Amino Acids (2013) 45:1353–1364
DOI 10.1007/s00726-013-1595-9

ORIGINAL ARTICLE

Enantiomer-specific selection of amino acids

Xueying Ren · Luis A. Tellez · Ivan E. de Araujo

Received: 23 April 2013 / Accepted: 10 September 2013 / Published online: 27 September 2013

Abstract Dietary intake of L-amino acids impacts on several physiological functions, including the control of gastrointestinal motility, pancreatic secretion, and appetite. However, the biological mechanisms regulating behavioral predilections for certain amino acid types remain poorly understood. We tested the hypothesis that, in mice, the potency with which a given glucogenic amino acid increases glucose utilization reflects its rewarding properties. We have found that: (1) during long-, but not short-, term preference tests, L-alanine and L-serine were preferred over their D-enantiomer counterparts, while no such effect was observed for L-threonine vs. D-threonine; (2) these behavioral patterns were closely associated with the ability of L-amino acids to promote increases in respiratory exchange ratios such that those, and only those, L-amino acids able to promote increases in respiratory exchange ratios were preferred over their D-isomers; (3) these behavioral preferences were independent of gustatory influences, since taste-deficient *Trpm5* knockout mice displayed ingestive responses very similar to those of their wild-type counterparts. We conclude that the ability to promote increases in respiratory exchange ratios enhances the reward value of nutritionally relevant amino acids and suggest a mechanistic link between substrate utilization and amino acid preferences.

Keywords Enantiomers · Food reward · Glucose metabolism · Sweet taste

X. Ren · L. A. Tellez · I. E. de Araujo (✉)
The John B Pierce Laboratory, 290 Congress Avenue, New Haven, CT 06519, USA
e-mail: iaraujo@jbpierce.org

X. Ren · L. A. Tellez · I. E. de Araujo
Department of Psychiatry, Yale University School of Medicine, New Haven, CT, USA

Introduction

Ingestion of amino acids, mainly in the form of digestible proteins, generates a set of physiologically active signals that impact on a number of biological functions including gastrointestinal motility, pancreatic peptide secretion, brain neurotransmission release, and appetite control (Peters and Harper 1987; Tome et al. 2009; Fromentin et al. 2012). Specifically, amino acid sensing in the gastrointestinal tract (Ritter 2004; Tome et al. 2009), as well as in brainstem, hypothalamic (Blouet and Schwartz 2012; Schwartz 2013) and cortical (Hao et al. 2005) circuits, plays critical roles in inducing satiation and regulating energy homeostasis. However, the precise signaling pathways linking the biochemical and physiological properties of amino acids to their reward value lack full characterization (Fromentin et al. 2012).

We have previously described a close relationship between glucose oxidation levels and taste-independent nutrient intake levels, with animals increasing intake as a function of increases in respiratory exchange ratios (Ren et al. 2010). Furthermore, microdialysis measurements revealed that intragastric infusions of glucose induced significantly higher levels of dopamine release compared with isocaloric amino acids in both ventral and dorsal striatum, an effect that was abolished by the administration of an anti-metabolic glucose analog (de Araujo et al. 2010; Ren et al. 2010). Based on these results, we aimed at determining in the present study whether similar physiological processes may apply to amino acid behavioral preferences.

More specifically, in the present study we tested the following interrelated hypotheses: (1) the appetitive properties of the glucogenic amino acids L-alanine, L-serine, and L-threonine (Pere et al. 1987) are dictated by the extent

to which these L-amino acids promote increases in respiratory exchange ratios; (2) the appetitive properties of glucogenic L-amino acids are greater than those of their corresponding D-isomers, since most amino acids of nutritional importance exist as L-isomers (Friedman and Levin 2012).

Materials and methods

Subjects

A total of 63 male mice on a C57BL/6 background were used. At the time of experiments, animals were 8–16 weeks old. A subgroup of these animals were homozygous for a partial deletion of the *Trpm5* gene (KO, Zhang et al. 2003) where a PGK-neor cassette specifically replaced exons 15–19 encoding the first five transmembrane domains of TRPM5, rendering the channel inactive. These animals were bred at the John B. Pierce animal facilities from mice generously donated by C. S. Zuker (Columbia University and Howard Hughes Medical Institute). Controls were littermate male wild-type C57BL/6. Genotype was confirmed by PCR amplification followed by gel electrophoresis. *Trpm5*-specific primers (forward 5′-ATTCTAGAGCCCACCCGCCCCATC-3′, reverse 5′-TTCACCTGCCCAGCCCTCATCTAC-3′) were used to amplify a segment >2 kb in WT animals and <2 kb in KO animals. All experiments were conducted in accordance with the J.B. Pierce Laboratory and Yale University regulations on usage of animals in research.

Stimuli and behavioral apparatus

All stimuli (sucralose, D-glucose, L-alanine, D-alanine, L-serine, D-serine, L-threonine, D-threonine, L-lysine, D-lysine) were obtained from Sigma (St Louis, MO) and prepared daily in distilled water at room temperature. The amino acid and glucose solutions were prepared using a weight/volume ratio, i.e., every 100 mL of solution contained 10 g of the relevant amino acid (or glucose) in water. Therefore, for every 1 mL consumed, 100 mg of the corresponding amino acid was ingested. Sucralose was prepared at 2 mM. To mask aversive taste properties in L-lysine and D-lysine, these solutions were mixed with 2 mM sucralose. Short-term gustatory tests were conducted in either one of two identical mouse behavior chambers enclosed in a ventilated and sound-attenuating cubicle (Med Associates Inc., St. Albans, VT). Each chamber is equipped with two slots for sipper tubing placements, at symmetrical locations on one of the cage walls. All sippers are connected to a contact-based licking detection device allowing for measurements of licking responses with 10 ms resolution. All lick timestamps were saved in a computer file for posterior analysis.

Short- and long-term two-bottle preference tests

Short-term (10 min) two-bottle preference tests between either of the stimuli and water, or between the L- and D-isomers of the amino acids, were used to determine the ability of *Trpm5* wild-type and knockout mice to display orosensory-driven preferences. The short duration of the test aims at minimizing post-ingestive influences. Once habituated to the behavioral chamber, each animal was presented with the choice between two given solutions. The number of licks on each sipper was recorded and used to calculate the preference ratio as follows:

$$\text{Preference ratio for Sipper1} = \frac{n(\text{Sipper1})}{n(\text{Sipper1}) + n(\text{Sipper2})}$$

where n(Sipper$x$) denotes the detected number of licks to sipper x during a given session. To eliminate the influence of side biases, mice were tested for four consecutive days with sipper positions being switched daily. For long-term preference tests, after habituation with two-bottle water exposure, mice were allowed 16-h access to the L- and D-isomers of each amino acid from graded (mL) sippers. Mice were food deprived overnight before the beginning of the experiments.

Indirect calorimetry

Energy expenditure was measured via indirect calorimetry using the Oxymax/CLAMS Animal Monitoring System (Columbus Intruments, Columbus, OH). This is a mouse-dedicated, four-cage system equipped with open-circuit calorimetry, contact lickometers (thus allowing for concomitant measurement of licking responses), and *XZ*-axis motor activity sensors. Metabolism-induced heat was derived by assessing the exchange of oxygen for carbon dioxide that occurs during metabolic processes (Jequier et al. 1987), as measured by the mass flow principle. Oxygen (O_2) measurement was performed via paramagnetic sensing and carbon dioxide (CO_2) by single beam non–dispersed IR. The respective volumes were determined as:

$$VO_2 = V_iO_{2i} - V_oO_{2o} \quad (1)$$

$$VCO_2 = V_oCO_{2o} - V_iCO_{2i} \quad (2)$$

where: V_i = mass of air at chamber input per unit time, V_o = mass of air at chamber output per unit time, O_{2i} = oxygen fraction in V_i, CO_{2i} = oxygen fraction in V_i, O_{2o} = carbon dioxide fraction in V_o, and CO_{2o} = carbon dioxide fraction in V_o.

The respiratory exchange ratio RER was calculated as

$$\mathrm{RER} = V\mathrm{CO}_2/V\mathrm{O}_2 \quad (4)$$

Heat was calculated by determining the calorific value of the food being metabolized. For the accepted range of nutritional RQs (0.707–1.0), the heat available is 4.686–5.047 kcal/L(O_2). The calorific value (C_v) is interpolated by straight line approximation for values within the RER range ($C_v = 3.815 + 1.232 \times \mathrm{RQ}$). The resulting calorific value is applied to the obtained figure for oxygen consumption for derivation of heat, followed by normalization of this quantity to the animal's body volume:

$$\mathrm{Heat} = (C_v \times V\mathrm{O}_2)/(\text{body weight})^{0.75} \quad (3)$$

Ambulatory activity was obtained from the total number of beam break counts of the XZ sensors.

Ketone bodies, and glucose and glycogen measurements

Naïve mice randomly allocated to seven different experimental groups were provided with one of the amino acids or water (i.e., seven stimuli in total) for 22 h under food deprivation in their individual home cages. Graded sippers were used to assess ingestion of these substances following 1-h-long ad libitum, after which animals were killed with an overdose of Euthasol (0.1 mL). Mice were decapitated and truncal blood and liver samples were harvested. Liver glycogen was immediately hydrolyzed from 100 mg of tissue using 2N HCL (0.6 mL) for 2 h at ~100 °C. Measurement of the resulting glucose units was carried out on an YSI Automated Glucose Analyzer by glucose oxidase (Yellow Springs Instruments, Inc., Yellow Springs, OH). Data are presented as glucosyl units in μmol/g wet wt. Plasma lactate and glucose were also measured by glucose oxidase. Plasma ketone bodies were measured by a beta-hydroxybutyrate ("beta-HB") assay kit (Abcam) that utilizes beta-HB dehydrogenase to generate a product reactant with a colorimetric probe (450 nm). Results are shown as nmol/μl beta-HB. The corresponding total number of licks from each session was then entered in regression analyses using the linear regression function of SPSS (see below).

Experimental conditions

To control for potentially confounding factors such as the presence of food in the gastrointestinal tract, we used food-deprived (non-thirsty) mice only. All experiments were preceded by overnight food deprivation (approximately, 14 h); for calorimetry experiments, a baseline period of 6 h was added to allow for signal stabilization and habituation to the metabolic cages. All experiments started at the late phase of the light cycle (around 3–5 pm) so that animals would be highly motivated to perform early in the experimental sessions. Food (i.e., other than amino acids) was not available during any of the experiments.

Intra-gastric amino acid infusions

Animals were anesthetized with an intraperitoneal injection of a ketamine/xylazine (100/15 mg/kg) and a midline incision was made into the abdomen. The stomach was exteriorized through the midline incision and a purse-string suture was placed in its non-glandular region, into which the tip of a MicroRenathane tubing (Braintree Scientific Inc., Braintree, MA) was inserted. The purse string was tightened around the tubing, which was then tunneled subcutaneously to the dorsum via a small hole made into the abdominal muscle; a small incision to the dorsum between the shoulder plates was then made to allow for catheter exteriorization. Incisions were sutured and thoroughly disinfected and the exterior end of the catheter plugged. Intra-gastric infusions were performed to rule out the possibility that differences in enantiomer-specific metabolic responses were due to enantiomer-specific differences in ingestion. After a period of baseline measurements in the calorimetry chambers as described above, a fixed 1.25 mL volume of the amino acid solutions used in the oral tests were infused intra-gastrically over a period of 3 min, after which mice were immediately replaced in the calorimetry chambers and measurements taken as usual. Results were interpreted as indicating that enantiomer-specific metabolic effects were not due to changes in oral patterns, amounts consumed, or motivational state.

Statistical analyses

Data analyses were performed using SPSS (PASW Statistics Release 18.0.0) and made use of linear mixed regression analyses (reported as the F-statistic of the corresponding fit) as well as three-, two-, and one-way mixed model or repeated-measures ANOVAs. Data are reported as mean ± SEM unless stated otherwise.

Results

Trpm5 wild-type, but not knockout, mice display short-term preferences for glucogenic L-amino acids and their D-isomers

We started by assessing the short-term, presumably taste-driven, responses to amino acids in both *Trpm5* wild-type

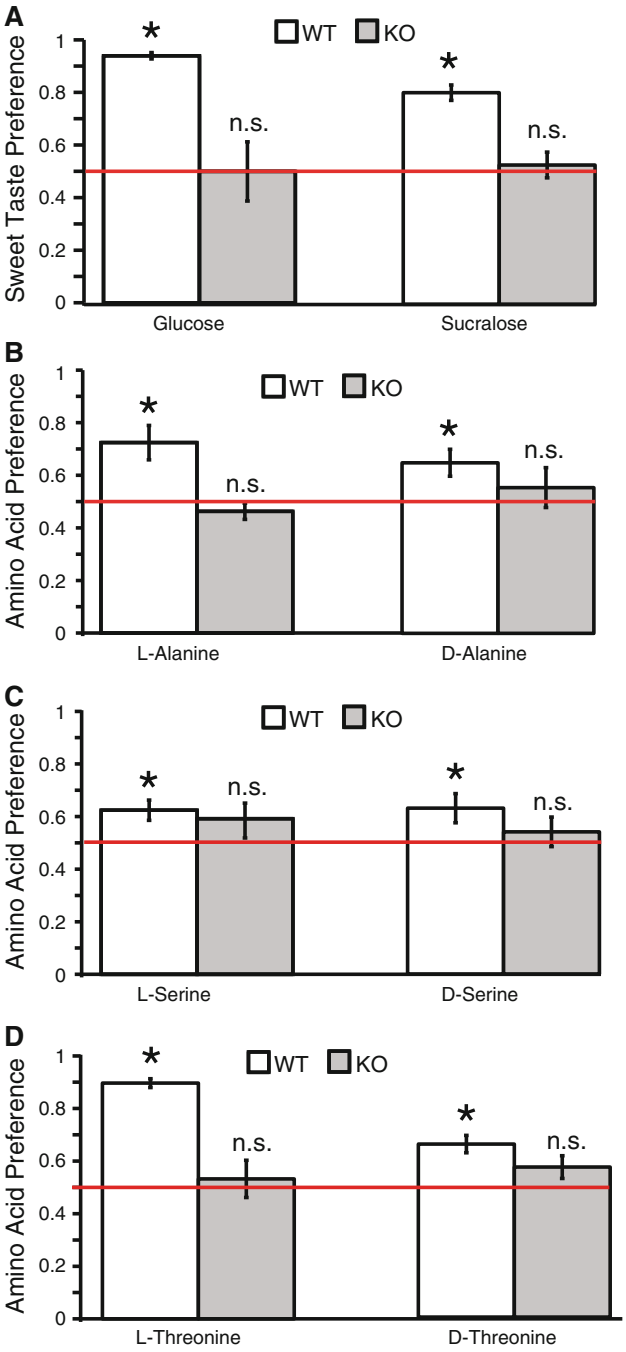


Fig. 1 *Trpm5* wild-type, but not knockout, mice display short-term preferences for glucogenic L-amino acids and their D-isomers. **a** Plots indicate mean ± SEM. While *Trpm5* wild-type ("WT") mice preferred glucose versus water in 10-min two-bottle gustatory tests, knockout mice ("KO") were indifferent to this choice (left, one-sample *t* test against indifference ratio of 0.5, WT $t[7] = 36.3$, $*p < 0.001$; KO $t[7] = -0.04$, $p = 0.99$); similar results were observed for the sweetener sucralose (right, WT $t[7] = 10.3$, $*p < 0.001$; KO $t[7] = -0.6$, $p = 0.56$). **b–d** WT, but not KO mice, showed preferences for all amino acids tested in 10-min two-bottle gustatory tests versus water: three-way mixed model ANOVA: amino acid type × genotype $F[2,28] = 4.0$, $*p < 0.03$; enantiomer type × genotype $F[1,14] = 8.9$, $p = 0.01$; amino acid type × enantiomer $F[2,28] = 0.9$, $p = 0.4$; genotype effect $F[1,14] = 16.8$, $p = 0.001$. Results are shown for alanine on **b**, serine on **c**, and threonine on **d**. *Red horizontal line* indicates the indifference ratio of 0.5; *n.s.* non-statistically significant

(henceforth "WT") and knockout ("KO") mice. We first confirmed the severe sweet taste impairment in KO mice (Zhang et al. 2003) by performing short-term (10 min) two-bottle preference tests between glucose and water. As expected, while WT mice displayed strong preferences for glucose, KO mice were indifferent to this choice (Fig. 1a left). These results were confirmed by using the potent non-caloric sweetener sucralose (Fig. 1a right). Similar tests were then performed for each of the amino acids selected for this study (L- and D- alanine, serine and threonine). Overall, WT mice preferred all forms of amino acid against water, whereas KO mice were equally indifferent to all of them (Fig. 1b: alanine, 1c: serine, 1d: threonine). We conclude that all amino acid forms elicited attractive oral sensations in WT, but not in KO, mice. The clear indifference of KO mice during the choice tests indicates that such sensory features relate to the presumed sweet/savory tastes of these amino acids (Treesukosol et al. 2011).

Both *Trpm5* wild-type and knockout mice display long-term preferences for glucogenic L-amino acids versus their D-isomers

We next inquired whether WT and KO mice had behavioral preferences for one of the two isomers when compared against each other during two-bottle choice tests. While both WT and KO mice were indifferent to the choice between L-alanine vs. D-alanine during short-term (10 min) tests, both genotypes displayed robust preferences for L-alanine over D-alanine during longer-term (16 h) tests (Fig. 2a). Similar results were obtained when serine was used: While both WT and KO mice were indifferent to the choice between L-serine *vs.* D-serine during short-term tests, both genotypes displayed preferences for L-serine over D-serine during longer-term tests (Fig. 2b). These longer-term preferences for L-alanine and L-serine are presumably accounted for by post-ingestive rewarding effects. Finally, and rather interestingly, both WT and KO mice were indifferent to the choice between L-threonine vs. D-threonine during both short- and long-term tests (Fig. 2c). We conclude that L-alanine and L-serine produce taste-independent physiological signals that increase the reward value of these solutions, and that such signals are either attenuated or absent from L-threonine solutions.

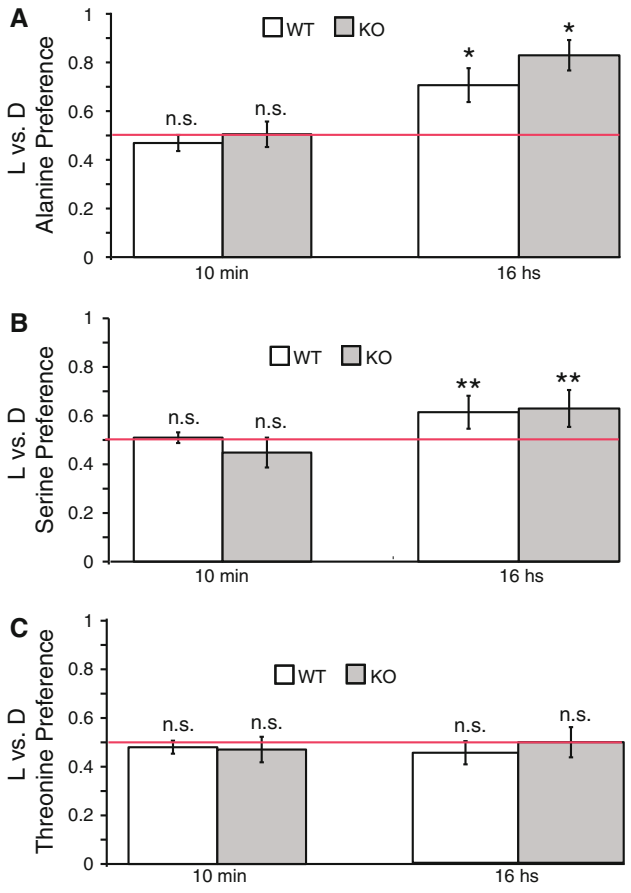


Fig. 2 Both *Trpm5* wild-type and knockout mice display long-term preferences for glucogenic L-amino acids versus their D-isomers. During short-term 10-min gustatory tests, both WT and KO mice were indifferent to the choice between the L- and D-enantiomers of alanine, serine, and threonine (two-way mixed model ANOVA: main effect of amino acid $F[2,28] = 0.27$, $p = 0.97$; genotype effect $F[1,14] = 0.98$, $p = 0.75$; amino acid × genotype $F[2,28] = 0.43$, $p = 0.64$). However, during longer term, 16 h, tests, both WT and KO mice preferred the L-isomers of alanine and serine, but not of threonine (two-way mixed model ANOVA: main effect of amino acid $F[2,28] = 15.7$, $p < 0.001$; genotype effect $F[1,14] = 0.77$, $p = 0.39$; amino acid × genotype $F[2,28] = 0.56$, $p = 0.57$. One-sample t tests against indifference ratio of 0.5, $t[15] = 5.5$, $^{*}p < 0.001$; $t[15] = 2.4$, $^{**}p = 0.02$). Results are shown for alanine on **a**, serine on **b**, and threonine on **c**. *Red horizontal line* indicates the indifference ratio of 0.5; *n.s.* non-statistically significant

Only L-alanine and L-serine promoted significant increases in respiratory exchange ratios during long-term ingestion tests

We then set out to determine what physiological signals may underlie these taste-independent preferences for L- vs. D-alanine and L- vs. D-serine (and whether the same signal accounts for the indifference between L- vs. D-threonine). As mentioned, in a previous work we had observed strong associations between the ability of a given nutrient to promote glucose utilization and its rewarding value (Ren et al. 2010). We then tested the hypothesis that similar principles apply to enantiomer-specific amino acid preferences.

Mice were placed in calorimetry chambers and given 24-h access to lickometer-equipped sippers mounted on the chambers, containing one of the amino acid solutions. Therefore, amino acid intake and respiratory exchange ratio ("RER", i.e., the ratio between the amount of CO_2 exhaled and O_2 inhaled) were measured concomitantly. We observed that RER values were significantly higher during L-alanine compared to D-alanine intake in both WT (Fig. 3a) and KO mice (Fig. 3b). In fact, linear model regression analyses revealed increasing RER values over time (possibly, but not necessarily, reflecting increased glucose oxidation) during L-alanine intake in both WT (standardized $\beta = 0.91$, $p < 0.001$) and KO ($\beta = 0.84$, $p < 0.001$) mice. On the other hand, no such effects were observed during D-alanine intake in either WT ($\beta = -0.09$, $p = 0.67$) or KO ($\beta = 0.04$, $p = 0.84$) mice.

Similar increases in respiratory exchange ratios were observed during L-serine intake in both WT (Fig. 3c) and KO (Fig. 3d) mice. In fact, similar regression analyses revealed increasing RER over time during L-serine intake in both WT ($\beta = 0.87$, $p < 0.001$) and KO ($\beta = 0.96$, $p < 0.001$) mice. Consistent with the enantiomer-related differences in RER, regression analyses revealed decreasing RER values over time during D-serine intake in both WT ($-\beta = -0.92$, $p < 0.001$) and KO ($-\beta = 0.88$, $p < 0.001$) mice.

Finally, for the case of threonine, higher RER values were observed during L-threonine compared to D-threonine intake in both WT (Fig. 3e) and KO (Fig. 3f) mice. However, these differences were not accounted for by increased respiratory exchange ratios during L-threonine intake (WT $\beta = 0.3$, $p = 0.14$; KO $\beta = 0.2$, $p = 0.24$), but rather to decreases during D-threonine intake (WT $-\beta = 0.82$, $p < 0.001$; KO $-\beta = 0.8$, $p < 0.001$).

In summary, the two preferred amino acids, L-alanine (vs. D-alanine) and L-serine (vs. D-serine), were the only ones to promote increases in respiratory exchange ratio over the course of the calorimetry experiments.

Only L-alanine and L-serine promoted greater intake compared to their D-isomers during long-term ingestion tests

We have analyzed the intake patterns associated with the RER changes described above. Consistent with the regression data above, we found that only L-alanine and L-serine promoted greater intake compared to their D-isomers during the calorimetry sessions (Fig. 4a–d). Interestingly, and consistent with the two-bottle tests results, L- and D-threonine were consumed at very similar levels by both WT (Fig. 4e) and KO (Fig. 4f) mice. Accordingly,

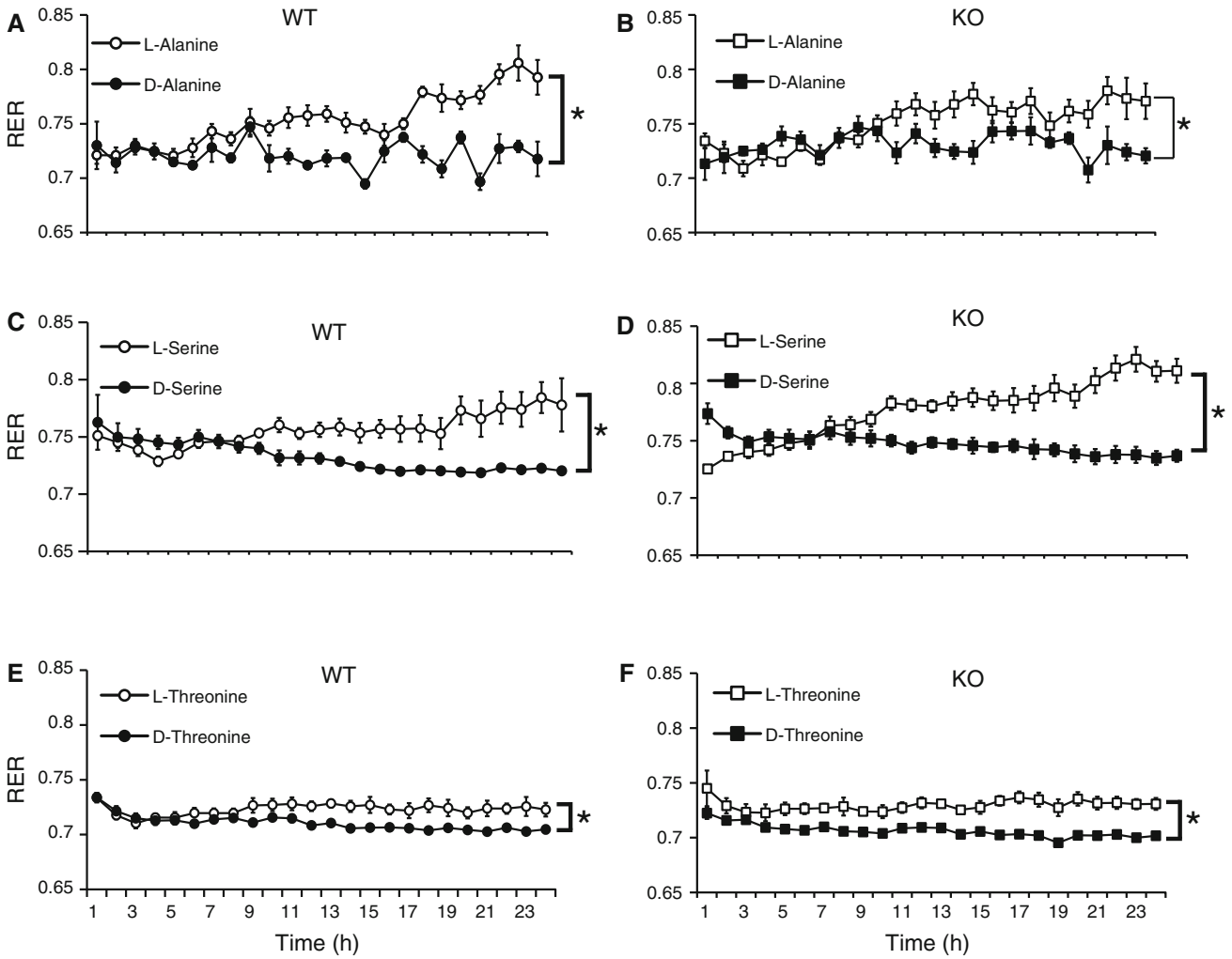


Fig. 3 Only L-alanine and L-serine promoted significant increases in respiratory exchange ratios during long-term ingestion tests. Mice were placed in calorimetry chambers and given 24-h access to lickometer-equipped sippers containing one of the amino acid solutions; therefore, amino acid intake and respiratory exchange ratio ("RER") were measured concomitantly. For calorimetry experiments, a baseline period of 6 h was added to allow for signal stabilization and habituation to the metabolic cages. All experiments started at the late phase of the light cycle (around 3–5 pm) so that hour #1 should correspond to 4–6 PM. **a** L-Alanine promoted higher respiratory exchange ratios compared to D-alanine in WT mice (two-way repeated-measures ANOVA enantiomer × time $F[23,161] = 6.2$, $^*p < 0.001$). **b** L-Alanine promoted higher respiratory exchange ratios compared to D-alanine in KO mice ($F[23,161] = 6.1$, $^*p < 0.001$). **c** L-Serine promoted higher respiratory exchange ratios compared to D-serine in WT mice ($F[23,161] = 9.7$, $^*p < 0.001$). **d** L-Serine promoted higher respiratory exchange ratios compared to D-serine in KO mice ($F[23,161] = 40.2$, $^*p < 0.001$). **e** L-Threonine promoted higher respiratory exchange ratios compared to D-threonine in WT mice ($F[23,161] = 5.1$, $^*p < 0.001$). **f** L-Threonine promoted higher respiratory exchange ratios compared to D-threonine in KO mice ($F[23,161] = 2.7$, $^*p < 0.001$). However, only L-alanine and L-serine promoted significant increases in RER values over time (see regression analyses in text)

when the cumulative intake patterns were contrasted to the corresponding RER profiles, we found that only L-alanine and L-serine cumulative intakes correlated positively with their corresponding RER values (see Fig. 4).

Ingestion of the non-gluconeonic amino acid lysine is not associated with enantiomer-specific increases in RER values or intake levels

We have performed additional experiments to support our conclusions that (1) lick rates and/or amounts consumed do not reflect RER values and could not have accounted for any of the enantiomer-specific RER patterns (since this relationship does not apply to any of the D-enantiomers used); and (2) these enantiomer-specific RER patterns are associated exclusively with gluconeogenic amino acids. We thus performed additional tests where the effects of intra-gastric infusions (see "Materials and methods" for details) of 1.25 mL L-alanine on RER values were compared to those produced by similar infusions of D-alanine (therefore controlling for factors such as lick numbers or rates). As anticipated, L-alanine infusions produced

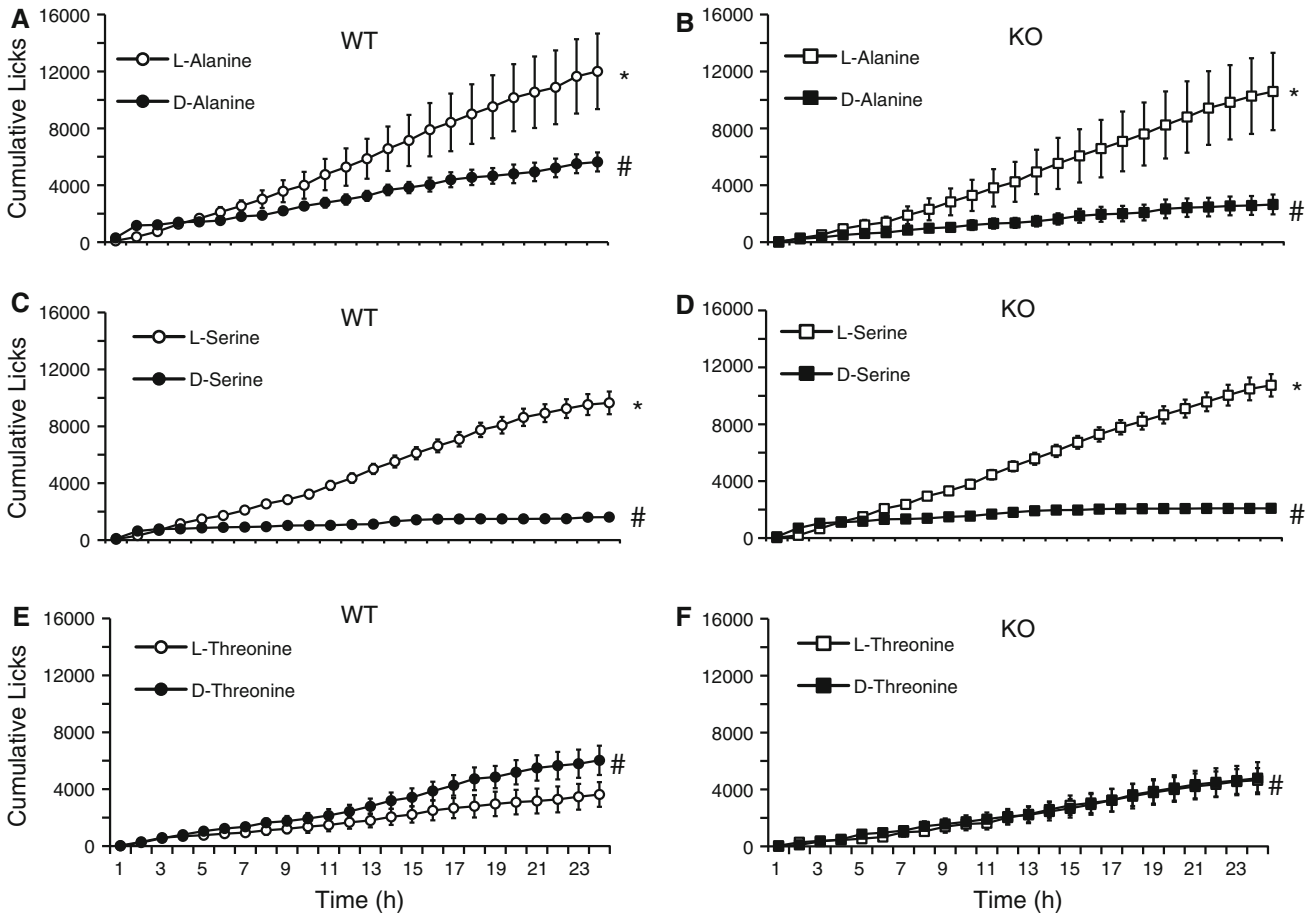


Fig. 4 Only L-alanine and L-serine intake levels correlated positively with the corresponding RER values. We analyzed the intake patterns associated with the RER changes described above in Fig. 3. For calorimetry experiments a baseline period of 6 h was added to allow for signal stabilization and habituation to the metabolic cages. All experiments started at the late phase of the light cycle (around 3–5 pm) so that hour #1 should correspond to 4–6 PM. **a** L-Alanine intake was linearly associated with RER values in WT mice (linear model fit $F[1,23] = 106.8$, $*p < 0.001$), but this effect was not observed for D-alanine ($F[1,23] = 0.2$, $\#p = 0.65$). **b** L-Alanine intake was significantly associated with RER values in KO mice ($F[1,23] = 48.9$, $*p < 0.001$), but this effect was not observed for D-alanine ($F[1,23] = 0.9$, $\#p = 0.76$). **c** L-Serine intake was significantly associated with RER values in WT mice ($F[1,23] = 64.3$, $*p < 0.001$), while D-serine intake levels were inversely correlated with RER values ($F[1,23] = 253.0$, $\#p < 0.001$). **d** L-Serine intake was significantly associated with RER values in KO mice ($F[1,23] = 258.7$, $*p < 0.001$), while D-serine intake levels were inversely correlated with RER values ($F[1,23] = 105.6$, $\#p < 0.001$). **e** L-Threonine intake was not associated with RER values in WT mice ($F[1,23] = 1.4$, $p = 0.24$), while D-threonine intake levels were inversely correlated with RER values ($F[1,23] = 43.4$, $\#p < 0.001$). **f** L-Threonine intake was not associated with RER values in KO mice ($F[1,23] = 1.9$, $p = 0.18$), while D-threonine intake levels were inversely correlated with RER values ($F[1,23] = 44.1$, $\#p < 0.001$)

significantly greater increases in RER values compared to equal infusions of D-alanine (Fig. 5a). These results establish that increased RER values were accounted for by physiological signals rather than differences in intake patterns.

Next, we performed similar experiments as above, but this time using a non-glucoeneogenic amino acid, lysine. According to the results presented thus far, we hypothesized that (1) intra-gastric L-lysine infusions would not produce greater increases in RER values compared to equal infusions of D-lysine; and (2) L-lysine would promote neither greater intake nor greater RER values compared to its D-isomer during calorimetry sessions. As anticipated, 1.25 mL L-lysine infusions did not result in significantly greater increases in RER values compared to equal infusions of D-lysine (in fact, a transient depression in values was observed immediately following the infusions; Fig. 5b). No enantiomer-specific differences in locomotor activity were detected for either alanine (Fig. 5c) or lysine (Fig. 5d). In the case of energy expenditure, however, whereas no enantiomer-specific effects were noted in response to alanine infusions (Fig. 5e), intra-gastric L-lysine infusions produced a significant drop in heat production compared to D-lysine (Fig. 5f), further suggesting

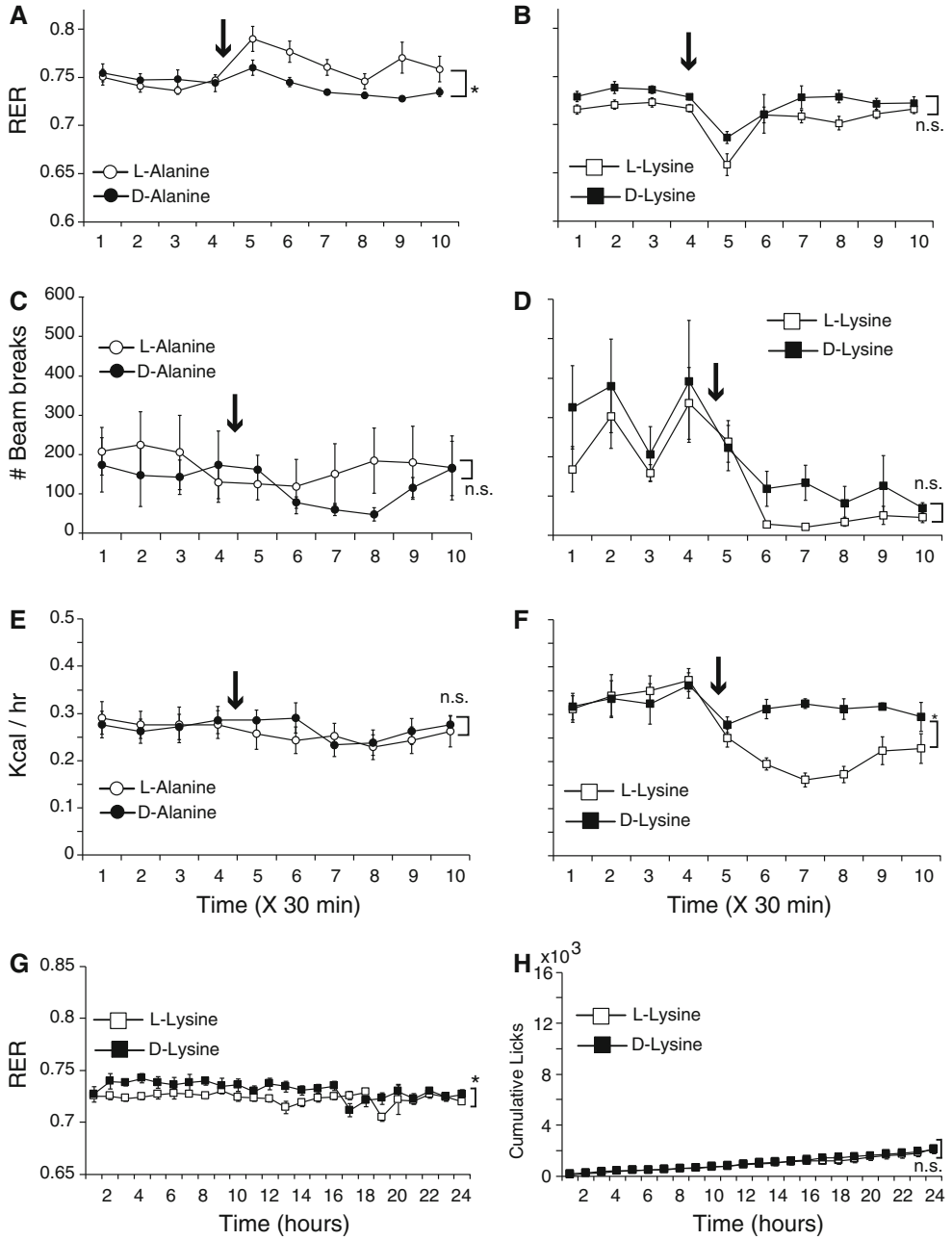


Fig. 5 Ingestion of the non-gluconeonic amino acid lysine is not associated with enantiomer-specific increases in RER values or intake levels. **a** Intra-gastric infusions of 1.25 mL L-alanine produced significantly greater increases in RER values compared to equal infusions of D-alanine (two-way repeated-measures ANOVA enantiomer × time $F[9,54] = 4.3$, $*p < 0.001$), establishing that increased RER values are accounted for by physiological signals rather than differences in intake pattern. *Downward arrow* indicates the point in time where intra-gastric infusions were performed. **b** Similar experiments involving the non-gluconeogenic amino acid lysine resulted in no enantiomer-specific changes in RER values; in fact, a transient depression in values was observed immediately following the infusions (two-way repeated-measures ANOVA enantiomer × time $F[9,45] = 0.83$, $p = 0.58$). **c** No enantiomer-specific differences in locomotor activity were detected between L- and D-alanine, as assessed by total detected beam break counts (two-way repeated-measures ANOVA enantiomer × time $F[9,54] = 0.6$ $p = 0.79$). **d** Similar results were observed between L- and D-lysine ($F[9,45] = 0.406$ $p = 0.92$). **e** In the case of energy expenditure, no enantiomer-specific effects were noted in response to alanine infusions ($F[9,54] = 0.606$ $p = 0.78$). **f** However, intra-gastric L-lysine infusions produced a significant drop in heat production compared to D-lysine ($F[9,45] = 5.14$, $*p < 0.001$). **g** During L-lysine intake, a slight depression in RER values was noted, whereas no changes were detected for D-lysine (two-way repeated-measures ANOVA enantiomer × time $F[23,115] = 1.8$, $*p = 0.02$). **h** Consistently, no enantiomer-specific differences in intake levels were noted during the calorimetry sessions ($F[23,115] = 0.4$, $p = 0.99$). *n.s.* non-statistically significant

important metabolic differences between glucogenic L-alanine and non-gluconeogenic L-lysine that did not depend on locomotion or amounts ingested.

Furthermore, during active intake in the calorimetry chambers, L- and D-lysine intake did not result in any detectable increases in RER values (in fact, a slight depression in RER values was noted during L-lysine intake, whereas no changes were detected for D-lysine, Fig. 5g), and were consumed at very similar levels (Fig. 5h). We note that both L-lysine and D-lysine (combined to sucralose to make them comparable in terms of taste with the other amino acids) were preferred over water in the short-term two-bottle tests (0.56 ± 0.01 0.56 ± 0.02 preference ratios against water for L- and D-lysine, respectively, $t[6] = 3.4$, $p = 0.01$ and $t[6] = 2.8$, $p = 0.03$ against indifference 0.5 ratio, respectively). These solutions were also isopreferred when compared directly (0.47 ± 0.06 for L- against D-lysine, $t[6] = 0.36$, $p = 0.72$). In sum, non-glucogenic amino acids do not appear to induce metabolic or behavioral enantiomer-specific patterns.

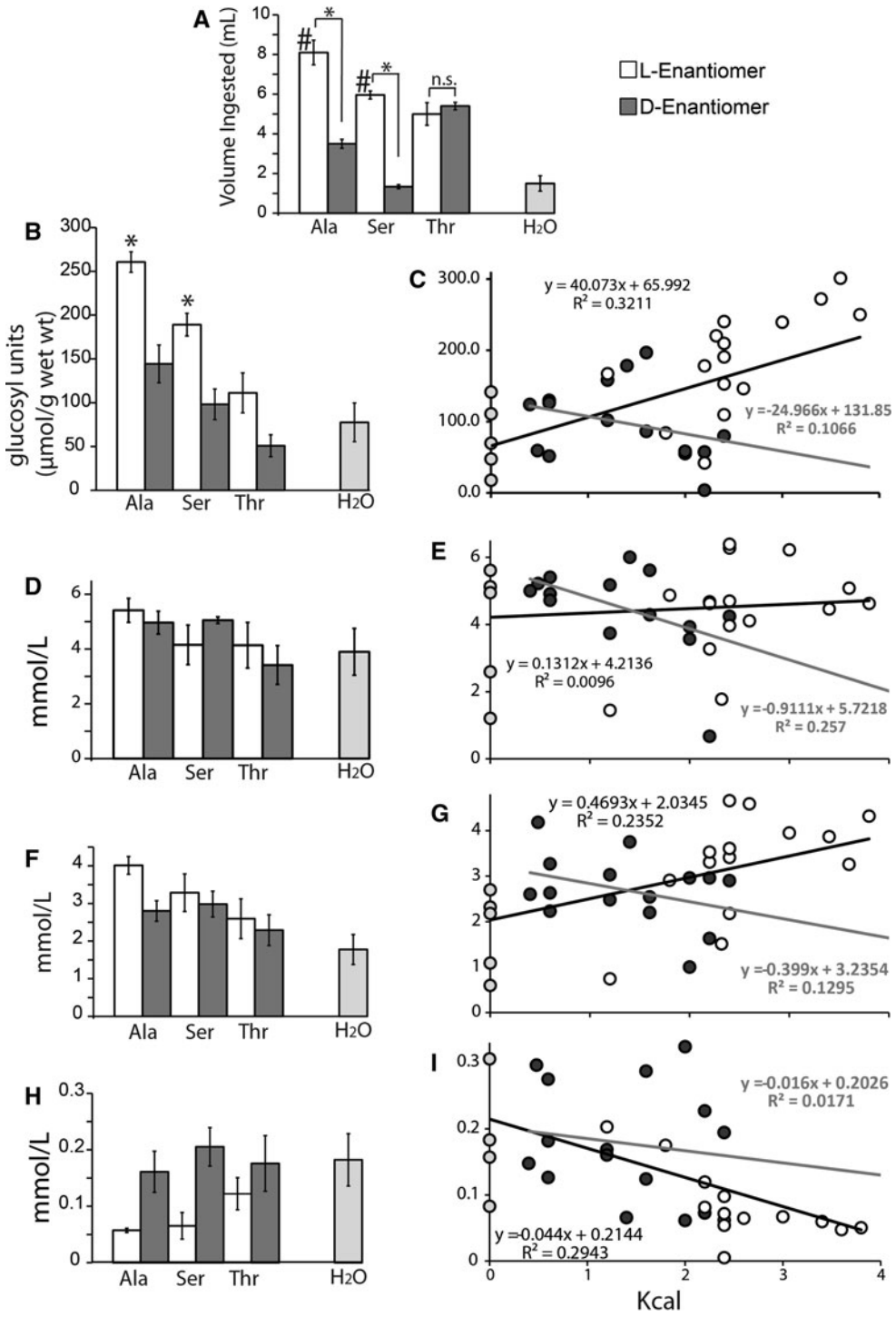
Liver glycogen, but not blood glucose, was significantly associated with amino acid intake levels

Wild-type mice were provided with one of the amino acids or with water (i.e., seven stimuli in total) for 22 h under food deprivation in their individual home cages. This was followed by immediate collection of blood (plasma) and liver samples. Intake levels during these tests are shown in Fig. 6a. As expected, both L-alanine and L-serine were consumed at higher levels than their D-isomer counterparts, whereas no such effect was observed for threonine. Also, plain water was consumed at lower levels than any of the L-enantiomers (L-alanine, L-serine or L-threonine). We then performed correlations between the measured metabolic factors and the corresponding intake levels. Liver glycogen levels produced by intake of both L-alanine and L-serine were significantly higher than those associated with water (Fig. 6b) and with their D-isomer counterparts, such that these levels were significantly associated with intake (Fig. 6c). However, no such association was observed for blood glucose levels (Fig. 6d, e). Significant associations were observed between blood lactate and intake levels (Fig. 6f, g). Finally, and consistent with all the above, plasma ketone bodies increased in inverse proportion to increases in liver glycogen (Fig. 6h), such that ketone bodies were inversely correlated to intake (Fig. 6i). Overall, these metabolic data indicate that the glucogenic L-amino acids, especially L-alanine and L-serine, significantly promoted glucose metabolism, consistent with the indirect calorimetry data. We do however stress that such effect was not simply a function of amounts of amino acid ingested, since no relationship is detected for any of these factors when the analyses are restricted to D-amino acid data points (see Fig. 6c, e, g, i).

Discussion

In this study, we have described a link between the extent to which a given glucogenic amino acid increases respiratory exchange ratios and its appetitive value. More specifically, we have found that: (1) during long-, but not short-, term preference tests, L-alanine and L-serine were preferred over their D-enantiomer counterparts, while no such effect was observed for L-threonine vs. D-threonine; (2) these behavioral preferences were closely associated with the ability of L-amino acids to promote increases in respiratory exchange ratios such that those, and only those, amino acids able to increase in respiratory exchange ratios were preferred; (3) these behavioral preferences were independent of gustatory influences, since taste-deficient *Trpm5* knockout mice displayed ingestive responses very similar to their wild-type counterparts; and (4) no such enantiomer-specific changes in metabolic or behavioral patterns were detected during intake of the non-glugenic amino acid lysine.

The actual biochemical mechanisms leading to greater exchange ratios by the intake of L-alanine and L-serine, compared to L-threonine, remain to be uncovered. However, specificities in the metabolic pathways associated with each amino acid may provide us with some clues. Thus, it is noticeable that while L-alanine and L-serine are readily converted into pyruvate, the carbon skeleton of threonine is degraded by pathways that yield succinyl-CoA, an intermediate of the citric acid cycle (Lehninger et al. 1993). It is therefore conceivable that low metabolic cost for conversion into pyruvate constitutes one principle regulating the overall rewarding value of glucogenic amino acids. This is consistent with the relatively high and low gluconeogenic capacities of L-alanine (Felig 1973) and L-threonine (Hetenyi et al. 1984), respectively (i.e., in this order the most and less preferred L-amino acids in comparison to their D-isomers). Similar concepts may be applied to the physiological pathways regulating the higher preferences for L-alanine and L-serine over their D-isomers. ^{1}H and ^{13}C NMR spectroscopic studies upon infusion of L- and D-alanine labeled with ^{13}C in fasted rats show that D-alanine is metabolized significantly more slowly than the correspondingly labeled l-enantiomer (Dolle 2000). In addition, the immediate product of D-serine metabolism is likely to be hydroxypyruvate, rather than pyruvate, since hydroxypyruvate was identified as a metabolite of D,L-serine, but not of L-serine, in rat liver (Sprinson and Chargaff 1946; Nadkarni et al. 1960). The above observations are also consistent with the observed differences in



the post-ingestive reward properties between distinct sugars. Specifically, as shown in flavor-nutrient conditioning paradigms (Holman 1968; Tordoff 1991; Sclafani 2001), intra-gastric glucose produces significantly stronger flavor conditioning than fructose (Ackroff et al. 2001), consistent with the different biochemical pathways used by these monosaccharides leading to conversion into pyruvate (Lehninger et al. 1993).

Whatever the actual mechanisms may be, the above naturally leads to the more critical question of where in the body substrate utilization is translated into reward. We note that blood glucose levels—unlike respiratory exchange rates and liver glycogen—failed to predict intake, a finding that closely replicates our previous report on glucose reinforcement (Ren et al. 2010). This is also consistent with previous reports that intra-venous glucose, i.e., bypassing

◀ **Fig. 6** Liver glycogen, but not blood glucose, was significantly associated with amino acid intake levels. We have performed additional experiments to ascertain that intake levels were associated with changes in glucose production, oxidation, and/or storage. Wild-type mice were provided with one of the amino acids or with water for 22 h under food deprivation in their individual home cages, followed by immediate collection of blood (plasma) and liver samples. **a** Intake levels following a 1-h-long ad libitum one-bottle assay (in mL ingested). Only L-alanine and L-serine were ingested at significantly higher levels than their D-isomer counterparts (*$p < 0.05$). Also, only L-alanine and L-serine were ingested at significantly higher levels than water (#$p < 0.05$). **b**, **c** Liver glycogen levels (**b**) were linearly correlated with intake levels (**c**; intake levels shown as Kcal ingested; *dark gray data points* represent observations of animals ingesting L-amino acids, *light gray data points* represent observations of animals ingesting water, and *white data points* represent observations of animals ingesting D-amino acids). Overall linear model fit was $F[1,34] = 15.6$, $p < 0.0001$). However, when the linear correlation analysis was performed for D-amino acids only, no relationship between intake levels and glycogen was found ($F[1,13] = 1.5$, $p = 0.23$). Also, only L-alanine and L-serine produced significantly higher glycogen levels than their D-isomer counterparts (*$p < 0.05$). **d**, **e** Plasma glucose levels (**d**) were not correlated with intake levels (**e**; $F[1,34] = 0.31$, $p = 0.57$). When the linear correlation analysis was performed for D-amino acids only, no relationship between intake levels and glucose was found ($F[1,13] = 4.4$, $p > 0.05$). **f**, **g** Plasma lactate levels (**f**) were linearly correlated with intake levels (G; $F[1,34] = 10.1$, $p = 0.003$). However, when the linear correlation analysis was performed for D-amino acids only, no relationship between intake levels and lactate was found ($F[1,13] = 1.9$, $p = 0.18$). **h**, **i** Circulating ketone levels (**h**, beta-hydroxybutyrate) were inversely correlated with intake levels (**i**; $F[1,34] = 12.9$, $p = 0.001$). However, when the linear correlation analysis was performed for D-amino acids only, no relationship between intake levels and beta-hydroxybutyrate quantities was found ($F[1,13] = 0.22$, $p = 0.64$)

the gastrointestinal tract, fails to induce flavor preferences (Ackroff et al. 2010). While it remains plausible that at least part of the sensing mechanism involves glucosensing neurons (Levin 2006), including those located in reward circuits (Levin 2000), other organs may generate behaviorally relevant signals upon increases in glucose utilization. For example, the liver has been proposed as one critical metabolic sensor involved in the formation of flavor preferences (Russek 1970; Tordoff and Friedman 1986). In this context, it is relevant to note that L-amino acids are metabolized in the liver, the major site of gluconeogenesis, whereas D-amino acids are more preferably metabolized in kidney and other non-gluconeogenic tissues (Lehninger et al. 1993). This fact may explain the increased RER values and intake levels associated with the glucogenic L-amino acids.

Finally, we stress that the reinforcing properties of glucogenic amino acids seem to be largely independent of taste transduction, as was found to be the case for glucose itself (Ren et al. 2010). In our behavioral studies, we made use of the fact that *Trpm5* knockout mice are reported to be unable to detect sweet and most forms of L-amino acid tastants (Zhang et al. 2003). Given our previous results showing sweet-blindness concomitant to sugar post-ingestive sensitivity in *Trpm5* knockout mice (de Araujo et al. 2008; Ren et al. 2010), our current findings are consistent with the idea that sweet L-amino acids and sugars may share common signaling pathways downstream to the membrane taste receptor (Zhang et al. 2003; Bachmanov and Beauchamp 2008; Treesukosol et al. 2011). In a second *Trpm5* knockout strain described by Damak et al. (2006), residual sensitivity to high-concentration sucrose and umami solutions has been reported at both behavioral and cranial nerve levels. However, Damak et al. (2006) observed that the dissimilar phenotypes displayed by the two strains might result from the different gene deletions used in each case. In any event, the central fact to be retained here is that the strain used in our study did not display any residual sensitivity to sugar, sweetener, or glucogenic amino acid solutions used in our tests, in conformity with previous findings (Zhang et al. 2003).

In summary, our findings reveal a link between increases in respiratory exchange ratio and the appetitive properties of glucogenic L-amino acids, an effect that was moreover largely independent of the gustatory properties of these amino acids. Uncovering the identity of the physiological pathways and tissues linking substrate utilization to reward remains an important area for future research.

Acknowledgments R01-DC009997 to IEA is acknowledged.

Conflict of interest The authors declare no conflicts of interest.

References

Ackroff K, Touzani K, Peets TK, Sclafani A (2001) Flavor preferences conditioned by intragastric fructose and glucose: differences in reinforcement potency. Physiol Behav 72:691–703

Ackroff K, Yiin YM, Sclafani A (2010) Post-oral infusion sites that support glucose-conditioned flavor preferences in rats. Physiol Behav 99:402–411

Bachmanov AA, Beauchamp GK (2008) Amino acid and carbohydrate preferences in C57BL/6ByJ and 129P3/J mice. Physiol Behav 93:37–43

Blouet C, Schwartz GJ (2012) Brainstem nutrient sensing in the nucleus of the solitary tract inhibits feeding. Cell Metab 16(5):579–587

Damak S, Rong M, Yasumatsu K, Kokrashvili Z, Perez CA, Shigemura N, Yoshida R, Mosinger B, Glendinning JI, Ninomiya Y, Margolskee RF (2006) *Trpm5* null mice respond to bitter, sweet, and umami compounds. Chem Sens 31:253–264

de Araujo IE, Oliveira-Maia AJ, Sotnikova TD, Gainetdinov RR, Caron MG, Nicolelis MA, Simon SA (2008) Food reward in the absence of taste receptor signaling. Neuron 57(6):930–941

de Araujo IE, Ren X, Ferreira JG (2010) Metabolic sensing in brain dopamine systems. Results Probl Cell Differ 52:69–86

Dolle A (2000) Metabolism of D- and L-[(13)C]alanine in rat liver detected by (1)H and (13)C NMR spectroscopy in vivo and in vitro. NMR Biomed 13(2):72–81

Felig P (1973) The glucose–alanine cycle. Metabolism 22:179–207
Friedman M, Levin CE (2012) Nutritional and medicinal aspects of D-amino acids. Amino Acids 42(5):1553–1582
Fromentin G, Darcel N, Chaumontet C, Marsset-Baglieri A, Nadkarni N, Tome D (2012) Peripheral and central mechanisms involved in the control of food intake by dietary amino acids and proteins. Nutr Res Rev 25(1):29–39
Hao S, Sharp JW, Ross-Inta CM, McDaniel BJ, Anthony TG, Wek RC, Cavener DR, McGrath BC, Rudell JB, Koehnle TJ, Gietzen DW (2005) Uncharged tRNA and sensing of amino acid deficiency in mammalian piriform cortex. Science 307(5716):1776–1778
Hetenyi G Jr, Anderson PJ, Kinson GA (1984) Gluconeogenesis from threonine in normal and diabetic rats. Biochem J 224(2):355–360
Holman GL (1968) Intragastric reinforcement effect. J Comp Physiol Psychol 69:432–441
Jequier E, Acheson K, Schutz Y (1987) Assessment of energy expenditure and fuel utilization in man. Ann Rev Nutr 7:187–208
Lehninger AL, Nelson DL, Cox MM (1993) Principles of Biochemistry. Worth Publisher, New York
Levin BE (2000) Glucose-regulated dopamine release from substantia nigra neurons. Brain Res 874:158–164
Levin BE (2006) Metabolic sensing neurons and the control of energy homeostasis. Physiol Behav 89(4):486–489
Nadkarni GB, Friedmann B, Weinhouse S (1960) Gluconeogenesis from glycine and serine in the rat. J Biol Chem 235:420–425
Pere MC, Gilbert M, Assan R, Battaglia FC (1987) Studies of gut and hepatic metabolism in conscious rabbits. Am J Physiol 252(5 Pt 1):E573–E580
Peters JC, Harper AE (1987) Acute effects of dietary protein on food intake, tissue amino acids, and brain serotonin. Am J Physiol 252(5 Pt 2):R902–R914
Ren X, Ferreira JG, Zhou L, Shammah-Lagnado SJ, Yeckel CW, De Araujo IE (2010) Nutrient selection in the absence of taste receptor signaling. J Neurosci 30:8012–8023
Ritter RC (2004) Gastrointestinal mechanisms of satiation for food. Physiol Behav 81:249–273
Russek M (1970) Demonstration of the influence of an hepatic glucosensitive mechanism on food intake. Physiol Behav 5:1207–1209
Schwartz GJ (2013) Central leucine sensing in the control of energy homeostasis. Endocrinol Metab Clin North Am 42(1):81–87
Sclafani A (2001) Post-ingestive positive controls of ingestive behavior. Appetite 36(1):79–83
Sprinson DB, Chargaff E (1946) The occurrence of hydroxypyruvic acid in biological systems. J Biol Chem 164:411–415
Tome D, Schwarz J, Darcel N, Fromentin G (2009) Protein, amino acids, vagus nerve signaling, and the brain. Am J Clin Nutr 90(3):838S–843S
Tordoff MG (1991) Metabolic bases of learned food preferences. Chemical Senses. Friedman MI, Tordoff MG, Kare MR. New York, Marcel Dekker. 4
Tordoff MG, Friedman MI (1986) Hepatic portal glucose infusions decrease food intake and increase food preference. Am J Physiol 251(1 Pt 2):R192–R196
Treesukosol Y, Smith KR, Spector AC (2011) The functional role of the T1R family of receptors in sweet taste and feeding. Physiol Behav 105(1):14–26
Zhang Y, Hoon MA, Chandrashekar J, Mueller KL, Cook BWD, Zucker CS, Ryba NJ (2003) Coding of sweet, bitter, and umami tastes: different receptor cells sharing similar signaling pathways. Cell 112:293–301