Sucrose consumption increases naloxone-induced c-Fos immunoreactivity in limbic forebrain

James D. Pomonis, David C. J. EWett, Catherine M. Kotz, Jacqueline E. Briggs, Charles J. Billington, and Allen S. Levine. Sucrose consumption increases naloxone-induced c-Fos immunoreactivity in limbic forebrain. Am. J. Physiol. Regulatory Integrative Comp. Physiol. 278: R712–R719, 2000.—Opioids have long been known to have an important role in feeding behavior, particularly related to the rewarding aspects of food. Considerable behavioral evidence suggests that sucrose consumption induces endogenous opioid release, affecting feeding behavior as well as other opioid-mediated behaviors, such as analgesia, dependence, and withdrawal. In the present study, rats were given access to a 10% sucrose solution or water for 3 wk, then they were injected with 10 mg/kg naloxone or saline. Brains were subsequently analyzed for c-Fos immunoreactivity (c-Fos-IR) in limbic and autonomic regions in the forebrain and hindbrain. Main effects of sucrose consumption or naloxone injection were seen in several areas, but a significant interaction was seen only in the central nucleus of the amygdala and in the lateral division of the periaqueductal gray. In the central nucleus of the amygdala, naloxone administration to those rats drinking water significantly increased c-Fos-IR, an effect that was significantly enhanced by sucrose consumption, suggesting an upregulation of endogenous opioid tone in this area. The data from this study indicate that the central nucleus of the amygdala has a key role in the integration of gustatory, hedonic, and autonomic signals as they relate to sucrose consumption, if not to food intake regulation in general. Furthermore, the data from this study lend further support to the hypothesis that sucrose consumption induces the release of endogenous opioids.

Central nucleus of the amygdala; diet palatability; bed nucleus of the stria terminalis; nucleus accumbens

FEEDING IS A COMPLEX behavior regulated by numerous brain regions and neuromodulators, including opioids. Opioid agonists stimulate feeding (12), and alterations in food intake or diet composition affect gene expression or peptide levels of endogenous opioids (42). Recent evidence has led to the hypothesis that opioid involvement in feeding may lie in the rewarding aspects of feeding, rather than in those related to energy requirement (20). The rewarding aspects of feeding are regulated not only by various neuromodulators, but by diet composition as well. Operant and free-feeding studies indicate that dietary sucrose is particularly rewarding (see Ref. 3 for review). Because sucrose consumption is considered to be rewarding and because the reward pathway in the brain involves endogenous opioids, it has been hypothesized that consumption of sucrose induces the release of endogenous opioids. Several studies support this contention, largely on the basis of observations that various behavioral effects of the opioid antagonist naloxone are enhanced after sucrose consumption. Because these effects of naloxone are observed in the absence of exogenously administered opioids, this increased potency of naloxone presumably reflects antagonism of elevated levels of endogenous opioids. For instance, chronic consumption of a high-sucrose diet increases the ability of naloxone to inhibit operant responding for a food reward (35). Similarly, rats allowed to drink a sucrose solution show increased sensitivity to the appetite-suppressive effects of naloxone compared with rats with no access to sucrose (14, 19).

Other studies indicate that acute sucrose ingestion induces an immediate release of endogenous opioids, which affects behaviors other than feeding. Rat pups receiving an intraoral infusion of a 7.5% sucrose solution showed decreased pain sensitivity compared with those drinking water (33). Similar analgesic effects of sucrose consumption have been reported in human infants (2, 5). Also, termination of sucrose feeding to morphine-dependent rats increases the severity of naloxone-precipitated withdrawal, suggesting that a regimen of sucrose feeding enhances opioid tone (36). Furthermore, acute or chronic sucrose consumption has been shown to enhance (4, 15) or decrease (10) the analgesic properties of morphine. These effects on morphine-induced analgesia apparently are specific to dietary sucrose or carbohydrates, inasmuch as alterations in dietary proteins or vitamins and minerals have no effect on morphine-induced analgesia (13).

The present study tested the hypothesis that sucrose consumption increases endogenous opioid release by examining c-Fos immunoreactivity (c-Fos-IR) after naloxone administration to rats that had been subjected to

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
prolonged exposure to a 10% sucrose solution. This methodology can be used to unmask brain regions under tonic opioid inhibition on the basis of the following rationale: Inasmuch as the effects of opioid agonists on cellular physiology are inhibitory, resulting in decreased activity of adenylate cyclase, increased K+ conductance, and decreased Ca2+ conductance (8, 27), interruption of these signals by naloxone would be expected to increase cellular activity and, hence, increase c-Fos-IR. An example of this hypothesis is as follows: A single opioid receptor-expressing cell with unoccupied opioid receptors that is treated with naloxone should show no alterations in cellular physiology. That same cell, when exposed to an opioid peptide, would be inhibited as described above. However, if this cell (when inhibited by an opioid peptide) is treated with naloxone, a release from inhibition would occur and may result in subsequent expression of immediate early genes such as c-fos. Thus elevated opioid tone due to sucrose ingestion may be seen as increased c-Fos-IR after naloxone injection (Fig. 1).

MATERIALS AND METHODS

Animals. Twenty-eight male Sprague-Dawley rats (Harlan, Madison, WI), weighing 225–240 g at the start of the experiment, were housed in individual cages in a temperature-controlled vivarium on a 12:12-h light-dark cycle (lights on at 0700). Rats were divided into two groups: the first group received normal chow and tap water ad libitum; the second group received normal chow and 10% sucrose solution ad libitum, with no access to water for 3 wk. Between 0900 and 1300 of the final experimental day, rats were given a subcutaneous injection of isotonic saline or 10 mg/kg naloxone hydrochloride (RBI, Natick, MA). Rats were not allowed to eat or drink for 1 h after injection. One hour after injection, rats were deeply anesthetized with pentobarbital sodium (Nembutal) and transcardially perfused as described previously (31). This design yields four treatments (n = 7/group): those drinking tap water and receiving a saline injection (water-saline), those drinking tap water and receiving a naloxone injection (water-naloxone), those drinking sucrose and receiving a saline injection (sucrose-saline), and those drinking sucrose and receiving a naloxone injection (sucrose-naloxone). One rat in the sucrose-naloxone group was excluded from the study because of illness before the completion of the experiment.

c-Fos immunohistochemistry. Frozen sections were cut on a cryostat at a thickness of 40 µm and were immediately placed in cryoprotectant (41) and subsequently stored at −20°C until staining. Sections were stained for c-Fos-IR, as described previously (31). Briefly, sections were rinsed in PBS, then incubated with anti-c-Fos antiserum (Oncogene Science, Cambridge, MA) at a dilution of 1:50,000 for 48 h at 4°C. After further rinses in PBS, sections were incubated in biotinylated anti-rabbit IgG at a dilution of 1:200 (Vector Laboratories, Burlingame, CA) for 1 h, rinsed, and then incubated for 1 h in Vectastain Elite ABC reagent (Vector Laboratories), rinsed, and then reacted with nickel sulfate-diaminobenzidine. To
control for possible variations in the chromogen reaction, all sections in a given neural region were processed at the same time.

Data collection and statistical analysis. c-Fos-IR was examined in specific forebrain, midbrain, and hindbrain areas that are involved in food intake regulation or opioid dependence/withdrawal. In the forebrain, c-Fos-IR was examined in the central (ACe), basomedial (BMA), and basolateral nuclei of the amygdala, the laterodorsal (BNSTLD) and medial divisions of the bed nucleus of the stria terminals, the caudate putamen (CPu), the ventral, intermediate, and dorsal divisions of the lateral septum, the core (NAcc) and shell (NAccSh) of the nucleus accumbens, the medial and lateral (latARC) divisions of the arcuate nucleus, and the dorsomedial (DMH), ventromedial (VMH), lateral, and paraventricular nuclei of the hypothalamus. In the midbrain and hindbrain, c-Fos-IR was examined in the ventral, intermediate, and lateral periaqueductal gray (latPAG), the intermediate and rostral divisions of the nucleus of the solitary tract, and the intermediate and rostral divisions of the ventrolateral medulla.

c-Fos-IR was measured as described previously (31). Briefly, the neuroanatomic region of interest was viewed using a charge-coupled device camera (Dage MTI, Michigan City, IN) attached to a Nikon ECLIPSE 400 microscope. With the image from the camera displayed on a computer screen and with Scion Image software, the area of interest was outlined and measured in square millimeters, and a threshold was set to denote individual immunoreactive nuclei. These nuclei were counted, and this number was divided by the area. Measurements in this fashion were taken bilaterally on two sections for each animal. The counts were averaged per animal and then per group. Data were analyzed by two-factor ANOVA for each animal. The counts were averaged per animal and then per group. Data were analyzed by two-factor ANOVA for each animal. The counts were averaged per animal and then per group.

RESULTS

Effects of sucrose consumption on c-Fos-IR. As shown in Table 1, there was a main effect of drink in a number of brain regions studied, typically described by an increase in c-Fos-IR in rats drinking sucrose compared with rats drinking water. The majority of these regions are located in limbic forebrain regions but also extended to the hindbrain. A significant main effect of drink was seen in NAccC [F(1,21) = 10.124, P = 0.0045], NAccSh [F(1,21) = 8.637, P = 0.0078], CPu [F(1,21) = 6.503, P = 0.0186], AEC [F(1,21) = 7.539, P = 0.0115; Fig. 2A], BNSTLD [F(1,21) = 5.00, P = 0.0353; Fig. 2B], and IPBN [F(1,21) = 8.240, P = 0.0102]. In all these regions, c-Fos-IR was significantly increased in rats drinking sucrose. There was also a significant main effect of drink in BMA [F(1,21) = 9.073, P = 0.0062]; however, in this region, sucrose consumption increased c-Fos-IR.

Effects of naloxone injection on c-Fos-IR. As shown in Table 1, there was a main effect of drug in a number of brain regions, but this effect was restricted to discrete nuclei within the hypothalamus and amygdala. In all these regions, the main effect was due to increased

| Table 1. Effect of sucrose consumption and naloxone injection on c-Fos-IR in selected brain regions |
|-----------------------------------------------|---------------------|---------------------|---------------------|
|                                | Water-Saline | Water-Naloxone | Sucrose-Saline | Sucrose-Naloxone |
|                                |              |                |                |                 |
| **Main effect of drink**       |              |                |                |                 |
| NAcC                          | 79.0 ± 11.3  | 76.3 ± 8.2     | 113.8 ± 4.3    | 101.5 ± 10.0    |
| NAcSh                         | 139.5 ± 12.0 | 181.1 ± 20.3   | 207.2 ± 29.5   | 262.6 ± 37.5    |
| CPu                           | 543.7 ± 78.1 | 455.5 ± 48.6   | 699.7 ± 120.7  | 759.2 ± 99.5    |
| AEC                           | 189.3 ± 29.4 | 586.2 ± 47.2   | 150.4 ± 26.0   | 858.3 ± 63.4    |
| BNSTLD                        | 225.5 ± 26.5 | 634.7 ± 123.1  | 336.1 ± 47.4   | 994.7 ± 178.1   |
| BMA                           | 137.2 ± 18.5 | 201.0 ± 29.6   | 100.0 ± 11.7   | 115.5 ± 15.6    |
| IPBN                          | 155.1 ± 13.0 | 187.7 ± 13.0   | 314.4 ± 48.5   | 252.5 ± 45.8    |

**Main effect of drug**

|                                |              |                |                |                 |
|                                |              |                |                |                 |
| ACE                           |              |                |                |                 |
| BNSTLD*                       |              |                |                |                 |
| DMH                           | 470.4 ± 25.5 | 541.9 ± 33.1   | 472.5 ± 57.4   | 601.7 ± 39.8    |
| VMH                           | 87.2 ± 6.2   | 97.4 ± 8.2     | 92.3 ± 11.0    | 132.9 ± 17.6    |
| latARC                        | 238.8 ± 27.4 | 309.0 ± 25.0   | 248.5 ± 30.1   | 376.4 ± 55.7    |
| Drink × drug interaction      |              |                |                |                 |
| Ace                           | 267.3 ± 24.9 | 390.3 ± 55.4   | 419.3 ± 37.1   | 350.6 ± 50.7    |

Values are means ± SE, expressed as mean c-Fos-immunoreactive (IR) nuclei per mm². See RESULTS for statistics and means comparisons. NAcC, core of nucleus accumbens; NAcSh, shell of nucleus accumbens; CPu, caudate putamen; AEC, central amygdala; BNSTLD, basolateral bed nucleus of stria terminals; BMA, basomedial amygdala; IPBN, lateral parabrachial nucleus; DMH, dorsomedial hypothalamus; VMH, ventromedial hypothalamus; latARC, lateral arcuate nucleus; latPAG, lateral periaqueductal gray. *See Main effect of drink.

c-Fos-IR in rats receiving naloxone injections compared with rats receiving saline injections. In the hypothalamus, a significant main effect of drug was seen in latARC [F(1,23) = 8.027, P = 0.0094], VMH [F(1,23) = 5.300, P = 0.0307], and DMH [F(1,23) = 6.005, P = 0.0223]. In the amygdala, there was a significant main effect of drug in AEC [F(1,23) = 169.00, P < 0.0001; Fig. 3] and BNSTLD [F(1,23) = 25.935, P < 0.0001; Fig. 4].

Sucrose consumption alters naloxone-induced c-Fos-IR in AEC and latPAG. Of all the brain regions examined in this study, only two showed a significant drink × drug interaction (Table 1). The most pronounced effect was seen in AEC [F(1,23) = 13.394, P = 0.0013]. c-Fos-IR in this area was significantly elevated in the water-naloxone group compared with the water-saline group [t(12) = 7.136, P < 0.0001], indicating a basal level of endogenous opioid release. c-Fos-IR was also significantly higher in the sucrose-naloxone group than in the sucrose-saline group [t(12) = 10.937, P < 0.001]. To determine whether sucrose consumption significantly enhanced this opioid release, c-Fos-IR in the sucrose-naloxone group was compared with that in the water-naloxone group. Indeed, c-Fos-IR was significantly higher in the sucrose-naloxone group than in the water-naloxone group [t(13) = 3.505, P = 0.0049]. Along with these qualitative differences, qualitative differences in the pattern of c-Fos-IR were also present. In AEC, c-Fos-IR in the water-naloxone group was generally localized to the lateral division, whereas c-Fos-IR in the sucrose-naloxone group was spread throughout the lateral, medial, and capsular divisions (Fig. 3).
A significant drink × injection interaction was also seen in latPAG (F(1,23) = 4.900, P = 0.0371; Table 1). Although there was a trend of higher c-Fos-IR in the water-naloxone group than in the water-saline group, this effect did not reach significance (t(12) = 2.023, P = not significant (NS)). Similarly, there was no significant difference between the sucrose-saline and sucrose-naloxone groups (t(11) = −1.117, P = NS) or between the saline-naloxone and sucrose-naloxone groups (t(11) = −0.522, P = NS).

**DISCUSSION**

Feeding influences on endogenous opioid tone in the amygdala. The data presented here provide indirect evidence in support of the hypothesis that sucrose consumption increases endogenous opioid tone in ACe. We propose that this elevated opioid tone, in turn, plays a significant role in the palatable and rewarding characteristics of sucrose.

Our observation that naloxone injection results in greater c-Fos-IR in ACe in rats drinking sucrose than in rats drinking water correlates with previous behavioral studies on the anorectic ability of naloxone. Naloxone’s anorectic effect is potentiated by prior exposure to a sucrose-based diet (35) or sucrose solutions in addition to normal chow (14, 19). It may be that the increased ability of naloxone to induce c-Fos-IR after sucrose administration reflects a portion of the cellular substrate for naloxone’s anorectic potential. Thus manipulations that result in altered amygdalar activity may have pronounced effects on the potency of naloxone and perhaps other anorectic agents.

The extended amygdala and, particularly, ACe are known to be important structures in the regulation of food intake, including the inhibition of feeding. For example, injection of naltrexone into ACe inhibits feeding induced by administration of neuropeptide Y into the paraventricular nucleus (11). Also, injection of the GABA_α agonist muscimol into ACe decreases spontaneous and deprivation-induced feeding (24). Several potent satiety agents may also use neural pathways involving ACe. Peripheral administration of the anorectic agents naloxone, naltrexone, leptin, satiety, or cholecystokinin induces c-fos expression in ACe (6, 31, 34, 39, 40).

Although limitations inherent to using c-Fos-IR to study patterns of neuronal activation do not allow us to distinguish primary sites of opioid release from regions that may be activated transsynaptically, κ-opioid receptors are found in ACe, albeit at a relatively low level (21).

The data from the present study also suggest that ACe is responsible for the significant amount of processing that occurs in naloxone-induced satiety. ACe is the only area examined that showed main effects of drink and drug as well as a significant drink × drug interaction (Fig. 5). In fact, BNSTLD was the only other region examined that showed more than one main effect. The patterns of c-Fos-IR in ACe and BNSTLD were nearly identical, except in BNSTLD, sucrose consumption did not significantly increase naloxone-induced c-Fos-IR. The similarities in responses of ACe and BNSTLD may be attributable, at least in part, to the ontogenetic and neurochemical similarities shared by these two regions (see Ref. 38 for review). It may be that BNSTLD shares a similar role with ACe in naloxone-mediated inhibition of feeding.

Convergence of sucrose- and naloxone-mediated signals on ACe. Although there were significant main effects of drink and drug in ACe and BNSTLD, there was a striking divergence of areas showing a main effect of drink and those showing a main effect of drug (Fig. 5). This separation of responsiveness correlates with previous data on the roles of these regions in behavioral and physiological roles in taste, reward, and feeding. A number of areas that showed a main effect of
drink have established roles in gustatory processing. After gustatory primary afferents enter the nucleus of the solitary tract, the taste pathway bifurcates at IPBN: one path follows a classic sensory thalamocortical route; the other projects to limbic regions, including ACe and BNSTLD (26). With the exception of BMA, the increased c-Fos-IR seen in these areas due to sucrose consumption was unaffected by naloxone injection, indicating involvement of non-opioid systems. If the increased c-Fos-IR in IPBN seen in the present study reflects activity of the taste pathway, then it is not surprising that such activity is unaffected by naloxone. Although naloxone has been shown to decrease intake of a 10% sucrose solution (29), it is unlikely that this effect is due to alterations in taste perception, inasmuch as naloxone does not alter the ability of rats to distinguish small amounts of sucrose from water in an operant discrimination task (28).

Regions showing a main effect of drug were restricted to the hypothalamus and amygdala and were largely separate from those regions showing a main effect of drink. The regions showing a main effect of drug have established roles in the regulation of feeding and energy expenditure (see Refs. 12 and 20 for reviews). Naloxone not only inhibits feeding but also blocks neuropeptide Y-induced decreases in energy expenditure (17, 18), indicative of autonomic effects of naloxone administration.
A significant drink × drug interaction was seen only in ACe and latPAG. Although the significant interaction seen in latPAG likely represents the role of this structure in the modification of analgesia due to sucrose consumption (33), the results in ACe suggest a broad role for ACe in a number of behavioral processes. Together with the main effects of drink and drug seen in ACe, the significant interaction seen here indicates that ACe may play a role in the integration and processing of taste, reward, and autonomic signals. Indeed, neuroanatomic studies indicate that a significant amount of intra-amygdaloid processing occurs before signals leave the amygdala via ACe (30).

Sucrose-induced alteration of opioid tone. If, as we contend here, sucrose consumption increases endogenous opioid tone, then prolonged sucrose consumption may lead to a situation that is in some way analogous to opioid dependence. Administration of naloxone to rats drinking sucrose resulted in increased c-Fos-IR in increased c-Fos-IR in some, but not all, of the same regions known to be activated after naloxone administration to morphine-dependent animals (1, 7, 9, 22, 32, 37). Although the pattern of c-Fos-IR seen in the sucrose-naloxone group resembles that seen in animals undergoing withdrawal, no changes in c-Fos-IR were seen in several prominent nuclei implicated in opioid dependence. Notable examples are LC and the septal nuclei. An absence of c-Fos-IR in LC in the sucrose-naloxone group is not entirely surprising, since the animals did not show withdrawal signs such as wet dog shakes, diarrhea, ptosis, weight loss, and jumping (data not shown). It appears that autonomic regions mediate these physiological responses of withdrawal, whereas limbic regions affect motivated behaviors. It has been shown that naloxone administration into LC induces severe physical withdrawal with relatively few changes in drug reward. However, naloxone injection into NAccC elicits almost no withdrawal behaviors but dramatically reduces drug reward (16). Furthermore, microinjection of methylnaloxonium (a congener of naloxone) into discrete brain sites induces some, but not necessarily all, withdrawal behaviors, depending on the site of injection. For instance, injection into LC induces severe withdrawal, whereas injection into ACe induces relatively mild withdrawal (23).

Dependence on endogenous opioids has been demonstrated previously, in the context of stress-induced eating due to repeated tail pinch. Rats subjected to repeated tail pinches for 10 days exhibited withdrawal behaviors, such as wet dog shakes and diarrhea, after naloxone administration (25). Inasmuch as neither the animals in the present study nor those in a preliminary study demonstrated any of the "classic" opiate withdrawal behaviors after naloxone administration, these animals are not opioid dependent. However, the sucrose diet does appear to have altered the endogenous opioid system in selected brain sites in a way similar to dependence conditions.

Perspectives

Although it is almost certain that the neuronal circuits involved in food intake regulation and reward are linked in some way, the data from the present study indicate that alterations in diet composition can induce changes in the neurochemistry of these systems. This diet-induced plasticity may, in turn, have profound effects on the response of the central nervous system to subsequent drug administration. This plasticity may have special relevance to the efficacy or potency of
anorectic agents. The data from the present study indicate that ACoE is among the most plastic of the brain regions studied and is also involved in integration of hedonic and autonomic signals relating to feeding.

This work was supported by National Institute on Drug Abuse Grant DA-03999, National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-50456, and the Department of Veterans Affairs.

Address for reprint requests and other correspondence: A. S. Levine, Research Service (151), VA Medical Center, One Veterans Dr., Minneapolis, MN 55417 (E-mail: allens@duh.umn.edu).

Received 28 April 1999; accepted in final form 28 September 1999.

REFERENCES

