Differences in BOLD Responses to Intragastrically Infused Glucose and Saccharin in Rats

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Abstract

The postingestive effect is different between caloric and noncaloric sweeteners. The gut administration of glucose induces a preference for flavored water which is paired with the intragastric infusion of glucose. However, a comparison of the brain response to the gut glucose and saccharin stimuli still remains to be demonstrated. Here, using functional magnetic resonance imaging, we investigated the blood oxygenation level-dependent signal response to gut glucose and saccharin in the brain of conscious rats. Glucose induced a positive signal increase in the amygdala and nucleus accumbens, both of which receive dopaminergic input from the ventral tegmental area. In contrast, saccharin administration did not activate these areas. Both glucose and saccharin increased the blood oxygenation level-dependent signal intensity in the insular cortex and the nucleus of the solitary tract. These results show that there were significant differences between postingestive glucose and saccharin-induced increases in the blood oxygenation level-dependent signal in rats. Together with previous findings, these results suggest distinct activation patterns in the brain for both glucose and saccharin, which is partially due to different changes of internal signals, including the blood glucose and insulin levels.

Key words: functional MRI, glucose, nucleus accumbens, rat, saccharin

Introduction

It is important for organisms to maintain their internal nutrient conditions by regulating the intake of nutrients in the brain. The sweet taste of carbohydrates generally indicates the caloric contents of foods and is palatable for a variety of organisms (Berridge 2003). In some cases, sweet caloric foods can lead to overeating and obesity. Therefore, currently, a lot of people use noncaloric artificial sweeteners, such as saccharin or aspartame, because they have no food energy and are as sweet as saccharide.

Ingested glucose or sucrose is absorbed in the gut, and then gut hormones and pancreatic hormones, such as “glucagon like peptide-1 and insulin,” are secreted. These circulating hormones affect the brain in several ways. Increased insulin evokes neuronal activity in the ventral tegmental area (VTA) and the limbic regions (Tsuji et al. 1996; Pardini et al. 2006). In addition to these observations, recent studies have revealed the existence of the sweet taste-sensing systems (T1R2 and T1R3) in the gastrointestinal (GI) epithelium (Jang et al. 2007). Glucose also activates the vagus nerve, which innervates the GI tract and conveys the nutrient information from the GI tract to the brain (Niijima 1991). These results indicate that chemosensing in the GI tract is also an important pathway for delivering sweet nutrient information to the brain.

Positively reinforcing postingestive effects are observed in rodents following the ingestion of glucose without taste sensing (Sclafani 2001). Note that previous study of brain mechanisms mediating sugar conditioning, intragastric infusions of 8% glucose conditioned preferences for flavored 0.2% saccharin solutions. Trpm5 knock-out mice, which lack the sweet taste sensation in the oral cavity, develop a preference for sucrose solution but not for sucralose solution (de Araujo et al. 2008). This report indicates that the postingestive effect is not due to the taste sensing.
One of the great advantages of functional magnetic resonance imaging (fMRI) is that all activation in entire brain regions can be investigated simultaneously. Previous fMRI studies have shown changes in the blood oxygenation-level dependent (BOLD) signal following the ingestion of glucose. In humans, glucose intake causes significant changes in BOLD signal in the hypothalamus (Liu et al. 2000; Smeets et al. 2005). In rodent studies, gut glucose increases the BOLD signal in several forebrain regions, including the nucleus accumbens (NAc) and the amygdale (AMG) (Tsurugizawa et al. 2009). However, it remains unclear whether the intragastric administration of glucose and saccharin precipitate different BOLD responses. The purpose of this study was, using awake fMRI, to compare the BOLD responses to gut 8% glucose (caloric) and 0.2% saccharin (noncaloric) stimulation without oral sensation. The concentration of the solution was determined from previous studies showing the postingestive behavior and c-fos immunoreactivity (Yamamoto and Sawa 2000; Touzani et al. 2008; Touzani et al. 2009a, 2009b). Although one of the advantages of fMRI is that we can simultaneously detect the brain regions which are related to the physiological response (Logothetis 2008; Tsurugizawa et al. 2013), an obstacle to the use of fMRI in rodents is the disturbance of neurovascular coupling by anesthetics (Tsurugizawa et al. 2010; Masamoto and Kanno 2012). Here, we successfully removed the effects of anesthetics on neurovascular coupling by developing awake fMRI methods for rats.

Materials and methods

Animals

Measurements of BOLD were performed using 18 male Sprague-Dawley rats (10 weeks old at the start of surgery, Charles River Laboratories, Kawasaki, Japan) for glucose (n = 6), saccharin (n = 6), or physiological saline (n = 6) infusion. The rats were housed individually in wire-mesh cages under controlled temperature (23 ± 0.5°C) and light (1:00–13:00) conditions with free access to water and food (CRF-1, Oriental Yeast, Tokyo, Japan). All animal procedures in this study were approved by the Committee for Animal Experiments at Ajinomoto Co. Inc. and were carried out in accordance with guidelines of the US NIH regarding the care and use of animals for experimental procedures.

Surgery

To perform awake fMRI measurements, rats underwent cranioplastic surgery under pentobarbital anesthesia (50 mg/kg body weight, i.p.) as described previously (Tsurugizawa et al. 2010). In brief, for intragastric cannulation, one end of a silicon tube was passed from the abdomen under the back skin and held on the head. Cranioplastic acrylic cement was applied to the skull with two holes molded on each side to serve as a receptacle for the four glass-fiber bars which are used for head fixation during the imaging session. The other end of the silicone tube was inserted into the gastric fundus and ligated with a silk thread. Following surgery, the rats were allowed to recover for >1 week.

Awake fMRI

We used the acclimation method as described previously (Tsurugizawa et al. 2010; Uematsu et al. 2014). To allow the rats to adapt to the awake MRI condition, they were trained for 5 days at the same time each day (13:00–17:00) to minimize effects due to circadian rhythm. During the first 3 days, a pseudo-MRI system was used, which consisted of a pseudo-MRI nonmagnet bore and head positioner. At first, the rats were lightly anesthetized for a short time using 2% isoflurane. The rats were then immediately set into the head positioner by fixing the cranioplastic acrylic mounting with four bars, and their bodies were gently restrained with elastic bands. To reduce stress due to noise, earplugs were used throughout the experiment. The rats were then left in the pseudo-fMRI apparatus for 30 min on the first day and for 90 min on the second and third days after recovery from anesthesia. These conditions and MRI machine were used for the next 2 days. Throughout training, the heart and respiration rates were measured using an MR-compatible monitoring system (Model 1025, SA Instruments, NY, USA). The respiration and heart rates on the fifth day were 85.3 ± 1 and 387.9 ± 4.5 beats/min, respectively, indicating that both had returned to normal levels. Thus, the measurement of physiological parameters in this study showed that acclimation training minimizes the stress induced via MRI scanning.

All MRI measurements were performed during the dark period after the rats were fasted for 12–15 h, and body temperature was maintained between 36 and 37 °C by circulating water throughout the experiment. The rats were anesthetized for a short time with 1.5% isoflurane. The cranioplastic acrylic mounting was then immediately fixed with four glass fiber bars (avoiding the painful insertion of ear bars) on a nonmagnetic stereotaxic apparatus. Scanning was then initiated. The studies utilized a Bruker Avance III system (Bruker, Ettlingen, Germany) with a 4.7 T/40 cm horizontal superconducting magnet equipped with gradient coils (100 mT/m, 26 cm diameter). The BOLD fMRI data were obtained using a T2*-weighted multislice fast low-angle shot sequence with the following parameters: time of repetition (TR) = 15 s, echo time (TE) = 10 ms, flip angle = 30°, field of view = 35 mm × 35 mm, acquisition matrix = 64 × 64, slice thickness = 1.3 mm, and slice number = 17. Structural images were obtained via the multislice rapid acquisition with relaxation enhancement (RARE) sequence using the following parameters: TR = 2500 ms, effective TE = 60 ms, RARE factor = 8, acquisition matrix = 128 × 128 and 4 averages. Twenty minutes after the scanning began, an 8% glucose (Wako, Osaka, Japan), 0.2% saccharin (Wako, Osaka, Japan), or 0.9% NaCl (Wako, Osaka, Japan) solution was delivered into the stomach via...
an implanted tube for 10 min at a rate of 1 mL/min/kg body weight by means of a syringe pump (CVF-3200, Nihon Kohden, Tokyo, Japan) to reduce the effects of gastric expansion (Figure 1). The prescan period (10–20 min before the infusion) was set to make rats adapt to the scanning situation.

Data processing

Spm5 software (Wellcome Trust Center for Neuroimaging, UK) was used for preprocessing, including realignment, coregistration to structural images, and the spatial normalization of functional data. Before preprocessing, template images coregistered to the Paxinos and Watson rat brain atlas were obtained (Paxinos and Watson 1998). The image sets containing motion artifacts were discarded.

The statistical analyses were conducted using a program written in MATLAB (Mathworks Inc., Tokyo, Japan) as described earlier (Tsurugizawa et al. 2009). The brain regions demonstrating significant BOLD changes were determined by applying boxcar functions. The “off” period of the boxcar function was the basal period, corresponding to the 10-min period prior to the start of nutrient administration, and the “on” period was the period of potential activity, which corresponded to the 10-minute period after the start of administration (Figure 1). A two-way repeated measures ANOVA was used to confirm that there were no significant changes in BOLD signal intensity during the 10-min period prior to administration both within and between groups. The first-level (fixed effect) analyses were performed on data from individual animals. To make inferences regarding group data, second-level (random effect) analyses were conducted using the results of the first-level analysis. We then compared the BOLD signals between glucose and saccharin groups in second-level analysis. Furthermore, the BOLD signal changes by glucose or saccharin infusion were compared with the physiological saline infusion to investigate the BOLD responses without physiological effects (i.e., gastric expansion). The areas that showed significant activation ($P < 0.05$, corrected for multiple comparisons using false discovery rate procedure) and had a cluster size >49 pixels were analyzed to construct the T-contrast images.

To perform the time-series analysis, regions of interest (ROIs) in each brain region were created using MRicron software. We drew the ROIs according to the rat brain atlas separated from fMRI study (Paxinos and Watson 1998). The time course of the changes in BOLD signals within a ROI was calculated as follows:

$$\% \text{ changes in BOLD signal intensity} = \left( \frac{\text{BOLD signals within ROI}}{\text{Averaged BOLD signals within ROI in basal period}} - 1 \right) \times 100,$$

where the basal period applies to the 5 min prior to infusion.

Results

Glucose-induced BOLD signal changes

The T-map images induced via the 8% glucose, 0.2% saccharin, and physiological saline at 0–5 min (period 1), 5–10 min (period 2), 10–15 min (period 3), and 15–20 min (period 4) after the start of administration are shown in Figure 2 and Supplementary Figures 1–3. The $t$-values and periods when the BOLD signal changed most significantly in each brain region are given in Tables 1 and 2. Glucose evoked a significant increase in the BOLD signal intensity in the bilateral anterior cingulate cortex, medial prefrontal cortex, insular cortex (IC), ventral/lateral orbital cortex, caudate putamen (CPu), NAc, VTA, substantia nigra, AMG, hippocampus (HIP), dorsomedial thalamus, ventroposteromedial thalamus, lateral hypothalamus, ventromedial hypothalamus (VMH), medial preoptic area, arcuate nucleus, and the left nucleus of the solitary tract. In most of the areas, BOLD signal increases were induced during glucose infusion; these increases persisted throughout period 4.

Saccharin and physiological saline-induced BOLD signal changes

Saccharin induced a significant increase in the BOLD signal intensity in the nucleus of the solitary tract, IC, and the CPu during the saccharin infusion (period 1). The BOLD signal
increase in the CPu was observed after saccharin infusion (period 4). The BOLD signal increase following the intragastric infusion of physiological saline, which served as a control for intragastric infusion, was also examined. The physiological saline infusion increased the BOLD signal intensity only in the bilateral CPu during infusion (Figure 2, Supplementary Figure 3, and Table 2). This result also supports the stability of the BOLD response in conscious rats throughout the experiment.

Comparisons of BOLD signal changes among glucose, saccharin, and physiological saline groups

The pixel-by-pixel comparisons of the BOLD signal changes among glucose, saccharin, and physiological saline groups were shown in Figure 3. Glucose-induced BOLD signal changes were significantly larger than saccharin and saline groups in the several regions including the cerebral cortex, NAc, hypothalamic regions, HIP, AMG, and VTA (Figures 3A and B). In contrast, saccharin-induced BOLD signals were significantly larger than physiological saline including the cerebral cortex, HIP, and VTA (Figure 3C). There was no pixel in which saccharin or physiological saline-induced BOLD signal changes were significantly larger than glucose.

Time-course of averaged BOLD response in the AMG, NAc, NTS, and VTA

Figure 3 shows the time course of the BOLD signal changes, averaged over 5-min periods, in the AMG, NAc, nucleus of the solitary tract, and the VTA, for the glucose, saccharin, and the physiological saline groups. In the AMG, glucose infusion significantly increased the BOLD signals in period 1 and BOLD increase was prolonged until period 4 (Figure 4A). In the NAc, glucose significantly increased only in period 2 (Figure 4B). Saccharin did not increase in the AMG and the NAc. In the nucleus of the solitary tract, glucose and saccharin increased the BOLD signals in period 1, 2, or 3, and no significant changes between glucose and saccharin groups were observed (Figure 4C). In the VTA, glucose significantly increased BOLD signals in period 3 and
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4, whereas saccharin did not increase (Figure 4D). There was no significant signal change in physiological saline group in all regions.

**Discussion**

This study demonstrates that BOLD response was different between intragastric infusion of glucose and saccharin. In addition, intragastric infusion of glucose increased the BOLD signals in the nucleus of the solitary tract, VTA, AMG, VMH, and the NAc, and those increases were prolonged until 10 min after the end of the intragastric infusion. The gut saccharin increased the BOLD signals in the nucleus of the solitary tract and IC but did not increase the BOLD signals in the VTA or NAc. These results are different from the oral sensation results, as chemosensation in the oral cavity in mice generally interprets glucose and saccharin to have the same sweet taste (Zhao et al. 2003).

Previous studies have shown that the intragastric administration of sucrose increased the number of c-Fos-positive cells in several brain regions including the central nuclei of the AMG. In contrast, 0.1% saccharin does not increase the expression of c-Fos in the central nuclei of the AMG (Yamamoto et al. 1997; Yamamoto and Sawa 2000). This result corresponds with our fMRI data showing that the BOLD signals in the AMG were increased via the intragastric infusion of glucose but not by saccharin. Additionally, glucose induced significant increases of the BOLD signal intensity in the dopaminergic pathways including the NAc and the VTA, whereas saccharin did not increase the BOLD signal intensity in these regions. It is important to note that the dopaminergic system and the hypothalamus are key regions for the control of food intake and preference. Dopaminergic fibers originating from the VTA project to the NAc and the AMG, and the dopaminergic actions in the NAc are associated with the positive motivation of sucrose intake (Berridge and Robinson 1998). Sucrose intake also increases dopamine release in the NAc and the AMG. Dr Touzani and his colleague have shown that lesions in the AMG eliminate the preference for flavored water paired with

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**Table 1** Brain regions showing the most significant increases \((P < 0.05, \text{corrected})\) in BOLD signal following intragastric administration of glucose or saccharin

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Glucose</th>
<th>Saccharin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Period (min) (L/R)</td>
<td>MAX T-value (L/R)</td>
</tr>
<tr>
<td>Anterior cingulate cortex</td>
<td>15–20/15–20</td>
<td>7.05/6.5</td>
</tr>
<tr>
<td>Medial prefrontal cortex</td>
<td>0–5/0–5</td>
<td>12.85/13.08</td>
</tr>
<tr>
<td>Ventral orbital cortex</td>
<td>10–15/10–15</td>
<td>9.31/9.45</td>
</tr>
<tr>
<td>Lateral orbital cortex</td>
<td>10–15/15–20</td>
<td>8.23/15.27</td>
</tr>
<tr>
<td>CPu</td>
<td>15–20/15–20</td>
<td>11.91/10.97</td>
</tr>
<tr>
<td>Nucleus of the solitary tract</td>
<td>10–15/ns</td>
<td>6.35/ns</td>
</tr>
<tr>
<td>NAc</td>
<td>10–15/10–15</td>
<td>8.85/8.75</td>
</tr>
<tr>
<td>Ventral tegmental area</td>
<td>15–20/15–20</td>
<td>7.06/8.61</td>
</tr>
<tr>
<td>Substantia nigra</td>
<td>15–20/15–20</td>
<td>9.77/7.24</td>
</tr>
<tr>
<td>AMG</td>
<td>10–15/10–15</td>
<td>13.85/11.77</td>
</tr>
<tr>
<td>HIP</td>
<td>10–15/15–20</td>
<td>15.38/13.74</td>
</tr>
<tr>
<td>Dorsomedial thalamus</td>
<td>10–15/10–15</td>
<td>8.57/8.05</td>
</tr>
<tr>
<td>Ventroposteromedial thalamus</td>
<td>0–5/0–10–15</td>
<td>13.04/7.74</td>
</tr>
<tr>
<td>Lateral hypothalamus</td>
<td>10–15/15–15</td>
<td>8.67/8.74</td>
</tr>
<tr>
<td>VMH</td>
<td>10–15/15–20</td>
<td>6.86/9.3</td>
</tr>
<tr>
<td>Medial preoptic area</td>
<td>10–15/15–20</td>
<td>15.11/6.57</td>
</tr>
<tr>
<td>Arcuate nucleus</td>
<td>10–15/10–15</td>
<td>10.25/10.44</td>
</tr>
</tbody>
</table>

ns, not significant.

**Table 2** Brain regions showing the most significant increases \((P < 0.05, \text{corrected})\) in BOLD signal following intragastric administration of saline

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Saline</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Period (min) (L/R)</td>
<td>MAX T-value (L/R)</td>
</tr>
<tr>
<td>CPu</td>
<td>15–20/15–20</td>
<td>11.91/10.97</td>
</tr>
</tbody>
</table>
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the intragastric administration of sucrose, indicating that the AMG is key to the expression of food preferences (Touzani and Sclafani 2005). Dr Sclafani and his colleagues reported that IG infusions of noncaloric sweetener (sucralose) failed to condition a flavor preference in mice (Sclafani et al. 2010). Furthermore, they also reported the sucrose-induced preference conditioning in T1R3 knockout mice, indicating that postingestive sucrose, without sweet taste, can induce the preference. The VMH, which is a nucleus in the hypothalamus referred to as the satiety center, controls food intake. An increase of the blood glucose level from the fasting state evokes the activation of glucose-sensitive neurons located in the ventromedial nucleus of the hypothalamus to induce the suppression of food intake (Oomura 1986). The medial prefrontal cortex was activated by intragastric infusion of glucose and saccharin. The medial prefrontal cortex has the important roles on evaluation caloric density (Rudenga and Small 2013; Kennedy and Dimitropoulos 2014), indicating that caloric density between ingested glucose and saccharin could be evaluated in the medial prefrontal cortex.

Although results of BOLD fMRI are consistent with the previous behavioral study, we did not directly compare the BOLD signal changes with behavioral changes. Therefore, there are still limitations to link the BOLD response with behavioral study. Another limitation of this study is that only one concentration of glucose and saccharin were investigated, and therefore, lower or higher concentration may produce different results. In addition, given that osmolality of 8% glucose is higher than those of 0.2% saccharin and physiological saline, there is a possibility of osmotic effects by 8% glucose. In the future, awake fMRI and the behavior measurement should be concurrently performed with other concentrations in the magnetic bore.

In human fMRI study, oral stimuli of caloric sweetener (sucrose) evoke the stronger BOLD response in the IC, striatum, and cingulate cortex rather than noncaloric sweetener (sucralose) (Frank et al. 2008). Especially, dopaminergic pathway is activated only by sucrose stimuli. This indicates the distinct quality of taste in the sweeteners. Ingested glucose induced significant changes in the hypothalamus (Liu et al. 2000; Little et al. 2014) and this signal changes were not evoked by aspartame (noncaloric sweetener) (Smeets et al. 2005). The comparison of caloric and noncaloric sweeteners in human fMRI is needed in the future study.

Why do glucose and saccharin produce varying BOLD signals? This is difficult to explain with gut chemosensation because both glucose and saccharin interact with the T1R2 and T1R3 receptors to induce sweet taste (Nelson et al. 2001;

Figure 3  The comparisons of the BOLD signal changes among glucose, saccharin, and physiological saline group (P < 0.05, corrected). The t-value shows the significant changes in BOLD signal intensity between two groups, respectively, (A) glucose > saccharin, (B) glucose > saline, and (C) saccharin > saline. Color bar, t-values.
One possibility could be due to the difference of the internal signals from the gut to the brain, such as insulin, GLP-1, or blood glucose. Recently, gut glucose sensors (SGLT1 and SGLT3) are thought to be important for the gut glucose sensing and subsequent hormone release (Raybould 2010; Gribble 2012). Intragastrically administered glucose in fasting rats induces the acute increase of circulating blood glucose, which triggers an increase in insulin levels. These internal signals affect several brain regions, including the VTA, hypothalamus, and the limbic system (Tsuji et al. 1996; Pardini et al. 2006; Solomon et al. 2006). In addition, we have shown earlier that BOLD signal changes in the AMG and NAc induced via the intragastric load of glucose were correlated with blood insulin fluctuations in rats, although we did not investigated with saccharin solution (Tsurugizawa et al. 2009). An intragastric load of glucose were correlated with blood insulin fluctuations in rats, although we did not investigated with saccharin solution (Tsurugizawa et al. 2009). An intragastric load of glucose were correlated with blood insulin fluctuations in rats, although we did not investigated with saccharin solution (Tsurugizawa et al. 2009). An intragastric load of glucose were correlated with blood insulin fluctuations in rats, although we did not investigated with saccharin solution (Tsurugizawa et al. 2009). An intragastric load of glucose were correlated with blood insulin fluctuations in rats, although we did not investigated with saccharin solution (Tsurugizawa et al. 2009).

As both glucose and saccharin increased the BOLD signal intensity in the nucleus of the solitary tract and IC, these areas could be related to the chemical sensing for sweet tastants. Although the neuronal pathway conveying chemosensory information of sweet tastants from the gut to the brain is present, the vagus nerve could possibly mediate the conveyance of chemosensory information of sweet tastants to the brain. Anatomically, the afferent vagus nerve enters the nucleus of the solitary tract in the medulla oblongata, and the neurons in the nucleus of the solitary tract connect to forebrain regions including the IC (Allen et al. 1991). Although electrophysiological studies have demonstrated vagus nerve stimulation by the infusion of glucose into the small intestine (Mei 1978), no study to date has investigated noncaloric sweeteners. Further study is needed to clarify the contribution of the vagus nerve to this phenomenon.

In conclusion, our data show that the intragastric administration of glucose, but not saccharin, activates the VTA–NAc pathway and components of the limbic system, including the VTA, AMG, and NAc. This is primarily via internal signals,
including the insulin and glucose signal, which conveys postingestive information of glucose and saccharin from the gut to the brain. Our data also indicate that there are different mechanisms for both oral sensation and GI perception of sweet tastants in the brain.

Supplementary material
Supplementary material can be found at http://www.chemse.oxfordjournals.org/

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Conflict of interest statement
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References


