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Experience-dependent escalation of glucose drinking and the development of glucose preference over fructose: association with glucose entry into the brain

Ken T. Wakabayashi, Laurence Spekterman, and Eugene A. Kiyatkin*

In-Vivo Electrophysiology Unit, Behavioral Neuroscience Branch, National Institute on Drug Abuse – Intramural Research Program, National Institutes of Health, DHHS, 333 Cassell Drive, Baltimore, MD 21224, USA

Abstract

Glucose, a primary metabolic substrate for cellular activity, must be delivered to the brain for normal neural functions. Glucose is also a unique reinforcer—besides its rewarding sensory properties and metabolic effects, which all natural sugars have, glucose crosses the blood-brain barrier and acts on glucoreceptors expressed on multiple brain cells. To clarify the role of this direct glucose action in the brain, we compared neural and behavioral effects of glucose with those induced by fructose, a sweeter yet metabolically equivalent sugar. First, by using enzyme-based biosensors in freely moving rats, we confirmed that glucose rapidly increases in the nucleus accumbens in a dose-dependent manner after its intravenous delivery. In contrast, fructose induced a minimal response only after a large-dose injection. Second, we showed that naive rats during unrestricted access consume larger volumes of glucose than fructose solution; the difference appeared with a definite latency during the initial exposure and strongly increased during subsequent tests. When rats with equal sugar experience were presented with either glucose or fructose in alternative order, consumption of both substances was initially equal, but only consumption of glucose increased during subsequent sessions. Finally, rats with equal glucose-fructose experience developed a strong preference of glucose over fructose during a two-bottle choice procedure; the effect appeared with a definite latency during the initial test and greatly amplified during subsequent tests. Our results suggest that direct entry of glucose in the brain and its subsequent effects on brain cells could be critical for the experience-dependent escalation of glucose consumption and the development of glucose preference over fructose.

Graphic Abstract

While glucose and fructose are calorically equal and fructose is sweeter than glucose, rats exhibit escalating glucose consumption and develop preference of glucose over fructose during repeated sessions of free drinking. These behavioral phenomena appear to be related to glucose entry to the brain and its action on glucose-sensitive central neurons. This neural effect of glucose could be critical in regulating consummatory behaviors with both sugary product and possibly different foods.

*Correspondence should be addressed to Eugene A. Kiyatkin at the above address. Fax: (443) 740-2155; tel.: (443) 740-2844; ekiyatki@intra.nida.nih.gov.

Keywords

Sugars; Taste; Reinforcement; Nucleus accumbens; Rats

INTRODUCTION

Rats quickly learn to drink glucose and fructose solutions without food or water deprivation. These two monosaccharides have equal caloric value but fructose appears to be sweeter than glucose (Hanover & White, 1993; Ramirez, 1996). Since it is generally believed that the drinking of sugary substances is determined by their rewarding sensory properties (Sclafani, 1987; de Araujo, 2011), the sweeter taste of fructose may result in greater consumption and preference over glucose. However, experimental data are controversial, suggesting a preference of glucose over fructose, and the role of learning in the development of this preference (Ackroff & Sclafani, 1991; Jacobs, 1962; Tordoff et al., 1990). These contradictory results could be related to profound differences between glucose and fructose in their metabolism and neural effects.

In contrast to fructose that appears in circulation only after its consumption and has very limited blood-brain barrier permeability (Oldendorf et al., 1971), glucose is normally present in the blood at relatively high and stable levels, continuously entering brain tissue by facilitated, gradient-dependent diffusion via GLUT-1 transporters (Duelli & Kuschinsky, 2001). In addition to its uptake by brain cells for metabolic use, glucose arriving to the brain after its consumption directly acts on neuronal glucoreceptors that are expressed primarily on hypothalamic neurons that are involved in regulating sleep/wakefulness, locomotion, energy expenditure, and feeding (Levin *et al.*, 2002; Burdakov et al., 2005). Different subtypes of neurochemically heterogeneous neurons are either excited or inhibited by physiological increases in local glucose levels (Routh, 2002), creating a specific neural effect. In contrast, fructose lacks specific neuronal receptors and, although ingested fructose is partially converted in the liver into glucose, this transformation takes time, resulting in minimal, if any, increases in blood and possibly brain glucose levels (Page et al., 2013).

This study was designed to explore the possible link between the entry of glucose in the brain and the behavioral phenomena associated with the consumption and preference of glucose and fructose solutions. First, we used glucose biosensors coupled with high-speed amperometry to examine how glucose appears in the brain's extracellular space after systemic delivery of glucose and fructose in freely moving rats. These measurements were conducted in the nucleus accumbens (NAc), a critical structure of the motivation-reinforcement circuit (Di Chiara, 2002; Wise & Bozarth, 1987) and an area with high density of GLUT-1 transporters (Zeller et al., 1997). Second, we conducted two behavioral experiments aimed to determine whether consumption of glucose and fructose differs during long-term repeated exposure, and whether rats prefer glucose over fructose. In the first experiment, we examined the time-course of consumption of equimolar solutions of glucose or fructose during unrestricted access during prolonged repeated sessions. We also examined how drinking behavior changed during substitution, when glucose-experienced rats were exposed to fructose and *vice versa*. In the second behavioral experiment, we examined how

consumption of glucose and fructose differed in equally sugar-experienced rats by daily alternating access to each sugar solution, and testing the preference to each sugar using a two-bottle choice paradigm.

MATERIALS AND METHODS

Animals and housing

35 male Long-Evans rats (440±40 g) supplied by Charles River Laboratories (Greensboro, NC) were housed individually in a temperature-, humidity- and light-controlled room (12 h/12 h light/dark cycle, lights on at 07:00) with free access to food and water. Protocols were performed in compliance with the Guide for the Care and Use of Laboratory Animals (NIH, Publication 865-23) and were approved by the NIDA-IRP Animal Care and Use Committee.

General structure of the study

This study combines the results of three experiments (I, II, III). In Experiment I (n=11 rats), we examined the changes in NAc extracellular glucose levels induced by intravenous (iv) injections of 10% solutions of glucose and fructose. Rats for this experiment were intensively habituated to the recording environment, surgically prepared, and electrochemical recordings with glucose biosensors were conducted during one daily session. Experiment II (n=16 rats) was conducted in intact rats divided onto two equal groups. In these animals, we examined the development of drinking behavior following unrestricted access to 10% solutions of either glucose or fructose (for 4 hrs/daily, 5 daily tests over a 2-week period). In this experiment we also examined how drinking behavior and consumption changed following reversal substitution, when glucose- and fructose-experienced rats were exposed to other sugar solution (i.e. fructose and glucose, respectively). In Experiment III (n=8 rats), we examined which of two solutions is preferred by rats: (a) first training rats to be equally experienced with both solutions by alternating their access to either sugar solution during daily repeated sessions and then (b) measuring their preference for either solution during a two-bottle choice paradigm.

Surgical preparations for Experiment I

Each rat used for Experiment I was surgically prepared for electrochemical recordings as described in detail previously (Kiyatkin & Lenoir, 2012; Wakabayashi & Kiyatkin, 2014, 2015a). Briefly, under general anesthesia (Equithesin 0.33 ml/100 g, ip), rats were implanted with a BASi cannula (Bioanalytical Systems, Inc.; West Lafayette, IN, USA) for future insertions of a biosensor in the medial sector of the nucleus accumbens (NAc shell). The target coordinates were: AP +1.2 mm, ML ±0.8 mm and DV 7.3 mm, according to the stereotaxic atlas of Paxinos and Watson (1998). The guide cannula hub was fixed to the skull with a head mount constructed from dental acrylic that was secured using three stainless steel bone screws. During the same surgical procedure, rats were also implanted with a chronic jugular catheter, which ran subcutaneously to the head mount and was secured to the same head assembly. Rats were allowed a minimum of 4 days of post-operative recovery; jugular catheters were flushed daily with 0.2 ml heparinized saline (15 units/ml) to maintain patency.

Fixed-potential amperometry with enzyme-based electrochemical sensors

Commercially produced glucose oxidase-based biosensors (Pinnacle Technology, Inc., Lawrence, KS, USA) coupled with fixed-potential amperometry have been extensively used in our previous studies (Kiyatkin & Lenoir, 2012; Kiyatkin et al., 2013; Kiyatkin & Wakabayashi, 2015; Wakabayashi & Kiyatkin, 2014, 2015a, b). These reports describe in detail multiple issues regarding the sensitivity/selectivity of these sensors, their *in vitro* and *in vivo* performance, and possible physical and chemical contributions that could be evaluated and controlled for providing high reliability and accuracy of electrochemical measurements of extracellular glucose fluctuations.

Briefly, glucose sensors are prepared from Pt-Ir wire of 180 μm diameter, with a sensing cavity of ~ 1 mm length on its tip. The active electrode is incorporated with an integrated Ag/AgCl reference electrode. On the active surface, glucose oxidase converts glucose to glucono-1,5-lactone and hydrogen peroxide (H_2O_2), which is detected as an amperometric oxidation current generated by a +0.6 V applied potential (Hu & Wilson, 1997). The potential contribution of ascorbic acid to the measured current is competitively reduced by co-localizing ascorbic acid oxidase enzymes on the active surface of the sensor. This enzyme converts ascorbic acid to non-electroactive dehydroascorbate and water. In addition, a negatively charged Nafion polymer layer under the enzyme layer serves to exclude endogenous anionic compounds (Hu & Wilson, 1997).

Glucose sensors were calibrated immediately before and after each *in vivo* experiment. *In vitro* calibrations were conducted in PBS (pH 7.3) by incrementally increasing the concentration of glucose (Sigma-Aldrich) from 0 to 0.5, 1.0, and 1.5 mM followed by a single addition of ascorbate (25 μM). Within this physiological range of glucose levels (Fellows & Boutelle, 1993; McNay et al., 2001), glucose sensors used in this study produced incremental linear current increases. Mean sensitivity to glucose at 22–23°C was 2.28 ± 0.15 nA/0.5 mM (or 4.46 ± 0.29 nA at 37°C). Glucose sensors showed low sensitivity to ascorbate (0.17 ± 0.06 nA/25 μM) and, as showed previously, they were only minimally sensitive to dopamine at its physiological levels (5–50 pA/10–100 nM). Glucose sensors remained equally sensitive to glucose and selective against ascorbate during post-recording *in vitro* calibrations (2.03 ± 0.16 nA/0.5 mM and $0.10 \text{ nA} \pm 0.03/25 \mu\text{M}$, respectively). As tested *in vitro*, glucose sensors were fully insensitive to fructose ($-0.01 \text{ nA} \pm 0.01/0.5 \text{ mM}$). Since our previous *in vitro* and *in vivo* tests with glucose- and glucose-null sensors revealed minimal contributions of chemical and physical (i.e., brain temperature fluctuations, negative trend in baseline currents) interferences to glucose currents, null sensors were not used in this study.

Protocol for Experiment I

In this experiment, we examined changes in NAc extracellular glucose levels ([glucose]) induced by iv injections of saline, glucose and fructose. Electrochemical recordings occurred during the day (9:00–18:00) in an electrically insulated chamber (38×47×47 cm) under continuous dim illumination (20 W red light bulb), with a room wide air filter fan providing background noise. Prior to recording, rats were habituated to the testing environment for a minimum of 6 hrs per day for 3 consecutive days.

At the beginning of each experimental session, rats were minimally anesthetized (<2 min) with isoflurane and a calibrated glucose sensor was inserted into the brain through the guide cannula. The sensor was connected to the potentiostat (Model 3104, Pinnacle Technology) via an electrically shielded flexible cable and a multi-channel electrical swivel. Additionally, the injection port of the jugular catheter on the head mount was connected to two plastic catheter extensions that allowed stress- and cue-free delivery of saline and the tested substance from outside the cage, thus minimizing possible detection of the iv injection procedure by the rat. Testing began ~140 min after insertion of the sensor when the baseline currents relatively stabilized and continued for 3–5 hrs.

We used 10% solutions of glucose and fructose (Sigma-Aldrich, St. Louis, MO) that were delivered iv in two doses (30 mg in 0.3 ml of saline delivered over 20 s and 60 mg in 0.6 ml saline delivered over 40 s). Taking into account that “normal” levels of glucose in blood of rats are within 5.4–7.0 mM (Smith & Pogson, 1977) and the blood volume is ~30 ml, iv delivery of 30 mg and 60 mg of glucose could maximally double or triple its blood levels (6 and 12 mM increases), respectively, when it is distributed within the entire blood volume. The real increase should be lower than these calculated maximal increases because of rapid glucose uptake in tissues and its slower transformation into other products in the liver. Intervals between individual injections were 60 min, which were well enough for full restoration of glucose current baselines. Although fructose is not normally present in the blood, its iv injection at 30 and 60 mg doses could maximally increase its blood levels to 5.5 and 11 mM, respectively.

We used two groups of rats for glucose (n=5) and fructose (n=6) injections, and each rat in each group received two injections of either substance at 30 mg and one injection at 60 mg dose. Prior to tests with glucose and fructose, rats received a single iv injection of saline (0.3 ml over 20 s) to assess the response to the procedure of injection. At the end of each session, rats were iv injected with Equithesin (0.8 ml by iv injection over 2 min) to induce deep general anesthesia. Then, the rat was disconnected from the potentiostat and the sensor was removed for post-recording calibrations. Rats were then transcardially perfused with room-temperature PBS (pH 7.4) followed by 10% formalin. Brains were later sectioned on a cryostat to a thickness of 45 μ m. The location of the sensors was verified using the stereotaxic atlas of Paxinos and Watson (1998).

Data processing and statistical analyses for Experiment I

Electrochemical data were sampled at 1 Hz (i.e. mean current over 1 s) using the PAL software (Version 1.5.0, Pinnacle Technology) and analyzed using two time resolutions. Slow changes in electrochemical currents were analyzed with 30-s quantification bins using an analysis window of 5 min before and 30 min after each iv injection. Rapid current changes were analyzed with 2-s bins for 30 s before and 120 s after the onset of iv injections of saline, glucose, and fructose. Since the baseline currents slightly varied in amplitude between individual glucose electrodes, absolute current changes were transformed into relative changes taking a basal value before each event (30 s for slow and 4 s for rapid time-course analyses) as 0 nA. These current changes were then transformed into glucose concentration (μ M) based on sensor sensitivity determined during pre-recording *in vitro*

calibrations and adjusted by the temperature coefficient (95.6%) determined in previous analytical tests (Kiyatkin et al., 2013). The data were also presented as a percent change based on previously determined basal levels of glucose in the NAc (range of 540–700 μ M; Kiyatkin & Lenoir, 2012; Wakabayashi & Kiyatkin, 2015).

Statistical data analyses included the use of one-way repeated measure (RM) ANOVAs to find time periods where there was a significant post-injection main effect. Fisher *post-hoc* tests were used for pair-wise comparisons, and the latency of the glucose response was determined based on the first data point significantly different from baseline ($p < 0.05$).

Protocol for Experiment II

This experiment was conducted on 16 intact rats, which were habituated to the plastic testing chambers (42×42×32 cm) before the start of the experiment for 3 daily sessions. For this experiment, rats were assigned to receive either glucose or fructose. At the start of the session, each rat was placed in the testing chamber for a 2.5-h habituation. Then they were given 4 hours of unrestricted access to a drinking tube that contained 10% solutions of their assigned sugar, and the volume consumed by each rat was recorded every 4 min (60 values). These tests were conducted every other day for two weeks for a total of 5 sessions. On the third week, the assigned sugar solution for each rat was switched (i.e. substituted) once, so that rats experienced with glucose were presented with fructose, and *vice versa*. The volume consumed of each substituted sugar solution was recorded during 4 daily 4 hr sessions given every other day.

Protocol for Experiment III

This experiment was conducted on 8 rats, which were habituated to the same environment as in Experiment II and were initially pre-trained to drink an equal mixture of glucose and fructose (5% glucose plus 5% fructose, or 10% a total sugar concentration) for three days. All 8 rats in this group showed consistent drinking behavior before the tests. Then each rat had one glucose and one fructose test session (both 4 hours) per week every other day for 4 weeks. To reduce possible order effects, each week the order of the glucose and sucrose sessions was stochastic for each rat. Similar to Experiment II, we monitored consumption volume every 4 min bin for 4 hours. After 4 weeks of testing (with 4 glucose and 4 fructose sessions), these equally experienced glucose and fructose rats were presented for 4 hours with two identical drinking tubes filled with 10% solutions of glucose and fructose placed adjacent to each other. These two bottle choice tests were conducted three times during one week, with one free day between sessions.

Statistical analyses for Experiment II and III

The primary parameter in these experiments was the volume of glucose or fructose consumed every 4 min over the 4-hr test. Two-way RM ANOVA with a Dunnett multiple comparison test was used to detect between-group differences in consumption. Day-to-day differences in dynamics of consumption were analyzed with one-way RM ANOVA.

RESULTS

Electrochemical experiment

Intravenous administration of glucose to quietly resting rats induced significant increases in NAc glucose, which were clearly dose-dependent (one-way RM ANOVA: 30 mg: $F_{9,549}=29.4$, 60 mg: $F_{10,610}=43.4$, both $p<0.001$ see Fig. 1A). At the 30 mg dose, glucose rapidly increased after the injection onset, peaked at ~3 min (82.2 ± 11.2 μM) and slowly decreased to or slightly below the pre-injection baseline. At the 60 mg dose, NAc glucose levels also rapidly increased, peaking at ~5 min at significantly higher levels (184.5 ± 18.1 μM , $p<0.05$ vs. 30 mg), and slowly returning to baseline (~30 min vs ~15 min for a lower dose). With respect to previously measured basal concentrations of glucose in the NAc (700 μM ; Wakabayashi & Kiyatkin, 2015), these increases were relatively modest, at ~10 and ~22% over the quiet resting baseline.

For more detailed analyses of the initial changes in glucose response, the data were analyzed at a 2-s time resolution for the 120 s following the onset of glucose injection (Fig. 1B). While highlighting the dose-dependent dynamics of the NAc glucose response, we found that glucose levels began to increase rapidly during the injection (~20 s after injection onset), creating a weak initial peak, evident for both injection doses. This surprising finding could be explained taking into account the inescapable damage of the blood-brain barrier that is associated with the acute insertion of the sensor into densely vascularized brain tissue. This initial peak appears to be an “echo” of the rapid, strong rise in blood glucose levels that was seen at ~30 and ~50 s after the injection start for 30 and 60 mg doses, respectively or ~10 s after the injection end. While clearly seen with each glucose injection, the magnitude of this increase (~50 and ~80 μM) suggests a relatively modest leakage of glucose from the blood taking into account much larger calculated blood glucose levels after its iv injections (6 and 12 mM). If the blood glucose phasically rises to 6,000 or 12,000 μM but brain glucose rises for only 50 and 80 μM , this “artifactual” increase due to leakage from the blood is very low, less than 1% for both doses. This initial peak was fully absent after saline injection, which did not result in any changes in [glucose].

In contrast to glucose, iv injections of fructose induced only minimal, if any, changes in NAc glucose (Fig. 2A). At the 30 mg dose, no significant increases in glucose was observed, while a 60 mg injection resulted in a small and slow increase in glucose (one-way RM ANOVA: $F_{5,305}=2.22$ $p<0.05$, first significant bin ~3 min post injection). In contrast to glucose injections, no increases in NAc glucose were observed at the 2-s bin time resolution during the first 120 s post-injections (Fig. 2B).

Behavioral experiment I

To assess the differences in the pattern of glucose and fructose consumption, two groups of rats were given unrestricted access to either fructose or glucose. Most rats exposed on Day 1 to a drinking tube containing a 10% solution of either glucose or fructose began drinking within one hour of access and continued drinking throughout the session. Five of 16 rats did not exhibit drinking on Day 1 (3 in fructose and 2 in glucose group), but two of them began drinking on Day 2. On Day 3, all rats except one consumed their respective solutions and

drinking was stable during all subsequent sessions. One rat that did not exhibit drinking during the entire experiment was excluded from the sample and our consumption averages shown in Fig. 3A were obtained from 8 and 7 rats for glucose and fructose, respectively.

As shown in Fig. 3A, consumption of glucose was significantly larger than consumption of fructose in each of five sessions. Two-way RM ANOVA revealed that the difference is minimal but significant on Day 1 and grows during subsequent days (Substance x Time interaction $F_{59,767}=2.92, 4.16, 7.21, 5.57$ and 12.01 for Days 1–5, respectively; $p<0.001$ each). Moreover, with day-to-day experience the between-group difference became significant at earlier times (106, 114, 46, 62, 66 min for Days 1–5). Finally, the total consumption of each substance significantly increased during repeated sessions (Fig. 3C; one-way RM ANOVA: $F_{4,39}=12.71, p<0.0001$ and $F_{4,34}=8.52, p<0.01$ for glucose and fructose, respectively). On the final day of exposure, rats consumed on average 34.05 ± 3.91 ml of glucose (range 16.2–47.5 ml) and only 16.09 ± 1.27 ml of fructose (range 11.3–22.0 ml). The mean consumption volumes in both groups gradually increased during Day 1–3, but stabilized during the last three sessions, resulting in relatively similar difference between glucose and fructose consumption.

However, dramatic changes in consumption occurred when substances were switched (Fig. 3B). During the first substitution test (Day 6), glucose-experienced rats that were given access to fructose significantly decreased consumption (10.60 ± 1.61 vs. 34.05 ± 3.91 ml, $p<0.001$), while fructose-experienced rats significantly increased consumption of glucose (24.97 ± 3.23 vs. 16.09 ± 1.27 ml, $p<0.01$). Due to these opposite changes, on the first substitution session (Day 6) there were no significant between-group differences in consumption (glucose vs. fructose $F_{1,13}=1.62, p=0.22$) and between-group difference in mean consumption volumes became evident only at the very end of the session (226 min). During subsequent sessions (Day 7–9), glucose consumption significantly exceeded the consumption of fructose (Substance x Time interaction $F_{59,767}=10.09, 14.97$ and 15.01 for Days 7–9, respectively; $p<0.001$ each). Importantly, fructose intake remained at stable levels (~17–19 ml) but intake of glucose progressively increased or accelerated ($F_{3,27}=11.41, p<0.01$), and the between-group differences became larger. At the final day of exposure (Day 9), previously fructose-experienced rats consumed on average 37.30 ± 2.65 ml of glucose (range 29.2–47.1 ml) and previously glucose-experienced rats consumed only 17.98 ± 2.54 ml of fructose (range 4.8–27.4 ml).

Behavioral experiment II

Since in Experiment I, each sugar was presented to rats repeatedly, changes in consumption could reflect learning or the development of conditioned association between specific taste of glucose and its post-ingestion entry into the brain. To minimize the possible contribution of learning, in this experiment rats were pre-trained to consume an equal mixture of glucose/fructose and the consumption of each sugar was assessed following its alternative presentation. To directly assess preference, we used a two-bottle choice procedure, where rats with equal glucose-fructose experience were simultaneously exposed to two identical drinking tubes with glucose and fructose solutions.

On Week 1, rats previously trained to consume glucose/fructose mixture did not show differences in consumption of glucose and fructose presented separately on alternating days (Fig. 4A–B). Both the effect of Substance (Glucose vs. Fructose) and Substance x Time interaction were not significant ($F_{1,13}=0.52$, $p=0.48$ and $F_{60,780}=1.23$, $p=0.11$). Rats quickly consumed ~10 ml of each sugar within the first 20 min of exposure, after which consumption slowed, reaching 15–20 ml at the end of the session.

However, consumption changed during the second and subsequent weeks (Fig. 4A–B). On Week 2, the rats initially showed identical consumption of both drinks but from ~2 hr, consumption of glucose accelerated, with the appearance of a significant between-group difference (Substance x Time interaction: $F_{60,780}=3.63$, $p<0.001$). This between-group difference became larger on Days 3 and 4 ($F_{60,780}=12.52$ and 6.08 , respectively; $p<0.001$), when rats on average consumed ~30 ml of glucose compared to ~15 ml of fructose. Similar to the first test, consumption was rapid during the first 10–12 min of exposure, then slowed, and finally accelerated for glucose. In addition, between-group differences in consumption became significant earlier in a session: at 180, 124, and 88 min from its start for Weeks 2, 3, and 4, respectively. Being analyzed by a total volume, consumption of glucose showed significant acceleration or escalation following repeated exposure (one-way RM ANOVA: $F_{3,31}=5.30$, $p<0.01$) but consumption of fructose remained relatively constant ($F_{3,31}=1.56$, $p=0.22$, NS)

When the rats with identical glucose/fructose experience were exposed for the first time to two drinking tubes containing these substances, consumption of glucose and fructose was equal at the beginning of the session, but as time progressed glucose consumption slowly outpaced fructose consumption (Fig. 4C). The difference appeared at ~30 min but became significant at 168 min from the session start. This preference for glucose over fructose became stronger during the next two tests. In these cases, glucose consumption significantly increased, fructose consumption decreased, and between-group difference in individual time points appeared from 8 and 76 min from the session start on Days 2 and 3, respectively. However, using two-way RM ANOVA with sliding time window, the between-group difference became significant from 8 min for Days 2 and 3. While consumption of glucose on Day 1 exceeded fructose consumption twofold, the ratio grew to 9:1 and 5:1 on Days 2 and 3, respectively. Therefore, rats are able to differentiate sugars based on their specific taste but a certain time is always necessary for this to occur. With repeated tests, this time for differentiation is shortened to 8 min—the time necessary for glucose to enter the brain after its drinking (Wakabayashi & Kiyatkin, 2015).

DISCUSSION

Glucose is a primary energetic substrate for metabolic activity of all living cells and its proper delivery into the extracellular space is critical for maintaining normal brain activity and functions. Although most glucose is synthesized from carbohydrate and non-carbohydrate precursors delivered into the body in consumed foods and drinks, pure glucose could also enter the circulation after consumption of high glucose-containing products. People and animals also consume other sugary substances, which have either no caloric value or are calorically equal to glucose itself. While consumption of non-caloric sweet

products (sugar substitutes) is determined entirely by their taste, suggesting that sweetness *per se* is rewarding, it is still unclear whether it is an innate feature or a result of previous life experience with natural sugar-containing products (Jacobs, 1972; Harris et al., 1933; Jacobs, 1962; Sclafani, 2004; Burke & Small, 2015). Appetitive responses of newborns to the oral sensation of sugars (Steiner & Glaser, 1984) are consistent with the idea that sweet taste is a primary reinforcer, but it is still unclear whether or not very early experience with the post-ingestional effects of mother's milk contributes to subsequent sweet preference. The primary reinforcing action of sweet taste is also questioned by a clear preference for nutritive but not non-nutritive sugars found in mice genetically engineered to lack the ability to sense sweet taste (Ren et al., 2010). In this study, we tried to establish the relationship between glucose entry into the brain and changes in consumption of equimolar solutions of glucose and fructose following initial and repeated presentations. These two sugars are calorically equal, thus excluding the contribution of general metabolic action, but fructose is sweeter in taste than glucose. If taste is the primary driver of consumption, rats should consume more fructose. If the post-ingestion entry of glucose into the brain and its action on central neurons is the primary factor determining consumption, glucose intake should exceed that of fructose.

In Experiment I, we quantitatively compared NAc glucose changes induced by the iv delivery of equimolar solutions of glucose and fructose solutions. In contrast to a glucose injection that resulted in a rapid and relatively large rise in its concentration in the extracellular space, fructose induced no changes in NAc glucose when estimated levels of fructose in blood doubled and only a minimal and delayed change when fructose blood levels in blood rapidly tripled. The latter change could be explained by the known partial conversion of fructose into glucose that occurs in the liver and takes a definite time. Due to similar transformations, brain glucose levels should also increase following consumption of different types of foods. Currently the direct data on brain glucose fluctuations induced by consumption of different foods are absent, but these changes should be slow, delayed, and directly dependent on the amount of consumed foods and their "glucose equivalent."

While the increases in NAc glucose after its iv injection were relatively small and transient (peak at 80 μ M or ~10% above baseline for ~10 min with a 30 mg dose), much stronger increases occurred during natural drinking behavior in trained rats (Wakabayashi & Kiyatkin, 2015). After the first drinking bout (6–8 ml of 10% glucose or 600–800 mg), NAc glucose increased much more (200–250 μ M or ~30% above baseline) and for a much longer time (~50–60 min). These increases always occurred with a 4–5-min latency, reflecting a slower appearance of glucose in blood after its ingestion. Importantly, these increases in NAc glucose coincided with the end of drinking, which was followed by a 40–50 min inactivity pause when glucose levels were increased. Eventually, when NAc glucose levels dropped to or below baseline, the rats initiated the second drinking bout that resulted in a second rise in NAc glucose. These findings suggest that changes in brain glucose are involved in regulating glucose-drinking behavior; the temporal aspects of these changes are important to explain why the differences in consumption always have consistent time delays.

Our current electrochemical recordings were conducted in the NAc shell, a critical structure of the motivation-reinforcement circuit and an area with dense expression of GLUT-1

transporters (Zeller et al., 1997), but it is highly likely that similar changes in extracellular glucose will occur in other brain structures, particularly in the hypothalamus and the ventral tegmental area. Both these structures also have extensive vascularization, a high density of GLUT-1 transporters (Zeller et al., 1997), and contain glucose-sensitive neurons (Routh et al., 2014; Sheng et al., 2014). Particularly, both orexin-containing lateral hypothalamic neurons and dopamine-containing ventral tegmental area neurons are inhibited by glucose (Routh et al., 2014; Liu et al., 2015; Kosse et al., 2015), and these neural effects could determine the pausing in glucose-drinking behavior consistently seen in trained rats after the initial and subsequent consumption bouts (Wakabayashi & Kiyatkin, 2015).

The rapid development of drinking behavior with glucose and fructose solutions shown in Experiment II suggest that both sugars act as reinforcers. However, glucose was consumed in larger volumes; the differences were small and appeared with a 40–60 min latency during the first session but grew substantially during subsequent sessions. Although consumption of both glucose and fructose increased during repeated sessions, the rise in glucose consumption was consistently larger. Therefore, due to its central action, glucose may serve as a stronger reinforcer than fructose despite its less sugary taste and equal metabolic value. This conclusion is supported by our findings obtained in the substitution experiment (see Fig. 3B), when glucose-experienced rats exposed to fructose decreased consumption presumably due to a lack of central action. In contrast, fructose-experienced rats significantly increased consumption of glucose due to appearance of its central effect.

According to the Pavlovian paradigm, increased glucose consumption and its day-to-day escalation could be viewed as the result of learning or the development of a conditioned association between its sensory action (specific sweet taste) and the delayed central action of ingested glucose in the brain. Although our electrochemical experiment revealed that NAC glucose rise induced by its iv injection is large, a comparatively weaker change found after a large-dose fructose injection suggests that the drinking of fructose could also induce a weaker and more delayed increases in brain glucose. While not directly tested so far, these increases could be substantially larger during natural fructose-drinking behavior due to a much larger amount of sugar consumed. This finding could explain the weak but significant day-to-day increase in fructose consumption found in our first behavioral experiment. When the influence of learning was reduced by pre-training with glucose-fructose mixture, the rats consumed equal volumes of each solution during the first session when either glucose or fructose was presented alone. However, even with alternative presentation, consumption of glucose but not fructose substantially increased during subsequent sessions, suggesting the development of conditioned association between the specific taste of glucose and its direct central action. Importantly, at the beginning of a session rats consumed similar volumes of glucose and fructose solutions but one to two hours later in a session glucose consumption increased but fructose consumption remained stable. This accelerated glucose consumption appeared only after a certain time that is obviously necessary for ingested glucose to enter the brain and induce its central effect.

These equally glucose- and fructose-experienced rats were then tested using a two-bottle choice procedure, which directly examined their preference for either sugar. Initially within the first session, rats showed identical consumption of both solutions, suggesting the lack of

differentiation and preference but consumption curves diverged from ~40–50 min, with significantly larger intake of glucose. Again, this delay could reflect the time necessary for ingested glucose to enter the brain and induce its neural effects, shifting consumption toward glucose at the expense of fructose. Therefore, the preference for glucose appears to be learned, reflecting the development of a conditioned association between the specific taste of glucose and its central action. This acutely developed association became stronger and the preference was shifted to earlier time points with each subsequent test. However, despite robust differences in total consumption, at least 8 min were always necessary for this change to occur. This time is close to the previously reported delay in glucose entry into the NAc after the initial consumption bout in trained rats (Wakabayashi & Kiyatkin, 2015).

The traditional dogma suggests that two primary factors, sweet taste and metabolic consequences (which are typically refer to caloric or nutritive value), are critical factors in determining consumption of sugar-containing products. While both of these factors are important, this study stresses the importance of a third factor: the direct action of glucose in the brain, which could make it a unique natural reinforcer. This action appears to be independent of general metabolic or nutritive effects of consumed foods and drinks because it differs from the more unspecific uptake of glucose by brain cells in its more specific neuronal targets and receptor mechanisms (Venner et al., 2012; Kosse et al., 2015). Since blood glucose levels increase after consumption of all caloric foods, the entry of glucose into the brain and its direct action on specific subsets of neurons via activation of glucoreceptors (but not the taste and more delayed metabolic effects) could be the primary source of reinforcement provided by consumed foods and drinks. In contrast to the undefined “post-ingestive” (Adolf, 1947), “need-reducing” (Hall, 1943) or “the deficit-relieving” effects of foods (Jacobs, 1962), this action is specific and could be objectively defined and studied at the molecular, cellular and neuronal circuit levels in terms of timing, parameters, neuronal targets, and physiological and behavioral effects. Therefore, glucose shares basic similarities with drug reinforcers, which by acting in the brain induce drug-taking behavior, preference of this behavior in expense of other natural behaviors, and eventually an experience-dependent escalation of drug intake (Ahmed et al, 2000; Vanderschuren & Ahmed, 2013; Guillem et al., 2014).

While our approach of comparing the central effects of glucose and fructose with the behavioral phenomena associated with repeated consumption of these sugars could suggest that glucose entry into brain tissue and its subsequent action on brain cells is a critical factor determining experience-dependent escalation of glucose drinking and the development of glucose preference over fructose, this association is in fact correlative. Further experiments are underway to establish causality of this association and clarify the role of glucose entering the brain in the regulation of feeding behavior.

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Abbreviations

ANOVA	analysis of variance
iv	intravenous
NAc	nucleus accumbens

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Figure 1. Change in NAc glucose concentration (μM) after an iv injection of glucose

Panel A shows the mean $\pm$ SEM changes in glucose concentration at 30-s bins for 30 minutes after the injection. Both 30 mg and 60 mg injections resulted in an overall significant rise in NAc glucose levels compared to baseline. Panel B shows the same data (mean $\pm$ SEM) analyzed at 2-s bin time resolution, and includes the change in glucose concentration after an iv saline injection (grey circles). Filled symbols in A denote individual bins significantly different from the pre-injection baseline by Fisher post-hoc test ($p < 0.001$). Vertical hatched lines show the injection onset (time 0) and offsets (B, 20 and 40 s for 30 and 60 mg doses).

Figure 2. Change in NAc glucose concentration (μM) after an iv injection of fructose

Panel A shows the mean±SEM changes in glucose concentration at 30-s bins for 30 minutes after the injections. While there was no significant change after 30 mg fructose dose, a 60-mg fructose injection resulted in a small, slow rise in NAc glucose that began ~3 min post injection. Panel B shows the same data at 2-s bin time resolution, where neither dose resulted in significant increases in NAc glucose. Filled symbols in A denote individual bins significantly different from the pre-injection baseline by Fisher post-hoc test ($p < 0.05$). Similar to Fig. 1, vertical hatched lines show the injection onsets (time 0) and offsets (time 20 and 40 s for 30 and 60 mg, respectively).

Figure 3. Differences in consumption of glucose and fructose during repeated exposure in naive rats

Panel A shows the time-course of consumption (mean ± SEM) during Days 1–5. Panel B shows changes in consumption (mean ± SEM) during substitution in glucose- and fructose-experienced rats. Panel C shows differences in mean (±SEM) volumes of glucose and fructose consumed in Experiment 1. Bold horizontal lines with asterisks in A and B show time intervals where difference in consumption was significant (Fisher LSD test after a significant effect assessed by two-way RM ANOVA). Asterisks inside the bars in C show significance (*, $p < 0.05$ and **, $p < 0.01$; Dunnett test after one-way RM ANOVA) of changes with respect to initial value on Day 1 and # reflects significant difference between marked bars. Data obtained from 8 and 7 rats for glucose and fructose, respectively.

Figure 4. Differences in consumption of glucose and fructose in two variants of preference test: stochastic presentation and two-bottle choice

A and C show the time-course of consumption volume ($\text{mean} \pm \text{SEM}$) and B and D show week-to-week and day-to-day differences in final consumption volumes. Bold horizontal lines with asterisks in A and C show time intervals, when both curves are different ($p < 0.05$ Fisher LSD test after significant Substance x Time interaction). Asterisks in B and D show significant (*, $p < 0.05$ and **, $p < 0.01$; Dunnett test after one-way RM ANOVA) changes in consumption relative to Week 1 and Day 1. # and ### show significant between-group differences ($p < 0.05$ and $p < 0.001$, respectively). Number of rats in each of two groups is 8.