Sucrose self-administration and CNS activation in the rat

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Figlewicz DP, Bennett-Jay JL, Kittleson S, Sipols AJ, Zavosh A. Sucrose self-administration and CNS activation in the rat. Am J Physiol Regul Integr Comp Physiol 300: R876 –R884, 2011. First published February 9, 2011; doi:10.1152/ajpregu.00655.2010.—We have previously reported that administration of insulin into the arcuate nucleus of the hypothalamus decreases motivation for sucrose, assessed by a self-administration task, in rats. Because the pattern of central nervous system (CNS) activation in association with sucrose self-administration has not been evaluated, in the present study, we measured expression of c-Fos as an index of neuronal activation. We trained rats to bar-press for sucrose, according to a fixed-ratio (FR) or progressive-ratio (PR) schedule and mapped expression of c-Fos immunoreactivity in the CNS, compared with c-Fos expression in handled controls. We observed a unique expression of c-Fos in the medial hypothalamus (the arcuate, paraventricular, retrochiasmatic, dorsomedial, and ventromedial nuclei) in association with the onset of PR performance, and expression of c-Fos in the lateral hypothalamus and the bed nucleus of stria terminalis in association with the onset of FR performance. c-Fos expression was increased in the nucleus accumbens of both FR and PR rats. Our study emphasizes the importance of both hypothalamic energy homeostasis circuitry and limbic circuitry in the performance of a food reward task. Given the role of the medial hypothalamus in regulation of energy balance, our study suggests that this circuitry may contribute to reward regulation within the larger context of energy homeostasis.

food reward; c-Fos; hypothalamus

THE MESOLIMBIC DOPAMINERGIC (DA) circuitry, including the ventral tegmental area (VTA) and projections to the striatum and cortical sites, has been identified as playing a critical role in the motivating or rewarding aspects of numerous classes of drugs of abuse (13, 22–24, 26, 48). Recent research from our laboratory and others suggests that this circuitry likewise plays a major role in the motivating or rewarding aspects of food. Functional and anatomical interaction with circuitry that regulates energy homeostasis is suggested by reports of the modulation of food reward by the nutritional status of animals (10, 14, 16, 43). Modulation of reward, including food reward, by nutritional or metabolic status, is strongly influenced by neural and endocrine signals, including insulin (15), leptin (11, 18, 21, 30, 32), ghrelin (35), melanin-concentrating hormone (MCH) (45), and orexin (5, 7): the presence of receptors, the biochemical and cellular efficacy, and the in vivo or behavioral efficacy of these signals in the central nervous system (CNS) have been abundantly demonstrated in recent years.

The extended limbic circuitry has likewise been shown to play a role in feeding and food reward (2, 19, 28). However, there are additional contributing CNS sites. Notably, the lateral hypothalamus (LH) has long been known to be a site mediating feeding and self-stimulation behaviors (4, 31). Orexigenic neurons and leptin signaling in the LH have been identified as important for feeding and food reward (5, 29, 30). We recently observed that insulin administered either into the third cerebral ventricle or into the arcuate nucleus of the hypothalamus (ARC) could decrease sucrose self-administration, but insulin administration into the VTA or nucleus accumbens had no effect on this specific reward paradigm (15). Thus, it appears that multiple hypothalamic sites may play a significant role in motivated food seeking and acquisition, and consistent with this, one would hypothesize that hypothalamic regions are substantially activated in association with food self-administration. To begin to test this hypothesis, we have mapped c-Fos expression in the CNS of rats trained in a sucrose self-administration paradigm, after fixed-ratios (FR) training, or after progressive ratios (PR) training, a more stringent task for assessing motivation (20).

MATERIALS AND METHODS

Subjects. Subjects were male Albino rats (325–425 g) from Simonsen (Gilroy, CA). Rats were maintained on Chow ad libitum. They were maintained on a 12:12-h light-dark cycle with lights on at 6 AM and were trained and tested between 7 AM and noon, in the postprandial and postabsorptive condition. All procedures performed on the rats followed the National Institutes of Health’s guidelines for animal care and were approved by the Animal Care and Use Subcommittee of the Research and Development Committee at the VA Puget Sound Health Care System.

Sucrose self-administration. Procedures were based upon our published methodology (15) and were carried out on fed rats. The experiment included three phases: autoshaping to initiate training, FR training, and progressive ratios (PR) training using the PR algorithm of Richardson and Roberts (38). The PR algorithm requires 1, 2, 4, 6, 9, 12, 16, 20, 28, 36, 48, 63, 83, 110, 145, 191, 251, 331, 437, 575, 759, 999, 999(etc) lever presses for succeeding reward deliveries within a session (38). Rats were trained to self-administer 5% sucrose (0.5 ml reward) delivered into a liquid drop receptacle. The operant boxes, controlled by a Med Associates (Georgia, VT) system, had two levers, but only one lever (an active, retractable lever) activated the infusion pump. Presses on the other lever (an inactive, stationary lever) were also recorded. As we have observed previously, the number of presses on the inactive lever was very low (less than 10 presses/session). The sucrose solution was delivered into a liquid drop receptacle for oral consumption (Med Associates, St. Albans, VT). Initial training was conducted during 1-h sessions under a continuous reinforcement schedule (FR1: each lever press was reinforced). Each session began with the insertion of the active lever and the illumination of a white house light that remained on for the entire session. A 5-s tone (2900 Hz, 20 dB above background) and light (7.5 W white light above the active lever) discrete compound cue accompanied each reward delivery, with a 20-s time out beginning with the sucrose delivery. FR training was carried out for 10 days; stable responding is...
achieved by the fifth session. PR training was carried out for a maximum possible 3 h/day for 10 days. PR sessions ended after 30 min of no active lever press responding, at which point the house light was turned off automatically and the active lever was retracted; rats were taken out of the chambers and returned to their home cages.

“Stop time” reported in Table 2 represents the time at which the system was turned off; therefore, the last active lever press would have occurred 30 min prior to the stop time. Behavioral data (Table 2) represent averages of sessions 6–10 for FR training, and sessions 1–9 for PR training. Control-handled rats were taken from the housing room and placed in a clean operant chamber with house light on for 60 min, within the procedure room, to simulate the handling and room experiences of the rats self-administering sucrose. They were not given anything to eat or drink while in the operant boxes, and had no access to levers.

On the final day, rats were placed in the chambers as per training days and were kept in the chambers for 90 min, after which they were removed, for anesthesia, perfusion, and subsequent immunohistochemistry. Control rats were likewise brought into the procedure room and kept in a clean operant chamber, as per training days, for 90 min, after which they were anesthetized and perfused. Immediately following that last 90-min session, rats were deeply anesthetized with isoflurane inhalation and perfused with 0.9% NaCl followed with cold 4% paraformaldehyde solution. The timing for anesthetic and euthanasia was based upon the known time course of peak expression of c-Fos protein at 90–120 min after the event. Thus, c-Fos expression would reflect the activation of the CNS at the onset of the behavioral task, rather than being the result of the animals’ experiencing the task and ingesting sucrose. Brains were removed and postfixed in paraformaldehyde for several days; then, they were subsequently placed in 20% sucrose-PBS, after which they were placed in 30% sucrose-PBS solutions. Brains were sectioned on a cryostat (Leica CM 3050S cryostat for immunohistochemistry.

**c-Fos immunohistochemistry and quantitation.** We used our established methodology to quantitate immunoreactive c-Fos protein in brain sections (12). Initial qualitative screen of the entire brain was conducted for c-Fos expression. Slide-mounted 12-μm whole-brain coronal sections were washed 3 times in PBS (Oxoid, Hampshire, UK). Sections were then blocked for 1 h at room temperature in PBS containing 5% normal goat or donkey serum. Sections were then washed multiple times in PBS and incubated overnight at 4°C in primary antibody solutions made up in PBS. Sections were washed three times in PBS and then incubated in the dark at room temperature in secondary antibody solution made up in PBS for 1 h. Sections were subsequently washed again in PBS and mounted and cover slipped in Vectashield hard set mounting medium (Vector Laboratories, Burlingame, CA) mounting medium. Digital images of sections were acquired using a Nikon Eclipse E-800 fluorescence microscope connected to an Optiphot camera and using Image Pro Plus (Media Cybernetics, Silver Spring, MD) software.

Subsequently, we focused upon a limited number of areas showing an apparent difference between conditions, for quantitation, and for neuronal phenotyping. Specifically, we focused upon nucleus accumbens core and shell (NAc); anterior and posterior bed nucleus of stria terminalis (aBNST, pBNST); medial hypothalamic regions [ventromedial nucleus (VMH), dorsomedial hypothalamus (DMH), paraventricular nucleus (PVN), retrosathmic area (RCh), and ARC]; lateral hypothalamus (LH), including dorsal and ventral regions and the perifornical (pef) area; VTA; brain stem [inferior olive, hypoglossal (nXII) nucleus of the solitary tract, lateral reticular nucleus, and C1/A1 adenine/noradrenaline nuclei]. Atlas-matched 12-μm sections were evaluated for c-Fos expression and quantitation in matched sections and regions, based upon the atlas of Paxinos and Watson (34). Please see Table 1 for specific stereotaxic coordinates. The primary focus of the assays was to compare each behavioral task with its respective control (PR vs. PRC; FR vs. FRC). To optimize possible differences based on behavior vs. control conditions, peak performers from the PR and FR groups were selected for analysis. Thus, 4/12 PR and 3/12 FR rats were analyzed: These rats had active lever press number (the primary behavioral endpoint) that was greater than one standard deviation above the mean for their respective behavioral group. A subcohort of the control rats (5 PRC and 3 FRC rats, present in the procedure room at the same time as the FR or PR rats) was also analyzed. An additional group of three rats was taken through the FR procedure (“FRext”) to mimic the added duration of the PR procedure (i.e., for a total of 20 days, as PR rats are taken through PR and then PR) to evaluate whether differences between FR and PR were due to the behavioral task or the duration of the procedure. The FRext brains were not analyzed and screened systematically, but specific regions of interest were assayed with the other four groups, to allow comparative quantitation, as indicated specifically in RESULTS.

For quantitation (at 40X magnification), atlas-matched regions were selected. ImagePro Plus software (Media Cybernetics) was utilized to capture an image of the desired area. An area was delineated for counting, and a threshold for positive cell counts was established. The identical area and background (threshold) were utilized for sections from the respective experimental groups, and software counting of positive cells (quantitation) was carried out in the same session for all experimental groups, to prevent between-session changes in background setting. For statistical analysis, counts were taken from an individual rat only if corresponding or complete sections through each area (as defined in Table 1) were available; data for a specific area were not taken from a rat if there were incomplete bilateral representation for that area.

**Qualitative double-labeled immunofluorescence analysis.** Brain sections were taken from the rats in which c-Fos was quantitated, for double-labeled immunohistochemistry. Because we did not wish to disturb the animals’ behavioral performance, they were not pretreated with colchicine in order to optimize visualization of peptide neuronal transmitters. Therefore, visualization of synaptic phenotypes activated in association with the self-administration task was limited. However, to begin the assessment of the phenotypes of activated neurons in a number of CNS locations, digital images (acquired as described in the section above) were taken at 20X, 40X, or 60X (as indicated in figure legends) magnification. The dual-staining procedure for glutamate decarboxylase (GAD), tyrosine hydroxylase (TH), CRF, neuropeptide Y (NPY), Agouti-related peptide (AgRP), and tryptophan hydroxylase was comparable to the assay of c-Fos-immu-
noreactivity on its own, except that a mixture of c-Fos-Ab and one of the other primary antibodies was used for overnight incubation at 4°C; likewise, both secondary antibodies were in the same solution and incubated for 1 h in the dark at room temperature. A 20-min 50% ethanol wash prior to the blocking step was utilized for the orexin assay. Initial optimization assays were carried out to determine an appropriate dilution of the primary antibodies. Primary antibodies used were rabbit anti-c-Fos (1:500) (sc-52) and mouse anti-c-Fos (1:800) (both from Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-GAD (1:1,000), mouse anti-tyrosine hydroxylase (1:500), and sheep anti-tryptophan hydroxylase (all from Chemicon, Temecula, CA); rabbit anti-CRF (1:500) (gift from Dr. Wylie Vale, Salk Institute, CA); rabbit anti-NPY (1:1,000), rabbit anti-AGRP (1:1,000), and mouse anti-GAD (1:1,000), mouse anti-tyrosine hydroxylase (1:500), used were rabbit anti-c-Fos (1:500) (sc-52) and mouse anti-c-Fos

Statistical analyses. Group data are presented as means ± SE in the text, tables, and figures. Significance is defined as P ≤ 0.05. Statistical comparisons are made between experimental groups (FR vs. PR) or between experimental groups and corresponding controls (FR vs. PR; FR vs. FRC; PR vs. FRC) using unpaired Student’s t-test. Pearson correlation coefficients between active lever presses and c-Fos expression in different brain regions, as well as correlation of c-Fos expression between various brain regions under identical experimental conditions, were calculated using the StatPlus:mac LE statistical analysis program for Mac OS version 2009 by AnalystSoft. We tested for linear correlations (Pearson’s r statistic) between c-Fos expression in different CNS regions. We also examined correlations between c-Fos expression in different activated CNS regions and behavior. FR and PR data from rats, for which c-Fos quantitation was carried out, were used for these correlations.

RESULTS

c-Fos quantitation. As we have observed previously, the number of active lever presses was significantly greater for FR vs. FR performance (Table 2), and the number of sucrose rewards was greater during FR performance. Session length for the PR rats was about 90 min (stop time – 30). Table 3 lists c-Fos immunoreactive cell counts in all CNS regions where quantitation was carried out. The pattern of c-Fos expression for the FR and PR rats is summarized in Fig. 1. There was significant activation of the medial hypothalamus (MHtot, a composite of ARC, PVN, RCh, DMH, and VMH) of rats engaged in PR lever pressing for sucrose, but no overall activation in rats engaged in FR lever pressing for sucrose, compared with respective controls. Within the medial hypothalamus of PR rats, this activation occurred in the PVN, ARC, and VMH (Fig. 2). FR lever pressing, but not PR lever pressing, was associated with significant activation within the LH (based predominantly on activation within the perifornical area). Both active lever presses and hypothalamic c-Fos expression were comparable between the FRext and FR groups (MHtot, 946 ± 26 and 911 ± 118; ARC, 176 ± 18 and 186 ± 10; LHtot, 468 ± 79 and 378 ± 34; LHpeF, 200 ± 31 and 173 ± 15, respectively), suggesting that the difference in expression pattern between FR and PR groups is related not to the duration of the training/experience but to the nature of instrumental task. For the FR group, there was a significant increase in c-Fos expression in the BNST, observed in both aBNST and pBNST. Both FR and PR lever pressing were associated with increased c-Fos-immunopositive neurons in the NAc shell; c-Fos counts were significantly increased in the NAc core from rats engaged in FR lever pressing, with a nonsignificant trend toward increased c-Fos expression in rats engaged in PR lever pressing. c-Fos was not increased in the VTA with the PR task, although a nonsignificant trend toward an increase was ob-

Table 2. Behavioral parameters for FR and PR rats

<table>
<thead>
<tr>
<th></th>
<th>Active Lever Presses</th>
<th>Sucrose Rewards*</th>
<th>Stop Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FR (n = 3)</td>
<td>60 ± 3</td>
<td>39.7 ± 1.2</td>
<td>N/A</td>
</tr>
<tr>
<td>PR (n = 4)</td>
<td>102 ± 13*</td>
<td>7.8 ± 0.4*</td>
<td>121 ± 6</td>
</tr>
</tbody>
</table>

*P < 0.05, Fixed ratio (FR) vs. progressive ratio (PR). N/A, not applicable.

Table 3. cFos Expression in the CNS

<table>
<thead>
<tr>
<th></th>
<th>FR</th>
<th>FR Control</th>
<th>PR</th>
<th>PR Control</th>
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</thead>
<tbody>
<tr>
<td>ARC</td>
<td>186 ± 10</td>
<td>180 ± 60</td>
<td>282 ± 31*</td>
<td>124 ± 19</td>
</tr>
<tr>
<td>PVN</td>
<td>308 ± 82</td>
<td>229 ± 49</td>
<td>227 ± 20*</td>
<td>136 ± 32</td>
</tr>
<tr>
<td>RCh</td>
<td>178 ± 2</td>
<td>24 ± 12</td>
<td>143 ± 38</td>
<td>84 ± 24</td>
</tr>
<tr>
<td>DMH</td>
<td>116 ± 27</td>
<td>213 ± 107</td>
<td>122 ± 28</td>
<td>159 ± 16</td>
</tr>
<tr>
<td>VMH</td>
<td>123 ± 13</td>
<td>258 ± 69</td>
<td>412 ± 19*</td>
<td>296 ± 34</td>
</tr>
<tr>
<td>MHtot</td>
<td>911 ± 118</td>
<td>903 ± 251</td>
<td>1235 ± 74*</td>
<td>783 ± 50</td>
</tr>
<tr>
<td>LHtot</td>
<td>378 ± 34*</td>
<td>215 ± 23</td>
<td>214 ± 15</td>
<td>194 ± 35</td>
</tr>
<tr>
<td>LHov</td>
<td>145 ± 37</td>
<td>70 ± 7</td>
<td>72 ± 12</td>
<td>63 ± 23</td>
</tr>
<tr>
<td>LHhyp</td>
<td>173 ± 15*</td>
<td>107 ± 12</td>
<td>80 ± 7</td>
<td>78 ± 9</td>
</tr>
<tr>
<td>NAc shell</td>
<td>254 ± 24*</td>
<td>114 ± 41</td>
<td>265 ± 27*</td>
<td>152 ± 13</td>
</tr>
<tr>
<td>NAc core</td>
<td>188 ± 9*</td>
<td>107 ± 15</td>
<td>151 ± 3</td>
<td>108 ± 21</td>
</tr>
<tr>
<td>VTA</td>
<td>208 ± 27</td>
<td>116 ± 40</td>
<td>38 ± 11</td>
<td>26 ± 2</td>
</tr>
<tr>
<td>aBNST</td>
<td>246 ± 69*</td>
<td>71 ± 10</td>
<td>75 ± 5</td>
<td>90 ± 12</td>
</tr>
<tr>
<td>pBNST</td>
<td>188 ± 5*</td>
<td>106 ± 14</td>
<td>44 ± 5</td>
<td>45 ± 9</td>
</tr>
<tr>
<td>n XII</td>
<td>121 ± 68</td>
<td>123 ± 35</td>
<td>221 ± 32*</td>
<td>79 ± 26</td>
</tr>
<tr>
<td>Inf. Olive</td>
<td>138 ± 71</td>
<td>165 ± 38</td>
<td>94 ± 34</td>
<td>141 ± 13</td>
</tr>
</tbody>
</table>

LHtot, lateral hypothalamus, total. *P < 0.05 vs. respective control (FR vs. FRC or PR vs. PRC).

Fig. 1. c-Fos immunopositive-cell counts in central nervous system (CNS) regions of fixed ratio (FR)- and progressive ratio (PR)-performing rats relative to handling controls. Cell counts for FR-control (FRC) and PR-control (PRC) were set to 100%. See Table 2 for raw data. Data are expressed as means ± SE. *P < 0.05 vs. corresponding FRC or PRC control groups.
served with the FR task. Finally, c-Fos was significantly increased in the hypoglossal (cranial nerve XII) nucleus in the brain stem of rats trained for PR, but not for FR.

C-Fos expression was observed in other CNS regions, including the amygdala and cerebral cortex (Fig. 3). However, expression was observed in both control conditions as well as in association with PR and FR tasks, suggesting that the nonspecific aspects of the procedure (handling, movement into the procedure room) might have resulted in this activation. Quantitation in these regions was not carried out. Likewise, activation within brain stem regions other than nXII was observed, but occurred in association with both control and task-related conditions, also suggesting a role in nonspecific arousal or behavioral activation.

We tested for correlations between c-Fos expression in different CNS regions. Combining data from lever-pressing groups, we found a negative correlation between c-Fos expression in the LH and the VMH; thus, activation of the VMH was associated with decreased overall activation of the LH (Pearson’s R, −0.7996; t = −3.7534; P = 0.0056). Also, we observed a significant positive correlation between c-Fos expression in the perifornical region of the LH and the VTA (Pearson’s R, 0.7772; t = 3.493; P = 0.0082), consistent with known monosynaptic connectivity between these two regions (see discussion in Refs. 2 and 13). We found a significant negative correlation between c-Fos expression in the VTA vs. the NAc-shell, whether tested separately for FR performance (Pearson’s R, −0.9262; t = −4.9125; P = 0.008) or for PR performance (Pearson’s R, −0.9897; t = −9.7624; P = 0.0103), consistent with known reciprocal inputs between striatal regions to the substantia nigra and VTA (2, 13). We also tested for correlations between c-Fos expression in different CNS regions, and behavior. Combining data from lever-pressing groups, we observed a significant positive correlation between c-Fos in the ARC, and active lever presses (Pearson’s R, 0.8208; t = 3.8017; P = 0.0067).

Identification of neurons activated with sucrose intake and motivation for sucrose. In the brain stem, c-Fos-positive neurons did not show positive immunostaining for TH, the rate-limiting enzyme for epinephrine and norepinephrine (and dopamine); thus, these catecholaminergic neurons did not appear to be activated by the FR or PR tasks. However, some c-Fos-positive neurons showed positive immunostaining for tryptophan hydroxylase, indicating that a population of serotonin neurons was activated. As shown in Fig. 4, in the ARC, c-Fos-positive cell bodies were surrounded by AGRP-stained fibers, and a similar pattern for NPY fiber/c-Fos immunostaining was observed (not shown). In the PVN, c-Fos-positive neurons appeared to surround CRF-positive neurons, but no colocalization was observed (data not shown). In Fig. 5 shows immunostaining for both orexin and MCH in the LH. Orexin neurons were found in both the dLH and peLH. Although we observed MCH-positive neurons in the peLH (Fig. 6, top), and very limited c-Fos colocalization with MCH in the vLH (Fig. 6, bottom). It should be reemphasized that both localization, and colocalization with c-Fos, may be underestimated for the peptide neurotransmitters such as CRH, because rats were not pretreated with colchicine. Finally, within the nucleus accumbens core and shell (Fig. 7), c-Fos coimmunostaining with GAD, the synthetic enzyme for the neurotransmitter GABA, was observed, for both FR and PR rats. There was robust staining for TH within the VTA; however, c-Fos-positive neurons were rarely observed and did not appear to exclusively colocalize with TH.

DISCUSSION

In the current study, we used expression of the immediate early gene, c-Fos, to evaluate the pattern of acute CNS activation associated with the onset of sucrose self-administration lever pressing activity, either as a relatively undemanding task (FR) or a progressively more challenging task thought to reflect motivated seeking of a reward, such as sucrose, and to strongly involve limbic circuitry (22, 24, 49) (PR). Hypothalamic patterns of activation differed between the two tasks, with LH/limbic activation predominating in the FR task and medial hypothalamic/limbic activation predominating in the PR task (see Fig. 1). There are several possible reasons for this. First, these paradigms might “map” as qualitatively different expe-
periences in the CNS. Rats trained in FR performance would be expecting an easy, high-reward activity. Anticipation of a rewarding food should heavily influence the c-Fos pattern observed in the FR rats. The apparent qualitative difference in activation pattern suggests that a second possibility—that the PR animals simply have more experience with the task—is less likely, and this was supported by our measurement of c-Fos in the hypothalamus of rats that received 20 FR sessions, which showed activity similar to the FR group, not the PR group. Both of these possibilities might be tested by systematically increasing the difficulty of FR training and evaluating changes in CNS activation, in which case, one would predict a qualitative change in activation pattern. However, whereas number of training experiences might not account for the CNS activation pattern, the average number of sucrose rewards in a session might: the PR task might simply be learned as a “less rewarding” experience, and this might be functionally linked with the lack of LH activation. Thus, the CNS activation pattern at the start of the session might reflect an interoceptive state, such as that of the conditioned place paradigm: strength of activation within limbic circuitry is tied to learning and to motivation. We did observe variability of c-Fos expression in the medial hypothalamus of the FRC animals. Particularly within the PVN, this variability might be masking activation in the FR rats, for which a trend toward increased c-Fos vs. FRC rats was observed (Table 3). However, overall medial hypothalamic activation did not differ between FR and FRC animals.

It should be noted that although our goal was to identify CNS sites that contribute to the onset of behavior, temporal resolution is somewhat of a consideration. As discussed below, it is now appreciated that different subcomponents of instrumental or operant behaviors are mediated by activation of different populations of neurons (13, 23, 33, 49). We cannot completely rule out that activation due to very immediate bar-pressing or licking of rewards might have contributed
somewhat to the activation patterns that we observed. Our findings do provide the basis for further investigation into the roles of specific CNS sites in different aspects or components of the self-administration task, and for such studies, measurement of other immediate early genes with different “on” and “off” timecourses (36) will be very useful.

The correlations we found in c-Fos expression between different brain regions support the known functional connectivity of hypothalamic and primary limbic regions for this particular reward task, such as between the LH and the VMH, and between the perifornical region of the LH and the VTA (see discussion in Refs. 2 and 13). We also examined correla-

![c-Fos colocalization](image1)

**Fig. 6.** c-Fos colocalization in an FR rat with orexin in the perifornical LH (AP −3.3) (**top**) and with MCH in the vLH (−AP-3.0) (**bottom**). ×40 magnification.

![GAD and c-Fos co-localization](image2)

**Fig. 7.** Colocalization of immunostaining for GAD (green) and c-Fos (red) in the nucleus accumbens core (**top**) and shell (**bottom**).
tions between c-Fos expression in different activated regions, and behavior. The correlation between c-Fos in the ARC and active lever presses fits with the well-defined role of ARC activity in food intake (1); with our previous observation that insulin injection specifically into the ARC decreased sucrose self-administration (15); with prior reports of the critical role of the ARC, and its endorphinergic neurons, in the acquisition and performance of cocaine self-administration (40–42); and with the identified projections from the ARC to the NAc (17). Thus, the ARC likely plays a key role in the motivated behavior to seek and obtain many types of rewarding stimuli, including, but not limited to, food. Finally, we observed significant activation of the PVN and VMH with the onset of PR sucrose-seeking. This is consistent with the well-characterized roles of these medial hypothalamic nuclei in the regulation of food intake, direct synaptic connectivity with the ARC, and identified connections with the limbic circuitry (1, 27, 37).

We found a significant negative correlation between c-Fos expression in the VTA vs. the NAc-shell, whether tested for FR or PR performance. It was somewhat surprising that stronger VTA activity was not observed in association with PR or FR sucrose self-administration (vs. respective controls). Perhaps this finding reflects the timing of our measurement, focusing on potential CNS sites active at the onset of the task, for which these animals were well trained. This would be consistent with the observations and thesis of Schultz (44), that dopamine neuronal activation serves as a marker of unexpected stimuli or rewards, and this activation decreases in association with training. However, striatal dopamine release during sucrose-taking in trained animals has been shown to occur as a very precise and temporally discrete event (39). Thus, it is possible that the trends that we observed would be strongly significant with a larger study group (i.e., more statistical power). We did observe NAc activation in association with onset of both FR and PR sucrose taking. Both activation and inhibition of NAC neurons have been reported in association with instrumental reward performance, and the pattern of activation/activity is dependent upon training and environment, and is associated with different components of the behavior (e.g., orienting, approach, intake) (6, 33, 50). As discussed above, measurement of c-Fos would not capture such specific activity. Carlezon has proposed that “reward” is predominantly associated with a decrease in activity of the NAc neurons, i.e., medium spiny neurons (3). This is not consistent with our observations—substantially enhanced NAc c-Fos compared with handling controls and c-Fos-positive neurons colocalized with GAD, consistent with activation of medium spiny neurons (GABAergic)—but we have not specifically assessed NAc neuronal “inhibition”. NAc activation and inhibition may both occur during instrumental tasks, with both anatomical and temporal specificity. From the perspective of this study, one may conclude that the NAc is involved in the onset of instrumental sucrose-taking, with the NAc core contributing to motoric activation and the NAc shell contributing to both motor and motivational aspects of the task.

We also observed activation of both major regions of the BNST (anterior and posterior) in FR rats. The BNST is a portion of limbic circuitry that modulates neuroendocrine responses to repeated stimulus experiences (8, 9), and in a larger sense, is associated with the learning about recurrent stimuli. Although its role has been elucidated most comprehensively in relation to repeated stressor experiences, our finding suggests a broader role for the BNST: The BNST may modulate CNS responses to recurring positive, as well as negative or stressful stimuli. Since we observed this activation at the onset of FR, but not PR, performance, BNST recruitment may be tied to the increased sucrose rewards of FR training. Our observation of no direct activation of CRF neurons suggests that instrumental responding for sucrose is not a major stressor; however, c-Fos expression in other PVN neurons is consistent with modulation of stress circuitry (46). In fact, Ulrich-Lai and colleagues have reported that, using a different diet/feeding paradigm, sucrose intake modulates PVN function (47). Finally, we observed activation of the nucleus of the hypoglossal nerve in association with PR but not FR performance. The significance of this can only be speculated upon; one possibility is that the taste relevance of sucrose may be heightened in rats that ingest fewer sucrose rewards.

Sucrose-seeking and sucrose-taking should be considered as a multimodality experience, dynamic in time, as ingestion would result in peripheral signals related to the caloric content of the sucrose, as well as habituation and within-session alliesthesia (25). While our research has focused upon the influence of peripheral endocrine signals, i.e., insulin and leptin, to modulate food reward, their effects may, in turn, be directly mediated centrally by transmitters and neuropeptides that play a role in short- or long-term feeding or food reward (see discussion in Ref. 14). The current study provides some insight into this; we observed some activation of neurons that express either MCH or orexin, two neuropeptides that are orexigenic. These findings may, in fact, underestimate the role of MCH or orexin in food reward, as immunocytochemistry in non-colchicine-treated rats no doubt limited the visualization of both of these neuropeptides. The identification of activated orexin neurons in the LH is consistent overall with the numerous studies implicating orexin neurons in feeding, food reward, and more generalized stimulus reward (e.g., 5, 7, 29). We observed activation of peFLH orexin neurons. Aston-Jones and colleagues (5) have dissected the roles of different populations of LH orexin neurons in reward behavior and have implicated peFLH orexin neurons in arousal, as opposed to reward per se. Our finding, thus, suggests a role for LH orexin in arousal, and perhaps orientation toward the active lever or cues for sucrose-taking.

Worthy of future consideration is the uniqueness or generalizability of sucrose as a rewarding stimulus. Whether the pattern of early CNS activation we report here is specific for food as a stimulus, or generalizes to other rewarding stimuli, remains to be determined. As alluded to above, particularly in the FR task, ingestion of a number of sucrose rewards would be expected to have metabolic consequences, with modulation of hormone release (for example, cholecystokinin, ghrelin, insulin, ghrelin) and changes in peripheral and CNS neural activation. These changes would not be expected to play a direct role in the early CNS activation patterns that we measured but may play a role in the learning about sucrose reward during training. Again, neuropeptides such as orexin may be critically implicated.

Our study represents, to our knowledge, the first demonstration of activation of specific medial hypothalamic nuclei at the onset of sucrose self-administration, including both the PVN, implicated in homeostasis and stress responsivity, and the
ARC, which is critical for energy homeostasis, nutrient sensing, and regulation of food intake. Importantly, we observed activation of the medial hypothalamus and the NAc, in association with PR onset, suggesting that both homeostatic and some limbic sites play a role in the initiation of sucrose self-administration. Additional limbic circuitry sites may be recruited at a later timepoint in the task.

**Perspectives and Significance**

Whereas, historically, studies of motivational and reward behaviors would most strongly implicate CNS limbic circuitry, a large body of evidence has accrued that emphasizes the critical functional interaction between limbic and energy homeostasis circuitry. The current study now suggests the likely importance of specific medial hypothalamic nuclei in motivated work for sucrose. Extrapolating from this study, future studies can evaluate whether the role of the medial hypothalamus is requisite and whether its activation is implicated in motivated seeking of other rewards such as drugs of abuse. Additionally, the findings of this study provide the rationale for studying alterations of motivated behaviors in circumstances concomitant with altered medial hypothalamic physiology, such as in obesity.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

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