

Video Article

Simultaneous Detection of c-Fos Activation from Mesolimbic and Mesocortical Dopamine Reward Sites Following Naive Sugar and Fat Ingestion in Rats

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Abstract

This study uses cellular c-fos activation to assess effects of novel ingestion of fat and sugar on brain dopamine (DA) pathways in rats. Intakes of sugars and fats are mediated by their innate attractions as well as learned preferences. Brain dopamine, especially meso-limbic and meso-cortical projections from the ventral tegmental area (VTA), has been implicated in both of these unlearned and learned responses. The concept of distributed brain networks, wherein several sites and transmitter/peptide systems interact, has been proposed to mediate palatable food intake, but there is limited evidence empirically demonstrating such actions. Thus, sugar intake elicits DA release and increases c-fos-like immunoreactivity (FLI) from individual VTA DA projection zones including the nucleus accumbens (NAC), amygdala (AMY) and medial prefrontal cortex (mPFC) as well as the dorsal striatum. Further, central administration of selective DA receptor antagonists into these sites differentially reduce acquisition and expression of conditioned flavor preferences elicited by sugars or fats. One approach by which to determine whether these sites interacted as a distributed brain network in response to sugar or fat intake would be to simultaneously evaluate whether the VTA and its major mesotelencephalic DA projection zones (prelimbic and infralimbic mPFC, core and shell of the NAC, basolateral and central-cortico-medial AMY) as well as the dorsal striatum would display coordinated and simultaneous FLI activation after oral, unconditioned intake of corn oil (3.5%), glucose (8%), fructose (8%) and saccharin (0.2%) solutions. This approach is a successful first step in identifying the feasibility of using cellular c-fos activation simultaneously across relevant brain sites to study reward-related learning in ingestion of palatable food in rodents.

Video Link

The video component of this article can be found at <https://www.jove.com/video/53897/>

Introduction

Brain dopamine (DA) has been implicated in central responses to intake of palatable sugars through proposed hedonic^{1,2}, effort-related³ and habit-based^{4,5} mechanisms of action. The primary DA pathway implicated in these effects originates in the ventral tegmental area (VTA), and projects to the nucleus accumbens (NAC) core and shell, the basolateral and central-cortico-medial amygdala (AMY), and the prelimbic and infralimbic medial prefrontal cortex (mPFC) (see reviews^{6,7}). The VTA has been implicated in sucrose intake^{8,9}, and DA release is observed following sugar intake in the NAC¹⁰⁻¹⁵, AMY^{16,17} and mPFC¹⁸⁻²⁰. Fat intake also stimulates DA NAC release²¹, and another DA-rich projection zone to the dorsal striatum (caudate-putamen) has been also associated with DA-mediated feeding^{22,23}. Kelley²⁴⁻²⁷ proposed that these multiple projection zones of this DA-mediated system formed an integrated and interactive distributed brain network through extensive and intimate interconnections²⁸⁻³⁴.

In addition to the ability of DA D1 and D2 receptor antagonists to reduce intake of sugars³⁵⁻³⁷ and fats³⁸⁻⁴⁰, DA signaling has also been implicated in mediating the ability of sugars and fats to produce conditioned flavor preferences (CFP)⁴¹⁻⁴⁶. Microinjections of a DA D1 receptor antagonist into the NAC, AMY or mPFC⁴⁷⁻⁴⁹ eliminate acquisition of CFP elicited by intragastric glucose. Whereas microinjections of either DA D1 or D2 receptor antagonists into the mPFC eliminates acquisition of fructose-CFP⁵⁰, the acquisition and expression of fructose-CFP are differentially blocked by DA antagonists in the NAC and AMY^{51,52}.

The c-fos technique^{53,54} has been employed to investigate neural activation induced by palatable intake and neural activation. The term "c-fos activation" will be used throughout the manuscript, and is operationally defined by increased transcription of c-Fos during neuronal depolarization. Sucrose intake increased fos-like immunoreactivity (FLI) in the central AMY nucleus, the VTA as well as the shell, but not core, of the NAC⁵⁵⁻⁵⁷. Whereas sucrose intake in sham-feeding rats significantly increased FLI in the AMY and the NAC, but not the VTA⁵⁸, intragastric sucrose or glucose infusions significantly increased FLI in the NAC and central and basolateral nuclei of the AMY^{59,60}. Repeated addition of sucrose to scheduled chow access increased FLI in the mPFC as well as the NAC shell and core⁶¹. A sucrose concentration downshift paradigm

revealed that the greatest FLI increases occurred in the basolateral AMY and NAC, but not the VTA⁶². Following conditioning, extinction of sugar-related natural reward behaviors increased FLI in the basolateral AMY and the NAC⁶³. Moreover, pairing sugar availability to a tone resulted in the tone subsequently increasing FLI levels in the basolateral AMY⁶⁴. High-fat intake also increased FLI in NAC and mPFC sites⁶⁵⁻⁶⁷.

Most of the previously cited studies examined sugar and fat effects on c-fos activation in single sites that do not provide information about identification of reward-related distributed brain networks²⁴⁻²⁷. Further, many of the studies also did not delineate the relative contributions of sub-areas of the NAC (core and shell), AMY (basolateral and central-cortico-medial) and mPFC (prelimbic and infralimbic) that could potentially be examined by the advantage of excellent spatial, single-cell resolution in c-Fos mapping⁶⁸. Our laboratory⁶⁹ recently used c-fos activation and simultaneously measured alterations in the VTA DA pathway and its projection zones (NAC, AMY and mPFC) after novel ingestion of fats and sugars in rats. The present study describes the procedural and methodological steps to simultaneously analyze whether acute exposure to six different solutions (corn oil, glucose, fructose, saccharin, water and a fat emulsion control) would differentially activate FLI in sub-areas of the NAC, AMY, mPFC as well as the dorsal striatum. This simultaneous detection of differences allowed confirmation of significant effects on FLI in each site and determination as to whether changes in one particular site correlated with changes in related sites, thereby providing support for a distributed brain network²⁴⁻²⁷. These procedures tested whether the VTA, the prelimbic and infralimbic mPFC, the core and shell of the NAC, and the basolateral and central-cortico-medial AMY as well as the dorsal striatum would display coordinated and simultaneous FLI activation after oral, unconditioned intake of glucose (8%), fructose (8%), corn oil (3.5%) and saccharin (0.2%) solutions.

Protocol

These experimental protocols have been approved by the Institutional Animal Care and Use Committee certifying that all subjects and procedures are in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

1. Subjects

1. Purchase and/or breed male Sprague-Dawley rats (260 - 300 g).
2. House rats individually in wire mesh cages. Maintain them on a 12:12 hr light/dark cycle with rat chow and water available *ad libitum*.
3. Assign appropriate sample sizes (e.g., $n \approx 6 - 8$) randomly into groups.

2. Testing Apparatus and Intake Procedures

1. Use calibrated centrifuge tubes with rubber stoppers and a 45° angle metal sipper tube to provide accurate measurement (± 0.1 ml) of the presented solutions. Secure them to the home cages by a taut metal spring to allow visibility of the calibrations.
2. Restrict food rations (~ 15 g/day) of the rats to reduce weight to 85% of their original body weight to increase motivation to consume the solutions. Note: The weight reduction should take between 3 - 5 days.
3. Provide pre-training solutions (10 ml) of 0.2% saccharin for four days over a 1 hr session to maximize the probability that rats will sample the subsequent test solutions with short (less than 1 min) latency.
4. Confirm flow through the centrifuge tube by spilling a few drops.
5. Weigh tubes before and after each session to obtain intake measurement.
6. Perform an intake test on the fifth day on subgroups receiving one of six solutions (10 ml, 1 hr): a) water, b) novel flavored (0.05% cherry flavor) 0.2% saccharin, c) 8% fructose, d) 8% glucose, e) 3.5% corn oil suspended in 0.3% xanthan gum, and f) 0.3% xanthan gum.
7. Ensure that the nutrient solutions are isocaloric; thus, the 3.5% corn-oil concentration is isocaloric to the 8% sugar solutions.
8. Ensure that rats sample solutions with short latency (less than 1 min). If this requirement is not met, then discard the subject from the study.

3. Tissue Preparation

1. Anesthetize each animal by an intraperitoneal injection of pentobarbital 90 min after initial exposure to each test solution. Confirm that animals are properly anesthetized by demonstrating that the animal is no longer responsive to such reflexes as withdrawal to foot pinch, blinking following direct corneal pressure or head shaking to deep pinna stimulation.
2. Perfuse each animal transcardially as described previously⁶⁹.
 1. Anesthetize rats with an overdose of sodium pentobarbital (65 mg/kg), remove the ribcage and expose the chest for free access to the heart⁶⁹.
 2. Place the needle in the apex of the left heart valve, and cut the vena cava. Administer phosphate buffer solution (PBS, ~ 180 ml) followed by a phosphate-buffered fixative containing 4% paraformaldehyde (~ 180 ml).
 3. Ensure that the animal actually is being perfused correctly by examining whether liquid is leaving other cavities, such as the nose, mouth, and genital areas. Note: Proper fixation with paraformaldehyde will be accompanied by large muscle movements. If this does not occur, re-adjust the needle until this reaction occurs.
3. Remove the brain from the skull quickly by cutting fur and skin away from the skull. Use rongeurs to crack and remove the bone from the brain moving from rear to front. Work initially in the area below and behind the cerebellum, ensuring that the rongeur is between the bone and meningeal pia mater. Once the top and sides of the skull are removed, use a small spatula to lift the brain from the base, and snip cranial nerves with small scissors. Be careful not to damage the brain while attempting to remove the bone.
4. Fix the brains in a 4% paraformaldehyde solution overnight at 4 °C. Place the brains in a 30% sucrose/70% PBS solution at room temperature until they settle at the bottom of the container.
5. Block the brain
 1. Remove the rostral part of the brain cutting transversely caudal to the olfactory bulb.
 2. Remove the caudal part of the brain cutting transversely at the level of the cerebellum and the pons.

6. Mount the brain coronally with the caudal part fixed to the stage of a sliding microtome, and cut coronal sections (40 μ m) through the mPFC (+2.86 – +2.20 mm rostral to bregma), the NAC core and shell and dorsal striatum (+1.76 – +1.60 mm rostral to bregma), the AMY (-2.12 – -2.92 mm caudal to bregma), and the VTA (-5.20 – -5.60 mm caudal to bregma). Use a rat brain atlas⁷⁰ for guidance.
7. Collect free-floating sections into individual wells of a 24-well plate filled with PBS for eventual immunohistochemical analysis⁷¹. Use Parafilm to seal the 24 well plate to make sure the PBS does not evaporate in the container and dry up the brain. Store the brain tissue in 4 °C.

4. c-fos Procedures (Adapted from ⁷¹)

1. Treat each section with 5 ml of 5% Normal Goat Serum and 0.2% Triton x-100 in PBS for 1 hr.
2. Incubate the treated sections with primary antibodies (rabbit anti-c-fos, 1:5,000) at 4 °C for 36 hr in wells containing 1 ml of PBS.
3. Rinse sections 3x with PBS (5 ml) for 10 min each.
4. Incubate with secondary antibodies (biotinylated goat anti-rabbit; 1:200) at RT for 2 hr in wells containing 1 ml of PBS.
5. Rinse each section 3x in PBS (5 ml) for 10 min each.
6. Incubate the rinsed sections for 2 hr in a commercially available avidin-horseradish peroxidase mixture that comes in a kit composed of Avidin DH (100 μ l) and Biotinylated Horseradish Peroxidase H (100 μ l) in 5 ml of PBS.
7. Re-rinse the sections 3x in PBS (5 ml) for 10 min each.
8. React the sections with 0.05% diaminobenzidine (DAB) in the presence of 0.0015% H₂O₂ for 5 - 10 min, depending on the reactivity of the tissue in wells containing 5 ml of the DAB solution.
9. Double-label the VTA sections. Incubate them with a tyrosine hydroxylase (TH) antibody (rabbit anti-rat TH, 1:2,000) in PBS (5 ml) overnight at 4 °C.
10. Rinse sections 3x in PBS (5 ml) for 10 min each.
11. Incubate with secondary antibodies (biotinylated goat anti-rabbit; 1:200) in PBS (5 ml) at room temperature for 2 hr.
12. Rinse sections 3x in PBS (5 ml) for 10 min each.
13. Visualize the antibodies by using a secondary antibody-peroxidase complex. React with a combination of 0.05% DAB and a 0.3% nickel sulfate solution, for 5 - 10 min, depending on the reaction of the tissue in wells containing 5 ml of the DAB/NiCl solution.
14. Ensure that the DAB solution is milky light green in color from its reaction with the 0.3% nickel sulfate. If the solution is too green, then the reaction will be too dark.
15. Mount all sections onto gelatin-coated slides. Let them dry overnight, and then cover-slip with a few drops of a Toluene-Based Solution (TBS).
16. Code slides so that the experimental condition is unknown to the observers.

5. Determination of c-fos Immunoreactive Counts

1. Assign pairs of unbiased observers to count Fos-positive neurons in these regions of interest (ROI): prelimbic mPFC, infralimbic mPFC, NAC core, NAC shell, basolateral AMY nuclei, central-cortico-medial AMY, dorsal striatum, and VTA. Delineate whether c-Fos immunoreactivity was present in TH+ and TH- cells in the VTA. **Figure 1** provides a screen-captured picture of the NAC from the microscope.

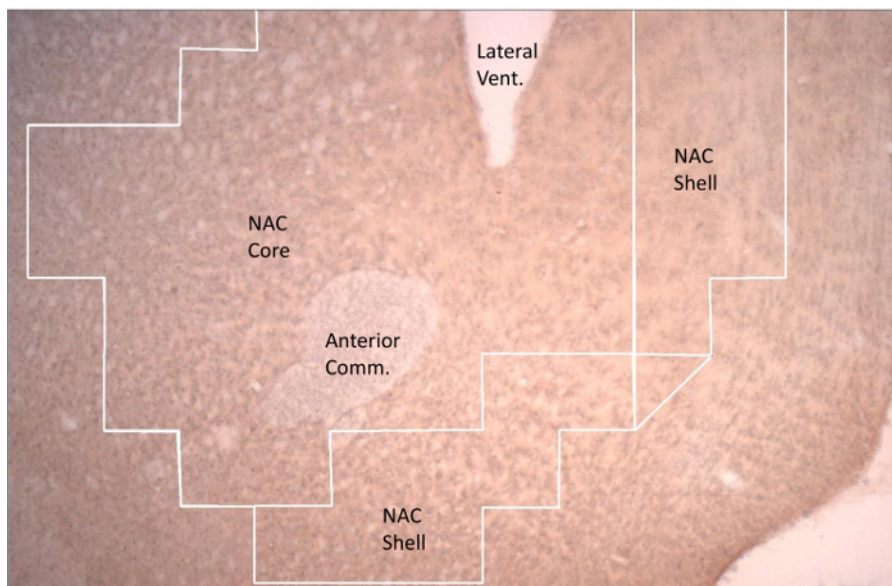


Figure 1. Representative Section of the Nucleus Accumbens (NAC) Displaying Regions of Interest Outlined by the Grid by which c-fos Counts are Made. The NAC shell is found medially and ventrally to the NAC core. The NAC core encircles the anterior commissure (Anterior Comm.). The ventral extent of the lateral ventricle (Lateral Vent.) is visible. [Please click here to view a larger version of this figure.](#)

2. Analyze at least three representative slices per site common to all animals in all of the testing conditions.
3. Use software and an optical microscope to analyze the entire region for each ROI by tracing an outline (**Figure 1**).
 1. For a given site, open the application and click on the acquisition drop-down menu and click "Live Image". Bring the ROI into focus and click the screen to establish a reference point. Then trace the chosen brain region using the grid as a guide. Once the trace is completed, count cells (steps 5.3.1.1 - 5.3.1.3).

1. Double-click the software icon. Go to the menu bar, click on "Acquisition" and then "Live Image". Bring the ROI into focus and click the screen to establish a reference point.
2. Go to the grid toolbar and click "Display Grid" and "Use grid labels". Outline the ROI with a predetermined trace.
3. Count all cells in each ROI area, select a "+" in the left hand sidebar to keep count of c-fos cells. Click each cell singly to register counts. Consider a cell positive for c-fos when a defined dark red circle is observed (**Figure 1**).

2. Repeat this process for each site.

3. Record counts in a lab notebook and on the computer for future analysis. Go to the menu bar, click on "File", "Save Data File" to save the tracing and counts.

4. Ensure that inter-rater reliability (using correlation of counts) of the two uninformed raters for each section in each ROI always exceeds 0.8.

6. Statistics

1. Evaluate baseline saccharin intakes over the first four days using a repeated-measures 1-way analysis of variance (ANOVA) comparing the saccharin intakes of Days 1, 2, 3 and 4⁶⁹.
2. Compare saccharin intake (Day 4) with test intakes (Day 5) of the six groups using a randomized-block 2-way ANOVA⁶⁹.
3. Use Tukey comparisons ($p < 0.05$) to determine individual significant effects⁶⁹.
4. Determine inter-rater reliability, and then use a common observer's counts.
5. Average c-fos counts for the three representative slices for each site⁶⁹.
 1. Perform a 1-way ANOVA of c-fos activation induced by intake of the six solutions (3.5% corn oil, 8% glucose, 8% fructose, 0.2% flavored saccharin, xanthan gum control and water) for the perirhinal mPFC⁶⁹.
 2. Repeat parallel analyses of the six groups for infralimbic mPFC, NAC core, NAC shell, basolateral AMY, central-cortico-medial AMY, VTA and dorsal striatum. Use Tukey comparisons ($p < 0.05$) to reveal individual significant effects⁶⁹.
6. Compare corn oil intake with both water intake and intake of its suspension agent, xanthan gum. Compare fructose and glucose intakes with both water intake and intake of the non-nutritive sweetener, saccharin.
7. Establish whether significant relationships between solution intakes and c-fos activation in each of the sites were observed using Bonferroni r correlations ($p < 0.05$).
 1. Systematically compare c-fos counts in the perirhinal and infralimbic pre-frontal cortex for each animal in the 3.5% corn oil group.
 2. Repeat systematically parallel analyses of each pair of the six sites (VTA, dorsal striatum, infralimbic mPFC, perirhinal mPFC, NAC core, NAC shell, basolateral AMY, central-cortico-medial AMY) for the 3.5% corn oil.
 3. Repeat systematically parallel analyses of these six sites for the other five experimental intake conditions (8% glucose, 8% fructose, 0.2% flavored saccharin, xanthan gum control and water).
8. Take advantage of the fact that the same animals within a solution condition were evaluated across all sites by determining significant relationships between c-fos activation across solutions and within each solution using Bonferroni r correlations ($p < 0.05$).

Representative Results

All representative results described below have been published previously⁶⁹, and are re-presented here to support "proof of concept" in indicating the effectiveness of the technique.

Solution Intakes

Significant differences in baseline saccharin intakes were observed over the first four days for all animals ($F(3,108) = 57.27$, $p < 0.001$) with intakes (Day 1: $1.3 (\pm 0.2)$ ml; Day 2: $3.9 (\pm 0.4)$ ml; Day 3: $5.9 (\pm 0.6)$ ml; Day 4: $7.1 (\pm 0.6)$ ml) significantly ($p < 0.05$, Tukey HSD test) and progressively increasing. Fructose and glucose intake, but not corn oil or saccharin intake on Day 5 significantly ($p < 0.05$, Tukey HSD test) increased relative to Day 4 saccharin intake ($p < 0.05$, Tukey HSD test) with fructose ($9.6 (\pm 0.4)$ ml) and glucose ($9.4 (\pm 0.6)$ ml) significantly higher than saccharin intake. Further, corn oil intake ($7.4 (\pm 0.6)$ ml) was significantly ($p < 0.05$, Tukey HSD test) higher than xanthan gum intake.

These results raised the possibility that solution intake per se might account for any observed c-fos activation in any of the sites. To examine this, Bonferroni r correlations were performed in which intake of the five solutions was related to c-fos activation in each of the six sites. Significant correlations failed to be observed between solution intake and c-fos activation in the core ($r(29) = 0.186$), shell ($r(29) = 0.029$) or total ($r(29) = 0.10$) NAc, the prelimbic ($r(29) = 0.23$), infralimbic ($r(29) = 0.30$) or total ($r(29) = 0.14$) mPFC, the VTA ($r(29) = 0.10$), the dorsal striatum ($r(29) = 0.14$) or the basolateral ($r(29) = 0.47$), the centro-cortico-medial ($r(29) = 0.48$) or total ($r(29) = 0.409$) AMY. Given the higher correlation between intake and AMY c-fos activation, further correlations were performed for each individual solution. Significant ($p < 0.05$, Tukey HSD test) relationships failed to be observed between intake and AMY FLI for fructose (basolateral ($r = 0.15$), centro-cortico-medial ($r = 0.13$), total ($r = 0.13$)), glucose (basolateral ($r = 0.17$), centro-cortico-medial ($r = 0.17$), total ($r = 0.13$)), saccharin (basolateral ($r = 0.42$), centro-cortico-medial ($r = 0.42$), total ($r = 0.42$)) or corn oil (basolateral ($r = 0.54$), centro-cortico-medial ($r = 0.59$), total ($r = 0.64$)). A significant ($p < 0.05$, Tukey HSD test) negative correlation was observed between xanthan gum intake and total AMY FLI ($r = 0.94$).

mPFC c-Fos Activation

Corn oil significantly ($p < 0.05$, Tukey HSD test) increased total (**Figure 2A**), infralimbic (**Figure 2B**) and prelimbic (**Figure 2C**) mPFC c-Fos counts relative to water (*) or the xanthan gum control (#). Fructose significantly ($p < 0.05$, Tukey HSD test) increased c-Fos counts in the infralimbic mPFC relative to water (*) or saccharin (+) (**Figure 2B**), but not total or prelimbic mPFC counts. In contrast, glucose or saccharin failed to alter total, perirhinal or infralimbic mPFC c-Fos counts. **Figure 3** displays representative mPFC sections of animals showing increased corn oil-induced FLI relative to water.

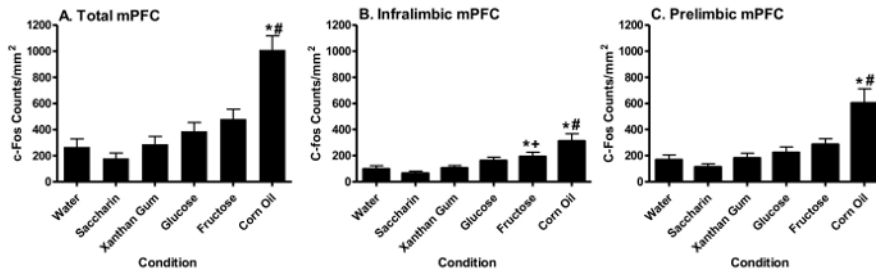


Figure 2. Fat or Sugar Intake Differentially Increases c-fos Activation in the Medial Prefrontal Cortex (mPFC). Changes in c-fos activation (mean \pm S.E.M.) are noted in the entire mPFC (**Panel A**), the infralimbic mPFC area (**Panel B**), and the prelimbic mPFC area (**Panel C**) following consumption (1 hr) of water, saccharin (0.2%), xanthan gum (control for corn oil), glucose (8%), fructose (8%) or corn oil (3.5%). (Previously published⁶⁹.) [Please click here to view a larger version of this figure.](#)

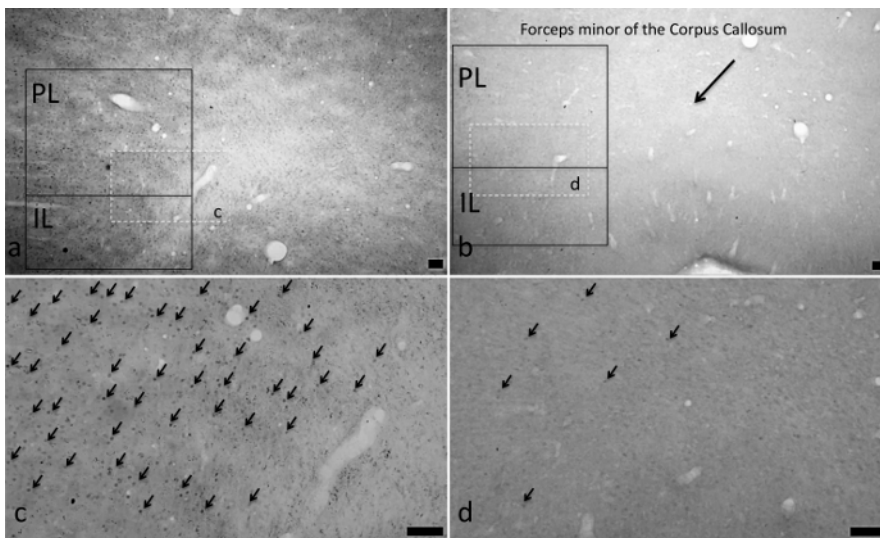


Figure 3. Actual mPFC c-fos Activation Following Fats and Sugars. C-fos activation was observed in animals exposed to intake of corn oil (**Panels A** (4-fold magnification) and **C** (10-fold magnification)) that was significantly greater than that of intake of water (**Panels B** (4-fold magnification) and **D** (10-fold magnification)). Representation of the delineated sub-areas of the Prelimbic (PL) and infralimbic (IL) mPFC are diagrammed in Panels A (corn oil) and C (water) as are the part of those panels (**A and C**) magnified to 10-fold magnification in the corresponding panels (**B and D**). Arrows in Panels C and D indicate representative c-fos positive cells. All scale bars are 100 μ m. (Previously published⁶⁹.) [Please click here to view a larger version of this figure.](#)

AMY c-Fos Activation

Corn oil significantly ($p < 0.05$, Tukey HSD test) increased total AMY (**Figure 4A**), basolateral (**Figure 4B**) and central-cortico-medial (**Figure 4C**) sub-area AMY c-Fos counts relative to water (*) or the xanthan gum control (#). Glucose also significantly ($p < 0.05$, Tukey HSD test) increased total (**Figure 4A**), basolateral (**Figure 4B**) and central-cortico-medial (**Figure 4C**) sub-area AMY c-Fos counts relative to water (*) or saccharin (+). Glucose and fructose significantly ($p < 0.05$, Tukey HSD test) increased AMY FLI relative to saccharin or water. Fructose significantly ($p < 0.05$, Tukey HSD test) increased total (**Figure 4A**) and central-cortico-medial sub-area (**Figure 4C**) of the AMY c-Fos counts relative to water (*) or saccharin (+), but not in the basolateral AMY sub-area. Saccharin failed to alter total, basolateral or central-cortico-medial AMY c-Fos counts relative to water. Detailed analyses of individual nuclei within the AMY revealed that the significant changes noted in the basolateral area of the AMY were also noted in the individual basolateral and lateral AMY nuclei. The significant changes noted in the central-cortico-medial area of the AMY were also noted in the individual central, cortical and medial AMY nuclei. **Figure 5** displays representative AMY sections of animals showing increased corn oil-, glucose-, and fructose-induced FLI relative to water. (Previously published⁶⁹.)

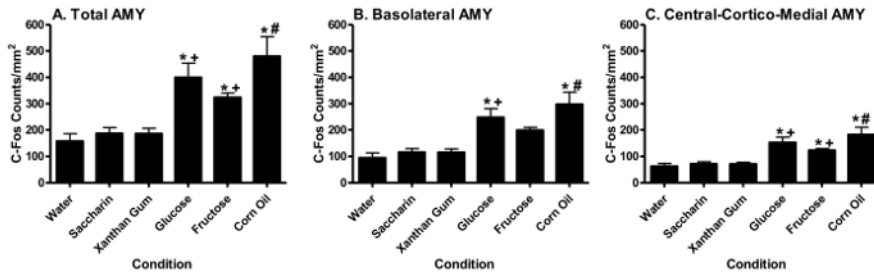


Figure 4. Fat or Sugar Intake Differentially Increases c-fos Activation in the Amygdala (AMY). Changes in c-fos activation (mean \pm S.E.M.) are noted in the entire AMY (**Panel A**), the basolateral AMY area (**Panel B**), and the central-cortico-medial AMY area (**Panel C**) following consumption (1 hr) of water, saccharin, xanthan gum, glucose, fructose or corn oil. The significant changes noted in the basolateral area of the AMY were also noted in the individual basolateral and lateral AMY nuclei. The significant changes noted in the central-cortico-medial area of the AMY were also noted in the individual central, cortical and medial AMY nuclei. (Previously published⁶⁹.) [Please click here to view a larger version of this figure.](#)

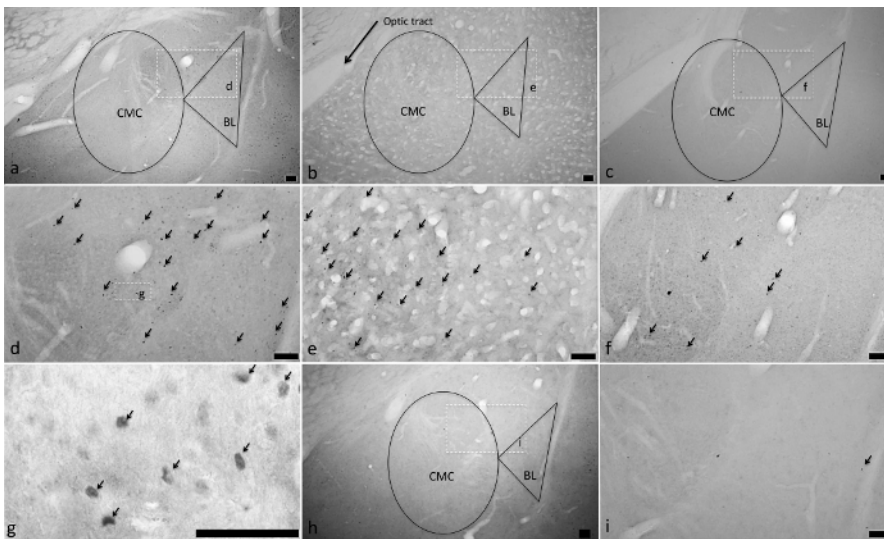


Figure 5. Actual AMY c-fos Activation Following Fats and Sugars. C-fos activation was observed in animals exposed to intakes of corn oil (**Panels A** (4-fold magnification), **D** (10-fold magnification) and **G** (60-fold magnification)), glucose (**Panels B** (4-fold magnification) and **E** (10-fold magnification)), and fructose (**Panels C** (4-fold magnification) and **F** (10-fold)) that were significantly greater than that of intake of water (**Panels H** (4-fold magnification) and **I** (10-fold magnification)). Representation of the delineated sub-areas of the central-cortico-medial (CMC) and the basolateral (BLA) AMY are diagrammed in Panels A (corn oil), B (glucose), C (fructose) and H (water) as are the part of those panels (**A**, **B**, **C** and **H**) magnified to 10-fold magnification in the corresponding panels (**D**, **E**, **F** and **I**). The delineated sub-area in Panel D (corn oil, 10-fold magnification) is magnified to 60-fold magnification in Panel G. Arrows in Panels D, E, F, G and I indicate representative c-fos positive cells. All scale bars are 100 μ m, except for Panel G (50 μ m). (Previously published⁶⁹.) [Please click here to view a larger version of this figure.](#)

NAC c-Fos Activation

Corn oil significantly ($p < 0.05$, Tukey HSD test) increased total (**Figure 6A**) and core (**Figure 6B**) NAC c-Fos counts relative to water (*), but not the NAC shell (**Figure 6C**). Glucose significantly ($p < 0.05$, Tukey HSD test) increased c-Fos counts in the NAC core (**Figure 6B**), but not in the total NAC or the NAC shell relative to saccharin (+) or water (*). In contrast, fructose and saccharin failed to differ from water in eliciting c-Fos activation in the NAC core and/or shell. **Figure 7** displays representative sections in the NAC core of animals showing increased corn oil- or glucose-induced FLI relative to water.

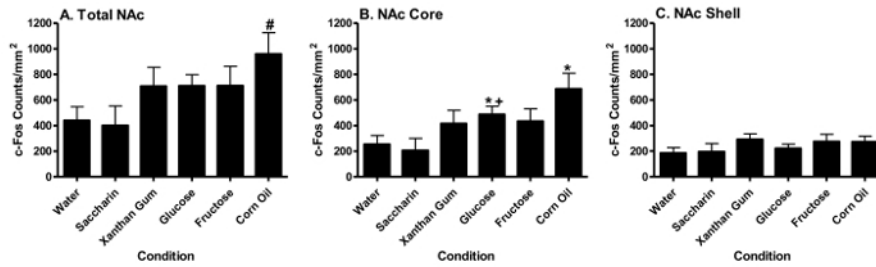


Figure 6. Fat or Sugar Intake Differentially Increases c-fos Activation in the NAC. Changes in c-fos activation (mean ± S.E.M.) in the entire NAC (**Panel A**), the NAC core (**Panel B**), and the NAC shell (**Panel C**) following consumption (1 hr) of water, saccharin, xanthan gum, glucose, fructose or corn oil. (Previously published⁶⁹.) [Please click here to view a larger version of this figure.](#)

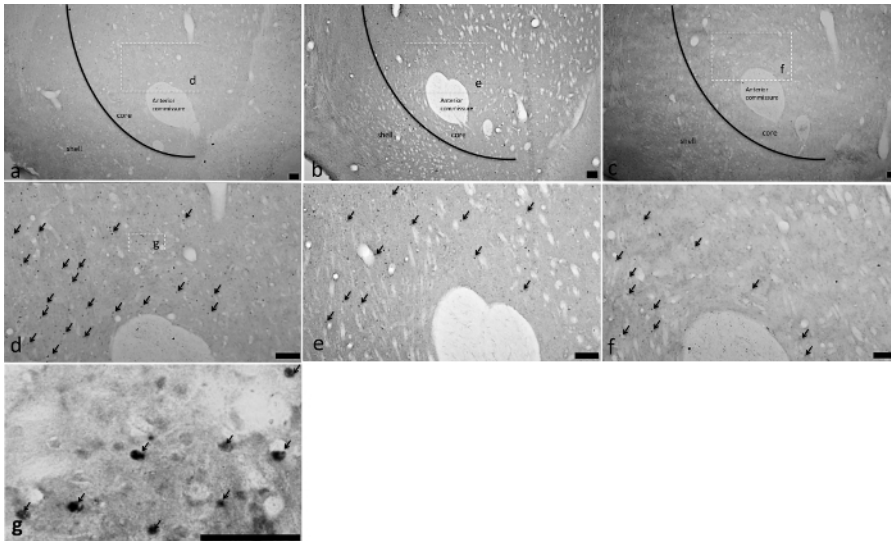


Figure 7. Actual NAC Core, but not NAC Shell c-fos Activation Following Fats and Sugars. C-fos activation was observed in animals exposed to intakes of corn oil (**Panels A** (4-fold magnification), **D** (10-fold magnification) and **G** (60-fold magnification)) and glucose (**Panels B** (4-fold magnification) and **E** (10-fold magnification)) that were significantly greater than that of intake of water (**Panels C** (4-fold magnification) and **F** (10-fold magnification)). Representation of the delineated sub-areas of the NAC core and the NAC shell are diagramed in Panels A (corn oil), B (glucose) and C (water) as are the part of those panels (**A, B, C and H**) magnified to 10-fold magnification in the corresponding panels (**D, E and F**). The delineated sub-area in Panel D (corn oil, 10-fold magnification) is magnified to 60-fold magnification in Panel G. Arrows in Panels D, E, F and G indicate representative c-fos positive cells. All scale bars are 100 µm. (Previously published⁶⁹.) [Please click here to view a larger version of this figure.](#)

Dorsal Striatal c-Fos Activation

Corn oil significantly ($p < 0.05$, Tukey HSD test) increased c-Fos counts in the dorsal striatum relative to water (*) or the xanthan gum (#) (**Figure 8A**). Glucose or fructose significantly ($p < 0.05$, Tukey HSD test) increased dorsal striatal FLI relative to saccharin (+) (**Figure 8A**). In contrast, saccharin failed to differ from water in eliciting dorsal striatal c-Fos activation. **Figure 9** displays representative dorsal striatal sections of animals showing increased corn oil-, glucose- or fructose-induced FLI relative to water.

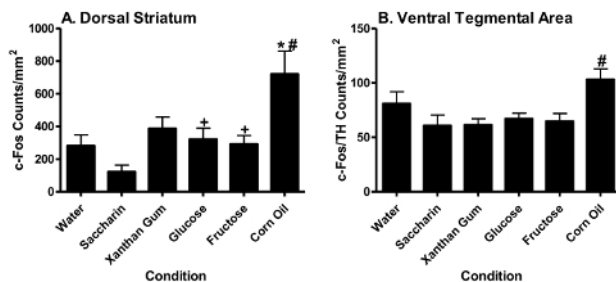


Figure 8. Fat or Sugar Intake Differentially Increases c-fos Activation in the Dorsal Striatum and Ventral Tegmental Area. Dorsal striatal (**Panel A**) and ventral tegmental area (**Panel B**) alterations were noted for c-fos activation (mean ± S.E.M.) following consumption (1 hr) of water, saccharin, xanthan gum, glucose, fructose or corn oil. (Previously published⁶⁹.) [Please click here to view a larger version of this figure.](#)

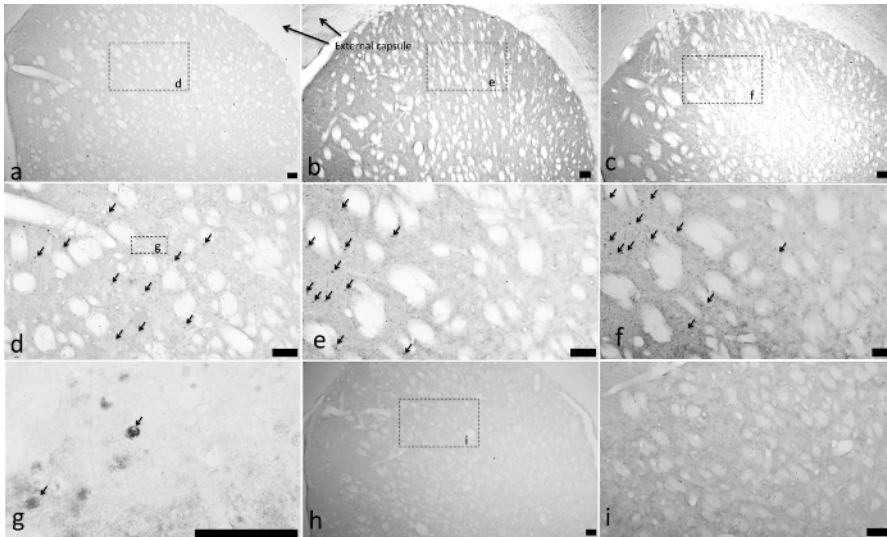


Figure 9. Actual Dorsal Striatal c-fos Activation Following Fats and Sugars. C-fos activation was observed in animals exposed to intakes of corn oil (**Panels A** (4-fold magnification), **D** (10-fold magnification) and **G** (60-fold magnification)), glucose (**Panels B** (4-fold magnification) and **E** (10-fold magnification)), fructose (**Panels C** (4-fold magnification) and **F** (10-fold magnification)) that were significantly greater than that of intake of water (**Panels H** (4-fold magnification) and **I** (10-fold magnification)). Delineated sub-areas of the dorsal striatum in Panels A (corn oil), B (glucose), C (fructose) and H (water) are indicated that magnified to 10-fold magnification in the corresponding panels (D, E, F and I). The delineated sub-area in Panel D (corn oil, 10-fold magnification) is magnified to 60-fold magnification in Panel G. Arrows in Panels D, E, F and G indicate representative c-fos positive cells. All scale bars are 100 μ m, except for Panel G (50 μ m). (Previously published⁶⁹.) [Please click here to view a larger version of this figure.](#)

VTA c-Fos Activation

Corn oil significantly ($p < 0.05$, Tukey HSD test) increased c-Fos counts in TH+ VTA cells relative to the xanthan gum control (#) (**Figure 8B**). In contrast, glucose, fructose or saccharin failed to alter c-Fos counts in the VTA relative to water. **Figure 10** displays representative TH+ and TH- and c-Fos-activated VTA cells of animals showing increased corn oil-induced FLI relative to water.

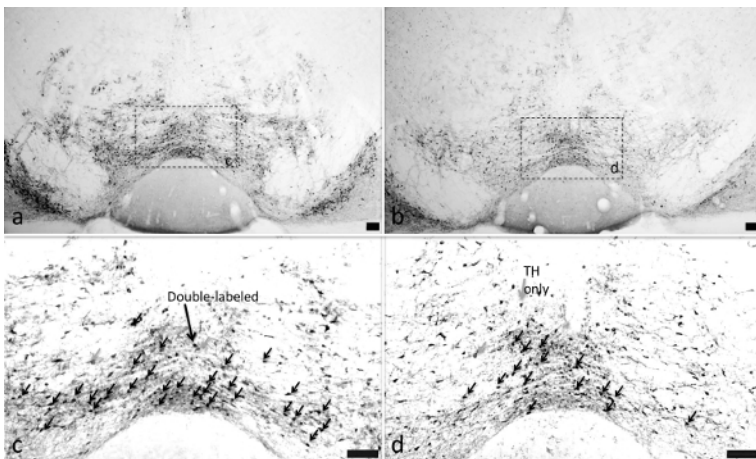


Figure 10. Actual Ventral Tegmental Area c-fos Activation Following Fats and Sugars. VTA c-fos activation was observed in animals exposed to corn oil (**Panels A** (4-fold) and **C** (10-fold)) and water (**Panels B** (4-fold) and **D** (10-fold)). Black arrows indicate representative double-labeled TH/c-fos positive cells, while gray arrows indicate representative c-fos only cells. All scale bars are 100 μ m. (Previously published⁶⁹.) [Please click here to view a larger version of this figure.](#)

Relationships of c-Fos Activation Among Sites and Solutions

The pattern of c-Fos counts in animals exposed to *corn oil* revealed significant ($p < 0.05$) positive correlations between the NAc core and either the NAc shell ($r = 0.971$) or entire mPFC ($r = 0.670$), between the prelimbic mPFC and either the infralimbic mPFC ($r = 0.940$) or dorsal striatum ($r = 0.849$), between the infralimbic mPFC and dorsal striatum ($r = 0.749$), between the basolateral and central-cortico-medial AMY ($r = 0.999$), and between the dorsal striatum and the VTA ($r = 0.723$). In contrast, the pattern of c-Fos counts in animals exposed to *corn oil* revealed significant ($p < 0.05$) negative correlations between the basolateral AMY and either the NAc core ($r = -0.712$) or shell ($r = -0.708$), and between the central-cortico-medial AMY and either the NAc core ($r = -0.712$) or shell ($r = -0.710$). The pattern of c-Fos counts in animals exposed to *glucose* revealed significant ($p < 0.05$) positive correlations between the prelimbic and infralimbic mPFC ($r = 0.930$), between the dorsal striatum and either the VTA ($r = 0.821$), basolateral ($r = 0.910$) or central-cortico-medial ($r = 0.911$) AMY, and between the basolateral and central-cortico-medial ($r = 0.999$) AMY. The pattern of c-Fos counts in animals exposed to *fructose* revealed significant ($p < 0.05$) positive correlations between the NAc core and either the NAc shell ($r = 0.969$) or prelimbic mPFC ($r = 0.740$), between the NAc shell and the prelimbic mPFC ($r = 0.733$), between the prelimbic and infralimbic mPFC ($r = 0.959$), and between the basolateral and central-cortico-medial AMY ($r = 0.996$). The pattern of c-Fos counts in animals exposed to *saccharin* revealed significant ($p < 0.05$) positive correlations between the NAc core and NAc shell ($r = 0.792$), between the NAc shell and dorsal striatum ($r = 0.715$), and between the prelimbic mPFC and infralimbic mPFC ($r = 0.999$).

Discussion

The goal of the study was to determine if the source (VTA) and forebrain projection targets (NAc, AMY, mPFC) of DA reward-related neurons were simultaneously activated after novel ingestion of fat and sugar in rats using the cellular c-fos technique. The present study is a detailed description of the protocols of a study published previously⁶⁹. It was hypothesized that the VTA, its major projection zones to the prelimbic and infralimbic mPFC, the core and shell of the NAc and the basolateral and central-cortico-medial AMY, as well as the dorsal striatum would act as a distributed brain network²⁴⁻²⁷, and display coordinated and simultaneous FLI following novel intake of glucose (8%), fructose (8%) or corn oil (3.5%) solutions relative to saccharin (0.2%), water and other control solutions. Corn oil, glucose and fructose, but not saccharin intake produced significant and differential FLI activation of the VTA, the prelimbic and infralimbic mPFC, the core and shell of the NAc, the basolateral and central-cortico-medial AMY, and the dorsal striatum. In addition to the c-fos technique, behavioral measures of sugar, fat and artificial sweetener intake were employed.

One critical step included timely sampling of intake such that they would be comparatively equal, thereby ensuring that any differences in c-fos activation across sites were due to the solution being consumed rather than the pattern or magnitude of intake. The four days of baseline saccharin intake ensured that the food-restricted animals sampled the solutions quickly, and thereby minimized non-specific effects. A second critical step was that the procedure caused minimal stress or novelty to the animals as changes in emotional valence independent of intake type could also produce c-fos activation. Therefore, the findings provide a convincing "proof of concept" for the effectiveness of this approach and protocols related to identify whether acute exposure to fat (e.g., corn oil), sugar (glucose and fructose) and non-nutritive sweetener (saccharin) solutions simultaneously activate DA-mediated ROI's in manner suggestive of a coordinated distributed brain system²⁴⁻²⁷.

Because optimal c-Fos activation requires time-sensitive responses prior to sacrifice^{51,52}, previously validated procedures^{42,44} maximized solution sampling with short latency in the 1-hr test. Thus, food restricted rats were trained with 0.2% saccharin solutions (10 ml, 1 hr) for 4 days, and given the test solution on the fifth day. Baseline saccharin intakes significantly and progressively increased, and fructose and glucose, but not corn oil or saccharin intakes on the fifth day were significantly higher than fourth day saccharin intake. Hence, solutions associated with increased FLI significantly increased (glucose, fructose) or failed to affect (corn oil) intake relative to previous saccharin training, and appeared to be mediated through a reward-related behavioral incentive mechanism. Careful consideration needs to be taken to ensure sampling and equality of behavior. Other researchers can effectively use this procedure to study other types of novel solutions or introduce variations in the paradigm to understand mechanisms related to adaptation and learning.

The advantage of the present protocol is the ability to compare effects of well-studied sugars (fructose, glucose) and fats (corn oil) and compare their c-fos activating effects with than of important controls (the non-nutritive sweetener, saccharin, a control emulsifier, xanthan gum, and water), and then examine these effects across six related brain sites. Although this approach has obvious benefits in allowing simultaneous examination across brain sites of the different palatable substances, it has the drawback of producing a potentially astronomical data set of cells displaying neuronal expression. To make this more manageable, we took the approach of analyzing three representative coronal slices per site common to all animals in all of the testing conditions. This of course is accompanied by the caveat of choosing the appropriate levels of each ROI in these three sections. Given the broad rostro-caudal extent of the AMY, NAc, mPFC, dorsal striatum and VTA, this caveat should not be taken lightly. Further, it is then incumbent upon the investigators to be consistent in accurately selecting each of the three representative sections across all animals across all sites. Minor mistakes in this choice can lead to "false positives" and "false negatives". Efficiency of counting also is a relevant variable. Our solution for this potential confound was to assign two uninformed raters for each section in each ROI, and then ensure that inter-rater reliability (using correlation of counts) always exceeded 0.8. This approach, while duplicative, gave us far greater assurance about accuracy as the inter-rater reliability easily exceeded this minimum criterion. Sub-regions of the NAc (core vs. shell), AMY (baso-lateral vs. central-cortico-medial) and mPFC (perilimbic vs. infralimbic) were analyzed. These regions could be divided further, particularly the individual AMY nuclei, the patch and matrix compartments of the dorsal striatum, and the NAc shell (vertex, arch, cone, intermediate zone). Because the NAc shell failed to consistently display changes in FLI following corn oil, glucose or fructose, further sub-analyses of this structure was not performed. Definitive examination of the patch and matrix zones of the dorsal striatum required further immunohistochemical techniques that were not employed in the present study, but would be an important follow-up study. Analyses of individual AMY nuclei within each sub-region would also be an additional future study.

Previous studies showed that sucrose intake increased FLI in the central AMY nucleus, the VTA as well as the shell, but not core, of the NAc, yet oral or IG saccharin infusions are largely ineffective^{55-57, 60-62}. Glucose and fructose intake elicited sugar-specific effects upon FLI with both effective in the central-cortico-medial AMY and dorsal striatum, the former effective in the NAc core and the basolateral AMY, and the latter effective in the infralimbic mPFC. Saccharin intake failed to elicit any changes in FLI in any site relative to water. Fat intake also increased FLI in

accumbal and mPFC sites in previous studies⁶⁵⁻⁶⁷, and produced simultaneous significant activation in the VTA, infralimbic and prelimbic mPFC, dorsal striatum, NAC core, and the basolateral and central-cortico-medial AMY.

Although previous studies demonstrated that sugar and fat intake induced FLI in forebrain meso-corticolimbic and nigro-striatal DA systems, the present study systematically evaluated simultaneous FLI activation in the VTA, basolateral and central-cortico-medial AMY, dorsal striatum, prelimbic and infralimbic mPFC, NAc core and shell following acute intake of corn oil, fructose, glucose or saccharin. Significant FLI increases were highly related to each other across forebrain sites, supporting the idea of distributed brain network activation mediating sugar and fat intake. Such protocols identifying simultaneous changes in multiple brain loci can be utilized under chronic and binge conditions as well as under conditioning and preferences. These studies show that a strong anatomical correlate (c-fos) can be effectively used in multiple brain sites simultaneously to identify candidates for mediating palatable intake and preferences in animals that may provide insights into human medical conditions related to obesity, diabetes and other eating disorders.

Disclosures

The authors have no competing financial interests.

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