SUPPORTING INFORMATION

Fluctuations in Nucleus Accumbens Extracellular Glutamate and Glucose during Motivated Glucose-drinking Behavior: Dissecting the Neurochemistry of Reward

by Ken T. Wakabayashi, Stephanie E. Myal, and Eugene A. Kiyatkin

(contains a full version of the Methods section, including supplementary Fig. S1 and additional references as well as two additional figures (Fig. S2 and S3) which are described in the main body of the manuscript).

METHODS

Subjects

Data from 23 male Long-Evans rats (Charles River Laboratories, Raleigh, NC, USA) weighing 440±40 g at the time of surgery were used in this study. Rats were housed individually in a climate-controlled vivarium maintained on a 12-12 hour light-dark cycle (lights on at 8:00AM), with food and water available *ad libitum*. All experiments complied with the "Guide for the Care and Use of Laboratory Animals" (8th edition, 2011, US National Research Council) and experimental protocols were approved by the Intramural Research Program (NIDA) Animal Care and Use Committee and the UK ARRIVE guidelines.

Motivated drinking behavior

In this study, we used a naturalistic model of motivated glucose-drinking behavior that required minimal training, was relatively uniform in all rats, and remained stable in individual animals. In contrast to food and water, which act as state-dependent reinforcers typically inducing eating or drinking only in food- or water-deprived conditions, glucose consumption is maintained without food or water deprivation, suggesting that its reinforcing properties are independent of deprivation state. In this model, rats were presented prior to surgery with 5mL of 10% glucose solution in a plastic cup (3.0 x 1.6 cm, secured to a metal plate for stability) for 2 hours twice per 6-hr session for 4 days. Only rats that learned to consume the glucose solution quickly and completely during the last training session were selected for surgery. Most naive rats quickly learned to consume the glucose solution after relatively limited experience (2-3 days) and continued to reliably perform this task during post-surgery retraining and the subsequent recording session. All behavioral procedures during training and subsequent recording were performed inside a Plexiglas chamber (32x47x47 cm) located inside an open-faced

plastic box (60x56x70 cm). The cage was illuminated continuously by a 20-W red light bulb, and a room-wide air filter fan provided background white noise. The bottom of the cage was covered with wood chip bedding, which was replaced daily. A small camera mounted above the cage permitted in-cage viewing and video recording of animal behavior, which was used later to synchronize behavior with electrochemical measurements.

Surgery

Surgical procedures for electrochemical experiments have been described in detail elsewhere (Wakabayashi and Kiyatkin 2012). Briefly, under general anesthesia (Equithesin 0.33 ml/100 g, ip; active ingredients: sodium pentobarbital, 32.5 mg/kg and chloral hydrate, 145 mg/kg), each rat was unilaterally implanted with a BASi cannula (Bioanalytical Systems, West Lafayette, IN, USA) for later insertion of the electrochemical sensor in the right NAc shell. The NAc is a critical brain structure involved in sensorimotor integration, behavioral regulation and reward (Mogenson *et al*. 1980; Wise and Bozarth 1987; Di Chiara 2002; Salamone and Correa 2012), it receives dense glutamate and DA inputs (Groenewegen *et al.* 1999, Ito and Hayen 2011), and its medial "shell" compartment is preferentially involved in mediating the primary reinforcing properties of natural stimuli and drugs of abuse (Cardinal 2003). This brain area has been well characterized by our previous electrophysiological (Kiyatkin and Rebec 1996, Kiyatkin and Rebec 1999), neurochemical (Kiyatkin and Gratton 1994; Kiyatkin *et al.* 2000; Kiyatkin and Lenoir 2012; Wakabayashi and Kiyatkin 2014) and temperature (Kiyatkin 2010) recordings. Moreover, glutamate responses elicited by novel arousing stimuli are clearly stronger in this compartment of the NAc compared to the core (Wakabayashi and Kiyatkin 2012). Target coordinates were: AP +1.2 mm, ML ±0.8 mm, and DV depth +7.6 mm from the skull surface, according to coordinates of Paxinos and Watson (1998). The cannula was secured with dental acrylic in a head mount anchored to the skull with three stainless steel screws. An obdurator was inserted into the cannula to prevent occlusions prior to use. After a minimum of 4 days post-operative recovery, rats underwent an additional 6 hr drinking session prior to the electrochemical recording on the next day.

Three rats, in addition to cannula implantation, were equipped with a chronic intragastric catheter during the same surgery. The catheter was implanted into the forestomach (the upper area with minimal vascularization) and tightly fixed to the stomach wall. After surgical closure of the abdominal muscular wall, the catheter was fed subcutaneously to an injection port on the head assembly. During recovery, the catheter was flushed daily with water to maintain patency.

Electrochemical sensors

Commercially produced glutamate oxidase-based glutamate, glucose oxidase-based glucose, and enzyme-free Null sensors (Pinnacle Technology, Lawrence, KS, USA) were used in this study. Each sensor is prepared from platinum-iridium wire (180μm diameter), with a ~1mm sensing cavity at the tip and a sensing area of ~0.56 mm^2. The active electrode is incorporated with an integrated Ag/AgCl reference electrode. On the active surface of glutamate sensors, glutamate oxidase converts glutamate to α-ketoglutarate and hydrogen peroxide (H_2O_2), which is detected as an amperometric oxidation current generated by a +0.6 V applied potential (Hu *et al.* 1994). Similarly, glucose oxidase on the sensor's active surface converts glucose to glucono-1,5-lactone and H_2O_2 on the active surface of glucose sensors, which is likewise detected as an oxidation current at +0.6 V (Hu & Wilson 1997). In both glutamate and glucose sensors, the contribution of ascorbate to the measured current is competitively reduced by co-localizing ascorbic acid oxidase on the active surface of the sensor to convert electroactive ascorbate to non-electroactive dehydroascorbate and water. A negatively charged Nafion polymer layer under the enzyme layer further helps to exclude endogenous anionic compounds. The currents from all sensors were passed to a computer via a potentiostat (Model 3104, Pinnacle Technology), and all electrochemical data were sampled at 1 Hz using the PAL software utility (Version 1.5.0, Pinnacle Technology).

As shown in our studies (Wakabayashi and Kiyatkin 2012, 2014; Kiyatkin and Lenoir 2012; Lenoir and Kiyatkin 2013) and recently reviewed (Kiyatkin *et al.* 2013), electrochemical currents detected *in vivo* by enzyme-based glutamate and, to a lesser degree, by glucose sensors used in this study are affected by three types of non-specific influences. Traditionally, the most important interferents are other cationic and anionic electroactive species present in the extracellular space that are oxidized by the same applied potentials. While the substrate-sensitive sensors are made to minimize these chemical contributions, each sensor used in this study was tested *in vitro* before and after *in vivo* recording to determine its actual substrate sensitivity and selectivity with respect to major oxidizable interferents. Second, electrochemical sensors are temperature-sensitive, showing increases or decreases in basal electrochemical currents with respective changes in temperature (Kiyatkin *et al.* 2013). Since brain temperature naturally fluctuates within 1-2°C under behavioral conditions (see Kiyatkin, 2010 for review) and consistent temperature changes within 0.5-1.0°C were reported during free feeding (Smirnov and Kiyatkin 2008) and drinking behavior (Smirnov and Kiyatkin 2010), this inescapable influence could significantly affect slow changes in electrochemical currents detected by substrate-sensitive sensors. Finally, electrochemical currents during single-session long-term *in vitro*

and *in vivo* recording show a consistent downward drift that could also affect slow changes in substrate-specific currents.

To reduce the influence of all these factors, we used Null sensors, which are similarly constructed but lack an active enzyme and, when used in the same type of *in vivo* experiment, are exposed to the same physical and chemical environment as substrate-sensitive sensors. As shown previously and confirmed in this study (see below), Null sensors are fully insensitive to either glutamate or glucose, but have similar temperature sensitivity, a similar downward current drift during long-term *in vitro* recordings, and a comparable sensitivity to ascorbate and DA, two possible chemical interferents to the electrochemical currents generated by glutamate and glucose sensors *in vivo*. Therefore, the difference in currents detected by the substrate-sensitive and Null sensors provides the best possible method of revealing actual changes in glutamate and glucose levels with this type of sensors. Electrochemical recordings employing different sensors were performed in separate rats to completely eliminate the possibility of electrical crosstalk between sensors during *in vivo* recording (Kiyatkin *et al.* 2013). Of course, the use of an independent group of rats introduced an additional source of between-group variability, requiring an equally large number of control animals with verified sensor locations to make reliable statistical evaluations.

Immediately before and after each *in vivo* experiment, all sensors were calibrated *in vitro* in PBS solution (pH 7.3, t° = 23°C). Glutamate sensors were calibrated by incrementally increasing the concentration of glutamate (L-glutamic acid monosodium salt hydrate; Sigma-Aldrich) from 0 to 2, 4, 6, and 16 µM followed by a single addition of 25 µM ascorbate ([+]sodium L-ascorbate; Sigma). Glucose sensors were calibrated by increasing glucose (D-[+]-glucose; Sigma-Aldrich) concentrations from 0 to 1, 2, and 3 mM followed by a single addition of 25 µM ascorbate. Null sensors were calibrated identically as respective substrate-sensitive sensors. Since the current response to glutamate and glucose directly depends upon temperature and this dependence as shown in multiple *in vitro* tests is very stable for different substrate-sensitive and Null sensors (Kiyatkin *et al.* 2013), all sensitivity values were corrected for 37°C (+84% for glutamate and +96% for glucose).

The glutamate sensors used in this study (n=9) varied slightly in their glutamate sensitivity (mean: 23°C=0.29±0.06 nA/µM; 37°C=0.54±0.11 nA/µM), producing incremental, highly linear (r=0.99) increases in current with increases in glutamate concentration ([glutamate]) during pre-recording calibrations (**Fig. 1a,c**). The *in vitro* detection limit of our electrodes was 0.02±0.002 nA, thus allowing us to detect ~38 nM glutamate after a single test, although the precision of glutamate detection is known to increase (as $\sqrt{n}$) with the averaging of repeated tests and also depends on the analysis quantification

bin. The response time measured *in vitro* following rapid glutamate delivery was ~1-3 s. Glutamate sensors showed current changes with addition of ascorbate (mean 0.07±0.02 nA/25 µM; mean selectivity ratio 1:104). Electrodes with low sensitivity to glutamate (<0.1 nA/1 µM) and/or low selectivity against ascorbate (<1:50) were not used. Post-recording calibrations of glutamate sensors revealed an approximately two-fold decrease in their sensitivity to both glutamate (0.13±0.03 nA/1 µM) and ascorbate (0.034±0.009 nA/25 µM), with virtually unchanged glutamate-ascorbate selectivity ratio (1:94). This decrease in sensitivity is consistent with other studies using sensors of similar design (Behrend *et al.* 2009, Naylor *et al.* 2011) and may be due in part to fouling of the active surface (Kulagina *et al.* 1999) or disruption of the enzyme layer during sensor removal from the brain. As confirmed in this study, glutamate sensors are sensitive to DA (0.24±0.01 nA/1 µM) but current changes within the range of basal DA levels detected by no-net flux microdialysis (~5-25 nM; Parsons and Justice 1992, Crippens *et al.* 1993) and its physiological fluctuations (~30-70 nM; Kiyatkin and Gratton 1994, Wise *et al.* 1995; Wightman *et al.* 2007; Owesson-White *et al.* 2012) are within the background noise (~12 and 24 pA for 50 and 100 nM DA change, respectively). The temperature sensitivity of glutamate sensors determined *in vitro* was 0.14±0.04 nA/1.0°C (within 35-38°C).

The glucose sensors used in this study (n=9) showed incremental increases in current with increases in glucose concentration (**Fig. 1b-d**). Within the range of extracellular glucose levels detected by microdialysis and electrochemistry in the rat brain (1.0-2.0 mM; Fellows *et al.* 1993; McNay and Gold 2002; Dunn-Meynell *et al.* 2009), substrate sensitivity of the glucose sensors averaged 6.13±1.18 nA/1 mM (22-23°C) or 11.98 nA/1 mM (37°C) and was highly linear. Similar to glutamate sensors, glucose sensors showed very small changes in current with addition of ascorbate (mean 0.03±0.01 nA/25 µM), lower sensitivity to DA (~0.1 nA/1 µM) but they were equally temperature-sensitive (0.13±0.03 nA/1.0°C). The mean post-recording calibration curve for glucose sensors was almost identical to the pre-recording curve, and sensitivity and selectivity remained virtually unchanged after 7-8 hours of *in vivo* recording. This robustness of glucose sensors has been shown previously in both single-day and multi-day recordings (Kiyatkin and Lenoir 2012; Dash *et al.* 2013).

As expected, Null sensors were fully insensitive to both glutamate and glucose (**Fig. 1c-d**) and they showed comparable but slightly weaker current responses to both ascorbate (0.02 nA/25 µM) and DA (0.03-0.15 nA/1 µM) than substrate-sensitive probes. As shown previously, Null sensors were equally temperature-sensitive (0.19±0.02 nA/1.0°C) as both glutamate and glucose sensors and they showed a similar downward trend in baselines following long-term in vitro and in vivo recordings.

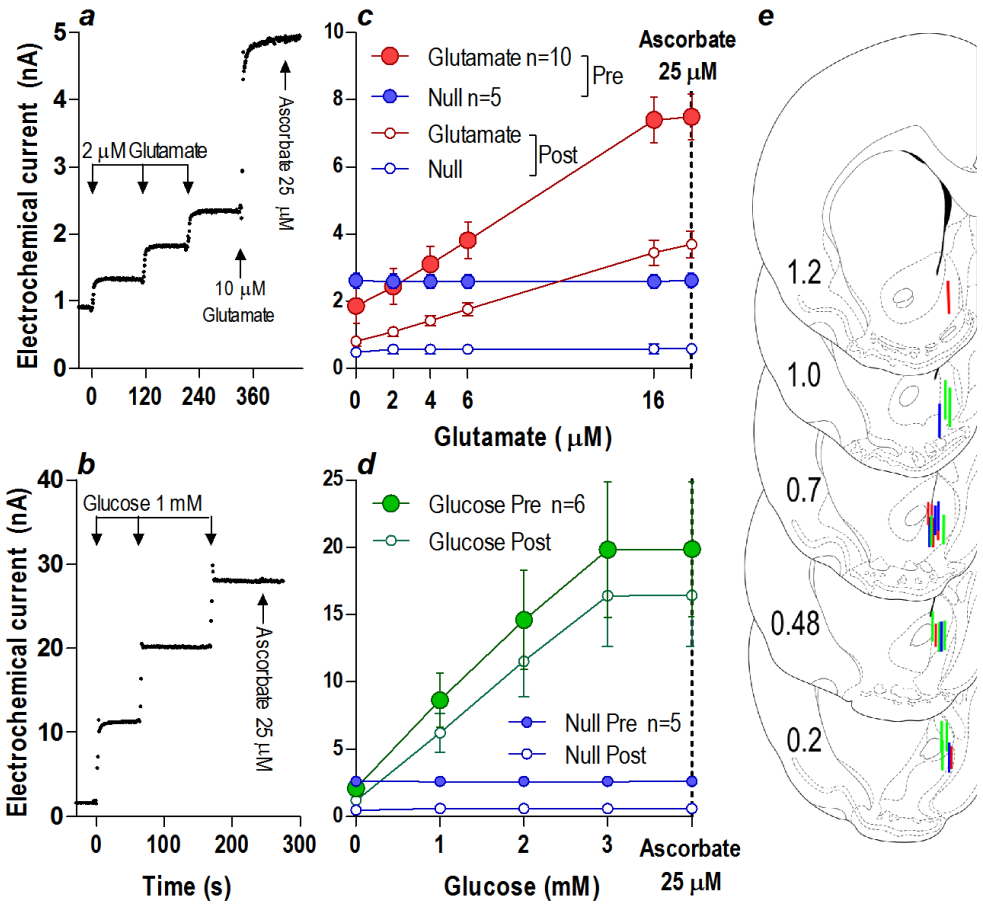

Fig. S1. Results of pre-recording and post-recording calibrations of glutamate, glucose and Null sensors (a-d) and their locations within the NAc (e). Left panel (**a** and **b**) shows examples of real-time changes in electrochemical currents of glutamate and glucose sensors during *in vitro* pre-recording calibrations. Arrows indicate the time test substances were added. Panels **c** and **d** show mean changes in currents versus substrate concentration during pre- and post-recording sensor calibration. During pre-recording calibrations, both types of substrate-sensitive sensors showed linear increases in electrochemical currents following repeated applications of either glutamate (2 µM) or glucose (1 mM) and weak increases following applications of ascorbate (25 µM). During post-recording calibrations, both basal currents and responses to substrates slightly decreased; the change was minimal for glucose sensors and stronger for glutamate sensors (see text for quantitative analyses). In contrast, Null sensors during both pre- and post-recording calibrations were fully insensitive to glutamate and glucose but showed a comparable response to ascorbate. Right panel (**e**) shows location of each sensor's active area (red, glutamate; blue, Null; green, glucose) in coordinates of the stereotaxic atlas of Paxinos and Watson (1998).

Experimental protocol

On the day of electrochemical recording, rats were minimally anesthetized (<2 min) with isoflurane and a calibrated sensor (glutamate, Null, or glucose) was inserted into the right NAc shell through the guide cannula. The rat was then placed in the testing chamber and the sensor was connected to the potentiostat via an electrically shielded cable and a multi-channel electrical swivel. Testing began a minimum of 150 min after sensor insertion to allow the baseline current to stabilize (see Results). First, to verify sensor functionality and the specificity of drinking-induced glucose responses, rats were exposed to control sensory stimuli that were not related to our behavioral task. They included a 3-min tail-touch, a mild, behaviorally activating somatosensory stimulus that induces phasic release of NAc glutamate and glucose (Wakabayashi and Kiyatkin 2012, 2014; Kiyatkin and Lenoir 2012). Since NAc glucose currents show even more rapid, transient increases following exposure to simple sensory stimuli (Kiyatkin and Lenoir 2012), in glucose sensor experiments rats were also exposed to a brief auditory stimulus (75 dB, 0.25 s) that induces rapid, transient EEG desynchronization and EMG activation, with no evident behavioral changes (Kiyatkin and Smirnov 2010). These control tests were important for evaluating the role of sensory input and arousal in determining certain components of changes in [glucose] found during drinking behavior. One hour after sensory stimulus controls, rats were presented with a cup containing 5 mL of 10% glucose solution, and the elapsed time from cup presentation to the beginning and end of drinking (after the entire 5-mL volume was fully consumed) was confirmed visually by two observers and by video recording. Prior to presentation, the glucose solution was warmed to 37^{o}C to control for possible changes in glutamate and glucose currents due to changes in brain temperature that may result from drinking cool liquids. Removal of the empty cup from the cage occurred a minimum of 40 min after its presentation and the second glucose cup was presented a minimum of 30 min after cup removal. After two presentations of 10% glucose, an identical volume of water (also warmed to 37^{o}C) was presented as a comparison condition. During this test, although rats approached the cup normally and began to drink, they quickly refused to consume more. Therefore, we could compare neurochemical dynamics under conditions of reward omission.

Since drinking of glucose solution should eventually result in its entry to the brain, we examined how glucose directly injected into the stomach at the same concentrations and volumes via an intra-gastric catheter would affect NAc glucose levels and how these changes differ from those occurring during drinking behavior. This test allowed us to distinguish the component of the glucose response related to behavior and reveal the contribution of behavior-associated metabolic use of glucose. In three rats equipped with chronic intra-gastric catheters, we examined changes in NAc glucose currents

during stress- and cue-free passive intra-gastric delivery of glucose at the same volume, concentration and rate (5 mL of 10% solution, warmed to 37°C and delivered over ~200 s) as consumed in behavioral experiments. Each rat typically received one water and two glucose injections at 120-min inter-injection intervals.

At the end of the recording, rats were removed from the cage and lightly anesthetized with isoflurane (<2 min), and the biosensor was removed for post-recording calibration.

Histological verification

After recording was completed, all rats were deeply anesthetized with Equithesin (0.4 mL/100 g, ip) and transcardially perfused with PBS followed by 10% formalin. Sensor placements were verified on 45 µm brain slices using the stereotaxic atlas of Paxinos and Watson (1998). Results of sensor placement verification for glutamate, glucose, and Null sensors are shown in **Fig. 1e.**

Data analysis

All data obtained in rats with incorrect sensor placements and all data samples that had technical complications during the recording were removed from further analysis. All recordings during drinking tests were verified to be free from extraneous artifacts before being included in the subsequent data analysis. Several approaches were used in statistical data analyses. First, we evaluated basal levels of extracellular glutamate and glucose in the NAc. We determined these values by taking the difference in mean absolute currents detected by substrate-sensitive and Null sensors before a cup presentation (quiet resting conditions), and then calculating the concentration based on the sensor's substrate sensitivity corrected for 37°C.

Since trained rats showed variable latencies to initiate drinking after cup presentation and exhibited different durations of drinking, we then analyzed relative changes in electrochemical currents preceding and following key behavioral events (peri-event analysis). For the glucose-drinking test, these events were: glucose cup presentation, initiation and end of drinking, and the removal of an empty cup. Since rats did not drink significant amount of water, when it was substituted for the glucose, only two behavioral events (water cup presentation and initiation of drinking) were analyzed and compared with the analogous events during the glucose-drinking test. For these analyses, we determined relative changes in current (vs. baseline=0 nA) detected by substrate-sensitive and Null sensors and, based on their difference, determined relative changes in [glutamate] and [glucose] preceding and following the events of interest. Different quantification bins (2-60 s, indicated in figure legends) and different sample durations were used for individual data analyses to accurately represent and properly statistically evaluate of both phasic and tonic changes. Two-way repeated-measures (RM) ANOVAs were initially

used for evaluating differences in the current dynamics produced by substrate-sensitive and Null sensors. Since currents were analyzed as a change from 0 nA baseline, the length of the effect was determined as the duration when either the substrate-specific currents were different from Null (main effect) or when the substrate-specific currents were changing with respect to the Null current (Current × Time interaction). We then evaluated the pattern and magnitude of changes in [glutamate] and [glucose] associated with these events by subtracting substrate-specific currents from the mean Null sensor current, and transforming this value into concentrations. These data were further analyzed using one-way (RM) ANOVA. If a significant effect of time was detected, individual data points were compared against the peri-event baseline point using Fisher post-hoc tests to detect the latency and duration of this change.

After conducting these individual peri-event analyses and determining mean basal values of [glutamate] and [glucose] immediately preceding each behavioral event, we evaluated the pattern of fluctuations in these neurochemical parameters during the entire glucose-drinking behavior taking into account their tonic changes and essential variability of natural animal behavior. Linear regression was then used to assess the relationship between [glutamate] and [glucose] during the entire behavior. For text clarity, some quantitative results of statistical evaluations are shown in figure captions.

Two-way (RM) ANOVA was also used to directly compare differences in [glucose] and [glutamate] responses to cup- and "tasting"-associated events associated with different outcomes (i.e. presentation of glucose- vs. water-containing cup vs. cup removal). Individual bins within periods where there were significant main effects or interactions were further assessed by Fisher post-hoc tests.

To evaluate the possible influence of ingested glucose on the extracellular glucose levels detected in the NAc, changes during a glucose-drinking session were compared to those occurring after passive intra-gastric delivery of glucose at the same volume. These data were also compared to those obtained with control intra-gastric water injections at the same volumes. Since extracellular glucose levels depend upon two variables, its inflow from peripheral blood and its cellular uptake for consumption, this comparison allowed us to shed light on metabolism-related glucose use during motivated glucose-drinking behavior.

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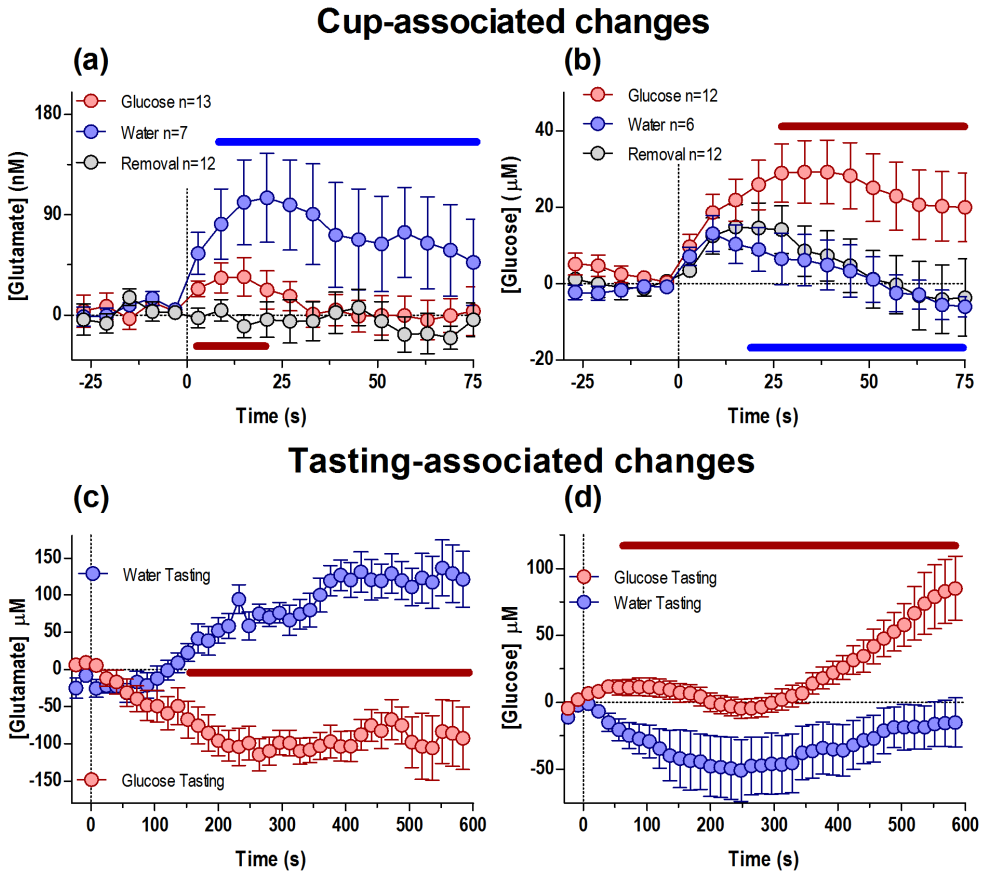

Fig. S2. NAc [Glutamate] and [Glucose] responses to similar types of events differ depending on the behavioral response. Top graphs show the mean (±SEM) changes in [glutamate] (a) and [glucose] (c) analyzed at 6-s bins after the presentation of a glucose-containing cup, water-containing cup and removal of empty cup. Bottom graphs show the mean (±SEM) changes in [glutamate] (c) and [glucose] (d) analyzed at 16-s bins after rats tasted the glucose solution or water. There was a significant difference in changes between presentation of the glucose-cup and removal of empty cup (glutamate, 15 s: main effect $F_{1,23}$=4.43, Event x Time interaction $F_{3,69}$=3.26; glucose, 75 s: Event x Time interaction $F_{13,286}$=2.15, all $p<0.05$). Individual bins that differed from each other denoted as a red line (Fisher test). Note that the individual differences in glutamate and glucose responses have different onsets. The response to the water cup differed significantly from the response to the glucose cup (75 s: Content x Time interaction, glutamate $F_{13,234}$=1.96; glucose $F_{13,208}$=2.19, both $p<0.05$). Individual bins that differed from each other denoted as a blue line (Fisher test). There was a significant difference between the glutamate response to water cup presentation and cup removal (75 s: main effect $F_{1,17}$=6.01, Event x Time interaction $F_{13,221}$=2.97, both $p<0.05$, all individual bins significantly different, Fisher test $p<0.05$), but no difference in the glucose response. After tasting glucose or water both neurochemical parameters differed significantly (584 s, main effect: glutamate $F_{1,18}$=42.0; glucose $F_{2,26}$=14.04, Content x Time interaction: glutamate $F_{37,666}$=7.27; glucose $F_{37,592}$=2.22, all $p<0.05$). Individual bins that differed from each other are denoted as a red line (Fisher test). Note that while all rats tasted both solutions, they fully consumed the glucose solution but refused to consume water.

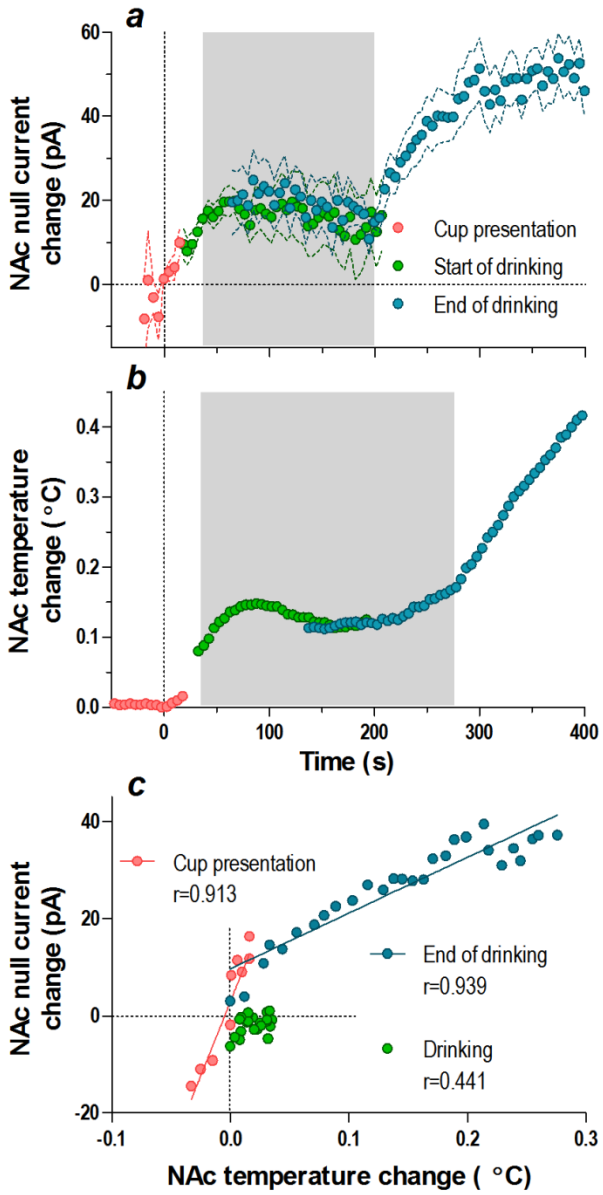

Fig. S3. Behavior-related changes in currents detected in the NAc by Null sensors tightly correlate with NAc temperatures. (a) Mean (±SEM) changes in electrochemical current detected in the NAc by Null sensors during glucose-drinking test. (b) Mean changes in NAc temperature during a similar behavioral test (Smirnov and Kiyatkin, 2010). Vertical lines at 0 s show the time of cup presentation and gray areas show duration of drinking. **(c)** The relationships between NAc Null electrochemical currents and NAc temperature during drinking behavior. Regression lines and correlation coefficients are shown separately for three time intervals (red, between cup presentation and start of drinking; green, during drinking; blue, post-consumption interval. While the correlation was strong when both parameters increased (cup presentation and post-drinking; r=0.913 and 0.939, $p < 0.01$), it was absent (r=0.44) during drinking behavior *per se*.