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Fat, carbohydrate and protein by oral gavage in the rat can be equally effective for satiation☆

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ABSTRACT

This study aimed to determine the relative efficacy of the macronutrients, protein, fat and carbohydrate to induce satiation and satiety in rats in relation to macronutrient activation of neurons in the nucleus of the solitary tract (NTS). Male Sprague Dawley rats were schedule-fed twice a day for 2 h, receiving 100% of daily ad-libitum energy intake. On test day 1, 30 min before the first scheduled meal of the day, rats were gavaged with an 8 kcal isocaloric, isovolumetric solution of a glucose, lipid or peptone macronutrient solution or a non-caloric saline solution. To assess satiation, thirty minutes later rats were given access to food for 2 h and food intake determined. A second 2 h food access period 3 h later was used for assessment of satiety. On the second test day, rats were gavaged as before and killed 90 min after food presentation. Blood was collected for measurement of circulating metabolic markers. Brains were removed for analysis of *c-Fos* expression by in situ hybridization in the NTS.

Rats which received saline consumed a similar amount of food compared to pre-gavage intakes. However, rats gavaged with a caloric macronutrient solution all reduced food intake by 18-20 kcal. Interestingly, the reduction in caloric intake was greater than the caloric value of the macronutrient solution gavaged and was sustained following the second scheduled meal. Quantification by in situ hybridization of *c-Fos* mRNA expression in the NTS 90 min post-gavage, showed a significant increase with each macronutrient, but was 24–29% higher with a lipid or peptone gavage compared to a glucose gavage. In conclusion, when delivered directly to the stomach, all macronutrients can be equally effective in inducing satiation with significant neuronal activation in the NTS of the hindbrain.

1. Introduction

Of the three macronutrients, protein, carbohydrate and fat, there is evidence from both rodent and human studies indicating that protein on a caloric basis, may provide the most effective satiation-satiety signal [1–3]. This is proposed to be due to protein-induced release of the gut hormones, PYY, GLP-1 and CCK from the small or large intestine [3–5], leading to a reported greater stimulatory effect on neuronal activity of the appetite regulatory neurons in the hindbrain when ingested high protein diet was compared to a normal protein diet [6,7]. Nevertheless, there persists uncertainty and debate over the relative efficacy of each dietary macronutrient in inducing satiation or satiety [8]. The differences in perspective have been further fuelled by a recent study providing evidence for fat as the principle regulator of energy intake [9]. This uncertainty of view may, in part, reflect the attributes of macronutrients when consumed orally, including smell, taste, structure and volume. Even when given via a gastric or intraduodenal route, the degree of suppression of subsequent nutrient intake can vary [10–12]. Comparative studies of macronutrient effect on energy intake are commonly performed with a pre-load of macronutrient, followed by an assessment of the effect on subsequent intake. For example, a study in rats suggested that gastric pre-load macronutrients had differential effects on satiety with lipid being ineffective [10].

Vagal afferent connections from the intestinal tract convey signals of food ingestion and nutrient composition to the hindbrain nucleus of the solitary tract (NTS) as a result of stomach enlargement or nutrient evoked release of gut hormones by intestinal endocrine cells, leading to satiation via activation of NTS neurons [13]. While there is good evidence to support vagal transmission of gastric distension and duodenal nutrient sensing, little information exists relating to how individual macronutrients engage vagal afferent signalling. Therefore the question arises whether the efficacy of different macronutrients to induce

☆ Macronutrient activation in the NTS

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satiation is a result of differential activation of vagal afferent pathways. The aim of this study was to investigate whether solutions of individual macronutrients of equal caloric content delivered to the stomach quantitatively differ in their activation of NTS neurons, assessed by activation of *c-Fos* gene expression, in the hindbrain and how this relative activity relates to subsequent energy intake. For our study, a scheduled feeding paradigm was employed modified from a study by Johnstone et al., designed to investigate sites of neuronal activation (c-FOS expression) in response to feeding [14]. This aforementioned study defined the peak time of c-*FOS* expression in the NTS after food intake to be between 90 and 150 min (peak 120 min) using a paradigm of one scheduled meal giving access for a 2 h period to facilitate prompt food access on presentation. The study was performed during the light phase to avoid confounding initiation of *c-Fos* expression by a photoperiodic cue. However, rats on this protocol only consumed 36% of ad-libitum diet and lost a significant amount of body weight. In our study we modified the scheduled feeding paradigm to two, two hour feeding periods during the day. This meant that the rats received 100% of ad-libitum intake as this was the total caloric value of the food provided across 2 meals (all food was consumed), and that growth was normal, but it also ensured that when food was presented it was consumed immediately, allowing intake to be accurately assessed and compared between treatments. It also defined a time point for the measurement of *c-Fos* mRNA which was chosen as 90 min post gavage, at the leading edge of the peak of c-FOS expression [14].

Studies on the ability of macronutrients to induce satiation or satiety may be confounded by the sensory properties of food, including smell, structure and taste [15,16]. In this study, to assess the impact of macronutrients in the gut on signalling to the hindbrain, while avoiding a contribution from gustatory or olfactory inputs to the regulatory centres of the brain, solutions containing individual macronutrients were delivered by gavage directly into the stomach.

2. Materials and methods

2.1. Animals

Thirty-two male Sprague Dawley rats of 7–8 weeks old were purchased from Charles River (Charles River, UK) and on arrival were housed in 12 h light: 12 h dark cycle with lights on at Zeitgeber (ZT) 0 and lights off at ZT12. The rats were acclimatized to an ad libitum standard chow diet (Special Diet Services, UK, #871505 CRM (P); 69% carbohydrate, 22% protein, 9% fat by energy, 3.77 kcal/g) for 7 days. They were then individually housed with ad libitum access to AIN-93 M, a stock diet composed of defined components (Special Diet Services, UK, 14.7% protein, 75.9% carbohydrate and 9.4% fat by energy at 3.77 kcal/g) diet for 12 days, with food intake measured daily. An average daily caloric intake on AIN-93 M was calculated for each individual. Water was available at all times throughout the study. Animal husbandry and experiments were carried out under a project licence approved by the Home Office and in accordance with the Animals (Scientific Procedures) Act of 1986. The project also received ethical approval from the Rowett Institute of Nutrition and Health and the University of Aberdeen Ethics Committee.

2.2. Dietary manipulations

This study was based on a scheduled feeding program to investigate feeding induced neuronal activation [14]. In contrast the study by Johnstone et al. in which food intake was ~36% of ad-libitum during one scheduled meal, our study enabled intake of 100% of ad libitum caloric intake of AIN-93 M over two daily scheduled meals.

For the first two days of the scheduled feeding protocol, a food ration of 100% of the previously determined ad libitum intake of AIN-93 M was given at ZT6 for 2 h to train the rats to expect food at this specific time. Following this training period, food was then made available for a 2 h period for the next 5 days at ZT6, with food left over from this meal being reintroduced at ZT11, again for 2 h. This facilitated the rapid consumption of food upon presentation and in a coordinated manner between all rats and thus enabled recovery of the brain from the rats at a precise interval of 90mins relative to the start of food consumption for analysis of *c-Fos* mRNA expression.

2.3. Gastric preload studies

After 7 days on the scheduled feeding protocol, the rats were gavaged 30 min prior to ZT6, with a 4 ml bolus, pre-warmed to 37 °C, containing either a saline solution, 2 kcal/ml glucose (0.5 g/ml dissolved in water), Intralipid 20% (containing 200 mg Soya oil, 12 mg purified egg phospholipids and 22 mg glycerol/ml, Fresenius Kabi, UK) or meat peptone (0.5 g/ml dissolved in water, Sigma Aldrich, UK). Thirty minutes after gavage, AIN-93 M stock diet was supplied for 2 h. Food not consumed was presented at ZT11 for 2 h. The quantity of food consumed was measured by weighing the food that remained at the end of the 2 h access period. Twenty four hours later, the gavage was repeated but food was withheld at the scheduled feed. Ninety minutes after gavage, the rats were euthanized by anaesthetising with isoflurane, followed by decapitation. Trunk blood was collected into 15 ml polypropylene tubes containing 50 I.U. heparin and 8 mM DPPIV inhibitor (KR-62436 hydrate, Sigma Aldrich, UK) and stored on ice until processed for plasma. Brains were removed and frozen on dry ice and stored at −80 °C until use. Blood was centrifuged at 1000*g* for 15 min at 4 °C and the plasma removed to microfuge tubes and stored in aliquots at −80 °C.

2.4. Circulating hormones and metabolic parameters

The plasma measurements of glucose, non-esterified fatty acids (NEFAs) and triglycerides were performed by the Analytical Department at the Rowett Institute using KONE calorimetric assays, in a Konelab 30 instrument (Thermo Fisher Scientific, Basingstoke, UK). GLP-1 and leptin were measured by the Core Biochemical Assay Laboratory, Cambridge using the Meso Scale Discovery Total GLP-1 Kit (K150JVC-1, Gaithersburg, MD, USA) for the measurement of amidated GLP-1 (7–36), and a previously published leptin assay [17]. Insulin was measured using a rat insulin ELISA (Mercodia AB, Uppsala, Sweden).

2.5. Brain gene expression

Brain tissue was cut on a cryostat at a thickness of 20 µm, with sections collected on poly D-lysine coated slides. Neuronal activation in the appetite regulatory areas of the brain was investigated using established in situ hybridization methods [18,19]. Sections spanning hindbrain regions which included the NTS were probed for *c-Fos* mRNA expression by RNA in situ hybridization of brain sections using a ^{35}S labelled anti-sense riboprobe for *c-Fos* mRNA. Following hybridization, slides were apposed to autoradiographic film for 7 days. Quantification expressed as integrated optical density (IOD), was achieved by image analysis, using Image Proplus v7.0 software with reference to a ^{14}C microscale exposed to film on the same film as the brain sections.

2.6. Statistical analysis

Statistical analysis was performed using SIGMAPLOT 11.0 software (Systat software Chicago, IL, USA). Data was analysed by a one-way ANOVA except in the case of Fig. 2A which was analysed by two-way ANOVA with repeated measures, with differences between the macronutrients being analysed using of a Holm-Sidak *post-hoc* test. Results are expressed as mean ± SEM of 8 animals per group. In all data, $P < 0.05$ was considered to be statistically significant.

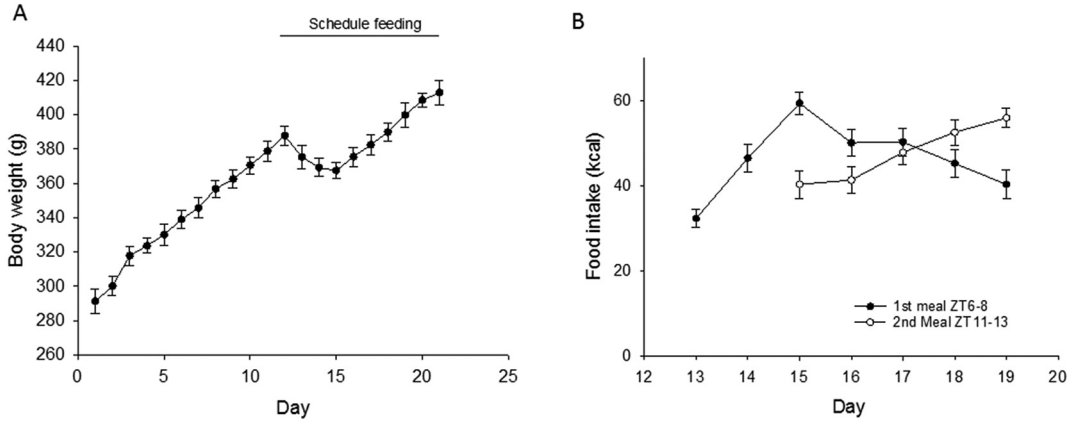

Fig. 1. Body weight and scheduled meal food intakes. (A) Body weight of rats prior to and during the scheduled feeding period. Bar indicates duration of scheduled feeding; (B) mean daily food intake of rats when presented with access to a pre-determined 100% of ad libitum AIN-93 M during the first schedule meal (ZT6-8) and from day 15, a second scheduled meal (ZT11-13) of the food remaining after the first meal. Data presented as mean ± SEM. All groups, $n = 8$.

3. Results

3.1. The effect of schedule meals on body weight and food intake

Bodyweight increased consistently during the 12 days of ad-libitum feeding. The introduction of scheduled feeding on day 13 where food was only available as a single meal of 2 h duration resulted in a reduction in body weight through to day 15. However, after the addition of the second meal from day 15 onwards, body weight increased again until the end of the study at day 21 (day 12: 387.8 ± 5.96 g; day 15: 367.50 ± 4.86 g; day 21: 412.8 ± 7.15 g; Fig. 1A). The feeding schedule increased intake of AIN-93 M at ZT6–8 during the first three days of presentation from 32.25 ± 2.15 kcal on day 13 to 59.34 ± 2.59 kcal on day 15, an increase that was reversed after the introduction of the second meal (day 19 40.25 ± 3.45 kcal, Fig. 1B). When food was available as 2 meals per day, all the remaining food available in their pre-determined 100% ration of daily caloric intake was consumed by the end of the second scheduled meal.

3.2. Gavage with all nutrient preloads reduced subsequent and total caloric intake

Twenty-four hours before gavage treatment (pre-gavage caloric intake), the rats consumed between 36 and 42 kcal in the 2 h scheduled feed at ZT6 (Fig. 2A). A two-way repeated measure ANOVA revealed there was no overall effect of macronutrient on AIN-93 M intakes between groups ($F_{3,28} = 1.38$, $P = 0.27$), but there was an effect of gavage ($F_{1,28} = 46.55$, $P < 0.001$), and an interaction of macronutrient and gavage ($F_{3,28} = 4.18$, $P = 0.014$). Post-gavage consumption of AIN-93 M in all macronutrient gavaged groups was reduced relative to saline gavaged rats (glucose gavage $P = 0.036$, peptone gavage $P = 0.022$ and lipid gavage P = 0.01). Rats gavaged with saline exhibited no reduction in food intake, compared to their pre-gavage intake (saline; pre: 35.85 ± 4.21 kcal vs. post: 33.95 ± 5.00 kcal, $P = 0.712$). However, glucose, lipid and peptone were all effective at reducing intake of AIN-93 M following gavage (Fig. 2A), but a post-hoc analysis revealed no differences in food intake with macronutrient type. No differences in intake were noted between the 4 treatment groups during the second scheduled meal at ZT11-13 (Fig. 2B). Combined caloric intake for the test day, including calories acquired as a result of gavage treatment, showed that rats that had received the saline bolus consumed significantly more total calories over the course of 24 h than the groups receiving the macronutrient preloads (saline: 81.88 ± 1.91 kcal vs. glucose: 72.42 ± 1.82 kcal; lipid: 70.79 ± 2.69 kcal; peptone: 72.51 ± 1.46 kcal, $P < 0.001$; Fig. 2C), with a Holm-Sidak *post-hoc* test revealing no differences in intake between the macronutrient groups.

3.3. Hormone profiling in response to macronutrient gavage

Leptin, GLP-1 and insulin are all peptide hormones, which possess anorexigenic aspects to their physiological function, that are released

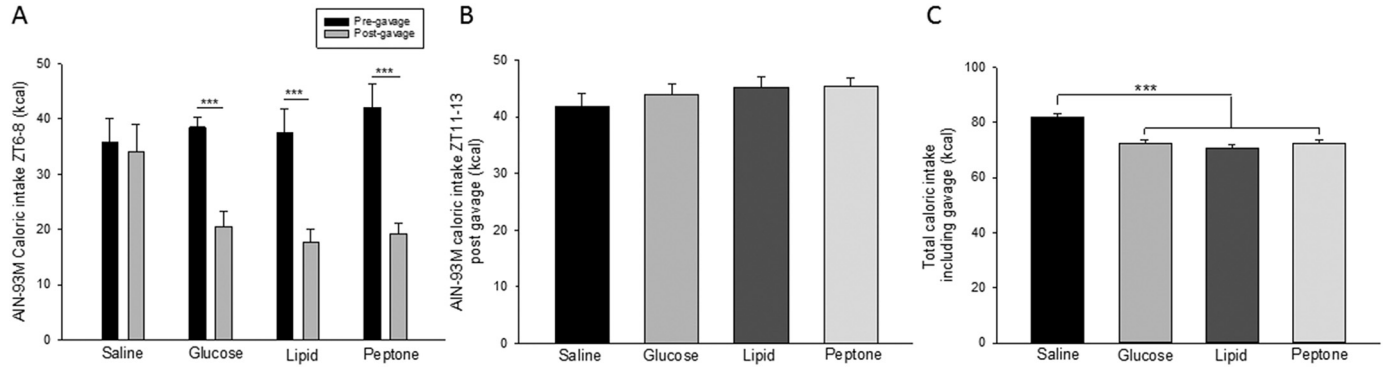

Fig. 2. The effect of macronutrient gavage on subsequent caloric intake. (A) Intake of AIN-93 M standard rat maintenance diet pre-gavage (intake of the previous day) and post-gavage of 4mls of saline or macronutrient solution with a caloric value of 8kcals. (B) AIN-93 M intake after the second post-gavage scheduled feed at ZT11-13 (C) Total caloric intake during both scheduled meals (ZT6-8 and ZT11-13) including the 8 kcal preload. Differences in caloric intake were analysed by two-way ANOVA with repeated measures (A) or One-Way ANOVA (B,C) both with post-hoc Holm-Sidak test (***$P < 0.001$). Results are expressed as mean ± SEM. All groups, $n = 8$.

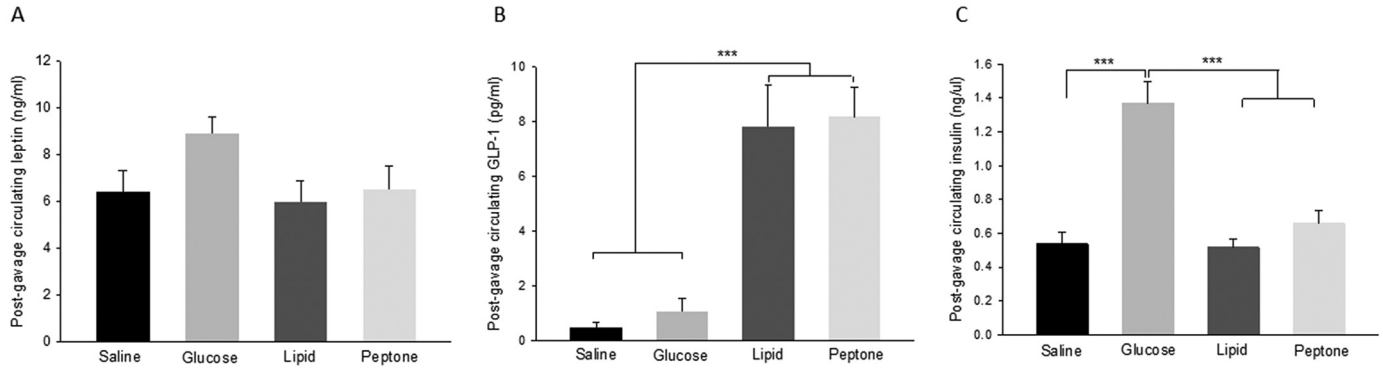

Fig. 3. Circulating leptin GLP-1 and insulin levels at 90 min post gavage of saline or macronutrient containing solutions. (A) Leptin, (B) GLP-1 and (C) insulin concentration in terminal blood samples 90 min after macronutrient pre-load gavage. Hormonal levels analysed by One-Way ANOVA with post-hoc Holm-Sidak test (*** P < 0.001). Data presented as mean ± SEM. n = 8 for each group.

post-prandially in response to nutrient stimulation from gastric chief cells [20], L-cells [21] and β-cells, respectively. Hormones were analysed in terminal blood samples at 90 min post-gavage on the second test day. Differences in circulating leptin levels between gavage treatments did not achieve statistical significance (glucose: 8.99 ± 0.71 ng/ml; lipid: 5.96 ± 0.91 ng/ml; peptone: 6.51 ± 1.00 ng/ml vs. saline: 6.44 ± 0. 88 ng/ml, $P = 0.095$; Fig. 3A). There was a significant increase of GLP-1 levels by both lipid and peptone gavage compared with saline (lipid: 7.83 ± 1.53 pg/ml; peptone: 8.19 ± 1.05 pg/ml vs. saline: 0.48 ± 0.17 pg/ml, $P < 0.001$; Fig. 3B) and glucose gavage (glucose: 1.07 ± 0.47 pg/ml, P < 0.001; Fig. 3B), but rats gavaged with glucose were not different to the saline group ($P = 0.264$). Insulin was significantly increased by the glucose gavage (glucose 1.37 ± 0.37 ng/μl; vs. saline 0.54 ± 0.67 ng/μl, P < 0.001; Fig. 3C), but rats gavaged with lipid or peptone were not different compared to saline (lipid 0.52 ± 0.05 ng/μl, $P = 0.868$; peptone 0.66 ± 0.08 ng/μl, $P = 0.562$; Fig. 3C).

3.4. Effect of macronutrient gavage on triglyceride and NEFA levels

Glucose, triglycerides and non-esterified fatty acids (NEFAs) were analysed in the terminal blood samples at 90 min post-gavage. One-Way ANOVA in conjunction with a Holm-Sidak *post-hoc* test revealed significant effects of macronutrient preload on all three metabolites. Only gavage of lipid produced a significant increase in glucose at this time point compared to saline (saline 10.78 ± 0.35 mmol/l vs lipid 12.84 ± 0.36 mmol/l, $P = 0.044$; Fig. 4A). Triglyceride levels were raised only in rats gavaged with lipid ($P < 0.001$; Fig. 4B). NEFAs were lower in rats that received glucose or peptone gavage relative to saline (saline: 0.41 ± 0.03 mmol/l vs. glucose: 0.18 ± 0.02 mmol/l; peptone: 0.24 ± 0.02 mmol/l, $P \leq 0.001$; Fig. 4C), but an increase in circulating NEFAs was observed with a lipid gavage compared to the saline control (lipid 0.55 ± 0.05 vs. saline: 0.41 ± 0.03 mmol/l; $P = 0.008$).

3.5. Macronutrient gavage increases c-Fos expression in the NTS

One-Way ANOVA revealed that there was a significant effect of preload gavage on *c-Fos* mRNA expression in hindbrain region encompassing caudal regions of the NTS. Relative to saline, all caloric gavages increased *c-Fos* mRNA expression in the NTS of the hindbrain (saline: 11.04 ± 0.78 IOD vs. glucose: 17.89 ± 0.98 IOD; lipid: 23.10 ± 0.63 IOD; peptone: 22.35 ± 1.16 IOD, $P < 0.001$). A post-hoc Holm-Sidak test comparing the effects of macronutrients revealed c-*Fos* mRNA expression in the glucose group was lower than the peptone and lipid groups (glucose: 17.89 ± 0.98 IOD vs. lipid: 23.10 ± 0.63 IOD, $P = 0.002$; peptone: 22.35 ± 1.16 IOD, $P = 0.01$), but there was no difference between lipid and peptone treatments (Fig. 5).

4. Discussion

This study aimed to assess whether a difference exists between dietary macronutrients in their ability to activate neurons of the NTS which constitute part of the relay pathway from the stomach to CNS appetite regulatory regions. A scheduled feeding paradigm was employed modified from a previous study designed to investigate feeding-induced neuronal (*c-FOS*) activation, which was found to peak around 90-150 min following food intake [14]. Although nocturnal animals such as rats eat most of their food in the dark phase, caloric

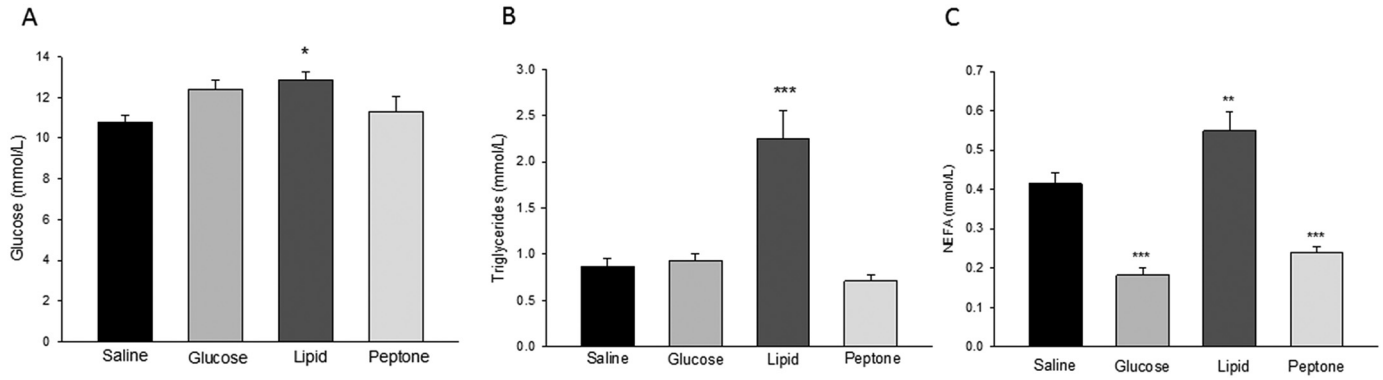

Fig. 4. Metabolic profile at 90 min post gavage of saline or macronutrient containing solutions. (A) Circulating glucose concentrations; (B) Circulating triglyceride concentrations (C) Circulating non-esterified fatty acids (NEFAs). Metabolite levels were analysed by One-Way ANOVA and post-hoc Holm-Sidak test. Asterisks indicate significant difference relative to Saline in each panel. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) Data presented as mean and ± SEM $n = 8$ for each group.

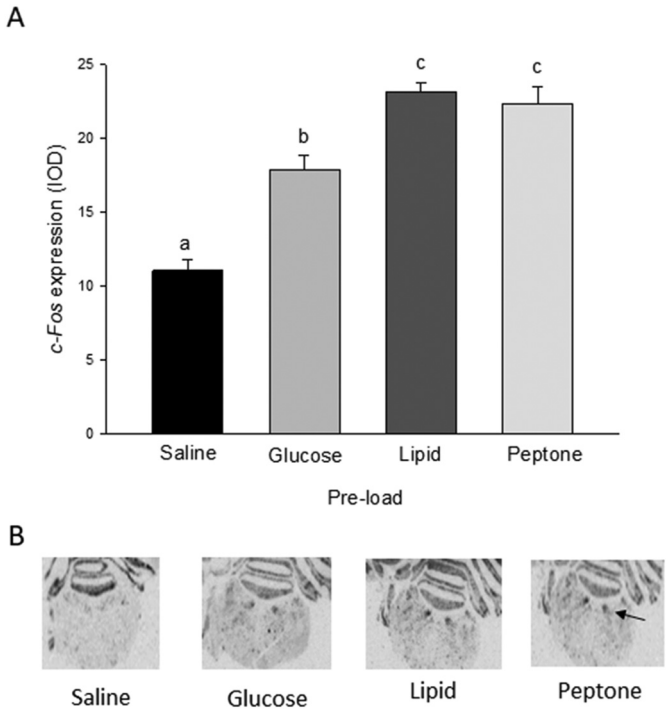

Fig. 5. Expression of *c-Fos* mRNA in the nucleus of the solitary tract of the hindbrain 90 min post gavage of saline or macronutrient containing solutions. (A) *C-Fos* expression (IOD) as measured by in situ hybridization and image analysis. (B) Representative autoradiograph images of *c-Fos* mRNA expression in the NTS. *C-Fos* mRNA expression analysed by data One-Way ANOVA and post-hoc Holm-Sidak test (different letter denote significant difference between groups; 'b' and 'c' compared to 'a' $P < 0.001$; 'b' compared to 'c', $P < 0.01$,). Data presented as mean ± SEM. n = 8 for each group. Arrow in (B) indicates c-fos expression.

compensation was previously found to be comparable whether performed during the light or dark phases [22]. We adopted this light phase feeding paradigm for the assessment of *c-Fos* mRNA expression in the NTS using the known kinetics and relationship between c-FOS mRNA and protein expression [23,24]. However, the previous study by Johnstone et al. [14] used a single scheduled meal, giving access for a 2 h period during the light phase and resulted in considerable weight loss. Consequently, in our study rats were given a meal twice per day to avoid a confounding weight loss.

After 7 days of scheduled feeding, rats were gavaged with an isocaloric (8 kcal), isovolumetric solution containing one of the three macronutrients, or received an isovolumetric, zero calorie bolus of saline, 30 min prior to the first meal of the day at ZT6. At the end of this scheduled meal, food intake was measured. Rats which received saline had a similar caloric intake of AIN-93 M to pre-gavage intake, demonstrating that gavage was not unduly stressful and did not affect intake of the scheduled meal. However, all rats which received a gavage containing an 8 kcal caloric solution of macronutrient reduced their intake of AIN-93 M by a similar amount (18-20 kcal), demonstrating that all macronutrients at the caloric dose tested, were equally effective at reducing subsequent food intake. However, as we have only assessed one caloric preload at a single dose, it could be that the relative degree of suppression of appetite could differ between nutrients at different preload doses, and this requires further investigation.

Unexpectedly, the caloric reduction in food intake of gavaged rats was greater by 10-12 kcal than the caloric value of the macronutrient solution delivered. Contributory factor to this reduction in food intake may be the liquid state of the gavaged macronutrients which would have required minimal digestion and may be available more quickly to activate release of gut hormones to induce satiation: or sensory inputs of smell and taste received via oral consumption, which could otherwise stimulate food reward mechanisms [15,16,25,26], were bypassed by this delivery route, allowing unhindered efficacy of satiation potential. Nevertheless, given that gavage per se does not affect subsequent food intake, these data would suggest that, when delivered directly to the stomach, each macronutrient elicited a similar response to reduce appetite.

These data compare, and to some extent contrast, with similar studies which have assessed effects of different macronutrients on food intake using a variety of paradigms, although these studies differ in the method of delivery of the preload and the observed efficacy of a preload on subsequent food intake [27–33].

Our macronutrient challenge was partly based on a study by Schwartz et al. [10], where scheduled access to a liquid Ensure diet was timed for the early afternoon with gavage of test macronutrient taking place in the morning after an overnight fast. This study found that gavage of intralipid up to a dose of 10 kcal had no effect on subsequent caloric intake compared to a saline control, measured by intake of a glucose solution during a 30 min access period starting 5 min after the gavage. However, both peptone and glucose solutions showed some dose dependency, being ineffective at preloads of 2.5 kcal and with a threshold at a load of 5 kcal to reduce subsequent glucose solution intake [10]. However, the dose dependency between a 5 kcal and a 10 kcal preload was not statistically significant, perhaps reflecting a limitation of assaying intake by oral intake of a glucose solution.

Our data shows a similar caloric reduction following the peptone, lipid and glucose gavage. This would be consistent with studies in non-human primates which have demonstrated glucose, lipid or protein preloads are equally effective at reducing caloric intake in a dose dependent manner [32,33]. Although we chose a single dose to compare neuronal activation in the NTS, we anticipated from previous studies [10,22] that at least for glucose and peptone, this would be greater than the threshold value to attenuate food intake at the first meal.

The absence of a reduction of glucose intake by intralipid in the study by Schwartz contrasts with the data presented here. One hypothesis for this difference is that our rats were given a 30 min interval after gavage before food was presented compared with a 5 min interval in the aforementioned study. As there may be a time differential in the passage of nutrients from the stomach with fat being retained longest [34], the longer duration between gavage and food presentation in our study may have given all macronutrient solutions time to exit the stomach into the small intestine [35]. This would then permit the caloric value of the food to be assimilated via nutrient sensing mediated pathways to effect an adjustment of intake of the food presented at the scheduled meal. This would be consistent with studies which have shown that timing of meals relative to preloads can have a subsequent effect on meal intake, with a longer interval between preload and meal, resulting in less compensation [36].

At the end second scheduled meal following gavage treatment, food intake was similar irrespective of macronutrient gavage. This contrasts with macronutrient type in the study of Giliebter (performed during the light period) who found sucrose, starch and protein suppressed food intake 3.5 h after a gastric load, but corn oil did not [29]. Surprisingly, we found no compensation for reduced caloric intake of the first meal occurred at the second meal. Indeed the cumulative intake between the two meals in our study (including the caloric value of the gavage), was lower by 9-11 kcal in those groups receiving an 8 kcal macronutrient gavage. This might be indicative of satiety which has not yet been compensated for at the second scheduled meal. However, earlier studies in rats suggest that caloric compensation would occur over time irrespective of whether the pre-load is given in the light or the dark phase [22,37]. This contrasts to a fasting state, where compensation for a fasted period can occur when food is offered [38].

Several metabolic hormones could contribute to satiation following the gavage. Plasma leptin levels are known to increase via secretion from the stomach following food intake and therefore contribute to the

onset of satiation [20], however in our study there was no significant increase in leptin levels at 90 min post-gavage.

GLP-1, an incretin hormone with known effects on satiety, is released from enteroendocrine cells in the small intestine following nutrient stimulation [21]. In contrast to leptin, total GLP-1 levels responded differentially to macronutrient content. At 90 min post-gavage of glucose, total GLP-1 values were not different from baseline (saline). A possible explanation for this finding, is not necessarily that circulating GLP-1 levels did not change, but that by 90 min post-gavage, the combination of a short transit time through the stomach, rapid absorption of glucose in the small intestine and few L-cells capable of secreting GLP-1 in the duodenum, may have resulted in a lower nutrient-induced GLP-1 secretion from the upper small intestine returning to baseline values more quickly. This is consistent with a previous finding of a rapid rise in total GLP-1 values following a glucose gavage [39,40]. However, by contrast, significant rises in GLP-1 were recorded with both lipid and peptone gavage. Lipids and peptides are likely to traverse further along the gut to the jejunum before absorption, exposing and stimulating larger numbers of L-cells along the small intestine thereby extending the time after gavage when an increase in GLP-1 might be observed [41–43]. Circulating insulin levels were only significantly increased following the glucose gavage. This is consistent with a relative temporal order of digestion and passage from the stomach and absorption via the intestinal tract. However, it is also plausible that the differences seen in the macronutrient stimulation of both insulin and GLP-1 at the 90 min point may also be attributed to how stimulatory these macronutrients are upon the different endocrine cell populations involved [44–46].

With respect to the effect of gavage on metabolic parameters, circulating glucose levels showed only a small, non-significant, increase with gavage of the glucose solution, probably as a result of rapid glucose absorption and subsequent utilization consistent with the increased circulating insulin level in glucose gavaged rats; similarly, the peptone gavage did not increase circulating glucose, but a significant rise was obtained with the lipid gavage. This may be due to impairment of insulin activity by increased circulating fatty acids and lipids after gavage [47]. No effect was obtained with the glucose or peptone gavage on TG levels, but both treatments decreased NEFAs, an effect which has been previously demonstrated for glucose intake [48], however, the mechanism of peptone action may reside in the gluconeogenic potential of absorbed amino acids [49,50].

A key determinant in the induction of satiation is integration by the brain of post-ingestive hormonal signals arising from the gut which, together with circulating metabolic signals, act to terminate or reduce further food intake. A principal site for the integration of hormonal signals from the gut is the NTS where neurons expressing relevant receptors, such as those for leptin, Cholecystokinin (CCK) and GLP-1, relay afferent signals from vagal nerves and the circulation through subsets of neurons expressing anorexogenic neuropeptides including POMC, GLP and CCK [51–53]. Analysis of *c-Fos* mRNA expression in the NTS revealed increased expression 90 min after gavage with all three macronutrient preparations compared with a gavage of saline. Glucose stimulated *c-Fos* mRNA expression, but values were 24–29% higher after the lipid and peptone gavages compared to glucose. It has previously been reported that there is good correlation between the number of neurons activated in the NTS and the amount of food consumed at a meal [54]. As the caloric value of each macronutrient gavage was identical in the current study, it might have been expected that *c-Fos* expression would be similar for each of the three macronutrients. Our findings raise the possibility that differences might exist between macronutrients in their ability, calorie for calorie, to activate NTS neurons. Further work is required to ascertain whether the lower activation with gavaged glucose reflects a reduction in the number of activated neurons or in the amount *of c-Fos* mRNA synthesised per neuron, or both. These findings, in turn, may reflect the extent to which each macronutrient stimulated release of gut peptides [55,56]. Alternatively the lower IOD value for *c-Fos* expression with the glucose gavage could be a reflection of the temporal order of macronutrient passage and absorption from the intestinal tract, and represent the early stages of post-activation waning of *c-Fos* expression. Although we have not defined the phenotype of the neurons expressing *c-Fos* mRNA following gavage, the data is consistent with findings of D'Agostino et al., who found increased *c-Fos* expression in CCK neurons of the NTS following a gavage of glucose or amino acids after an overnight fast [57]. Accordingly, these neurons are likely to be involved in the suppressive effect on food intake following gavage of macronutrient.

All three macronutrients gavaged individually at equal caloric value appear to have the same potential for satiation. This poses the question as to why meals of different macronutrient composition but of an equal caloric content do not have the same outcome on satiation? One contributory factor could be activation of reward mechanisms overriding inhibitory signals of the gut-brain axis. Such signals are generated by sensory perceptions of food, including smell and taste, which are known to stimulate reward mechanisms promoting food intake [25,26]. Another contributory factor may be different rates of processing and access of consumed macronutrients to the intestine resulting in differences in conveying a satiation signal to central mechanisms controlling food intake. Together, both reward and meal processing time could limit the potential of the caloric value of a meal to induce satiation.

In conclusion, all three macronutrients when gavaged individually at equal caloric value may have the same potential for satiation and induce similar levels of neuronal activation in the NTS, a key nucleus of the hindbrain in the relay of intestinal satiation signals.

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Author contributions

NC, RA and DW performed the experiments. NC, JGM and PB were involved in experimental design. NC and PB analysed the data. NC, JGM and PB wrote the manuscript.

Conflict of interest

The authors have no conflict of interest to declare.

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