# Protein appetite drives neural activity in the ventral tegmental area

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**Abstract:** Amino acids are essential for almost all biological processes and many cannot be synthesized *de novo*. Thus, it is crucial that all animals, including humans, acquire an adequate amount of protein in their diet. Here, we show that rats maintained on a protein-restricted diet develop a strong preference for protein, relative to carbohydrate. In addition, this preference was associated with increased neural activation in ventral tegmental area while rats were consuming protein. These changes were relatively persistent as when protein levels were restored by switching back to regular chow, behavioral preference and elevated neural activity to protein did not completely disappear. This study provides the first indication that activity in mesolimbic circuitry is involved in generating an appetite for protein in times of need.

**Keywords:** Rat, protein appetite, photometry, feeding behavior, VTA

## Introduction

Ensuring appropriate intake of each of the nutrients needed for survival is a compelling problem for all animals, including humans. However, the neural and physiological processes that control such nutrient-specific intake are poorly understood. Of the three macronutrients, intake of protein is thought to be the most tightly regulated. This is important as many amino acids cannot be synthesized *de novo* (Berthoud et al., 2012). Concordantly, many species adjust their behavior to ensure adequate intake of dietary protein. For example, when provided with diets differing in protein content, rats will adjust their consumption of each diet such that their protein intake remains stable (Theall et al., 1984). In invertebrates, maintenance on diets with differing protein content shifts the food choices of predatory spiders and beetles (Mayntz et al., 2005). Furthermore, in humans, inadequate protein in diet may contribute to obesity, by leveraging up the amount of calories consumed from fats and sugar (Simpson and Raubenheimer, 2005). Recently, we demonstrated that rats maintained on a protein-restricted diet developed a strong preference for a protein-rich

solution, relative to a carbohydrate-rich solution (Murphy et al., 2018), indicating that animals can specifically direct feeding behavior towards protein sources in times of need. However, the neural mechanisms by which diets that are low in protein might shift behavior are not understood.

The ventral tegmental area (VTA) and its projections play a central role in food-seeking behaviors, food preference, and in the motivation to eat (Berridge, 2007; Bromberg-Martin et al., 2010; Ikemoto and Panksepp, 1999). Direct stimulation of neurons within the VTA modulates food intake, cue-food reward associations, and reward-seeking behaviors (Adamantidis et al., 2011; Saunders et al., 2018; Steinberg et al., 2013; van Zessen et al., 2012). Interestingly, VTA neurons have been shown to be sensitive to post-ingestive signals of nutrient intake (e.g. from fat and carbohydrates; Sclafani et al., 2011) and to peripheral feeding-related hormones including insulin, ghrelin, glucagon-like peptide 1 (GLP-1) and amylin (Di Chiara and Abizaid, 2009; Cone et al., 2014; Mebel et al., 2012; Mietlicki-Baase et al., 2013, 2014) allowing the formation of future food preferences. Moreover, the caloric value of foods, independent of their taste, may be signaled by populations of VTA neurons (De Araujo et al., 2008; Beeler et al., 2012; Domingos et al., 2011; Ferreira et al., 2012; McCutcheon et al., 2012a) allowing integration of current physiological state to guide food-seeking behaviors. Interestingly, despite abundant data on the involvement of VTA activity in mediating responses to fat- or carbohydrate-containing food, the role of this region in regulation of protein appetite is still unexplored.

Here, we use *in vivo* fiber photometry to record the activity of VTA neurons during consumption of isocaloric protein- and carbohydrate-containing solutions in an animal model of protein preference (Murphy et al., 2018). We find that, in protein-restricted animals, protein consumption is associated with elevated neural activation, relative to carbohydrate consumption. Moreover, the behavioral and neural changes induced by protein restriction are persistent and are not immediately reversed when physiological state is changed by repletion of protein status.

## Results

First, all rats were injected with an AAV encoding the calcium sensor, GCaMP6s, in the ventral tegmental area and implanted with a fiber optic in the same location to allow fluorescence changes related to neural activity to be monitored *in vivo* (Figure 1A-B). After 3-4 weeks, to allow for transgene expression, protein-restricted (PR) rats (n=6) were switched to low protein diet (5% casein) while control, non-restricted (NR) rats (n=5), remained on regular chow. Body weight data for the subsequent two weeks – before conditioning sessions started - are shown in Figure S1 and show that both PR and NR rats gained weight at a similar rate across days, as shown in our previous work (Murphy et al., 2018).

### No Nutrient-specific Appetite is Apparent during Sessions When Only One Nutrient Is Available

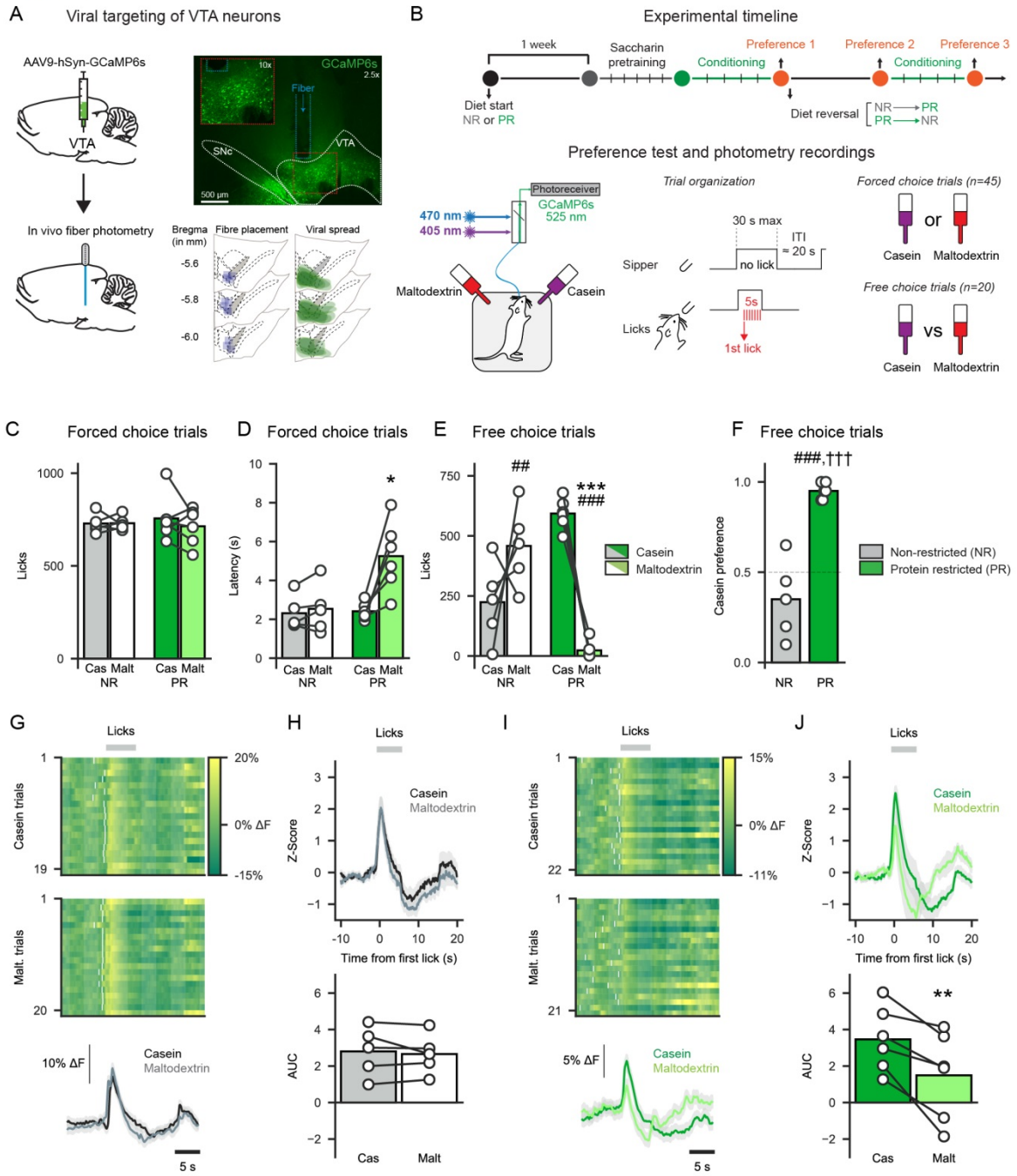
The artificial sweetener, saccharin, was used to familiarize rats with the retractable sippers in the behavioral chambers. Following five saccharin sessions, rats experienced four daily conditioning sessions in which they were given access to one bottle containing either distinctly-flavored casein (protein) or maltodextrin (carbohydrate) solutions, alternated from day to day (Figure S2). For both solutions, rats licked more on the second session in which it

was experienced. However, there were no differences in the amount each group licked for either casein or maltodextrin. Thus, rats in both physiological states experienced the same exposure to casein and maltodextrin solutions in advance of the preference test session.

## Protein-restricted Rats Show a Strong Preference for Protein over Carbohydrate

Following conditioning sessions, all rats underwent a preference test in which casein and maltodextrin were available during a single session (Figure 1B). This session was structured such that rats first experienced 45 trials in which only one bottle was available at a time (termed *forced choice trials*), similar to conditioning sessions. Following these forced choice trials, rats were presented with twenty trials in which both bottles were available at the same time (termed *free choice trials*). On both forced choice and free choice trials, sippers were

Figure 1



programmed to extend for 30 s maximum or for 5 s after the initiation of licking before retracting, as in conditioning trials.

Across all forced choice trials, there was no difference in the number of licks rats made for casein compared to maltodextrin and no difference between diet groups (PR vs. NR; Figure 1C), although PR rats did show greater latencies to drink from the maltodextrin sipper than the casein sipper (Figure 1D). In contrast, in free choice trials, the number of licks rats made for casein, relative to maltodextrin, was strongly influenced by diet group (Figure 1E). As such, PR rats licked more for casein than NR rats did and less for maltodextrin. In addition, when preference of rats was examined, by considering how many times out of the twenty free choice trials each rat chose casein, we found a significant difference between PR and NR rats, with NR rats showing no preference for one solution but PR rats displaying a strong preference for casein (Figure 1F).

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**Figure 1. Protein-restricted Rats Show a Strong Preference for Protein over Carbohydrate that Is Associated with Elevated Neural Activity**

(A) Schematic showing targeting of ventral tegmental area (VTA) by GCaMP6s and implantation of optic fiber (*left*). Expression of virus in VTA and fiber track are shown in photomicrograph (*top right*) and location of expression and fiber placements are shown for all rats (*bottom right*).

(B) Schematic showing experimental timeline (*top*), fiber photometry set-up (*bottom left*), and trial structure of preference tests (*bottom right*).

(C) On forced choice trials, there was no difference in number of total licks for each solution in either diet group. Two-way repeated-measures ANOVA: no main effect of Diet ($F(1,9)=0.016$, $p=0.903$), Solution ($F(1,9)=0.511$, $p=0.493$), and no Diet x Solution interaction ($F(1,9)=0.583$, $p=0.465$).

(D) On forced choice trials, latency to drink from each sipper was influenced by diet group. Two-way repeated-measures ANOVA: main effect of Diet ($F(1,9)=5.163$, $p=0.049$), main effect of Solution ($F(1,9)=15.800$, $p=0.003$), and a Diet x Solution interaction ($F(1,9)=11.558$, $p=0.007$). PR rats had increased latency to drink from maltodextrin than casein (Sidak: $p=0.024$) and showed a tendency for increased latency vs. NR rats ($p=0.055$). *, $p<0.05$ vs. casein.

(E) On free choice trials, PR rats licked more for casein than maltodextrin whereas NR rats did not. Two-way repeated-measures ANOVA: main effect of Diet ($F(1,9)=12.722$, $p=0.006$), main effect of Solution ($F(1,9)=5.675$, $p=0.041$), and a Diet x Solution interaction ($F(1,9)=32.564$, $p<0.001$). PR rats licked more for casein than maltodextrin (Sidak: $p<0.001$) and, relative to NR rats, licked more for casein ($p=0.002$) and less for maltodextrin ($p<0.001$). ***, $p<0.001$ vs. casein; ##, ###, $p<0.01$, 0.001 vs. NR rats.

(F) On free choice trials, diet group had an effect on choice. Unpaired t test, $t(9)=6.731$, $p<0.001$. PR rats chose to drink casein on more free choice trials than maltodextrin (one sample t-test vs. 0.5, $t(5)=24.648$, $p<0.001$) whereas NR rats showed no preference ($t(4)=1.560$, $p=0.194$). ###, $p<0.001$ vs. NR rats; †††, $p<0.001$ vs. 0.5.

(G) Heat map for a single representative NR rat showing normalized fluorescence changes evoked by consumption of casein (*top*) or maltodextrin (*middle*) on forced choice trials. White lines show time of sipper extension. Average fluorescence change across all trials is shown with solid line as mean and shaded area is SEM (*bottom*).

(H) Group data from forced choice casein and maltodextrin trials showing z-score calculated from fluorescent changes aligned to first lick and averaged across all NR rats (top). Solid line is mean and shaded area is SEM. AUC from 0-2 seconds following first lick in each trial from data in top panel (*bottom*) compared to data from PR rats (J) shows that diet influences neural activation during intake of casein and maltodextrin. Two-way repeated-measures ANOVA: no main effect of Diet ($F(1,9)=0.060$, $p=0.812$), but main effect of Solution ($F(1,9)=14.893$, $p=0.003$), and a Diet x Solution interaction ($F(1,9)=11.023$, $p=0.009$). Planned comparisons show greater activation to casein than maltodextrin in PR rats (Paired t test: $t(5)=4.252$, $p=0.008$) but no difference in NR rats ($t(4)=0.678$, $p=0.535$).

(I) Representative data from a PR rat. Conventions are as in G.

(J) Group data from all PR rats show greater neural activation to casein consumption than maltodextrin. Conventions are as in H and statistical analysis is reported in H. **, $p<0.01$ vs. casein.

### Elevated Neural Activity in Ventral Tegmental Area Is Associated With Protein Preference

Next, we examined whether neural activity in the VTA evoked during consumption of each solution was associated with the differing preference observed across diet groups. We focused our analysis on forced choice trials, as across rats these yielded a similar number of trials per solution. All neural data were aligned to the first lick in each trial (Figure 1G-J). Examination of heat maps showing multiple trials for a representative rat in each group suggests that casein and maltodextrin trials evoked a similar level of VTA activity in NR rats (Figure 1G). By contrast, there was greater activation of VTA to casein than to maltodextrin in PR rats (Figure 1I). This finding was borne out by analysis of group data (Figure 1H and J), which showed that when the neural activation (AUC from 0-2 s after start of lick bout) was considered, for PR rats there was significantly elevated neural activation for casein consumption, relative to maltodextrin, whereas for NR rats there was no difference between solutions.

### Changing Diets Leads to Rapid Changes in Behavior toward Nutrients

Following this preference test, we tested the stability of nutrient preferences and neural signals in the face of changing dietary conditions. In other words, would a protein preference persist in the protein-restricted group even once protein levels were restored? Equally, would non-restricted rats switch their preference towards protein once they were maintained on the protein-restricted diet?

To test these possibilities, immediately following the preference test, all rats had their diet switched so that non-restricted rats were now given low protein diet (NR → PR) and protein-restricted rats were given regular chow (PR → NR). After one week in the home cage – with no intervening experience of the solutions – rats were tested for their preferences while we recorded neural activity with fiber photometry.

Behaviorally, we found that on the second preference test, on forced choice trials, rats continued to lick equally for both casein and maltodextrin with no differences due to dietary group (Figure 2B). Interestingly, latency to approach the sipper on these trials continued to differ with PR → NR rats still showing increased latency to drink from the maltodextrin sipper than the casein sipper (Figure 2C). In addition, PR → NR rats were slower to start drinking from the maltodextrin sipper than NR → PR rats. On free choice trials, in contrast to the first preference test, rats from both diet groups did not differ from each other. As such, their licking and choice behavior was similar with consumption of casein being greater than maltodextrin across both groups (Figure 2D-E). In fact, now the preference of NR → PR rats had shifted towards casein whereas for PR → NR rats, preference had shifted away from casein. In summary, after a switch in diets but even without any intervening experience with nutrient solutions, behavior towards casein and maltodextrin had changed in both groups reflecting their new physiological state.

### Activity in VTA is slower to respond to new protein status

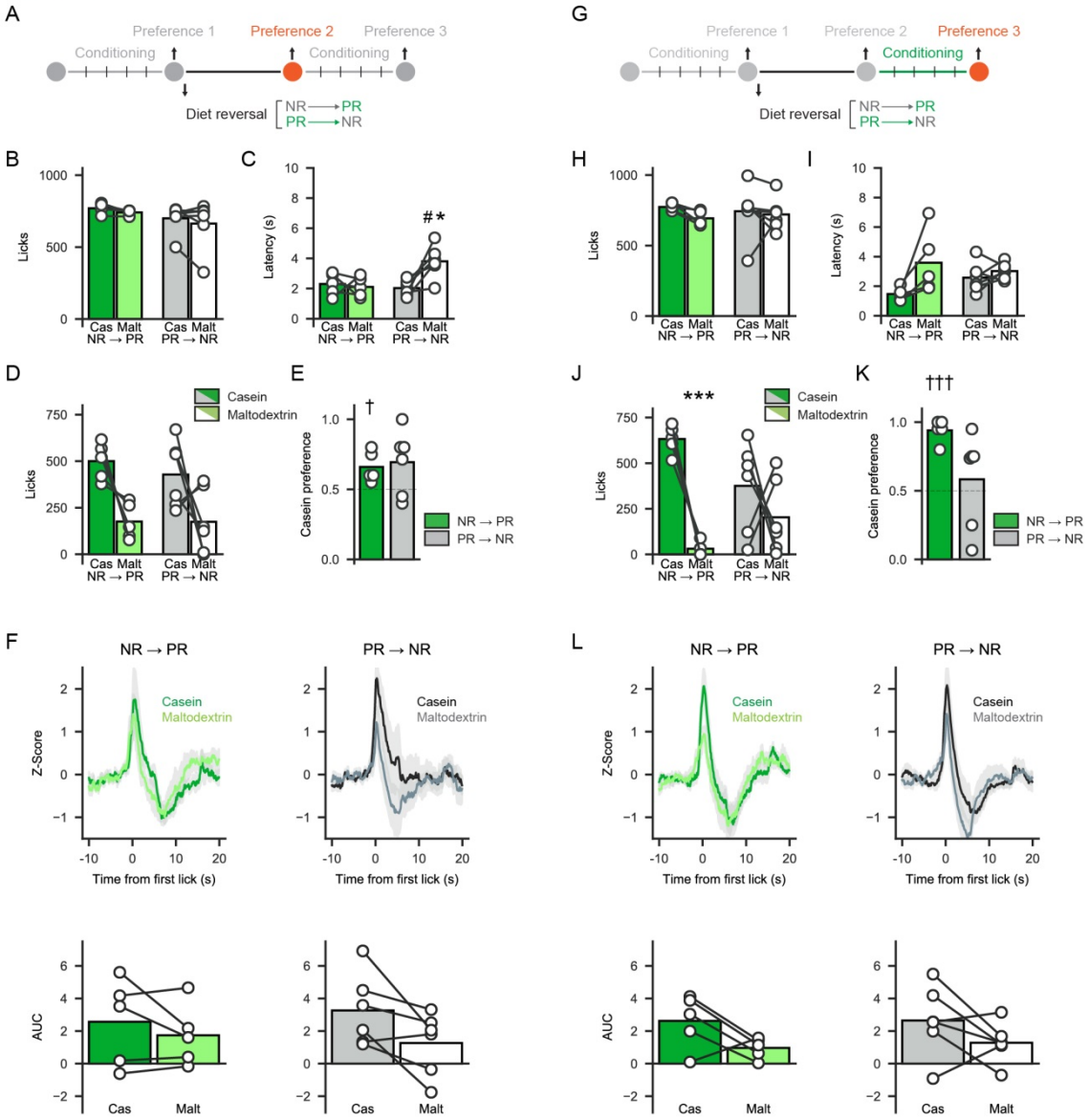
Despite changes in protein preference in the two diet conditions, a parallel change in VTA activity was not observed. (Figure 2F). Analysis of the neural activation evoked by licking during forced choice trials revealed a greater VTA activation for casein, than for maltodextrin consumption, across both diet groups. However, restricted analyses for each diet group

showed no difference in VTA activation between the two solutions in NR → PR rats despite their preference for casein exhibited later in the session during free choice trials. By contrast, there was still a tendency for a greater VTA activation evoked by casein consumption in PR → NR rats despite their slight preference shift away from the protein solution. Taken together, these results suggest the fast behavioral adaptation of food preference according to the current protein status is associated with slower changes in the activation of VTA neurons in response to nutrient consumption.

## Additional Experience with Nutrients Leads to Further Changes in Behavior but Not Neural Activity

Finally, we asked whether further experience with each solution – and thus pairing the flavored solutions with the new diet state – would further alter behavior and associated neural signals. We therefore exposed all rats to four more conditioning sessions in which only one solution was available – two sessions for casein and two sessions for maltodextrin

Figure 2



– and then performed a third, final preference session (Figure 2G).

As in other preference sessions, we found that there was no difference in consumption during forced choice trials with all rats drinking casein and maltodextrin regardless of diet (Figure 2H). Latency to approach the sipper was different on these forced choice trials, however, with rats overall being a little slower to respond to the sipper with maltodextrin, relative to casein (Figure 2I).

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### Figure 2. Changing Diets Leads to Rapid Changes in Behavior toward Nutrients

(A) Schematic showing experimental timeline for Preference Test 2 (before additional conditioning sessions).

(B) On forced choice trials, there was no difference in licks for casein and maltodextrin in either diet group. Two-way repeated-measures ANOVA: no effect of Diet ($F(1,9)= 1.470$, $p=0.256$), Solution ($F(1,9)=2.598$, $p=0.141$), and no Diet x Solution interaction ($F(1,9)=0.044$, $p=0.839$).

(C) On forced choice trials, latency to drink from each sipper was affected by diet. Two-way repeated-measures ANOVA: a tendency towards an effect of Diet ($F(1,9)=5.055$, $p=0.051$), and Solution ($F(1,9)=4.900$, $p=0.054$), as well as a significant Diet x Solution interaction ($F(1,9)=7.510$, $p=0.023$). PR → NR rats showed increased latency to drink maltodextrin, relative to casein (Sidak: $p=0.049$), and relative to NR → PR rats ($p=0.042$). *, $p<0.05$ vs. casein; #, $p<0.05$ vs NR → PR rats.

(D) On free choice trials, both groups drank more casein than maltodextrin. Two-way repeated-measures ANOVA: no main effect of Diet ($F(1,9)=2.928$, $p=0.121$), but a main effect of Solution ($F(1,9)=11.300$, $p=0.008$), and no Diet x Solution interaction ($F(1,9)=0.167$, $p=0.692$).

(E) Choice behavior was similar across groups. Unpaired t-test: $t(9)=0.424$, $p=0.767$) with NR → PR rats showing a preference for casein vs. maltodextrin (one sample t-test vs. 0.5: $t(4)=3.301$, $p=0.030$) and PR → NR rats only showing a weak tendency towards a casein preference ($t(5)=2.074$, $p=0.092$). †, $p<0.05$ vs. 0.5.

(F) In NR → PR rats, no difference in neural activation between casein and maltodextrin. Z-score calculated from fluorescence changes aligned to first lick in all forced choice trials is shown (*top panels*) and AUC from 0-2 s following first lick in each trial (*bottom panels*) for NR → PR rats (*left*) and PR → NR rats (*right*). Solid line is mean across all rats in group and shaded area is SEM. Two-way repeated measures ANOVA: no effect of Diet ($F(1,9)=0.008$, $p=0.929$), but a main effect of Solution ($F(1,9)=6.231$, $p=0.034$), and no Diet x Solution interaction ($F(1,9)=1.053$, $p=0.332$). Planned comparisons revealed a tendency for casein to evoke greater neural activation than maltodextrin in PR → NR rats ($t(5)=2.496$, $p=0.055$) but no difference in NR → PR rats ($t(4)=1.061$, $p=0.349$).

(G) Schematic showing experimental timeline for Preference Test 3 (after additional conditioning sessions).

(H) On forced choice trials on preference session 3, there was no difference in licks for casein or maltodextrin due to diet. Two-way repeated-measures ANOVA: no effect of Diet ($F(1,9)=0.001$, $p=0.979$), no effect of Solution ($F(1,9)=1.759$, $p=0.217$) and no Diet x Solution interaction ($F(1,9)=0.560$, $p=0.473$).

(I) Latency to approach each sipper was affected by solution but not diet. Two-way repeated-measures ANOVA: no effect of Diet ($F(1,9)=0.233$, $p=0.641$), a main effect of Solution ($F(1,9)=7.874$, $p=0.021$), but no Diet x Solution interaction ($F(1,9)=3.326$, $p=0.101$).

(J) On free choice trials, licks for each solution were affected by solution but not diet. Two-way repeated-measures ANOVA: a main effect of Solution ($F(1,9)=14.104$, $p=0.005$), and a tendency for an effect of Diet ($F(1,9)=3.666$, $p=0.088$), and a Diet x Solution interaction ($F(1,9)=4.330$, $p=0.067$). NR → PR rats lick more for casein than maltodextrin (Sidak: $p<0.001$) but no difference for PR → NR rats ($p=0.154$). ***, $p<0.001$ vs. casein.

(K) Diet groups showed a tendency towards differing in their choices (Unpaired t-test: $t(9)=2.243$, $p=0.052$). NR → PR rats showed a preference for casein vs. maltodextrin (one sample t-test vs. 0.5: $t(4)=11.975$, $p<0.001$). PR → NR rats showed no preference ($t(5)=0.598$, $p=0.576$). †††, $p<0.001$ vs. 0.5.

(L) Casein evokes greater activity than maltodextrin across all rats without an effect of diet. Figure conventions are as in F. Two-way repeated-measures ANOVA: no effect of Diet ($F(1,9)=0.058$, $p=0.816$), main effect of Solution ($F(1,9)=5.826$, $p=0.039$), and no Diet x Solution interaction ($F(1,9)=0.057$, $p=0.816$). Planned comparisons showed no difference in neural activation between casein and maltodextrin in NR → PR rats ($t(4)=2.085$, $p=0.105$) or in PR → NR rats ($t(5)=1.468$, $p=0.202$).

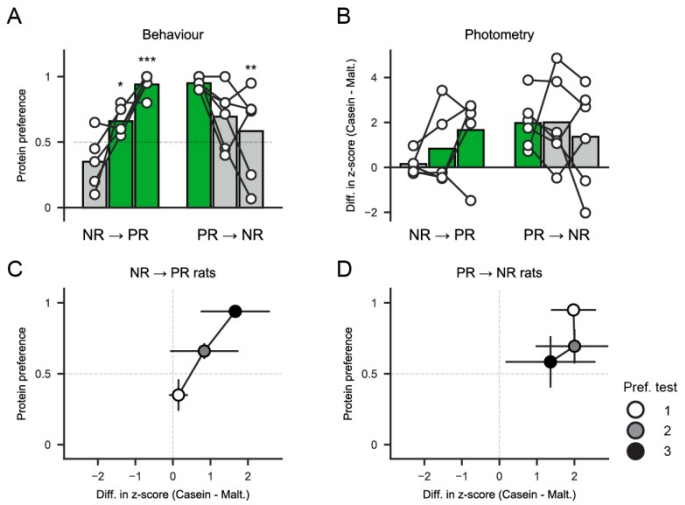
On free choice trials, behavior was influenced by physiological state with NR → PR rats now licking more for casein than maltodextrin whereas there were no differences in intake of each solution for PR → NR rats (Figure 2J). This was reflected in rats' choices as the groups differed significantly with NR → PR rats showing a strong preference for casein and PR → NR rats showing no preference (Figure 2K).

Analysis of photometry data from forced choice trials during this third preference test showed that, across all rats, casein evoked greater neural activation than maltodextrin, but that this was not affected by the rats' dietary status (Figure 2L). Thus, the difference in neural activity observed in PR → NR rats, which were initially protein-restricted, was relatively persistent and was not reversed in all rats even after restoring protein levels and gaining additional experience with each solution.

### Behavior and VTA Activity Become Uncoupled After Diet Switch

To compare across all sessions, we calculated two difference scores to quantify the behavioral and the neural difference between responses to casein and maltodextrin in each group. Behaviorally, we considered the percent of times that rats chose casein over maltodextrin as an index of their preference for protein. For photometry we calculated the difference in evoked fluorescence between casein and maltodextrin aligned to first lick in each bout on forced lick trials. These data are shown in Figure 3. Comparing behavior across all sessions, we found that protein preference was strongly influenced by physiological state in a time-dependent manner (Figure 3A). As such, NR → PR rats' protein preference increased remarkably from the first session, when they were not restricted, through to the second and third sessions, when they had been placed on protein-restricted diet. By contrast PR → NR rats presented an opposite behavioral pattern across tests with a gradual decrease of their protein preference after the diet switch to a non-protein restricted state. When fiber photometry data were considered, although visual inspection of the data suggested that neural activation shifted moderately across days, there was no significant change in signals indicating that changes in neural activation are less flexible (Figure 3B). Considering changes in neural activity as a function of protein preference, NR → PR rats appeared to show a gradual shift in neural activation reflecting the change in protein preference, whereas an opposite effect was not seen in PR → NR rats (Figure 3C-D).

Figure 3



## Discussion

Over the years a number of studies have shown that animals prioritize protein intake over the intake of other macronutrients (Morrison and Laeger, 2015). However, the neural mechanisms underpinning this behavioral process are not known. Here, for the first time, we show that, under conditions of protein restriction, neural activity in the VTA reflects the preference for protein over carbohydrate. Specifically, rats maintained on a low protein diet show a marked preference for casein-containing solution and this is paralleled by an increase in VTA neural activity when licking for casein vs. licking for maltodextrin. Furthermore, we also demonstrate that protein preference is dependent on current physiological state and can be rapidly induced in response to protein needs. Interestingly, VTA neural activity is slower than the behavioral response to adapt to new protein status, especially when protein appetite is decreased.

### Protein preference is sensitive to current phsyiological state

Consistent with our earlier studies (Murphy et al., 2018), protein-restricted rats developed a strong preference for protein-containing solution over carbohydrate-containing solution when they faced a choice between the two nutrients. As previously observed, rats consumed similar amounts of both casein and maltodextrin during conditioning and forced choice trials, when only one bottle was available at a time. These results strongly suggest that the development of protein preference in protein-restricted rats does not coincide with a general aversion to other nutrients, such as carbohydrates. This result differs to the responses seen to diets lacking single amino acids that can lead to the development of conditioned taste aversion for foods with imbalanced amino acid content (Gietzen and Aja, 2012; Maurin et al., 2005). The reason for this discrepancy remains unclear but may be related to the palatability of the maltodextrin solution adulterated with saccharin, which promotes consumption even when the only option available. Interestingly, even though protein-restricted rats consumed the same amount of each solution on forced choice trials as non-restricted rats, their latency to approach and consume maltodextrin was increased, relative to casein.

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**Figure 3. Behavior and VTA Activity Become Uncoupled After Diet Switch**

(A) Preference for casein vs. maltodextrin shifts differently in each diet group. Bars are mean and circles are individual rats. Two-way repeated-measures ANOVA: no effect of Diet ($F_{1,9}=1.329$, $p=0.279$), no effect of Session ($F_{2,18}=1.035$, $p=0.375$), but a significant Diet x Solution interaction ($F_{2,18}=17.523$, $p<0.001$). Post hoc Dunnett's tests showed for NR → PR rats both Session 2 ($p=0.034$) and Session 3 ($p<0.001$) differed from Session 1 and for PR → NR rats Session 2 showed a tendency to differ ($p=0.057$) and Session 3 differed ($p=0.007$) from Session 1.* $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. Session 1.

(B) Neural activation for casein vs. maltodextrin does not shift significantly across sessions in either diet group. Bars are mean and circles are individual rats. Two-way repeated-measures ANOVA: no effect of Diet ($F_{1,9}=1.517$, $p=0.249$), no effect of Session ($F_{2,18}=0.288$, $p=0.753$), and no Diet x Session interaction ($F_{2,18}=1.509$, $p=0.248$). Planned comparisons on each diet group separately showed no significant effect of Session.

(C-D) Behavioral preference for casein vs. maltodextrin (y-axis) plotted as a function of difference in neural activation (z-score) to consumption of each solution (x-axis) in NR → PR rats (C) and PR → NR rats (D). Circles connected by black solid lines show mean ± SEM.

Consistent with this result, we previously observed that casein consumption in protein-restricted rats is driven by an increase in casein palatability, relative to maltodextrin (Murphy et al., 2018). Thus, despite both solutions being consumed in similar amounts, casein solution seems to have greater motivational and hedonic properties than maltodextrin when rats are in a state of protein deficiency, which may drive casein preference during free choice trials.

Changes in protein status result in different behavioral adaptations depending on the direction of diet shift. Rats experiencing a new protein deficiency (NR → PR) rapidly shifted their preference toward casein even without prior experience of the casein solution in this new motivational state. On the other hand, rats returning to a protein repleted state after initial learning under protein deficiency required more extensive exposure to extinguish their protein preference. These results differ from previous work using carbohydrates (e.g. sucrose) in the context of incentive learning, where it is necessary to experience the nutrient in a new motivational state for learning to take place (Balleine, 2005; Wassum et al., 2009). Conversely, the results in our study after diet switch (Figure 2; NR → PR) suggest that protein appetite can manifest independently of prior experience. Interestingly, previous studies have demonstrated that an immediate specific appetite exists for another essential nutrient, sodium (Krause and Sakai, 2007). As such, upon sodium depletion there are immediate and unlearned alterations in how sodium is perceived and how animals respond to stimuli previously associated with sodium (Robinson and Berridge, 2013). However, sodium appetite is rapidly terminated once sodium levels are restored (Krause and Sakai, 2007). Such fine regulation was not observed with protein intake (Figure 2; PR → NR) and, indeed, exposure to both protein- and carbohydrate-containing solution seemed required before protein appetite waned. This discrepancy between nutrients may result from different processes underlying sodium and protein sensing or might result from long-lasting alterations induced after several weeks of protein deficiency. Whether the switch toward protein intake affects the intake of other macronutrients and general regulation of food intake remains to be investigated.

## VTA neural activity is driven by protein sensing

Neural substrates regulating protein appetite remain largely unknown (Morrison and Laeger, 2015). Here we recorded the activity of VTA neurons during both protein and carbohydrate consumption. We first show that VTA activation is modulated by both the macronutrient content of the food and the rats' protein status during the initial preference test (Figure 1). Interestingly these differences in VTA activity are observed during forced choice trials, in which only one solution is provided, and this difference in activity reflects future food preference in the later free choice trials. This design allowed us to analyze neural activity evoked by consumption of both types of solution even in rats who only drank one solution during free choice trials. Of note, we demonstrate that, in PR rats, casein consumption induces an elevated activation of VTA neurons compared to the consumption of maltodextrin. Importantly, as discussed above, this difference is not the result of different behavioral activation as rats exhibited similar levels of licking. Previous studies have demonstrated that sugar conditioned flavor preferences induced by oral or post-ingestive processes are dependent on VTA activity and dopamine release (Araujo et al., 2012; Hsu et al., 2018; McCutcheon, 2015; Sclafani et al., 2011). Here we show for the first time that

protein conditioned flavor preference also involves VTA activity. The VTA plays a central and complex role in the control of food-related behaviors. In our study, the nutrient-dependent activation of VTA neurons may be the result of several processes. The role of VTA neurons, especially dopamine cells, in prediction error processes is extensively described (Day et al., 2007; Schultz, 2007) and the VTA activity reported here may be the result of sipper extension during discrete trials acting as a conditioned stimulus. However, our data were aligned to the licking response and so are unlikely to be related to prediction of specific protein or carbohydrate solution. Moreover, the difference in VTA activity may reflect both incentive processes (Berridge, 2007; Bromberg-Martin et al., 2010; Salamone and Correa, 2012) and differences in hedonic value (McCutcheon et al., 2012b; Roitman et al., 2008) as suggested by the shorter latency for casein consumption (Figure 1&2) and increased casein palatability in protein-restricted rats reported previously (Murphy et al., 2018).

Following a switch in diet and change in protein status, the response of VTA neurons to both casein and maltodextrin is more complex and does not immediately follow changes in protein preference. During the second preference test, performed before any additional experience of casein or maltodextrin solutions in their new physiological state, newly protein-restricted rats (NR → PR) exhibited similar VTA activation during casein and maltodextrin consumption despite their increased preference for the protein solution. By contrast, rats that were now protein-repleted maintained an elevated VTA response to casein than maltodextrin. Furthermore, subsequent exposure to both solutions during additional conditioning sessions did not seem sufficient to significantly reverse the pattern despite elevated activity during casein consumption in both groups. These data starkly contrast with those from studies of sodium appetite where there is an immediate sensitivity of the VTA dopamine system to changes in physiological state (Cone et al., 2016). Interestingly, this gradual change in VTA responses is more similar to what is observed in response to food-related cues and stimuli after motivational changes that require extensive and state-dependent experience of the food in the new state before approach behaviors adapt (Wassum et al., 2009, 2011).

It is important to note that in this study we used a strategy to target neurons that was not-selective for dopamine neurons. As such, it is likely that some of the signal we recorded resulted from the activity of non-dopamine populations of VTA neurons including local GABA interneurons and projecting GABA or glutamate neurons (Dobi et al., 2010; Morales and Margolis, 2017) although, by number, dopamine neurons represent the largest proportion of VTA neurons (Nair-Roberts et al., 2008). In addition, the increases in neural activity evoked by behavioral events are qualitatively similar to those others have observed when recording dopamine neurons specifically (e.g. using transgenic TH::Cre rats; Parker et al., 2016) or when recording dopamine release using fast-scan cyclic voltammetry (Phillips et al., 2003). As other VTA neuronal populations are involved in different aspects of food-related behaviors (Morales and Margolis, 2017), future cell specific targeting experiments will be required to tease apart responses from these neuronal subtypes.

A key remaining question is how VTA midbrain circuits detect the nutrient content of food and integrate this with current protein status to regulate protein homeostasis. Previous work suggests that, as dopamine is sensitive to palatability, the VTA must receive taste information (Hajnal et al., 2004; McCutcheon et al., 2012b; Roitman et al., 2008) and protein can be detected via umami receptors expressed on taste buds (Chaudhari et al., 2009; Liman et al., 2014). However, VTA circuits – and especially the mesolimbic dopamine

system – are not only sensitive to taste and palatability but also to the caloric content of food (De Araujo et al., 2008; Beeler et al., 2012; Domingos et al., 2011; Ferreira et al., 2012; McCutcheon et al., 2012a) and this information is relayed to forebrain regions controlling food-seeking behaviors (Tellez et al., 2016). Whether VTA neurons are also sensitive to protein or amino acids directly is not known but individual amino acid levels can be detected by limbic regions connected to the VTA (Anthony and Gietzen, 2013; Heeley and Blouet, 2016; Karnani et al., 2011). VTA populations are also highly sensitive to feeding related hormones and gut-derived signals including ghrelin and GLP1 (Di Chiara and Abizaid, 2009; Cone et al., 2014; Mebel et al., 2012; Mietlicki-Baase et al., 2013, 2014), which may also be released by protein in the diet (Morrison and Laeger, 2015). Finally, with respect to the signal relaying protein restriction, recent work showed that fibroblast growth factor 21 (FGF21), a hepatic hormone, is released in humans and rodents in response to reduction in dietary protein (Laeger et al., 2014). A centrally-mediated effect of FGF21 in inducing behaviors associated with protein restriction has not been tested but central FGF21 has been shown to alter glucose metabolism and energy expenditure (Liang et al., 2014; Sarruf et al., 2010). Thus, there are multiple signals that could influence the VTA activity reported in this study and these remain to be investigated. We can hypothesize that the prolonged exposure to low protein diet combines with detection of protein content during licking to activate VTA and induce short-term changes in activity and long-lasting circuit alterations.

### Conclusions

In summary, we show that under the physiological state of protein restriction, rats develop a strong preference for protein over carbohydrate and that this preference is associated with elevated activation of VTA during consumption of protein. Reversal of diets leads to novel learning about the value of protein in newly restricted animals but for previously restricted rats this learning is blunted and the preference for protein is persistent. Moreover, VTA activation becomes uncoupled from behavioral responses during diet switch suggesting the involvement of a broader circuits regulating protein appetite. Given the potential contribution of inadequate protein diet *in utero* or after birth on neurodevelopmental disorders (Gould et al., 2018; Grissom and Reyes, 2013) and obesity (Simpson and Raubenheimer, 2005), our results highlight neurobiological substrates that may underlie protein appetite in normal and pathological conditions.

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## Author Contributions

Conceptualization, J.E.M.; Formal Analysis, G.C., F.N. and J.E.M.; Investigation, G.C., F.N., K.Z.P., E.M.S.S. and J.E.M.; Writing – Original Draft, F.N. and J.E.M.; Writing – Review & Editing, G.C., F.N., K.Z.P., E.M.S.S. and J.E.M.; Funding Acquisition, J.E.M.

## Declaration of Interests

The authors declare no competing interests.

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## Figure Legends

### Figure 1. Protein-restricted Rats Show a Strong Preference for Protein over Carbohydrate that Is Associated with Elevated Neural Activity

(A) Schematic showing targeting of ventral tegmental area (VTA) by GCaMP6s and implantation of optic fiber (*left*). Expression of virus in VTA and fiber track are shown in photomicrograph (*top right*) and location of expression and fiber placements are shown for all rats (*bottom right*).

(B) Schematic showing experimental timeline (*top*), fiber photometry set-up (*bottom left*), and trial structure of preference tests (*bottom right*).

(C) On forced choice trials, there was no difference in number of total licks for each solution in either diet group. Two-way repeated-measures ANOVA: no main effect of Diet ($F(1,9)=0.016$, $p=0.903$), Solution ($F(1,9)=0.511$, $p=0.493$), and no Diet x Solution interaction ($F(1,9)=0.583$, $p=0.465$).

(D) On forced choice trials, latency to drink from each sipper was influenced by diet group. Two-way repeated-measures ANOVA: main effect of Diet ($F(1,9)=5.163$, $p=0.049$), main effect of Solution ($F(1,9)=15.800$, $p=0.003$), and a Diet x Solution interaction ($F(1,9)=11.558$, $p=0.007$). PR rats had increased latency to drink from maltodextrin than casein (Sidak: $p=0.024$) and showed a tendency for increased latency vs. NR rats ($p=0.055$). *, $p<0.05$ vs. casein.

(E) On free choice trials, PR rats licked more for casein than maltodextrin whereas NR rats did not. Two-way repeated-measures ANOVA: main effect of Diet ($F(1,9)=12.722$, $p=0.006$), main effect of Solution ($F(1,9)=5.675$, $p=0.041$), and a Diet x Solution interaction ($F(1,9)=32.564$, $p<0.001$). PR rats licked more for casein than maltodextrin (Sidak: $p<0.001$) and, relative to NR rats, licked more for casein ($p=0.002$) and less for maltodextrin ($p<0.001$). ***, $p<0.001$ vs. casein; ##, ###, $p<0.01$, 0.001 vs. NR rats.

(F) On free choice trials, diet group had an effect on choice. Unpaired t test, $t(9)=6.731$, $p<0.001$. PR rats chose to drink casein on more free choice trials than maltodextrin (one sample t-test vs. 0.5, $t(5)=24.648$, $p<0.001$) whereas NR rats showed no preference ($t(4)=1.560$, $p=0.194$). ###, $p<0.001$ vs. NR rats; †††, $p<0.001$ vs. 0.5.

(G) Heat map for a single representative NR rat showing normalized fluorescence changes evoked by consumption of casein (*top*) or maltodextrin (*middle*) on forced choice trials. White lines show time of sipper extension. Average fluorescence change across all trials is shown with solid line as mean and shaded area is SEM (*bottom*).

(H) Group data from forced choice casein and maltodextrin trials showing z-score calculated from fluorescent changes aligned to first lick and averaged across all NR rats (top). Solid line is mean and shaded area is SEM. AUC from 0-2 seconds following first lick in each trial from data in top panel (*bottom*) compared to data from PR rats (J) shows that diet influences neural activation during intake of casein and maltodextrin. Two-way repeated-measures ANOVA: no main effect of Diet ($F(1,9)=0.060$, $p=0.812$), but main effect of Solution ($F(1,9)=14.893$, $p=0.003$), and a Diet x Solution interaction ($F(1,9)=11.023$, $p=0.009$). Planned comparisons show greater activation to casein than maltodextrin in PR rats (Paired t test: $t(5)=4.252$, $p=0.008$) but no difference in NR rats ($t(4)=0.678$, $p=0.535$).

(I) Representative data from a PR rat. Conventions are as in G.

(J) Group data from all PR rats show greater neural activation to casein consumption than maltodextrin. Conventions are as in H and statistical analysis is reported in H. **, $p<0.01$ vs. casein.

### Figure 2. Changing Diets Leads to Rapid Changes in Behavior toward Nutrients

(A) Schematic showing experimental timeline for Preference Test 2 (before additional conditioning sessions).

(B) On forced choice trials, there was no difference in licks for casein and maltodextrin in either diet group. Two-way repeated-measures ANOVA: no effect of Diet ($F(1,9)= 1.470$, $p=0.256$), Solution ($F(1,9)=2.598$, $p=0.141$), and no Diet x Solution interaction ($F(1,9)=0.044$, $p=0.839$).

(C) On forced choice trials, latency to drink from each sipper was affected by diet. Two-way repeated-measures ANOVA: a tendency towards an effect of Diet ($F(1,9)=5.055$, $p=0.051$), and Solution ($F(1,9)=4.900$, $p=0.054$), as well as a significant Diet x Solution interaction ($F(1,9)=7.510$, $p=0.023$). PR → NR rats showed increased latency to drink maltodextrin, relative to casein (Sidak: $p=0.049$), and relative to NR → PR rats ($p=0.042$). *, $p<0.05$ vs. casein; #, $p<0.05$ vs NR → PR rats.

(D) On free choice trials, both groups drank more casein than maltodextrin. Two-way repeated-measures ANOVA: no main effect of Diet ($F(1,9)=2.928$, $p=0.121$), but a main effect of Solution ($F(1,9)=11.300$, $p=0.008$), and no Diet x Solution interaction ($F(1,9)=0.167$, $p=0.692$).

(E) Choice behavior was similar across groups. Unpaired t-test: $t(9)=0.424$, $p=0.767$) with NR → PR rats showing a preference for casein vs. maltodextrin (one sample t-test vs. 0.5: $t(4)=3.301$, $p=0.030$) and PR → NR rats only showing a weak tendency towards a casein preference ($t(5)=2.074$, $p=0.092$). †, $p<0.05$ vs. 0.5.

(F) In NR → PR rats, no difference in neural activation between casein and maltodextrin. Z-score calculated from fluorescence changes aligned to first lick in all forced choice trials is shown (*top panels*) and AUC from 0-2 s following first lick in each trial (*bottom panels*) for NR → PR rats (*left*) and PR → NR rats (*right*). Solid line is mean across all rats in group and shaded area is SEM. Two-way repeated measures ANOVA: no effect of Diet ($F(1,9)=0.008$, $p=0.929$), but a main effect of Solution ($F(1,9)=6.231$, $p=0.034$), and no Diet x Solution interaction ($F(1,9)=1.053$, $p=0.332$). Planned comparisons revealed a tendency for casein to evoke greater neural activation than maltodextrin in PR → NR rats ($t(5)=2.496$, $p=0.055$) but no difference in NR → PR rats ($t(4)=1.061$, $p=0.349$).

(G) Schematic showing experimental timeline for Preference Test 3 (after additional conditioning sessions).

(H) On forced choice trials on preference session 3, there was no difference in licks for casein or maltodextrin due to diet. Two-way repeated-measures ANOVA: no effect of Diet ($F(1,9)=0.001$, $p=0.979$), no effect of Solution ($F(1,9)=1.759$, $p=0.217$) and no Diet x Solution interaction ($F(1,9)=0.560$, $p=0.473$).

(I) Latency to approach each sipper was affected by solution but not diet. Two-way repeated-measures ANOVA: no effect of Diet ($F(1,9)=0.233$, $p=0.641$), a main effect of Solution ($F(1,9)=7.874$, $p=0.021$), but no Diet x Solution interaction ($F(1,9)=3.326$, $p=0.101$).

(J) On free choice trials, licks for each solution were affected by solution but not diet. Two-way repeated-measures ANOVA: a main effect of Solution ($F(1,9)=14.104$, $p=0.005$), and a tendency for an effect of Diet ($F(1,9)=3.666$, $p=0.088$), and a Diet x Solution interaction ($F(1,9)=4.330$, $p=0.067$). NR → PR rats lick more for casein than maltodextrin (Sidak: $p<0.001$) but no difference for PR → NR rats ($p=0.154$). ***, $p<0.001$ vs. casein.

(K) Diet groups showed a tendency towards differing in their choices (Unpaired t-test: $t(9)=2.243$, $p=0.052$). NR → PR rats showed a preference for casein vs. maltodextrin (one sample t-test vs. 0.5: $t(4)=11.975$, $p<0.001$). PR → NR rats showed no preference ($t(5)=0.598$, $p=0.576$). †††, $p<0.001$ vs. 0.5.

(L) Casein evokes greater activity than maltodextrin across all rats without an effect of diet. Figure conventions are as in F. Two-way repeated-measures ANOVA: no effect of Diet ($F(1,9)=0.058$, $p=0.816$), main effect of Solution ($F(1,9)=5.826$, $p=0.039$), and no Diet x Solution interaction ($F(1,9)=0.057$, $p=0.816$). Planned comparisons showed no difference in neural activation between casein and maltodextrin in NR → PR rats ($t(4)=2.085$, $p=0.105$) or in PR → NR rats ($t(5)=1.468$, $p=0.202$).

### Figure 3. Behavior and VTA Activity Become Uncoupled After Diet Switch

(A) Preference for casein vs. maltodextrin shifts differently in each diet group. Bars are mean and circles are individual rats. Two-way repeated-measures ANOVA: no effect of Diet ($F1,9)=1.329$, $p=0.279$), no effect of Session ($F(2,18)=1.035$, $p=0.375$), but a significant Diet x Solution interaction ($F(2,18)=17.523$, $p<0.001$). Post hoc Dunnett's tests showed for NR → PR rats both Session 2 ($p=0.034$) and Session 3 ($p<0.001$) differed from Session 1 and for PR → NR rats Session 2 showed a tendency to differ ($p=0.057$) and Session 3 differed ($p=0.007$) from Session 1.* $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. Session 1.

(B) Neural activation for casein vs. maltodextrin does not shift significantly across sessions in either diet group. Bars are mean and circles are individual rats. Two-way repeated-measures ANOVA: no effect of Diet ($F(1,9)=1.517$, $p=0.249$), no effect of Session ($F(2,18)=0.288$, $p=0.753$), and no Diet x Session interaction ($F(2,18)=1.509$, $p=0.248$). Planned comparisons on each diet group separately showed no significant effect of Session.

(C-D) Behavioral preference for casein vs. maltodextrin (y-axis) plotted as a function of difference in neural activation (z-score) to consumption of each solution (x-axis) in NR → PR rats (C) and PR → NR rats (D). Circles connected by black solid lines show mean ± SEM.

### Figure S1. Protein Restriction Leads to Increased Weight Gain

Body weight increases throughout the experiment and this increase is greater in protein-restricted rats. Two-way repeated measures ANOVA: main effect of Day ($F(14,126)=13.811$, $p<0.001$), no main effect of Diet ($F(1,9)=0.120$, $p=0.737$), and a Day x Diet interaction ($F(14,126)=1.972$, $p=0.025$).

### Figure S2. No Nutrient-specific Appetite is Apparent during Sessions When Only One Nutrient Is Available

Rats lick more in second conditioning sessions with each solution than in the first session. This is not influence by diet group or solution type. Three-way repeated-measures ANOVA: main effect of Session ($F(1,)=19.862$, $p=0.002$), no effect of Diet ($F(1,10)=1.435$, $p=0.262$), no effect of Solution ($F(1,10)=1.994$, $p=0.192$), and no significant interactions (all p's > 0.05).

### Figure S3. Neural signals measured by fiber photometry shows lick-evoked activations

Uncorrected fiber photometry traces showing signals resulting from 470 nm (blue trace) and 405 nm (violet trace) excitation of GCaMP. The three dashed grey boxes on the upper trace are expanded in the three lower panels. Data are represented as a percentage change in total level of fluorescence across the session. Behavioral events (sipper extension and individual licks) are shown in lower traces.

# Methods

## CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and request for reagents should be directed to and will be fulfilled by the Lead Contact, Dr. James E. McCutcheon (jem64@le.ac.uk).

## EXPERIMENTAL MODEL AND SUBJECT DETAILS

### Subjects

Adult male Sprague Dawley rats (Charles River Laboratories, n=11) weighing 250-300g on arrival were used. Rats were housed in pairs in individually ventilated cages (46.2 x 40.3 x 40.4 cm), in a temperature (21 ± 2˚C) and humidity (40- 50%) controlled environment with a 12 h light/dark cycle (lights on at 7:00 AM) and with water and food available *ab libitum*. All testing occurred in the light phase. Data are not reported for three rats due to lack of photometry signal resulting from poor expression or misplacement of fiber. Two rats were removed from the study due to aggressive behavior in the week following the initial dietary manipulation, which lead to them being singly housed, rather than in pairs. Procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986 and carried out under Project License 70/8069.

### Virus injection and fiber implantation

For fiber photometry recording, rats received a unilateral injection of a GCaMP6s expressing virus in the VTA and were implanted with an optical fiber targeting the injection site. One-two weeks after their arrival, rats were anesthetized with isoflurane (5% induction, 2-3% maintenance) and mounted in a stereotaxic frame (David Kopf Instruments) in a flat skull position. The scalp was shaved, cleaned with chlorhexidine and locally anaesthetized with bupivacaine (150 µl, s.c.). Rats also received i.p. injection of non-steroidal anti-inflammatory meloxicam (1 mg/kg). Core body temperature, oxygen saturation and heart rate were monitored throughout the surgery. A hole was drilled above the VTA at the following coordinates: AP -5.8 mm, ML +0.7 mm relative to Bregma (Paxinos and Watson, 2007). A 10 µl Hamilton syringe placed in a motorized syringe pump (Harvard Apparatus Pump 11 Elite) was loaded with the GCaMP6s virus (AAV9.Syn1.GCaMP6s.WPRE.SV40, ≈$1.9 \times 10^{13}$ GC/ml, Penn Vector Core) and was slowly lowered into VTA (-8.1 relative to brain surface). 1 µl of virus was delivered over 10 minutes (100 nl/min) and the syringe was left in place for 5 additional minutes before being slowly removed. An optic fiber cannula (ThorLabs CFM14L10, 400 µm, 0.39 NA, 10 mm length) was implanted at the same coordinates, 0.1 mm above the injection site (DV -8.0 mm relative to brain surface). The cannula was secured in place by dental cement (C&B Supabond followed by regular dental acrylic, Prestige Dental) overlaying 4 small skull-screws. Rats were housed in pairs immediately for recovery. Rats were allowed at least 4 weeks to recover before the start of behavioral testing to allow ample time for virus expression.

### Diets

All rats were initially maintained on standard laboratory chow diet (EURodent Diet 5LF2, LabDiet) containing 14% protein. Four weeks after surgery, seven of the rats were randomly assigned to the Protein Restricted diet condition (PR). For these rats, standard chow was switched to a modified AIN-93G diet containing 5% protein from casein (#D15100602,

Research Diets; Murphy et al., 2018). Remaining rats were maintained under standard laboratory chow diet (Non Restricted group, NR). Behavioral testing started 1 week following protein restriction.

## METHOD DETAILS

### Flavor conditioning and casein preference tests

Animals were trained in two identical conditioning chambers (30.5 x 24.1 x 21.0 cm; Med Associates), each located inside a sound- and light-attenuated aluminum outer chamber (1200 x 700 x 700 cm). Each conditioning chamber was equipped with a house light located on the left wall, 2 retractable sippers located on the right wall and 2 light cues located above each sipper hole. Each bottle placed on a retractable sipper was connected to a contact lickometer (Med Associates) used to measure intake of flavored solution. The house light was turned on at the beginning of each daily session and turned off at the end of it. Conditioning chamber apparatus was controlled via a computer running Med- PC IV Software Suite (Med Associates).

Initially, all rats were pretrained with 2 bottles containing 0.2% sodium saccharin (Sigma). First, rats had continuous access to both bottles in the chambers until they reached >1000 licks during the daily 60 min session (1-3 days). Then, each saccharin bottle was presented individually in a pseudorandom order (inter-trial interval 10-30 s, mean 20 s) during 45 trials On each trial, if no licks were made, then sippers remained available for 30 s. However, once a lick was made, sippers remained extended for 5 s before retraction (Figure 1B). This protocol trained rats over a small number of sessions to approach and drink avidly from sippers when available. Coincident with sipper activation, the cue light located above the sipper hole was turned on and remained on until the sipper was retracted. Sippers took approximately 2 s from activation until the rat could reach them to drink. Rats were trained with 0.2 saccharin in both bottles until they reached the criteria of >1000 licks across the session. Following saccharin pre-training, during the next 4 days, all rats were trained to associate a specific flavored solution (0.05% cherry or grape Kool-Aid with 0.2% sodium saccharin) with a different nutrient in daily sessions lasting a maximum of 60 min. (**Conditioning sessions**). During conditioning sessions, only one bottle was available and was presented during 45 individual trials, as described above. Bottles were filled with either protein-containing solution (4% casein, 0.21% L-methionine, 0.2% sodium saccharin, 0.05% flavored Kool-Aid) or isocaloric carbohydrate-containing solution (4% maltodextrin, 0.2% sodium saccharin, 0.05% flavored Kool-Aid), as previously described (Murphy et al., 2018). Bottle positions, presentation order, and flavor-macronutrient associations were counterbalanced between rats. Bottle position was alternated between days.

Twenty-four hours after the last conditioning session, rats received a first preference test (**Pref test 1**). Both casein and maltodextrin-flavored solutions were available during the test. The test started with 45 trials during which each bottle was presented in pseudorandom order (**Forced choice trials**; 20 sec variable inter-trial interval). These trials were followed by 20 presentations of the two bottles simultaneously (**Free choice trials**).

Immediately after Preference test 1, diet conditions were switched between experimental groups. Non-restricted rats were now given protein restricted diet (**NR→PR**) while protein restricted rats were given standard chow diet (**PR→NR**). Seven days after the diet switch, a second preference test was conducted (**Pref test 2**). This test was followed by 4 days of

additional conditioning sessions, as described above, before a final preference test (**Pref test 3**).

### Photometry recordings

To assess the activity of VTA neurons during the consumption of differently-flavored macronutrient solutions, the 'bulk' fluorescence signal generated by GCaMP6s expressing cells was recorded using fiber photometry (Gunaydin et al., 2014; Lerner et al., 2015). Signal processing and acquisition hardware (RZ5P; Tucker Davis Technologies) was used to control two light sources: a 470 nm LED (ThorLabs, M470F3) modulated at 211 Hz and a 405 nm LED (ThorLabs, M405F1) modulated at 539 Hz. A fluorescence minicube (Doric Lenses) combined both wavelengths, which were transmitted through an optical patch cable to the rats' optic cannula implant. LED power was set at 30-60 µW. Emitted light was delivered through the same patch cable back to the minicube where it was filtered for GFP emission wavelength (525 nm) and sent to a photoreceiver (#2151 Femtowatt Silicon Photoreceiver, DC-750 Hz; Newport). Demodulation of the two light sources allowed dissociation of calcium-dependent GCaMP6s signals (470 nm) and calcium-independent changes resulting from autofluorescence and motion artefacts (isosbestic 405 nm wavelength). All signals were acquired using Synapse Essentials software (Tucker Davis Technologies). Signals were sampled at 6.1 kHz (before demodulation) and 1017 Hz (after demodulation). Behavioral events (e.g., licks and sipper presentations) were time stamped by registering TTLs generated by the Med-PC system. The demodulated signals were filtered using a Matlab script (kindly provided by Vaibhav Khonaur) that used FFT to convert each signal from the time domain into the frequency domain, subtracted the 405 signal from the 470 signal, and then converted back into the time domain. This corrected signal was expressed as a change in fluorescence relative to total fluorescence and used for all further analysis.

### Histology

After completion of behavioral testing and recordings, rats were deeply anaesthetized using 5% isoflurane followed by pentobarbital (50 mg/ml) before being transcardially perfused with cold 0.1 M phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA) solution. Brains were then post-fixed overnight in ice cold 4% PFA before being transferred in 0.1 M PBS solution with 30% sucrose for at least 48 h at 4°C. Serial coronal sections (40 µm thick) were cut on a freezing microtome and stored in PBS solution containing 0.02% sodium azide. VTA-containing sections were selected to check virus spread and the position of the fiber track. Free-floating sections were transferred to 6-well plates filled with PBS. First, sections were rinsed in 0.1 M PBS (3 x 5 min) before being incubated for 1 h in blocking solution (3% goat serum, 3% donkey serum, 3% Triton in 0.1 M PBS). Next, sections were incubated overnight at room temperature with primary antibody to detect GCaMP (chicken anti-GFP, A10262, ThermoFisher Scientific; 1:1000 in blocking solution). After rinses in 0.1 M PBS (3 x 5 min), sections were incubated with secondary antibody solution (goat anti-chicken IgG Alexa Fluor 488 conjugate, A-11039, ThermoFisher Scientific, 1:250 in 0.1 M PBS) for 90 min at room temperature. Finally, sections were rinsed with 0.1 M PBS (3 x 5 min) and mounted in VectorShield Hard Set mounting medium and cover-slipped. Images were taken using an epifluorescence microscope (Leica DM2500) using 2.5x, 10x and 20x objectives and a R6 Retiga CCD camera (QImaging). Fiber position and virus spread were determined according to neuroanatomical landmarks (Paxinos and Watson, 2007).

## QUANTIFICATION AND STATISTICAL ANALYSIS

Behavioral data (lick timestamps) were extracted from data files and analyzed using custom Python scripts that measured numbers of licks for each solution and latencies from sipper extension.

For the analysis of fiber photometry recordings, data were divided into discrete trials by alignment with timestamps representing the first lick in each trial and binning into 100 ms bins. Z-scores were calculated for each trial by taking the mean divided by the standard deviation of a baseline period lasting for 10 seconds preceding the first lick in each trial.

For statistical analysis of within session behavioral and neural variables, two-way mixed repeated measures ANOVA was used with Diet group as a between-subject variable (e.g. protein-restricted vs non-restricted) and Solution as a within subject variable (casein vs. maltodextrin). Choice data were analyzed using one-sample t-tests vs. no preference (0.5).

For summary data, across all sessions, two-way mixed repeated measures ANOVA was used with Diet as a between-subject variable and Session as a within-subject variable.

For data from conditioning sessions, three-way mixed repeated measures ANOVA was used with Diet group as a between-subject variable (e.g. protein-restricted vs non-restricted) and Solution and Session as within subject variables (casein vs. maltodextrin; session 1 vs. session 2). For food intake, two-way mixed repeated measures ANOVA was used with Diet as a between-subject variable and Day as a within-subject variable.

Significant effects and interactions were followed with subsequent ANOVAs, post hoc tests, or t-tests as appropriate with correction for multiple comparisons. All repeated measures ANOVA were checked for sphericity of data using Mauchly's test and, if this was significant, the Greenhouse-Geisser corrected values were used.

## DATA AND SOFTWARE AVAILABILITY

All data files are available at Figshare (doi: 10.25392/leicester.data.7636268). These experiments used a combination of software tools: Matlab (data extraction), Python (analysis and plotting), and R (statistics). All code is available at Github (https://github.com/mccutcheonlab/PPP_analysis/tree/Submission1 and https://github.com/mccutcheonlab/tdt-convert).

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| **Antibodies** | | |
| Anti-GFP chicken | ThermoFisher Scientific | Cat#: A10262; RRID: AB_2534023 |
| Goat anti-chicken IgG(H+L), Alexa Fluor 488 conjugate, secondary antibody | ThermoFisher Scientific | Cat#: A-11039; RRID: AB_2534096 |
| **Bacterial and Virus Strains** | | |
| AAV9.Syn1.GCaMP6s.WPRE.SV40 | Penn Vector Core / Addgene | Cat#: 100843-AAV9; RRID:Addgene_100843 |
| **Chemicals, Peptides, and Recombinant Proteins** | | |
| Sodium saccharin | Sigma-Aldrich | Cat#: s1002 |
| Kool-Aid flavors | Kool-Aid | Cat#: 220 and 224 |
| Casein | Sigma-Aldrich | Cat#: C8654 |
| Maltodextrin | Sigma-Aldrich | Cat#: 419672 |
| L-methionine | Sigma-Aldrich | Cat#: 419672 |
| Phosphate buffered saline | Sigma-Aldrich | Cat#: P4417 |
| Paraformaldehyde | VWR | Cat#: 28794.295 |
| VectoShield Hard Set mounting | VectorLabs | Cat#: H-1400 |
| **Deposited Data** | | |
| Raw and analyzed behavioral and photometry data | This study | doi: 10.25392/leicester.data.7636268 |
| **Experimental Models: Organisms/Strains** | | |
| Sprague-Dawley male rats | Charles River | RRID: 5651135 |
| **Software and Algorithms** | | |
| Python 3.6 | Python | RRID: SCR_008394 |
| Python scripts | This study | https://github.com/mccutcheonlab/PPP_analysis/tree/Submission1 and https://github.com/mccutcheonlab/tdt-convert |
| R package | GraphPad | RRID: SCR_001905 |
| Adobe Illustrator | Adobe | RRID: SCR_010279 |
| **Other** | | |
| Standard chow diet | LabDiet | Cat#: EURodent Diet 5LF2 |
| 5% protein diet | Research Diets | Cat#: D15100602 |

## Supplemental Information

### Figure S1

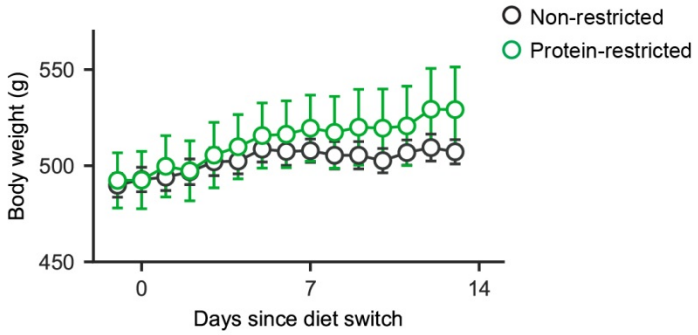


**Figure S1. Protein Restriction Does Not Lead to Changes in Weight Gain**

Body weight increases throughout the experiment and this increase is similar in both groups of rats. Two-way repeated measures ANOVA: main effect of Day ($F(1.4,12.7)=13.811$, $p=0.001$), no main effect of Diet ($F(1,9)=0.120$, $p=0.737$), and no Day x Diet interaction ($F(1.4,12.7)=1.972$, $p=0.184$).

Figure S2

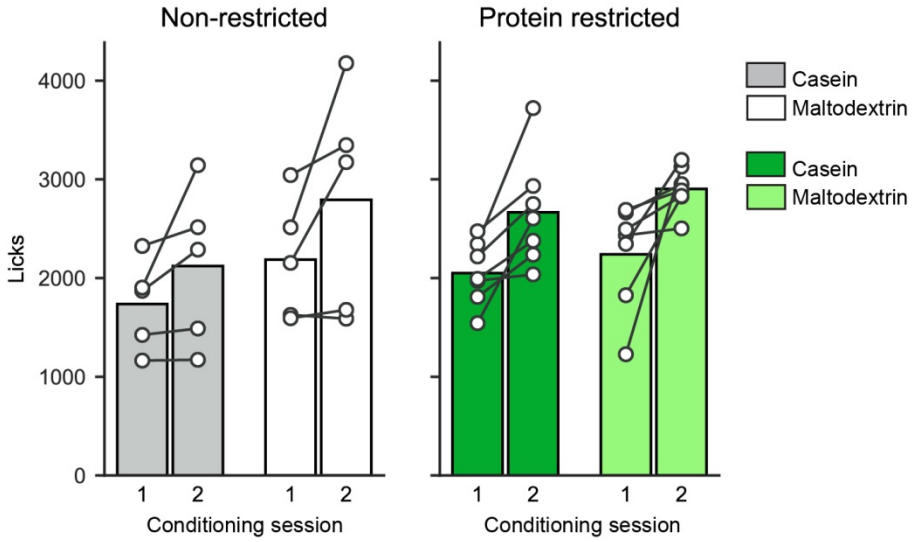


**Figure S2. No Nutrient-specific Appetite is Apparent during Sessions When Only One Nutrient Is Available**

Rats lick more in second conditioning sessions with each solution than in the first session. This is not influenced by diet group or solution type. Three-way repeated-measures ANOVA: main effect of Session ($F(1,10)=19.862$, $p=0.002$), no effect of Diet ($F(1,10)=1.435$, $p=0.262$), no effect of Solution ($F(1,10)=1.994$, $p=0.192$), and no significant interactions (all p's > 0.05).

Figure S3

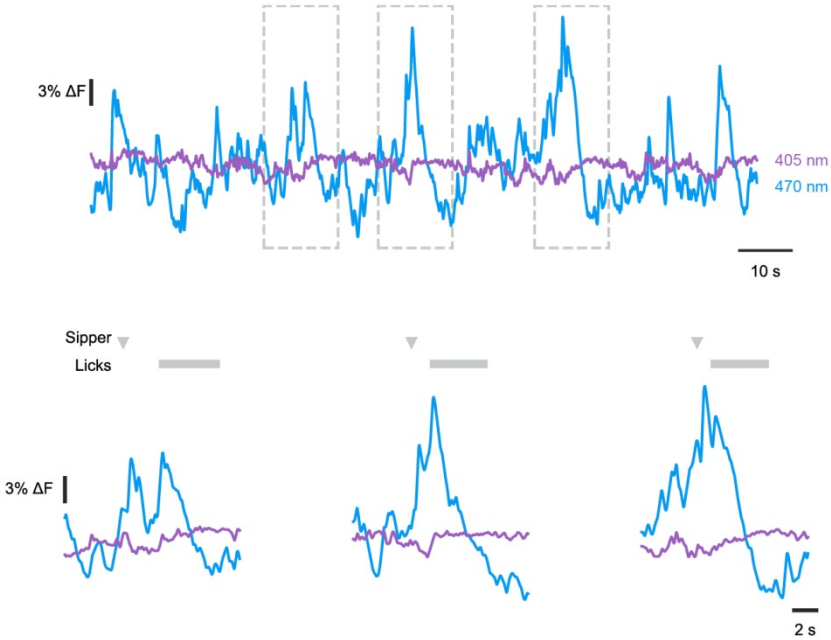


**Figure S3. Neural signals measured by fiber photometry shows lick-evoked activations**

Uncorrected fiber photometry traces showing signals resulting from 470 nm (blue trace) and 405 nm (violet trace) excitation of GCaMP. The three dashed grey boxes on the upper trace are expanded in the three lower panels. Data are represented as a percentage change in total level of fluorescence across the session. Behavioral events (sipper extension and individual licks) are shown in lower traces.