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Multiple Brain Sites Sensitive to Feeding Stimulation by Opioid Agonists: A Cannula-Mapping Study

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STANLEY, B. G., D. LANTHIER AND S. F. LEIBOWITZ. *Multiple brain sites sensitive to feeding stimulation by opioid agonists: A cannula-mapping study.* PHARMACOL BIOCHEM BEHAV 31(4) 825–832, 1988.—Evidence suggests that brain opioid receptors of the mu, delta and kappa subtypes may be involved in the control of feeding behavior. However, limited information is available regarding the specific anatomical location of these feeding relevant opioid receptors. To address this problem, we microinjected three opioid agonists, morphine, (D-Ala^2)-Met-enkephalinamide (DALA) or MR 2034, into one of 15 different brain areas and measured the subsequent feeding responses of satiated rats. Morphine (25 nmol) and DALA (6.8 nmol) both elicited strong feeding responses from the same five brain areas, namely, the paraventricular, dorsomedial and lateral hypothalamus, as well as from sites within the septum and amygdala. No other brain sites yielded significant responses to these opioid receptor agonists. In contrast to this anatomically specific pattern of effects, the opioid agonist MR 2034 (8.6 nmol) produced a feeding response which was generally smaller in magnitude and had little anatomical specificity. These findings suggest that opioid receptor systems for stimulating feeding exist in multiple discrete brain areas. Of the regions tested, specific sites within the hypothalamus, septum and amygdala are distinguished as being most sensitive to feeding stimulation by morphine and DALA.

Eating Opioids Morphine Enkephalin Paraventricular nucleus Dorsomedial nucleus Lateral hypothalamus Septum Amygdala

SINCE the discovery of opioid peptides and receptors in the brain, evidence has accumulated to suggest that they play important roles in the regulation of various physiological and behavioral processes, including stimulation of eating behavior [for recent reviews, see (1, 3, 8, 40, 41)]. Although some studies suggest that peripheral opioids regulate feeding behavior and energy balance (55) the preponderance of evidence argues that their stimulatory effects on food intake are largely due to an action within the brain (18). This view is supported by studies demonstrating that: 1) brain levels of some endogenous opioids are altered by manipulations, such as food deprivation, which normally affect eating behavior (15, 43, 44, 51); 2) intracerebroventricular injection of opioid agonists and antagonists alter eating behavior at doses that are ineffective peripherally (28, 81); and 3) peripherally administered opioids are most effective in altering feeding when they are able to penetrate the blood-brain barrier (6, 7, 9).

In addition, several opioid agonists and antagonists have been shown to affect eating behavior when administered directly into specific sites in the brain (22, 39, 46, 47, 69, 77). The majority of these central microinjection studies have focused on the hypothalamic paraventricular (PVN) and ventromedial (VMH) nuclei (21, 22, 39, 48, 70, 77), while a few studies have revealed a reliable eating response with extra-hypothalamic opioid injections, namely, into the nucleus accumbens septi, ventral tegmental area, and amygdala (46, 52, 59). Whereas this work suggests that the opioids may be effective in multiple areas within the brain, only a few sites have been tested in these studies, and, therefore, the full extent of opioid sensitivity remains to be determined. Moreover, since no systematic comparison of a large number of different brain sites has been made within the same study, we are greatly limited in our ability to determine the specific nature and differential contributions of these different areas and to formulate an overall hypothesis regarding the neural substrates of opioid-induced eating. The present study, previously presented in preliminary form (37,73), addresses this issue by testing the ability of three different opioid agonists to stimulate eating when injected into multiple hypothalamic and extra-hypothalamic brain sites.

METHOD

Subjects

Male Sprague-Dawley rats weighing 350–400 g were anesthetized with Metofane and stereotaxically-implanted with chronic 26-gauge (o.d.=0.46 mm) stainless steel guide cannulas, targeted approximately 1 mm dorsal to one of the

TABLE 1
STEREOTAXIC COORDINATES FOR EACH INJECTION SITE

Brain Structure	Incisor	A	L	V*
Paraventricular n.	3.0	−0.4 B	0.4	7.2
Perifornical hypo.	−2.5	−2.0 B	1.3	7.1–7.6
Lateral hypothalamus	−2.5	6.0	1.5–1.8	8.2
Dorsomedial n.	−2.5	5.4	0.5	7.9
Ventromedial n.	−2.5	5.4–5.0	0.5	8.6
Caudate Putamen	−2.5	−1.5–1.0 B	0.5–2.2	4.2–4.8
Septum	−2.5	8.0	0.7	4.7
Thalamus	−2.5	6.8	0.4–1.8	7.0–7.4
Amygdala	−2.5	5.0–6.2	3.8	8.2
Hippocampus	−2.5	−4.5–2.6 B	1.6–4.3	2.8–7.0
Periaqueductal gray	−2.5	0.0–1.4	0.5	5.0–5.6
Midbrain tegmentum	−2.5	2.2	1.0	6.6–7.8
Ventral tegmental area	−2.5	3.2	0.8	8.0
IV ventricle	−2.5	−3.1	0.4	6.6

A—anterior to interaural line (numbers followed by B are relative to bregma), L—lateral to the midsagittal sinus, V—ventral to the surface of the skull. *These ventral coordinates were 1 mm dorsal to the injection site to compensate for the injector cannula which projected 1.0 mm beyond the guide cannula.

15 different brain areas listed in Table 1. During the 7-day postsurgical recovery period, animals were repeatedly handled and mock-injected to habituate them to the testing procedure.

Procedure

Subjects were maintained and tested on a sweet milk-mash diet, consisting of a mixture of 46% Purina rat chow powder, 37% sucrose and 17% Carnation evaporated milk. This diet was prepared daily and given 1 hr prior to the tests to ensure that the subjects were fully satiated. Tests were conducted during the light phase of a 12/12 hr light/dark cycle, with vehicle (0.9% saline) and drug tests administered on alternate days. Injections (0.3 μl) were given directly into each of the brain sites (Table 1), through a 33-gauge (o.d.=0.2 mm) stainless steel injector which projected 1.0 mm beyond the guide cannula.

Three opioids were tested: morphine sulfate (17.0 μg or 25.4 nmol; Merck); the long acting enkephalin analogue (D-Ala^2)-Met-enkephalinamide (DALA; 4.0 μg or 6.8 nmol; Sigma), and the universal opioid receptor agonist (30) MR 2034 TA (4.0 μg or 8.6 nmol; Boehringer Ingelheim). The moderate-to-high doses of each agonist were chosen, based on previous dose-response studies (48, 70, 81), to yield near maximal feeding responses in order to minimize the possibility of false negative results. Food intake was measured 2 and 3 hr after morphine injection and 1 and 2 hr after DALA and MR 2034 administration, times which have been shown to yield maximal eating responses with each of these agonists (48, 70, 81). Each drug was administered in a block of 4–7 tests/subject, with statistical analysis conducted on the average scores of each drug and its vehicle. To facilitate comparisons between the different agonists, all three drugs were usually tested in each subject in counterbalanced order.

After behavioral testing was completed, subjects were sacrificed with an overdose of Nembutal and perfused transcardially with saline followed by 10% formalin. Brains were removed, 100 μm coronal sections were cut and then stained with cresyl violet. Injection sites, as determined by an experimenter blind to the behavioral results, were analyzed with reference to the atlas of Paxinos and Watson (63) and defined as on target if 50% or more of the tip of the cannula track was within the intended brain region.

Statistical analysis was conducted separately for each agonist at each postinjection time. Initially, a two-way ANOVA compared drug to vehicle scores by brain site, to determine whether each drug significantly increased food intake. In subsequent analyses, each subject's vehicle scores were substracted from its drug scores to eliminate the impact of any variability in individual vehicle baselines. These data were analyzed by one-way ANOVA, to determine whether these increases were significantly different in different brain sites. This was followed by a Duncan's New Multiple Range test, to determine the specific brain sites in which food intake scores were significantly increased. Correlations between the effects of the different agonists in the same subjects was determined using a Pearson Product Moment correlation.

RESULTS

Statistical analysis revealed that morphine (96 subjects), DALA (91 subjects) and MR 2034 (64 subjects), compared to their respective vehicle injection scores (0.9 g/hr overall average), each produced highly significant ($p<0.001$) increases in eating behavior at each postinjection test interval. The increases in food intake over vehicle baselines were clearly site specific for morphine, $F(14,81)=6.69$, $p<0.001$, and for DALA, $F(13,77)=3.45$, $p<0.01$. Although MR 2034 also significantly increased food intake, this effect failed to exhibit significant site specificity, $F(12,51)=1.19$, N.S.

As shown in the top panels of Figs. 1 and 2, morphine elicited strong, highly site-specific feeding, with 3-hr food

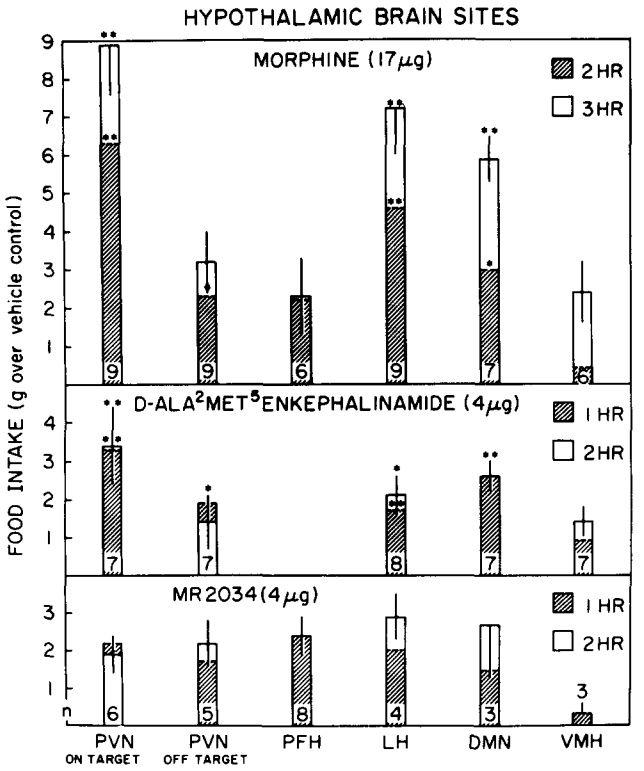

FIG. 1. Average increase in food intake over vehicle baseline (each subject's vehicle score at a particular postinjection interval was subtracted from its drug score at that interval) elicited by morphine (top panel), D-Ala²-Met-enkephalinamide (middle panel), or MR 2034 (bottom panel) microinjected into various hypothalamic brain sites. The measurement intervals were 2 and 3 hr after morphine injection, and 1 and 2 hr after DALA and MR 2034 injection. The number of subjects in each group are indicated at the base of each bar. PVN—paraventricular nucleus, PFH—perifornical area (anterior region), LH—lateral hypothalamus, DMN—dorsomedial nucleus, VMH—ventromedial nucleus. $*p<0.05$, $**p<0.01$ by Duncan's New Multiple Range test.

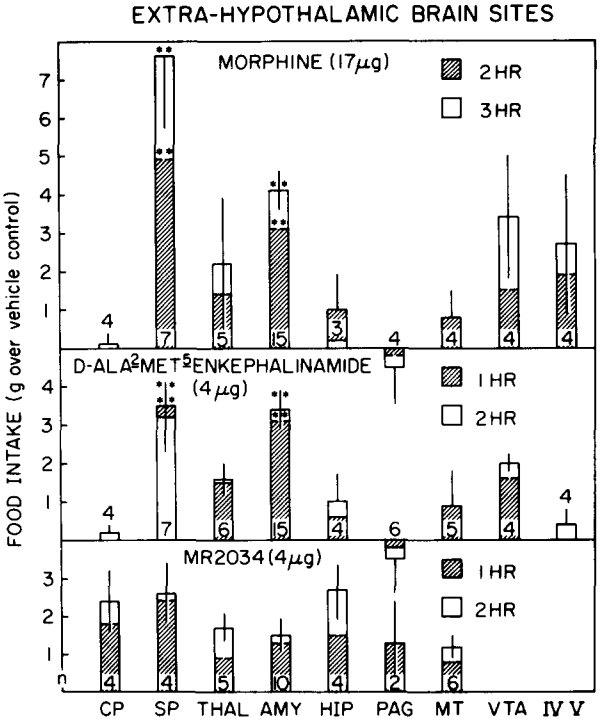

FIG. 2. Average increase in food intake elicited by opioid microinjections into extra-hypothalamic brain areas (see legend of Fig. 1 for details). CP—caudate putamen, SP—septum, THAL—thalamus, AMY—amygdala, HIP—hippocampus, PAG—periaqueductal gray, MT—midbrain tegmentum, VTA—ventral tegmental area, IV V—IV ventricle.

intake scores ranging from 8.9 g greater than vehicle down to 0.5 g less than vehicle. The strongest response was obtained from the PVN (8.9 g), followed by the septum (7.6 g), lateral hypothalamus (LH, 7.2 g), dorsomedial nucleus (DMN, 5.9 g), and amygdala (4.1 g). There was also a small but significant effect obtained from the group designated "PVN off target," which consisted of animals with cannulas within 1 mm anterior or posterior to this nucleus. There were no statistically significant effects in the other 9 brain areas tested. However, in the ventral tegmental area (3.4 g), two of the four subjects showed strong consistent eating responses of more than 4 g over their vehicle scores. Although not statistically significant, this is noteworthy, since it supports previous work demonstrating that some opioids injected specifically into this midbrain area may induce or facilitate feeding responses in satiated rats (23, 24, 27, 59).

As shown in the middle panels of Figs. 1 and 2, DALA elicited a substantially smaller, but still site-specific, feeding response, ranging from 3.5 g greater than vehicle down to 0.5 g less than vehicle. This opioid peptide's stimulatory effects on 2-hr food intake scores were significant in the same five brain areas, namely, the PVN (3.4 g), amygdala (3.4 g) septum (3.2 g), DMN (2.6 g) and LH (2.1 g), where morphine also elicited feeding. Furthermore, the same brain regions unresponsive to morphine (see Figs. 1 and 2) were similarly unresponsive to DALA. Despite the difference in magnitude and duration of the feeding responses induced by DALA and morphine, comparison of these responses across all brain regions revealed that, within a set of animals, the effects of these opioids on feeding were highly correlated in magnitude (e.g., $r=+.86$, $p<0.001$, for 2-hr DALA as compared to 3-hr morphine scores).

As shown in the bottom panel of Figs. 1 and 2, MR 2034 also elicited a significant eating response. However, in contrast to the other two opioid agonists, the effects of this opioid agonist were not anatomically specific, with the responses from the 8 most effective brain regions ranging from only 2.0 to 2.9 g above vehicle baselines. Furthermore, there were no significant correlations in the magnitude of the feeding responses elicited by MR 2034 and those evoked by either of the other two opioids.

Representative injection sites for each of the brain regions, where significant eating was elicited, are presented in Fig. 3. As shown in this figure, the area of necrotic tissue produced by repeated microinjections is usually minimal. At the injection site, the diameter of the necrotic tissue ranged from 0.2 to 0.6 mm. Dorsally, the guide cannula left a track

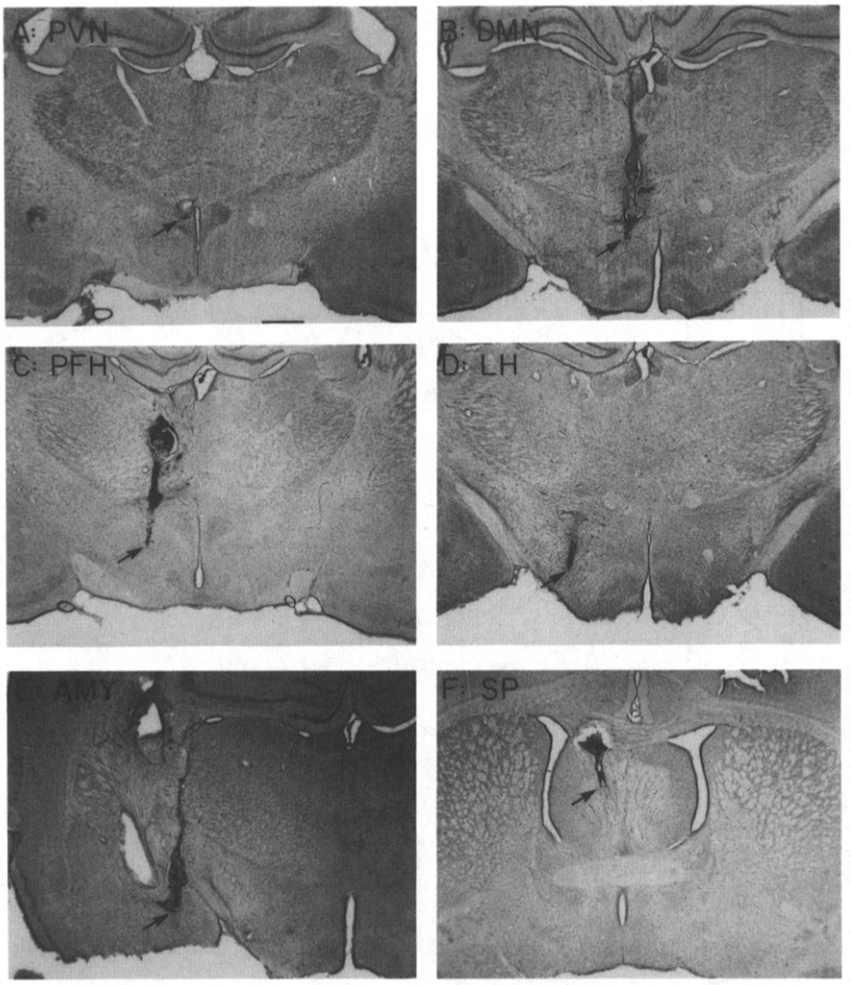

FIG. 3. Photomicrographs of coronal, cresyl violet-stained sections of the rat brain showing six representative injection sites as indicated by arrows. The rest of the cannula track cannot be seen because these sections were cut out of the plain of the cannula track. The placement of the injection sites were determined from the brain section in a particular subject which contained the most ventral damage. (A) PVN—paraventricular nucleus; (B) DMN—dorsomedial nucleus; (C) PFH—perifornical area (anterior region); (D) LH—lateral hypothalamus; (E) AMY—amygdala; (F) SP-septum. The bar at the base of panel A is 1.0 mm in length.

ranging from 0.5 to 1.4 mm in diameter. As can be seen, opioid-induced feeding was obtained in widely distributed brain sites, which in many cases were separated by or adjacent to the ineffective areas. For example, injections of morphine into the PVN (panel A) were highly effective in stimulating eating, whereas injections immediately lateral, into the anterior portion of the perifornical hypothalamus (PFH; panel C), did not produce significant eating. However, morphine injections were effective in LH sites (panel D), which were just lateral and slightly posterior (at the level of the VMH) to these ineffective anterior PFH sites. Likewise, morphine administered into the DMN (panel B) elicited a strong eating response (5.9 g), whereas injections into the VMH, less than 1 mm further ventral, produced a small response (2.4 g) which did not reach statistical significance.

A typical placement within the amygdaloid complex is illustrated in panel E. Effective injection sites for morphine within this structure were widely distributed, ranging from the level of the PVN anteriorly to the posterior hypothalamic nucleus caudally, and also covering virtually the entire medial-lateral and dorsal-ventral extent of the amygdala. A septal injection site is shown in panel F. The placements in this structure were focused on the lateral septal areas, with the tip of the injector cannula occasionally entering into the area of the fornix.

DISCUSSION

This study confirms previous work indicating that morphine and the Met-enkephalin analogue DALA produce a strong feeding response when injected into the PVN or

amygdala (46,81). In addition, we demonstrate that both these opioid agonists are effective in three other brain areas, namely, the DMN, LH, and lateral septum, and further that the effects of these two opioids are highly correlated across subjects. We also confirm that MR 2034 elicits a reliable feeding response when injected into the PVN (70) and find that this response is smaller and less anatomically specific than that observed with morphine and DALA. These results are consistent with previous work suggesting that opioid peptides act within multiple hypothalamic and extrahypothalamic brain regions to modulate feeding behavior (21, 22, 39, 46, 48, 52, 59, 69, 76, 81), and are supported by other studies indicating that the five areas found here to be responsive to opioid stimulation contain significant concentrations of endogenous opioid peptides and their receptors (10, 12, 14, 17, 33, 34, 49, 54, 58, 66, 75, 79) and also have some role in the control of ingestive behaviors (see below).

Previous studies focused on the PVN have suggested a role for this nucleus in opioid modulation of eating behavior. Consistent with our present data, other reports have demonstrated that injections of endogenous opioids or opioid agonists into the PVN stimulate feeding, whereas local administration of naloxone or β-endorphin antisera suppresses feeding (39, 48, 69, 81). Moreover, electrolytic lesions of the PVN attenuate, but do not abolish, the eating induced by peripheral morphine injection (71). This nucleus is also important for the feeding stimulatory effects of norepinephrine (38), and evidence suggests that norepinephrine and opioid peptides may interact here to control eating behavior (39). Met-enkephalin levels in the PVN are found to be increased by food deprivation (45), suggesting that this peptide may act in the PVN to control normal feeding. The present experiment, demonstrating that the PVN is the most sensitive site for feeding stimulation by morphine and DALA, is consistent with this suggestion.

It is clear, in part from lesion studies (71), that the PVN is not the sole area involved in opioid control of feeding. Another area suggested by our study is the DMN, where a strong eating response was elicited by morphine and DALA. There is little previous evidence that this nucleus is involved in opioid stimulation of eating behavior, and the evidence that does exist is conflicting in nature. One study shows that lesions in the DMN attenuate, but do not abolish, the feeding suppression produced by the opioid antagonist naloxone (4), while another study fails to reveal any change in food intake after naloxone injection into the DMN (81). Whereas the reasons for these differences are not clear, our data, demonstrating a feeding stimulatory effect with opioid agonists in this nucleus, suggest that DMN opioid receptors are involved in mechanisms of feeding behavior. It may be noteworthy that the DMN is immediately dorsal to the VMH, a site in which others have observed opioid-induced eating behavior [e.g., (22,77)]. While we also obtained a small response (2.4 g with morphine) from the VMH in the present study, this effect failed to reach statistical significance, as we reported earlier (81), and it was substantially less than the response we obtained in the DMN (5.9 g). Given the anatomical proximity of these two areas, it is likely that the small effect in the VMH, shown here as well as in other publications (22,77), is due in part to the reflux of injected opioids up the guide cannula shaft, from the VMH to the DMN or possibly into the PVN. The evidence that electrolytic lesions of the VMH fail to attenuate the anorectic effect of peripherally injected naloxone (35) is also consistent with this suggestion.

With regard to the LH, our demonstration that opioid injections here can stimulate eating is consistent with evidence that LH neurons are critical to the control of eating behavior and that opioid input may be important in these control mechanisms. Specifically, eating can be elicited by electrical stimulation of the LH, and this response may be mediated by endogenous opioids, as it is attenuated by naloxone (7). Furthermore, injections of naloxone directly into the LH have been shown to suppress eating induced by food deprivation (78). It has also been shown that iontophoretic application of Met-enkephalin or morphine into the LH can decrease the activity of local glucose-sensitive neurons which are believed to participate in the control of eating behavior (61,62). This evidence, together with our present microinjection results, support the proposal that opioids may act on LH neurons to control natural feeding. This LH site may be distinguished from the PFH area. This latter area runs along the fornix at the medial border of the LH and extends from anterior to the PVN to posterior to the VMH. In the present study, morphine injections into the anterior portion of the PFH, just lateral to the PVN, failed to induce a significant feeding response; whereas previous work from our laboratory has revealed a strong effect with injections into the posterior portion of the PFH, just lateral to the VMH (81). It remains to be established whether these different results are in fact due to differences in the anterior-posterior placement of the PFH injections, or whether the responsiveness of the posterior PFH, shown with the use of larger guide cannulas and injection volumes (81), reflects the spread of morphine into effective brain sites (PVN, DMN and LH) that bracket the PFH.

In the septum, which is heavily innervated by opioid fibers and contains high levels of opioid receptors (10, 12, 14, 34, 49, 54, 79), studies have revealed a circadian variation of β-endorphin content, with peak levels in the middle of the dark phase when eating behavior also peaks (32). Further, it has been shown that electrolytic lesions in this brain region cause considerable disruption in the normal meal patterns (13). It may also be relevant to note that there is a dense projection of enkephalin containing neurons from the LH to the lateral septum (66), suggesting a possible interaction of opioid mechanisms in these two brain areas. These studies, taken together with our finding that intraseptal opioid microinjections elicit a strong eating response, suggest a role for the septum in opioid mechanisms of eating behavior.

There is considerable evidence for involvement of the amygdala in opioid control of eating. The amygdaloid complex is densely innervated by intrinsic and extrinsic opioid-containing neurons (12, 33, 34, 65, 79) and is also rich with several types of opioid receptors (17, 49, 54, 58). It has been shown that the activity of glucose-sensitive neurons in the amygdala is affected by opioids in a higher proportion than nonglucose-sensitive neurons (36,60). Moreover, Met-enkephalin levels are increased in the amygdala by as little as 4 hr of food deprivation (68). The present study suggests that activation of opioid receptors in the amygdaloid complex may contribute to the process of eating initiation, and recent work, demonstrating that mu agonists in this structure are more effective than either delta or kappa agonists (19), suggests that these receptors may be mu in nature.

In addition to multiple sites of action, the findings that morphine and DALA act in similar brain sites argues that these agonists may act via similar mechanisms to stimulate feeding. This possibility is supported by the similarities in macronutrient intake patterns, namely, enhanced protein

and fat intake, associated with PVN injection of these different opioid agonists (72). Although the different agonists act within the same brain sites and have similar effects on macronutrient intake patterns, it is not clear to what extent they act on different receptor subtypes to induce eating.

Previous work has suggested that stimulation of several different opioid receptor subtypes may elicit eating. The majority of work has focused on mu, delta, and kappa subtypes, and there is considerable evidence suggesting that each may play a role in control of feeding behavior (20, 26, 31, 41, 57, 77). The agonist MR 2034 is believed to act on each of these receptor subtypes (30,80), and this might partially account for its widespread sites of action. Morphine and DALA are typically described as mu and delta opioid receptor agonists, respectively, but there is considerable crossover in their effects (16, 53, 74, 82), and the doses used here may be too high to produce selective effects on specific receptor subtypes. There is some evidence, however, that, at least in the PVN, these two opioid agonists are acting on different receptor subtypes to stimulate eating. Specifically, in the PVN, the eating stimulatory effects of morphine, but not DALA, appear to be partially dependent on circulating corticosterone (5,47). This dissociation suggests that in the PVN these agonists may act at least in part through different receptor subtypes to potentiate eating. However, since physiologically functional mu and delta receptors have been shown to exist on the same neuronal cell bodies (11), the possibility that activation of different opioid receptor subtypes in a particular brain area may stimulate eating through a similar mechanism(s) needs to be strongly considered.

One of the major concerns with central injections is the extent of drug spread from the cannula tip. Depending upon the substance injected and specific procedures employed, diffusion may range from a fraction of a mm to several mm throughout or even outside of the brain (29,38). In fact, drug spread might account for the relative lack of anatomical specificity of the MR 2034. It does not appear, however, that drug spread could account for the multiple sites at which morphine and DALA are effective. This is indicated by the findings that morphine's diffusion is minimal subsequent to intracerebral microinjection (42,64), that DALA and morphine exhibit similar sites of action despite their chemical dissimilarities and probable differential diffusion patterns, and, more importantly, that effective and ineffective brain sites are frequently in close anatomical proximity or even adjacent to each other. These findings strongly suggest that morphine and DALA act near the injection site to stimulate a feeding response.

To conclude, this study suggests that the neuroanatomical substrates underlying opioid controls of eating behavior are complex and multifaceted, involving a wide variety of different brain areas, including the PVN, DMN, LH, septum and amygdala. Additionally, this study supports previous results suggesting that multiple opioid receptor subtypes might mediate feeding stimulation produced by opioid injections. The finding that opioids can act in multiple brain sites to stimulate eating behavior may have important implications for the future design of studies examining opioid brain control mechanism of eating behavior. While many studies have employed peripheral or intracerebroventricular injections to elucidate these mechanisms, the likelihood that multiple, yet distinct, opioid systems exist within different brain areas would appear to require that direct tissue injection procedures be employed to determine their differential functions.

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