

Research report

Opioid site in nucleus accumbens shell mediates eating and hedonic ‘liking’ for food: map based on microinjection Fos plumes

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Abstract

Microinjection of opioid agonists, such as morphine, into the nucleus accumbens shell produces increases in eating behavior (i.e. ‘wanting’ for food). This study (1) reports direct evidence that activation of accumbens opioid receptors in rats also augments food ‘liking’, or the hedonic impact of taste, and (2) identified a neural site that definitely contains receptors capable of increasing food intake. Morphine microinjections (0.5 μ g) into accumbens shell, which caused rats to increase eating, were found also to cause selective increases in positive hedonic patterns of behavioral affective reaction elicited by oral sucrose, using the ‘taste reactivity’ test of hedonic palatability. This positive shift indicated that morphine microinjections enhanced the hedonic impact of food palatability. The accumbens site mediating morphine-induced increases in food ‘wanting’ and ‘liking’ was identified using a novel method based on local expression of Fos induced directly by drug microinjections. The plume-shaped region of drug-induced increase in Fos immunoreactivity immediately surrounding a morphine microinjection site (Fos plume) was objectively mapped. A point-sampling procedure was used to measure the shape and size of ‘positive’ plumes of Fos expression triggered by microinjections of morphine at locations that caused increases in eating behavior. This revealed a functionally ‘positive’ neural region, containing receptors directly activated by behaviorally-effective drug microinjections. A subtraction mapping procedure was then used to eliminate all surrounding regions containing any ‘negative’ Fos plumes that failed to increase food intake. The subtraction produced a conservative map of the positive site, by eliminating regions that gave mixed effects, and leaving only a positive region that must contain receptors capable of mediating increases in food intake. The resulting mapped ‘opioid eating site’ was contained primarily within the medial caudal subregion of the nucleus accumbens shell, and did not substantially penetrate either into the accumbens core or into other subregions of the shell. Several other structures outside the nucleus accumbens (such as rostral ventral pallidum), immediately medial and adjacent to the shell, also appeared to be included in the functional site. Opioid receptors within this site thus are capable of mediating morphine-induced increases in eating, in part by enhancing the hedonic reward properties of food. © 2000 Elsevier Science B.V. All rights reserved.

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Topics: Ingestive behavior; Motivation and emotion

Keywords: Nucleus accumbens; Mesolimbic; Shell; Core; Morphine; Opiate; Opioid; Striatum; Dopamine; Naloxone; Agonist; Antagonist; Hyperphagia; Palatability; Appetite; Food intake; Hedonic; Reward; Pleasure; Motivation; Addiction; Genes (fos); Transcription factors

1. Introduction

Opioid brain systems are crucial to both eating behavior and reward, and activation of opioid systems can cause

increases in food intake [34,48,51,53,85]. Opioid brain systems have been suggested to control food intake in part by mediating the palatability, or reward aspects, of the taste of food [21,54]. Direct evidence that opioid systems specifically mediate food *palatability* has come from findings that systemic administration to humans of naltrexone or other antagonists suppresses subjective ratings of food palatability [10,26,30,84]. Other direct evidence for a role of opioid systems in palatability has come from

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animal studies of affective taste reactivity patterns. Taste reactivity patterns are hedonic and aversive clusters of behavioral affective reactions to taste, which can be used to measure the hedonic impact of a taste (food 'liking') in human infants or in animals [7,78]. Opioid-focused taste reactivity studies have demonstrated that systemic administration of naloxone causes behavioral affective reactions elicited by sweet or bitter tastes to become less positive or more aversive, and conversely that morphine, administered either systemically or into the lateral ventricles, causes taste reactivity patterns to become more positive or hedonic [19,25,70,72,74]. Less direct but also supportive evidence that the opioid system mediates palatability has come additionally from the many studies of effects of opioid agents on animal food preference and consumption patterns [21,54].

It is not yet known which brain structure(s) contains the opioid systems that can mediate hedonic palatability. Local microinjection of opioid agonists has been reported to cause increases in food intake in many brain structures, including the nucleus accumbens, amygdala, striatum, hypothalamus, tegmentum, and hindbrain [4,33,58,66,77,86]. It is not necessary that all structures that cause increases in intake or other aspects of food 'wanting' must also mediate hedonic impact or 'liking' [6,9]. There has been no direct evidence so far that any one of these brain structures specifically mediates the role of opioid systems in hedonic impact, as opposed to some other process involved in food intake.

One often suggested candidate for mediating the hedonic properties of food and other rewards, however, is the nucleus accumbens [46,51,69]. Although there is now ample evidence that mesotelencephalic dopamine projections to the nucleus accumbens are not needed to mediate the hedonic impact of food rewards [9], there is no reason why other neurochemical components of the nucleus accumbens, such as intrinsic neurons containing opioid receptors, might not genuinely mediate hedonic palatability or 'liking' [6]. The nucleus accumbens is rich in mu, delta, and kappa opioid receptor binding and mRNA [61,62]. Numerous neurobehavioral studies have shown that delta, kappa, and especially mu opioid agonists and antagonists delivered to the accumbens can alter food intake. Intra-accumbens microinjections of opioid mu or delta agonists increase food intake [3,5,27,32,51,58,66,77,86]. Conversely, intra-accumbens microinjections of opioid antagonists decrease food intake [11,51].

The nucleus accumbens is divided anatomically into core and shell regions [42,63,64]. The accumbens core and shell differ in their anatomical projection patterns of connectivity [2,67,82], and in their intrinsic organization regarding cell morphology, gene expression, and distribution of neurotransmitters and receptors [1,17,63]. Given the many neurochemical, morphological, and anatomical differences between the core and shell of the nucleus accumbens, it is not surprising that functional differences also exist between the two subregions.

Regarding food intake in particular, a number of studies chiefly by Kelley and co-workers have indicated differences between the roles of nucleus accumbens shell and the core in eating behavior [52,59,60,79–81]. Several neurotransmitter manipulations seem clearly able to alter food intake more powerfully when directed to the accumbens shell than to the core. These include the intake-stimulating effects of GABA_A and GABA_B agonists, and of AMPA and kainate glutamate antagonists [60,80]. Microinjections of opioid agonists and antagonists into the accumbens are also sufficient to alter food intake, although whether the shell has an advantage over core has not been as clear for opioid-induced eating [51,79,88]. However, studies by several other groups have indicated at least that *reward* properties of opioid agonists may be more significant in the accumbens shell than in the core [13–15,47,48].

Our aim in this study was to more accurately map the site in the nucleus accumbens that mediates morphine-elicited eating, based on an objective measure of the extent of neuronal activation induced by a microinjection of morphine, and to identify its role in hedonic 'liking' for food, based on hedonic taste reactivity patterns. It has sometimes been known how far a drug *physically diffuses* from a microinjection site, but the extent of *functional activation* of neuronal receptors has generally not been known. Point-sampling of c-Fos immunohistochemistry was used to identify plumes of neural tissue activated by morphine. The goal of this mapping technique was to allow the visualization of the actual region of functional alteration triggered directly by a drug after microinjections that increased eating behavior. Measurement of Fos plumes thus provides information on the spatial extent of functional receptor activation caused by an effective drug microinjection.

Further, by using a subtraction mapping procedure, the Fos plume measure also allows elimination of nearby 'negative' or 'mixed' neural regions that contain Fos plumes which *fail* to change behavior. The combined Fos plume point-sampling and subtraction mapping procedure therefore allows one to identify with confidence a definite 'positive' region, or in other words, a region that must contain neural substrates able to mediate the behavioral effect.

In this study, Fos plumes from microinjections that increased eating were compared to plumes from nearby ineffective microinjection sites. Our results indicate that the accumbens opioid eating site is localized primarily to a medial caudal subregion of the accumbens shell and to adjacent ventromedial structures. We also used the 'taste reactivity' technique for measuring palatability or food 'liking' [7,38], to assess whether morphine microinjections within the 'positive site' actually increased the hedonic impact of the taste of sucrose or quinine. The results showed that morphine microinjection in the accumbens shell positive site increased the hedonic reaction patterns elicited by oral infusions of sucrose.

2. Methods

2.1. Subjects

Fifty-two male Sprague–Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) weighing 275–325 g at the time of surgery, were housed in pairs in plastic tub cages with wood shaving bedding on a 12:12 light/dark cycle with lights on at 07:00 h, and maintained on ad libitum food and water throughout the experiment. All behavioral tests took place during the light phase of the cycle (09:00 h to 15:00 h). For several days before testing, the rats were habituated to the palatable cereal diet used in feeding tests until intake stabilized in 3-h sessions.

2.2. Surgery: *accumbens* (microinjection) cannulae and oral (taste reactivity) cannulae

Rats were pretreated with atropine methyl nitrate (5 mg/kg i.p.) and penicillin (0.1 ml i.m.), and were anesthetized with a mixture of ketamine HCl (87 mg/kg i.p.) and Rompun (13 mg/kg i.p.). Each rat was bilaterally implanted with two intracranial microinjection guide cannulae (22 gauge) aimed 2 mm above the intended injection sites. Twelve placements were targeted: six placements within the *accumbens* shell region, and six placements within the *accumbens* core. Every rat was assigned to a particular microinjection placement, which was the same for both its left and right microinjection cannulae. The stereotaxic coordinates for *accumbens* shell sites were: $A = +2.2$ (seven rats), $+1.6$ (nine rats) or $+1.0$ (nine rats) mm; $L = \pm 0.9$ mm; $V = -5.0$ (seven rats), -6.0 (nine rats), or -7.0 (nine rats). Coordinates for core sites were: $A = +2.2$ (eight rats), $+1.6$ (10 rats) or $+1.0$ (nine rats) mm; $L = \pm 2.0$ mm; $V = -5.0$ mm (nine rats), -6.0 mm (nine rats), or -7.0 mm (nine rats; all coordinates with respect to bregma; skull positioned so that bregma and lambda were level). Stylets were inserted into the guide cannulae to prevent occlusion. Guide cannulae were anchored to the skull with sterile stainless-steel screws and acrylic cement.

Twelve of the animals that had shell microinjection cannulae were implanted in addition with two bilateral chronic oral cannulae to permit taste-reactivity testing [38]. Oral cannulae (heat-flared PE 100 tubing) entered the mouth just lateral to the first maxillary molar, ascended lateral to the skull, and exited the head at the dorsal part of the skull, where they were attached to a 19-gauge steel tubing. These cannulae allow the direct infusion of solutions into the mouth for taste reactivity tests, and do not interfere with normal eating behavior.

2.3. Microinjections

Morphine sulfate solutions (1 mg/ml or 2 mg/ml concentrations in sterile 0.9% sodium chloride; Sigma

Chemicals, MO) were freshly prepared 30 min before each behavioral test. Rats received either microinjections of vehicle or morphine (0.5 μ g in 0.25 μ l or 0.5 μ l). The 0.5 μ g dose was chosen because it is among the lowest morphine doses reported to reliably evoke eating behavior [5,28]. Use of a low dose activates receptors in a more restricted area, and allows localization of well defined, localized *c-Fos* plumes of neural tissue around the microinjection site. The two volumes allowed an initial comparison of drug diffusion as a function of microinjection volume. Each rat received counterbalanced administration of either morphine or vehicle on separate test days (spaced at least 2 days apart). Microinjections were carried out using a 1- μ l Hamilton syringe connected to a syringe pump (0.5 μ l/min), while the animals were gently hand-held. The injector tip (30 gauge) extended 2.0 mm below the tip of the guide cannulae. A 1-min period was allowed after the microinjection before removal of injectors and replacement of stylets. To habituate animals to the testing procedures, all rats were given several ‘mock’ microinjections, which consisted of removing the stylets and placing the dummy injector in the guide cannulae, on 3 days prior to their first test. Microinjections were spaced at least 48 h apart.

2.4. Behavioral food intake tests

Behavioral tests began 1 week after surgery. After a microinjection of morphine or vehicle, animals were placed immediately in the food intake test chamber. A dish containing freshly-mixed and palatable commercial baby cereal was presented (Gerber; mixed with water in a 1 to 3 ratio; pre-weighed), and the rats were allowed to eat ad libitum for 3 h. Water was also available. Food intake (g) was recorded at 15 min, 1 h, 2 h and 3 h. The order of morphine and vehicle administration was counterbalanced. Food intake tests were spaced 48 h apart. For the purpose of the analyses below, a morphine microinjection was classified as causing increased intake if the rat’s food consumption at 2 and 3 h was at least 33% greater than its baseline intake at those times after vehicle microinjection.

2.5. Naloxone antagonism of opioid-stimulated feeding

To confirm whether the morphine-induced feeding enhancement was mediated by opioid receptors, we tested whether systemic naloxone would block feeding induced by morphine microinjections in 10 rats that had been found to show the increased food intake effect. These rats were re-tested for food intake after morphine microinjection (0.5 μ g in 0.5 μ l on each side) 30 min after receiving either naloxone (2.0 mg/kg, i.p. in a volume of 1 ml/kg) or a control vehicle injection. Every rat was tested twice, receiving both naloxone and vehicle 48 h apart, in counterbalanced order.

2.6. Taste reactivity test of enhanced palatability

To assess whether intra-accumbens morphine was capable of specifically increasing ‘liking’ for a food reward, or the positive hedonic impact of the taste, in rats that ate after the microinjection, we used the ‘taste reactivity’ test of palatability developed by Grill and Norgren [38]. The taste reactivity test measures hedonic and aversive patterns of species-typical reactions elicited by sweet and bitter tastes, and is useful for assessing changes in palatability produced by neural or pharmacological manipulations [6,36]. Seven rats of the 12 that had been implanted with oral cannulae had shown increases in food intake after morphine microinjections, and were used for taste reactivity testing. Each rat again received bilateral microinjections of either morphine sulfate (0.5 µg/0.5 µl) or the vehicle (0.5 µg/0.5 µl). The order of drug and vehicle was counterbalanced across animals, and the two tests were conducted 48 h apart. A rat’s oral cannula was connected immediately after each microinjection to a stimulus delivery tube (PE 50 tubing attached to a PE 10 nozzle), and the rat was placed in a plexiglass test chamber. A mirror positioned beneath the transparent floor of the chamber reflected a view of the rat’s face and mouth into the close-up lens of a video camera. After a 15-min habituation period, a 1-ml volume of 0.06 M sucrose was infused into the rat’s mouth through the oral cannula by syringe pump during a 1-min period. Affective reactions elicited by the sucrose taste were videotaped for subsequent analysis. The taste reactivity test was repeated again at 1 h and 2 h after the microinjection. In order to assess the effect on aversive reactivity, the taste reactivity tests were also run (vehicle and morphine in balanced order) using oral infusions of 2.7×10^{-4} M quinine HCl as the tastant. Sucrose and quinine trials were counterbalanced and spaced 48 h apart. Quinine solutions typically elicit aversive reaction patterns from rats, allowing comparison of intra-accumbens morphine on positive hedonic versus aversive patterns of affective reactions.

2.7. Videoscoring of taste reactivity

Hedonic and aversive reaction patterns were scored in a slow-motion video analysis (1/30 s frame-by-frame to 1/10th actual speed). Positive hedonic reactions included rhythmic midline tongue protrusions, lateral tongue protrusions, and paw licking. Aversive reaction patterns included gapes, chin rubs, face washes, forelimb flails, paw treads, and locomotion. Neutral reactions (less strongly linked to hedonic/aversive evaluations) were rhythmic mouth movements, passive drip of the solution, and ordinary grooming characterized by rapid sequential alternation between face wash and paw lick behaviors (<1 s duration of each behavior type between transitions). Separate occurrences were scored for each lateral tongue protrusion, gape, and chin rub, and for each bout of face washing, forelimb flails and locomotion. Time bin scoring procedures were used to

score behaviors that are emitted in longer continuous bouts such as tongue protrusions (2 s bins), and rhythmic mouth movements and paw licks (5 s bins). Use of these procedures allows multiple components to be combined into an overall hedonic reaction score and an overall aversive reaction score. For more information on scoring criteria for each component, scoring procedures, and the classification of components into hedonic/neutral/aversive categories see Refs. [7,8,36].

2.8. Histology

C-fos gene expression was used as a measure of local impact induced by morphine [43,44]. After vehicle microinjections, there was a range of 6 to 13 neurons that expressed Fos in each $625 \mu\text{m}^3$ block of tissue, and the baseline varied from rat to rat. Because of the inter-individual variation in baseline Fos expression across rats, we used a within-subject procedure to compare baseline to morphine-induced Fos. To make a within-subject comparison of vehicle versus morphine effects on Fos, each rat received a unilateral microinjection of vehicle on one side of the brain and a unilateral microinjection of morphine on the other side. Preliminary observations suggested that Fos expression on one side after a unilateral microinjection of morphine was similar to localized Fos expression after bilateral drug injections. Pilot observations indicated that a criterion of Fos doubling would reliably distinguish morphine from vehicle microinjections. Twice as many neurons had to express Fos in a block on a rat’s morphine side as on its vehicle side in order for the morphine to be considered as having elevated Fos expression. This criterion minimized ‘false positives’ and produced clearly defined plumes of Fos expression (below).

Several days after the conclusion of the behavioral experiment, 21 rats received a microinjection of 5 µg morphine on one side of the brain (in 0.5 µl vehicle for seven rats; 0.25 µl for 14 rats) and of vehicle on the other side (same volume). The rats were left undisturbed for 60 min after the microinjection (without food) before being deeply anesthetized with sodium pentobarbital (100 mg/kg i.p.), and perfused transcardially with 250 ml of 0.2 M sodium phosphate buffered saline containing 0.1% sodium nitrite, followed by 250 ml of 4% paraformaldehyde in 0.1 M sodium phosphate buffer (NaPB). Brains were removed, post-fixed for 1 h at room temperature in the paraformaldehyde solution, and cryoprotected in a 20% sucrose solution in 0.1 M NaPB at 4°C. Coronal sections (40 µm) were cut on a freezing microtome and incubated for Fos immunocytochemistry (at room temperature unless otherwise noted) in: (1) rabbit anti-c-fos antiserum (diluted 1:1000 in potassium phosphate buffered saline [KPBS] with 0.3% Triton; Ab-2 (Santa Cruz Biotechnology, CA) for 48 h at 4°C; (2) biotinylated donkey anti-rabbit IgG (diluted 1:100 in KPBS with 0.3% Triton; Jackson ImmunoResearch Labs, PA) for 1 h; (3) avidin–biotin complex (Vectastain ABC Kit) for 1 h, and (4) 0.0125%

diaminobenzidine (DAB) solution containing 0.06% hydrogen peroxide and 0.015% nickel chloride in KPBS for 10 min. Sections were mounted and coverslipped on gelatin-coated slides. Adjacent sections were stained with cresyl violet and examined under light microscopy to confirm anatomical placement of injection sites.

2.9. Mapping of shell opioid eating site: subtraction procedure

First, an objective quantification was obtained of the Fos plumes of neuronal activation induced by morphine microinjections. Plumes of neuronal activation were considered to be the area in which the quantitative elevation of c-Fos immunohistochemical density induced by morphine microinjection exceeded 200% of baseline density. We used a point sampling procedure to quantify baseline and morphine-induced Fos-like immunoreactivity (FLI) at selected locations surrounding each microinjection, based on a ‘modified fractionator’ procedure devised earlier for quantifying the extent of neuron death after excitotoxin lesions [24,40].

2.10. Point sampling of c-fos elevation

Point samples (Fig. 1) were taken of the number of neurons that exhibited Fos-like immunoreactivity (FLI) within a $125\ \mu\text{m} \times 125\ \mu\text{m} \times 40\ \mu\text{m}$ block (or ‘point’) of tissue at selected points spaced at $125\ \mu\text{m}$ intervals, along each of seven radial arms plotted to emanate from the center of the microinjection site (45° , 90° , 135° , 180° , 225° ,

270° , 315° ; the 0° or 360° radial arm was not sampled because it was occupied by the microinjection cannulae track).

To take a point sample, the microscope was focused at $400\times$ magnification power over a point location, and the $125\ \mu\text{m} \times 125\ \mu\text{m}$ borders of the point sample were delineated with an eyepiece grid (Fig. 1). The grid was focused first on the surface of the slice and all neurons in clear focus that showed FLI were counted by an observer who was blind to the morphine/vehicle condition. Then in order to include neurons that were deeper in the block, the focal plane was then adjusted to a deeper focus, so that the counted neurons became blurry, and deeper neurons became clear. These FLI neurons were added to the count for that point sample, and the procedure was repeated until the entire $40\text{-}\mu\text{m}$ depth had been sampled.

Vehicle microinjections were associated with c-Fos counts per $125 \times 125 \times 40$ block of tissue that typically began high (84.5 ± 15.7 ; mean \pm S.E.M.) within a narrow $200\ \mu\text{m}$ strip immediately adjacent to the microinjection, but fell dramatically to an average of only 9.3 ± 4.2 once past $200\ \mu\text{m}$ from the center.

2.11. Identification of morphine-induced fos elevation

Morphine microinjections markedly increased FLI counts within a 0.5 to $1.5\ \text{mm}$ radius. For the purpose of mapping a particular morphine microinjection, high Fos activation was considered to be any FLI count that exceeded 400% of baseline for a particular point location (Fig. 2). This $>400\%$ FLI criterion was used to establish

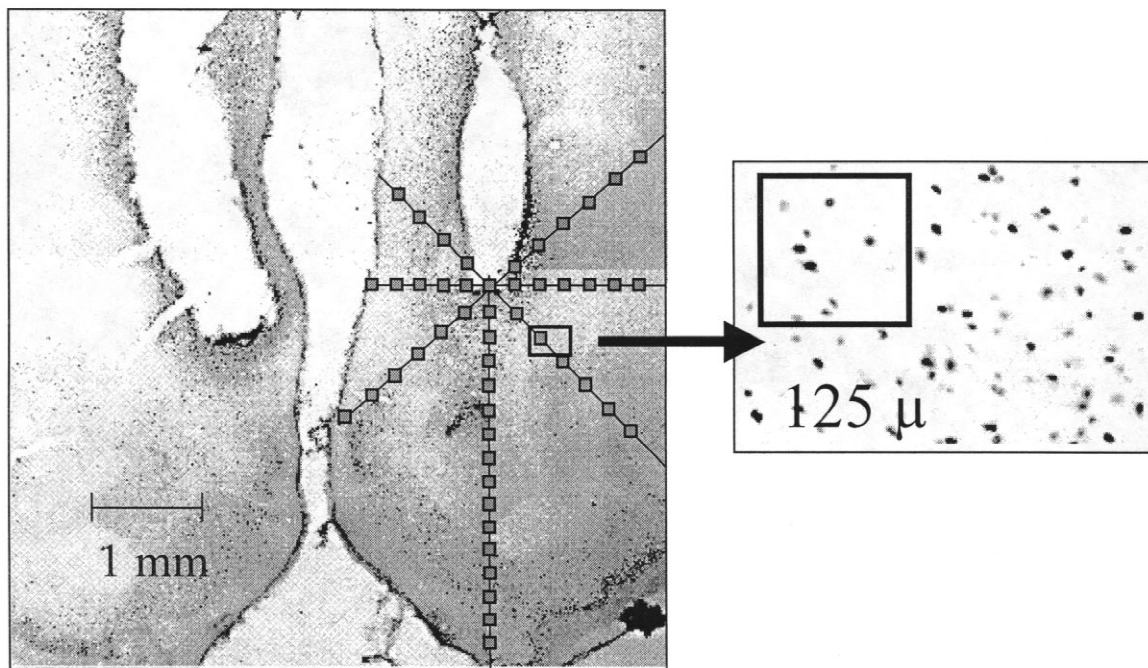
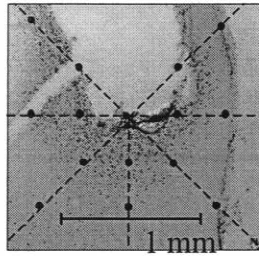


Fig. 1. Core sample method of Fos analysis. The locations where samples were taken are shown unilaterally on radial arms surrounding a microinjection site (left; $160\times$). The expanded box (right; $400\times$) shows a single core sample region ($125 \times 125 \times 40\ \mu\text{m}$ square), in which Fos-expressing neurons were counted.

Sample points (Vehicle)



Morphine Fos Plumes

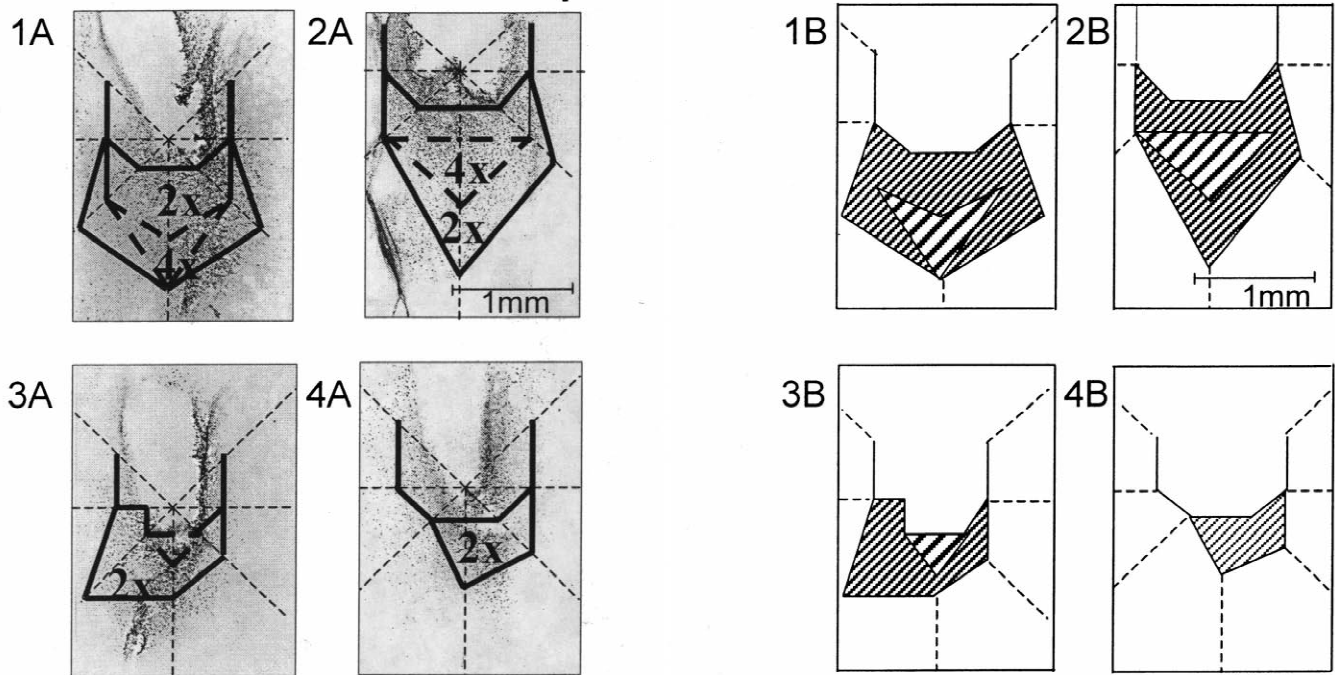


Fig. 2. Examples of Fos plumes. Top photograph shows sparse Fos staining after a vehicle microinjection. Dots depict sample points used to establish vehicle baselines for Fos expression. Photographs at left (1A–4A) show plumes of elevated Fos expression induced by morphine microinjections. Region of Fos $>200\%$ elevation over baseline ($2\times$) is contained within the heavy line. Region of Fos $>400\%$ elevation is contained within the dashed line. Diagrams at right (1B–4B) show computer images of the corresponding Fos plumes for each photograph. Fine hatch lines show regions of Fos $>200\%$ elevation, and coarse hatch lines show regions of 400% elevation.

the inner region of morphine microinjection plumes. Moderate Fos activation was considered to be any FLI count between 200% and 400% of vehicle baseline for a point location. This 200% $>400\%$ FLI region defined the outer border of morphine Fos plumes.

The extent of high and moderate Fos elevation was plotted for each morphine microinjection on a digitized image of that injection site. These plots showed that a morphine microinjection produced an outer c-Fos plume of $>200\%$ FLI that resembled a pentagon in shape (typically pointing down; Fig. 2). Approximately one of four plumes failed to contain a $>400\%$ inner region after the smaller 0.25 μl morphine microinjection, but even these plumes contained an inner plume of relatively dense FLI, which generally reached at least $>300\%$ above baseline.

It was necessary to reconstruct the Fos plumes produced

by microinjections in the behavioral tests on the basis of plumes observed after the final microinjections (since each plume had a slightly different shape, and it would have been impossible to simultaneously collect behavioral data and Fos data after the same microinjection). We constructed plume 'prototype shapes' based on averages of the final Fos measurements, and used the plume prototype diameters to reconstruct the microinjections produced earlier in the behavioral tests. Pentagon shapes were constructed separately for each microinjection volume, based on measured height and width of $>200\%$ Fos expression. The prototype '0.25 μl plume pentagon' had a coronal surface area of 0.52 mm^2 (vertical diameter=0.74 mm; horizontal diameter=0.63 mm), which reflected the extent of moderate FLI for a 0.25- μl volume microinjection of morphine. The prototype '0.5 μl plume pentagon'

had a surface area of 1.15 mm^2 (vertical diameter=0.85 mm, horizontal diameter=0.75 mm). These were used to construct the >200% Fos map of the ‘opioid eating site’ described below. Finally, a prototype ‘>400% FLI plume pentagon’ was constructed to map the smaller zones of high FLI activation triggered by most $0.5 \mu\text{g}/0.5 \mu\text{l}$ morphine microinjections (surface area= 0.34 mm^2 ; vertical diameter=0.59 mm; horizontal diameter=0.50 mm).

2.12. Mapping of ‘positive site’ from plume plots

For all ‘positive’ microinjection sites that increased food intake to at least 133% of vehicle baseline levels (Fig. 4), morphine plume pentagons were mapped onto a computerized rat brain atlas [71]. A ‘positive’ eating microinjection zone was constructed by combining all the overlapping positive plumes (i.e. all morphine microinjections that increased food intake). This provided a preliminary map of the region activated physiologically, according to c-Fos criteria, by morphine microinjections that stimulated food intake (Fig. 5).

Although the resulting preliminary map showed the total area activated by ‘positive’ microinjections that increased food intake, only a part of this initial site was likely to contain the accumbens neural receptors responsible for triggering opioid-stimulated eating behavior. Diffusion of morphine would produce FLI in bordering regions too, even if those regions did not contribute to the behavior effect. Since it was desirable to remove such non-contributing regions from the ‘eating site’ map, a negative map of ‘failed sites’ was made of surrounding c-Fos plumes that had failed to increase food intake (Fig. 5). As a third step, negative regions that overlapped with positive plumes were *subtracted* from the positive microinjection map (because fos activation of these ineffective sites was clearly not a sufficient cause for feeding). The result was a nearly final map of the ‘positive opioid eating site’ that included only contributing portions of behaviorally-effective microinjection sites, and gave an objective boundary between positive and negative regions. The subtraction procedure deliberately ensured that this boundary map would be a conservative estimate of the actual neural substrate, since regions of mixed positive/negative effect were all eliminated.

A final step combined the left and right hemisphere maps (Fig. 6), and plotted the intensity of eating behavior evoked within the site (Fig. 7). Regions that were identified as ‘eating sites’ in both the left and right maps were marked as ‘bilateral zones’ of high confidence whereas regions that were identified on only one site were marked as ‘unilateral zones’ (Fig. 6). Within this anatomical map, the amount of food intake stimulated at each microinjection site was plotted along a color scale of intensity to create a functional map of eating intensity evoked within the site (Fig. 7). This functional plot constituted the final map of the opioid eating site (Fig. 7).

3. Results

3.1. Feeding test

Morphine microinjections ($0.5 \mu\text{g}$) into the nucleus accumbens enhanced food intake overall compared to vehicle ($F(1,51)=53.799$, $P<0.01$; three-factor ANOVA: within-subject factors were drug treatment (morphine versus vehicle) and time of test (15 min, 1 h, and 2 h after microinjection); between-subject factor was the general anatomical location of microinjection (accumbens core versus shell)). Shell and core placements differed in their ability to support morphine-elicited increases in eating (drug \times site interaction ($F(2,102)=24.601$, $P<0.01$; Fig. 3)). Microinjections into the accumbens shell of morphine generally increased food intake ($F(1,3)=51.68$, $P<0.01$; $159.16\pm 5.92\%$, mean \pm S.E.M. elevation over vehicle baseline) but microinjections into the accumbens core did not increase intake significantly (N.S.; $115.3\pm 10.92\%$; Fig. 3). Within shell sites, $0.5 \mu\text{l}$ and $0.25 \mu\text{l}$ volumes of the $0.5 \mu\text{g}$ dose were equally effective at increasing food intake ($0.5 \mu\text{l}=158.7\pm 5.8\%$, $0.25 \mu\text{l}=152.6\pm 9.7\%$). Food intake after accumbens shell morphine microinjections was significantly elevated at 1 h ($F(1,50)=10.570$, $P<0.01$), 2 h ($F(1,50)=28.946$, $P<0.01$), and 3 h ($F(1,50)=51.09$, $P<0.01$), but not at 15 min after morphine microinjections. In the second series of tests (naloxone plus morphine versus morphine alone) naloxone suppressed the increase in food intake normally produced by accumbens shell morphine ($F(1,5)=20.024$, $P<0.01$; morphine without naloxone = $137.03\pm 2.57\%$ increase over baseline; morphine plus naloxone = $114.00\pm 6.7\%$ increase) (Figs. 4–7).

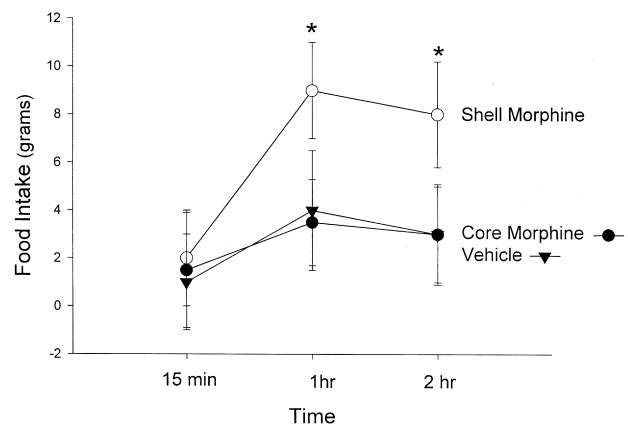


Fig. 3. Noncumulative food intake. Increase in amount eaten (mean \pm standard error grams of food) over preceding measure after either vehicle microinjections or morphine microinjections into either the core or the shell regions of the nucleus accumbens. Vehicle microinjections into core and shell were averaged into a single vehicle score. Food intake was increased after morphine microinjections into the accumbens shell at 1 h and at 2 h, but not after only 15 min after the microinjection. Statistical significance from vehicle denoted by *.

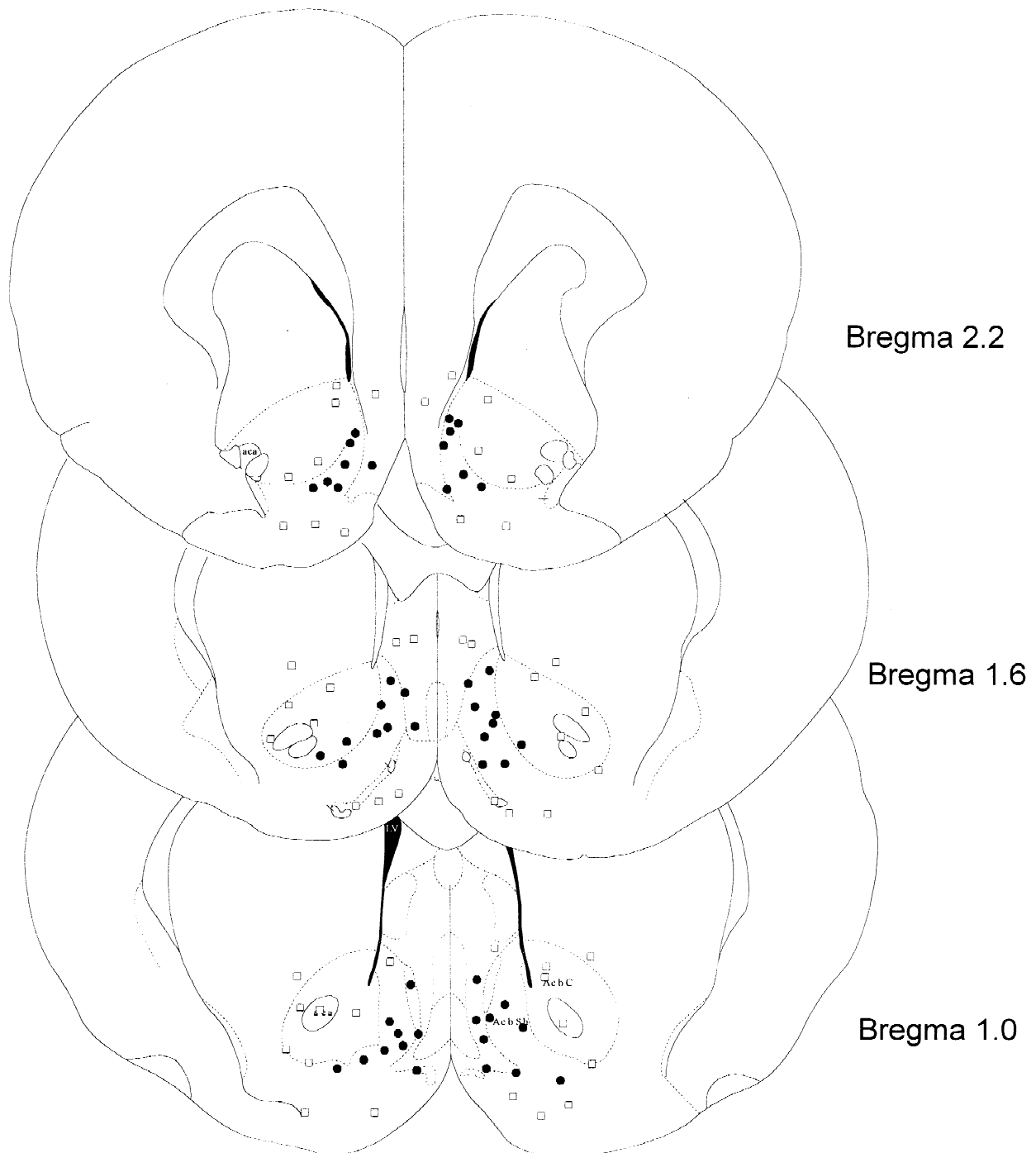


Fig. 4. Point locations of microinjections. Locations where morphine microinjection caused increased food intake are marked with filled circles. Locations where morphine failed to produce an increase are marked with open squares.

3.2. Mapping results

The 'opioid eating site' was restricted principally to a medial caudal subregion of the nucleus accumbens shell (Fig. 6). Viewed in coronal section, each side of the accumbens shell contained a diagonal wedge-shaped opioid eating site. The dimensions of the mapped opioid eating site were ~ 3 mm (from ventrolateral to dorsomedial corners) \times 2 mm (from ventromedial to dorsolateral corners) \times 1 mm (from anterior to posterior edges), mapped

on the basis of a $>200\%$ Fos elevation for defining microinjection plumes.

The opioid eating site was co-extensive with the *shell* of the nucleus accumbens at least to a large extent, and did not overlap substantially with any portion of the accumbens core. The mapped site began rostrally as a narrow strip (0.5 mm lateral diameter and 2.0 mm vertical diameter) in the nucleus accumbens shell at 2.5 mm in front of bregma, and expanded caudally in width to 2.0 mm, reaching a maximal diameter at the caudal border of

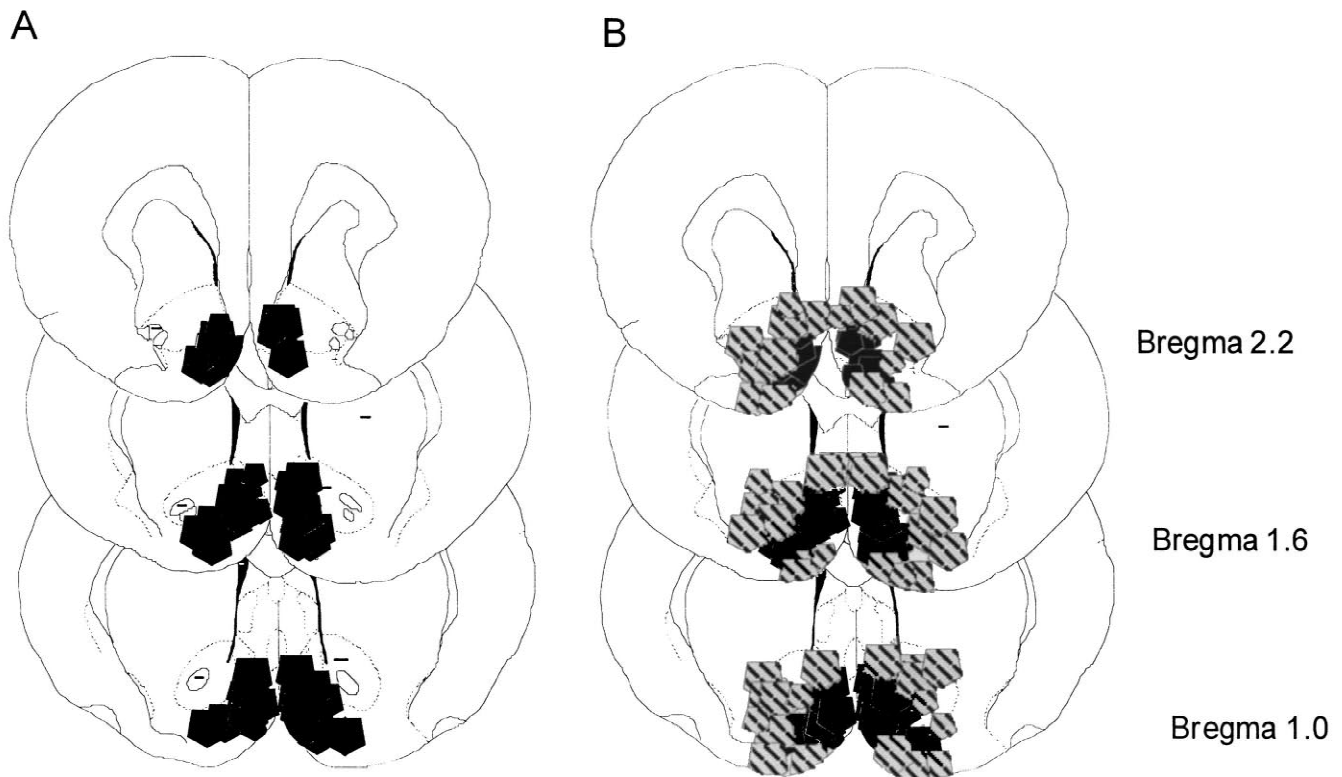


Fig. 5. Functional Fos mapping procedure: positive and negative plumes. The region where morphine microinjections caused increase in eating behavior are shown by the solid plume symbols (A). Negative regions, where morphine-induced Fos failed to be accompanied by increased food intake, were subtracted from the positive region, and are shown by the striped plumes (B).

the nucleus accumbens, 1.0 mm in front of bregma. The magnitude of food intake stimulated by morphine in rostral portions of the mapped site was relatively lower (130% to 150% increase over vehicle baseline intake) compared to microinjections in more caudal (and especially medial caudal) portions of the eating site (160% to 200% increase in food intake; Fig. 7). The lateral edge of the shell opioid eating site touched the medial edge of the accumbens core especially in rostral planes, but the mapped eating site rarely penetrated into the core at any point.

Despite this general localization within the accumbens shell, several aspects of the opioid eating site did *not* correspond simply to the anatomical borders of the accumbens shell (Fig. 6). First, as noted above, eating as a function was not distributed equally within the shell. The mapped eating site did not extend throughout the entire accumbens shell, but was instead restricted to its medial zone (and did not include dorsomedial or ventrolateral portions of the shell). Further, there was a functional ‘hot spot’ in the medial caudal portion of the mapped site (Fig. 7). Thus, the opioid eating substrate constituted only about one-third to one-half of the accumbens shell’s total volume.

The boundary of the opioid feeding site further appeared to extend outside the nucleus accumbens to include several other structures that were ventromedial to it (Fig. 6). Most prominent among these external structures was a narrow strip of the rostral ventral pallidum, which is immediately

ventromedial and adjacent to the accumbens shell (containing the most medial component of the medial forebrain bundle) and the nucleus of the vertical limb of the diagonal band. Also included in the mapped eating site were the ventral portion of the lateral septal (intermediate) nucleus, and portions of the islands of Calleja along the ventromedial edge.

The anterior, dorsal, ventral, lateral and medial boundaries of the opioid eating site were all identified objectively as described above, on the basis of FLI transitions from the plumes of behaviorally effective microinjections to the plumes of ineffective microinjections. However, the most caudal boundary of the opioid eating site was not explicitly mapped: eating was elicited even at the most posterior edge of the nucleus accumbens, and we did not make microinjections in structures that were caudal to it. Thus our results imply that the posterior edge of the opioid eating site extends *at least* to the caudal border of the nucleus accumbens. It is very likely that the eating site extends further caudally past the nucleus accumbens boundary [4]. Future studies will be needed to make a Fos-based map of positive sites in brain structures posterior to the accumbens.

3.3. Hedonic reaction patterns to sucrose enhanced by morphine in opioid eating site

Hedonic reactions elicited by the taste of sucrose were

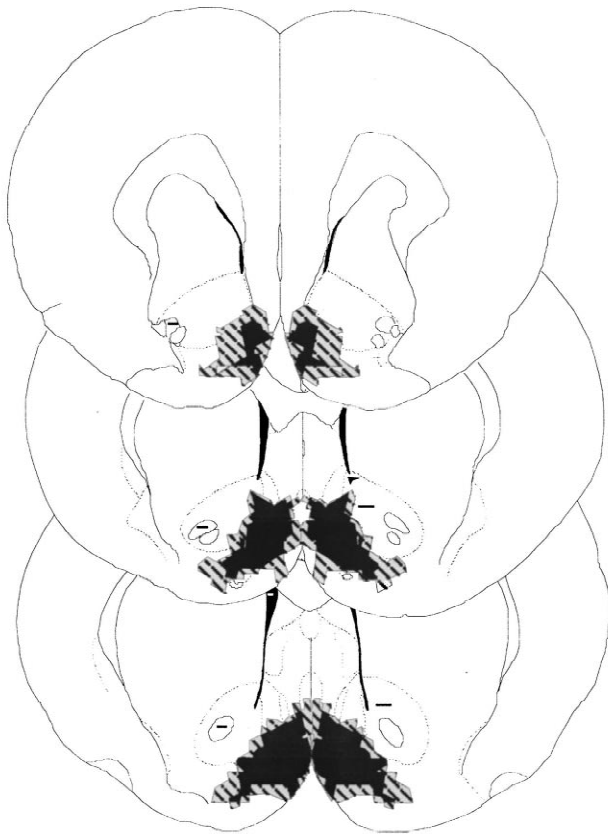


Fig. 6. Anatomical map: boundaries of opioid 'eating site'. This final Fos-based map shows the result of the subtraction mapping procedure, using a Fos >200% elevation criterion. The opioid eating site corresponds largely to the shell of the nucleus accumbens, especially ventromedial portions. The site may also extend to encompass other structures along the medial rim of the brain (although note that subtraction of potential negative midline region was not done, because all medial sites evoked eating). Solid black region shows zone of 'high confidence' based on areas that were activated bilaterally (increased Fos expression on both sides of the brain). Surrounding striped zone shows zone of 'moderate confidence' based on Fos in areas that were activated unilaterally.

enhanced after microinjections of morphine into the accumbens shell opioid eating site, for rats that ate more in response to morphine, compared to after vehicle microinjections ($F(1,5)=13.207$, $P<0.05$; Fig. 8). Hedonic reaction patterns to the taste of sucrose were enhanced by morphine both at 15 min after the microinjection ($F(1,6)=6.437$, $P<0.05$) and at 1 h after the microinjection ($F(1,6)=10.711$, $P<0.05$; Fig. 8). The most elevated hedonic reaction components were rhythmic tongue protrusions and nonrhythmic lateral tongue protrusions. Overall hedonic reactions were higher at both 15 min and 1 h after morphine ($F(1,32)=5.789$, $P<0.022$). The hedonic enhancement waned by the third test at 2 h after microinjection, and was no longer significant ($P=0.093$). Few aversive components (gape, forelimb flail, etc.) were ever elicited by sucrose, and no aversive component was elevated by morphine microinjections.

Quinine infusions always elicited predominantly aversive reactions and few hedonic reactions. Aversive re-

actions elicited by quinine were not significantly altered by morphine microinjection ($F(1,6)=0.495$, $P=0.54$), although there appeared to be a slight trend towards suppression of aversion by morphine. Thus morphine in the accumbens shell opioid eating site appeared to selectively enhance hedonic reactions, and this positive hedonic enhancement was most evident with sucrose, which elicited the most hedonic reactions to begin with. Aversive aspects of palatability appeared either unchanged or else diminished if changed at all.

4. Discussion

These results indicate that the nucleus accumbens is functionally heterogeneous regarding opioid-induced eating behavior. The site for morphine-elicited eating was limited primarily to a medial and caudal subregion of the accumbens shell, according to the results of our Fos-based mapping procedure. Within the mapped site a clear medial caudal 'hot spot' of behavioral effect could be discerned, in terms of the magnitude of the food intake increase, which corresponded to accumbens shell regions known to contain high levels of mu, delta, and kappa opioid receptor binding and mRNA expression [1,61,62].

4.1. Connectivity of opioid eating site in accumbens shell

The opioid eating site in the accumbens shell is well positioned to modulate food reward. The nucleus accumbens receives gustatory information via direct connections from the nucleus of the solitary tract [12], which is the first taste sensory relay nucleus within the brainstem [68], and from gustatory cortical areas, such as the anterior insular cortex [12]. The accumbens shell sends projections primarily to the medial ventral pallidum, in contrast to the accumbens core which projects to the lateral ventral pallidum [18,42]. The *medial* ventral pallidum (including what has controversially been called substantia innominata [41]) has been suggested to make a special contribution to behavior related to food motivation and to reward [49,50]. The accumbens shell receives reciprocal projections back from the medial ventral pallidum, whereas the lateral ventral pallidum projects to both shell and core [12,39].

The connectivity of the accumbens shell subregion, which corresponds best to our mapped eating site, is of special interest. The *caudal ventromedial portion of the accumbens shell* that contains the mapped site projects to the ventromedial pallidum and substantia innominata [82]. Interestingly, damage within that ventral pallidal target site can cause aphagia, increased food aversion, and suppression of hedonic taste reactivity patterns [23,65], which further suggests this circuit may mediate the hedonic palatability of foods. Other special connectivity features of the mapped portion of the accumbens shell include projections to the pontine parabrachial nucleus [82], which is an

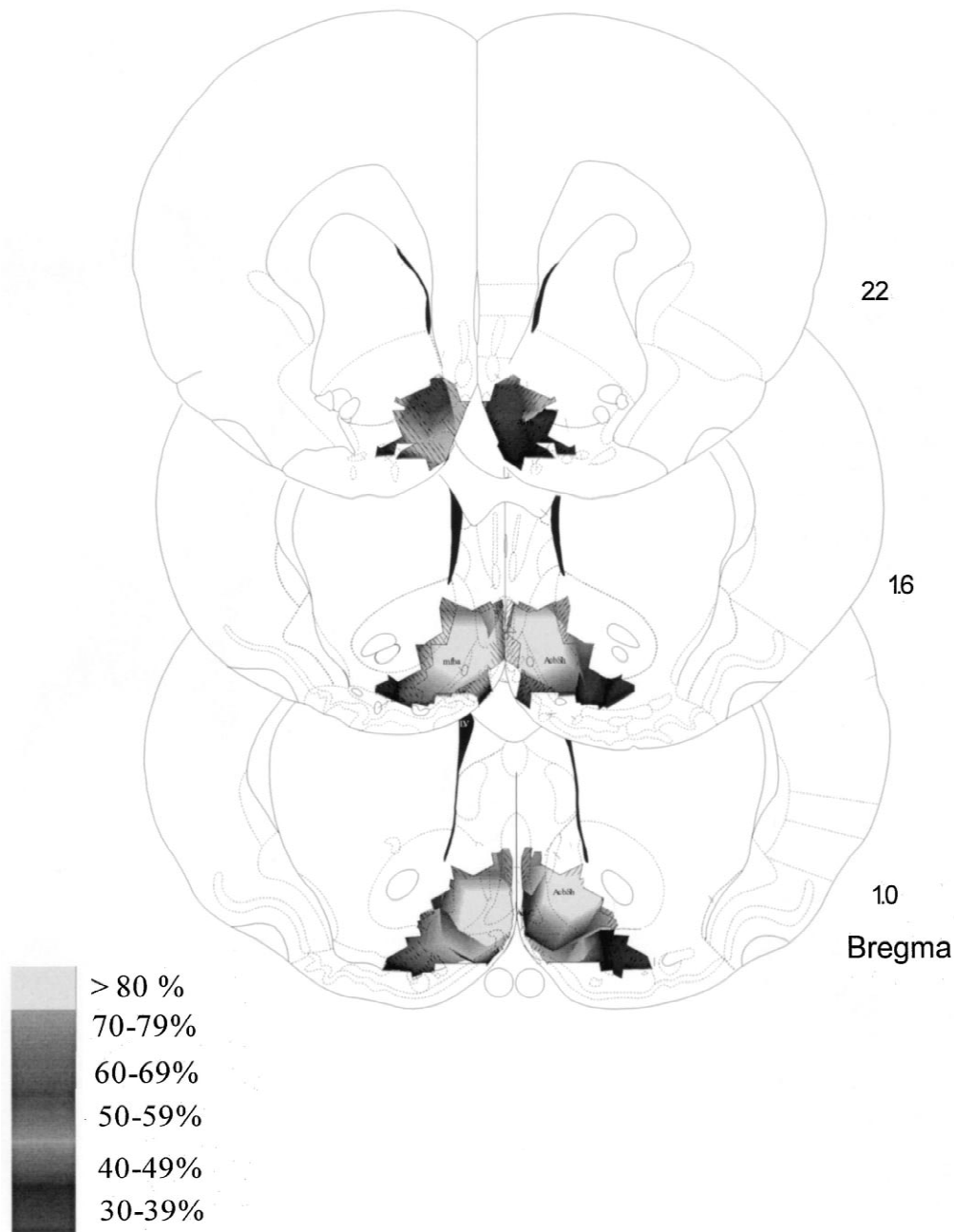


Fig. 7. Functional map: intensity of eating behavior evoked by morphine microinjections within borders of Fos map. The largest increases in eating behavior were produced by microinjections in the medial and caudal portion of the 'opioid eating site'. The magnitude of the increase in food intake caused by morphine is plotted as a percentage increase compared to each rat's baseline food intake after vehicle microinjection, using a color density scale.

additional gustatory relay nucleus at least in rodent brains [68,76].

The amygdala also sends inputs from the caudal magnocellular basal amygdala fibers, and accessory basal nucleus specifically to ventral parts of the accumbens shell relevant to the site mapped here [83]. Further, the 'extended amygdala' system, a column of neurons extending from the central and medial amygdala to the bed nucleus of the stria terminalis, has a special relationship to the portion of accumbens shell that contains the opioid eating site [41]. Heimer et al. note that 'the caudomedial part (of shell) is continuous with the extended amygdala' [41, p. 976], and

the caudal part of the shell certainly would overlap with the Fos-mapped site identified here.

4.2. Caveats for a Fos-based functional map

There are several limitations regarding the use of c-fos early gene activation as a measure of functional activation of neurons, which imply caveats for our map. Not all neurons express Fos, and for those neurons that do, Fos expression varies over time after stimulation [16,44,56]. We sampled Fos expression at the 1 h time corresponding to a significant behavioral increase in food intake, on the

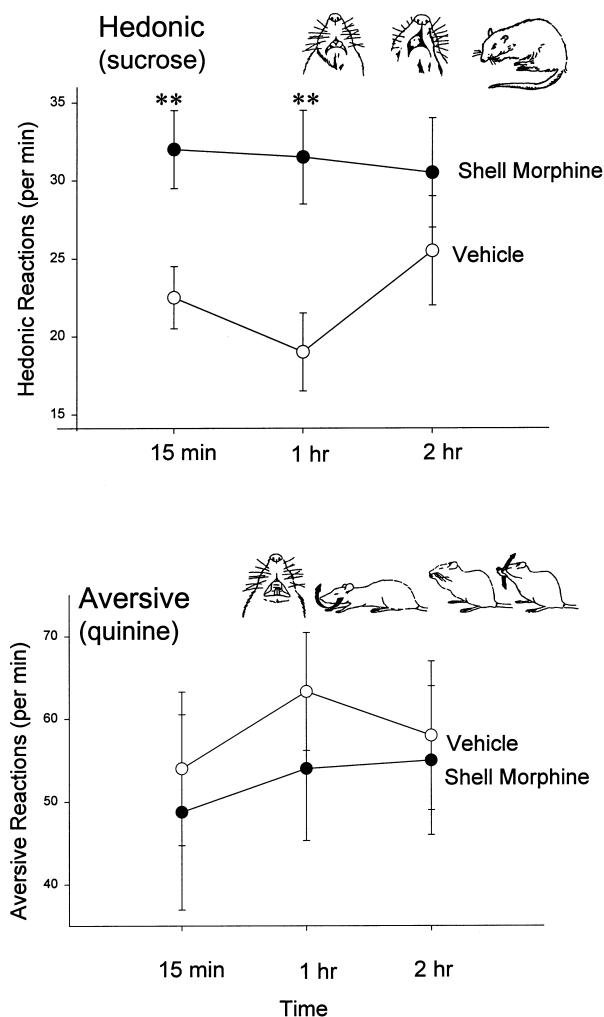


Fig. 8. Enhancement of hedonic reactions to sucrose by morphine microinjection. Hedonic taste reactivity components (midline tongue protrusions, lateral tongue protrusions, and paw licks; each drawn at upper right) elicited by an infusion of sucrose solution into the mouth were increased by microinjections of morphine into the 'opioid eating site'. Hedonic reactions were enhanced at 15 min and at 1 h. Aversive reactions elicited by either sucrose or quinine infusions were never significantly changed by morphine microinjection, and are not shown. Statistical symbols as in Fig. 3.

grounds that neurons activated then might be most relevant to the behavioral effect. However, one cannot be sure that the neurons which expressed Fos at 1 h were those that mediated the opioid-mediated eating behavior. It would be of interest to remap the site boundaries using procedural variations that sampled Fos at different times after microinjection.

Other methodological issues should be kept in mind too. Our mapping procedures used c-fos as the gene transcription factor, morphine as the opioid agonist to increase food intake, and a within-subject analysis of the effect of morphine versus vehicle. In order to gain further confidence about site boundaries it would be of interest to remap with alternative procedures. For example, one could

use another gene transcription factor such as c-jun. The results of such studies would reveal whether site boundaries remain stable across different markers of neuronal gene transcription. Similarly, it would be of interest to compare the present site map with one obtained using a different opioid agonist, such as DAMGO, to elicit eating, in order to assess whether there is one 'opioid eating site' or whether accumbens opioid receptor subtypes involved in eating may have partly different spatial distributions. Finally, it would be worthwhile to re-map using a between-subject procedure that compared bilateral agonist microinjections to bilateral vehicle.

The relation of Fos expression to neuronal function is important for interpreting any Fos-based map. Agonist activation of mu, delta, and kappa receptors leads to an extracellular signal regulated kinase cascade known to be a main inducer of c-fos transcription [1]. Morphine induction of c-Fos in the accumbens has been suggested to require co-activation of NMDA glutamate receptors and D₁ dopamine receptors [57]. Since co-localization of opioid and glutamate receptors has been reported to occur in neurons within the nucleus accumbens shell [35], it is possible that neurons which expressed Fos were those that were directly stimulated by morphine. This may seem paradoxical, as Fos expression has often been associated with electrophysiological depolarization of neurons [43], whereas activation of opioid receptors on medium spiny neurons in the nucleus accumbens is generally regarded to produce hyperpolarization. However, there may be exceptions to the association of Fos expression and depolarization, as well as possibilities for modulation of neuronal polarization by other neurotransmitter receptors [43]. It is conceivable that morphine directly triggers transcription of the fos gene in neurons that possess opioid receptors. An alternative possibility is that a synapse intervenes between neurons that have opioid receptors and neurons that express Fos, and that the FLI we observed reflected secondary neuronal activation triggered via local circuits. There are additional possibilities for indirect activation via other types of receptors, such as melanocortin-4, since opioid blocking of melanocortin receptors could increase food intake [73,75] (though sites other than nucleus accumbens might mediate such effects [37]). At present, the precise cellular mechanism by which morphine triggers Fos expression in local neurons remains an open issue.

In any case, the observation that morphine microinjections produced localized plumes of Fos activation indicates that Fos expression was limited to the region of tissue immediately affected by the microinjection. The FLI plume borders were distinct, and the transition from >200% elevated levels of Fos expression to normal baseline levels typically occurred within a short 250 micron distance. Since the Fos plumes were identified entirely on the basis of objective criteria, the site map was constructed without need of subjective judgment. Further, the site remained stable across several variations of the

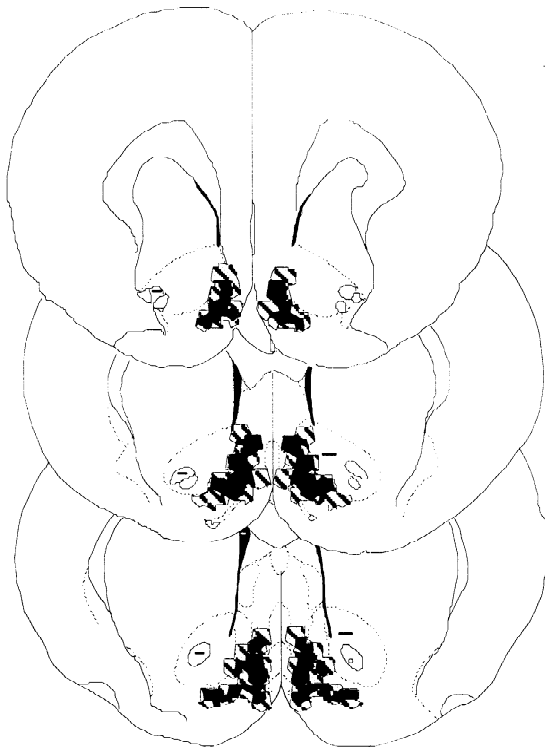


Fig. 9. Effect of changing Fos criteria: $>400\%$ Fos elevation map. A comparison map similar to Fig. 6, except that smaller plumes were plotted based on a criterion of Fos expression of at least 400% over vehicle baseline levels. The total map is very similar to Fig. 6 in outer boundaries and location. The chief difference is that the high confidence zone (bilateral Fos expression) has shrunk, and the moderate confidence zone (unilateral Fos expression) has expanded, due to the more stringent criterion for Fos elevation.

mapping procedure employed here. Even when the criterion for elevated Fos expression was changed from $>200\%$ over vehicle baseline to $>400\%$ over baseline, the location of the functional site did not change, and only the zone of ‘high confidence’ contracted markedly (because there was less bilateral overlap between regions of $>400\%$ Fos elevation; Fig. 9). Indeed, our informal observations showed the site did not change markedly even if the criterion for morphine-induced neuronal activation was lowered to $>150\%$ elevation from baseline or raised to $>500\%$ elevation, probably because changes in the size of ‘positive plumes’ resulting from criteria shifts were matched by changes in the size of surrounding ‘negative plumes’ from sites that failed to elicit feeding (subtraction sites).

4.3. Comparison of fos-mapped ‘opioid eating site’ to other studies

The location of the opioid ‘eating site’ mapped in this study overlaps partly with the sites identified by studies of Kelley and co-workers for elevated food intake produced by microinjections into the shell of the nucleus accumbens of opioid agonists [4,87,88], although they also report

effects from accumbens core sites, from more lateral sites within accumbens shell, and at more posterior sites in the striatum. Our findings do not in any way contradict the existence of posterior striatal eating sites or of any other opioid eating sites *outside* the nucleus accumbens. The present study mapped only within the accumbens itself, and simply did not test posterior sites or other sites outside the accumbens. It is likely that the ‘eating site’ mapped here continues caudally into the ventral striatum as described by Kelley and co-workers, and possibly even the ventral pallidum, since the most intense increases in eating behavior were elicited at the caudal edge of the accumbens site.

More interesting is the partial discrepancy *within* the nucleus accumbens between sites reported by Kelley and co-workers [4,87,88] and the smaller region mapped here. It is possible that this partial discrepancy in site boundaries could be explained by various methodological differences. First, it must be kept in mind that the Fos plume subtraction procedure used here is designed explicitly to produce a conservative map of the functional zone. It is intended to find a *brain region that contains receptors definitely capable of mediating morphine-elicited eating*. The subtraction procedure eliminates all peripheral zones containing mixed positive/negative effects, since all regions containing a single ‘negative’ Fos plume are eliminated from the positive map. This naturally produces a smaller map of the positive zone than conventional mapping procedures. What remains after subtraction can be considered as positive with high confidence, but what is outside the site may not be completely negative. A second potential reason for the partial discrepancy is that the present study used a low dose of morphine ($0.5 \mu\text{g}$), in order to produce small Fos plumes, whereas other studies have included additional doses up to $20 \mu\text{g}$, which would activate larger regions and so allow a larger positive site. Finally, in a recent mapping study by Zhang and Kelley [88], which found positive sites throughout the accumbens lateral shell and core, DAMGO was used as the opioid agonist for microinjection rather than morphine. If DAMGO is more potent than morphine at eliciting eating behavior in the accumbens, then the difference between site maps may result from the difference between agonists. It would be desirable to directly compare DAMGO and morphine using the Fos plume subtraction mapping procedure. It is possible that all discrepancies in map boundaries would be resolved if agonist, dose, microinjection, and analysis procedures were equated.

In short, the procedure used here produced a deliberately conservative map of the opioid eating site within the accumbens. Regions within it can be regarded as definitely able to mediate eating behavior produced by accumbens morphine. But these results do not exclude a functional contribution under some conditions either from opioid receptors outside the positive eating site. It is interesting, in any case, that the accumbens map for morphine-elicited

eating identified in this study closely corresponds to the region reported by Kelley and co-workers to mediate eating elicited by other GABA agonists and glutamate antagonists [52,79,80]. The meaning of this anatomical correspondence for different neurochemical systems involved in food intake remains to be clarified.

4.4. Functional role: hedonic enhancement of food reward (taste 'liking' as well as 'wanting')

Our observation of enhanced positive hedonic reactions to sucrose indicates that opioid-stimulated food intake from it may be due in part to enhancement of the rewarding properties of the taste of food. This is consistent with suggestions that opioid systems mediate food palatability [20,21,29,55]. Those suggestions have been based on the effects of opioid agents in rats on food intake and choice tests [22,31,55,86], on the reciprocal effects of opioid agonists and antagonists in rats on affective taste reactivity patterns [19,25,45,70,72,74], and on the effects of opioid antagonists in humans on subjective palatability ratings [26,84,85].

Our present results are the first demonstration of enhanced positive taste reactivity after opioid agonist stimulation delivered directly to the nucleus accumbens. Affective reactions were shifted in the positive hedonic direction, indicated by the enhancement of hedonic reaction patterns to sucrose. Aversive reaction patterns to quinine were not enhanced by morphine microinjections in the same site, but instead were suppressed if changed at all. This pattern of change suggests a specific functional or psychological mechanism for the effect on food intake, namely, that rats eat and 'want' food more after morphine microinjections in the accumbens shell in part because opioid stimulation at this site makes them 'like' food more. This may partly explain why opioid-induced increases in eating typically are delayed (e.g. up to 1 h after administration). Increased food intake may follow upon the rat's experience with enhanced palatability after opioid stimulation of the accumbens site. In the present study, hedonic taste reactivity to sucrose was significantly enhanced at 15 min after the morphine microinjection, whereas food intake was not significantly increased until the 1 h measurement. Thus the opioid-induced enhancement in palatability may precede and be a cause of the opioid-induced enhancement in intake.

What remains unknown is whether the entire accumbens shell opioid eating site is capable of supporting increases in hedonic reaction patterns, or whether the accumbens opioid 'eating site' and the 'hedonic enhancement site' only partly overlap. In other words, whether 'liking' and 'wanting' enhancements are both mediated by the same identical site, or whether some accumbens site can mediate 'wanting' without 'liking' or vice versa. It also remains to be elucidated how opioid accumbens substrates for hedonic

'liking' of food and other rewards interact with other neural systems to produce 'wanting' for those rewards [9].

5. Conclusion

Our mapping results show that the accumbens shell is functionally heterogeneous with regard to opioid-stimulated increases in food intake. The 'opioid eating site' was contained primarily within a medial caudal subregion of the nucleus accumbens shell, and did not substantially penetrate into the accumbens core or into other subregions of the shell. Increased food intake appears to be accompanied by increased positive hedonic reactions to the taste of food for at least some microinjections within the eating site, indicating that palatability enhancement may be a partial explanation for increased food intake produced by opioid agonists.

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