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O-GlcNAc cycling mediates energy balance by regulating caloric memory

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ABSTRACT

Caloric need has long been thought a major driver of appetite. However, it is unclear whether caloric need regulates appetite in environments offered by many societies today where there is no shortage of food. Here we observed that wildtype mice with free access to food did not match calorie intake to calorie expenditure. While the size of a meal affected subsequent intake, there was no compensation for earlier under- or over-consumption. To test how spontaneous eating is subject to caloric control, we manipulated O-linked β-N-acetylglucosamine (O-GlcNAc), an energy signal inside cells dependent on nutrient access and metabolic hormones. Genetic and pharmacological manipulation in mice increasing or decreasing O-GlcNAcylation regulated daily intake by controlling meal size. Meal size was affected at least in part due to faster eating speed. Without affecting meal frequency, O-GlcNAc disrupted the effect of caloric consumption on future intake. Across days, energy balance was improved upon increased O-GlcNAc levels and impaired upon removal of O-GlcNAcylation. Rather than affecting a perceived need for calories, O-GlcNAc regulates how a meal affects future intake, suggesting that O-GlcNAc mediates a caloric memory and subsequently energy balance.

1. Introduction

Energy need has long been considered a dominating driver of appetite (Adolph, 1947; Richter, 1927; Saper, Chou, & Elmquist, 2002; Waterson & Horvath, 2015). Physical activity and resting metabolism are believed to create a caloric deficit that spurs motivation to seek and then consume food until the need has been satisfied (Berridge, 2004; Burnett et al., 2016; Dietrich, Zimmer, Bober, & Horvath, 2015; Flier, 2004; Mrosovsky; K. Williams & Elmquist, 2012). Sociocultural factors are known to influence when humans decide to eat. Most caloric regulation instead results from how much food is ingested per meal (Moran, 2009; Woods, Seeley, Porte, & Schwartz, 1998). The timing of satiation depends on a cascade of neuroendocrine signals from the gut, adipose tissue and other organs that carry information about dietary calories and body energy stores (Fig. 1A) (Chaudhri, Salem, Murphy, & Bloom, 2008; Grill, 2010). Specialized neurocircuitry in the brain processes the information and then stops the eating (Abizaid & Horvath, 2008; Sohn, Elmquist, & Williams, 2013). Need-based accounts of food intake often have these circuits calculating need by encoding a target level – or set point – of body fatness against which deviations are measured and corrected (Berridge, 2004; J. M.; Friedman, 1998; Keesey & Hirvonen, 1997; Kennedy, 1953). If body fatness may be an indirect measure of caloric availability other accounts define need as a function of direct caloric availability such as glucose utilization (Hopkins & Blundell, 2017; Mayer, 1953). For example, eating increases blood glucose and the following surge in cellular adenosine triphosphate (ATP) has been proposed as a meal stopper (M. I. Friedman, 1995). Body fatness levels are comparatively stable but adipokine storage signals such as leptin fluctuate repeatedly over a day, have effects on food intake within 1 h and gauge the sensitivity to cholecystokinin and other meal-dependent signals (Barrachina, Martinez, Wang, Wei, & Tache, 1997; Boden, Chen, Mozzoli, & Ryan, 1996; Licinio et al., 1997; McMinn, Sindelar,

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Havel, & Schwartz, 2000; Morton et al., 2005; Morton, Meek, & Schwartz, 2014; Sinha et al., 1996; D. L.; Williams, Baskin, & Schwartz, 2006). These different theories can be assumed under a general need-based account that we call here the energy deficit model of food intake. The energy deficit model of food intake predicts that energy balance is protected on a meal-to-meal basis if not perturbed by non-metabolic factors (Chambers, Sandoval, & Seeley, 2013; Heisler & Lam, 2017; Le Magnen & Devos, 1970; Schwartz, 1997; Schwartz, Woods, Porte, Seeley, & Baskin, 2000; Speakman et al., 2011; West, Fey, & Woods, 1984). Although hedonic aspects may influence feeding, need-driven regulation of feeding is often argued to work near perfectly as body weight in humans fluctuates on average less than 1 kg per year (Lewis et al., 1997; Ochner, Barrios, Lee, & Pi-Sunyer, 2013; Rosenbaum, Kissileff, Mayer, Hirsch, & Leibel, 2010).

Criticism has been raised against a deficit-driven account of metabolic control of feeding behavior, especially in situations where there is no shortage of food as in environments offered by many societies today (Speakman, 2018). Under such living conditions a nutrient supply so low that it would threaten the life of the individual is rare (Woods, 2004). Several papers have shown that free-living humans do not match their daily intake to energy expenditure (EE) (Edholm et al., 1970; Edholm, Fletcher, Widdowson, & McCance, 1955). Bouts of exercise usually decrease hunger-feelings and are not compensated by increased eating. Day-to-day calorie intake across cultures and ages varies by around 25% (Balogh, Kahn, & Medalie, 1971; Bingham et al., 1994; Champagne et al., 2013; Fukumoto et al., 2013; Nelson, Black, Morris, & Cole, 1989; Oh & Hong, 1999; Thackray, Deighton, King, & Stensel, 2016). The prevalence of obesity, a state of excessive fat accumulation resulting from eating beyond one's metabolic need, since the 1970's has reached epidemic proportions. Obesity is now a major health problem worldwide (Collaboration, 2017; Ng et al., 2014). From associated conditions such as cardiovascular disease and cancer, obese patients run a higher risk of premature death (Global et al., 2016; Prospective Studies et al., 2009). Today a majority of patients lack effective treatment (Booth, Prevost, & Gulliford, 2015; Rodgers, Tschop, & Wilding, 2012). While many metabolic signals that regulate food intake have been characterized they have been difficult to translate into therapy, in part due to confusion over how they combine to regulate information processing in the brain (Rodgers et al., 2012; K. W.; Williams & Elmquist, 2012; Woods & Langhans, 2012).

The presumed set point was suggested as explanation for why food intake tends to increase and body weight stabilize quickly after periods of starvation (Keesey & Hirvonen, 1997; Kennedy, 1953). Modeling shows, though, that a caloric memory function that records past caloric ingestion to affect future intake without calculating caloric need is sufficient to account for body weight stability and fasting-induced hyperphagia (Allcroft, Tolkamp, Glasbey, & Kyriazakis, 2004; Speakman, Stubbs, & Mercer, 2002). Caloric memories as a register of the number of calories consumed are distinct from episodic memories of situational cues surrounding a meal. However, there is very little biological data on whether caloric memories exist and mediate energy homeostasis but they may involve glucose metabolism (Davidson, Jones, Roy, & Stevenson, 2019; Ritter, Roelke, & Neville, 1978; Zhang et al., 2015).

Some memories are thought to be encoded by the brain through

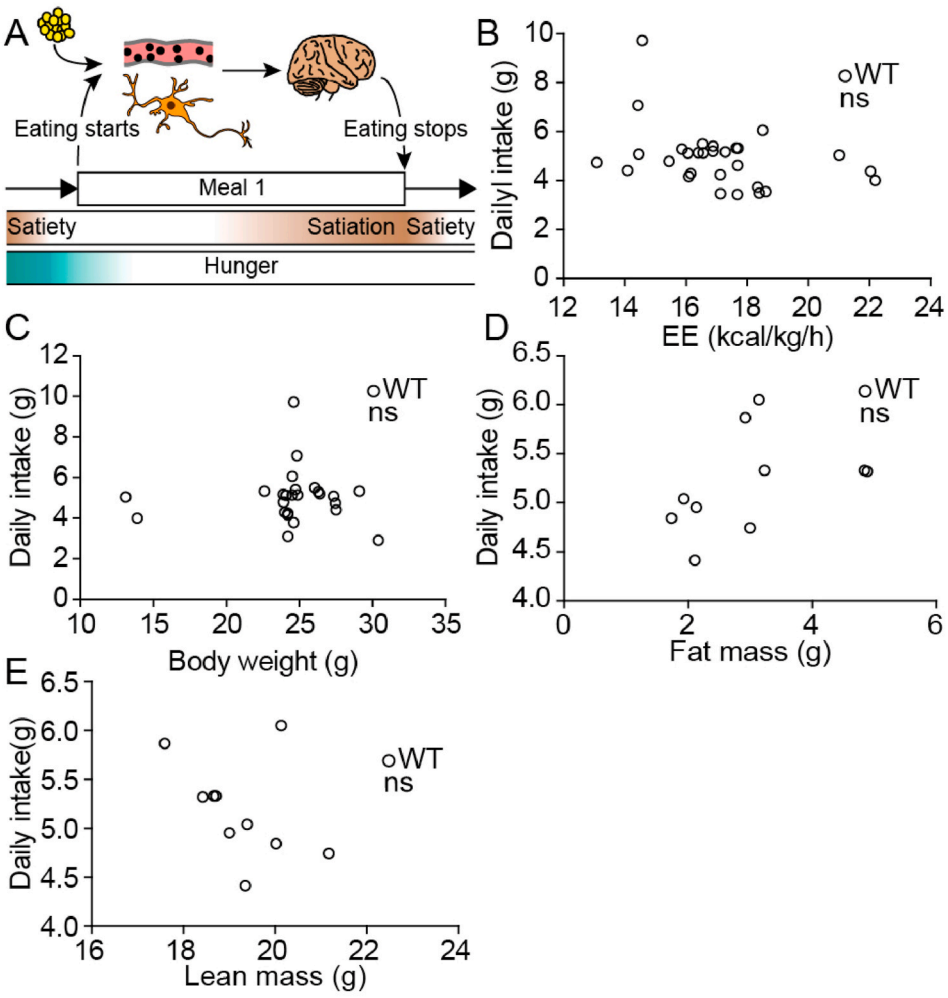

Fig. 1. Daily intake does not match energy expenditure or body constitution. (**A**) Schematic representation of the neuroendocrine cascade regulating satiation (yellow: adipose and other peripheral tissues; red: blood-borne factors; orange: neuronal signaling; brown: brain processing of peripheral signals). (**B-E**) Relationships in Wt mice between daily food intake and (**B**) energy expenditure (n = 29, r = −0.37 $P > 0.05$), (**C**) body weight (n = 25, r = 0.006, $P > 0.05$), (**D**) fat mass (n = 10, average daily intake, r = 0.42, $P > 0.05$), (**E**) lean mass (n = 10, average daily intake, r = −0.39, $P > 0.05$). All quantifications were based on Pearson's correlation coefficient (r). . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

synaptic plasticity, a process in which the number or strength of contacts change between neurons (Bhatt, Zhang, & Gan, 2009; Kessels & Malinow, 2009; Shepherd & Huganir, 2007). Many proteins that regulate synaptic plasticity, *e.g.* calcium-/calmodulin-dependent kinase II α (CaMKIIα) and SynGAP, are modified by β-N-acetylglucosamine (GlcNAc) (Alfaro et al., 2012; Lagerlof & Hart, 2014; Trinidad et al., 2012; Vosseller et al., 2006). GlcNAc becomes covalently attached to the hydroxyl group of specific serines and threonines (O-GlcNAc) by O-GlcNAc transferase (OGT) (Banerjee, Lagerlof, & Hart, 2016). Once attached, O-GlcNAc can be removed by O-GlcNAcase (OGA). Depending on the protein, O-GlcNAc cycles on and off on a time scale of minutes (e.g. on CaMKIV) to hours (e.g. αB-crystallin) (Chou, Smith, & Omary, 1992; Hart, Slawson, Ramirez-Correa, & Lagerlof, 2011; Roquemore, Chevrier, Cotter, & Hart, 1996; Song et al., 2008; Yuzwa et al., 2008). Its absolute levels depend on flux through the hexosamine biosynthesis pathway (HBP) which converts glucose to uridine diphosphate (UDP)-GlcNAc, the substrate of OGT (Hart et al., 2011). Food intake affects O-GlcNAcylation in the brain (X. Li, Lu, Wang, & Gong, 2006; Liu, Iqbal, Grundke-Iqbal, Hart, & Gong, 2004). Brain O-GlcNAcylation is modulated by direct cellular uptake of glucose but also from regulation of OGT by metabolic hormones such as ghrelin (Lagerlof et al., 2016; Pekkurnaz, Trinidad, Wang, Kong, & Schwarz, 2014; Ruan et al., 2014; Zimmerman & Harris, 2015). We and others have shown previously that deleting OGT in αCaMKII-positive neurons in the brain leads to increased food intake and subsequent obesity (Dai, Gu, Liu, Iqbal, & Gong, 2018; Lagerlof et al., 2016). The hyperphagia results from impaired feeding-induced activation of αCaMKII neurons in the paraventricular nucleus of the hypothalamus (PVN) which normally terminate feeding (Lagerlof et al., 2016). The lost response to food probably stems at least in part from attenuated excitatory synaptic input by downregulation of the glutamate-gated α-amino-3--hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor (Hwang & Rhim, 2019; Lagerlof, Hart, & Huganir, 2017; Lagerlof et al., 2016; Tallent et al., 2009; Taylor et al., 2014; Yang et al., 2017). Genetic deletion experiments and pharmacological manipulation targeting OGA also affect synaptic plasticity (Lagerlof, 2018). Both associative and non-associative memory in tasks not related to food intake have been shown to be regulated by O-GlcNAcylation (Ardiel et al., 2018; Xie et al., 2016; Yang et al., 2017).

Here we investigated how caloric signaling affects appetite. First, we tested in wildtype mice the prediction of the energy deficit model that caloric ingestion is matched to caloric expenditure if variation in all major environmental influences over food intake is removed. We find that intake in stable environments neither varies according to energy expenditure nor compensates for earlier over- or under-consumption. Then three different mouse models of O-GlcNAcylation (OGT deletion, OGA deletion and pharmacological inhibition of OGA) were used to manipulate caloric regulation of food intake. Rather than affecting a perceived need for calories, our results favor a model where O-GlcNAcylation mediates a caloric memory by which the size of a meal affects future intake and subsequently energy balance.

2. Methods

2.1. Animals

The animals were used in previously published articles; here we analyzed their meal-to-meal food intake (Keembiyehetty et al., 2015; Lagerlof et al., 2016; Tan et al., 2017). Full details of the methods are found in them.

All animal work was done according to the guidelines of and approved by the Johns Hopkins University Animal Care and Use Commmittee (OGT KO animals), National Institutes of Health (OGA Het animals) and the University of Kansas Medical Center Animal Care and Use Committee (TMG treated animals). All animal work adhered to the ARRIVE Guidelines including the principles of the 3Rs. *OGT KO animals*: Fully backcrossed (C57Bl/6N, N > 11) floxed OGT (OGT^{FL}) and αCaMKII-$CreER^{T2}$ mice were mated and injected with tamoxifen at about 6 weeks of age (Erdmann, Schutz, & Berger, 2007; O'Donnell, Zachara, Hart, & Marth, 2004). 2 mg tamoxifen was injected twice per day for 5 days. Control mice were injected with vehicle (sunflower seed oil injected into OGT^{FL} x αCaMKII-$CreER^{T2}$ mice). The animals had free access to standard chow (24% protein, 18% fat, 58% carbohydrate) in powder form and kept on a 12 h/12 h light/dark cycle. Metabolic data were acquired by subjecting the mice about two weeks after OGT deletion to comprehensive laboratory animal monitoring system (CLAMS, Columbus Instruments, Columbus, OH, USA) that detects food intake, physical activity and energy expenditure through indirect calorimetry in real-time. The formulas used to calculate energy expenditure can be found at www.colinst.com. Only males were used (controls, n = 6 and knockouts, n = 7) and they were acclimatized to the metabolic cages before data acquisition started. Daily intake values for the OGT mice in Fig. 3B have been published previously (Lagerlof et al., 2016). *OGA Het animals*: OGA was knocked out by crossing OGA^{FL} mice (C57Bl/6) with a mouse line that expresses Cre recombinase in the oocyte (the murine mammary tumor LTR-virus line; C57Bl/6–129 hybrid). These mice have been described previously (Keembiyehetty et al., 2015; Wagner et al., 2001). Adult heterozygotic female OGA KO animals (controls n = 4 and OGA HET KO n = 5) were then subjected to CLAMS for metabolic data acquiring as discussed for the OGT KO animals. Daily intake values for the OGA mice in Fig. 3B of this paper have been published previously (Keembiyehetty et al., 2015) with copyrights belonging to the American Society for Biochemistry and Molecular Biology. *TMG treatment*: Adult male wildtype C57Bl/6J mice were acclimatized to individual housing for 5 days and then transferred to metabolic cages (Promethion High Definition Continuous Respiratory system for mice, Sable Systems Inc.) for real-time food intake and energy expenditure measurements. They were then transferred back to their home cage (still individually housed) to receive intraperitoneal injections with TMG (50 mg/kg, SD Specialty Chemicals) or saline every other day for 15 days (8 doses in total; saline n = 8 and TMG n = 8 animals). During the last 5 days they were transferred back to the metabolic cages to measure intake and expenditure as before. The mice were offered normal chow diet in pellet form. No animals had been exposed to any other experimental manipulation prior to this experiment.

2.2. Body composition

Fat and lean mass were measured using EchoMRI (EchoMRI, Houston, TX, USA).

2.3. Meals analysis

A "meal" was defined as a period of 15 min where the cumulative amount of intake was 50 mg or more and the previous 15 min had no registered intake. The end of a meal was defined as when no intake had been registered for another 15 min. Meal frequency was defined as the number of meals consumed per day. 'Snacks' were considered as any other intake continuous in time. The raw intake data were recorded in 1 min bins. A subset of all the data were used to evaluate the performance of these conditions (Fig. S1). The rules were coded in 'Python' and the software automatically identified meals and snacks from the raw intake data. The code is available on Github. All data in this paper is available upon request. Eating speed was defined as amount consumed during the length of each meal.

2.4. Statistics

Statistical analyses were done primarily in Prism 5. Some analyses were done using Excel. Sometimes negative food intake values were recorded. Negative values occur when animals, *e.g.*, defecate in the food bowl. No difference was seen between wildtypes and O-GlcNAc

modulated mice (data not shown) and the negative values were removed from analyses. For analyses of wildtype feeding behavior, the wildtypes in the OGT and OGA experiments were pooled. In Fig. 3D wildtypes from the TMG treated group were used. When testing the effect of O-GlcNAc cycling manipulations, the data were compared against the respective wildtype group for each manipulation. All Student's *t*-tests were two tailed and unpaired. For Fig. 3C–E one-way ANOVA was used with follow-up comparisons of significant differences between intra-meal bins according to the Bonferroni multiple comparisons method. When analyzing correlation, Pearson's- and Spearman's correlation were performed as well as linear regression analysis. Asterisk signifies $P < 0.05$. All error bars represent mean ± standard error of the mean (SEM). A table of complementary statistics used for each figure is included in the Supplemental information (Table S1).

3. Results

3.1. Body energy status does not predict variations in spontaneous feeding

If it is the case that energy deficiency drives appetite, then food intake has been expected to closely match energy expenditure, or a derivate thereof such as adipose tissue, when not perturbed by environmental stimuli (Blundell et al., 2012; Blundell, Gibbons, Caudwell, Finlayson, & Hopkins, 2015). Here we subjected adult mice to metabolic cages in stable environments. The mice ate *ad libitum* and their energy expenditure was measured by indirect calorimetry. In contrast to the predictions of the energy deficit model, we could not detect a clear match in wildtypes (Wt) between daily food intake and energy expenditure (Fig. 1B). Neither females nor males matched their daily intake to calories spent (Fig. S1A-B). Likewise, correlates to body energy status, body weight, or fat or lean mass did not predict daily intake (Fig. 1C–E). Animals tend to consume food in bouts (Fig. S2A) (Brobeck, 1955; Richter, 1927). The amount of food consumed per bout is thought to be the primary target for caloric regulators of food intake (Moran, 2009; Woods et al., 1998). During feeding bouts rodents eat but also drink and explore their immediate surroundings (Barnett, 1956; Blundell, Rogers, & Hill, 1985; Rodgers, Holch, & Tallett, 2010). Depending on the length of time spent not eating during *versus* between bouts, eating events can be organized into meals (Allcroft et al., 2004; Le Magnen & Devos, 1980; Tolkamp et al., 2011). Mathematical analysis has been instrumental to show that meal definitions that accept shorter breaks to occur during meals satisfy predictions of how satiety (the inhibition of appetite in the intermeal interval) affects meal behavior (Tabarin et al., 2007; Tolkamp et al., 2011; Zorrilla et al., 2005). The concept of satiety suggests that physical activity decreases after a meal has finished and that the probability to initiate a second meal just after finishing the first meal is low. The probability to start another meal then increases with time as satiety decreases (Richter, 1922; Rodgers et al., 2010; Tolkamp, Allcroft, Austin, Nielsen, & Kyriazakis, 1998). While there are no universally accepted criteria in the feeding literature, many authors argue that these satiety-dependent behavioral predictions should be used to verify meal criteria (Tabarin et al., 2007; Tolkamp et al., 2011; Zorrilla et al., 2005). Very small intakes, or "snacks", may not induce behavioral satiety or be energetically regulated in the same way as meals (Chapelot, 2011; Martire, Holmes, Westbrook, & Morris, 2013). In accordance with previous mathematical analyses of meal behavior, here we generated an algorithm in 'Python' that automatically classified intake events as 'meals' or 'snacks' by accepting short breaks during meals once a minimum amount of food (50 mg) had been consumed. Physical activity decreased in the post-meal period (Fig. S2B). Immediately after finishing a meal, the likelihood to start another meal was close to zero and rose with time (Fig. S2C). Several papers have shown a smaller meal size during the daytime compared to nighttime (Le Magnen & Devos, 1980; Richard, Tolle, & Low, 2011). We also observed that meal, but not snack, size was smaller during light hours (Fig. S2D). These data indicate that the meals identified here map satiety-dependent eating events. Apart from meal frequency and fat mass, meal size and frequency varied without a clear correlation to body weight or body constitution (Fig. S3A-F). These observations suggest that body energy status cannot explain variations in *ad libitum* feeding behavior in stable environments.

3.2. Feeding behavior is unstable over time

While body energy status did not predict variations in spontaneous feeding behavior, day-to-day intake changed 22% (±3.6%). Daily intake varied over time for the same mouse as much as it did between mice of different body constitutions (Fig. 2A). There was neither any difference in the intra- and inter-individual variance for meal and snack frequency (Fig. 2A). The size of meals and snacks fluctuated over time more than the size did between individuals (Fig. 2A). Satiety has long been thought to give a calorie-dependent structure to meal-to-meal behavior by making the postprandial interval a function of the preceding meal (Le Magnen & Devos, 1980). Over the course of a whole day, meal frequency and meal size were negatively correlated (Fig. 2B). In contrast, the size of a large meal predicted that the next meal would also be large and a small meal was followed typically by another small meal (Fig. 2C–D). The larger meal size, the shorter break until the next meal started (Fig. 2C and E). Overall, our data indicate that feeding behavior is structured across meals. This structure, however, does not work to compensate preceding deviations from a mean level of intake.

3.3. O-GlcNAc cycling regulates daily intake by affecting meal size

To test how caloric signaling affects spontaneous feeding behavior, we manipulated O-GlcNAc cycling in mice (Fig. 3A). OGT, the enzyme that catalyzes the final step in O-GlcNAc synthesis, was knocked out (KO) in αCaMKII brain neurons to abolish O-GlcNAcylation by injecting adult OGT^{FL} x αCaMKII-CreER mice with tamoxifen (Lagerlof et al., 2016). Deleting OGT in αCaMKII neurons increased daily food intake (Fig. 3B) (Dai et al., 2018; Lagerlof et al., 2016). The OGT KO mice ate bigger meals and snacks without changing meal or snack frequency (Fig. 3B) (Lagerlof et al., 2016). We elevated protein O-GlcNAcylation by knocking out OGA, the enzyme that removes O-GlcNAc, through a cross between OGA^{FL} mice and mice that express Cre recombinase in the oocyte (the murine mammary tumor LTR-virus line) (Keembiyehetty et al., 2015). Heterozygotic (HET) OGA KO mice develop normally without any gross structural or other developmental defects and preserve fertility (Keembiyehetty et al., 2015). These animals are haploinsufficient for OGA (Keembiyehetty et al., 2015). Total intake or meal and snack size and frequency were not altered in haploinsufficient OGA animals (Fig. 3B). In contrast, raising O-GlcNAc levels acutely by intraperitoneal injections with Thiamet-G (TMG), a highly specific inhibitor of OGA, elevated daily intake (Fig. 3A–B) (Tan et al., 2017). Similar to adult deletion of OGT, meal size and not meal frequency was affected (Fig. 3B). These effects occurred independently of current energy status as, apart from OGT deletion introducing a significant correlation between body weight and meal frequency, no variations in daily intake, meal size or frequency could be predicted by body weight, fat or lean mass in the OGT KO, haploinsufficient OGA or TMG animals (Fig. S4A-G). There was also no correlation between daily intake and energy expenditure in the O-GlcNAc mice (Fig. S5A-C). Instead comparing when during a meal food is consumed, intake was binned according to time from the start to finish of each meal. When offered a powdered diet, wildtypes ate more in early and late phases of the meal (Fig. 3C). This intra-meal structure was abolished by deleting OGT or OGA (Fig. 3E). A uniform eating pattern was observed also in animals, both wildtypes and after TMG-treatment, that ate food in pellet form (Fig. 3D–E). The eating rate in wildtypes was almost identical between meals of different sizes, as indicated by the strong correlation between meal size and length (Fig. 3F) (Le Magnen & Devos, 1980). Whereas O-GlcNAc manipulations did not affect the size-length correlation (Fig. 3G–I), removing OGT or OGA increased meal speed (Fig. 3K). These

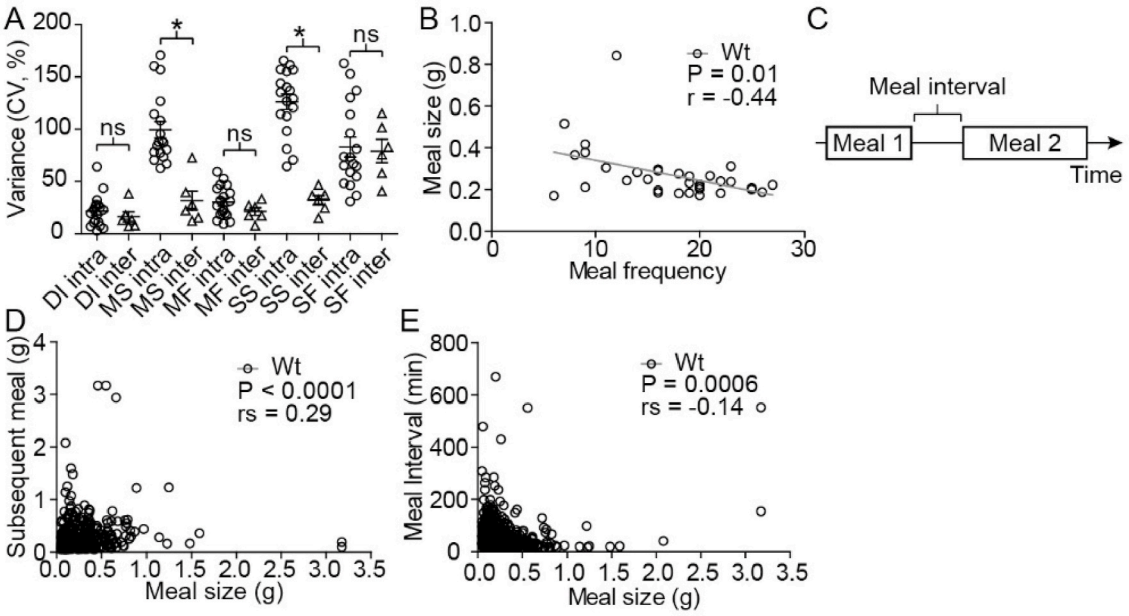

Fig. 2. Meal-to-meal behavior is unstable over time. **(A)** Wt intra-individual (intra) and inter-individual (inter) coefficient of variance (CV) for daily intake (DI), meal size (MS), meal frequency (MF), snack size (SS) and snack frequency (SF) (intra: n = 18, inter: n = 6; two-tailed *t*-test $*P < 0.05$). **(B)** Pearson's correlation (r) between meal size and meal frequency (meals/day) with linear regression (gray line) (n = 33 pairs of daily meal size and meal frequency). **(C)** Cartoon of meal-to-meal correlations shown in (D-E). **(D–E)** Spearman's correlations (rs) between two subsequent meals (D) or a meal and the subsequent meal interval (E) (n = 569 pairs of meal size and subsequent meal size/interval). Error bars represent mean ± SEM.

data suggest that O-GlcNAcylation alters daily food intake by regulating meal size at least in part through affecting eating speed.

3.4. O-GlcNAc cycling mediates energy balance across meals

The selective effect of O-GlcNAc cycling on meal size suggests that O-GlcNAcylation regulates satiation (meal termination) rather than satiety or hunger (Lagerlof et al., 2016). We hypothesized that if impaired satiation upon manipulating O-GlcNAc cycling reflects an increased caloric need to satisfy, then not only would the average meal size increase but also the frequency of relatively large meals. However, there was no change in meal size distribution upon modulation of O-GlcNAcylation (Fig. 4A). Genetically altering OGT or OGA or TMG treatment affected neither the intra-individual variance for daily intake, meal size, meal frequency, snack size nor snack frequency (Fig. 4B). Surprisingly, after OGT removal the amount consumed in a meal no longer affected the amount the animal ate in the subsequent meal (Fig. 5A). OGT deletion also uncoupled the time between two meals from the size of the first meal (Fig. 5B). Heterozygotic KO of *Oga* improved the meal-to-meal size correlation (Fig. 5C) but eliminated the meal-to-meal interval correlation (Fig. 5D). Upon TMG treatment, the amount consumed in the first meal did not predict subsequent meal size (Fig. 5E) but did predict the following inter-meal interval (Fig. 5F). Next we investigated the effect on energy balance across days. No animals showed correlation between day-to-day variations in body weight and food intake (Fig. S6A-C). Instead, we observed a negative correlation between daily fluctuations of intake and expenditure in male wildtype animals that ate powdered food (Fig. 5G). Deleting OGT abolished the connection between changes in day-to-day intake and expenditure (Fig. 5G). During the same period the OGT KO mice rapidly developed obesity (Lagerlof et al., 2016). Females, whether wildtypes or haploinsufficient OGA animals, did not correlate food intake with calories spent across days (Fig. S6D). Neither was there any correlation between daily intake and expenditure changes when male wildtypes were offered pelleted food (Fig. 5H). In contrast, TMG treatment conferred a significant positive correlation between day-to-day variations in intake and expenditure (Fig. 5H). The TMG mice also gained less weight than wildtypes (Tan et al., 2017). Our observations together favor a model where O-GlcNAcylation mediates a process by which the caloric content of a meal affects future intake - caloric memory - and thus long-term energy balance (Fig. 6).

4. Discussion

Our results indicate that cycling of the posttranslational modification O-GlcNAc regulates appetite by mediating a caloric memory that is critical to maintain energy balance and protect against obesity.

These observations suggest a new model of how metabolic signaling controls appetite. It was thought previously that caloric need defending a homeostatic set point mediates feeding. The old model predicted that food intake will be correlated to energy expenditure on an hourly basis if environmental influence over feeding such as cultural factors and energy-dense food choices are removed (Berridge, 2004; Chambers et al., 2013; J. M.; Friedman, 1998; Heisler & Lam, 2017; Keesey & Hirvonen, 1997; Kennedy, 1953; Le Magnen & Devos, 1970; Schwartz et al., 2000; Speakman et al., 2011; West et al., 1984). In contrast, our data showed that spontaneous daily intake during stable living conditions was not accommodated to energy expenditure, body weight, or fat or lean mass. The size and timing of meals depended on previous intake but not such that eating corrected earlier over- or under-consumption. Sometimes negative food intake values were recorded. Negative values occur when animals, *e.g.*, defecate in the food bowl. No difference was seen between wildtypes and O-GlcNAc modulated mice (data not shown) and the negative values (20% of all values) were removed from analyses. Our analysis builds on correlative studies and thus cannot rule out that, e.g., fat mass drove intake but in addition to the lack of significant correlations the modest r-values shown here suggest at least that another major factor than caloric need is at play. Hence, even though all major environmental influence had been removed and the animals were offered a single diet of regular chow, there is no evidence in our data that caloric need explains daily wildtype feeding behavior.

Caloric need can explain neither daily wildtype feeding behavior nor the effect on food intake by O-GlcNAc. Deleting OGT specifically in αCaMKII brain neurons or inhibiting OGA with TMG increased daily

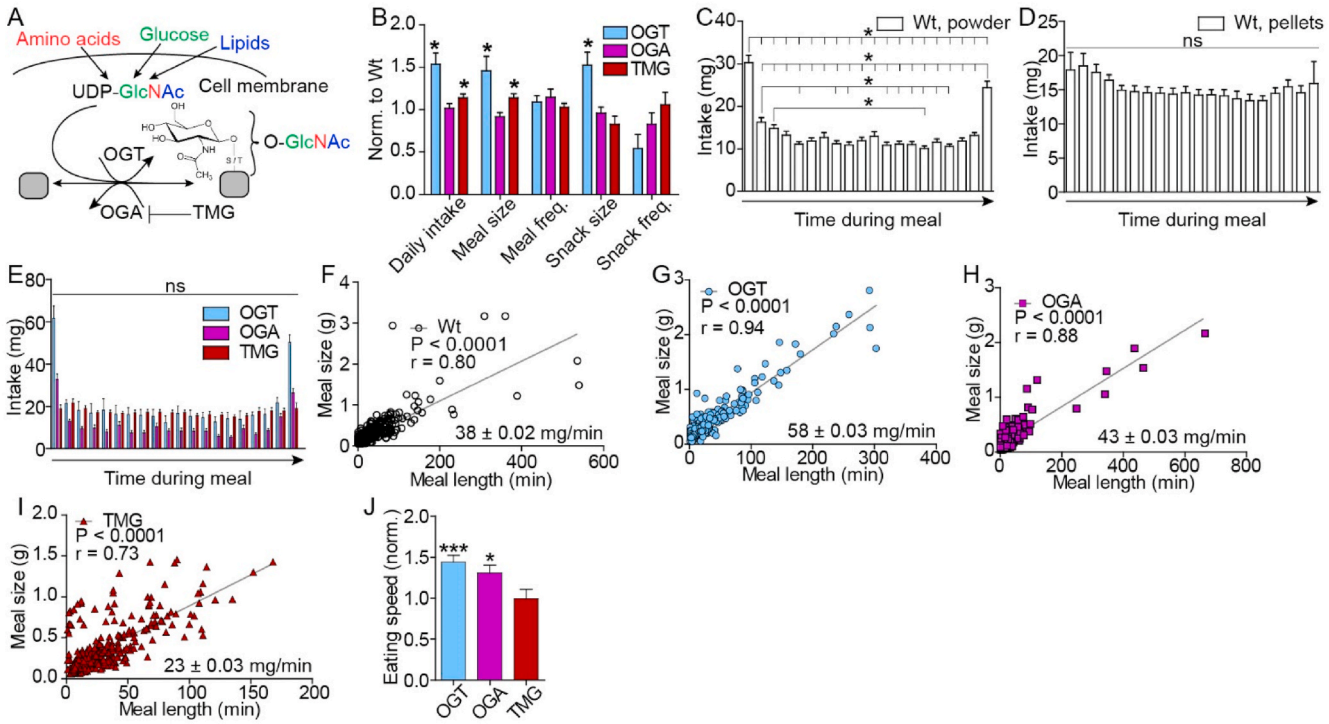

Fig. 3. O-GlcNAc regulates daily intake by affecting meal size. **(A)** Cartoon of the regulation and function of the posttranslational modification O-GlcNAc. **(B)** The effect on different food intake components upon OGT deletion (OGT) (Daily intake: Wt n = 17, KO n = 20. Meal size: Wt n = 325, KO n = 418. Meal frequency: Wt n = 17, KO n = 20. Snack size Wt n = 280, KO n = 178. Snack frequency: Wt n = 17, KO n = 20.), heterozygotic OGA deletion (OGA) (Daily intake: Wt n = 16, HET KO n = 20. Meal size: Wt n = 239, HET KO n = 342. Meal frequency: Wt n = 16, HET KO n = 20. Snack size Wt n = 374, HET KO n = 387. Snack frequency: Wt n = 16, HET KO n = 20.) or TMG treatment (TMG) (Daily intake: Wt n = 36, TMG n = 36. Meal size: Wt n = 336, TMG n = 346. Meal frequency: Wt n = 36, TMG n = 36. Snack size Wt n = 146, TMG n = 179. Snack frequency: Wt n = 36, TMG n = 36.) (two-tailed *t*-test $*P < 0.05$). **(C–D)** Food intake during meals for wildtypes eating powdered (n = 936 for each bin) or pelleted (n = 357 for each bin) chow where intake during each meal was allocated to 20 bins. Each bin was compared to the others using one-way ANOVA with Bonferroni's multiple comparison test ($*P < 0.05$). **(E)** Food intake during meals for OGT KO and OGA HET KO animals eating powdered or TMG treated animals eating pelleted chow where intake during each meal was allocated to 20 bins. Each bin was compared to the others within the same condition, *e.g.* between all OGT KO bins (OGT: n = 424, OGA n = 343, TMG n = 358 for each bin, one-way ANOVA with Bonferroni's multiple comparison test, $P > 0.05$). **(F–I)** Pearson's correlation (r) between meal length and meal size with linear regression (gray line) for (F) Wt (n = 579), (G) OGT KO (n = 422, (H) OGA HET KO (n = 342), (I)TMG treatment (n = 358). **(J)** Eating speed for OGT KO (WT n = 340, OGT KO n = 391), OGA HET KO (Wt n = 239, OGA HET KO n = 342) or TMG treatment (Wt n = 360, TMG n = 358) normalized to Wt eating speed (two-tailed *t*-test, $*P < 0.05$). Error bars represent mean ± SEM.

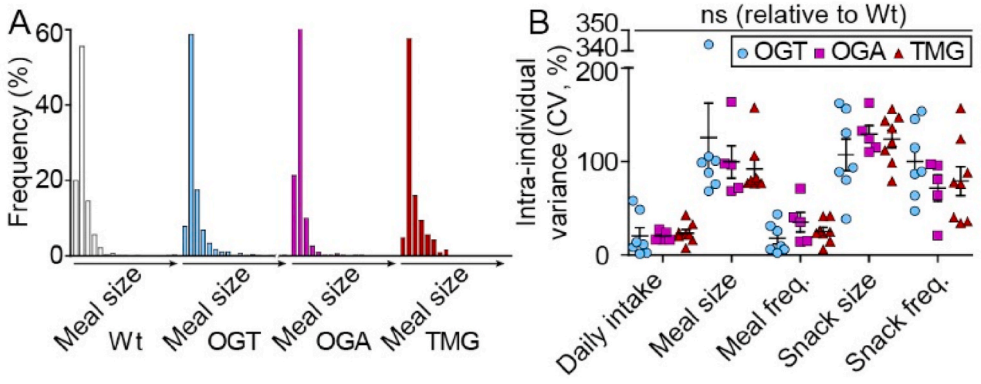

Fig. 4. Food intake variability not affected by O-GlcNAc. **(A)** Meal size frequency distribution (Wt n = 579; OGT KO n = 423; OGA HET KO n = 342, TMG n = 358). **(B)** Intra-individual coefficient of variance (CV) for OGT KO animals (Wt n = 6, KO n = 7), OGA HET animals (Wt n = 4, HET n = 5) and TMG treated animals (Wt n = 8, KO n = 8). Two-tailed *t*-test, $P > 0.05$. Error bars represent mean ± SEM.

food intake. The increased food intake was explained by larger meal size. Had the larger meal size been the result of a greater need for calories, then it is likely that meal frequency would have been higher as well. A preference for only large meals and consistently high daily intake also would have argued for a change in need. However, meal frequency was not affected and daily intake and meal size variation and distribution were not affected by OGT KO, OGA HET KO or TMG treatment. While TMG treatment increased food intake, TMG improved the balance between intake and expenditure. Indeed, TMG limited body weight increase (Tan et al., 2017). This suggests that the TMG-dependent increased intake was not the result of an up-shifted set point. Neither is an up-shifted set point a likely explanation for the

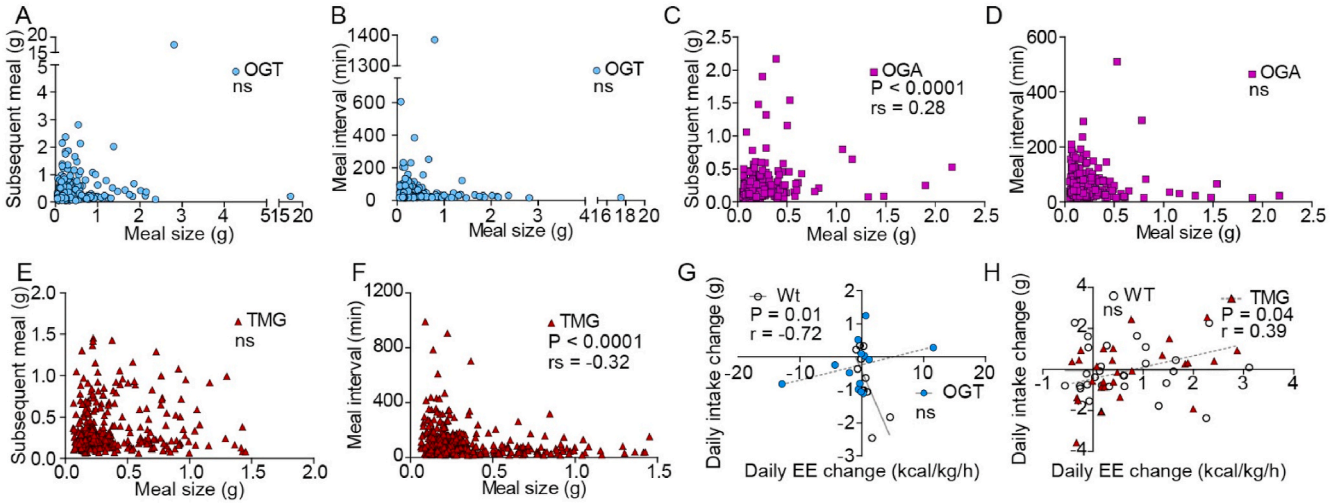

Fig. 5. O-GlcNAc matches food intake to energy expenditure across meals. **(A–F)** Relationship between two subsequent meals (A, C, E) or a meal and the subsequent meal interval (B, D, F) (Spearman's correlation (rs)). Fig. 2D–E shows the meal-to-meal relationships for pooled wildtype data. (A) Wt, n = 334 meal pairs rs = 0.35 $P < 0.0001$; OGT KO, n = 416 meal pairs rs = 0.070 $P > 0.05$. (B) Wt, n = 334 meal/meal interval pairs rs = −0.14 $P < 0.05$; OGT KO, n = 416 meal/meal interval pairs rs = −0.070 $P > 0.05$. (C) Wt, n = 235 meal pairs rs = 0.16 $P < 0.05$; OGA HET KO, n = 337 meal pairs rs = 0.28 $P < 0.0001$. (D) Wt, n = 235 meal pairs rs = −0.19 $P < 0.01$; OGA HET KO, n = 337 meal pairs rs = −0.07 $P > 0.05$. (E) Wt, n = 349 meal pairs rs = 0.21 $P < 0.0001$; TMG, n = 350 meal pairs rs = 0.043 $P > 0.05$. (F) Wt, n = 349 meal pairs rs = −0.31 $P < 0.0001$; TMG, n = 350 meal pairs rs = −0.32 $P < 0.0001$. **(G–H)** Pearson's correlation (r) between the daily change in intake and energy expenditure (G: Wt n = 11 r = −0.72 $P < 0.05$, OGT KO n = 13 r = 0.34 $P > 0.05$. H: Wt n = 28 r = 0.0087 $P > 0.05$, TMG n = 28 r = 0.39 $P < 0.05$).

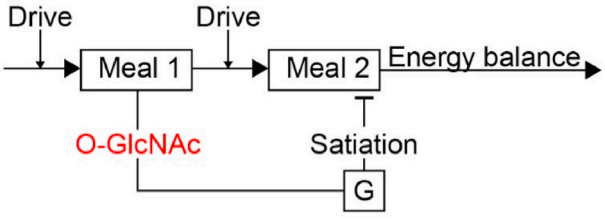

Fig. 6. O-GlcNAc cycling mediates energy balance by regulating caloric memory. Irrespective of what initiates feeding (Drive), O-GlcNAc mediates a caloric memory by which the caloric content of a meal affects future intake through adjusting satiation threshold (G) and subsequently long-term energy balance.

rapidly developing obesity upon deleting OGT in αCaMKII neurons in adult animals shown previously by us and others, as OGT deletion also leads to increased energy expenditure when fed *ad libitum* and stable body weight when pair-fed (Dai et al., 2018; Lagerlof et al., 2016). Hence, these and our previous data indicate that the effects on eating by modulation of O-GlcNAc cycling are the product of something other than a change in need for calories.

Instead of need, both wildtype spontaneous feeding behavior and the role of O-GlcNAc can be resolved by a model based on caloric memory. Memories are thought to form through synaptic plasticity, or changes in the synaptic number and strength between neurons (Bhatt et al., 2009; Kessels & Malinow, 2009; Shepherd & Huganir, 2007). We and others have shown that synaptic plasticity is regulated by OGT and OGA (Lagerlof, 2018). Deleting OGT in $PVN^{\alpha CaMKII}$ neurons decreased their excitatory glutamatergic synaptic input. These neurons normally become activated by eating to inhibit further consumption. The loss of excitation contributes to a larger meal size and explains the observed hyperphagia in the OGT KO mice (Lagerlof et al., 2016). Glutamatergic stimulation in the PVN decreases food intake (Fenselau et al., 2017; Hettes et al., 2003). Stimulating the $PVN^{\alpha CaMKII}$ neurons optogenetically did not affect the latency to eating but attenuated the amount food consumed during the first meal after start of stimulation (Lagerlof et al., 2016). Both OGT deletion and TMG treatment increased meal size indicating that it is the cycling of O-GlcNAc on and off proteins that regulate meal size and not other effects from OGT deletion. Whereas the manipulations have opposite effects on global O-GlcNAc levels it has been shown repeatedly that increased as well as decreased O-GlcNAcylation can decrease excitatory synaptic transmission (Hwang & Rhim, 2019; Lagerlof, 2018). We would expect thus bigger meals from both manipulations. Here we observed that the larger meal size is at least in part the result of a faster eating rate. Heterozygotic KO of OGA in the embryo did not affect meal size in adult mice although exhibiting elevated eating rate. This was probably due to developmental compensation which can occur in feeding circuits in the brain (Qian et al., 2002). As OGA was deleted in females and males were used for the OGT and TMG experiments the sex of the animals may also have contributed to the difference. Altered eating structure during meals may have added to the increased size. As the heterozygotic deletion of OGA occurred in the whole body and TMG injected systemically, effects in peripheral tissues contributed to the metabolic phenotype of these mice (Bond & Hanover, 2013; Keembiyehetty et al., 2015; Tan et al., 2017). The effects on feeding behavior by OGA haploinsufficiency and systemic TMG treatment may thus have been affected in part by non-brain mechanisms (M. D. Li et al., 2018). Withal, these data together with the selective effect of O-GlcNAc cycling on meal size indicate that O-GlcNAc cycling mediates satiation (meal termination) during individual meals. While satiation operates on single meals, we observed in addition that O-GlcNAc cycling regulated feeding behavior across meals. Deleting OGT removed the effect of the size of the first meal on the postprandial interval and the size of the next meal. Heterozygotic deletion of OGA and TMG treatment perturbed the coordination between the timing and size of subsequent meals, respectively. Moreover, OGT removal uncoupled changes across days in energy intake from changes in energy expenditure. Conversely, inhibition of OGA using TMG improved the day-to-day balance between intake and expenditure. OGT removal and TMG treatment both decrease excitatory synaptic transmission as discussed above. However, deleting OGT leads to a reduction in excitatory synapse number mainly and thereby possibly inhibiting memories (Lagerlof et al., 2017; Wang, Jensen, Rexach, Vinters, & Hsieh-Wilson, 2016). In contrast, TMG treatment may improve memory through long-term depression of excitatory synapse strength (which you would not expect from OGA haploinsufficiency) (Taylor et al., 2014; Yang et al., 2017). OGT overexpression in the hippocampus enhances fear

memory (Wheatley et al., 2019). The regulation of satiation on the one hand and feeding behavior over time on the other hand can be reconciled by a model in which O-GlcNAc adjusts satiation in subsequent meals depending on the caloric content of previous intake. Thus, these data indicate that O-GlcNAc incorporates a memory function in the threshold at which satiation occurs by regulating excitatory synaptic function in $PVN^{\alpha CaMKII}$ neurons.

Its basis in the hypothalamus suggests that satiation memory is a non-declarative type of memory – a behavioral disposition that becomes encoded without conscious representational content or need for active recall (Squire & Dede, 2015). This implicit memory stands in contrast to previous observations that explicit memory including working and episodic memory affects feeding behavior (Davidson et al., 2019; Higgs & Spetter, 2018). These different memory systems may however interact. There are, for example, connections between the hypothalamus, hippocampus and neocortex that may together shape the individual's adaptive responses to feeding stimuli (Cenquizca & Swanson, 2006; Cui, Gerfen, & Young, 2013; Saper, 2000). A recent article reporting that fruit flies and some mice can learn to distinguish between normal and high caloric food showed that the memory formation depended on learning and memory genes in both hippocampal and hypothalamic neurons (Zhang et al., 2015). While it has been recognized previously that representations of past meals can be affected by metabolic signals and be associated with clues as to how many calories the meal contained, O-GlcNAc-mediated regulation of satiation memory introduces a mechanism by which a memory specifically of a past meal's caloric content is encoded and affects subsequent meal behavior (Davidson et al., 2019).

Identifying a caloric memory function in satiation unifies metabolic and environmental influences over appetite. Experiences of one's surrounding environment can modulate the decision of when and how much to eat by learned associations between particular cues and motivational feeding drives (Berridge, 2004; Morales & Berridge, 2020). Much evidence shows that hedonic properties of food can lead to a strong motivation to eat (Morales & Berridge, 2020). It also has been hypothesized set point or other feedback signals can be associated with environmental stimuli to produce anticipatory feeding and avoid caloric deficits (Woods & Ramsay, 2007, 2011). If and in what way satiation memory relates to associative learning remains to be investigated but the model does not rely on any specific driver of food intake. Whereas presumed set points, using actual or anticipated metabolic error signals, can be understood as homeostatic motivational drives regulating when hunger and satiation occur, our data indicate that satiation is regulated by caloric memories (Berridge, 2004; Morales & Berridge, 2020; Woods, 2009; Woods & Ramsay, 2011). Caloric memories record the caloric content of a consumed meal. The information about past caloric consumption can then be activated and affect future intake when eating has begun and stimulated the $PVN^{\alpha CaMKII}$-dependent satiating pathway (Lagerlof et al., 2016; Speakman et al., 2002). Satiation memory hence accommodates potential metabolic and non-metabolic drivers by attenuating or enhancing their power according to past nutrient intake and other metabolic signals that have influenced O-GlcNAcylation in $PVN^{\alpha CaMKII}$ neurons. In our account there is no regulated property such as adiposity or body weight. Instead, body weight settles on a level influenced by metabolic as well as non-metabolic factors. We show here that O-GlcNAc controls energy balance across days. As this mode of metabolic control was identified without subjecting the animal to periods of starvation it is likely relevant in obesogenic societies (Hill, Wyatt, Reed, & Peters, 2003). O-GlcNAc-dependent obesity has been associated with impaired glucose tolerance and increased insulin levels, effects exacerbated by high-fat diet (Dai et al., 2018; Keembiyehetty et al., 2015). The considerable genetic background to body weight, where many obesity genes affect neuronal and synaptic function, may contribute to the regulation of satiation memory (Locke et al., 2015).

While our data identify caloric memory to mediate effects of caloric signaling on food intake, the data do not rule out set point-derived motivational regulation. Such regulation may work on time scales longer than two weeks and may thus not be apparent in our data (Muller & Bosy-Westphal, 2013). Although compatible, set point-derived motivational regulation does not explain the behavior or metabolic regulation of *ad libitum* food intake observed here.

In conclusion, this paper finds evidence for a model in which the posttranslational modification O-GlcNAc affects appetite by regulating satiation memory. The O-GlcNAc pathway is genetically linked to common forms of obesity (Gutierrez-Aguilar, Kim, Woods, & Seeley, 2012; Speliotes et al., 2010; Wolosker et al., 1998). If perturbed, massive obesity develops rapidly (Dai et al., 2018; Lagerlof et al., 2016). Identifying the targets that mediate appetite control by O-GlcNAcylation presents a much needed and novel approach to treat obesity - one of the world's most devastating health problems.

Declaration of interest

This study was funded by the Diabetes Research and Wellness Foundation (720-1597-16 PG), Swedish Society of Medicine (SLS-789011), Knut and Alice Wallenberg Foundation, Region Västerbotten, Umeå University, Stockholm Health Care Services and Karolinska Institutet (O.L.). None of the funding sources had any impact on the collection, analysis or interpretation of the data.

Ethical statement

All animal work was done according to the guidelines of and approved by the Johns Hopkins University Animal Care and Use Commmittee (OGT KO animals), National Institutes of Health (OGA Het animals) and the University of Kansas Medical Center Animal Care and Use Committee (TMG treated animals). All animal work adhered to the ARRIVE Guidelines including the principles of the 3Rs.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.appet.2021.105320.

Author contributions

B.A. designed all experiments and performed all data analysis. O.L. designed experiments and wrote the manuscript. C.S. and J.A.H wrote the manuscript. O.L., EP.T., S.R.M., U.A. contributed animal data. All authors have read and approved the final version of the manuscript.

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