Restriction of dietary protein leads to conditioned protein preference and elevated palatability of protein-containing food in rats

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Graphical Abstract

Abstract

The mechanisms by which intake of dietary protein is regulated are poorly understood despite their potential involvement in determining food choice and appetite. In particular, it is unclear whether protein deficiency results in a specific appetite for protein and whether influences on diet are immediate or develop over time. To determine the effects of protein restriction on consumption, preference, and palatability for protein we assessed patterns of intake for casein (protein) and maltodextrin (carbohydrate) solutions in adult rats. To induce a state of protein restriction, rats were maintained on a low protein diet (5% casein) and compared to control rats on non-restricted diet (20% casein). Under these dietary conditions, relative to control rats, protein-restricted rats exhibited hyperphagia without weight gain. After two weeks, on alternate conditioning days, rats were given access to either isocaloric casein or maltodextrin solutions that were saccharin-sweetened and distinctly flavored whilst consumption and licking patterns were recorded. This allowed rats to learn about the post-ingestive nutritional consequences of the two different solutions. Subsequently, during a preference test when rats had access to both solutions, we found that protein-restricted rats exhibited a preference for casein over carbohydrate whereas non-restricted rats did not. Analysis of lick microstructure revealed that this preference was associated with an increase in cluster size and number, reflective of an increase in palatability. In conclusion, protein-restriction induced a conditioned preference for protein, relative to carbohydrate, and this was associated with increased palatability.
1. Introduction

There is considerable evidence that of the three macronutrients dietary protein is most tightly regulated [1–3]. As such, when presented with diets that differ in macronutrient content, rats will adjust their consumption to ensure that protein intake meets a baseline level [4]. The mechanisms by which these adjustments occur are still not fully understood.

An important outstanding question is whether the drive for protein is immediate and innate or whether there is a role for learning using post-ingestive consequences [5,6]. Some evidence suggests that when protein-restricted a specific appetite for protein arises, similar to the appetite for sodium that arises under conditions of sodium depletion. Rats have been shown to rapidly increase their intake of a number of protein sources when protein-restricted in a manner that precludes using post-ingestive effects to guide their intake [7]. Further research suggested these rapid effects on protein appetite were driven by olfactory cues [8]. However, a large body of evidence indicates that adjustments to protein intake are slow, require experience with each food/diet, and likely involve post-ingestive feedback. For example, justedments to protein intake are slow, require experience with each protein-restricted a specific protein:carbohydrate ratio (Table 1) but were isocaloric (4.1 kcal/g). Non-restricted diet (#D11051801, Research Diets, New Brunswick, NJ) contained 20% casein whereas protein-restricted diet (#D11092301, Research Diets) contained 5% casein. Body weight data were collected daily throughout the experiments. As rats were group-housed, food intake data were collected by cage and divided by the number of rats in the cage to give an average intake per animal. Conditioning experiments started 2 weeks following diet switch.

Table 1

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>D11051801 (control, 20% casein)</th>
<th>D11092301 (protein-restricted, 5%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g/kg</td>
<td>g/kg</td>
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<tr>
<td>Casein</td>
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<tr>
<td>L-Cystine</td>
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<td>Corn starch</td>
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<td>485</td>
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<td>Maltodextrin 10</td>
<td>125</td>
<td>150</td>
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<tr>
<td>Sucrose</td>
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<td>107.1</td>
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<tr>
<td>Cellulose</td>
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<td>50</td>
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<tr>
<td>Soybean oil</td>
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</tr>
<tr>
<td>Lard</td>
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<td>75</td>
</tr>
<tr>
<td>Mineral mix S10022C</td>
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<td>3.5</td>
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<td>Calcium carbonate</td>
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<td>8.7</td>
</tr>
<tr>
<td>Calcium phosphate, dibasic</td>
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<td>5.3</td>
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<td>Potassium citrate</td>
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<td>2.48</td>
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<tr>
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</tr>
<tr>
<td>Sodium chloride</td>
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<td>2.59</td>
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<tr>
<td>Vitamin mix V10037</td>
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</tr>
<tr>
<td>Choline Bitartrate</td>
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<td>2.5</td>
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<tr>
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<tr>
<td>FD&amp;C Red dye #40</td>
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</tbody>
</table>

2. Materials and methods

2.1. Animals

Forty adult male Sprague-Dawley rats were used for experiments (Charles River; > 275 g at start of experiment). Twenty-four of these rats were used for the main behavioral experiment and a further sixteen contributed to the food intake data. Rats were group-housed (2–3 per cage) in IVCs with bedding materials as recommended by NC3R guidelines. Temperature was 21 ± 2 °C and humidity was 40–50% with 12 h:12 h light/dark cycle (lights on at 07:00). Water was available ad libitum; chow containing different protein:carbohydrate ratio was available ad libitum (details below). All experiments were covered by the Animals [Scientific Procedures] Act (1986) and carried out under the appropriate license authority (Project License: 70/8069).

2.2. Diet manipulations

All rats were initially maintained on standard laboratory chow containing 20% dietary casein. To induce a state of protein restriction in half of the rats, standard chow was switched for one of two experimental diets based on modified AIN-93G that differed in protein:carbohydrate ratio (Table 1) but were isocaloric (4.1 kcal/g). Non-restricted diet (#D11051801, Research Diets, New Brunswick, NJ) contained 20% casein whereas protein-restricted diet (#D11092301, Research Diets) contained 5% casein. Body weight data were collected daily throughout the experiments. As rats were group-housed, food intake data were collected by cage and divided by the number of rats in the cage to give an average intake per animal. Conditioning experiments started 2 weeks following diet switch.

2.3. Behavioral testing

All testing took place within standard operant chambers (in cm: 30.5L, 24.1D, 21.0H; Med Associates, St. Albans City, VT) equipped with a house light and two bottles. Each bottle was connected to a contact lickometer calibrated to detect individual licks. Licks were recorded on a computer for all sessions as a measure of intake. All sessions lasted for one hour. For one to three days at the start of each experiment, rats were placed in the chambers with 0.2% sodium saccharin in both bottles to familiarize them with the apparatus. Following this, rats underwent a series of conditioning sessions and a preference test. In conditioning sessions, which occurred in a block of 4 days, only one bottle each day was available and was filled with either protein-containing solution (4% casein + 0.21% methionine + 0.2% sodium saccharin + 0.05% flavored Kool-Aid) or an isocaloric carbohydrate-containing solution (4% maltodextrin + 0.2% sodium saccharin...
Food intake data from the eight cages of rats (three rats per cage) that participated in the main study are shown in Fig. 1B and visual inspection suggests a slight increase in intake (hyperphagia) in rats on protein-restricted diet. However, the small number of data points precludes statistical analysis. To address this, we combined this data set with food intake data from a pilot experiment in which an additional eight cages of rats were monitored (two rats per cage) and examined this extended data set (Fig. 1C). Statistical analysis of these data showed that protein-restricted rats did increase their intake of the low protein diet, relative to intake of non-restricted rats (t(15) = 3.179, p = 0.007). Thus, restriction of dietary protein resulted in hyperphagia without changes in body weight.

3.2. Protein restriction leads to development of preference for protein-containing solutions

Next, we asked whether rats would display a greater preference for protein-containing solutions over carbohydrate-containing solutions when they were protein-restricted. Our experiment was divided into conditioning days when only one solution was available – casein or maltodextrin – followed by a preference test day when both solutions were available. First, we analyzed data from conditioning days for both casein and maltodextrin to look for effects of diet or conditioning day (Fig. 2).

Analysis of data from casein conditioning days (Fig. 2A and B) revealed a significant interaction between Diet and Day (F(1,22) = 7.222, p = 0.014) with no main effects of Diet (F(1,22) = 1.19, p = 0.287) or Day (F(1,22) = 0.633, p = 0.435). Further analysis of each day separately revealed that on Day 1 there was a trend towards protein-restricted rats drinking more casein than non-restricted rats (t(11) = 1.97, p = 0.061). Interestingly, visual inspection of the time course data (Fig. 2A) suggests that on Day 1 protein-restricted rats showed a different pattern of casein consumption than non-restricted rats. As such, both groups of rats drank a similar amount in the first twenty minutes of the session but in the final forty minutes, casein consumption appeared to increase in protein-restricted rats, relative to non-restricted rats. Casein consumption on the second conditioning day did not differ between protein-restricted and non-restricted rats (t(11) = 0.160, p = 0.874).

Analysis of consumption during maltodextrin conditioning sessions (Fig. 2C and D) showed that protein-restricted rats drank more maltodextrin on both conditioning days than non-restricted rats. As such, there was a main effect of Diet (F(1,22) = 4.825, p = 0.039) with no main effect of Day (F(1,22) = 0.222, p = 0.642) and no significant interaction (F(1,22) = 2.343, p = 0.140).

Finally, when we analyzed total consumption of casein and...
maltodextrin across conditioning sessions (Fig. 2E) we found no significant differences between protein-restricted and non-restricted rats although there was a trend for protein-restricted rats to drink more of both solutions than non-restricted rats. As such, two-way mixed ANOVA revealed a trend towards a main effect of Diet (F(1,22) = 3.609, p = 0.071) but no main effect of Solution (F(1,22) = 1.203, p = 0.285) and no interaction between Diet and Solution (F(1,22) = 2.087, p = 0.163). In summary, there were subtle differences in consumption between diet groups when only one solution was available there was no clear difference in preference for protein over carbohydrate on conditioning days.

Following these four conditioning sessions, rats were given access to both solutions during the same session (Fig. 3). In this session, protein-restricted rats drank more casein than maltodextrin and this elevated intake appeared to occur in the first twenty minutes of the session (Fig. 3A). Furthermore, protein-restricted rats showed a significant preference for casein over maltodextrin whereas non-restricted rats did not (Fig. 3B & 3C). As such, two-way ANOVA revealed that there was a main effect of Solution (F(1,22) = 7.466, p = 0.012) and an interaction between Solution and Diet (F(1,22) = 11.677, p = 0.002).

Subsequent analysis of each diet group individually showed that protein-restricted rats licked more for casein than maltodextrin (t(11) = 4.630, p < 0.001) but non-restricted rats did not (t(11) = 0.458, p = 0.656). In addition, we calculated a casein preference score by dividing casein licks by total licks (Fig. 3D) and found that protein-restricted rats showed a greater protein preference, relative to non-restricted rats (t(21) = 2.660, p = 0.015).

### 3.3. Palatability of protein-containing solutions is increased by protein-restriction

Finally, we used analysis of lick microstructure [10] to examine whether the palatability of protein-containing solutions was affected by the state of protein restriction. Lick patterns were divided into clusters, separated by interlick intervals > 500 ms. An increased number of licks per cluster is generally thought to reflect increased palatability. We found that the state of protein restriction influenced palatability of casein, relative to maltodextrin (Fig. 4). As such, two-way ANOVA
revealed a significant interaction between Solution and Diet (F(1,22) = 7.099, p = 0.014).

Further analysis of each diet group separately showed that casein and maltodextrin had similar palatability in non-restricted rats (t(11) = 0.761, p = 0.463) but the palatability of casein was elevated relative and maltodextrin had similar palatability in non-restricted rats (t(11) = 2.688, p = 0.021).

In addition, the number of clusters was also influenced by the state of protein-restriction as two way ANOVA revealed a main effect of Solution (F(1,22) = 5.677, p = 0.026) and an interaction between Solution and Diet (F(1,22) = 7.119, p = 0.014). Analysis of each diet group separately showed that in non-restricted rats there were the same number of clusters for both casein and maltodextrin (t(11) = 0.203, p = 0.843) whereas protein-restricted rats had an increased number of clusters for casein, relative to maltodextrin (t(11) = 3.550, p = 0.005).

4. Discussion

Here, we examined the effect of protein restriction on development of preference and palatability of protein- vs. carbohydrate-containing solutions. We found that maintenance on a protein-restricted diet resulted in rats developing a preference for protein vs. carbohydrate when given a choice between the two. Moreover, the increase in protein intake was associated with an increase in palatability of the protein-containing solution, relative to the carbohydrate-containing solution.

We monitored food intake and body weight for the two weeks following the change to a protein-restricted diet but before beginning behavioral sessions. Previous studies have found that rats on diets that are moderately low in protein show hyperphagia without weight gain [9,15,16]. In support of these studies, we found that protein-restricted rats increased food intake, relative to controls, without changing their body weight. It is of note, however, that the slight increase in food intake we observe is still far below what is needed to match the protein intake of control, non-restricted rats. In our studies, we used a low protein diet that contained 5% protein whereas other studies using rats have found effects on behavioral and metabolic parameters using diets containing 10% protein [15]. Our choice of 5% was based on pilot experiments, in which we found no effects of 10% protein diet on food intake or conditioned preferences in adult rats (data not shown). The likely explanation for this variation in effective dietary manipulations is different protein requirements during development. Many studies have used late adolescent or young adult rats rather than mature animals and differences in the effects of low protein diets across age and development are well documented [9,17].

In conditioning sessions, over four days rats were given one type of solution - containing either protein or carbohydrate - and lick patterns were monitored. Although in general, rats from both dietary conditions drank similar amounts of casein and maltodextrin during these sessions subtle differences between the groups were apparent. For example, on the first casein conditioning day there was a suggestion that protein-restricted rats drank more casein than non-restricted rats especially during the late part of the session. This late consumption of casein could reflect an appetitive post-ingestive effect of casein. In addition, analysis of maltodextrin consumption or total consumption suggested that in other circumstances protein-restricted rats drank slightly more of both solutions than control rats. This may reflect a moderate form of hyperphagia, similar to home cage intake reported above. Intriguingly, in these conditioning sessions, when only one solution was available, consumption was increased for the carbohydrate-containing solution meaning that protein-restriction may also generate a hyperphagic response that disregards the macronutrient content of the food on offer.

One potential explanation for this lack of preference in conditioning sessions when only one bottle is available may be that rats are consuming close to their maximal intake during these hour-long sessions and thus satiety mechanisms are engaged that prevent further consumption. Another possibility is that when both solutions are present together the comparison generates a negative contrast effect whereby the value of the maltodextrin solution is reduced, relative to the casein solution [18].
In the preference test, when rats were given access to both solutions, we found a strong preference towards the protein-containing solution in protein-restricted rats. This preference was not present in control rats. This finding corroborates other work showing that protein-restricted rats can direct their behavior to increase protein intake. In addition, we have extended these previous studies by analyzing the precise temporal patterns of licking to assess how lick macrostructure and microstructure are affected by protein restriction. By analyzing lick microstructure during the preference test, we found that palatability of the protein-containing solutions increased in protein-restricted rats indicating that this might be a mechanism that drives increased intake of protein-containing foods. This situation parallels studies that examined palatability after flavor-nutrient conditioning. When flavored saccharin is paired with intragastric glucose infusions, palatability of the paired flavor is elevated [12]. Our studies used a similar paradigm in which solutions were sweetened with saccharin and distinctly-flavored with Kool-Aid, as is common in studies of flavor-nutrient conditioning [19]. Thus, increased palatability (flavor evaluation) might be a mechanism that drives increased intake by promoting more meals and longer meals.

The presentation of macronutrients in combination with saccharin and flavoring means that we do not know whether the changes in palatability that we observe reflect a change in palatability of individual components of the solution or the combination. When rats are made sodium-deficient, the nutrient itself, sodium, immediately becomes more palatable in an experience-independent manner [11]. This shift is profound as it applies to high concentrations of sodium, which are normally evaluated as aversive in sodium-replete animals. Moreover, sodium-evoked dopamine signals and appetitive behavioral responses to sodium-associated cues also emerge with no experience of sodium in a depleted state [20, 21]. Literature suggests that appetite for protein may differ from sodium appetite. For example, when rats are maintained on a diet deficient in a single essential amino acid (lysine), they develop compensatory responses, which increase their intake of lysine, but these responses take ~30 min to emerge and longer if they are required to discriminate between two different amino acids [14]. Interestingly, in this study no evidence of an increase in palatability, assessed by bout size, was observed. In addition, this delayed rather than immediate time course for the development of protein-directed responses suggests that learning about the post-ingestive consequences of protein ingestion is essential in a manner that is fundamentally different from sodium appetite. Our data, although not conclusive, support this idea as casein consumption on the first day of experience trends towards being different between protein-restricted and non-restricted rats but consumption of the two dietary groups only begins to diverge late (20–30 min) into the session.

A further point of consideration is our use of casein as the sole source of protein in these studies. We have used casein as it is the most commonly studied protein source in experiments of this kind and it comprises the major protein source in most standard laboratory diets for rodents. It has a relatively well-balanced profile of amino acids with the exception of cysteine/methionine, which is why we added methionine to all casein-containing solutions [3]. It will be of great interest to determine in future studies whether other protein sources drive behavioral preference to a similar level as other sources with different sensory and absorptive properties may differ. Indeed, previous studies indicated variable responses to different protein sources including soybean, gluten, and gelatin, that may be driven by their olfactory properties [7, 8].

Protein is a vital source of essential amino acids which are crucial precursors of neurotransmitters and are involved in almost all essential bodily processes. The importance of individual amino acids and their roles in regulating protein appetite and satiation is an active area of study. The avoidance of toxic levels of amino acids may underlie the aversive nature of very high protein diets – and their subsequent anorectic effects on consumption [22]. Conversely the maintenance of a minimum or optimal level of particular amino acids may contribute to the effects of protein restriction on increasing intake [6].

Specific amino acids can be detected by orosensory cues such as taste and by specific receptors, for example glutamate directly interacts with its own metabotropic receptors [23]. They are also sensed at the level of the duodenum and intestine and affect gut hormones [24]. Rats given diets deficient in single amino acids are capable of distinguishing and selecting for that specific amino acid in choice tests [25, 26]. The existence of a central amino acid sensing mechanism has been suggested involving the anterior piriform cortex [22, 27] and there appears to be a role for both central and peripheral mediators. Whether levels of individual amino acids contribute to the changes in consumption we have observed during dietary protein restriction remains to be elucidated.

One of the most thought-provoking theories developed to explain the obesity crisis is the protein leveraging hypothesis [28, 29]. This theory posits that a steady decrease in the proportion of protein in Western diets occurring over the last few decades has resulted in carbohydrate and fat being overconsumed. The relatively minor role of protein in overall energy intake (generally < 20%) produces this leveraging ability and means that compensating for even small changes
in protein can lead to significant overconsumption of energy from fat and carbohydrate. An important assumption of this hypothesis is that deficiencies in specific nutrients influence our feeding behavior by triggering consumption but that this consumption is indiscriminate and not well-targeted to replenish the nutrient in deficit. Contrary to this assumption, our data suggest that, at least in rats, protein-restriction does recruit mechanisms that enable rats to guide their behavior towards consumption of protein-rich food. However, our studies are far from modelling the human situation and there are numerous important discrepancies to be addressed. First, the level of protein restriction is likely more severe in our protocol than that which most humans in the developed world encounter. Second, the choice of food provided in our studies was limited (protein vs carbohydrate with similar sweetness but distinct flavor) and did not include foods that contained a mixture of macronutrients. Third, the pattern of experience (each solution separately on alternate days) was designed to maximize the ability of rats to discriminate post-ingestive effects and learn about the nutritional value of each solution. In the human situation, where foods contain mixtures of macronutrients and other flavorings, fine discrimination of nutritional consequences of ingestion is likely far more difficult. Moreover, numerous other factors influence our intake such as social setting, cultural norms and access, which may bias us against choosing food stuffs based solely on nutritional outcome. Future studies will attempt to address the ability of rats to develop protein preferences in more challenging situations that better model the human context.

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Data statement

All raw data files will be published with this manuscript alongside the Python scripts used to perform analysis. These are deposited on Github (https://github.com/jaimemcc/murphy-2017) and Mendeley Data (doi:http://dx.doi.org/10.17632/wgd83v3nth.1).

References
