Title: Protein appetite drives macronutrient-related differences in ventral tegmental area neural activity

Abbreviated Title: Protein appetite in the VTA

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Abstract

Control of protein intake is essential for numerous biological processes as several amino acids cannot be synthesized *de novo*, however, its neurobiological substrates are still poorly understood. In the present study, we combined in vivo fiber photometry with nutrient-conditioned flavor in a rat model of protein appetite to record neuronal activity in the ventral tegmental area (VTA), a central brain region for the control of food-related processes. In adult male rats, protein restriction increased preference for casein (protein) over maltodextrin (carbohydrate). Moreover, protein consumption was associated with a greater VTA response relative to carbohydrate. After initial nutrient preference, a switch from a normal balanced diet to protein restriction induced rapid development of protein preference but required extensive exposure to macronutrient solutions to induce greater VTA responses to casein. Furthermore, prior protein restriction induced long-lasting food preference and VTA responses. This study reveals that VTA circuits are involved in protein appetite in times of need, a crucial process for all animals to acquire an adequate amount of protein in their diet.

Significance Statement

Acquiring insufficient protein in one's diet has severe consequences for health and ultimately will lead to death. In addition, a low level of dietary protein has been proposed as a driver of obesity as it can leverage up intake of fat and carbohydrate. However, much remains unknown about the role of the brain in ensuring adequate intake of protein. Here, we show that in a state of protein restriction a key node in brain reward circuitry, the ventral tegmental area, is activated more strongly during consumption of protein than carbohydrate. Moreover, although rats' behavior changed to reflect new protein status, patterns of neural activity were more persistent and only loosely linked to protein status.

Introduction

Ensuring appropriate intake of the three main macronutrients (carbohydrate, fat, protein) is a compelling problem for survival of all animals, including humans. Of the three macronutrients, protein intake is thought to be the most tightly regulated, as many amino acids cannot be synthesized *de novo* (Berthoud et al., 2012). Concordantly, many species, including invertebrates (Mayntz et al., 2005) and mammals (Theall et al., 1984), adjust their behavior to ensure adequate intake of dietary protein. In humans, inadequate protein levels in diet may contribute to obesity, by leveraging up the amount of calories consumed from fats and sugar (Simpson and Raubenheimer, 2005; Hall, 2019; Raubenheimer and Simpson, 2019). Recently, we developed a rodent model of protein appetite in which animals rodents 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; see also Hill et al., 2019), indicating that animals can specifically direct feeding and food-seeking 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 (Ikemoto and Panksepp, 1999; Berridge, 2007; Bromberg-Martin et al., 2010). VTA neurons are sensitive to numerous food-related signals, including ingestive and post-ingestive processes (de Araujo et al., 2008; Domingos et al., 2011; Beeler et al., 2012; Ferreira et al., 2012; McCutcheon et al., 2012a; Alhadeff et al., 2019), and peripheral hormones (Di Chiara and Abizaid, 2009; Mebel et al., 2012; Mietlicki-Baase et al., 2013; Cone et al., 2014; Mietlicki-Baase et al., 2014), allowing the formation of future food preferences (Sclafani et al., 2011). 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.

50 Here, we use *in vivo* fiber photometry to record the activity of VTA neurons during consumption
51 of isocaloric protein- and carbohydrate-containing solutions in an animal model of protein
52 preference (Murphy et al., 2018; Naneix et al., 2019, 2020). We find that, in protein-restricted
53 animals, protein consumption is associated with elevated neural activation, relative to
54 carbohydrate consumption. We then show that when physiological state is reversed
55 behavioral protein preference shifts to reflect the new state more rapidly than neural activity.

56 Materials and Methods

57 *Subjects*

58 Adult male Sprague Dawley rats (Charles River Laboratories, n=15) weighing 250-300g on
59 arrival were used. Rats were housed in pairs in individually ventilated cages (46.2 x 40.3 x 40.4
60 cm), in a temperature (21 ± 2˚C) and humidity (40- 50%) controlled environment with a 12 h
61 light/dark cycle (lights on at 7:00 AM) and with water and food available *ab libitum*. All testing
62 occurred in the light phase. Data are not reported for seven rats due to poor or non-existent
63 photometry signal resulting from lack of viral expression, misplacement of fiber, or poor
64 connection between patch cable and ferrule. Two rats were removed from the study due to
65 aggressive behavior in the week following the initial dietary manipulation, which led to them
66 being singly housed, rather than in pairs. Procedures were performed in accordance with the
67 Animals (Scientific Procedures) Act 1986 and carried out under Project License 70/8069 /
68 PFACC16E2.

69 *Virus Injection and Fiber Implantation*

70 For fiber photometry recording, rats received a unilateral injection of a GCaMP6s expressing
71 virus in the VTA and were implanted with fiber optic cannulas targeting the injection site (Fig.
72 1A). One-two weeks after their arrival, rats were anesthetized with isoflurane (5% induction,
73 2-3% maintenance) and mounted in a stereotaxic frame (David Kopf Instruments) in a flat skull
74 position. The scalp was shaved, cleaned with chlorhexidine and locally anaesthetized with
75 bupivacaine (150 µl, s.c.). Rats also received i.p. injection of non-steroidal anti-inflammatory

76 meloxicam (1 mg/kg). Core body temperature, oxygen saturation and heart rate were
77 monitored throughout the surgery. A hole was drilled above the VTA at the following
78 coordinates: AP -5.8 mm, ML +0.7 mm relative to Bregma (Paxinos and Watson, 1998). A 10
79 µl Hamilton syringe placed in a motorized syringe pump (Harvard Apparatus Pump 11 Elite)
80 was loaded with the GCaMP6s virus (AAV9.Syn1.GCaMP6s.WPRE.SV40, ≈1.9×10^{13} GC/ml,
81 Penn Vector Core; RRID: Addgene_100843) and was slowly lowered into VTA (DV -8.1 mm
82 relative to brain surface). 1 µl of virus was delivered over 10 minutes (100 nl/min) and the
83 syringe was left in place for 5 additional minutes before being slowly removed. An optic fiber
84 cannula (ThorLabs CFM14L10, 400 µm, 0.39 NA, 10 mm length) was implanted at the same
85 coordinates, 0.1 mm above the injection site (DV -8.0 mm relative to brain surface). The
86 cannula was secured in place by dental cement (C&B Supabond followed by regular dental
87 acrylic, Prestige Dental) overlaying 4 small skull-screws. Rats were housed in pairs
88 immediately for recovery. Rats were allowed at least 4 weeks to recover before the start of
89 behavioral testing to allow ample time for virus expression.

90 ***Diets***

91 All rats were initially maintained on standard laboratory chow diet (EURodent Diet 5LF2,
92 LabDiet) containing 14% protein. Four weeks after surgery, eight of the rats were randomly
93 assigned to the protein-restricted diet condition (PR). For these rats, standard chow was
94 switched to a modified AIN-93G diet containing 5% protein from casein (#D15100602,
95 Research Diets; Murphy et al., 2018). Remaining rats were maintained under standard
96 laboratory chow diet (non-restricted group, NR). Behavioral testing started 1 week following
97 protein restriction.

98 ***Flavor Conditioning and Casein Preference tests***

99 Animals were trained in two identical conditioning chambers (30.5 x 24.1 x 21.0 cm; Med
100 Associates), each located inside a sound- and light-attenuated aluminum outer chamber (1200
101 x 700 x 700 cm). Each conditioning chamber was equipped with a house light located on the

102 left wall, 2 retractable sippers located on the right wall and 2 light cues located above each
103 sipper hole. Each bottle placed on a retractable sipper was connected to a contact lickometer
104 (Med Associates) used to measure intake of flavored solution. The house light was turned on
105 at the beginning of each daily session and turned off at the end of it. Conditioning chamber
106 apparatus was controlled via a computer running Med- PC IV Software Suite (Med
107 Associates). Sessions were video recorded at either 5 Hz or 10 Hz using a webcam (Microsoft
108 LifeCam) that interfaced with fiber photometry software.

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

128 macronutrient associations were counterbalanced between rats. Bottle position was alternated
129 between days.

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

135 Immediately after Preference test 1, diet conditions were switched between experimental
136 groups. Non-restricted rats were now given protein restricted diet (**NR→PR**) while protein
137 restricted rats were given standard chow diet (**PR→NR**). Seven days after the diet switch, a
138 second preference test was conducted (**Pref test 2**). This test was followed by 4 days of
139 additional conditioning sessions, as described above, before a final preference test (**Pref test**
140 **3**).

141 ***Fiber Photometry Recordings***

142 To assess the activity of VTA neurons during the consumption of differently-flavored
143 macronutrient solutions, the 'bulk' fluorescence signal generated by GCaMP6s expressing
144 cells was recorded using fiber photometry (Fig. 1; Gunaydin et al., 2014; Lerner et al., 2015).
145 Signal processing and acquisition hardware (RZ5P; Tucker Davis Technologies) was used to
146 control two light sources: a 470 nm LED (ThorLabs, M470F3) modulated at 211 Hz and a 405
147 nm LED (ThorLabs, M405F1) modulated at 539 Hz. A fluorescence minicube (Doric Lenses)
148 combined both wavelengths, which were transmitted through an optical patch cable to the rats'
149 optic cannula implant. LED power was set at 30-60 μW. Emitted light was delivered through
150 the same patch cable back to the minicube where it was filtered for GFP emission wavelength
151 (525 nm) and sent to a photoreceiver (#2151 Femtowatt Silicon Photoreceiver, DC-750 Hz;
152 Newport). Demodulation of the two light sources allowed dissociation of calcium-dependent
153 GCaMP6s signals (470 nm) and calcium-independent changes resulting from

154 autofluorescence and motion artefacts (isosbestic 405 nm wavelength). All signals were
155 acquired using Synapse Essentials software (Tucker Davis Technologies). Signals were
156 sampled at 6.1 kHz (before demodulation) and 1017 Hz (after demodulation). Behavioral
157 events (e.g., licks and sipper presentations) were time stamped by registering TTLs generated
158 by the Med-PC system. The demodulated signals were filtered by using FFT to convert each
159 signal from the time domain into the frequency domain, subtracting the 405 signal from the
160 470 signal, and then converting back into the time domain (Konanur et al., 2020). This
161 corrected signal was expressed as a change in fluorescence, relative to total fluorescence,
162 and used for all further analysis.

163 Subsequently, data were divided into discrete trials by alignment with timestamps representing
164 the first lick in each trial and binning into 100 ms bins. Z-scores were calculated for each trial
165 by taking the mean divided by the standard deviation of a baseline period lasting for 10
166 seconds preceding the first lick in each trial. Area under the curve (AUC) was calculated for
167 the 5 seconds following the first lick before the sipper retracted.

168 ***Histology***

169 After completion of behavioral testing and recordings, rats were deeply anaesthetized using
170 5% isoflurane followed by pentobarbital (50 mg/ml) before being transcardially perfused with
171 cold 0.1 M phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA) solution.
172 Brains were then post-fixed overnight in ice cold 4% PFA before being transferred in 0.1 M
173 PBS solution with 30% sucrose for at least 48 h at 4°C. Serial coronal sections (40 µm thick)
174 were cut on a freezing microtome and stored in PBS solution containing 0.02% sodium azide.
175 VTA-containing sections were selected to check virus spread and the position of the fiber
176 track. Free-floating sections were transferred to 6-well plates filled with PBS. First, sections
177 were rinsed in 0.1 M PBS (3 x 5 min) before being incubated for 1 h in blocking solution (3%
178 goat serum, 3% donkey serum, 3% Triton in 0.1 M PBS). Next, sections were incubated
179 overnight at room temperature with primary antibody to detect GCaMP (chicken anti-GFP,
180 A10262, ThermoFisher Scientific; RRID: AB_2534023; 1:1000 in blocking solution). After

181 rinses in 0.1 M PBS (3 x 5 min), sections were incubated with secondary antibody solution
182 (goat anti-chicken IgG Alexa Fluor 488 conjugate, A-11039, ThermoFisher Scientific; RRID:
183 AB_2534096; 1:250 in 0.1 M PBS) for 90 min at room temperature. Finally, sections were
184 rinsed with 0.1 M PBS (3 x 5 min) and mounted in VectorShield Hard Set mounting medium
185 and cover-slipped. Images were taken using an epifluorescence microscope (Leica DM2500)
186 using 2.5x, 10x and 20x objectives and a R6 Retiga CCD camera (QImaging). Fiber position
187 and virus spread were determined according to neuroanatomical landmarks (Paxinos and
188 Watson, 1998).

189 ***Experimental Design and Statistical Analysis***

190 Behavioral data (lick timestamps) were extracted from data files and analyzed using custom
191 Python scripts that measured numbers of licks for each solution and latencies from sipper
192 extension. Position of rats in the chamber was determined using DeepLabCut (Mathis et al.,
193 2018; Nath et al., 2019) to track body parts (nose, ears, base of tail) of rats in every frame
194 across the preference session.

195 For statistical analysis of within session behavioral and neural variables, two-way mixed
196 repeated measures ANOVA was used with Diet group as a between-subject variable (e.g.
197 protein-restricted vs non-restricted) and Solution as a within subject variable (casein vs.
198 maltodextrin). Choice data were analyzed by comparing diet groups using an unpaired t-test
199 and for preference within each diet group using one-sample t-tests vs. no preference (0.5).

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

202 For data from conditioning sessions, three-way mixed repeated measures ANOVA was used
203 with Diet group as a between-subject variable (e.g. protein-restricted vs non-restricted) and
204 Solution and Session as within subject variables (casein vs. maltodextrin; session 1 vs.
205 session 2). For body weight, two-way mixed repeated measures ANOVA was used with Diet
206 as a between-subject variable and Day as a within-subject variable and planned t-tests were

207 used to compare groups on the first and last day. For food intake, unit of statistic was 'cage'
208 as all rats were group housed and average food intake per rat across all days was compared
209 with t-test.

210 Significant effects and interactions were followed by estimating effect sizes between
211 subgroups. Effect sizes were determined by comparison to bootstrapped sampling
212 distributions, which are shown in lower panels for each comparison. 5000 bootstrap samples
213 were taken. Confidence intervals are bias corrected and accelerated and are shown on the
214 same plots and reported in the text. Reported p-values are permutation p-values resulting from
215 t-tests comparing 5000 reshuffles.

216 ***Data and Software Availability***

217 All data files are available at Figshare (doi: 10.25392/leicester.data.7636268). These
218 experiments used a combination of software tools: Python (data extraction, analysis and
219 plotting), and R (statistics). Estimation plots were adapted from *dabest v0.3.01* (Ho et al.,
220 2019). All code is available at Github
221 (https://github.com/mccutcheonlab/PPP_analysis/releases/tag/v0.1).

222 Results

223 VTA neurons were targeted by injecting an AAV encoding the calcium sensor GCaMP6s
224 (under control of the synapsin promoter) and a fibre optic was implanted above the injection
225 site to record neural activity in freely moving rats (n = 14; Fig. 1A-B). Three to four weeks after
226 surgery, a subset of rats were switched to low protein diet (5% protein from casein; PR group,
227 n=8) while the remaining animals remained on regular chow (14% protein; NR group, n=6).
228 Analysis of body weight data for the subsequent two weeks – before conditioning sessions
229 started - revealed that PR and NR rats gain weight at a slightly different rate across days
230 (Extended Data 1A; two-way ANOVA, Diet: $F(1, 13)=0.09$, $p=0.767$; Day: $F(14, 182)=25.02$,
231 $p<0.0001$; Diet x Day: $F(14, 182)=3.97$, $p<0.0001$). However, the difference between diets
232 was minimal as planned comparisons of PR and NR rats on either the first or last day did not

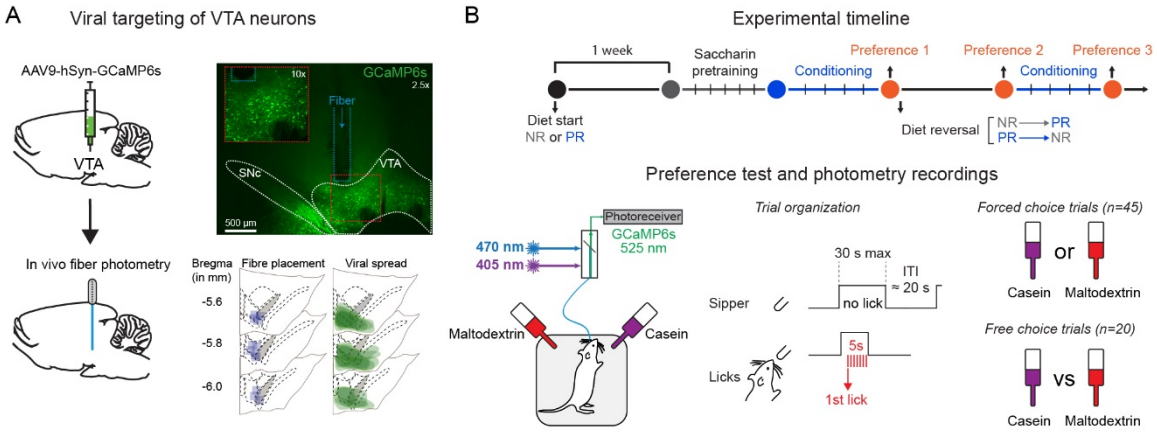

Figure 1. Experimental procedures and timeline. ***A.*** Schematic showing targeting of ventral tegmental area (VTA) by GCaMP6s and implantation of optic fiber (*left*). Expression of virus in VTA and fiber track are shown in photomicrograph (*top right*) and location of expression and fiber placements 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*).

reveal a difference in body weight between groups (Day 1: $t(13)=0.72$, $p=0.486$ and Day 14: $t(13)=0.15$, $p=0.881$). Analysis of food intake showed that PR rats exhibited a mild hyperphagia as has been previously reported (Extended Data 1B; mean difference in food intake between NR and PR rats: 3.77 g [95%CI 1.28, 6.88], $p=0.042$) (Laeger et al., 2014).

Following five days of saccharin pre-training, rats received four daily conditioning sessions in which they had access to distinctly-flavored solutions containing either casein (protein) or maltodextrin (carbohydrate; one session per day), alternated from day to day (Fig. 1B). Both groups similarly increased their consumption throughout conditioning (Extended Data 2; three-way ANOVA, Session: $F(1,13)=22.308$, $p<0.0001$) for both casein and maltodextrin (all Fs < 1; all Ps > 0.1). Thus, rats in both physiological states experienced the same exposure to casein and maltodextrin solutions in advance of the preference test session.

Protein preference is associated with elevated VTA response to protein over carbohydrate

Following conditioning sessions, we then recorded VTA responses during a test session (Fig. 1B). Rats first experienced 45 trials in which only one bottle was available at a time (*forced choice trials*), similar to conditioning sessions.

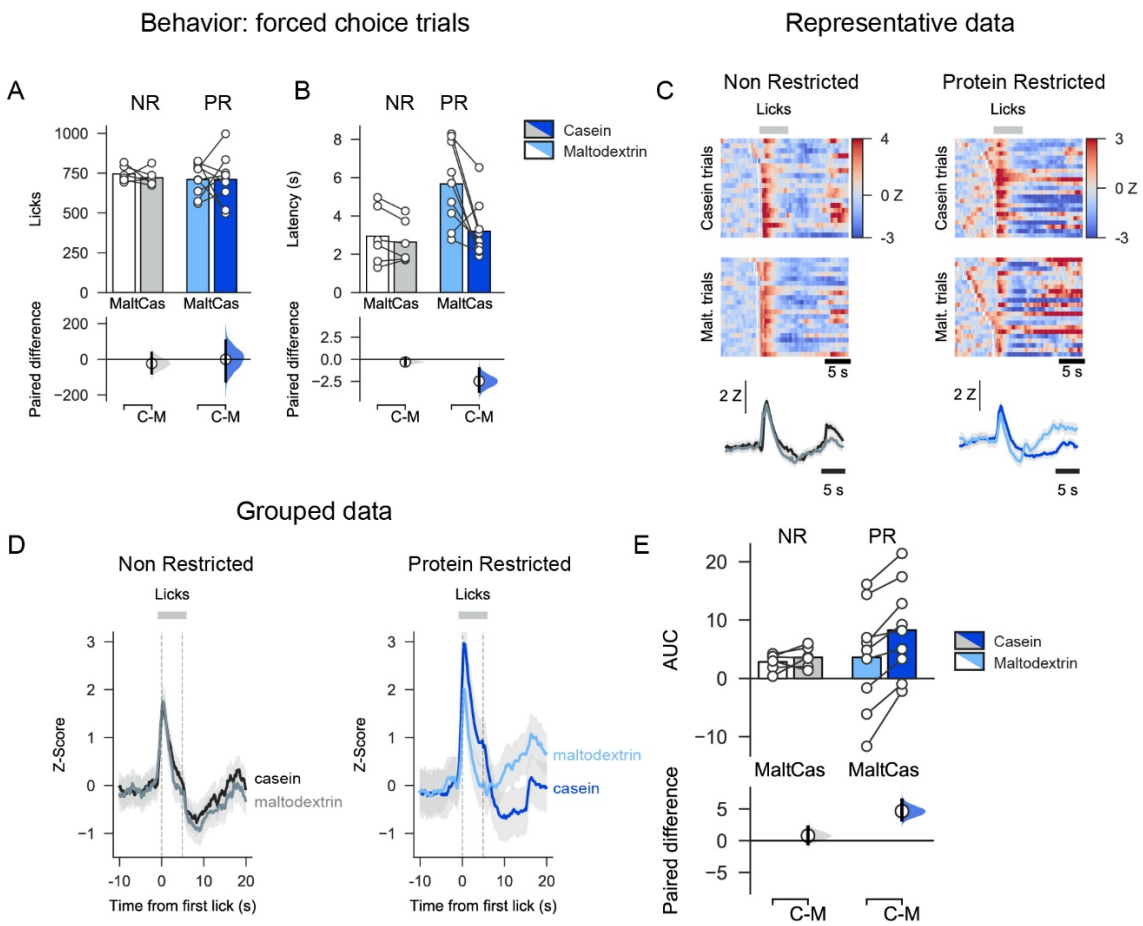

Figure 2. Increased neural activity in VTA of protein-restricted rats during casein consumption than maltodextrin. ***A.*** On forced choice trials, there was no difference in number of total licks for maltodextrin (Malt) vs. casein (Cas) in non-restricted (NR) or protein-restricted (PR) rats. ***B.*** Latency to drink from each sipper was influenced by diet group with PR rats showing shorter latencies on casein trials than maltodextrin trials. ***C.*** Heat maps for a single representative NR rat (*left*) and PR rat (*right*) showing normalized fluorescence changes (Z-scored) evoked by consumption of casein (*top*) or maltodextrin (*middle*) on forced choice trials. Trials are sorted by latency between sipper extension and first lick and 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*). ***D.*** Group data from forced choice casein and maltodextrin trials showing Z-score calculated from fluorescent changes aligned to first lick and averaged across all non-restricted rats (*left*) and protein-restricted rats (*right*). Solid line is mean and shaded area is SEM. ***E.*** Greater neural activation to casein consumption than maltodextrin in PR rats but not NR rats as shown by area under curve (AUC, 0-5 seconds following first lick). In A, B, and E, upper panels show mean as bars and data from individual rats as circles while lower panels show mean difference as a bootstrap sampling distribution with mean differences depicted as dots and 95% confidence intervals indicated by the ends of the vertical error bars.

Across all forced choice trials, rats exhibited similar licking behavior for casein and maltodextrin (Fig. 2A; two-way ANOVA: all Fs < 1 and all Ps > 0.1). However, PR rats did show shorter latencies to drink for casein than for maltodextrin (Fig. 2B; two-way ANOVA, Diet: $F(1,13)=4.83$, $p=0.047$; Solution: $F(1,13)=9.52$, $p=0.009$; Diet x Solution: $F(1,13)=5.83$, $p=0.031$; paired mean difference in latency between casein and maltodextrin for PR rats: -

2.48 s [95%CI -3.65, -1.03], p=0.011). In addition, PR rats on average spent more time closer to the casein sipper than the maltodextrin sipper (Extended Data 3; two-way ANOVA, Diet: F(1,12)=0.20, p=0.661; Solution: F(1,12)=0.50, p=0.492; Diet x Solution: F(1,12)=5.03, p=0.045; paired mean difference in distance to casein and maltodextrin sippers in NR rats: -33.2 pixels [95%CI -96.3, 59.5], p=0.375; paired mean difference in PR rats: 63.9 pixels [95%CI -11.3, 90.1], p=0.026).

Photometry recordings of VTA neurons during consumption of each solution (Fig. 2C-E) showed that casein and maltodextrin consumption evoked similar VTA responses in NR rats (paired mean difference in AUC between casein and maltodextrin in NR rats: 0.80 [95%CI -0.46, 2.17], p=0.354). In contrast, although PR rats licked similarly for both solutions (Fig. 2A), casein consumption is associated with a higher VTA response than for maltodextrin (Fig. 2E; two-way ANOVA, Diet: F(1,13)=0.60, p=0.454; Solution: F(1,13)=20.73, p=0.0005; Diet x Solution: F(1,13)=10.39, p=0.007; paired mean difference in AUC between casein and maltodextrin in PR rats: 4.66 [95%CI 3.27, 6.41], p=0.0026).

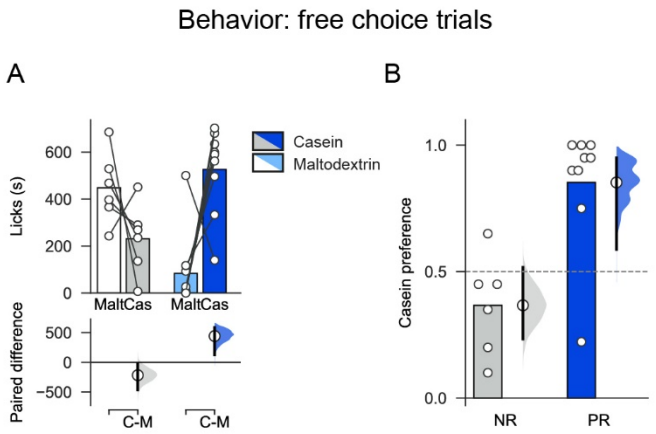

Figure 3. Protein-restricted rats show a strong preference for protein over carbohydrate that is not seen in control rats. ***A,*** On free choice trials, protein-restricted (PR) rats licked more than casein than maltodextrin but there was no difference in licking between the solutions in non-restricted (NR) rats. ***B,*** When number of choices for each solution were considered, PR rats showed a strong preference for casein relative to maltodextrin. Bars show mean and circles are data from individual rats. Bootstrapped sampling distributions are used to show mean paired difference in lower panel of A and difference vs. 0.5 to the right of bars in B. Means of distributions are shown as dots and 95% confidence intervals indicated by the ends of the vertical error bars.

Following these forced choice trials, rats were presented with twenty trials in which both bottles were available at the same time (*free choice trials*) to confirm the existence of protein preference in the PR group (Murphy et al., 2018; Naneix et al., 2019). In free choice trials, PR rats significantly licked more for casein than for maltodextrin (Fig. 3A; two-way ANOVA, Diet: F(1,13)=5.12,

281 p=0.041; Solution: F(1,13)=1.75, p=0.208; Diet x Solution: F(1,13)=14.96, p=0.002; mean
282 paired difference in licks between casein and maltodextrin for PR rats: 442.22 [95%CI 127.33,
283 587.22], p=0.006), whereas NR rats did not (mean paired difference in licks between casein
284 and maltodextrin for NR rats: -216.67 [95%CI -464.67, -16.16], p=0.121). Consistent with this
285 result, PR and NR rats exhibited differential casein preference, as calculated by the number
286 of times they chose casein during the free choice trials (mean difference in choice preference
287 between NR and PR rats: difference between groups: 0.49 [95%CI 0.23, 0.66], p=0.004). As
288 such, NR rats showed no preference for one solution over the other (preference for NR rats:
289 0.37 [95%CI 0.23, 0.52], p=0.121 vs. 50%) but PR rats displayed a strong preference for
290 casein (Fig. 3B; preference for PR rats: 0.85 [95%CI 0.58, 0.95], p=0.0064 vs. 50%).

291 ***Preference towards Protein Develops with Minimal Experience in a Newly Protein***
292 ***Restricted State***

293 Next, we were interested in what would happen to behavior and neural activity when rats'
294 protein needs changed. First, we investigated what happened when rats from the control group
295 were switched to the protein-restricted diet (hereafter, NR → PR rats). Importantly, we re-
296 tested rats at two time points: one week after diets were switched but before any intervening
297 experience of the casein and maltodextrin solutions (Fig. 4A; Pref. Test 2) and one week after
298 this once rats had experienced an extra block of conditioning sessions (Fig. 4G; Pref. Test 3).

299 As reported in Pref Test 1 (see above), animals licked similarly for casein and maltodextrin
300 during forced choice trials in Pref Test 2 (Fig. 4B; mean paired difference in licks between
301 casein and maltodextrin: 3.5 [95%CI -69.5, 36.0], p=0.817) but slightly increased the licking
302 for casein in Pref Test 3 (Fig. 4H; mean paired difference: 81.50 [95%CI 50.00, 111.17],
303 p<0.001). Similarly, analysis of latencies indicated no difference during Pref Test 2 (Fig. 4C;
304 mean paired difference in latency between casein and maltodextrin: -0.07 s [95%CI -0.94,
305 0.73] p=0.974), but showed shorter latencies to drink from the casein sipper during Pref Test
306 3 (Fig. 4I; mean paired difference: -2.22 s [95%CI -3.91, -1.23], p=0.030).

On free choice trials NR → PR rats licked more for casein than maltodextrin during both Pref Test 2 (Fig. 4D; mean paired difference in licks between casein and maltodextrin: 330.00 [95%CI 176.33, 440.17], p<0.001) and Pref Test 3 (Fig. 4J; mean paired difference: 623.17 [95%CI 511.17, 689.83], p<0.001). As expected, this pattern resulted in strong casein preference over maltodextrin on Pref Test 2 (preference: 0.71 [95%CI 0.60, 0.83], p=0.030 vs. 50%) and Pref Test 3 (preference: 0.95 [95%CI 0.83, 0.98], p=0.030 vs. 50%).

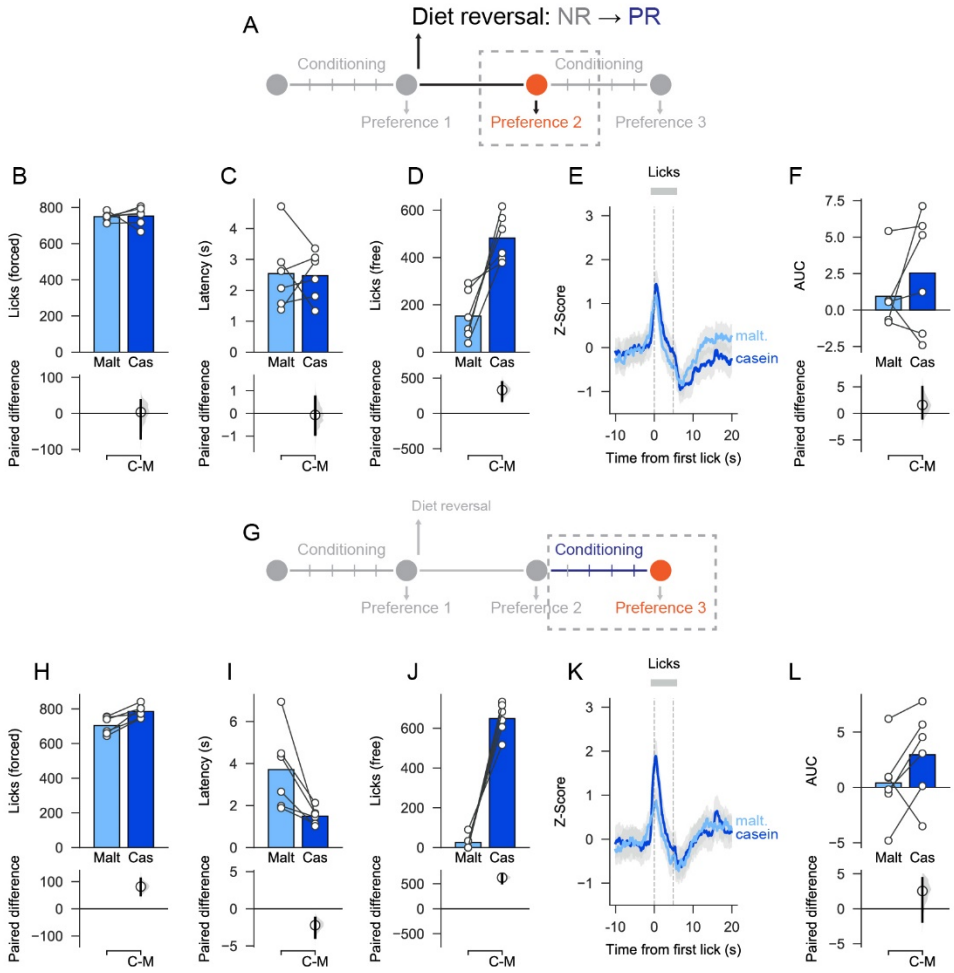

Figure 4. Changing from control diet to low protein diet leads to changes in behavior toward nutrients. ***A,*** Schematic showing experimental timeline for Preference Test 2 (before additional conditioning sessions). ***B-C,*** On forced choice trials, there was no difference in licks for casein and maltodextrin or in latency to drink from each sipper. ***D,*** On free choice trials, rats licked more for casein than maltodextrin. ***E-F,*** As a group, VTA neural activity was similar between casein and maltodextrin trials but there was a large amount of variability. ***G,*** Schematic showing experimental timeline for Preference Test 3 (after additional conditioning sessions). ***H-I,*** On forced choice trials, there was a small increase in licks for casein relative to maltodextrin and latency to drink was shorter on casein trials than maltodextrin trials. ***J,*** On free choice trials, rats licked more for casein than maltodextrin. ***K-L,*** VTA neural activity was not different between casein and maltodextrin trials although, as with the previous test, there was a high degree of variability. Upper panels show mean as bars and data from individual rats as circles while lower panels show mean difference as a bootstrap sampling distribution with mean differences depicted as dots and 95% confidence intervals indicated by the ends of the vertical error bars.

314 The casein preference reported in Pref Test 2 and Pref Test 3 in NR → PR rats strongly
315 contrasts with behavior during the first preference test (Fig. 3). Interestingly, photometry
316 recordings during forced choice trials did not show any difference in VTA responses to
317 casein and maltodextrin in either Pref Test 2 (Fig. 4E-F; mean paired difference in AUC
318 between casein and maltodextrin: 1.59 [95%CI -0.92, 4.95] p=0.381) or Pref Test 3 (Fig. 4K-
319 L; mean paired difference: 2.53 [95%CI -1.84, 4.37], p=0.097).

320 In summary, NR → PR rats developed a rapid behavioral preference to protein over
321 carbohydrate that was observed even before they had gained extensive experience with each
322 solution. Activity in VTA, however, was slower to change to reflect the rats new physiological
323 state and behavior.

324 ***Protein Preference and Differences in Associated VTA Activity Disappear After***
325 ***Experience with Nutrient Solutions In Protein Replete State***

326 We also investigated the effect of protein repletion on casein preference and VTA responses
327 using a similar diet switch design in rats that were initially protein restricted were changed to
328 non-restricted diet (hereafter, PR → NR rats). Again, rats were tested one week following the
329 diet switch but before being given additional experience with solutions (Pref Test 2; Fig. 5A)
330 and then, again, after a block of conditioning sessions (Pref Test 3; Fig. 5G).

331 During forced choice trials there was no difference in the number of licks for casein and
332 maltodextrin in Pref Test 2 (Fig. 5B; mean paired difference in licks between casein and
333 maltodextrin: 34.67 [95%CI -42.44, 100.44], p=0.386) or Pref Test 3 (Fig. 5H; mean paired
334 difference: -25.00 [95%CI -141.78, 64.33], p=0.682). The latency to drink from the casein
335 sipper was still shorter than the latency for maltodextrin in Pref Test 2 (Fig. 5C; mean paired
336 difference in latency between casein and maltodextrin: -2.11 s [95%CI -2.95, -1.22], p=0.003)
337 but this difference disappeared in Pref Test 3 after additional conditioning sessions (Fig. 5I
338 mean paired difference: -0.24 s [95%CI -0.91, 0.65], p=0.561).

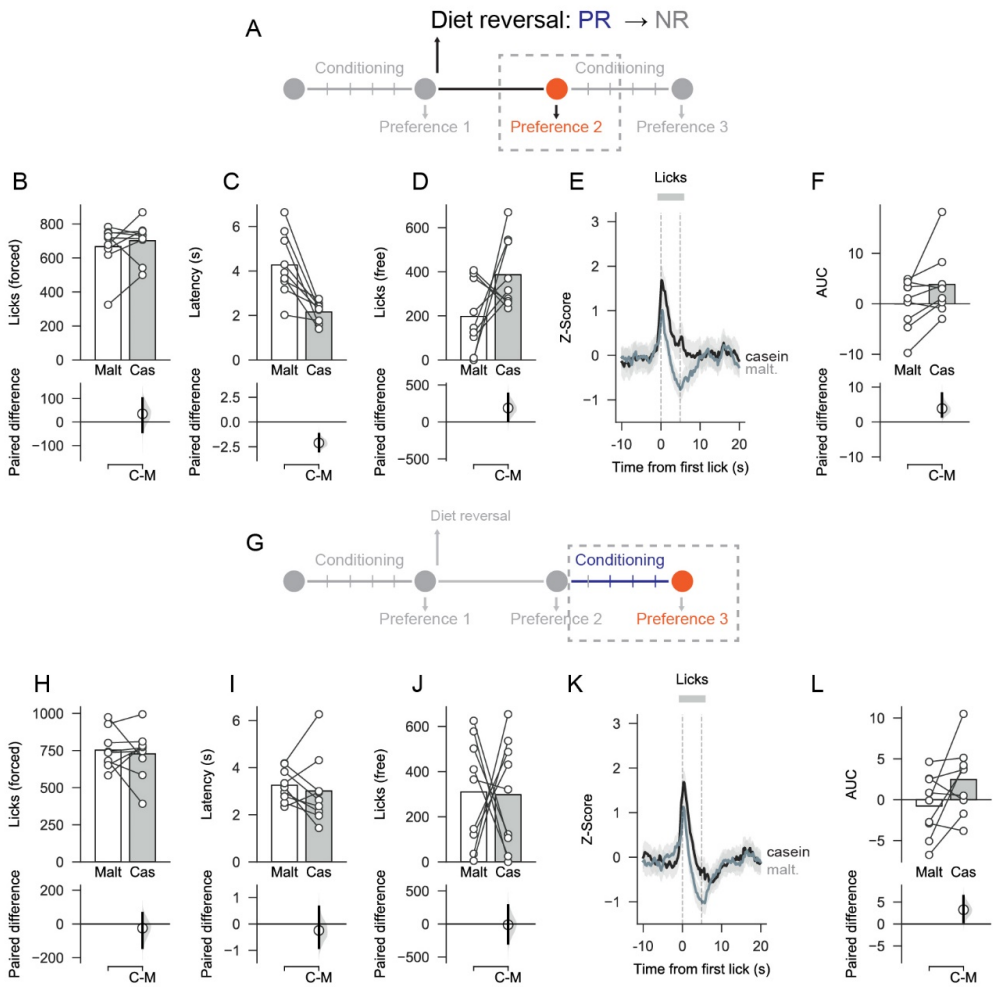

Figure 5. Changing from low protein diet to control diet leads to changes in behavior toward nutrients. ***A,*** Schematic showing experimental timeline for Preference Test 2 (before additional conditioning sessions). ***B-C,*** On forced choice trials, there was no difference in licks for casein and maltodextrin but latency to drink was shorter on casein trials than on maltodextrin trials. ***D,*** On free choice trials, number of licks was similar for casein and maltodextrin although rats chose the casein sipper more than the maltodextrin (see Results). ***E-F,*** VTA neural activity was elevated on casein trials vs. maltodextrin trials. ***G,*** Schematic showing experimental timeline for Preference Test 3 (after additional conditioning sessions). ***H-I,*** On forced choice trials, the number of licks and latencies were similar for casein and maltodextrin trials. ***J,*** On free choice trials, number of licks was similar for casein and maltodextrin. ***K-L,*** VTA neural activity was no longer different between casein and maltodextrin trials. Upper panels show mean as bars and data from individual rats as circles while lower panels show mean difference as a bootstrap sampling distribution with mean differences depicted as dots and 95% confidence intervals indicated by the ends of the vertical error bars.

On free choice trials there was now no significant difference in the number of licks between casein and maltodextrin during Pref Test 2 (Fig. 5D; mean paired difference in licks between casein and maltodextrin: 189.22 [95%CI 19.89, 380.44], p=0.099) although when number of choices was considered, as a group, PR → NR rats still showed a moderate preference for casein over maltodextrin (preference: 0.68 [95%CI 0.57, 0.79], p=0.020 vs. 50%). In Pref Test 3 after additional conditioning sessions, casein preference was completely abolished for both

346 licking (Fig. 5J; mean paired difference in licks between casein and maltodextrin: -11.78
347 [95%CI -294.11, 279.67], p=0.922) and choices (preference: 0.48 [95%CI 0.26, 0.68], p=0.889
348 vs. 50%).

349 When VTA neural activity was analyzed during forced choice trials we found that there was
350 still greater VTA activation on casein trials than maltodextrin trials during Pref Test 2 although
351 the effect size was more variable than on the first preference test (Fig. 5E-F; mean paired
352 difference in AUC between casein and maltodextrin 3.86 [95%CI 1.54, 8.17], p=0.028).
353 Consistent with the abolition of casein preference reported during Pref Test 3, analysis of VTA
354 neural activity also now showed no reliable difference between casein and maltodextrin in
355 forced choice trials although there was a high degree of variability (Fig. 5K-L; mean paired
356 difference in AUC between casein and maltodextrin 3.24 [95%CI 0.47, 6.37], p=0.091). Thus,
357 the protein preference and associated VTA responses that developed when rats were protein-
358 restricted was markedly reduced once rats had gained additional experience with the nutrient
359 solutions in the new protein replete state.

360 ***Behavior and VTA Activity Become Uncoupled after Diet Switch***

361 To compare across all sessions for each group of rats, we examined how protein preference
362 changed from preference test 1 to test 3. After the switch from non-restricted to protein-
363 restricted state (NR → PR rats), there was a clear shift in behavior across the three sessions
364 as shown by a main effect of Session (Fig. 6A; one-way repeated measures ANOVA:
365 F(2,10)=27.01, p<0.0001). Further comparisons showed that after diet switch NR → PR rats'
366 behavior differed both before additional conditioning sessions (mean paired difference in
367 preference between Pref. Test 2 and Test 1: 0.34 [95%CI 0.16, 0.52], p=0.007) and after
368 (mean paired difference between Pref. Test 3 and Test 1; 0.58 [95%CI 0.43, 0.73], p=0.001).
369 However, consistent with our earlier analysis, VTA responses to casein and maltodextrin did
370 not significantly change between the three preference tests (Fig. 6B; two-way repeated

ANOVA: Session (F(2,10)=0.72, p=0.508; Solution (F(1,5)=2.07, p=0.21); Session x Solution (F(2,10)=3.02, p=0.094)).

In contrast, protein repletion (PR → NR rats) induced a gradual decrease in casein preference across the three tests (Fig. 6D; one-way repeated ANOVA: F(2,16)=5.99, p=0.011). Between sessions comparisons showed that casein preference in second test session, when rats had not received additional conditioning, was no different to the first test session (mean paired difference in preference between Pref. Test 2 and Test 1: -0.17 [95%CI -0.31, 0.09], p=0.119). However, by the third test session there was a significant decrease in casein preference compared to the first session (mean paired difference in preference between Pref. Test 3 and Test 1: -0.37 [95%CI -0.60, -0.09], p=0.018). This shift in casein preference is associated with a trend towards a decrease in VTA responses to casein and maltodextrin through the three

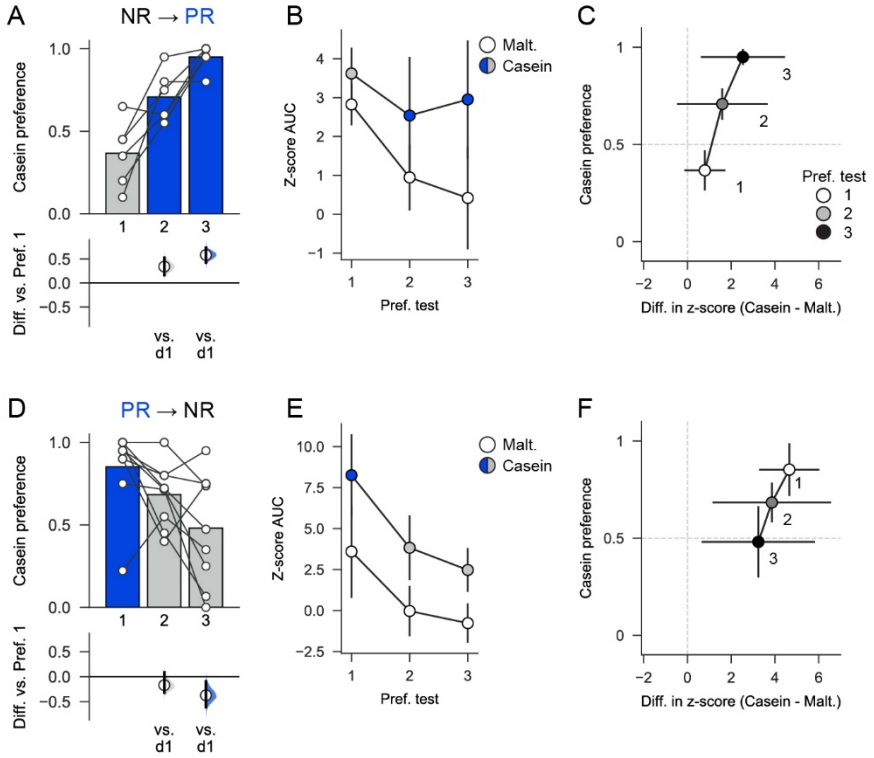

Figure 6. Behavior and VTA activity become uncoupled after diet switch. ***A,*** In NR → PR rats, preference for protein increases after diet switch in both preference test 2, without additional conditioning, and in preference test 3. Bars are mean and circles show data from individual rats with mean differences of bootstrapped sampling distributions shown in lower panel vs. preference test 1. ***B,*** Neural activity in VTA on casein and maltodextrin trials is not affected by diet switch. ***C,*** Behavioral preference for casein vs. maltodextrin (y-axis) plotted as a function of difference in neural activation (z-score AUC) associated with consumption of each solution (x-axis) in NR → PR rats. Circles connected by black solid lines show mean ± SEM. ***D,*** In PR → NR rats, behavior changes after diet switch but requires additional conditioning sessions for protein preference to shift, relative to preference test 1. Plotting conventions as in A. ***E,*** Neural activity in VTA is consistently elevated on casein trials, relative to maltodextrin trials. ***F,*** Preference vs. difference in neural activation for PR → NR rats with plotting conventions as in C.

398 sessions (Fig. 6E; two-way repeated ANOVA: Session $F(2,16)=4.57$, $p=0.06$; Session x
399 Solution $F(2,16)=0.42$, $p=0.666$). However, VTA responses to casein remained higher than
400 responses to maltodextrin (Solution: $F(1,8)=14.25$, $p=0.005$). The relationship between casein
401 preference and neural activation to each solution is summarized in Fig. 6C and 6F. Thus,
402 despite a clear decrease of casein preference during protein repletion, VTA neurons continued
403 to discriminate between the two nutrient solutions and to reflect the initial physiological need
404 for protein.

405 Discussion

406 Animals prioritize protein intake over the intake of other macronutrients (Morrison and Laeger,
407 2015). However, the neural mechanisms underpinning this behavioral process are not well
408 understood. Here, for the first time, we show that, under conditions of protein restriction, neural
409 activity in the VTA reflects the preference for protein over carbohydrate. Specifically, rats
410 maintained on a low protein diet show a marked preference for casein-containing solution and
411 this is associated with an increase in VTA neural activity during casein consumption vs.
412 maltodextrin. Furthermore, we also demonstrate that protein preference is dependent on
413 current physiological state and can be induced or abolished after an initial preference
414 according to protein needs. Interestingly, VTA nutrient-related responses are slower than
415 behavior to adapt to new physiological status, especially when protein appetite is reduced
416 after an initial preference.

417 *Protein appetite is associated with increased VTA activity*

418 Consistent with our earlier studies (Murphy et al., 2018; Naneix, Peters, McCutcheon, 2019),
419 protein-restricted rats developed a strong preference for protein-containing solution over
420 carbohydrate-containing solution when given a choice between the two nutrients. However,
421 protein preference does not coincide with a general aversion to other nutrients as rats
422 consumed similar amounts of both casein and maltodextrin during conditioning and forced
423 choice trials. This result differs from the responses seen to diets lacking single amino acids

424 that can lead to the development of conditioned taste aversion for foods with imbalanced
425 amino acid content (Maurin et al., 2005; Gietzen and Aja, 2012).

426 VTA neurons play a complex role in the control of food-related behaviors (Berridge, 2007;
427 Bromberg-Martin et al., 2010; Brown et al., 2012; Zessen et al., 2012; Root et al., 2020).
428 Previous studies have demonstrated that dopamine signaling originating in the VTA is involved
429 in the establishment of carbohydrate-based flavor preferences (Sclafani et al., 2011; de Araujo
430 et al., 2012; McCutcheon, 2015; Hsu et al., 2018), and we recently showed that protein
431 restriction altered dopamine release in the nucleus accumbens (Naneix et al., 2020). Here,
432 we show for the first time that protein appetite also involves VTA circuits and that VTA
433 activation is modulated by both the macronutrient content of the food and the rats' protein
434 status during the initial preference test (Fig. 2). Specifically, VTA responses are greater during
435 consumption of protein-containing solution (casein) compared to carbohydrate containing
436 solution (maltodextrin) selectively in protein-restricted rats. Interestingly these differences in
437 VTA activity are observed during forced choice trials, in which only one solution is provided,
438 and this difference in activity reflects future food preference in the later free choice trials.
439 Importantly, as discussed above, this difference is not the result of different behavioral
440 activation as rats exhibited similar levels of licking. Conversely, differences in VTA responses
441 to the consumption of different nutrient flavor solution may reflect the reward value of each
442 food and be used to guide food preferences (Berridge, 2007; Bromberg-Martin et al., 2010;
443 Salamone and Correa, 2012) (Roitman et al., 2008; McCutcheon et al., 2012b). In addition,
444 protein-restricted rats exhibited a shorter latency for casein consumption (Fig. 2) suggesting
445 an increase in incentive properties of this solution (Barbano and Cador, 2005). We have also
446 previously reported that protein appetite was associated with increased casein palatability
447 (Murphy et al., 2018; Naneix et al., 2019).

448 ***VTA responses do not follow changes in initial protein preferences***

449 Changes in protein status after an initial nutrient preference resulted in different behavioral
450 adaptations depending on the direction of diet shift. Rats experiencing a new protein deficiency
451 (NR → PR; Fig 4) rapidly shifted their preference toward casein even without additional
452 conditioning sessions, suggesting that protein appetite can manifest independently of prior
453 experience of protein-containing food in a protein-restricted state. Previous studies have
454 demonstrated that an immediate specific appetite exists for another essential nutrient, sodium
455 (Krause and Sakai, 2007). As such, upon sodium depletion there are immediate and unlearned
456 alterations in how sodium is perceived and how animals respond to stimuli previously
457 associated with sodium (Robinson and Berridge, 2013). However, sodium appetite is rapidly
458 terminated once sodium levels are restored (Krause and Sakai, 2007). Such fine regulation
459 was not observed with protein intake (Fig. 5; PR → NR) as casein preference only decreased
460 in newly protein-replete rats after experiencing additional conditioning sessions.

461 VTA responses to both casein and maltodextrin also become more complex and do not
462 immediately follow changes in protein preference. Newly protein-restricted rats (NR → PR;
463 Fig. 4) exhibited slow and delayed changes in VTA responses to casein and maltodextrin
464 consumption, despite their increased preference for the protein solution. Previous studies
465 have shown that the unconditioned response of the VTA dopamine system to food or specific
466 nutrients (Cone et al., 2014, 2016) updates immediately, independently of prior experience of
467 the physiological state (*e.g.* sodium depletion, hunger). In contrast, dopamine responses to
468 food- or nutrient-predictive cues require multiple associations under physiological conditions
469 in which the food is rewarding (Bassareo and Di Chiara, 1997; Day et al., 2007; Cone et al.,
470 2016). Thus, our behavioral and neurobiological results suggest that VTA activity may track
471 the value of the flavor paired with protein rather than the protein content itself (Sclafani et al.,
472 2011; McCutcheon, 2015).

473 On the other hand, protein repletion (PR → NR; Fig. 5) had a delayed impact on VTA activity,
474 as rats continued to show higher VTA responses to casein despite a progressive decrease of
475 their protein preference. These results contrast starkly with those from studies of sodium

476 appetite where VTA dopamine responses to conditioned cues are flexibly expressed in a state-
477 dependent manner once they are learned (Cone et al., 2016). Instead, the maintenance of
478 higher VTA response to casein relative to maltodextrin once an initial protein preference was
479 established, and even after this preference was reversed, may suggest some long-lasting
480 neurobiological impact of the protein restriction which may require extended time and longer
481 learning processes to be reversed.

482 ***Methodological considerations***

483 It is important to note that in this study we used a strategy to target neurons that was not
484 selective for dopamine neurons. As such, it is likely that some of the signal we recorded
485 resulted from the activity of non-dopamine populations of VTA neurons including local GABA
486 interneurons and projecting GABA or glutamate neurons (Dobi et al., 2010; Morales and
487 Margolis, 2017) although, by number, dopamine neurons represent the largest proportion of
488 VTA neurons (Nair-Roberts et al., 2008). In addition, the increases in neural activity evoked
489 by behavioral events are qualitatively similar to those others have observed when recording
490 only dopamine neurons (e.g. using transgenic TH::Cre rats; Parker et al., 2016) or when
491 recording dopamine release using fast-scan cyclic voltammetry (Phillips et al., 2003). As other
492 VTA neuronal populations are involved in different aspects of food-related behaviors (Brown
493 et al., 2012; Zessen et al., 2012; Morales and Margolis, 2017; Root et al., 2020), future cell
494 specific targeting experiments will be required to tease apart responses from these neuronal
495 subtypes.

496 ***Conclusions***

497 A key remaining question is how VTA midbrain circuits detect the nutrient content of food and
498 integrate this with current protein status to regulate protein homeostasis. Previous work
499 suggests that the VTA must receive taste information (Hajnal et al., 2004; Roitman et al., 2008;
500 McCutcheon et al., 2012b). Protein can be detected via umami receptors expressed on taste
501 buds (Chaudhari et al., 2009; Liman et al., 2014) but the link between protein sensing by the

502 tongue and VTA neuronal populations remain to be explored. VTA circuits are also sensitive
503 to the caloric content of food (de Araujo et al., 2008; Domingos et al., 2011; Beeler et al., 2012;
504 Ferreira et al., 2012; McCutcheon et al., 2012a) and this information is relayed to forebrain
505 regions controlling food-seeking behaviors (Tellez et al., 2016). Whether VTA neurons are
506 also sensitive to protein or amino acids directly is not known but individual amino acid levels
507 can be detected by hypothalamic, cortical, and hindbrain regions connected to the VTA
508 (Karnani et al., 2011; Anthony and Gietzen, 2013; Heeley and Blouet, 2016; Tsang et al.,
509 2020). Furthermore, recent work showed that fibroblast growth factor 21 (FGF21), a hepatic
510 hormone, is released in response to reduction in dietary protein (Laeger et al., 2014) and its
511 central action is necessary for development of protein preference in mice (Hill et al., 2019).

512 Given the potential effects of inadequate protein diet *in utero* or after birth on
513 neurodevelopmental disorders (Grissom and Reyes, 2013; Gould et al., 2018) and obesity
514 (Simpson and Raubenheimer, 2005), our results highlight neurobiological substrates that may
515 underlie protein appetite in normal and pathological conditions.

516

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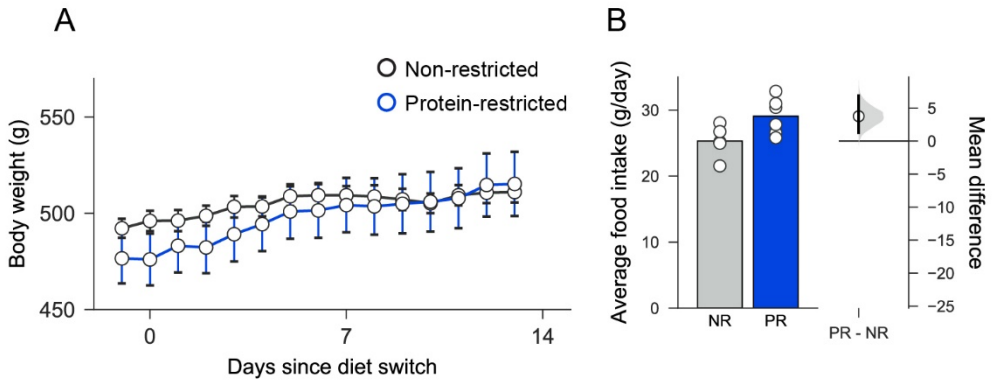
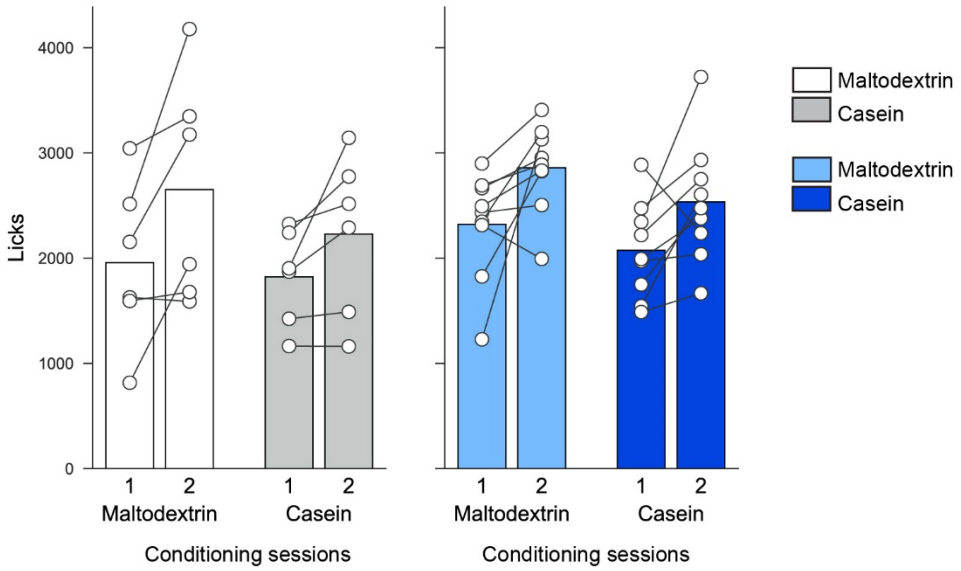
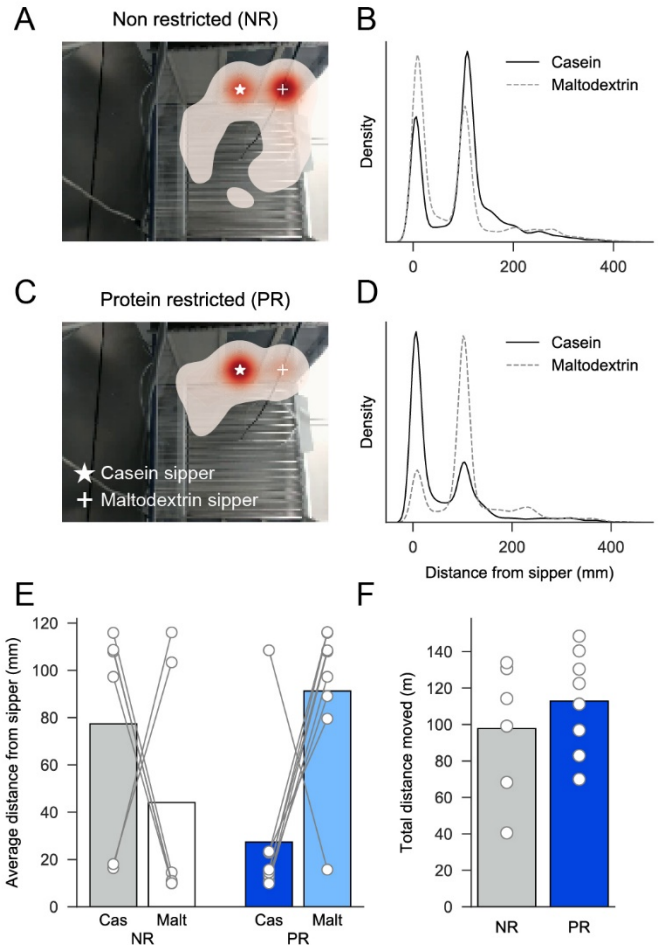
Extended Data



Extended Data 1. Body weight and food intake data. ***A***, Similar changes in body weight increase were seen in protein-restricted (PR) and non-restricted (NR) control rats. Circles show mean for each day and error bars are SEM. ***B***, Mild increase in food intake was seen in PR rats relative to NR rats. Left panel, bars are mean and circles are individual data points (cages). Right panel, mean difference as a bootstrap sampling distribution with mean difference depicted as dot and 95% confidence intervals indicated by the ends of the vertical error bars.



Extended Data 2. Data from conditioning sessions show that for both solutions more was consumed on the second conditioning day than on the first day but there were no differences between diet groups or solutions. Bars are mean and circles are indvidual data points (rats).



Extended Data 3. Position in chamber is determined by diet. ***A*,** Heatmap showing position of non-restricted (NR) rat in chamber when tracked across entire session with red colors representing increased time. Casein and maltodextrin sippers are marked with white star and white cross, respectively. ***B*,** Kernel density estimate for all tracked video frames showing distance from casein sipper (black solid line) and maltodextrin sipper (grey dashed line) for position data shown in *A*. ***C-D*,** Same analysis as in *A* and *B* but for a representative protein-restricted (PR) rat. ***E*,** Average distance from each sipper for all rats shows that protein-restricted rats spend more time near the casein sipper than the maltodextrin sipper. Diet x Substance interaction: $F(1,12) = 5.03$, $p=0.045$. ***F*,** Total distance moved in entire session is not different between NR and PR rats ($t(13)=0.87$, $p=0.400$).