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Feeding specific glutamate surge in the rat lateral hypothalamus revealed by low-flow push–pull perfusion

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Abstract

Substantial evidence implicates the lateral hypothalamus (LH) in the control of ingestive behavior and previous studies have found that glutamate release within the LH increases during meals. It is not known, however, whether this effect is selective for feeding, or whether similar changes are also seen during drinking. In this work, we examined this question using low-flow push–pull perfusion which allows sampling from small tissue volumes. Presentation of highly palatable solid or liquid foods to food-deprived rats resulted in an immediate increase in glutamate output of more than 200% over baseline. The response was maximal immediately after food presentation. In contrast, significant changes in glutamate output were not seen when water was presented to water-deprived animals, despite the occurrence of vigorous drinking. These findings confirm reports of feeding related glutamate release in the LH and demonstrate that this effect is specific to feeding, rather than being a general concomitant of all ingestive behaviors. The push–pull technique described here may allow the relevant region of the LH to be identified with greater precision than other methods.

Keywords: Feeding; Drinking; Food; Thirst; *In vivo* sampling; Glutamate; Glutamic acid; Excitatory amino acid; Perifornical region

1. Introduction

Obesity is a major health problem, contributing to disorders such as diabetes, heart disease, hypertension and cancer (Angelopoulos et al., 2005; Dhillo, 2007; King et al., 2007; Morton et al., 2006; Wilding, 2002) and which is associated with increased mortality (Adams et al., 2006; Conway and Lip, 2004). The prevention and treatment of obesity, and other disorders of food intake, clearly requires a better understanding of the neural mechanisms underlying ingestive behavior than is currently available. A great deal of evidence indicates that the lateral hypothalamic area (LH) plays an important role in the control of food intake. Classical studies indicated that electrolytic lesions of the LH lead to long lasting reductions in food intake and body weight and, conversely, electrical stimulation of this region was shown to induce feeding. Although these results could have been due to damage to fibers of passage, more recent work has confirmed that disturbances in feeding behavior can be produced by axon sparing lesions of the LH and by injections of a variety of neurotransmitters into the LH. In particular, Stanley and his coworkers have shown that injections of the excitatory amino acid glutamate into the lateral hypothalamus are able to induce robust feeding (Stanley et al., 1993, 1996). Similar effects can be observed following injections of glutamate agonists (Duva et al., 2002) and mapping studies have shown the central, tuberal, portion of the LH is more sensitive to this effect than are a number of adjacent regions (Stanley et al., 1993). Conversely, repeated local injections of NMDA type glutamate receptor antagonists are able to

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reduce food intake and body weight (Stanley et al., 1996). The possibility that glutamate in the LH is involved in the normal, physiological, control of feeding is further by studies which have used *in vivo* microdialysis to demonstrate that glutamate release in the tuberal LH is dramatically increased at the onset of feeding (Rada et al., 1997, 2003). In contrast, glutamate output from the nucleus accumbens shell has been shown to decrease in association with feeding (Rada et al., 1997), demonstrating that the response observed in the LH is not present globally throughout the brain.

The LH is clearly involved in the control of feeding, but a substantial body of work using lesioning, electrical stimulation, and imaging techniques indicates that it also exerts a pronounced influence on water intake. It is striking, therefore, that intra-LH injections of glutamate agonists have no effect on water intake, in marked contrast to their robust effects on feeding (Duva et al., 2002; Stanley et al., 1993). These results raise the possibility that glutamate in the tuberal LH is specifically involved in the control of food, but not water, intake. In evaluating this possibility, it would be interesting to know whether drinking, like feeding, is associated with increased LH glutamate output, but such information is not available at this time.

In the current study, we examined the relation between ingestive behavior and LH glutamate release using the method of low-flow push–pull perfusion (LFPP) combined with capillary electrophoresis (CE) to monitor glutamate output. This technique allows sampling from much smaller tissue volumes that would be possible using other techniques, such as *in vivo* microdialysis (Kottegoda et al., 2002; Stanley et al., 1993), and has been previously used to measure the extracellular content of a number of neuroactive compounds (Cellar et al., 2005; Gao et al., 2004, 2007; Kottegoda et al., 2002, 2007; Thongkhao-on et al., 2004; Zhao et al., 2003, 2004). In this work, we have examined feeding behavior during LFPP sampling to determine extracellular glutamate levels in the perifornical region of the LH. In particular, we examined glutamate output during the consumption of solid food and during the drinking of water. In addition, to determine whether differences observed between these to conditions might reflect the occurrence of chewing versus licking behavior, we examined glutamate output in animals consuming Ensure®, a highly palatable liquid diet. A brief version of some of these results has previously been presented in abstract form (Thongkhao-on et al., 2006).

2. Methods

2.1. Subjects and surgery

2.1.1. Subjects

Adult, male Sprague–Dawley rats weighing from 290 to 450 g at the time of surgery were used. These rats were bred in the Psychology Department of the University of Illinois at Chicago, and were descended from rats obtained from Charles River. They were individually housed in a temperature controlled room on a 12 h:12 h light:dark cycle (lights on at 06:00) and allowed free access to standard Purina rat chow pellets and tap water, except as noted below. All experiments were performed under a protocol reviewed and approved by the UIC Institutional Animal Care and Use Committee.

2.1.2. Surgery

The rats were anesthetized with Nembutal (50 mg/kg) (Abbott Laboratories, North Chicago, IL), and unilateral 22-gauge stainless steel guide cannulae (Plastics One, Roanoke, VA) were implanted using standard stereotaxic techniques. The guide cannulae were aimed so that they terminated in the LH at coordinates anterior–posterior −2.6 mm, lateral–medial 1.8 mm, dorsal–ventral −8.0 mm from bregma (Paxinos and Watson, 1998) and were held in place using stainless steel screws and dental cement. A stainless steel obturator, extending 1 mm beyond the end of the guide cannula, was inserted into the lumen of each cannula to help maintain patency. All rats were allowed to recover in their home cages for at least 7 days before the start of experiment.

Behavioral testing was conducted using the Raturn Interactive Caging System (BAS, West Lafayette, IN), in which rotary movements of the animal trigger an opposite rotation of the bowl in which the animal is placed, thus preventing tangling of the infusion and withdrawal lines. On each of the three or four days prior to testing, animals were transferred to a Raturn bowl (BAS, West Lafayette, IN) containing a Honey Graham cracker (Keebler, Battle Creek, MI) and water for few hours to familiarize the animal with the experimental bowl and test food. Before animals were returned to their home cages, the push–pull vacuum pump was turned on to provide the sound environment as the real testing conditions for 30 min.

2.2. Probe construction

A detailed description of the low-flow push–pull perfusion system is provided elsewhere (Kottegoda et al., 2002). The concentric single internal cannula push–pull probes were made from a 12 mm long, 28 gauge stainless steel cannulae (Plastic One, Roanoke, VA) into which an inner withdrawing cannula made of 50/150 μm (i.d./o.d.) fused-silica capillaries (Polymicro Technologies, Phoenix, AZ) was inserted.

2.3. Animal sampling

Animals were food or water deprived for 20 h prior to the sampling experiment. Details of the low-flow push–pull sampling technique can be found elsewhere (Kottegoda et al., 2002). Briefly, each animal was gently removed from its home cage, the obturator was then removed and replaced by the 28-gauge concentric probe push–pull probe which extended 1.0 mm beyond the end of the guide. The animal was placed in Raturn bowl. A PHD 2002 programmable syringe pump (Harvard Apparatus, Holliston, MA) was used to pump Kreb-Ringer Buffer (KRB; 3 mM KCl, 145 mM NaCl, 1.2 mM $CaCl_2$, 1 mM $MgCl_2$, 1.61 mM $NaHPO_4$, and 0.4 mM NaH_2PO_4) through the infusion capillary and the withdrawal capillary was connected to a vacuum pump (Barnant Co. Barrington, IL). Infusion rates and vacuum strength were adjusted to generate flow rates of 35–50 nl/min. Samples were collected every 5 min for analysis.

Collected sample volumes were immediately determined by measuring a length of tygon tubing.

2.4. Capillary electrophoresis (CE)

Experiments were carried out on a laboratory-built CE system with a commercial high-voltage power supply (Spellman, Hauppage, NY, USA) and a ZETALIF laser induced fluorescence (LIF) detector (Picometrics, Paris, France) operating with an argon ion laser (Coherent, Santa Clara, CA, USA) at 488 nm. We have previously described the analytic methods in detail (Thongkhao-on et al., 2004). Briefly, the samples were reacted with 3-(4-carboxybenzoyl) quinoline-2-carboxaldehyde (CBQCA) and potassium cyanide for 2 h and then run on the CE system. The running buffer was a 20 mM borate buffer with 50 mM sodium dodecylsulfate, 55 mM β-CD. The blanks, amino acid standards or brain samples were injected by gravity for 10 s at an 8.0 cm displacement and the separation was performed at 540 V/cm. All CE experiments were performed at room temperature (25 °C) with 38 cm × 360 μm o.d. × 50 μm i.d. (effective length of 30 cm) fused-silica capillary tubing (BioTAQ, Gaithersburg, MD, USA). The detector signal was collected by a custom LabVIEW (National Instruments, Austin, TX, USA) program and the data were analyzed using Microsoft Excel. The peaks for analytes were identified by matching the migration time with those in the spiked samples and measured by comparing their peak height ratios with standards. Fig. 1 shows a typical electropherogram of a brain perfusate.

2.5. Histological analysis

Following sampling, animals were allowed to survive with the probe in place for 4–7 days after which time they were deeply anesthetized with Nembutal and perfused transcardially with 50 ml of a 0.15 M saline solution, followed immediately by 500 ml of a 10% buffered formalin solution. The brains were removed from the skull and placed in the same fixative for at least 24 h. They were then frozen, and 50 μm coronal sections were taken throughout the extent of the injection sites. The sections were stained with cresyl violet, and the injection sites were examined for placement accuracy and excessive damage. Data from rats with misplaced cannulae were not included in the analyses.

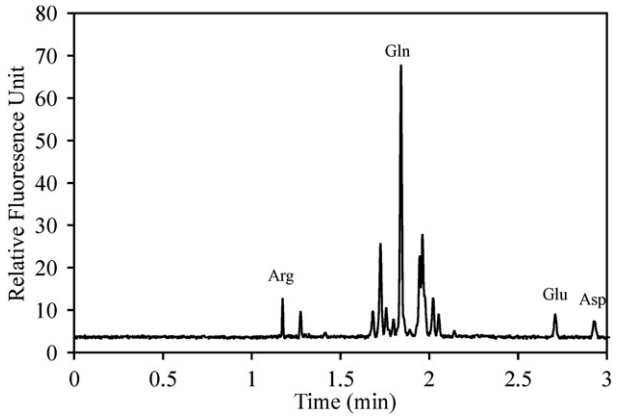

Fig. 1. Electropherogram of a LH brain perfusate. Separation was performed with a capillary length of 38 cm (with 30 cm to detection window) and a working voltage 21.5 kV. The run buffer consisted of 20 mM sodium borate (pH 9.3), 50 mM sodium dodecylsulfate, 55 mM β-cyclodextrin.

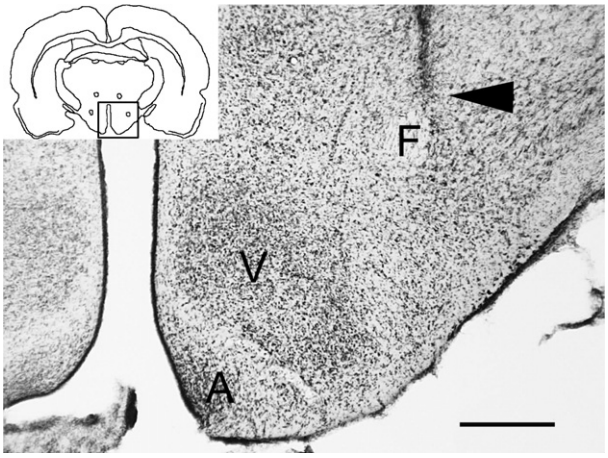

Fig. 2. Photomicrographs of coronal, Cresyl violet-stained sections of the rat brain showing a representative sampling site as indicated by arrow Abbreviations: F, fornix; A, arcuate hypothalamic nucleus; V, ventromedial hypothalamic nucleus. Coronal section is adopted from Paxinos and Watson (1998). Scale bar = 250 μm.

2.6. Behavioral analysis

2.6.1. Experiment 1

The probes were inserted and animals were placed in the testing bowl for 2–2.5 h prior to the presentation of test objects. Animals were then given access to the test objects for a period of 20 min after which they were removed. Data collection continued for at least 15 min following test object removal. Three groups of subjects were studied. Group 1 consisted of animals that were given a plastic Nylabone (Nylabone Products, Neptune, NJ) for 20 min at 2 to 2.5 h after probe was inserted. This group was intended to serve as a control. Group 2 animals were presented with several Honey Graham Crackers and group 3 subjects with tap water. Subjects in groups 1 and 2 were food deprived at the time of testing, whereas group 3 subjects were water deprived.

2.6.2. Experiment 2

Subjects in this experiment were treated identically to those in group 2 above, except that they were presented with a drinking tube containing Ensure®. These animals were also allowed to acclimate to the Ensure® solution on pretesting days, and were tested while food and water deprived.

3. Results

3.1. Experiment 1. Glutamate output during intake of solid food or water

Histological examination indicated that the tips of the push–pull probes were located within the tuberal portion of the LH in all subjects. An example of a typical probe location is shown in Fig. 2.

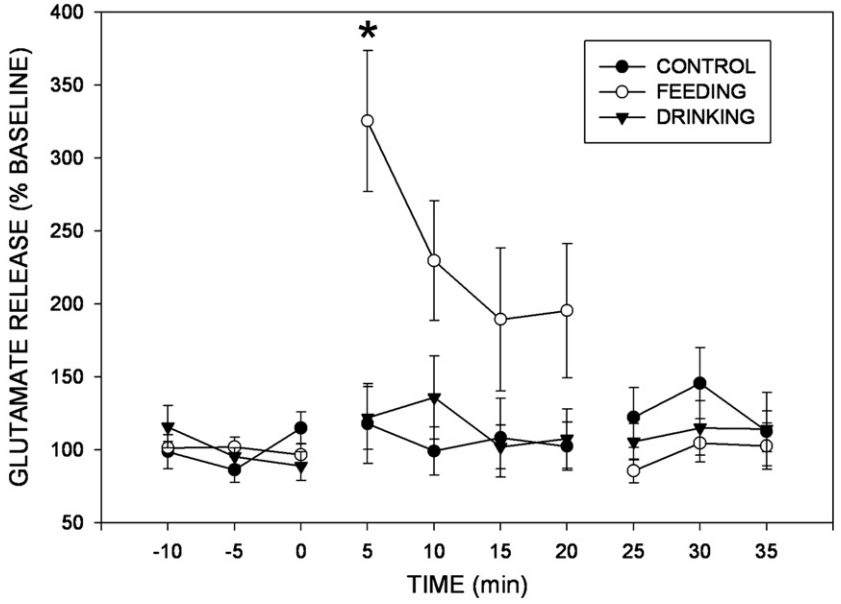

Fig. 3. Perifornical LH perfusate glutamate level (% basal±S.E.M.) as a function of ingestate type. Data are shown for the 15 min before and after ingestate presentation as well as during the presentation period. The control condition consisted of a 20 min presentation of Nylabones. The feeding condition was a 20 min presentation of Graham crackers. The drinking condition was a 20 min presentation of a water bottle. The glutamate level was significantly increased ($P<0.001$) during presentation of food, but not in the control and water conditions. The glutamate level at the first time point following presentation (*) is significantly increased relative to the preceding time point ($P<0.003$).

All subjects given food or water began to eat or drink within 1 min of presentation of the ingestates. Most subjects ate or drank continuously for several minutes and then stopped, and sometimes began subsequent bouts of ingestion at later times. Most subjects either ignored the Nylabones, or oriented to them only briefly when they were first presented.

Glutamate output across the period extending from 15 min before to 15 min after food presentation is shown in Fig. 3, where data are expressed as a percentage of mean perfusate level during the 15 min period preceding presentation of ingestates. The mean baseline glutamate perfusate level (not corrected for recovery) across all subjects was 1.71±0.38 μM and no significant differences between groups were seen. As can be seen, presentation of food, but not of water or of Nylabones, resulted in an immediate increase in glutamate output which was maximal during the first post-presentation time bin. These data were analyzed by two different approaches: First, we examined responses during the four time bins during which food, water or Nylabones were present by means of a 3×4 (experimental group×time bin) analysis of variance (ANOVA) with repeated measures on the second factor. This analysis indicated a significant effect of group ($F(2,17)=16.6$, $P<0.001$), but not of time ($F(3,51)=1.94$, $P>0.1$). There was a trend towards a group×time interaction, but this effect did not reach significance ($F(6,51)=1.95$, $P>0.09$). Post hoc comparisons using the Tukey technique indicated that glutamate levels of the animals given food was significantly higher across the post-presentation period than that of subjects given water or Nylabones ($P<0.001$ in both cases). In contrast, the responses of animals given water or Nylabones did not differ significantly from each other ($P>0.5$).

In order to determine whether glutamate output following food presentation was significantly increased compared to baseline levels, we conducted a one-way, repeated measures ANOVA on data collected from the time bin before and the four time bins after food presentation. Planned contrasts were used to compare output during the bin preceding presentation of the test objects to that occurring during each of the post-presentation bins. This analysis indicated that glutamate output was significantly increased during the first time bin following food presentation ($F(1,5)=29.5$, $P<0.003$), but that the difference from baseline was not statistically significant during any of the remaining three time bins ($0.07<P<0.2$). Similar analyses performed on data from animals given water or Nylabones indicated that there were no significant differences from baseline glutamate output during any of the four post-presentation time bins. In contrast to the effects observed on glutamate release, perfusate levels of arginine and glutamine were not significantly altered in response to feeding (data not shown).

In order to confirm the finding that feeding is associated with increased glutamate release from the LH, and to provide functional verification that the cannulae in the control subjects were appropriately placed, food was presented to five of the control animals 40 min following removal of the Nylabones. As can be seen in Fig. 4, food presentation was again associated with an immediate increase in glutamate release. The data for the time bin before and the four time bins following food presentation were analyzed with a one-way, repeated measures, planned comparisons ANOVA. This analysis indicated that food presentation was associated with an increase in perfusate glutamate which significant during the first ($F(1,4)=17.6$, $P<0.02$) and second ($F(1,4)=8.8$, $P<0.05$) time bins, but not at later times ($P>0.1$).

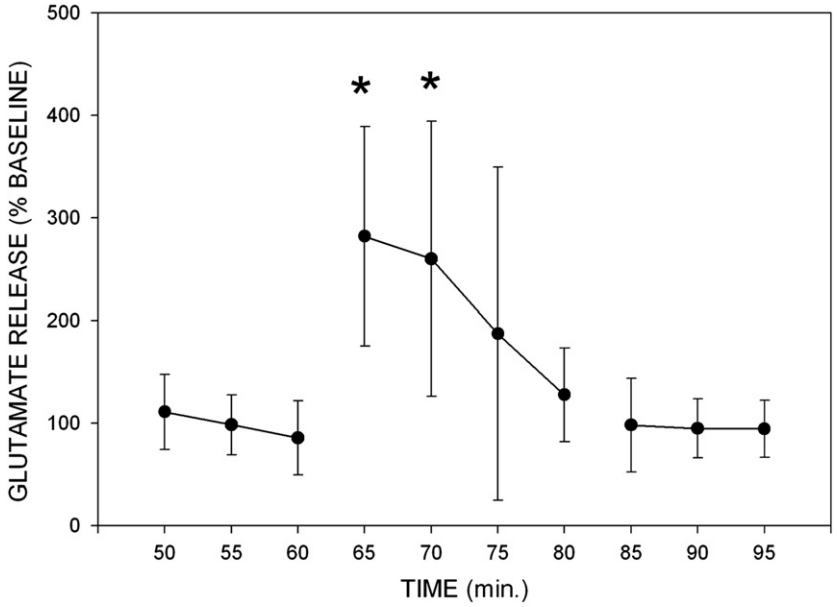

Fig. 4. Perifornical LH perfusate glutamate level (% basal ± S.E.M.) in the control animals shown in Fig. 3 who were presented with food 40 min after removal of the Nylabones. (The scale on the abscissa continues that used in Fig. 3 and represents time since the presentation of the Nylabones.) The glutamate level is increased with the presentation of food. Glutamate levels in the first ($P<0.02$) and second ($P<0.05$) samples after food presentation are significantly higher than those at the time point before food presentation (*).

3.2. Experiment 2. Glutamate output of subjects drinking Ensure®

Histological analysis indicated that probe placements in these subjects were very similar those studied in the first experiment. All animals began drinking in the first time bin following Ensure® presentation, and most subjects drank, with only brief interruptions, for most of the remainder of the 20 min presentation period. Data for glutamate output is shown in Fig. 5 and was analyzed using a one-way, repeated measures, planned comparisons, ANOVA. This analysis showed that, relative to baseline, glutamate levels were increased during the first time bin following Ensure® presentation, ($F(1,5)=7.4$, $P<0.05$), but not at later times ($P>0.2$).

4. Discussion

We demonstrate here for the first time that low-flow push–pull perfusion combined with CE-LIF analysis can be used to

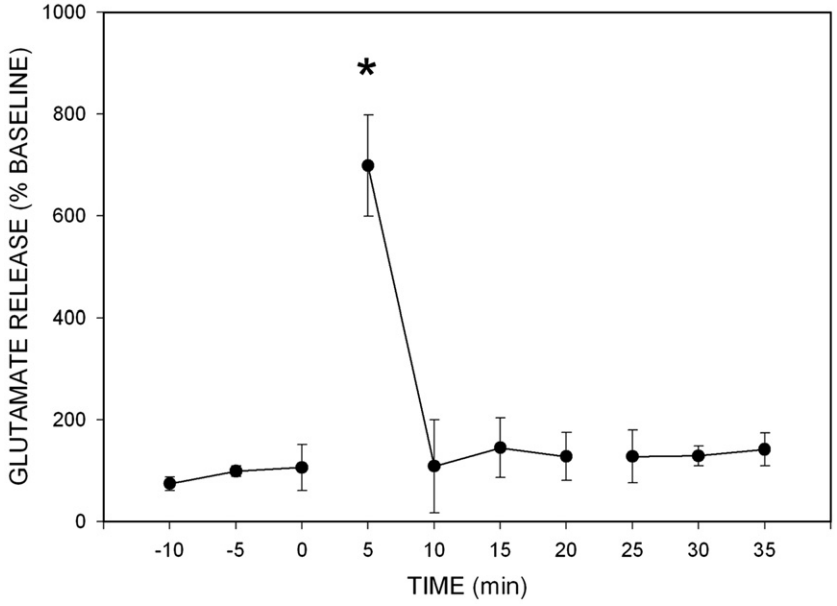

Fig. 5. Perifornical LH perfusate glutamate level (% basal + S.E.M.) during presentation of Ensure® and for the 15 min periods before and after ingestate presentation. The glutamate level from the first sample after Ensure® presentation is significantly higher than that in the sample immediately preceding presentation of the ingestate ($P<0.05$) (*).

monitor extracellular glutamate levels in the brain of awake, freely behaving animals. As we have discussed elsewhere (Kottegoda et al., 2002) it is likely that this method allows for sampling from substantially more restricted volumes than would be possible using conventional *in vivo* microdialysis. In agreement with previous studies (Rada et al., 2003) which used *in vivo* microdialysis, we observed that glutamate release in the LH is markedly increased in during feeding. The effects observed here tended to be of larger magnitude that those observed in microdialysis studies, perhaps resulting from the fact that the method we employed allowed us to sample more selectively from the relevant region of the hypothalamus. It was striking that increased glutamate release could be detected in animals consuming either a solid or a liquid diet; suggesting that the response was related to food consumption per se, and not to the production of particular motor patterns.

Previous studies suggested that glutamate output increases at the onset of the meal, and then declines even though feeding continues. A similar pattern was clearly present in the current experiments in the subjects tested while consuming Ensure®; most of these animals drank throughout almost all of the testing period, and yet glutamate release was increased only during the first time bin. An analogous trend could be seen in the rats consuming solid food; in these animals the increase in glutamate output, relative to baseline, was only significant during the first time bin. A trend towards an increase was, however, still seen during the later time bins, which might be related to the fact that many of the subjects tested under these conditions took several discrete meals during the sampling period. It would be interesting, in future studies, to examine the relation between glutamate output and the precise temporal structure of feeding in more detail.

It is difficult to specify, from the data currently available, the precise way in which lateral hypothalamic glutamate is involved in feeding. Reports that intra-hypothalamic injections of glutamate antagonists suppress feeding under a number of conditions (Stanley et al., 1996; Stratford and Kelley, 1999) suggest that glutamate release plays an important role in the production of ingestive behavior. The finding that glutamate output does not stay elevated throughout the entire duration of meals, however, suggests that feeding can occur in the absence of increases in glutamate release. One possibility is that activation of glutamate receptors may be necessary for the initiation, but not the maintenance, of ingestive behavior. Alternatively, it should be remembered that the rate of ingestive behavior is usually greatest at the start of a meal, after which it tends to decline; perhaps stimulation of glutamate receptors plays an especially important role in the vigorous consumption seen at meal onset. It should also be noted that the precise temporal relation between feeding and glutamate release remains to be clarified. For example, while it is possible that glutamate release may increase just prior to the onset of feeding, as would be required if it were a "command signal" for ingestion, the available data are also consistent with the possibility that changes in release occur after the start of the meal, perhaps in response to gustatory stimulation.

The most interesting aspect of the current studies may be our observation that glutamate output from the perifornical region of the LH was not significantly altered during drinking behavior. Since a significant increase was seen in animals drinking Ensure®, this discrepancy cannot be attributed to differences in motor output. The failure to see changes in glutamate output during drinking is surprising as many studies have implicated the LH in the control of fluid intake (Gonzalez-Lima et al., 1993; Tabuchi et al., 2002); in fact, drinking is typically more severely affected by lesions of the LH than is feeding (Teitelbaum and Epstein, 1962). It is possible that different regions of the LH are involved in feeding and drinking behavior and that our probes were located in a region specifically related to feeding. Alternatively, it is possible that LH glutamate release is specifically linked to feeding, and that other transmitters in the same region may be more closely related to drinking behavior. This possibility receives strong support from reports that injections of glutamate agonists into the tuberal LH increase food, but not water, intake (Duva et al., 2002; Stanley et al., 1993). It is also possible that the differential effects seen in animals consuming water and Ensure® might be related to differential activation of taste systems by these two ingestates, or to their different nutrient content.

In summary, the current findings demonstrate that lateral hypothalamic glutamate output measured by LFPPP increases during feeding but not drinking behavior. Many questions about the nature of these effects remain to be answered, but the current findings demonstrate low-flow push–pull perfusion provides a novel and powerful tool for addressing them.

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