Peptides that Regulate Food Intake
Appetite-inducing accumbens manipulation activates hypothalamic orexin neurons and inhibits POMC neurons

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FEW DOUBT THAT THE RAPID INCREASE in prevalence of obesity is related to changes in environment and lifestyle. Increased advertisement and availability of highly palatable and calorically dense foods are some of the components of a new obesigenic environment (30) that might overwhelm endogenous systems regulating energy homeostasis. The “liking” and “wanting” associated with this increased awareness and availability of food is thought to be processed in corticolimbic structures such as the prefrontal cortex, amygdala, and ventral striatum, whereas homeostatic controls have been assigned mainly to the hypothalamus (52).

Therefore, crosstalk between the two systems might be critical for understanding how environmental factors can override regulatory mechanisms.

More than 20 years ago, Mogenson (41) proposed that the nucleus accumbens is involved in the translation of motivation into action, but the functional relationship between this limbic forebrain area and the food intake circuits of the hypothalamus is still poorly understood. Hoebel and collaborators, measuring changes in nucleus accumbens shell (AcbSh) transmitter release and receptor binding induced by lateral hypothalamic (LH) electrical stimulation and excessive sugar intake, found that increased dopamine and acetylcholine were correlated to the rewarding or aversive character of the stimulation, respectively, and that this ratio was modulated by opioids (13, 14, 47). On the other hand, interruption of glutamatergic transmission with AMPA/kainate receptor antagonists or activation of GABA-ergic transmission with the GABA agonist muscimol in the rostral shell of the nucleus accumbens elicits robust feeding in satiated rats (38, 48, 58). As the shell area is also distinguished by unique projections to the LH and ventral pallidum (25), it is possible that feeding responses depend on these projections. Microinjection of the GABA agonist muscimol into the LH completely blocked the feeding response induced by rostral shell injection of the AMPA glutamatergic antagonist DNQX, suggesting that GABA-ergic shell neurons projecting to the LH may be disinhibited by DNQX and that muscimol reverses this disinhibition (38). Because blocking GABA_A receptors in the ventral pallidum also elicits a feeding response, and considering that the ventral pallidum in turn

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projects to the LH, it is also possible that the accumb ens shell affects LH activity and food intake via the ventral pallidum (59). With the use of Fos as a marker for neuronal activation, it was shown that muscimol injected to the nucleus accumbens shell strongly activated neurons in the LH and in other areas as well, including the paraventricular nucleus (PVN), lateral septum, ventral tegmental area, and nucleus of the solitary tract (57).

It has long been known that electrical and chemical stimulation of the LH, particularly the perifornical area, elicits strong feeding responses in satiated rats (55, 56). More recently, neuron populations expressing specific orexigenic peptides have been identified in the hypothalamus. Neurons expressing melanin-concentrating hormone (MCH) are distributed over most of the LH and zona incerta (5, 7, 20). MCH neurons project widely to almost every brain area (4). Intracerebroventricular MCH stimulates food intake (50), and MCH overexpression in the LH of mice leads to higher fat intake, mild obesity, and insulin resistance (37). MCH knockout mice exhibit reduced food intake and lower body weight (53). A separate population of neurons in the LH coexpresses orexin (20, 46) and dynorphin (12) and projects equally widely to most brain areas. Microinjection of orexin-A into the third or lateral ventricles (18, 61) or into the perifornical hypothalamus (17, 60) also increases food intake.

In the arcuate nucleus, one population of neurons coexpresses mRNA for neuropeptide Y (NPY) and agouti-related protein (AgRP) (1, 40), with major projections to the paraventricular nucleus and perifornical hypothalamus. Both peptides potently stimulate food intake (27, 54). Another, distinct population of neurons expresses proopiomelanocortin (POMC) and cocaine-amphetamine-related transcript (CART) (20, 35). These neurons can release α-melanocyte-stimulating hormone (α-MSH), β-endorphin, adrenocorticotropin hormone, and CART from their terminals in hypothalamic and many extrahypothalamic areas (1, 33, 34). Both α-MSH and CART peptide have been shown to suppress food intake (35, 39).

Given the strong hypothalamic Fos response induced by chemical inhibition of the AcbSh, the aim of the present study was to determine the extent of the recruitment of neurons expressing such feeding-related peptides. As AcbSh inhibition elicits robust feeding, we hypothesized that it works by activating neurons expressing the orexigenic peptides NPY, orexin, and MCH, and by inhibiting neurons expressing the anorexigenic peptides CART, POMC/α-MSH, and oxytocin. The AcbSh was inhibited by microinjection of the GABA agonist muscimol, and the effect on food intake was determined in satiated rats. Rats that significantly increased food intake with muscimol compared with saline were then given a final injection of either muscimol or saline in the absence of food, and the hypothalamus was processed for Fos/peptide double immunohistochemistry.

MATERIALS AND METHODS

All experimental protocols were approved by the Institutional Animal Care and Use Committee (Pennington Biomedical Research Center) and were conducted in compliance with United States Department of Agriculture regulations and with the “Guiding Principles for Research Involving Animals and Human Beings” of the American Physiological Society.

Animals and housing. Adult male Sprague-Dawley rats (Harlan Industries, Indianapolis, IN) weighing 280–320 g at the time of surgery were housed individually in hanging wire mesh cages in a climate-controlled room (22 ± 2°C) on a 12:12-h light cycle with lights on at 0700 and lights off at 1900. Food and water were available ad libitum except as specified below.

Intra-accumbens injections. Animals were anesthetized with ketamine-acepromazine-xylazine (80/1.6/5.4 mg/kg ip) and given atropine (1 mg/kg ip). Bilateral 24-Ga stainless steel guide cannulas were aimed at the rostral shell of the nucleus accumbens (antero-posterior 1.4 mm, mediolateral 0.8 mm, dorsoventral 6.0 mm). These rostral shell coordinates have been shown to yield the most robust food intake (48) and project to the LH (25). Twelve to fifteen days were allowed for recovery from the surgery, at which time the animals went through two to three mock injections with 31-Ga injectors extending 2.0 mm beyond the tip of guide cannulas. The correct placement of the injector tips was initially tested by the feeding response to muscimol. At the end of the experiment, the lipophilic dye Dil (0.2 μl, 2%) was injected just before perfusion. Injection sites were histologically verified by checking the dye deposits in 50-μm coronal sections. All injection sites in animals used for the present experiment were located in the rostral half of the nucleus accumbens shell.

Experimental protocol and measurement of food intake. On the experimental days, between 0900 and 1000, food was removed from the hopper 30 min before the injections, and muscimol (100 ng in 500 nl sterile saline) or saline alone as a control was infused bilaterally over a period of 1 min. This dose of muscimol has been used previously to stimulate food intake and c-Fos expression (57). After infusion, the injectors were left in place for an additional 1 min to prevent backflow. A preweighed amount of rat chow was provided 3 min after the end of injections, and intake was measured at 30 min and 1 h. For the purpose of measuring food intake, injections were counterbalanced, with each rat serving as its own control. In a final test, either muscimol or saline was infused as above, and rats were returned to their home cages in the absence of food. Ninety minutes later the animals were euthanized.

Tissue processing and immunohistochemistry. Rats were deeply anesthetized with pentobarbital sodium (120 mg/kg) and transcardially perfused with heparinized saline (20 U/ml) followed by ice-cold, 4% phosphate-buffered (pH 7.4) paraformaldehyde. Brains were extracted, blocked, and postfixed in the same fixative overnight. Tissue was immersed for 24 h in 25% sucrose in 4% paraformaldehyde before cryosectioning. Frozen sections of 30 μm were cut in a cryostat, separated into five series, and either processed immediately or stored in cryoprotectant solution at −20°C.

Four complete series (10–15 sections) of one-in-five sections stretching the entire rostrocaudal dimension of the hypothalamus were processed for c-Fos immunohistochemistry using the avidin-biotin complex (ABC) procedure (v 3400.3, 3-diaminobenzidine tetrahydrochloride (DAB) method. All processing was done on free-floating sections. Briefly, the tissue was prewashed with a solution of 1% sodium borohydride in PBS. Appropriate washes in PBS followed this and subsequent
incubations. For quenching endogenous peroxidase, sections were treated with 3% hydrogen peroxide-methanol (1:4) before blocking for 2 h in a solution of PBS with 0.5% Triton X-100 (PBS/T) containing 5% normal goat serum (NGS) and 1% bovine serum albumin (BSA). Incubation in c-Fos primary antibody (Table 1) was for 20 h at room temperature (RT) and was followed by 2 h in biotinylated goat anti-rabbit secondary antibody (1:500; Jackson ImmunoResearch, West Grove, PA). The sections were then incubated for 1 h in ABC (1:500; Vectastain ABC Elite Kit, Vector Labs, Burlingame, CA). The blue-black nuclear Fos was visualized using a metal-enhanced DAB substrate kit (Pierce Chemical, Rockford, IL).

Fos staining was followed by immunofluorescent labeling for CART, MCH, NPY, oxytocin, orexin-A, and α-MSH (Table 1). Briefly, sections were incubated with the appropriate blocking solution before incubation in the primary antiseraum for 20 h at RT or 40 h at 7°C. Alexa 594 goat anti-rabbit IgG (1:2,000; Molecular Probes, Eugene, OR) was applied for 2 h at RT in the dark for all peptides except α-MSH, which was labeled with Cy3 donkey anti-sheep IgG (1:600, Jackson ImmunoResearch, West Grove, PA). After 1 h in 70% glycerol, the sections were mounted in 100% glycerol with the anti-fade agent 5% n-propyl gallate.

Counting procedures, imaging, and statistical analysis. For the quantitative assessment of Fos expression in the LH, three to five sections from −2.5 to −3.8 mm (from Bregma) were selected, and images were generated through a rectangular field captured for each side of each section to include an extended perifornical area as shown in Fig. 2. Fos-positive cell nuclei were visualized using the 633-nm line of an internal He/Ne laser in the transmitted light mode and stored in one channel (green). Red fluorescent peptide immunoreactivity within a 10-μm slice (centered around the plane used for the Fos image) was captured in the confocal mode using the 568-nm line of an Ar/Kr laser by collapsing 10 optical sections, 1 μm apart, into one horizontal plane, and stored in a second channel (red).

Images were then displayed on a full screen using imaging software that allowed tagging each individual neuron (Image-Pro Plus, Media Cybernetics, Silver Spring, MD). Neurons were either classified as Fos only, peptide only, or double-labeled according to the presence above background of fluorescence in the cytoplasm and dark nucleus were also counted. Raw counts from both hemispheres of four comparable sections in each rat were averaged, and the percentage of peptide-positive neurons expressing Fos was calculated.

Separate one-way ANOVAs were used for all dependent measures for statistical evaluation of group means.

RESULTS

Food intake. Six rats that consumed significantly more chow after AcbSh muscimol injection compared with saline injection were selected for the final muscimol injection and the analysis of Fos expression. They consumed 2.86 ± 0.72 g within 1 h after muscimol compared with 0.52 ± 0.19 g after saline (P < 0.01), with most of the intake occurring during the first 30 min (Fig. 1). Of the four rats selected for final saline injection, two rats consumed significantly more chow after muscimol than after saline, and two rats did not significantly increase chow intake after muscimol injection. On average, they ingested 1.19 ± 0.72 g after muscimol and 0.18 ± 0.18 g after saline (P = 0.22; not significant (NS)). Eaters and non-eaters showed similar patterns and magnitudes of Fos expression in the LH and arcuate nucleus after AcbSh saline injection.

Fos expression in orexin and MCH neurons of the perifornical LH. Compared with saline injection, AcbSh muscimol injection resulted in increased Fos expression in several areas of the LH (Fig. 2). Fos expression was significantly increased in the perifornical area (muscimol 94 ± 12, saline 41 ± 3 cells/section, P < 0.01, Fig. 3A). Many orexin and MCH neurons were present in the dorsal perifornical area but very few in the medial tuberal area ventrolateral to the fornix (Fig. 2, C and E). Within the dorsal perifornical
Fig. 2. Effects of nucleus accumbens muscimol or saline injection on Fos expression in orexin and melanin-concentrating hormone (MCH) neurons of the perifornical/lateral hypothalamus. A and B: muscimol injection induces significant Fos-like immunoreactivity (black dots) mainly in areas dorsomedial and ventrolateral to the fornix (fx) of the tuberal hypothalamus (−2.5 to −3.5 mm from bregma, section shown at −3.0 mm, A) compared with control saline injection (B). The box in B indicates the area used for counting double-labeled neurons in the perifornical (PeF) hypothalamus. C and D: representative sections from muscimol injected rat showing distribution of Fos-like immunoreactivity (black) and orexin-like immunoreactivity (red) at low magnification (C), and an area dorsal to the fornix at higher magnification (D). Note the presence of many Fos/orexin double-labeled neurons in D, E and F: sections from muscimol injected rat showing distribution of Fos-like immunoreactivity (black) and MCH-like immunoreactivity (red) at low magnification (E), and an area dorsal to fornix and in the far lateral hypothalamus (inset) at higher magnification (F). Note the absence of any double-labeled neurons. Color images were obtained with scanning microscope using the red channel for the peptides in confocal mode and the green channel for Fos in transmitted light mode. The higher magnification images (red channel) represent 6–10 optical sections scanned 1 μm apart and collapsed into the horizontal plane.

area, many orexin neurons expressed Fos after muscimol (Fig. 2D). Based on a similar number of orexin neurons/rat analyzed for each group (saline 93 ± 18; muscimol 90 ± 20, NS, Fig. 3C), the percentage of orexin neurons with Fos was significantly higher after muscimol than after saline (62.2 ± 4.4 vs. 31.5 ± 6.5%, P < 0.01, Fig. 3B). In contrast, only the rare double-labeled MCH neuron (<1% of all MCH neurons) was present.
Fos expression in POMC/CART and NPY neurons of the arcuate nucleus. Most POMC neurons in the rat arcuate nucleus and retrochiasmatic area have been shown to coexpress CART (19). We found CART-immunoreactive and α-MSH-immunoreactive neurons mainly in the ventrolateral part of the arcuate nucleus. There was some Fos expression in this area in saline-injected control rats (Fig. 4A), as well as many CART/Fos and POMC/Fos double-labeled neurons (Fig. 4, B and C). Quantitative analysis of sections stained for CART and Fos revealed that a significantly greater percentage of CART neurons expressed Fos in saline-treated control rats compared with rats treated with muscimol [51 ± 5.4 vs. 9.8 ± 1.7%, F(1,9) = 76.3, P < 0.01, Fig. 5A]. There was no difference in the number of CART-immunoreactive neurons per section between saline and muscimol-treated rats (114 ± 8 vs. 112 ± 8, NS, Fig. 5B).

In contrast to the ventrolateral portion, AcbSh muscimol induced Fos expression in the dorsomedial portion of the arcuate nucleus (E), in many NPY-immunoreactive neurons (red, F). For details of image acquisition, see legend to Fig. 2.
tion of the arcuate nucleus (Fig. 4E), which contains most of the NPY neurons. Muscimol induced Fos expression in many NPY neurons (Fig. 4F), but because immunohistochemical visualization of NPY neuronal perikarya is difficult, we did not attempt to quantify them.

Fos expression in oxytocin and CART neurons of the PVN. Accumbens shell muscimol injection significantly increased Fos expression in the PVN (Fig. 6, A and B), confirming earlier observations (57). Very few (<5%) of the CART neurons located within the medial parvocellular subnucleus showed muscimol-induced Fos expression (Fig. 6, E and F).

Similarly, double-labeling for oxytocin showed that muscimol-induced Fos expression occurred in only a small percentage (<5%) of oxytocin neurons (Fig. 6, C and D), and quantification was not attempted.

DISCUSSION

Muscimol injections into the nucleus accumbens shell that stimulated food intake induced a specific pattern of Fos expression in hypothalamic peptidergic neuron populations. These results confirm and extend observations by Stratford and Kelley (57). Compared with saline control injections, Fos expression was increased in orexin neurons and suppressed in CART/POMC neurons of the arcuate nucleus. In addition, Fos expression appeared to be increased in NPY neurons of the arcuate nucleus, but weak and variable staining for NPY prevented quantitative analysis. No significant changes in Fos expression were found in oxytocin and CART neurons of the PVN, and no Fos expression at all was detected in MCH neurons. These results suggest that one possible mechanism by which accumbens shell muscimol injection induces feeding may be by differentially recruiting orexigenic and anorexigenic hypothalamic peptide systems. However, because the phenotypes of many neurons induced to express Fos were not identified in this study, other peptides and transmitters could account for accumbens-induced feeding. Furthermore, the results do not reveal whether these effects are due to direct or indirect projections from the nucleus accumbens to the hypothalamus.

Stimulation of orexin neurons. Orexin and MCH are expressed in separate populations of neurons with overlapping anatomic distributions throughout the LH (3, 7, 20, 45, 46). Orexin neurons coexpress dynorphin (12) and project widely in the brain, notably to areas involved in the sleep/wake cycle, arousal, autonomic outflow, and food intake (16, 28, 31, 46, 62). Orexin null

Fig. 6. Effects of nucleus accumbens muscimol or saline injection on Fos expression in oxytocin and CART neurons of the paraventricular nucleus of the hypothalamus. A and B: strong induction of Fos-like immunoreactivity after muscimol (A) compared with saline control (B). C and D: overall, few oxytocin-like immunoreactive neurons (red) expressed Fos-like immunoreactivity (black) after muscimol as well as after saline (not shown). An area with a few double-labeled neurons in the ventral parvocellular subnucleus is shown at higher magnification in D (arrows). E–G: CART-immunoreactive neurons were mainly restricted to the medial aspect of the PVN, and few were double-labeled (arrows) with Fos-immunoreactivity (black) both after muscimol (F) and after saline (G). For details of image acquisition, see legend to Fig. 2.
mice and rats with lesions of orexin neurons in the LH exhibit episodes of narcolepsy (11, 24). Fos expression is increased in LH orexin neurons during the dark period of the diurnal cycle (22) and after modafinil administration, a drug used by narcoleptics (51). Orexin-A injected into the perifornical and lateral hypothalamus increased food intake in sated rats (17, 60), but the effect was only observed during the light period (21, 61), which suggests that at least part of orexin’s stimulatory effect on ingestion is secondary to its effects on arousal. However, an additional, more direct effect was indicated by the anorectic, satiety-enhancing action of a selective orexin receptor antagonist (29, 49). It is interesting to note that the ability of antipsychotic drugs to induce weight gain in humans is tightly correlated with their ability to induce Fos in rat LH orexin neurons (23).

Our demonstration that Fos expression in hypothalamic orexin neurons nearly doubled after muscimol treatment suggests that orexin plays a role in the robust feeding response observed after injections in the AcbSh. The presence of direct projections to the LH is one of the features of the accumbens shell that distinguishes it from the core (25). The observation that local LH muscimol injection blocked the feeding response emphasizes the importance of projections to the hypothalamus for the activation of this response to AcbSh muscimol injection (38). However, direct proof of orexin involvement in the accumbens-initiated feeding response will require the use of orexin receptor antagonists or animals without functional orexin neurons.

In addition to the antipsychotic drugs (23) discussed above, we have recently shown that intracerebroventricular injection of the endogenous melanocortin receptor antagonist AgRP, which induces hyperphagia for several days, can stimulate Fos expression in orexin neurons that persists for 23 h after injection (63). Furthermore, hypoglycemia induced by insulin (10) and cytophagocypenia induced by 2-deoxyglucose (6) stimulate Fos expression and increases in cytosolic calcium concentration (43) in orexin neurons. Thus it appears that orexin neurons constitute a final common pathway by which various metabolic and psychological factors engage the neural substrate that orchestrates feeding behavior.

As MCH has been shown to be crucially involved in food intake and energy homeostasis, we expected activation of MCH neurons by AcbSh muscimol (37, 50, 53). However, there was no activation at all of MCH neurons. Lack of MCH activation was also observed with AgRP (63) or antipsychotic drugs (23), suggesting that MCH serves a different aspect of feeding behavior, possibly related to sex hormones (44).

Suppression of POMC/CART and stimulation of NPY/AgRP neurons. AcbSh manipulation with muscimol produced relatively subtle changes in arcuate nucleus Fos expression. Muscimol injection shifted Fos expression from the ventrolateral to the dorsomedial portions of this nucleus. In the rat, the former area mostly contains neurons with the POMC/CART phenotype, whereas the latter region mostly contains neurons of the NPY/AgRP phenotype (1). Our stimulus resulted, therefore, in a significant reduction of Fos double-labeled POMC/CART neurons, and in an increase of double-labeled NPY neurons. As melanocortin signaling through α-MSH and the MC3/4R melanocortin receptors is thought to be one of the central physiological modulators of food intake (see Ref. 9 for recent review), inhibition of POMC neurons should lead to potent disinhibition of food intake. In general, intracerebroventricular CART-peptide injections have also been reported to suppress food intake (e.g., Ref. 35), but the specific role for CART in arcuate POMC neurons has not been identified. AcbSh muscimol-stimulated feeding may partially depend on its inhibitory action on arcuate POMC/CART neurons.

As no direct accumbens-arcuate nucleus projections have been identified to date, it is possible that inhibition of POMC neurons is mediated by orexin neurons in the LH, which have been shown to produce direct synaptic inputs to POMC neurons (26). However, because orexin depolarizes and thus excites most neurons (8, 31, 32), it is not clear how this would lead to suppression of Fos expression, unless there is involvement of local GABA neurons identified in the arcuate nucleus electrophysiologically (15).

We have not attempted to quantify increased Fos expression in arcuate nucleus NPY neurons because it is difficult to visualize NPY-positive cell bodies with the available antibodies. We have detected increased Fos expression in the dorsomedial portion of the nucleus, an area that has been shown to harbor most NPY/AgRP neurons (1). We found Fos expression in many of the stronger labeled NPY neurons in muscimol-treated rats (Fig. 4). Because the arcuate NPY system is one of the strongest orexigenic pathways, activation of this system may also be responsible for the robust feeding induced by AcbSh muscimol.

PVN oxytocin and CART neurons are not involved. AcbSh muscimol increased Fos expression in the PVN, confirming earlier observations (57). We found a small proportion of oxytocin and CART neurons expressing Fos after both AcbSh muscimol and saline injections and did not attempt to quantify the effects. Because both peptides are known to inhibit food intake, we might have expected decreased activity after muscimol. The low basal Fos expression may have precluded detection of a further decrease. Not withstanding this, it appears that neurons expressing these two peptides are not involved in the AcbSh muscimol-induced feeding response.

Unidentified neuronal phenotypes. Of all the neurons activated by AcbSh muscimol, we have phenotypically identified only a fraction. In the hypothalamus, large unaccounted populations exist in the area ventrolateral to the fornix, also referred to as medial tuberal nucleus, in the PVN, and in the dorsomedial nucleus. Some of the neurons in the medial tuberal nucleus and PVN express galanin, a well-characterized orexigenic peptide (36). In addition, we also confirmed the earlier finding (57) that AcbSh muscimol induces Fos in neurons of the dorsal vagal complex in the medulla oblon-
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