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Activating μ -opioid receptors (MOPRs) in the lateral parabrachial nucleus increases c-Fos expression in forebrain areas associated with caloric regulation, reward and cognition

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Abstract

The pontine parabrachial nucleus (PBN) has been implicated in the modulation of ingestion and contains high levels of μ -opioid receptors (MOPRs). In previous work, stimulating MOPRs by infusing the highly selective MOPR agonist [d-Ala²,N-Me-Phe⁴,Gly⁵-ol]enkephalin (DAMGO) into the lateral parabrachial region (LPBN) increased food intake. The highly selective MOPR antagonist d-Phe-Cys-Trp-Arg-Thr-Pen-Thr-NH₂ (CTAP) prevented the hyperphagic action of DAMGO. The present experiments aimed to analyze both the pattern of neural activation and the underlying cellular processes associated with MOPR activation in the LPBN. Male Sprague-Dawley rats received a unilateral microinfusion of a nearly maximal hyperphagic dose of DAMGO into the LPBN. We then determined the level of c-Fos immunoreactivity in regions throughout the brain. MOPR activation in the LPBN increased c-Fos in the LPBN and in the nucleus accumbens, hypothalamic arcuate nucleus, paraventricular nucleus of the thalamus and hippocampus. Pretreatment with CTAP prevented the increase in c-Fos translation in each of these areas. CTAP also prevented the coupling of MOPRs to their G-proteins which was measured by [³⁵S]GTP γ S autoradiography. Together, these data strongly suggest that increasing the coupling of MOPRs to their G-proteins in the LPBN disinhibits parabrachial neurons which subsequently leads to excitation of neurons in regions associated with caloric regulation, ingestive reward and cognitive processes in feeding.

Keywords

obesity; brainstem; nucleus accumbens; arcuate nucleus; hippocampus; paraventricular nucleus of the thalamus

Numerous studies have examined the role of μ -opioid receptors (MOPRs) in the neural control of feeding behavior. Opioid receptor ligands have been administered centrally into multiple brain regions such as the nucleus tractus solitarius (NTS), parabrachial nucleus (PBN), hypothalamus, amygdala, ventral tegmental area (VTA) and nucleus accumbens (NAcc), in rats (Carr et al., 1991, Kotz et al., 1997, Giraudo et al., 1998b, Lamonte et al.,

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2002, MacDonald et al., 2003, Will et al., 2003, Wilson et al., 2003, Bodnar, 2004, Levine and Billington, 2004). Generally, the administration of an opioid receptor agonist promoted feeding whereas an opioid receptor antagonist decreased feeding. Additionally, interconnections between the aforementioned brain areas have been demonstrated (Giraud et al., 1998a, Giraud et al., 1998b, MacDonald et al., 2003, Quinn et al., 2003, Kim et al., 2004). Bidirectional μ -opioid-opioid signaling pathways have been reported between the paraventricular nucleus of the hypothalamus (PVH) and the VTA (Quinn et al., 2003) and also between the central nucleus of the amygdala and the NAcc (Kim et al., 2004). Another study demonstrated that increased fat intake induced by administration of a MOPR agonist into the NAcc was blocked reversibly by infusing the GABA-A agonist muscimol into any of four distinct regions, including the lateral hypothalamus, dorsomedial nucleus of the hypothalamus, ventral tegmental area and the intermediate region of the NTS (Will et al., 2003). Clearly, opioid receptors are part of a complex neural circuitry system involved in the regulation of food intake.

Our laboratory is interested particularly in the role of MOPRs located in the PBN in the modulation of food intake. The PBN, located in the pons, serves as a relay for gustatory signaling in rats (Norgren and Leonard, 1973) and also receives visceral sensory information from the gut via second-order vagal afferents (Norgren and Leonard, 1971). We have previously reported that the microinfusion of the MOPR agonist [D-Ala(2), N-MePhe(4), Gly-ol(5)]-enkephalin (DAMGO) into the lateral PBN (LPBN) increases food consumption (Wilson et al., 2003), regardless of the hedonic properties of the diet (i.e., standard chow or pellets high in fat and sucrose content) (Ward and Simansky, 2006, DiPatrizio and Simansky, 2008). Pretreatment with the MOPR antagonist, d-Phe-Cys-Trp-Arg-Thr-Pen-Thr-NH₂ (CTAP) blocked the orexigenic effect of DAMGO. Furthermore, infusion of the irreversible MOPR antagonist, β -funaltrexamine (β -FNA), into the LPBN prevented the action of parabrachial administration of DAMGO to increase feeding and, in parallel, prevented the action of this MOPR agonist to stimulate G-protein coupling *ex vivo*. In that study (Ward & Simansky, 2006), we used autoradiography followed by quantitative densitometry to measure the amount of [³⁵S] guanosine 5'-O-[gamma-thio]triphosphate ([³⁵S]GTP γ S) incorporated into coronal sections of PBN as an index of the coupling of MOPRs to their associated G-proteins elicited by DAMGO.

The present study was designed to link the local cellular actions of a hyperphagic dose of DAMGO in the PBN to neuronal activation in areas associated with the control of feeding. We used immunohistochemical detection of the c-Fos protein in combination with the cellular [³⁵S]GTP γ S autoradiography assay for this purpose. Immunohistochemical visualization of the c-Fos protein, a product of the *c-fos* gene, has been used to identify sites in which neuronal activity increases in response to the administration of agents targeting opioid receptors in other areas of the brain (Carr et al., 1999, Pecina and Berridge, 2000, Zhang and Kelley, 2000, Li et al., 2006). Here, we employed the reversible, competitive and selective MOPR antagonist CTAP to confirm a role for this receptor subtype in the actions of DAMGO to activate c-Fos and the coupling of MOPRs to their G-proteins in the PBN. In association with this strategy applied to the local circuit where DAMGO was infused, we analyzed the sites where c-Fos increased in the forebrain.

Experimental Procedures

Male Sprague-Dawley rats (Taconic Farms, Germantown, NY) were housed individually in suspended wire-mesh cages (43 cm length $\times$ 22 cm width $\times$ 18 cm height) in a temperature controlled (23 $\pm$ 2°C) room in an AAALAC-approved facility. The rats were maintained on a 12hr light/dark cycle, and all experiments were conducted during the light phase (0600h – 1800h). 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 (IACUC) of Drexel University. All rats had ad lib access to standard pelleted chow and water except during the 2hr experiment.

Immunohistochemical Study

c-Fos Immunohistochemistry—For the immunohistochemistry study, one week of adaptation after arrival was allowed prior to surgery. On the day of surgery, rats were anesthetized with Equithesin® (3.6mL/kg, ip) formulated to deliver pentobarbital (35mg/kg) and chloral hydrate (160mg/kg). Stainless steel guide cannulas (26 gauge; Plastics One, Roanoke, VA) were aimed at the lateral PBN (LPBN) using a stereotaxic apparatus (Kopf Instruments, Tujunga, CA) secured to the skull surface using three stainless steel jeweler's screws and cemented into place using dental acrylic. The stereotaxic coordinates were determined from the rat atlas of Paxinos and Watson (1998) and were 9.5 mm posterior to bregma, 1.8 mm lateral to the midline suture, and -4.8 mm ventral, with the skull level between lambda and bregma. Following surgery, obturators (33ga wire stylets; Plastics One) were inserted into the guide cannulas to prevent occlusion. All rats were allowed at least 7 days to recover after surgery.

The MOPR agonist DAMGO (2nmol/0.5µL; MW=514; Tocris Cookson, Ellisville, MO) and the MOPR antagonist CTAP (1nmol/0.5µL; MW=1104; Tocris Cookson, Ellisville, MO) were dissolved in 0.9% saline solution. We have previously demonstrated that unilateral administration of this dose of DAMGO (approximate ED₇₀) reliably and robustly increased chow intake and that this dose of CTAP blocked this hyperphagic effect (Wilson et al., 2003). These agents were infused by a Harvard microdrive pump (Harvard Apparatus Model 975, South Natick, MA) connected by polyethylene tubing (PE-20; Becton Dickinson, Sparks, MD) at a rate of 0.33µL/min for 90sec. The 33-ga injectors extended 1mm past the tip of the guide cannula and remained in place for 30sec following infusion.

In order to separate responders from nonresponders, we probed all rats with a single microinfusion of DAMGO. Testing began by providing each animal with approximately 60g of standard chow (3.34 kcal/g physiological fuel value, 28% protein, 12% fat, 60% carbohydrate; Purina 5001; St. Louis, MO). Measurements of food intake were recorded, to the nearest 0.1g, 30, 120 and 240min after DAMGO infusion and corrected for spillage. Responders were defined as rats that increased their food intake by 2g or more during the 240min period following DAMGO administration.

Rats classified as responders were divided into four groups. All groups received two infusions 15 min apart. One group (VEH/VEH) received only vehicle infusions. A second group (VEH/DAMGO) received a vehicle infusion followed by DAMGO. A third group (CTAP/VEH) received CTAP followed by vehicle. The final group (CTAP/DAMGO) received an infusion of CTAP followed by DAMGO. Food and water were not available to the rats after infusion. Ninety min following the last infusion, all rats were overdosed with Equithesin and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde using a peristaltic pump. The brains were removed, postfixed in 4% paraformaldehyde for approximately 1h, and then transferred to 0.1 M sodium phosphate buffer (PBS) containing 30% (wt/vol) sucrose for at least 24 h.

The protocol used for immunohistochemistry has been described previously by our laboratory (Nicklous and Simansky, 2003, DiPatrizio and Simansky, 2008). The brains were blocked, frozen at -16°C, and 30-µm-thick coronal sections (Leica cryostat model CM3050, Deerfield, IL) were collected in PBS. After a series of washes in PBS, the sections were incubated in PBS containing 10% normal goat serum (NGS; Vector Laboratories,

Burlingame, CA) and 0.3% Triton X-100 for 30min at room temperature to reduce background staining. Sections were then incubated with the primary antibody, rabbit anti-c-Fos antibody (Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1:5,000 in 4% NGS/PBS for 24h at room temperature. After three 10min washes with PBS, sections were incubated with biotinylated goat anti-rabbit secondary antibody (Vector Laboratories) diluted 1:200 in 4% NGS/PBS for 1h. Sections were again washed in PBS three times for 10min each after which the sections were exposed to an avidin/biotinylated enzyme complex (ABC) (Vectastain ABC kit; Vector Laboratories). After rinsing with PBS, staining was visualized using a 3,3'-diaminobenzidine (DAB) substrate kit (Vectastain DAB kit; Vector Laboratories; 3–5 min). Sections were rinsed and mounted on slides subbed with gelatin and chromium potassium sulfate dodecahydrate, dehydrated, and placed under coverslips with Permount. Sections were viewed under a Leitz Aristoplan (Wetzlar, Germany) microscope, and digital pictures were taken using a Leica DC-200 (Deerfield, IL) camera linked to Leica DC Viewer software. The number of c-Fos immunoreactive (c-Fos-IR) nuclei was semi-quantitatively measured using Image Pro-Plus® Version 5.1 software (MediaCybernetics; Newburyport, MA).

A unique area of interest was used to quantify each brain region. The total number of c-Fos-IR cells was divided by the area examined (0.15mm^2) to derive scores for counts per mm^2 . The ImagePro-Plus program provided the height and width of all elliptical shapes used to quantify each brain region.

MAP2/tau Immunofluorescence—Naïve, male Sprague-Dawley rats were overdosed with Equithesin and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde using a peristaltic pump. The brains were removed, postfixed in 4% paraformaldehyde for approximately 1h, and then transferred to 0.1 M sodium phosphate buffer (PBS) containing 30% (wt/vol) sucrose for at least 24 h. The brains were blocked, frozen at -16°C , and 30- μm -thick coronal sections (Leica cryostat model CM3050, Deerfield, IL) were collected in PBS. After a series of washes in PBS, the sections were incubated in PBS containing 10% normal donkey serum (NDS; Vector Laboratories, Burlingame, CA) and 0.3% Triton X-100 for 30min at room temperature to reduce background staining. For MAP2 and MOPR immunofluorescence, sections were incubated simultaneously with MAP2 and MOPR primary antibodies for 24hrs at room temperature. Dr I. Fischer generously provided the monoclonal antibody (1:5000 dilution in 4% NDS/PBS) (Crandall and Fischer, 1989) and was linked to rhodamine for immunofluorescence. MAP2 is highly localized to dendrites in the brain. The guinea pig anti-rat MOPR polyclonal antibody was directed against a 14-amino acid sequence towards the C-terminus of the receptor (1:5000 dilution in 4% NDS/PBS; Chemicon International, Temecula, CA).

For tau and MOPR immunofluorescence, sections were incubated simultaneously with tau and MOPR primary antibodies for 24hrs at room temperature. The anti-tau, polyclonal antibody (1:5000 dilution in 4% NDS/PBS) was generously provided by Dr. I. Fischer (Black et al., 1996) and was raised in rabbits against fusion protein constructs (rat) containing discrete regions near the carboxyl terminus of the protein (aa 616–686). Tau is localized preferentially, but not specifically, in axonal processes. The guinea pig anti-rat MOPR polyclonal antibody was directed against a 14-amino acid sequence towards the C-terminus of the receptor (1:5000 dilution in 4% NDS/PBS; Chemicon International).

After three 10-min washes with PBS, sections with tau and MOPR primary antibodies were incubated simultaneously with two secondary antibodies for two hours 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 TRITC (both secondary antibodies from Jackson ImmunoResearch Laboratories, West Grove, PA). 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, Burlingame, CA). Fluorescently labeled sections were visualized with a fluorescent microscope (Leitz Asistoplan) and digital pictures were obtained using a Leica DC-200 camera linked to Leica DC Viewer software.

Autoradiographical Study

[³⁵S]GTPγS Autoradiography—Naïve rats were decapitated by guillotine and the brains were removed immediately, frozen on dry ice and stored at −80°C. The procedure for [³⁵S]GTPγS autoradiography was based on an established method (Sim et al., 1995) as modified by our laboratory (Ward and Simansky, 2006). Twenty-micron, frozen coronal sections were cut throughout the PBN (Leica cryostat model CM3050, Deerfield, IL) and thaw-mounted onto slides subbed with gelatin and chromium potassium sulfate dodecahydrate. Slides were preincubated in slide mailers for 40min in assay buffer (50mM Tris–HCl, 4mM MgCl₂, 0.3mM EGTA, 100mM NaCl, pH 7.4) at 25°C then transferred into 2mM guanosine diphosphate (GDP) in assay buffer at 25°C for 20 min. Finally, sections were incubated for 2h at 25°C in assay buffer containing both [³⁵S]GTPγS (0.04 nM, 1,250Ci/mmol; Perkin Elmer, Boston, MA) and 2mM GDP (Sigma-Aldrich, St. Louis, MO). Each mailer contained either no drug (basal condition), 1μM of the MOPR agonist DAMGO, 100nmol of the MOPR antagonist CTAP (a concentration which produces maximal inhibition of DAMGO-stimulated coupling in vitro (Chaijale et al., 2008)), both DAMGO and CTAP, or unlabeled GTPγS (10μM; guanosine-S'-O-(γ-thio)-triphosphate; Sigma-Aldrich, St. Louis, MO) determine nonspecific binding. After incubation, slides were rinsed in cold (4°C) 50mM Tris–HCl buffer (pH 7.4) twice for 2 min each, rinsed briefly in cold deionized water, then dried immediately with a cool stream of air and desiccated overnight. Slides were then exposed to Kodak Biomax MS film (Sigma-Aldrich, St. Louis, MO) for 24h. Images from the developed films were scanned and quantified using Image Pro-Plus Version 5.1 software (MediaCybernetics).

We determined the densities of [³⁵S]GTPγS incorporated into six regions (see Figure 1) of the PBN (ventral, central 1, central 2, and external lateral PBN; external and central medial PBN) in each 20-μm section from each rat (Paxinos & Watson, 1998). For image analysis, the ventral region of interest included the ventral and internal subnuclei; central 1 included part of the central and dorsal subnuclei; central 2 included part of the central, crescent, and external lateral subnuclei; and external lateral included the external lateral subnucleus. Data were analyzed as femtomoles per milligram tissue after conversion from optical density (OD) units using nonlinear standard curves generated from [¹⁴C] standards (31–883nCi/g; Amersham Biosciences, Piscataway, NJ) placed in each cassette. All OD values (including nonspecific binding) were corrected for background of the film, but not for the different path lengths of isotopic emissions from ³⁵S (tissue sections) and ¹⁴C (standards). Each anatomical set of sections per rat contained alternating sections for the nonspecific, basal, DAMGO-stimulated, CTAP and CTAP/DAMGO conditions through the PBN. Nonspecific binding was subtracted from each of the conditions. Data are expressed as percent of basal incorporation of the [³⁵S]GTPγS.

Statistical analysis—Values from c-Fos quantification and from densitometry were analyzed by ANOVA of the c-Fos counts/ mm² tissue and after conversion to percent stimulation of basal incorporation of [³⁵S]GTPγS. The Student-Newman–Keuls test was used for *post hoc* comparisons of means. An alpha level of *P* < 0.05 was the threshold for statistical significance for all tests (Sigma Stat version 3.1; Systat, Richmond, CA).

Results

Figure 1 maps the subregions of the PBN that were examined in both the immunohistochemical and autoradiographical studies. The LPBN was divided into ventral, central (1&2) and external regions. The medial PBN (mPBN) was divided into central and external regions.

Following a single, unilateral infusion of the MOPR agonist, DAMGO, into the central region of the LPBN, the average number of c-Fos-immunoreactive (c-Fos-IR) nuclei increased in each subregion of the LPBN quantified compared to the corresponding subregion on the contralateral side that was not infused with DAMGO (Figure 2). A two-way repeated measures ANOVA on the data for each subregion on the contralateral and ipsilateral sides with respect to the infusion site revealed a significant effect of Side (contralateral v. ipsilateral) [$F(1,6)=10.52$; $p<0.05$], a difference among LPBN subregions [$F(3,18)=3.65$; $p<0.05$] but no significant interaction [$F(3,18)=1.13$; $p>0.05$]. There was no change in the mPBN in the ipsilateral or contralateral side. Similarly, there was no change in c-Fos expression in the PBN of three rats classified as nonresponders ($LC1 = 371 \pm 36$ c-Fos-IR neurons/ mm^2 , $LC2 = 368 \pm 18$ c-Fos-IR neurons/ mm^2 , $LE=157 \pm 86$ c-Fos-IR neurons/ mm^2). Cannula placements in these rats were determined to be rostral to the LPBN.

Representative sections of the LPBN of a rat from each group after immunohistochemical staining are shown in Figure 3. Increased c-Fos expression in the LPBN ipsilateral to the side of infusion was observed in the VEH/DAMGO condition compared to the VEH/VEH condition. Pretreatment with the μ -opioid antagonist, CTAP (CTAP/DAMGO) blocked the increase in c-Fos expression following DAMGO infusion. CTAP alone (CTAP/VEH) did not change c-Fos-IR in the LPBN.

Quantification data from the immunohistochemical study are graphed in Figure 4. DAMGO infusion increased c-Fos-IR in the LPBN in the VEH/DAMGO group $F(3,21)=4.62$, $p<0.05$. This increase varied by region $F(5,99)=14.04$, $p<0.001$ and there was a significant interaction $F(15,99)=4.01$, $p<0.001$. This increase in c-Fos expression was blocked by pretreatment with CTAP when CTAP was administered 15 min prior to DAMGO infusion (CTAP/DAMGO). The c-Fos expression levels did not differ among the VEH/VEH, CTAP/VEH and CTAP/DAMGO groups. Additionally, no change in c-Fos-IR was observed in the MPBN in any group (data not shown).

Pretreatment with CTAP not only blocked the increase in c-Fos-IR in the LPBN, CTAP also blocked DAMGO-stimulated G-protein coupling in the LPBN (Figure 5). When tissue sections were incubated with DAMGO alone, [^{35}S]GTP γ S incorporation increased in the LPBN compared to basal (no agonist present) level. When tissue sections were incubated with both CTAP and DAMGO, CTAP blocked DAMGO-stimulated G-protein coupling. CTAP alone did not alter the incorporation of [^{35}S]GTP γ S.

Quantification data from [^{35}S]GTP γ S autoradiography are graphed in Figure 6. Incubation with DAMGO increased [^{35}S]GTP γ S incorporation from basal conditions ($p=0.01$). No changes from basal conditions were observed in the conditions in which tissue was incubated with CTAP in combination with DAMGO.

The PBN is reported to project directly to the NAcc (Brog et al., 1993). Therefore, c-Fos expression was also examined in the NAcc core and shell. Figure 7 maps the subregions of the NAcc core and shell region. Representative sections through the NAcc after immunohistochemical staining for the c-Fos protein from the VEH/VEH group and the VEH/DAMGO group are shown in Figure 8.

In addition to increased c-Fos expression in the LPBN after DAMGO infusion, c-Fos expression also increased in the dorsomedial shell (DM shell), ventromedial shell (VM shell), and dorsal core (D core) of the NAcc of rats ($p < 0.001$, $p < 0.01$, $p < 0.05$ respectively) in the VEH/DAMGO group (Figure 9). Whereas c-Fos expression in the PBN increased unilaterally, ipsilateral to infusion, c-Fos expression increased bilaterally in the regions of the NAcc noted above after infusion of DAMGO. (The insert in Fig. 9 illustrates the data for bilateral c-Fos expression in the DM shell.) Furthermore, pretreatment with CTAP blocked the increase in c-Fos expression in the NAcc.

Because of its role in monitoring energy homeostasis, the hypothalamus was examined for changes in c-fos-IR after DAMGO infusion (Figure 10). There was an increase in c-Fos-IR in the arcuate nucleus (Arc) after DAMGO infusion compared to vehicle ($p < 0.05$; Figure 11). No significant differences were seen in the ventromedial hypothalamus (VMH), paraventricular nucleus of the hypothalamus (PVH), lateral hypothalamus (LH), perifornical area (PFA) or dorsomedial hypothalamus (DMH).

The VTA was examined because of its well-known dopaminergic projection to the reward circuitry of the NAcc (Figure 10). The central nucleus of the amygdala (CeA) and the basolateral amygdala (Will et al., 2003) were also examined because of reported connections to the PBN and NAcc (see Discussion) (Figure 10). The administration of DAMGO into the LPBN failed to alter c-Fos-IR in any of these regions.

Visual inspection of the immunohistochemical staining of all sections taken throughout the brain revealed obvious bilateral increases in c-fos-IR in the region of the paraventricular nucleus of the thalamus (PVT) and the hippocampus (Figure 12 and Figure 13 respectively). Quantification of these data confirmed significant increases in c-Fos-IR in the PVT and in the CA1, CA3 and dentate regions of the hippocampus (Figure 10).

Discussion

Our laboratory has shown previously that microinfusion of DAMGO into the LPBN increased consumption of standard pelleted chow in rats. In addition, we also showed that CTAP blocked this DAMGO-induced hyperphagia (Wilson et al., 2003). Here, unilateral infusion of the MOR agonist DAMGO into the central region of the LPBN increased c-Fos-IR in the ventral, central and external subregions of the LPBN compared to vehicle infusion. Incubating tissue sections with DAMGO increased incorporation of [35 S]GTP γ S in the central and external LPBN. In our immunohistochemical study, pretreatment with the MOR antagonist CTAP prevented this DAMGO-induced increase in c-Fos-IR. In addition, CTAP blocked DAMGO-stimulated G-protein coupling in our autoradiographic study.

The NAcc has been studied extensively for its role in food intake (Kelley, 2004, Kelley et al., 2005) and is largely implicated in motivation and reward. A direct connection from the PBN to the NAcc has been previously identified by tract tracing (Brog et al., 1993). After infusion of fluoro-Gold, a widely used neuronal retrograde tracer, into the medial shell of the NAcc, labeled cells were observed in the medial and lateral parabrachial regions. Of interest, the labeling in the parabrachial region was bilateral. In the present study, after DAMGO administration in the LPBN, c-Fos-IR increased unilaterally in the LPBN, ipsilateral to the infusion site. However, c-Fos-IR increased bilaterally within the DM shell of the NAcc.

Of course, the PBN and NAcc may also communicate via multisynaptic pathways. There is evidence to suggest that the external subregion of the LPBN projects to the central nucleus of the amygdala (Karimnamazi and Travers, 1998, Chamberlin et al., 1999) and microinfusion of DAMGO into the central nucleus of the amygdala has been shown to

increase c-Fos-IR within the NAcc shell (Levine et al., 2004). We report, however, no increase in c-Fos-IR in the central nucleus of the amygdala after microinfusion of DAMGO in the LPBN. Our findings also discount excitation of the VTA, another brain region that communicates extensively with the NAcc. Essentially no c-fos-IR was observed in the VTA of neural tissue from any rats in any of the treatment groups. C-Fos-IR did increase, however, in the paraventricular nucleus of the thalamus. This region communicates heavily with the nucleus accumbens, especially the shell (Su and Bentivoglio, 1990, Parsons et al., 2006). Thus, it is possible that direct and indirect (i.e., multisynaptic) pathways mediate the neural activation of the NAcc shell by infusion of DAMGO into the LPBN. It is interesting that the PVT has already been suggested to modulate feeding and arousal by conveying information from neuropeptides from the hypothalamus to the shell of the nucleus accumbens (Parsons et al., 2006).

The arcuate nucleus, a brain region that has a well established role in regulating energy balance (Zigman and Elmquist, 2003, Berthoud, 2007, Nogueiras et al., 2008) was the only region where we detected an increase in c-Fos expression in the hypothalamus. Examination of nuclei within the hypothalamus revealed a significant increase in c-Fos-IR within the Arc but not within the VMH, PVH or LH after DAMGO infusion into the LPBN. The PBN has been reported to communicate reciprocally with the arcuate nucleus of the hypothalamus (Moga et al., 1990, Li et al., 1999). The arcuate nucleus contains proopiomelanocortin/cocaine- and amphetamine-regulated transcript (POMC/CART)-expressing neurons. Activation of these neurons suppresses feeding. In contrast, the activation of a second population of arcuate neurons, neuropeptide Y/agouti-related protein (NPY/AgRP)-expressing neurons, stimulates feeding (Nogueiras et al., 2008). Thus, it appears that the PBN communicates with a region that has a well established role in energy balance as well as with the reward circuit. Future studies should identify the phenotype of those arcuate neurons which are activated secondarily to infusing DAMGO into the LPBN.

C-Fos expression was increased also in the dentate gyrus as well as the CA1 and CA3 of the hippocampus. These hippocampal regions are generally associated with learning and memory processes, including in ingestion (Davidson et al., 2007). It is possible that this increased c-Fos-IR was due to the prior experience of being infused with DAMGO as all rats were probed with DAMGO as a positive control. This initial DAMGO probe allowed rats that responded to DAMGO to be separated from nonresponding rats. Rats infused with vehicle for the c-Fos determination, after the initial probe with DAMGO, did not, however, display an increase in the expression of the early immediate gene. Thus, it appears that the increase in c-Fos expression in these regions of the hippocampus was not due to the infusion process itself. Furthermore, the PBN has been shown to be essential in some ingestion-related learning (Reilly et al., 1993, Grigson et al., 1998a, Grigson et al., 1998b, Reilly and Trifunovic, 2000). No data were found in the literature to suggest a direct connection of the LPBN with the hippocampus. It is possible that these regions are interconnected via the amygdala or the medial thalamus (Su and Bentivoglio, 1990).

In the present studies, all rats were sacrificed 90 min after vehicle or drug infusion. The expression of the c-fos protein reportedly becomes detectable within 45 min and is maximal between 90 and 150 min after manipulation (MacDonald et al., 1993). It is important to note that rats were not allowed access to food or water following infusion in order to avoid any confounding effects from food-related stimuli or from the act of ingestion itself. We have already established that cellular actions of DAMGO infusion to enable eating occur without the presence of food (Wilson et al., 2003).

MOPRs couple to inhibitory G_i/G_o G-proteins (Law and Loh, 1999). In the PBN, these receptors appear to be expressed on dendrites (see descriptive digital images suggesting

colocalization with dendritic marker, MAP2, Figure 14) and neuronal axons (see apparent colocalization with axonal tau). Activation of these MOPRs leads to the activation of potassium channels and inhibition of adenylate cyclase and calcium channels (Law and Loh, 1999). The net effect of these actions results in the inhibition of the neuron. Increases in c-Fos mark elevated neuronal cellular activity (Kovacs, 2008). Therefore, the cells that displayed increased expression of c-Fos following the administration of DAMGO into the LPBN were presumably second-order neurons to those expressing MOPRs and had become disinhibited by the administration of a MOPR agonist. Future studies should aim to identify the phenotypes of cells that are indirectly activated by DAMGO and the cellular localization of MOPRs. The immunofluorescent images shown in Figure 14 certainly raise the possibility that the MOPRs of interest are either located dendritically or on axons/terminals of critical parabrachial neurons. It must be acknowledged that c-Fos expression only maps potential sites where neurons are excited in pathways that may mediate orexigenic actions of parabrachial infusions of DAMGO. Also, c-Fos-IR is only one of several immediate early genes that could be studied for this purpose. Thus, additional work is required to fully identify candidate projection areas that support effects of parabrachial MOPR action on feeding. We are also investigating neural substrates that may be inhibited in this ingestive action of MOPR stimulation in the brainstem.

In conclusion, MOPRs have been implicated in mediating a distributed neural network that promotes reward, caloric regulation and cognitive activity. The roles of MOPRs in these processes may involve either overlapping or identical circuitry. These current data demonstrate communication between the LPBN and the NAcc, the LPBN and the Arc which implicates the LPBN as a link to affect both the rewarding and homeostatic aspects of food intake. The effect of parabrachial opioids on activation of medial thalamic and hippocampal cells suggests further influence of the LPBN on arousal and cognitive functions related to feeding. Therefore, the LPBN appears to act as an important node in the MOPR-mediated feeding and reward network connecting the brainstem and the forebrain.

Glossary

DAMGO	[D-Ala(2), N-MePhe(4), Gly-ol(5)]-Enkephalin
MOPRs	μ -opioid receptors
Arc	arcuate nucleus
BLA	basolateral amygdala
β-FNA	beta-funaltrexamine
CeA	central nucleus of the amygdala
c-Fos-IR	c-Fos-immunoreactivity
CTAP	d-Phe-Cys-Trp-Arg-Thr-Pen-Thr-NH ₂
GDP	guanosine diphosphate
LH	lateral hypothalamus
LPBN	lateral parabrachial nucleus
mPBN	medial parabrachial nucleus
NPY/AgRP	neuropeptide Y/agouti-related protein
NGS	normal goat serum
NAcc	nucleus accumbens

OD	optical density
PBN	parabrachial nucleus
PVH	paraventricular nucleus of the hypothalamus
PVT	paraventricular nucleus of the thalamus
POMC/CART	proopiomelanocortin/cocaine- and amphetamine-regulated transcript
PBS	sodium phosphate buffer
VTa	ventral tegmental area
VMH	ventromedial hypothalamus

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Figure 1.

Coronal section through the parabrachial nucleus. Regions of interest quantified are the ventral lateral (LPBV), central lateral (LPBC1 and LPBC2, respectively), external lateral (LPBE), central medial (MPBC) and external medial (MPBE) PBN.

Figure 2.

Unilateral infusion of DAMGO into the LPBN (N=7) increased the expression of c-Fos in the LPBN on the side ipsilateral to the infusion site. Data are represented as the mean number of c-Fos positive cells identified (mean ± SEM) in each subregion of the LPBN/mm² on the ipsilateral and contralateral sides of the brain. Asterisks indicate significant difference from corresponding value for vehicle: *p<0.05, **p<0.01, Student-Newman-Keuls test after two-factor repeated measures ANOVA.

Figure 3. Coronal sections through the PBN after immunohistochemical staining for c-Fos. Groups include those infused: twice with vehicle (VEH/VEH); vehicle followed by DAMGO (VEH/DAMGO); the MOR antagonist CTAP followed by vehicle (CTAP/VEH); and CTAP followed by DAMGO (CTAP/DAMGO). Bar in lower right corner of panels indicates scale (0.5mm).

Figure 4.

Infusion of the MOPR agonist DAMGO into the LPBN robustly increased the number of c-Fos positive nuclei found in the LPBV, LPBC1, LPBC2, and LPBE subregions. Data are represented as the mean number of c-Fos positive cells (means $\pm$ SEM) identified in each subregion of the LPBN/mm². Groups include VEH/VEH (N=7), VEH/DAMGO (N=7; same rats as in Fig. 2 above), CTAP/VEH (N=6) and CTAP/DAMGO (N=5). Asterisks indicate significant difference from corresponding value for vehicle: * p <0.05, ** p <0.01, Student-Newman-Keuls test after two-factor repeated measures ANOVA.

Figure 5. Autoradiograms of [^{35}S]GTP γ S incorporation in coronal sections from the PBN of a male Sprague–Dawley rat after incubation in vitro with no drug (Basal), 1 μM DAMGO, both 1 μM DAMGO and 100nM CTAP or unlabeled GTP γ S (Non-Specific). Arrows indicate [^{35}S]GTP γ S incorporation in the external lateral subregion of the LPBN in one set of autoradiograms from one rat.

Figure 6.

The MOPR antagonist CTAP blocks G-protein coupling stimulated by DAMGO in the external LPBN. Data are shown as percentage of basal level of GTP γ S incorporation (means $\pm$ SEM). Data (N=3) were calculated from individual means of multiple sections from three rats (total of 42 sections for all rats). Asterisks indicate significant difference from corresponding value for vehicle: **p<0.01, Student-Newman-Keuls test after two-factor repeated measures ANOVA.

Figure 7. Coronal section through the nucleus accumbens (NAcc). Regions of interest quantified include the dorsomedial shell (DM shell), ventromedial shell (VM shell), dorsal core (D core), ventral core (V core), and lateral core (L core) ac = anterior commissure.

Figure 8.
Coronal sections through the NAcc. Sections are from VEH/VEH and VEH/DAMGO conditions. Bar in lower right corner of panels indicates scale (0.5mm).

Figure 9.

Infusion of the MOPR agonist DAMGO into the central region of the LPBN increases the number of c-Fos-IR nuclei in the dorsomedial shell (DM shell), ventromedial shell (VM shell) and dorsal core (D core) of the NAcc (Veh/Veh N=9; Veh/DAMGO N=7). No difference was found in the number of c-Fos-IR nuclei between the contralateral and ipsilateral sides of the NAcc (see figure insert). Data are represented as the average number of c-Fos positive cells (means $\pm$ SEM) identified in each subregion of the NAcc/mm².

Asterisks indicate significant difference from corresponding value for vehicle: * p <0.05, ** p <0.01, Student-Newman-Keuls test after two-factor repeated measures ANOVA.

Figure 10.

Infusion of the MOPR agonist DAMGO into the central subregion of the LPBN increased c-Fos-IR in the arcuate nucleus of the hypothalamus but not in the ventromedial nucleus of the hypothalamus (VMH), paraventricular nucleus (PVH) or lateral hypothalamus (LH) (Veh/Veh N=4; Veh/DAMGO N=4) (see Figure 11). Similarly, there was no change in c-Fos-IR nuclei in the VTA or the central nucleus or basolateral nucleus of the amygdala. Increased c-Fos-IR was also seen in the CA1, CA3 and dentate regions of the hippocampal formation (see Figure 12). Data are represented as the average number of c-Fos positive cells (means $\pm$ SEM)/ mm². Groups include VEH/VEH and VEH/DAMGO. Asterisks indicate significant difference from corresponding value for vehicle: * $p < 0.05$, Student-Newman-Keuls test after two-factor repeated measures ANOVA.

Figure 11.
Coronal sections through the hypothalamus. Sections are from VEH/VEH and VEH/
DAMGO conditions. Bar in lower right corner of panels indicates scale (0.5mm) f = fornix.

Figure 12. Coronal sections through the hippocampal formation. Sections are from VEH/VEH and VEH/DAMGO conditions. Bar in lower right corner of panels indicates scale (0.5mm).

Figure 13. Coronal sections through the paraventricular nucleus of the thalamus. Sections are from VEH/VEH and VEH/DAMGO conditions. Bar in lower right corner of panels indicates scale (0.5mm) 3V=third ventricle.

Figure 14.

A. Immunolabeling of the parabrachial nucleus primarily in the external lateral subnucleus (scp, superior cerebellar peduncle), by antibodies directed against MOPRs and microtubule-associated protein 2 (MAP2). The postage stamp insert in the upper right corner shows staining only for MAP2. The larger image shows double labeling for MOPRs (FITC, green) and MAP2 (linked to rhodamine). Note the extensive overlap of MOPR and MAP2 staining (yellow), thereby suggesting that MOPRs are localized to dendrites within this region, as reported by others. B. Immunolabeling of a section adjacent to that shown for MOPR/MAP2 (A), showing staining for the microtubular protein tau (TRITC) (upper right inset). Note the much smaller degree of apparent overlap of tau with the MOPRs (larger image) when compared to MAP2/MOPR double labeling in A. These non-quantitative data are for descriptive purposes, indicating the possibility of either dendritic or axonal/terminal localization of MOPRs in mediating the cellular and behavioral actions of DAMGO.