Effects of the opioid antagonist naltrexone on feeding induced by DAMGO in the ventral tegmental area and in the nucleus accumbens shell region in the rat

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Running head: Feeding effects of opioids in VTA and NAcc
Abstract

The Nucleus Accumbens shell region (sNAcc) and the Ventral Tegmental Area (VTA) are two major nodes in the mesolimbic dopamine pathway, which mediates reward for various survival behaviors including feeding. Opioids increase and maintain food intake when injected peripherally and centrally. Opioids in the VTA cause increased release of dopamine in the sNAcc, and when injected into either site, cause an increase in food intake. Animals in this study were double cannulated in the VTA and in the sNAcc and injected with various combinations of naltrexone (NTX) (2.5, 5, and 25μg/side) and Tyr-D-Ala-Gly-(me) Phe-Gly-ol (DAMGO) (0.1, 0.3, 1, 3, and 5 nmol/side) in both sites. DAMGO was found to dose-dependently increase intake to an equal extent when injected into either site. DAMGO-induced increases in food intake when injected into the VTA were blocked to control levels with the highest dose of NTX injected bilaterally into the sNAcc; however, increases in intake when injected into the sNAcc were blocked only partially by the highest dose of NTX injected bilaterally into the VTA. These results indicate opioid-opioid communication between the two sites; however, the communication may be quite indirect, requiring other sites and transmitters to elicit a change in behavior.

Keywords: Food intake, mesolimbic, microinjection, reward, opioid antagonist
1. Introduction

Food intake results from the behavioral output of a diverse network of sites that integrates metabolic, emotional, and hedonic demands for increased or decreased consumption. Several peptides and neurotransmitters are responsible for different aspects of this behavior and appear to exert their effects differently in various brain sites (15). Opioids are thought to prolong intake, particularly of highly palatable foods, but not to initiate intake (9, 10), and this is not based on post-absorptive signals (11, 12). Opioid antagonists have been shown to inhibit intake of highly preferred foods much more robustly than less preferred foods, strengthening the argument that opioids are involved in the response to the quality of the food, rather than to hunger (16, 29). Since opioids are also well known hedonic agents, their actions in brain reward systems have been examined with regard to food intake.

The VTA and the sNAcc are part of the mesolimbic Dopamine (DA) pathway that mediates responses to rewarding stimuli such as drugs of abuse, electrical stimulation, sex, and food intake (8). When opioids bind to their receptors in the VTA, they inhibit GABA-ergic inhibitory interneurons that synapse on DA cells projecting to the sNAcc. In this way, opioids injected or released into this site increase DA release in the sNAcc. This is thought to be part of the process that maintains food intake once it has been initiated (27). Indeed, DAMGO, a µ-opioid agonist, injected into the VTA stimulates food intake, and this intake can be blocked by injection of µ and κ, but not δ, antagonists just prior to DAMGO in the same site (13). Likewise, µ, δ-1, δ-2, and κ-1 antagonists, as well as non-selective antagonists similarly block DAMGO-induced feeding in the sNAcc
In addition, when DAMGO is injected into feeding-related regions of the NAcc, the VTA is one of the sites that showed increased c-Fos. DA D-1 and, to a lesser extent, D-2 antagonists injected prior to DAMGO in the sNAcc also block DAMGO-elicited food intake. The VTA and the sNAcc both contain enkephalin- and dynorphin-expressing cells and receive input from β-endorphin cells; the sources of dynorphin and enkephalin inputs are varied, and may include reciprocal connections between the two sites. While the dopaminergic connections are fairly well known in this region, opioid communication between the two sites is less well understood.

We hypothesized that feeding induced by microinjection of DAMGO is equally robust whether injected into the VTA or the sNAcc, and that communication between the two sites is required for this behavior change. More specifically, is food intake triggered by opioids in the VTA dependent on opioid signaling in the sNAcc, and vice versa? Or do opioids in these sites influence food intake independently of each other? In addition, we generated a dose-response curve for DAMGO in each site, to compare whether one site is more effective than the other at eliciting food intake.
2. Materials and Methods

Male Sprague-Dawley rats (Charles River, Wilmington, MD), weighing 225-250g were individually housed in conventional hanging cages with a 12h light/12h dark photoperiod (lights on at 0700) in a temperature-controlled room (21-22°C). Rats were anesthetized with sodium pentobarbital (60mg/kg) injected intraperitoneally and fitted with either unilateral or bilateral (2mm apart) 26 gauge stainless steel guide cannulae (Plastics One, Austin, TX) in the VTA and/or the sNAcc. Stereotaxic coordinates taken from the rat brain atlas by Paxinos and Watson(20) were as follows: VTA—1.0mm lateral, 5.0mm posterior to Bregma, and 7.1mm below the skull surface; sNAcc—1.0mm lateral, 1.7mm anterior to Bregma, and 6.4mm below the skull surface. The injector (33 gauge) extended 1 mm beyond the tip of the cannula, and the incisor bar was set at 3.3mm below the ear bars. Seven to ten days elapsed between surgery and the first injection. Injections were administered in volume of 0.5µL over a period of 30s, with a 30s wait before removing the injector. Naltrexone was injected just prior to DAMGO.

Food and water were present at all times, and food was weighed in the hopper just before injection and again 1, 2, and 4 hours later and subtracted from initial weight and spillage to calculate the amount eaten.

Experiment 1: Rats unilaterally cannulated in the VTA and the sNAcc were injected with four doses of DAMGO (0.1, 0.3,1, and 3 nmol) or saline into either of the two sites in a repeated-measures, counter-balanced design. In this way, every dose in each site was given each day, to eliminate day effects and to allow direct comparison of the strength of stimulation in each site within the same animal. Data were analyzed by a
two factor repeated measures ANOVA for site and dose and means were compared using Fisher’s Protected LSD test. Data are expressed as means ± S.E.M.

Experiment 2: Rats unilaterally cannulated in the VTA and the sNAcc were stimulated to eat with an injection of 5 nmol DAMGO in one site, preceded by injection of 25µg NTX in the opposite site. There were eight treatments, as follows: (a) VTA saline, sNAcc saline; (b) VTA NTX, sNAcc saline; (c) VTA saline, sNAcc NTX; (d) VTA DAMGO, sNAcc saline; (e) VTA DAMGO, sNAcc NTX; (f) VTA saline, sNAcc DAMGO; (g) VTA NTX, sNAcc DAMGO; and (h) VTA DAMGO, sNAcc DAMGO. Again, repeated-measures, counter-balanced design was used to eliminate day effects. Data were analyzed by a one factor repeated measures ANOVA and means were compared using Fisher’s Protected LSD test. Data are expressed as means ± S.E.M.

Experiment 3: Two groups of rats were used. One group was unilaterally cannulated in the VTA and bilaterally cannulated in the sNAcc, and the other group was bilaterally cannulated in the VTA and unilaterally cannulated in the sNAcc. Rats were stimulated to eat by injection of 5 nmol DAMGO into the unilaterally cannulated site, preceded by one of three doses of NTX (2.5, 5, and 25µg per side) or saline injected bilaterally in the opposite site. Data were analyzed by a one factor repeated measures ANOVA and means were compared using Fisher’s Protected LSD test. Data are expressed as means ± S.E.M. After all experiments were complete, brains were dissected and stored in a 10% formaldehyde solution for later placement verification by histological examination (Figure 1).
3. Results

DAMGO stimulated feeding in a dose-responsive manner after four hours, with the two highest doses in both sites and the third highest dose in the sNAcc increasing intake by more than 50% (Figure 2). After one hour (Fig. 2a), there appeared to be a main effect of site, but this was not significant \( [F_{(1,9)} = 4.61, P = 0.06] \). At this time point, there was not a significant effect of dose \( [F_{(4,36)} = 1.80, P = 0.15] \), but there was a significant site X dose interaction \( [F_{(4,36)} = 2.92, P = 0.03] \). After four hours (Fig. 2b), there was a main effect of dose \( [F_{(4,36)} = 5.12, P = 0.002] \), but there was no main effect of site \( [F_{(1,9)} = 0.33, P = 0.58] \) or interaction of site X dose \( [F_{(4,36)} = 0.40, P = 0.81] \).

In the second experiment, unilaterally cannulated animals were stimulated to eat with DAMGO (5nmol) injected into either site, and this was preceded by NTX (25µg/side) injected unilaterally into the opposite site. While there was a significant treatment effect \( [F_{(7,91)} = 4.54, P = 0.0002] \), NTX failed to block DAMGO-induced food intake in either direction (Fig.3). When DAMGO was injected into both sites, there was neither an additive nor a synergistic effect on intake.

In the third experiment, animals cannulated unilaterally in the VTA and bilaterally in the sNAcc were stimulated to eat with 5nmol DAMGO injected into the VTA. This DAMGO-induced feeding was significantly inhibited by all three doses of NTX injected bilaterally into the sNAcc during the 1-2 hour time period (Table 1) and by the two highest doses after four hours (Fig. 4a), (2.5 µg/side, \( p=0.16 \); 5µg/side, \( p=0.04 \), 25µg/side, \( p=0.01 \), all compared to DAMGO control). In addition, the two highest doses reduced intake to control level (2.5µg/side, \( p=0.026 \); 5µg/side, \( p=0.11 \); 25µg/side, \( p=0.29 \), compared to saline control at four hours).
Animals cannulated unilaterally in the sNAcc and bilaterally in the VTA were stimulated to eat with 5nmol DAMGO in the sNAcc, which was preceded by three doses of NTX in the VTA. After the first hour, intake was significantly inhibited by the highest and lowest doses of NTX, and intake was inhibited by all three doses during the 2-4 hour time period (Table 1). After 4 hours, at least two of the doses of NTX blocked intake significantly (2.5µg/side, p=0.01; 5µg/side, p=0.11; 25µg/side, p=0.001, compared to DAMGO control) but remained significantly increased above saline control levels (2.5µg/side, p=0.009; 5µg/side, p=0.0005; 25µg/side, p=0.001, compared to saline intake).
4. Discussion.

DAMGO, a μ-opioid agonist, has been injected into several brain sites to experimentally induce food intake. In this study, DAMGO was shown to dose-dependently increase food intake when injected into both the VTA and the sNAcc, as seen previously (1, 2). However, the repeated-measures design of this study allowed comparison of the strength of stimulation in each site within animals, rather than between two groups of differently cannulated animals. We showed that after one hour, in spite of the appearance of a trend, DAMGO injected into the sNAcc was not significantly more effective at eliciting intake than when injected into the VTA. After four hours, the effect was equal between the sites, and the two highest doses (1 and 3 nmol) were most effective in both sites. The middle dose (0.3 nmol) significantly increased intake when injected into the sNAcc, but not into the VTA, but this effect did not alter the significance of the site effect analysis.

Microinjection techniques are commonly used to determine the effects of drugs in individual brain sites. The results of studies with this technique give very specific information about each site, but do not address the question of how sites are interconnected. Since food intake is modulated by a number of sites, all of which appear to be part of a network, it is necessary to determine the nature of connections between these sites. Giraudo et al. (5) found that food intake elicited by DAMGO injected into either the nucleus of the solitary tract (NTS) or the central nucleus of the amygdala (CeA) was blocked by injection of NTX into the opposite site, indicating a bi-directional opioid signaling pathway. In another study, Giraudo et al. (4) studied the connection between the CeA and the paraventricular hypothalamic nucleus (PVN) using DAMGO and NTX.
While DAMGO increased intake when injected into either site, NTX blocked this intake only when injected into the PVN, indicating a unidirectional opioid connection in which CeA opioid transmission is not necessary for PVN opioid-induced intake, but PVN opioid transmission is required for CNA opioid-induced intake. This type of study can be used to determine similar connections between any of the sites that modulate food intake, including the VTA and the sNAcc.

The opioid-dopamine connection between the VTA and the sNAcc is well documented, as shown by microinjection/microdialysis studies in which µ-opioid agonists injected into the VTA cause an increase in DA release in the NAcc (18, 24, 26, 27). Since it is clear that there are dopaminergic cells projecting from the VTA to the sNAcc and that opioids in the VTA regulate DA release in the sNAcc, the question arises as to whether there is a bi-directional opioid communication pathway between these sites as well, as in the case of the CeA and PVN opioid connection in Giraudo’s study (4). Both sites contain opioid receptors and terminals (19) but it is not clear whether the terminals in one site belong to cells that originate in the opposite site or elsewhere.

Opioid antagonists injected into either site have been shown to block the feeding effect of opioid agonists in that same site (13, 17), but this has not been shown between the sites. We sought to determine whether the feeding elicited by injection of DAMGO into one site was dependent on opioid signaling in the opposite site. In the second experiment, we observed the interaction of a high dose of NTX in one site and a high dose of DAMGO in the opposite site, using the same unilaterally cannulated animals as described in the dose-response experiment. NTX injected unilaterally into either site was ineffective at blocking intake stimulated by DAMGO into the opposite site, presumably due to bilateral
communication that was not antagonized by unilateral injections of NTX. We also injected into both sites doses of DAMGO that had been shown to be effective in each site individually to determine whether this had synergistic or additive effects on intake and saw no change from single-site stimulation.

To address the issue of bilateral communication, the third experiment used three doses of NTX, injected bilaterally, to determine whether DAMGO-elicited food intake from one site was dependent on opioid signaling in the opposite site. It has been shown that NTX injected into each of these sites dose-dependently inhibits opioid-elicited food intake (13, 22), but this has not been done to assess the interaction between these two sites specifically. When NTX was injected bilaterally into the sNAcc, the two highest doses blocked VTA DAMGO-induced intake to control levels. However, in the reverse direction, VTA NTX blocked sNAcc DAMGO-induced intake only to an intermediate level, rather than to control levels. From these results, it may be concluded that bilateral sNAcc NTX injections are necessary to block VTA opioid-induced intake, and are sufficient at the higher doses to block intake to control levels. In fact, a recent study confirms this result with VTA DAMGO-induced feeding significantly reduced by NAcc mu, kappa, and delta opioid antagonists (14). In contrast, though bilateral NTX injections into the VTA are necessary to block sNAcc opioid-induced intake, even the highest dose is not sufficient to block intake to control levels. The unusual dose-response curve in this study appears to be due to an animal that ate just under the amount required to qualify as an outlier in the 5 µg dose of NTX.

The question arises as to whether the interactions seen in the present study are due to direct opioid communication between the sites or to indirect communication via other
sites and other neurotransmitters. It is possible that there are direct opioid connections between the two sites, since the NAcc contains pro-Enkephalin and pro-Dynorphin cell bodies and the VTA contains pro-Enkephalin bodies and terminals and pro-Dynorphin terminals(19). In the case of this study, then, opioid injection in one site may enhance release of opioids in the opposite site, which is effectively blocked by NTX in that site. Another possibility for the mechanism of the apparent opioid interdependence is communication via other neurotransmitters. Opioids appear to interact with GABA in eliciting food intake from both the VTA and the sNAcc (3, 32) and there are descending GABA-ergic pathways from the NAcc to the VTA directly (6, 7, 28) and indirectly via the hypothalamus (8). In fact, it has been shown that GABA-ergic cells in the NAcc, which project to the VTA and are inhibited by opioids in the NAcc, may decrease GABA release in the VTA, thereby creating a mechanism to explain NAcc opiate-triggered DA release from VTA cells (28, 30). In addition, the GABA cells from the NAcc that project to the hypothalamus may inhibit hypothalamic opioid cells that project to the VTA. Therefore, it is possible that the interactions seen between the two sites in this study are not due to direct opioid-opioid communication, but are indirectly modulated by the interaction of GABA and opioids within and between multiple sites. On the other hand, since opioids primarily exert their effects on food intake which has already been initiated (9, 10), the inhibition of NAcc DAMGO feeding by NTX in the VTA may simply be due to inhibition of continued food intake after the initial intake stimulated by DAMGO in the NAcc.

In summary, we found that DAMGO increases feeding to a similar extent when injected into the VTA and NAcc. We also noted that naltrexone injected into the sNAcc
decreased feeding stimulated by intra-VTA DAMGO administration. Injection of naltrexone into the VTA also decreased feeding stimulated by intra-sNAcc DAMGO, perhaps to a lesser extent than the opposite injection protocol. These results support the idea that opioids in the VTA and sNAcc modulate food intake and that communication between these two brain sites—either mono- or poly-synaptically—is required for such behavioral effects. Examining multi-site interactions, such as those described in the present study, will help elucidate the complex distributed neural network involved in feeding behavior.
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5. References


6. Figure Legends

Figure 1. Photomicrographs of representative sections with cannula tracks; A) nucleus accumbens shell region, B) ventral tegmental area. Abbreviations: AC = anterior commissure, AQ = cerebral aqueduct, cNAcc = core region of nucleus accumbens, CP = caudate and putamen, CT = cannula track, ML = medial lemniscus, SN = substantia nigra, VTA = ventral tegmental area.

Figure 2. Dose-response curve for DAMGO-induced food intake in the VTA and sNAcc. Black bars indicate a significant increase in intake above control levels (p<0.05). A) 0-1 hour intake. B) 0-4 hour intake.

Figure 3. Unilateral (ipsilateral) injection of 25 µg NTX and 5 nmol DAMGO. Notation for injection combination is in the form VTA/sNAcc, where s = saline, n = NTX, d = DAMGO. Black bars are significantly greater than both shaded and white bars (p<0.05), and shaded bars are significantly greater than white bars (p<0.05).

Figure 4. A) Bilateral NTX in sNAcc dose-dependently blocks intake stimulated by 5 nmol DAMGO in VTA [F(4,44) = 3.91, P = 0.008]. Black bars are significantly greater than white bars (p<0.05) and shaded bar is not different from black or white bars. B) Bilateral NTX in VTA partially blocks intake stimulated by 5 nmol DAMGO in sNAcc, but not to control levels [F(4,48) = 7.95, P < 0.0001]. Black bar is significantly greater than white bar (p<0.05) and lightly shaded bar (p<0.05); darkly shaded bar is not different from black or lightly shaded bars, but is significantly greater than white bar.
(p<0.05); lightly shaded bars are significantly greater than white bar. Abbreviations are as described in Figure 3; numbers indicate dose of NTX injected into each side of VTA.
Figure 1
Figure 2.
Figure 3
Figure 4.
Data are presented as means ± SE. *Significant (p<0.05) increase above saline controls. ‡Significant (p<0.05) inhibition of feeding compared to DAMGO controls. Treatments are expressed in the form VTA/sNAcc, where d=DAMGO, s=saline, n=NTX, and numbers indicate the dose of NTX.

Table 1. *Naltrexone inhibition of DAMGO-induced feeding from opposite site.*

<table>
<thead>
<tr>
<th>NTX site/dose</th>
<th>0-1 hour</th>
<th>1-2 hours</th>
<th>2-4 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>sNAcc (n=12)</td>
<td>$F_{(4,44)} = 0.96$ P = 0.44</td>
<td>$F_{(4,44)} = 4.02$ P = 0.007</td>
<td>$F_{(4,44)} = 1.52$ P = 0.21</td>
</tr>
<tr>
<td>s/s</td>
<td>0.25±0.10</td>
<td>0.17±0.05</td>
<td>0.13±0.10</td>
</tr>
<tr>
<td>d/s</td>
<td>0.40±0.17</td>
<td>1.30±0.30*</td>
<td>0.64±0.34</td>
</tr>
<tr>
<td>d/2.5n</td>
<td>0.35±0.13</td>
<td>0.47±0.15</td>
<td>0.83±0.28</td>
</tr>
<tr>
<td>d/5n</td>
<td>0.20±0.05</td>
<td>0.54±0.24</td>
<td>0.59±0.22</td>
</tr>
<tr>
<td>d/25n</td>
<td>0.16±0.08</td>
<td>0.54±0.27</td>
<td>0.37±0.14</td>
</tr>
</tbody>
</table>

| VTA (n=13)    | $F_{(4,48)} = 3.81$ P = 0.009 | $F_{(4,48)} = 1.50$ P = 0.22 | $F_{(4,48)} = 6.85$ P = 0.0002 |
| s/s           | 0.05±0.02          | 0.02±0.01          | 0.08±0.02          |
| s/d           | 0.81±0.27*         | 0.62±0.16          | 0.76±0.19*         |
| 2.5n/d        | 0.35±0.16          | 0.59±0.28          | 0.25±0.12          |
| 5n/d          | 0.53±0.23*         | 0.84±0.38          | 0.20±0.07          |
| 25n/d         | 0.29±0.16          | 0.45±0.27          | 0.15±0.06          |

Data are presented as means ± SE. *Significant (p<0.05) increase above saline controls. ‡Significant (p<0.05) inhibition of feeding compared to DAMGO controls. Treatments are expressed in the form VTA/sNAcc, where d=DAMGO, s=saline, n=NTX, and numbers indicate the dose of NTX.