intake was recorded from graduated burets with sipper spouts. Prandial drinking was prevented by removal of food during the drinking test.

The third study investigated the time-response function for an acute dose of SR 141716. Twenty-four adult male rats (ca 6 months old) were adapted to consuming sweet milk dessert (100 g sucrose plus 100 g powdered milk, both from local food stores, per liter of water) for 1 h per day. In this and subsequent studies, chow was available continuously. Milk was presented in 50-ml graduated tubes fitted with stopper and sipper spout. When baseline intakes were stable (±20%, the normal day-to-day variation), four matched groups (average 3 days baseline) were formed and the next day received SR 141716 (1 mg/kg i.p.) either 0.5, 1, or 2 h before access to milk. One group served as a control and received the vehicle (propylene glycol) 1 h before access to milk. Intakes were recorded after 1 h, converted to percentages of individual baselines, and analyzed by one-way analysis of variance (ANOVA; SigmaStat; SPSS) and then Newman-Keuls *post hoc* group comparisons ($P<0.05$).

The same rats were used in the fourth study. Following the previous study, milk was presented for the next 6 days to establish new baselines, and four new matched groups were formed. The next day, they received either 1, 2, or 3 mg/kg SR 141716 or the propylene glycol vehicle (1 ml/kg i.p.). Milk was presented 1 h after injections and intakes were recorded after 1 h. Intakes were again converted to percent baselines and analyzed by one-way ANOVA. Further, linear regression analysis was performed and a 50% inhibitory dose (DI_{50}) and 95% CI values computed (SigmaPlot; SPSS).

Experiment 2: acute effect of SR 141716 in combination with other anorectic agents

Two classes of anorectic agent were tested in combination with SR 141716 and the results examined for synergy using an isobologram (Berenbaum 1989). In the first combination study, the serotonin (5-HT)-releasing and uptake-inhibiting agent, dexfenfluramine was examined. We used the same 24 rats as in the last part of experiment 1, but ca 1 month later. They were readapted to the sweet milk dessert regimen for a few days until 1 h baseline intakes were stable. DI_{50} values (and 95% CI range) for the individual drugs under identical conditions were 1.8 (1.4–2.3) mg/kg for SR 141716 (experiment 1) and 2.5 (1.3–3.6) mg/kg for dexfenfluramine (Roth and Rowland 1999). On the test day, 0.5 h before access to milk, rats received one of three fixed combinations (1:1, the approximate ratio of the DI_{50}s) of SR 141716 dexfenfluramine (*viz* 0.25+0.25, 0.5+0.5, 1.0+1.0 mg/kg) or the vehicles (propylene glycol i.p. and saline s.c., respectively). As before, 1 h intakes were measured and the DI_{50} for the combination estimated. This DI_{50} was plotted on an isobologram showing 95%CI for the components.

In the second study, 12 female rats (ca 3 months of age) were adapted to the sweet milk dessert for 6 days, and then were divided into three groups matched for baseline intakes. On 3 test days, each separated by at least 3 days baseline without drug, rats received either vehicle (saline, 1 ml/kg s.c.) or 1 or 3 mg/kg naloxone. They were injected 0.5 h before access to milk, and each subgroup received the doses in different order. The 1-h intakes were combined from each test day and were analyzed by repeated measures one-way ANOVA, and the DI_{50} estimated by linear regression.

The third study started a few days later, using the same rats and an identical procedure. The rats received in random order fixed combinations (1:2.5, based on DI_{50}s) of SR 141716 and naloxone (*viz* 0.1+0.25, 0.2+0.5, 0.4+1.0 mg/kg) or the vehicles (propylene glycol i.p. and saline s.c., respectively). The DI_{50} for the combination was determined by linear regression and plotted on an isobologram.

Experiment 3: repeated or continuous administration of SR 141716

Two studies were performed. In the first, 12 adult male rats (450–570 g) were adapted to daily sweet milk dessert presentation for 1 h as before. After stable baselines were established they were divided into two matched groups. On days 1, 3, and 5 thereafter, one group was injected with the vehicle (dimethylsulfoxide, 1 ml/kg i.p.) 15 min before access to milk. The other group received SR 141716 (1 mg/kg). On intervening days all rats received milk but no injections.

In the second study, SR 141716 was given orally by mixing it in the food. Eleven retired breeder female rats (ca 400 g) were fed powdered PMI 5001, provided ad libitum in glass jars inside the cages. Spillage, which was minimized by a rim on the jar, was collected on paper under the cages. Intakes, corrected for spillage, were computed daily. After baseline intakes were established, two matched groups were formed. One group (n=6) received chow to which SR 141716 (60 mg/kg food) was added and mixed thoroughly. This dosage was derived from baseline intakes (ca 20 g per day) and body weight, intended to give ca 3 mg/kg body weight per day, but because food intake was reduced, the dosage was ca 1.5 mg/kg per day. This regimen was maintained for 3 days. The second group was a control, fed chow without additive during these 3 days. At the end of this time, the controls were given a

Table 1 Effect of SR 141716 (1 mg/kg i.p.) given at various intervals prior to sweet milk access

Interval	Intake (% baseline; mean ± SE)
Vehicle	117.8±13.5
30 min	79.4±7.6*
60 min	69.5±7.4*
120 min	80.7±8.5*

* P<0.05 vs vehicle

24-h two-jar choice between regular and SR 141716-containing food; the purpose of this test was to determine whether rats could discriminate SR 141716-added chow using chemosensory cues.

Drugs

SR 141716 [*N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide] was a generous gift from Sanofi-Synthelabo Recherche (Montpellier, France). Dexfenfluramine and naloxone, both hydrochloride salts, were purchased from Sigma Chemicals (St. Louis, Mo., USA). All other reagents were from Fisher Scientific (Orlando, Fla., USA).

Results

Experiment 1: behavioral specificity, anorectic dose, and time-response of SR 141716

In the food deprivation study, mean ± SE chow intakes after vehicle and SR 141716 (1 mg/kg) treatments were 6.2±8 and 4.3±1.0 g, respectively, indicating a significant (P<0.05) 31% suppression by SR 141716. In the fluid deprivation study, corresponding water intakes were 9.6±7 and 7.9±1.1 ml, which did not differ significantly.

In the time-response study, intakes of all three drug-treated groups differed (P<0.05) from control but did not differ among themselves (Table 1). The effect was slightly greater when the drug was given 1 h before food than at either 0.5 or 2 h, so 1 h was adopted in most of the remaining studies.

The results of the dose-response study are shown in Fig. 1. SR 141716 suppressed food intake in a dose-related manner, but with little additional effect above 2 mg/kg. The results of the linear regression are shown in Fig. 1 and gave a DI_{50} of 1.8 mg/kg with 95% CI of 1.4–2.3 mg/kg. Other analyses, including linear regression omitting the 3 mg/kg data and a quadratic fit yielded similar DI_{50} values. This result is consistent with the results of the two foregoing studies in which 1 mg/kg produced less than 50% suppression of food intake.

Experiment 2: acute effect of SR 141716 in combination with other anorectic agents

The dose-inhibition data for the combination of SR 141716 and dexfenfluramine are shown in the *left panel* of Fig. 2. Linear regression yielded a DI_{50} of 1.64 mg/kg that, at the 1:1 ratio used, was 0.82 mg/kg SR 141716 plus 0.82 mg/kg dexfenfluramine. This is plotted on the DI_{50} isobologram in the *right panel* of Fig. 2 and falls within the 95% confidence quadrilateral of statistical additivity.

The mean ± SE intakes, as percent baseline, after 0, 1, and 3 mg/kg naloxone were 108±8, 61±11, and 49±2 mg/kg, respectively. Because tripling the dose had little additional effect, linear regression included only the 0 and 1 mg/kg data and yielded a DI_{50} of 1.2 mg/kg (95% CI 0.9–2.3 mg/kg). This is a conservative estimate compared with a value of ca 3 mg/kg that we obtained using either a quadratic function or linear regression involving all doses; using this lowest estimate is the most stringent test for synergy.

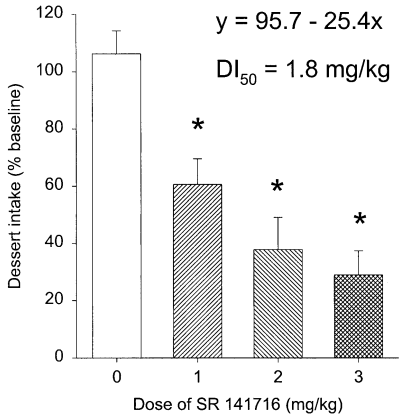

Fig. 1 Dose-related inhibition of sweet milk intake by SR 141716 in undeprived rats. Shown are mean ± SE intakes, expressed as percent of baseline on previous days; * indicates P<0.05 versus control. The result of the linear regression analysis and estimated 50% inhibitory dose (DI_{50}) also are shown

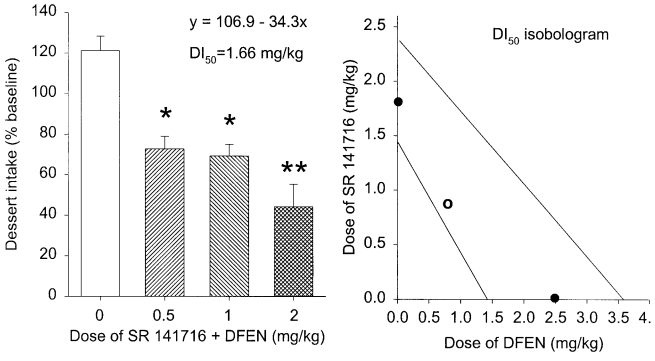

Fig. 2 *Left panel* shows the effect of fixed dose combinations of dexfenfluramine (*DFEN*) and SR 141716 on sweet milk intake in undeprived rats. * P<0.05 versus control; ** P<0.05 versus all other groups. The result of the linear regression and estimated DI_{50} also are shown. *Right panel* shows the DI_{50} isobologram. The *x*- and *y*-axes show DI_{50}s (95% CI) for DFEN and SR 141716, respectively, and the *trapezium* defines the area of statistical (95%) additivity. The *open circle* in the trapezium indicates the DI_{50} observed for the combination as determined from the data in the *left panel*

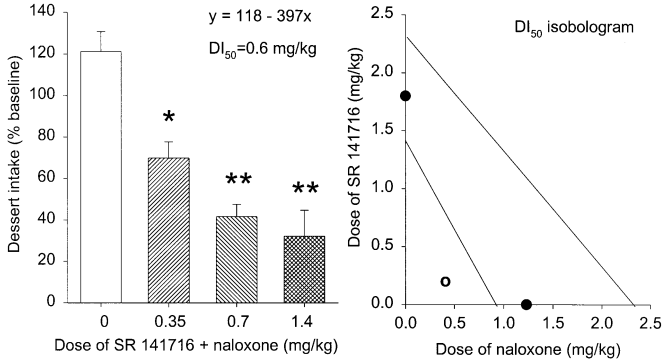

Fig. 3 *Left panel* shows the effect of fixed dose combinations of naloxone and SR 141716 on sweet milk intake in undeprived rats. * $P<0.05$ versus control; ** $P<0.05$ versus all other groups. The result of the linear regression and estimated DI_{50} also are shown. *Right panel* shows the DI_{50} isobologram. The *x*- and *y*-axes show DI_{50}s (95% CI) for naloxone and SR 141716, respectively, and the *trapezium* defines the area of statistical (95%) additivity. The *open circle* below the trapezium indicates the DI_{50} observed for the combination as determined from the data in the *left panel*

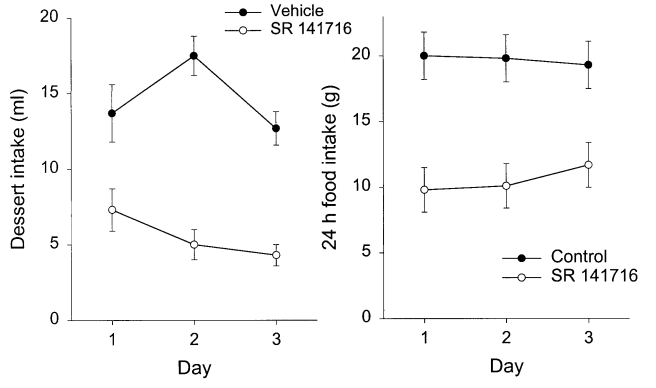

Fig. 4 *Left panel* shows the effects of repeated administration at 2-day intervals of SR 141716 (1 mg/kg i.p.) on 1 h sweet milk intake (mean ± SE). *Right panel* shows 24-h food intake in groups of rats fed ad libitum with either regular food (*Control*) or food containing SR 141716. For data in both panels, all drug-treatment values are less ($P<0.05$) than the corresponding vehicle or control values

The dose-inhibition data for the combination of SR 141716 and naloxone are shown in the *left panel* of Fig. 3. Linear regression yielded a total or combination DI_{50} of 0.6 mg/kg that, at the 1:2.5 ratio, is 0.17 mg/kg SR 141716 plus 0.42 mg/kg naloxone. This is shown on the DI_{50} isobologram in the *right panel* of Fig. 3 and falls below the 95% confidence quadrilateral of statistical additivity. This indicates that, at least at this ratio of components of 1:2.5, their combination was significantly more effective than predicted by a dose-additive model.

Experiment 3: repeated or continuous administration of SR 141716

Repeated acute administration of SR 141716 in the dessert protocol yielded equivalent and significant suppressions of food intake on each of the 3 days (Fig. 4 *left panel*). When the drug was administered chronically in the food, a similar result was obtained; 24-h intake was suppressed on each of 3 consecutive days (Fig. 4 *right panel*). In the two-jar choice test, the group mean intakes of regular and SR 141716-added chow were 50.4% and 49.6% of the total, respectively, indicating that rats were not avoiding the drug-added chow on the basis of either a taste or odor cue.

Discussion

Arnone et al. (1997) were the first to show that antagonism of endogenous cannabinoids via use of the CB1 receptor antagonist, SR 141716, produced a dose-related suppression of both sucrose and alcohol intakes in free-feeding rodents. Our present data using the dessert protocol is consistent with their results, suggesting a DI_{50} of ca 1 mg/kg. A similar result was found in marmosets (Simiand et al. 1998). However, Arnone et al. (1997) found no effect of SR 141716 on intake of rats on a restricted feeding regimen and Kirkham and Williams (2001) also found no significant effect in rats deprived of food for 1 h near the start of the night. We found a reduction of chow intake after food deprivation, an effect that has also been observed recently in wild-type mice (Di Marzo et al. 2001). In that study, as expected, SR 141716 did not suppress intake of CB1 receptor knockout ($CB1^{-/-}$) mice, indicating selectivity of its effect and as also suggested by our data on water intake. Collectively, these data show in several species of mammals that peripheral administration of SR 141716 at doses less than 3 mg/kg reduces intakes of bland or palatable foods, but that effect might be masked under schedules of restricted or peak nocturnal feeding.

Colombo et al. (1998) reported suppression of 24-h chow intake in free-feeding rats by high doses (2.5–10 mg/kg i.p.) of SR 141716 given once daily near the beginning of the nocturnal period. They also found that the anorectic action disappeared within 3–6 days, suggesting that tolerance occurred, although weight loss was sustained. The interpretation of those data is complicated by the possibilities that these higher doses may have adverse (but adapting) side-effects, rats may learn to increase their daily food intake during the subsequent light period when drug levels would be lower, and because weight loss provides an incremental stimulus to eat. Our first repeated dosing study was designed to overcome all of these difficulties. We found that anorexia was unchanged across three injections, a protocol that has been associated with profound tolerance to dexfenfluramine anorexia (Rowland et al. 2001). It is possible, of course, that tolerance might have developed using other doses or longer term treatments. We were unable to use osmotic minipumps in this study due to the low solubility of SR 141716. Nonetheless, our chronic oral dosing protocol shows that intermittent administration of SR 141716, to avoid large excursions in tissue levels, is

effective for 3 days. We did not continue this for longer because accumulative weight loss (not measured) potentially would have compromised the interpretation as well as the rats' welfare. Thus, over 3 days, SR 141716 anorexia shows no tolerance in either an acute palatable dessert protocol or under free-feeding conditions. Also, it is active orally in rats as previously has been shown after acute administration to marmosets (Simiand et al. 1998).

SR 141716 is insoluble in water so we first used dimethylsulfoxide, in which it is readily soluble, as vehicle (the chronological order in which we did the studies is different from the order in which the studies have been presented). Despite the fact that baselines were unaffected using this vehicle, we were concerned about toxicity with repeated injections. The lowest dose of SR 141716 was soluble in warmed 50% aqueous propylene glycol but we were unable to keep higher doses in solution unless we used 100% propylene glycol. Neither of these vehicles adversely affected food intakes of controls.

In terms of interaction with other feeding systems, Williams and Kirkham (1999) reported that the 5-HT releasing agent dexfenfluramine did not reverse THC-induced hyperphagia. This is surprising because most forms of experimental overeating are attenuated by treatment with dexfenfluramine (Rowland and Carlton 1988). In the present study we found that dexfenfluramine and SR 141716 anorexias are additive, which is consistent with independent mechanisms of action. In contrast, we found a supra-additive anorectic effect of SR 141716 with the opioid antagonist, naloxone. This result is consistent with a recent report by Kirkham and Williams (2001), although they used quite different feeding protocols and did not use isobolographic analysis. We have previously used isobolograms to investigate interactions of several pairs of pharmacological agents. These include phentermine and dexfenfluramine (Roth and Rowland 1999), norepinephrine and serotonin uptake inhibitors (Rowland et al. 2000), and dehydroepiandrosterone and monoaminergic agents (Rowland et al. 2001), and in each case the effects have been statistically additive. Thus, the finding of supra-additivity here is quite unusual. We should emphasize that we made conservative estimates of the DI_{50}s for the individual agents, and this makes the interaction even more impressive. Further support for an opioid-cannabinoid interaction includes the finding that THC-induced hyperphagia is reversed by low doses of naloxone (Williams and Kirkham 2000; see also Ledent et al. 1999; Welch and Eads 1999).

The location of the anorectic effect of SR 141716, or its interaction with naloxone, in feeding is not at all clear, because CB1 receptors are not exclusive to the brain. Indeed, not only was SR 141716 more effective orally than we had anticipated, but also in preliminary studies we were unable to obtain suppressions of food intake using s.c. injection of either SR 141716 or it analog AM 281. This high effectiveness of i.p. and oral routes of administration indicate that further studies on gut mechanisms of action are warranted. Because SR 141716 crosses the blood-brain barrier, most investigators have assumed that its anorectic effects are central in origin, but the only direct evidence for this is a report that cerebroventricular administration of AM 281 reduces intake in food-deprived rats (Koch and Werner 2000). Recently, Di Marzo et al. (2001) presented evidence for a negative and downstream regulation of endocannabinoids by leptin. They found that i.v. injection of leptin to mice produced a rapid decrease in hypothalamic endocannabinoid levels. It is also important to note that cannabinoid agents, either alone or in combination with opioid agents, may be important in the intake of other potentially abused substances. For example, SR 141716 decreases intake and motivation for alcohol containing beverages (Arnone et al. 1997; Freedland et al. 2001; Gallate and McGregor 1999). These data indicate that endocannabinoids are an important signaling system in the regulation of food and alcohol intake, and pharmacological intervention in this system may be clinically effective and useful. However, further studies on the sites of action and interactions with other systems are needed.

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