Behavioral/Systems/Cognitive

Activating Parabrachial Cannabinoid CB_1 Receptors Selectively Stimulates Feeding of Palatable Foods in Rats

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The endocannabinoid system is emerging as an integral component in central and peripheral regulation of feeding and energy balance. Our investigation analyzed behavioral roles for cannabinoid mechanisms of the pontine parabrachial nucleus (PBN) in modulating intake of presumably palatable foods containing fat and/or sugar. The PBN serves to gate neurotransmission associated with, but not limited to, the gustatory properties of food. Immunofluorescence and *in vitro* [^{35}S]GTPγS autoradiography of rat tissue sections containing the PBN revealed the presence of cannabinoid receptors and their functional capability to couple to their G-proteins after incubation with the endocannabinoid 2-arachidonoyl glycerol (2-AG). The selective cannabinoid 1 receptor (CB_1R) antagonist AM251 [*N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide] prevented the response, demonstrating CB_1R mediation of 2-AG-induced coupling. Microinfusions of 2-AG into the PBN in behaving rats robustly stimulated feeding of pellets high in content of fat and sucrose (HFS), pure sucrose, and pure fat (Crisco), during the first 30 min after infusion. In contrast, 2-AG failed to increase consumption of standard chow, even when the feeding regimen was manipulated to match baseline intakes of HFS. Orexigenic responses to 2-AG were attenuated by AM251, again indicating CB_1R mediation of 2-AG actions. Furthermore, responses were regionally specific, because 2-AG failed to alter intake when infused into sites ~500 μm caudal to infusions that successfully stimulated feeding. Our data suggest that hedonically positive sensory properties of food enable endocannabinoids at PBN CB_1Rs to initiate increases in eating, and, more generally, these pathways may serve a larger role in brain functions controlling behavioral responses for natural reward.

Key words: **brainstem; cannabinoids; CB_1 receptor; eating; feeding; opioid; parabrachial; reward**

Introduction

Feeding and energy balance are regulated by a complex network of brain regions and diverse neurochemical mechanisms, including those of the endocannabinoid system (Matias and Di Marzo, 2007). The recent identification of natural ligands (endocannabinoids) (Devane et al., 1992; Mechoulam et al., 1995; Sugiura et al., 1995), receptors responsible for their signaling (Devane et al., 1988; Munro et al., 1993), and proteins modulating their synthesis and inactivation (Piomelli, 2003) has afforded considerable pharmacological precision in elucidating various molecular, cellular, and behavioral actions of the cannabinoid system. Endocannabinoid mechanisms are present in all brain regions regulating feeding, including the ventral striatum, hypothalamus, and brainstem. Although serving diverse physiological roles, the influences of endocannabinoids on the neural substrates of feeding are only beginning to be identified. Orexigenic responses in rats were reported after infusion of endocannabinoids into the nucleus accumbens shell, a limbic forebrain region strongly implicated in the motivational mechanisms for feeding (Kirkham et al., 2002; Kelley et al., 2005; Soria-Gomez et al., 2007). In addition, inhibiting the endocannabinoid degradative enzyme fatty acid amide hydrolase increased endocannabinoid concentrations within the nucleus accumbens shell and stimulated feeding via cannabinoid 1 receptors (CB_1Rs) (Soria-Gomez et al., 2007). Orexigenic responding has been further reported after CB_1R stimulation in multiple hypothalamic nuclei regulating food intake (Anderson-Baker et al., 1979; Jamshidi and Taylor, 2001; Verty et al., 2005). Together with experiments describing increased levels of limbic forebrain endocannabinoids during fasting (Kirkham et al., 2002) and decreased hypothalamic levels after systemic administration of the anorexigenic mediator leptin (Di Marzo et al., 2001), the evidence clearly suggests a role for forebrain endocannabinoids in controlling food intake.

Investigations have focused on these forebrain regions traditionally associated with feeding, whereas sparse attention has been given to endocannabinoid activity in the hindbrain. Nonetheless, infusions of a synthetic cannabinoid agonist into the fourth ventricle modestly increased feeding (Miller et al., 2004). Furthermore, CB_1Rs were upregulated in the nodose ganglia of rats during food deprivation and downregulated during refeeding (Burdyga et al., 2004). Systemic administration of the satiety-related peptide cholecystokinin blocked the fasting-induced upregulation of CB_1Rs, whereas a cholecystokinin antagonist

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blocked their downregulation after feeding. These results suggest roles in feeding for neuronal cannabinoid mechanisms extending from the periphery through the hindbrain and into the most rostral regions of the nervous system. The hindbrain lies at the convergence of sensory information for the viscera and pathways distributing to regions influencing caloric regulation and caloric intake. The sites, specific mechanisms, and behavioral functions of hindbrain endocannabinoids, therefore, remain to be elucidated.

The pontine parabrachial nucleus (PBN) gates neurotransmission associated with, but not limited to, the gustatory properties of food and viscerally derived satiety signaling. Multiple receptor systems in the PBN have been implicated in modulating feeding. For example, we have shown that the local activation of serotonergic and opioidergic signaling pathways decreased (Simansky and Nicklous, 2002) and increased (Wilson et al., 2003) food intake, respectively. CB_1Rs and their mRNA have been identified in the PBN (Herkenham et al., 1991; Mailleux and Vanderhaeghen, 1992); however, their functional characteristics and behavioral roles in feeding have not been studied. This investigation aimed to evaluate PBN CB_1R G-protein activity and analyze behavioral roles for the system in controlling feeding. We report anatomically selective actions of an endocannabinoid in the PBN to increase consumption of foods with high hedonic value. Furthermore, response characteristics to endocannabinoids at CB_1Rs differed from those for opioid ligands at PBN μ-opioid receptors (MOPRs). Our results support a role for the PBN endocannabinoid system in providing an added layer of control to the complex neural network modulating feeding, energy balance, and natural reward.

Materials and Methods

Animals. Sixty-five male Sprague Dawley rats (Taconic Farms) weighing between 300 and 375 g at time of surgery were used for these experiments. Animals were housed individually in plastic hanging cages with wire-mesh floors (43 × 22 × 18 cm) and maintained on a standard 12 h light/dark cycle (lights on at 7:00 A.M.) at 23 ± 2°C. Standard laboratory chow (see below, Surgical procedures) and water were provided *ad libitum*, unless otherwise noted. All experimental procedures complied with the *Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research* of the National Research Council (2003) and were approved by the Institutional Animal Care and Use Committee of Drexel University.

Surgical procedures. Rats were implanted bilaterally, under pentobarbital (35 mg/kg) and chloryl hydrate (160 mg/kg) anesthesia (Equithesin), with bilateral 26 gauge stainless steel guide cannulas (3.8 mm center-to-center; Plastics One) aimed centrally within the lateral PBN (lPBN). Guide cannulas were secured to the skull using three stainless steel screws (Small Parts) and orthodontic resin (Dentsply). Twenty-eight gauge obturators (Plastics One) were placed into the guide cannulas immediately after surgery to prevent occlusion. Stereotaxic coordinates for cannulas placement were determined according to Paxinos and Watson (1998) using standard flat-skull technique (from bregma to lambda): 9.5–9.8 mm caudal to bregma, 1.9 mm lateral to the midline suture, and 4.8 mm ventral. For pain management, animals were administered ketoprofen (1 mg/kg at 2 mg/ml United States Pharmacopeia grade; Sigma-Aldrich) just before and 24 h after surgery. All animals were allowed 7–10 d to recover from surgery before testing commenced.

Drugs. The CB_1R agonist 2-arachidonoyl glycerol (2-AG) (molecular weight, 378), the CB_1R antagonist AM251 [*N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide] (molecular weight, 555), the MOPR agonist DAMGO ([D-Ala^2, *N*-Me-Phe^4, Gly^5-ol] enkephalin) (molecular weight, 514), and the MOPR antagonist CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2) were obtained from Tocris Cookson. The adenosine A_1 receptor antagonist DPCPX (1,3-dipropyl-8-cyclopentylxanthine) (molecular weight, 304) was purchased from Sigma-Aldrich. Because of the highly lipophilic nature of cannabinoid ligands, 2-AG was first solubilized in dimethylsulfoxide (DMSO) (Sigma-Aldrich), and then 0.9% (w/v) NaCl was slowly added to yield a final concentration of 25% DMSO for the vehicle. AM251 was solubilized in 100% DMSO. DAMGO and CTAP were solubilized in 100% sterile saline. Drugs were prepared freshly at the appropriate concentration just before experimentation. Infusions were made in a total volume of 0.5 μl with a Harvard infusion pump (Harvard Apparatus) using a 10 μl Hamilton microsyringe attached to a 33 gauge injector with PE20 polyethylene tubing (Becton Dickinson). Injector tips extended 2.5 mm past the tips of the guide cannulas. Bilateral infusions were made over 90 s, beginning between 9:00 A.M. and 10:00 A.M., with the injector left in place for 30 s after infusion of drug or vehicle to minimize backflow of liquid.

Experimental procedure. Experiments began after 1 week of daily habituation to a feeding schedule consisting of 4 h access to one of the test diets. At this time, baseline intakes were stable and did not vary by >10% during the last 3 d of this habituation. Except when noted, all testing occurred according to the following schedule: vehicle infusions on day 1, drug on day 2, no infusion on day 3, and then repeat cycle. For experiments investigating the ability of AM251 to block 2-AG-induced feeding, the group received all conditions in a counterbalanced manner as follows, with infusions 20 min apart and at least 48 h between conditions: vehicle + vehicle, AM251 + vehicle, vehicle + 2-AG, or AM251 + 2-AG. The same design was used to test whether CTAP would block the action of 2-AG. For confirming the action of CTAP to block DAMGO-induced feeding, the conditions were as follows: vehicle + vehicle, CTAP (0.01 or 0.03 nmol) + vehicle, vehicle + DAMGO, and CTAP (0.01 or 0.03 nmol) + DAMGO. A third dose of CTAP was not tested because the cannulas did not remain patent in all rats beyond these treatments.

After infusion of vehicle or drug, standard laboratory rodent chow was removed from the home cages and replaced with preweighed quantities (30 g) of one of the test diets. Test diets included the following: high-fat/sucrose (HFS) pellets [5.56 kcal/g (58% kcal from fat, 16% protein, 26% carbohydrate); catalog #D12331; Research Diets]; solid Crisco [9 kcal/g (100% kcal from fat); J. M. Smucker Co.]; 45 mg sucrose pellets [4 kcal/g (100% kcal from carbohydrate); Bio-Serv]; and standard pelleted laboratory chow [3.34 kcal/g (12% kcal from fat, 28% protein, 60% carbohydrate); Purina 5001]. HFS and standard chow pellets were simply placed on the floor in the front of the cage, and Crisco and sucrose pellets were given in a small ceramic bowl in the front of the cage. Intakes, adjusted for spillage, were measured 0.5, 2, and 4 h after infusion. After completion of the 4 h test, remaining diet was removed and replaced with access to 60 g of standard chow *ad libitum*. In one of our experiments, animals were rationed their standard chow to raise 30 min baseline intakes to near HFS quantities (see further description in Results).

Our laboratory has demonstrated routinely that the MOPR agonist DAMGO increases consumption of food when infused into this site in the lPBN (Nicklous and Simansky, 2003; Wilson et al., 2003). For later comparison with cannabinoids, we initially probed all groups of animals with DAMGO (1 nmol/side), except when we analyzed attempts at pharmacological blockade of CB_1Rs with AM251 and with CTAP. Significant responses to PBN infusion of DAMGO for all diets tested were found by 4 h after infusion (see Results).

Immunohistochemistry. Three rats were deeply anesthetized and perfused transcardially with 10% phosphate-buffered Formalin, pH 7.4 (Fisher Scientific) using a peristaltic pump (Cole Parmer Instruments). Brains were removed, immersed in phosphate-buffered Formalin for 1 h, and then transferred to 0.1 M PBS containing 30% (w/v) sucrose for 48 h. Brainstems were blocked and frozen at −16°C, with 30-μm-thick sections (Leica cryostat model CM3050) containing the PBN collected in PBS. After washing sections in PBS three times for 10 min each wash, sections were incubated in PBS containing 10% normal donkey serum (NDS) (Vector Laboratories) and 0.3% Triton X-100 for 30 min at room temperature to reduce background staining. For CB_1R and MOPR immunofluorescence, sections were incubated simultaneously with CB_1R and MOPR primary antibodies for 24 h at room temperature. The rabbit anti-human CB_1R polyclonal antibody was directed against a 14 amino acid sequence toward the extracellular N terminus of the receptor (1:800

dilution in 4% NDS/PBS; Alpha Diagnostics International). The guinea pig anti-rat MOPR polyclonal antibody was directed against a 14 amino acid sequence toward the C terminus of the receptor (1:5000 dilution in 4% NDS/PBS; Millipore Bioscience Research Reagents). The CB_1R primary was prepared 24 h ahead of the assay for the following two conditions: (1) one tube was prepared containing the correct amount of CB_1R primary antibody needed to obtain a 1:800 dilution in the assay, along with 50 times this concentration of the control peptide; and (2) a second tube was prepared containing only the CB_1R primary antibody, and all tubes were allowed to remain at room temperature for 2 h and then subsequently stored at 4°C for 24 h to allow for preabsorption of the primary antibody to the control peptide. After three 10-min washes with PBS, sections with CB_1R and MOPR primary antibodies were incubated simultaneously with two secondary antibodies for 2 h at room temperature (each diluted 1:200 in 4% NDS/PBS): donkey anti-rabbit IgG conjugated with FITC and donkey anti-guinea pig conjugated with tetramethylrhodamine isothiocyanate (TRITC) (both secondary antibodies from Jackson ImmunoResearch). All secondary antibodies were cross-adsorbed by the manufacturer to ensure specificity for primary antibodies raised in rabbit and guinea pig, respectively. After three 10-min washes in PBS, sections were mounted onto chrome-alum subbed glass slides and placed under coverslips with Vectashield mounting medium (Vector Laboratories). Fluorescently labeled sections were visualized with a fluorescent microscope (Leitz Aristoplan), and digital pictures were obtained using a Leica DC-200 camera linked to Leica DC Viewer software.

In situ *[^{35}S]GTPγS autoradiography.* The procedure for [^{35}S]GTPγS autoradiography was based on our previously published methods (Ward and Simansky, 2006; Ward et al., 2006) and modified for investigation of cannabinoid receptor mechanisms (Savinainen et al., 2001). Twenty-micrometer-thick sections containing the PBN (~9.5–9.8 mm caudal to bregma) were cut and thaw mounted onto chrome-alum subbed slides. Slides were preincubated in slide mailers for 20 min in assay buffer (in mM: 50 Tris-HCl, 4 $MgCl_2$, 0.3 EGTA, and 100 NaCl, pH 7.4) at 25°C, followed by 40 min incubation in 2 mM GDP assay buffer at 25°C. Sections were then incubated for 45 min at 25°C in assay buffer containing both [^{35}S]GTPγS (0.04 nM, 1250 Ci/mmol; PerkinElmer Life and Analytical Sciences) and 2 mM GDP (Sigma-Aldrich). Each mailer contained either no drug (basal condition), 2-AG (50 μM) (Savinainen et al., 2001), AM251 (50 μM), both, or unlabeled GTPγS (guanosine-*S*′-*O*-(γ-thio)-triphosphate) (10 μM; MP Biomedicals) to determine nonspecific binding. All conditions were incubated with the adenosine A_1 antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) (1 μM) for reduction of basal activity (Savinainen et al., 2001). After incubation, slides were rinsed twice for 2 min each in cold (4°C) 50 mM Tris-HCl buffer, pH 7.4, rinsed briefly in cold deionized water, and then dried immediately with a cool stream of air and dessicated overnight. Slides were exposed to Kodak Biomax MS film (Eastman Kodak) for 24 h. Autoradiograms were scanned and quantified using Image Pro-Plus Version 4.5 software (MediaCybernetics). We determined the optical densities [optical density unit (ODU)] of [^{35}S]GTPγS incorporated into the PBN for each 20 μm section from each rat. All ODU values (including nonspecific binding) were corrected for background of the film. Nonspecific binding was subsequently subtracted from each condition. Each set of sections per rat consisted of alternating sections of nonspecific, basal, 2-AG-stimulated, AM251-stimulated, and both. Four sections per animal for each condition were used for quantification, with the ODU units of each section obtained by averaging stimulation in left and right hemispheres.

Histology. After completion of all experiments, animals were killed by guillotine, and brains were removed, blocked to isolate hindbrain, and then immediately frozen to −18°C. Thirty-micrometer-sections were obtained containing the infusion site then slide mounted. Slides containing the tissue sections were projected onto templates of coronal sections of the brain with a Camera Lucida (Bausch and Lomb) for anatomical verification. The circle in Figure 1 (top row, left panel) indicates the boundaries in which the injector tips were located for infusion.

Statistical analysis. For experiments investigating the actions of 2-AG on intake of HFS, standard chow, Crisco, and sucrose, the mean intakes after infusion of vehicle were averaged together. This single value (vehicle as a condition) was used for statistical comparison versus intakes after infusion of each dose of 2-AG at each cumulative time point (0.5, 2.0, and 4.0 h after infusion) using a two-way repeated measures ANOVA with *post hoc* evaluation by the Student–Newman–Keuls test. For the experiments investigating the actions of DAMGO on the intake of the standard chow and HFS and the experiment evaluating anatomical specificity of the orexigenic response to 2-AG, a single dose was infused, and therefore a single vehicle value from the previous day (vehicle or drug condition vs 0.5, 2.0, and 4.0 h) was used for statistical comparison with a one-way ANOVA and *post hoc* evaluations for comparisons among means by Student–Newman–Keuls test. For our blockade experiments using AM251 or CTAP, statistical comparison of conditions at 30 min was made by ANOVA. An α level of $p < 0.05$ was the threshold for significance for all statistical tests.

Results

Immunofluorescence identifies the presence of CB_1Rs and MOPRs throughout the PBN

CB_1R-like immunoreactivity can be seen throughout the PBN (Fig. 1, middle row, left panel) and is absent when the CB_1R primary antibody was preabsorbed with the control peptide (top row, right panel). As reported previously (Nicklous and Simansky, 2003), MOPRs are found throughout the PBN (middle row, middle panel). Overlay of images shows sparse areas of overlap (middle row, right panel). At a higher magnification (bottom row) of the area in which we made infusion for our behavioral studies (lateral PBN), both types of receptors appear to be localized on neuritic processes (line arrow) and cell bodies (open arrow). Nonetheless, from these immunofluorescence data, only very few instances of potential colocalization of MOPRs and CB_1Rs appear to exist. In preliminary work from this laboratory, MOPRs appear to colocalize with both the dendritic marker MAP2 and the terminal marker TAU. Additional investigations using confocal and/or electron microscopy will be necessary to definitively identify the cellular locations of CB_1Rs in the PBN and of their common expression with MOPRs.

[^{35}S]GTPγS autoradiography reveals CB_1R-mediated G-protein coupling in the PBN, an effect blocked by CB_1R antagonism

Figure 2 (left panel) shows autoradiograms of tissue sections containing the PBN, with conditions identified as basal (top left; no ligand), 2-AG (top right; 50 μM), AM251 (bottom left; 50 μM), and both 2-AG and AM251 together (bottom right). Optical densities were quantified and represented in Figure 2 (right panel). Incubation of sections with 2-AG stimulated G-protein coupling ($p < 0.01$), an effect blocked by coincubation of 2-AG with the CB_1R-selective antagonist AM251 ($p < 0.01$), thereby supporting the mediation of 2-AG-stimulated coupling by CB_1Rs.

Parabrachial infusions of 2-AG stimulate feeding of a high-fat/sucrose diet, an action blocked by CB_1R antagonism

2-AG stimulated feeding of HFS pellets during the first 30 min after infusion (Fig. 3, $p < 0.05$; $p < 0.01$). Cumulative intakes, however, were not significantly different from those for vehicle treatment at 2 and 4 h, indicating that animals compensated for their 30 min orexigenic responses by decreasing consumption at later time points. There was an apparent inverted U dose effect for 2-AG as indicated by the failure of the highest

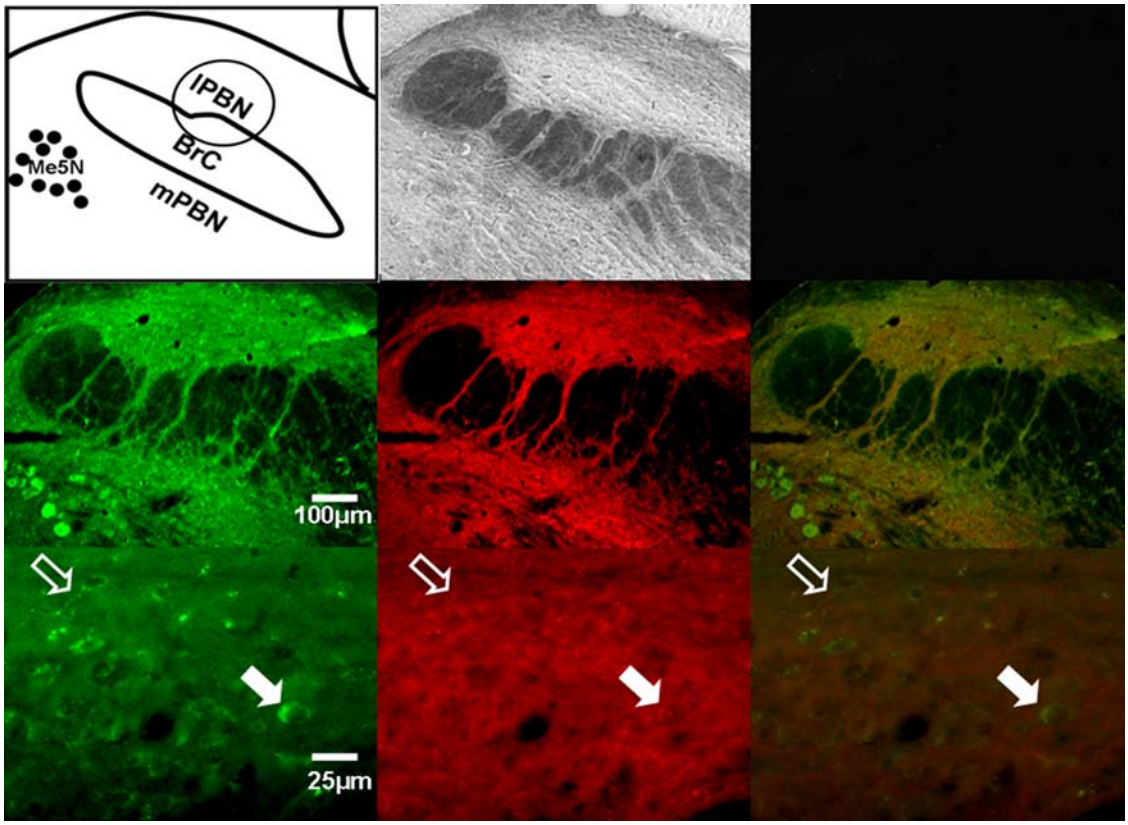

Figure 1. Immunofluorescence identifies the presence of CB_1Rs and MOPRs throughout the PBN. The bright-field image (top row, middle panel) shows the tissue section in which preabsorbing the CB_1R primary antibody with the control peptide completely blocked immunofluorescence for CB_1Rs, indicating the specificity of the antibody for this protein (top row, right panel). Green CB_1R-like immunoreactivity (FITC conjugate) can be seen throughout the PBN (middle row, left panel). Red MOPRs (TRITC conjugate) are found throughout as well (middle row, right panel). Overlaying the images revealed some small areas of overlap (yellow; middle row, right panel). In the higher-power images (bottom row), open arrows indicate what appear to be neuritic processes. Filled arrows indicate what appear to be cell bodies. Overlaying the images reveals only partial areas of potential colocalization (yellow; bottom row, right panel). Infusion sites were located within the black circle in the central lateral PBN (top row, left panel).

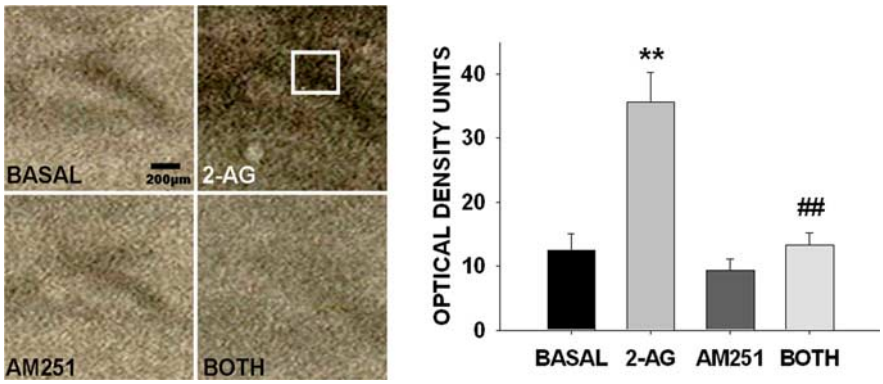

Figure 2. GTPγS autoradiography reveals G-protein coupling stimulated by 2-AG in the central lateral PBN *in vitro* is blocked by the CB_1R antagonist AM251. Left shows typical autoradiograms for 2-AG (50 μM), AM251 (50 μM), and the combination of 2-AG and AM251. All tissue was incubated in the presence of the adenosine A_1 antagonist DPCPX (1 μM) to reduce adenosine-mediated basal activity. Right shows values in optical density units + SEM for incorporation of [^{35}S] GTPγS for tissue from four rats studied *in vitro*. $^{**}p < 0.01$ indicates significant differences between value of 2-AG and all of the other conditions; $^{\#\#}p < 0.01$ indicates significant differences between value for 2-AG and the combination of 2-AG with AM251 (BOTH); ANOVA followed by Student–Newman–Keuls test. Area of quantification is within the white square (2-AG panel).

(2 nmol) and lowest (0.25 nmol) doses to significantly increase feeding. Informal observations noted decreased motor activity in the animals when infused with 2 nmol 2-AG. Importantly, in a separate group of animals ($n = 5$), pretreatment with the CB_1R antagonist AM251 prevented the action of 2-AG (1 nmol) to stimulate 30 min intake (Fig. 4, 2-AG vs vehicle, $p < 0.05$; both 2-AG and AM251 vs 2-AG alone, $p < 0.05$), implicating CB_1Rs in the actions of 2-AG. This dose of 2-AG did not alter water intake during the testing period. Furthermore, in a separate group of animals, AM251 alone failed to alter intake at any dose (0.1, 1.0, and 10.0 nmol) and time point during testing (Fig. 4, inset). The doses stated for all compounds used throughout this series of studies represent the amount of ligand infused into each side of the brain (bilaterally).

The action of 2-AG to increase feeding of HFS is anatomically specific

To assess the anatomical specificity of the locus for orexigenic actions of 2-AG, animals ($n = 7$) were implanted ~10.2 mm caudal to bregma, which is ~400–700 μm further caudal to the sites in which infusion of 2-AG increased feeding of HFS. In these control animals, the concentration of 2-AG (1 nmol) that maximally stimulated HFS intake (Fig. 3) failed to alter feeding of HFS at any time point during testing (Fig. 5).

Parabrachial infusion of 2-AG increased feeding of pure fat and sucrose

2-AG increased consumption of fat and sucrose (in separate groups of animals) by 30 min versus vehicle treatment (Fig. 6 left

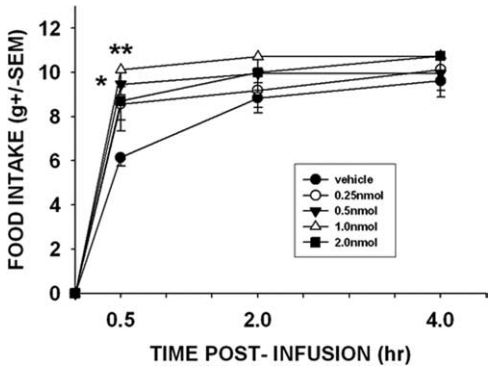

Figure 3. 2-AG stimulated feeding of high-fat/sucrose pellets during the first 30 min after infusion into the central lateral PBN. 2-AG stimulated the intake of high fat/sucrose pellets by 30 min after infusion ($n = 7$). *$p < 0.05$ and **$p < 0.01$ indicate significant differences between values of 2-AG and vehicle.

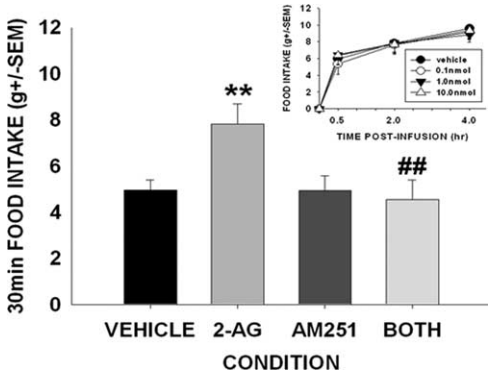

Figure 4. The orexigenic actions of 2-AG are blocked by CB_1R antagonism. The CB_1R antagonist AM251 (1 nmol) completely blocked the actions of 2-AG ($n = 5$). In a separate group of animals ($n = 8$), AM251, at all concentrations tested, failed to alter the intake of high-fat/sucrose pellets (inset). **$p < 0.01$ indicates significant differences between value for 2-AG versus vehicle. ##$p < 0.01$ indicates significant differences between value of 2-AG and AM251 (BOTH) versus 2-AG alone; ANOVA followed by Student–Newman–Keuls test.

and right panels, respectively; $p < 0.05$). Orexigenic responses for sucrose persisted until 2 h for only 2 nmol 2-AG ($p < 0.05$) and were absent by 4 h.

Parabrachial infusion of 2-AG failed to increase intake of standard rodent chow

In contrast to all other test diets, 2-AG failed to increase intake of standard chow (Fig. 7, bottom); however, baseline intakes were nearly absent at 30 min and only nominal by 4 h. To address whether higher baseline intakes influenced the ability for 2-AG to increase HFS feeding, a schedule of rationed feeding was used. Under this feeding schedule, animals were given daily access to 30 g standard chow (matching ~100% of their normal daily intake at 10:00 A.M.). Although not calorically restricting the animals, this feeding schedule increased baseline intakes during the initial 30 min period of access to the food to levels similar to those obtained at 30 min for HFS (Fig. 3). Intakes remained unaltered compared with vehicle at any concentration of 2-AG and time point (Fig. 7, top) when animals were maintained on this feeding schedule.

Parabrachial infusion of the MOPR agonist DAMGO stimulated feeding of HFS and standard chow at later time points

DAMGO (2 nmol) increased intake of the HFS diet by 4 h after infusion (Fig. 8, top; $p < 0.05$). Additionally, intakes for standard

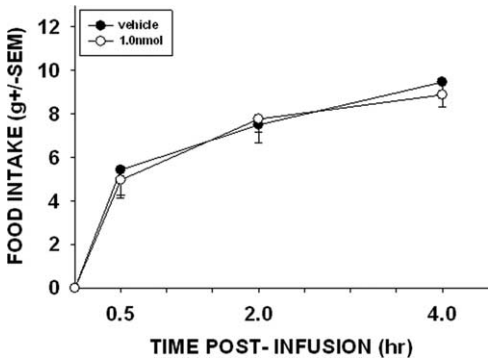

Figure 5. 2-AG failed to alter intake of high fat/sucrose pellets in off-target anatomical controls. The concentration of 2-AG that gave maximal stimulation of high fat/sucrose pellets intake (1 nmol; see Fig. 3) was infused ~400–700 μm caudal to infusions successfully stimulating intake ($n = 6$). In these anatomical controls, 2-AG failed to alter intake at any time point.

chow were elevated by 2 h after infusion (bottom; $p < 0.01$) and persisted at 4 h. Intakes of all diets remained unaltered 30 min after infusion versus vehicle. This delayed onset of eating, which contrasted with the early onset after 2-AG, agreed with our previous results for DAMGO-stimulated consumption of standard chow (Wilson et al., 2003). DAMGO also increased intake of the other diets (Crisco and sucrose pellets) with the same delayed onset (data not shown).

Blocking parabrachial MOPRs with the MOPR antagonist CTAP failed to affect the actions of 2-AG to stimulate feeding of HFS

DAMGO (2 nmol) increased the 4 h intake of HFS ($n = 7$, means ± SEM: vehicle, 9.8 ± 0.6 g; DAMGO, 13.5 ± 0.5 g; $p < 0.01$). Rats pretreated with CTAP (0.01 nmol) ate 12.6 ± 0.5 g. Increasing the dose of CTAP to 0.03 nmol reduced the DAMGO-stimulated orexigenic responses (13.5 ± 0.5 to 11.1 ± 0.06 g; $p < 0.01$). We calculated the difference from baseline intake for each condition on the basis of the data for each animal. DAMGO increased feeding by 3.8 ± 0.6 g. The two doses of CTAP reduced this by 27 ± 9% to 2.8 ± 0.6 g and by 75 ± 19% to 1.3 ± 0.9 g, respectively. Thus, a dose as small as 0.03 nmol of CTAP markedly reduced feeding stimulated by an MOPR agonist.

Given that 0.03 nmol CTAP successfully blocked orexigenic responses to DAMGO, we chose a dose of CTAP 10-fold higher (0.3 nmol) and tested its ability to affect the actions of 2-AG (1 nmol) to stimulate the 30 min intake of HFS in a separate group of animals. Blocking MOPRs with CTAP failed to affect the actions of 2-AG to stimulate the intake of HFS ($n = 5$; means ± SEM: vehicle, 6.5±.8 g; 2-AG, 10.1 ± 1.1 g; CTAP, 6.3 ± 0.8 g; 2-AG + CTAP, 10.5 ± 1.2 g; 2-AG vs vehicle; $p < 0.01$).

Discussion

These are the first data implicating a functional role for parabrachial endocannabinoid mechanisms in modulating food intake. The results confirmed the presence of CB_1R protein in the pontine region, demonstrated the ability of an endocannabinoid to stimulate coupling of these receptors to their G-proteins, and provided pharmacological evidence at the cellular level that CB_1Rs mediate increases in eating. Furthermore, parabrachial CB_1Rs appear to be linked to selectively enhancing consumption of foods presumed to have pleasurable sensory properties. Thus, the present study identified a new node in a series of sites along the neuraxis in which endocannabinoids increase food intake.

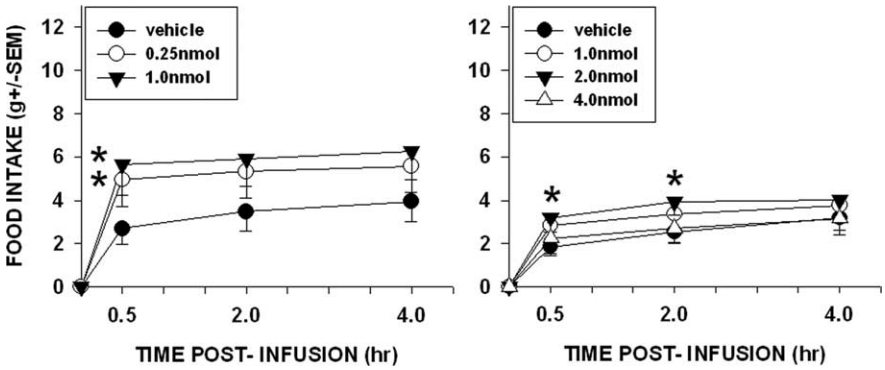

Figure 6. 2-AG increased intake of pure fat (Crisco) and pure sucrose (pellets). 2-AG (0.25 and 1 nmol) increased fat intake by 30 min after infusion ($n = 6$; left). Additionally, 2-AG (2 nmol) increased sucrose pellet intake at 0.5 and 2.0 h ($n = 6$; right). *$p <$ 0.05 indicates significant differences between values of 2-AG versus vehicle; ANOVA followed by Student–Newman–Keuls test.

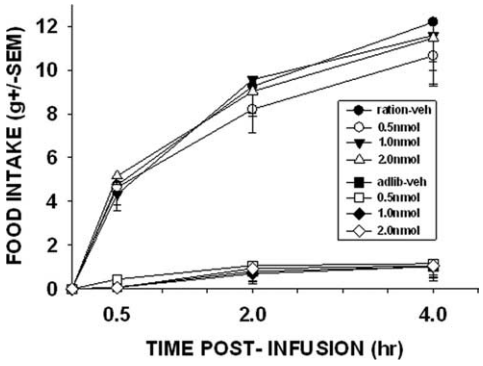

Figure 7. 2-AG failed to alter intake of *ad libitum* fed standard chow, regardless of baseline intake. In animals fed with *ad libitum* access to standard chow (adlib), 2-AG failed to alter intake at any time point tested ($n = 7$). 2-AG also failed to alter intake in a separate group of animals maintained on a schedule with rationed feeding (ration), in which their baseline intakes approximated those of animals eating high fat/sucrose pellets ($n = 7$).

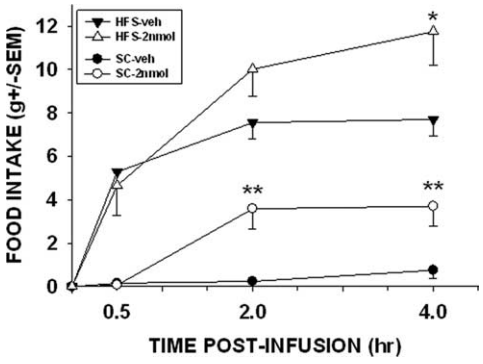

Figure 8. Stimulating parabrachial MOPRs with DAMGO (2 nmol) increased feeding of both high fat/sucrose pellets (HFS) and standard chow (SC) at later time points. Data represent mean of two groups of six rats each, with each rat infused with vehicle and DAMGO. *$p < 0.05$ and **$p < 0.01$ indicate significant differences between values of DAMGO versus vehicle; ANOVA followed by Student—Newman–Keuls test.

Moreover, we offer systematic evidence for a specialized role in the brainstem in which CB_1Rs may integrate eating with reward.

2-AG stimulated feeding of HFS pellets, pure fat, and sucrose. The endocannabinoid agonist appeared to selectively increase these diets with presumably pleasurable stimulus properties, because 2-AG failed to alter intake of standard chow, regardless of baseline. The energy density of sucrose (4 kcal/g) is considerably lower than that of the other palatable diets (HFS, 5.56 kcal/g; pure fat, 9 kcal/g) but similar to that of standard chow (3.34 kcal/g). Therefore, the positive hedonic value of the test diets, rather than their energy densities, were likely responsible for the orexigenic responses. Furthermore, the actions of 2-AG were limited to the first 30 min after infusion, and cumulative food intake returned to near baseline levels for most diets by 4 h. This indicated a shift in consumption to the beginning of the test period rather than persistent increases in consumption. The results may reflect a role for local CB_1Rs in initiating feeding responses to palatable foods. Alternatively, responses confined to the first 30 min may simply reflect the pharmacokinetic properties of 2-AG, especially rapid enzymatic degradation (Piomelli, 2003).

CB_1Rs and their mRNA have been identified in the PBN (Herkenham et al., 1991; Mailleux and Vanderhaeghen, 1992); however, their behavioral roles in feeding and functional characteristics have not been investigated previously in this brain region. We report a wide distribution of CB_1Rs throughout the PBN and their functional capacity to couple to their G-proteins, *in vitro*, after stimulation with 2-AG. Importantly, in parallel with behavioral effects, coupling induced by 2-AG was mediated by CB_1Rs, because coincubation with AM251 completely blocked the actions of this cannabinoid agonist. AM251 has been reported (Pertwee, 2005) to possess inverse agonist properties at CB_1Rs (i.e., reductions in basal activity in a constitutively active system). In the present study, however, it failed to decrease basal coupling, which may reflect that parabrachial CB_1Rs do not operate constitutively under our conditions. This agrees with work using rat cerebellar membrane homogenates (Savinainen et al., 2003). In that study, micromolar concentrations of AM251, or its analog SR141716 [*N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole carboxamide] (rimonabant), also did not reduce basal activity.

Systemic administration of the cannabinoid antagonists inhibits CB_1R activity throughout the brain and decreases food intake (Arnone et al., 1997; Colombo et al., 1998). In the present study, however, AM251 alone did not reduce baseline intakes of HFS under our test conditions, questioning the physiological necessity for intact endogenous signaling in the PBN to initiate or maintain feeding. This suggests that cannabinoid receptors in multiple brain regions must be antagonized to reduce feeding. As in our work, infusing antagonists into other single sites within the brain also failed to inhibit feeding. For example, an absence of anorectic actions for CB_1R antagonists has been reported when microinfused into the nucleus accumbens shell (Kirkham et al., 2002; Soria-Gomez et al., 2007), hypothalamic paraventricular nucleus (Verty et al., 2005), and lateral cerebral ventricles (Gomez et al., 2002) of rats. A lack of effect on baseline feeding for AM251 under our conditions suggests that endogenous activity at parabrachial CB_1Rs is not necessary to facilitate intake of HFS, but activating CB_1Rs is clearly sufficient.

We have identified a specific node in the brainstem in which CB_1Rs are present, functional, and serve a role in modulating feeding. The only previous report investigating brainstem cannabinoid mechanisms in feeding found a modest stimulation of consumption of sweetened condensed milk in rats after fourth ventricular infusions of the synthetic CB_1R agonist 2-[(1*S*,2*R*,5*S*)-5-hydroxy-2-(3-hydroxypropyl)cyclohexyl]-5-

(2-methyloctan-2-yl)phenol (Miller et al., 2004). Ventricular administration of ligands, however, fails to identify discrete brainstem sites in which endocannabinoid mechanisms may act.

The present work identifies the PBN as a discrete pontine site in which activating CB1Rs increases feeding and, in fact, does so selectively for palatable diets. Previous studies have rarely addressed the influence of diet on the orexigenic actions of CB_1R agonists. It has been reported, however, that lateral ventricular infusions of Δ^9-tetrahydrocannabinol (Δ^9-THC) in rats selectively increased intake of a chocolate cake batter rather than standard chow (Koch and Matthews, 2001). In contrast, standard chow intake was enhanced after activation of CB_1Rs directly by infusing the endocannabinoid agonist anandamide into the shell of the nucleus accumbens or indirectly by blocking enzymatic degradation or cellular uptake of native endocannabinoids (Soria-Gomez et al., 2007). Kirkham et al. (2002) also found orexigenic responses to standard chow when 2-AG was infused into the nucleus accumbens shell. Similarly, infusion of CB_1R agonists into a number of nuclei of the hypothalamus in rats increased intake of standard chow (Anderson-Baker et al., 1979; Jamshidi and Taylor, 2001; Verty et al., 2005). Together, the results suggest that forebrain mechanisms involving CB_1Rs modulate food intake regardless of dietary properties. However, the intracerebroventricular infusions may have gained access to pontine sites. Thus, the PBN may be the site in which endocannabinoids serve as the first (possibly only) gateway to circuits that respond to pleasurable food-related stimuli during ingestion.

A role for CB_1Rs in the PBN in modulating the intake of foods with hedonically positive sensory properties seems fitting given the anatomical positioning and known functions for this brain region. The PBN gates neurotransmission associated with, but not limited to, such stimuli. It communicates reciprocally with widely distributed brain loci identified in regulating feeding, including the ventral striatum and central nucleus of the amygdala, multiple hypothalamic nuclei, and viscerally derived satiety signals (Norgren, 1974, 1976; Norgren and Pfaffmann, 1975; Moga et al., 1990). Primary gustatory efferents from the tongue and mouth carry information associated with sensory properties of food along cranial nerves X, IX, and VII, synapsing in the nucleus of the solitary tract (NTS) (Norgren and Leonard, 1971; Contreras et al., 1982; Hamilton and Norgren, 1984). Second-order neurons of the NTS transmit this information onto the PBN, in which third-order neurons project to other areas of the brain for processing, including ventral striatal reward centers (Herbert et al., 1990). The ventral striatum has been proposed to assign hedonic value to sensory input via interactions with afferent gustatory signals (Norgren et al., 2006). Gustatory information travels through the waist region of the PBN, including portions of the lateral PBN and medial PBN that border the brachium conjunctivum (Norgren and Pfaffmann, 1975; Karimnamazi and Travers, 1998; Karimnamazi et al., 2002). Lesions confined mostly to the gustatory region of the medial PBN (mPBN) (Hajnal and Norgren, 2005) dramatically reduced nucleus accumbens dopamine levels in animals licking sucrose, supporting their view that the ventral striatum assigns positive affective value to sensory input transmitted by the gustatory PBN. Our target region for infusion was in the central lateral PBN (Fig. 1, left panel); however, the relatively large 0.5 μl infusion volume likely diffused to neighboring areas, including the mPBN gustatory region. Therefore, we are confident that 2-AG infusions bathed large portions of the gustatory PBN.

Cannabinoid and opioid mechanisms have been reported to interact in a variety of cellular and behavioral functions (Cota et al., 2006). In ingestive behavior, for example, Kirkham and colleagues used the general opioid receptor antagonist naloxone to block the CB_1R-dependant orexigenic responses to Δ^9-THC in rats. Both drugs were given systemically (Williams and Kirkham, 2002). This study suggested that functional interplay occurred between cannabinoid and opioid systems; however, the systemic administration of ligands failed to address interactions within specific brain sites. Furthermore, naloxone nonselectively antagonizes MOPRs and other opioid subtypes. We report the presence of MOPRs throughout the PBN in a similar distribution to CB_1Rs. Pharmacological blockade of parabrachial MOPRs failed to affect the actions on feeding of stimulating CB_1Rs. These results argue against functional interactions between the two receptor systems specifically within the PBN to modulate feeding of a palatable diet. The possibilities remain that this direct interaction occurs within other sites or that a series of neurons across sites allow for $MOPR/CB_1R$ interactions

Importantly, the actions of 2-AG greatly contrasted with those for the synthetic MOPR agonist DAMGO in the PBN. In concordance with data previously reported by our laboratory (Nicklous and Simansky, 2003; Wilson et al., 2003), DAMGO stimulated standard chow intake between 0.5 and 2.0 h after infusion and persisted until at least 4 h. Likewise, DAMGO increased feeding of palatable HFS over 4 h, indicating that DAMGO indiscriminately increased feeding of all test diets, regardless of their sensory properties. Unlike 2-AG, orexigenic responses to DAMGO were confined to later time points, highlighting a very different temporal component to MOPR actions on feeding when compared with those for CB_1Rs. It has been proposed (Ward and Simansky, 2006) that parabrachial MOPRs may be involved in the general maintenance of feeding behaviors through a dampening of satiety signaling transmitted by vagal afferents to the PBN, as evidenced by the delayed onset of response and nonselectivity for test diets. It should be noted, however, that irreversible blockade of MOPRs in the lateral PBN selectively reduces consumption of standard but not palatable chow (Ward and Simansky, 2006)

Our work lays the foundation for future investigations, including the use of appropriate behavioral paradigms to address more directly a role for parabrachial CB_1Rs in reward and convergent approaches testing the physiological role for parabrachial CB_1Rs in feeding, such as blocking enzymatic degradation of natively released endocannabinoids to enhance their local actions in the PBN. Overall, our data suggest that hedonically positive sensory properties of food enable endocannabinoids at parabrachial CB_1Rs to initiate increases in eating and, more generally, that these pathways may serve a larger role in brain functions controlling behavioral responses for natural reward.

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