AGE DEPENDENT EFFECTS OF PROTEIN RESTRICTION ON DOPAMINE RELEASE

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ABSTRACT

Despite the essential role of protein intake for health and development, very little is known about the impact of protein restriction on neurobiological functions, especially at different stages of the lifespan. The dopamine system is a central actor in the integration of food-related processes and is influenced by physiological state and food-related signals. Moreover, it is highly sensitive to dietary effects during early life periods such as adolescence due to its late maturation. In the present study, we investigated the impact of protein restriction either during adolescence or adulthood on the function of the mesolimbic (nucleus accumbens) and nigrostriatal (dorsal striatum) dopamine pathways using fast-scan cyclic voltammetry in rat brain slices. In the nucleus accumbens, protein restriction in adults increased dopamine release in response to high frequency trains of stimulation (10-20 Hz) with no effect on single pulse evoked release. By contrast, protein restriction performed at adolescence decreased nucleus accumbens dopamine release evoked by both single pulses and trains of stimulation pulses (5-10 Hz). In the dorsal striatum, protein restriction has no impact on dopamine release when performed at adulthood but in adolescent rats we observed frequency-dependent increases in stimulated dopamine release. Taken together, our results highlight the sensitivity of the different dopamine pathways to the effect of protein restriction, as well as their vulnerability to deleterious diet effects at different life stages.

INTRODUCTION

The regulation of food intake in an everchanging environment is a central survival process. Healthy diet requires a balanced intake of the three main macronutrients (carbohydrates, fat, and proteins) [1]. Protein intake is especially important as amino acids are essential for many biological functions (growth and maintenance, synthesis of nucleic acids and hormones, immune response, cellular repair) and many amino acids cannot be synthesized *de novo*. In humans, protein deficiency and a low protein diet are associated with muscle wasting, stunted growth and increased vulnerability to infections, but may also, to some extent, contribute to obesity by generally increasing appetite [2–4]. Furthermore, protein deficiency and severe protein malnutrition are especially detrimental during development and early life when demand is highest [5–7]. Numerous species, including humans and rodents, regulate their food intake and food-related behaviors to avoid protein deficiency [8–13]. Increasing evidence implicates broad hypothalamic and limbic circuits in the regulation of protein appetite [10,13–15]. However, the impact of protein imbalance (high or low protein diet) on the function of these neurobiological circuits remains undescribed, especially when protein deficiency occurs during a critical period of early development.

The dopamine system plays a central role in food-seeking behaviors, food preference, and in the motivation to eat [16–19]. Recent data show that dopamine neurons integrate current physiological state (*i.e.* hunger, nutrient deficiency) to guide food-seeking behaviors [20–23]. Dopamine neurons are especially sensitive to the nutrient content of ingested food [24–28], through gut-to-brain axis [29,30] and peripheral feeding hormones [31–35]. Furthermore, exposure to specific diets, such as high-carbohydrate and/or high-fat, impacts dopamine signaling within the nucleus accumbens (NAc) and the dorsal striatum [36–39]. However, the impact of low protein diet on the function of dopamine circuits is still largely unexplored.

Early life periods like childhood and adolescence are periods of particular vulnerability to the deleterious impact of various diets on corticolimbic circuits and reward-related processes [40–46]. Interestingly, the dopamine system undergoes delayed maturation taking place during adolescence making it vulnerable to external insults [46–52]. The impact of prolonged inadequate protein consumption on dopamine signaling remains unknown but may be exacerbated during adolescence when protein demand is increased to support rapid growth [53].

Here, we investigated the impact of protein restriction either during adolescence or adulthood on the function of the mesolimbic (NAc) and nigrostriatal (dorsal striatum) dopamine pathways using fast-scan cyclic voltammetry (FSCV) in rat brain slices. We found that protein restriction induced opposite effects on NAc dopamine release depending on age, with restriction

increasing dopamine release in adults but decreasing it in adolescents. In the striatum, however, dopamine function following protein restriction was increased only in adolescents and not adults.

MATERIAL AND METHODS

Subjects

Male Sprague Dawley rats (Charles River Laboratories) were received either at weaning (approximately P21, 50-70 g) for Adolescent groups (n = 13) or at adulthood (P60, 200-250 g) for Adult groups (n = 15). Rats were housed in groups of 2-3 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 food and water available *ab libitum*. All testing and tissue harvesting occurred in the light phase. Procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986 and carried out under Project License PFACC16E2.

Diets

All rats were initially maintained on standard laboratory chow diet (Teklad global #2918, Envigo) containing 18% protein. One week after arrival rats either continued on standard laboratory chow diet (Non Restricted group; Adolescents-NR n = 6, Adults-NR n = 8) or were switched to a modified AIN-93G diet containing 5% protein from casein (#D15100602, Research Diets; Protein Restricted group: Adolescents-PR n = 7, Adults-PR n = 7) [11]. Rats had *ad libitum* access to their assigned diet. Protein restriction was maintained for 12 to 14 days either during adolescence (from P28 to P42) or during adulthood (> P70). Tissue was collected for voltammetry recordings immediately after this period (**Figure 1A**).

Slice preparation

Rats were deeply anesthetized with chloral hydrate (400 mg/kg i.p., Sigma-Aldrich), decapitated, and brains were removed and transferred to ice cold artificial cerebrospinal fluid (aCSF) containing in mM: 126 NaCl, 10 glucose, 26 $NaHCO_3$, 2 KCl, 2.4 $CaCl_2$, 2 $MgCl_2$, 1.4 NaH_2PO_4. Acute 300 µm thick coronal slices, containing both the NAc and the dorsal striatum were prepared in ice-cold aCSF buffer using a vibratome (Leica VT1200S). Slices were kept

at room temperature (20-22ºC) in aCSF saturated with 95% O_2 and 5% CO_2 for at least 1 h before the start of recordings.

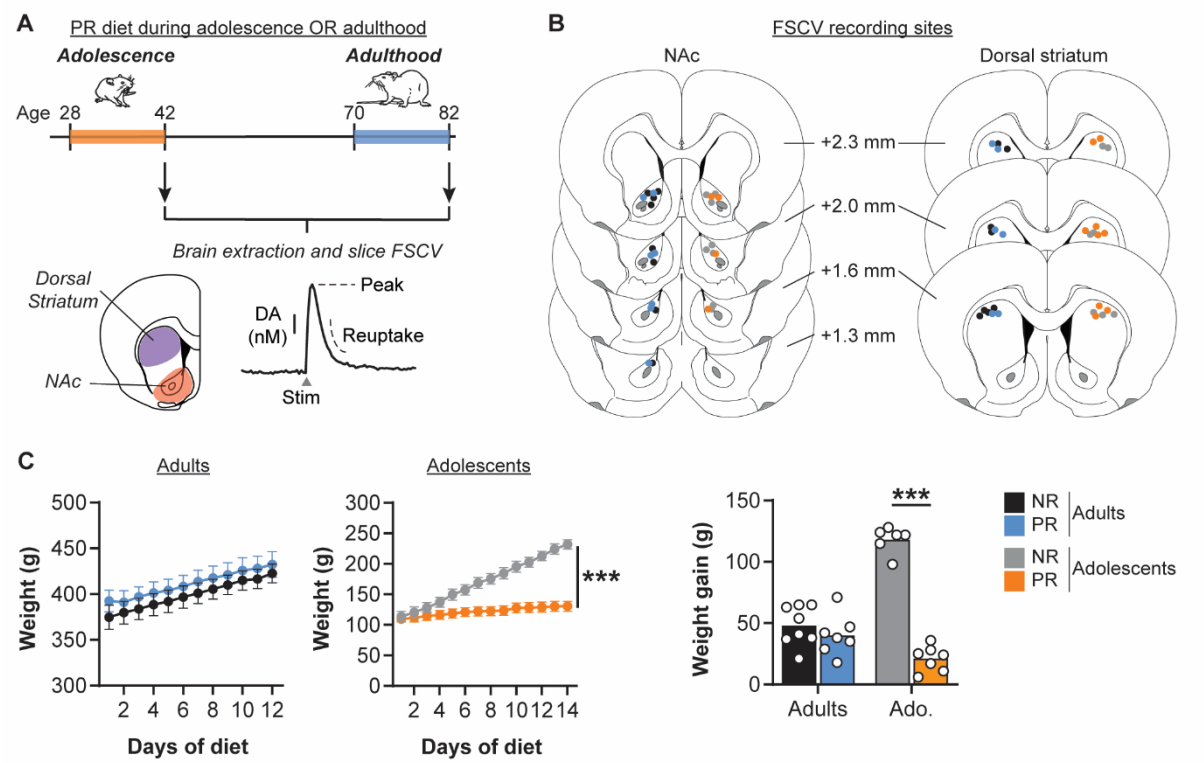

Figure 1. . (A) Schematic representation of the experimental design. Rats had access to either control chow diet (18% protein; non-restricted, NR) or protein-restricted diet (5% protein; PR) during either adolescence (post-natal day 28 to 42) or adulthood (post-natal-day 70 to 82). At the end of the diet exposure, brains were extracted to perform FSCV recordings of electrically evoked dopamine (DA) release in the NAc and dorsal striatum. (B) Coronal brain sections (modified from [76]) representing the recording sites in the NAc (*left*) and the dorsal striatum (*right*) for NR (Adults: black circles; Adolescents: grey circles) and PR groups (Adults: blue circles; Adolescents: orange circles). Numbers indicate the distance from Bregma. (C) Protein restriction during adolescence but not during adulthood altered weight (*left and middle*) and weight gain (*right*). Adults-NR, n = 8, black symbols; Adults-PR, n = 7, blue symbols; Adolescents-NR, n = 6, grey symbols; Adolescents-PR, n = 7, orange symbols. Data are mean ± SEM (weight curves) or mean with individual data points (weight gain). *** $p < 0.001$ Diet effect (two-way ANOVA or Student's unpaired t-test).

Fast scan cyclic voltammetry recordings

Unilateral slices were transferred to the recording chamber and superfused at 2 ml/min with aCSF saturated with 95% O_2 and 5% CO_2 at 30ºC. Slices were allowed to equilibrate for 30 min prior to recordings. A twisted stainless steel bipolar stimulating electrode (MS303T/2-B/SPC, P1 Technologies) was placed at the surface of the slice within the NAc or the dorsal striatum (**Figure 1B**). A homemade glass capillary carbon-fiber microelectrode (tip length 50-100 µm) was positioned in the slice approximately 100 µm beneath the tissue surface and 100-200 µm from the stimulating electrode [54,55]. For FSCV recordings, a triangular voltage waveform was applied (-0.4 to +1.3 V and back versus an Ag/AgCl reference electrode; 400 V/s) using a custom-built headstage circuit (University of Washington Electronics and Materials Engineering Shop, Seattle, WA) and TarHeel voltammetry software (Chapel Hill, University of North Carolina [56]). The waveform was initially applied at 60 Hz for 10 min, to

condition the electrode outside of the tissue, and then applied at 10 Hz while all experiments were being conducted. Dopamine release was evoked by monopolar stimulation pulses (0.7 mA, 0.2 ms) [57]. Electrical stimulations were repeated at 3 min intervals to ensure consistent release. Stimuli were either single pulses (1 p) or trains of five pulses (5 p) at frequencies ranging from 'tonic' (1, 5 or 10 Hz) to 'phasic' burst frequencies (20 Hz) of dopamine neurons reported *ex vivo* and *in vivo* [58–60]. Each stimulation was repeated 3 times in pseudo-random order and averaged to obtain the individual value for this frequency. Each slice yielded an individual recording site.

Extracellular dopamine levels ($[DA]_o$) were confirmed by examining current-voltage plots showing oxidation (approximately +0.6 V) and reduction (approximately -0.2 V) peaks using TarHeel software. Dopamine currents (in nA) were then converted to dopamine concentration (in nM) using the calibration of each electrode against a known standard dopamine concentration. $[DA]_o$ peaks were measured following any stimulation artefacts. Recording electrodes were calibrated after use using 1-2 µM dopamine solution in a flow cell system [61] and in the recording chamber.

Statistical analysis

Weight and weight gain were analyzed using two-way repeated measures ANOVA with Diet (non-restricted, NR; protein-restricted, PR) as between-subject factor and Day of diet as within-subject factor or using unpaired t-tests. For single pulse stimulation, $[DA]_o$ peak and T_{80} clearance time (time for 80% decay from peak amplitude) were analyzed using two-way ANOVAs with Age (Ado, Adult) and Diet (NR, PR) as between-subject factors. Frequency distribution of $[DA]_o$ peaks in response to single pulse were analyzed using Kolmogorov-Smirnov test. $[DA]_o$ peaks from frequency-response curves were analyzed using three-way and two-way repeated measures ANOVA using Age (Ado, Adult) and Diet (NR, PR) as between-subject factors and Frequency (1, 5, 10, 20 Hz) as within-subject factor. 5 p/ 1 p $[DA]_o$ ratios were calculated by dividing the average $[DA]_o$ peak value at 20 Hz by the average $[DA]_o$ peak value at 1 Hz for the same recording site, and were analyzed using two-way ANOVA with Age (Ado, Adult) and Diet (NR, PR) as between-subject factors. Sidak and Dunnett *post hoc* tests were performed when required.

Statistical analyses were conducted using GraphPad Prism 8. All values were expressed as mean ± standard error of the mean (SEM). The alpha risk for the rejection of the null hypothesis was 0.05.

Upon publication, all data analyzed in this paper will be available on Figshare (10.25392/leicester.data.c.4911678).

RESULTS

Age dependent impact of protein restriction on weight

We first investigated the impact of protein restriction (PR) during either adolescence or adulthood on weight and weight gain (**Figure 1C**). As we previously observed [11], protein restriction at adulthood did not significantly affect either the weight of rats (two-way repeated measures ANOVA: Diet $F(1,13) = 0.5$, $p = 0.5$; Day $F(11, 143) = 85.5$, $p < 0.001$; Diet x Day $F(11, 143)= 0.4$, $p = 1.0$) or weight gain (NR: +48 g ± 6 / PR: +40 g ± 6; Diet $t(13) = 0.9$, $p = 0.4$). In contrast, protein restriction during adolescence significantly decreased weight gain, relative to control diet (Diet $F(1,11) = 19.8$, $p < 0.001$; Day $F(13, 143) = 478.7$, $p < 0.001$; Diet x Day $F(13, 143)= 234.0$, $p < 0.001$). Control, non-restricted, rats showed substantial weight increases through adolescence (+118 g ± 4), indicating a normal developmental growth whereas protein-restricted rats showed only a modest increase in their weight (+ 21 g ± 4; Diet $t(11) = 16.6$, $p < 0.001$), demonstrating that protein restriction in adolescence disrupted normal growth.

After two weeks of protein restriction, we then assessed the neurobiological impact of this diet on dopamine release in both the NAc and the dorsal striatum using *ex vivo* FSCV in brain slices.

Age dependent impact of protein restriction on NAc dopamine release

<u>*Single pulse evoked NAc dopamine release*</u>

In the NAc, protein restriction had a different impact on dopamine release evoked by single pulse stimulation depending on the life stage (**Figure 2A-B**; Two-way ANOVA: Age, $F(1,24) = 1.4$, $p = 0.2$; Diet $F(1,24) = 0.2$, $p = 0.7$; Diet x Age $F(1,24) = 7.6$, $p < 0.05$). Protein restriction at adulthood induced a non-significant increase (+87 % ± 42) in NAc dopamine release in response to single pulse stimulation compared to non-restricted control rats ($t(13) = 1.9$; $p = 0.07$). Moreover, protein restriction at adulthood did not affect the frequency distribution of $[DA]_o$ peaks (**Figure 2C**; Kolmogorov-Smirnov test: $D(13) = 0.5$, $p = 0.2$). In contrast, protein restriction during adolescence significantly decreased NAc dopamine release evoked by single pulse stimulation (**Figure 2A-B**; -31% ± 4; $t(11) = 2.2$, $p < 0.05$), inducing a significant left shift of the frequency distribution of $[DA]_o$ peaks in the protein restricted group (**Figure 2C**; Kolmogorov-Smirnov test: $D(11) = 0.8$, $p < 0.05$). Neither protein restriction at adulthood or during adolescence altered dopamine uptake measured by T_{80} (**Figure 2D**; Two-way ANOVA:

Diet, F(1,24) = 0.1, p = 0.7; Diet x Age, F(1,24) = 2.7, p = 0.1). However, T_{80} was significantly shorter in adolescents (1.1 s ± 0.1) compared to adults (1.7 s ± 0.2).

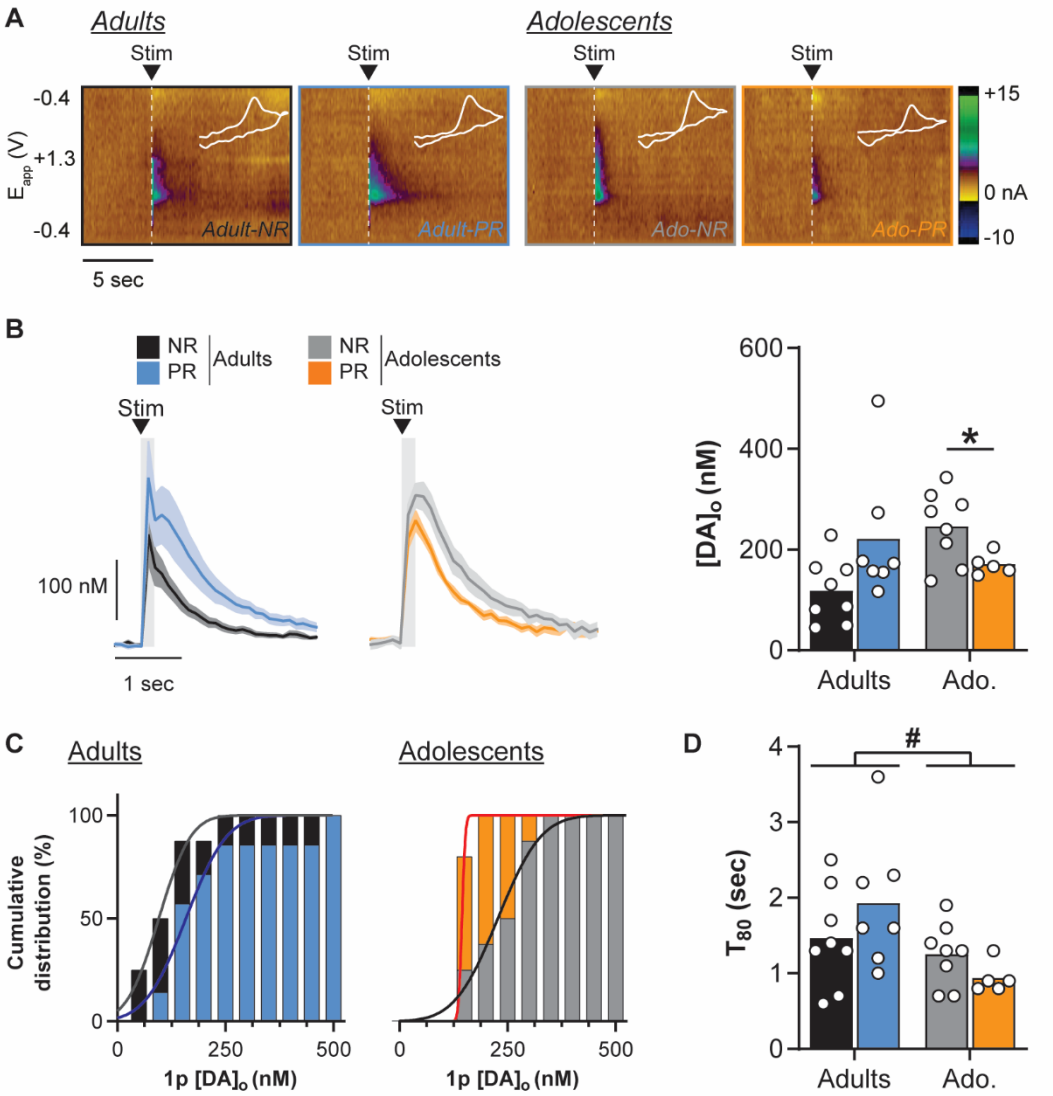

Figure 2. Age-dependent impact of protein restriction on NAc dopamine release evoked by single pulse. (A) Representative FSCV color plots for each diet group (non-restricted, NR; protein-restricted, PR) depicting current changes (color) over time (x-axis; in sec) as a function of the recording electrode holding potential (y-axis; -0.4 to +1.3 V and back) in response to single pulse electrical stimulation (0.7 mA, 0.2 ms; vertical white dashed lines). White line insets represent voltammograms for each color plot. (B) *Left*: NAc $[DA]_o$ versus time (in nM; mean ± SEM) in slices from Adult and Adolescent PR and NR rats, aligned to the single pulse electrical stimulation (black arrow); *Right*: Mean $[DA]_o$ peaks evoked by single pulse stimulation in the NAc. (C) Cumulative distribution of single pulse evoked NAc $[DA]_o$ peaks in Adult (*left*) and Adolescent (*right*) groups. (D) Average T_{80} (time for 80% decay from $[DA]_o$ peak) in the NAc. Adults-NR (black, n = 8), Adults-PR (blue, n = 7), Adolescents-NR (grey, n = 9) and Adolescents-PR (orange, n = 5). Bars show means and circles show individual (e.g. recording site) data points. * $p < 0.05$ Diet effect (Student's unpaired t-test), # $p < 0.05$ Age effect (two-way ANOVA).

Frequency-dependent NAc dopamine release

Dopamine neurons *in vivo* show a range of responses from low-frequency firing (< 10 Hz, *tonic* mode) to brief bursts of action potentials at high frequency (15-25 Hz, *phasic* mode) [57–60].

We therefore investigated the effect of protein restriction on dopamine release at different stimulation frequencies ranging from 1 to 20 Hz (1 p = 1 Hz, or 5 p at 5, 10 or 20 Hz; **Figure 3**). Evoked dopamine release increased with the stimulation frequency (three-way repeated measures ANOVA: Frequency, $F(3,72) = 8.7$, $p < 0.001$) similarly in Adolescent and Adult groups (Age, $F(1,24) = 1.2$, $p = 0.3$). Protein restriction did not affect the frequency-dependent effect (Frequency x Diet, $F(3,72) = 2.2$, $p = 0.1$; Frequency x Diet x Age, $F(3,72) = 2.1$, $p = 0.1$). However, protein restriction did differentially affect NAc dopamine release depending on age (Diet, $F(1,24) = 1.4$, $p = 0.2$; Age x Diet $F(1,24) = 7.2$, $p < 0.01$). In adult rats, protein restriction increased dopamine release in response to the range of stimulation frequencies (two-way repeated measures ANOVA: Diet, $F(1,13) = 4.7$, $p < 0.05$; Frequency, $F(3,39) = 30.0$, $p < 0.001$; Diet x Frequency, $F(3,39) = 2.6$, $p = 0.06$). This increase is especially noticeable for the higher stimulation frequencies (10-20 Hz; Sidak's *post hoc* tests all $p < 0.05$). Conversely, protein restriction during adolescence significantly decreased evoked NAc dopamine release (two-way repeated measures ANOVA: Diet, $F(1,11) = 9.8$, $p < 0.01$; Frequency, $F(3,33) = 35.6$, $p < 0.001$; Diet x Frequency, $F(3, 33) = 1.3$, $p = 0.3$). In adolescent rats, protein restriction appeared to especially decrease dopamine release in response to low frequency stimulations (Sidak's *post hoc* tests: $p < 0.05$ and $p < 0.01$ for 5 Hz and 10 Hz, respectively). At high frequency (20 Hz), dopamine release is similar between protein-restricted and non-restricted rats' slices (Sidak's *post hoc* test $p < 0.09$) but represents a higher relative increase in protein-restricted groups (% of 1p release: PR, 197 ± 14 % vs. NR, 157 ± 10 %).

The relationship between dopamine release during tonic and phasic activity is a central process in the signaling of significant environmental events and learning [60,62,63]. We examined whether protein restriction during either adolescence or adulthood affected the 'phasic/tonic ratio' of NAc dopamine release (5 p at 20 Hz / 1 p, **Figure 3C**). Adolescent rats exhibited a lower ratio than adult rats (Two-way ANOVA: Age, $F(1,24) = 13.9$, $p < 0.01$). However, protein restriction did not alter this ratio at either age (Diet, $F(1,24) = 0.2$, $p = 0.7$; Age x Diet, $F(1,24) = 0.6$, $p = 0.5$).

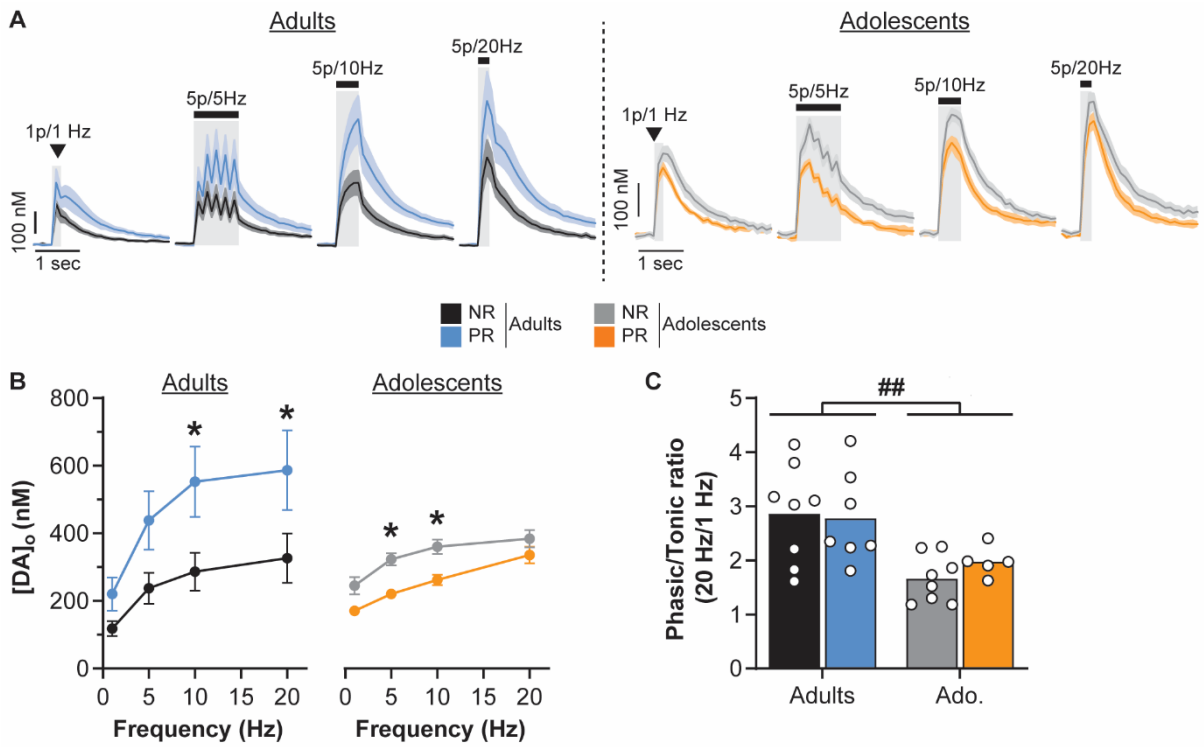

Figure 3. Age-dependent impact of protein restriction on frequency-dependent NAc dopamine release. (A) NAc [DA]o versus time (in nM, mean ± SEM) for each diet group (non-restricted, NR; protein-restricted, PR) aligned to the electrical stimulation (black symbol) at 1 Hz (single pulse), 5, 10 or 20 Hz (5 pulses; 0.7 mA, 0.2 ms). (B) Protein restriction increased frequency dependent NAc DA release in Adult rats (left) but decreased it in Adolescent rats (right; mean $[DA]_o$ peaks ± SEM). (C) Protein restriction has no impact on $[DA]_o$ Phasic/Tonic ratios. Adults-NR (black, n = 8), Adults-PR (blue, n = 7), Adolescents-NR (grey, n = 9) and Adolescents-PR (orange, n = 5). Bars show means and circles show individual (e.g. recording site) data points. * $p < 0.05$ Diet effect (two-way ANOVA followed by Sidak's *post hoc* tests), $p < 0.01$ ## Age effect (two-way ANOVA).

Age dependent impact of protein restriction on striatal dopamine release

Single pulse evoked striatal dopamine release

In the dorsal striatum, protein restriction had no effect on dopamine release evoked by single pulse stimulation whether rats were exposed to the diet during adolescence or adulthood (**Figure 4A-B**; two-way ANOVA: Age, $F(1,28) = 0.08$, $p = 0.8$; Diet, $F(1,28) = 0.04$, $p = 0.8$; Diet x Age, $F(1,28) = 1.4$, $p = 0.2$). In addition, diet did not alter the frequency distribution of $[DA]_o$ peaks (**Figure 4C**; Kolmogorov-Smirnov tests: Adult groups, $D(14) = 0.4$, $p = 0.3$; Adolescent groups, $D(14) = 0.6$, $p = 0.1$). Protein restriction at adulthood or during adolescence also did not affect dopamine uptake measured by T_{80} (**Figure 4D**; Two-way ANOVA: Diet, $F(1,28) = 0.03$, $p = 0.9$; Age, $F(1,28) = 0.9$, $p = 0.4$, Diet x Age, $F(1,28) = 2.1$, $p = 0.2$).

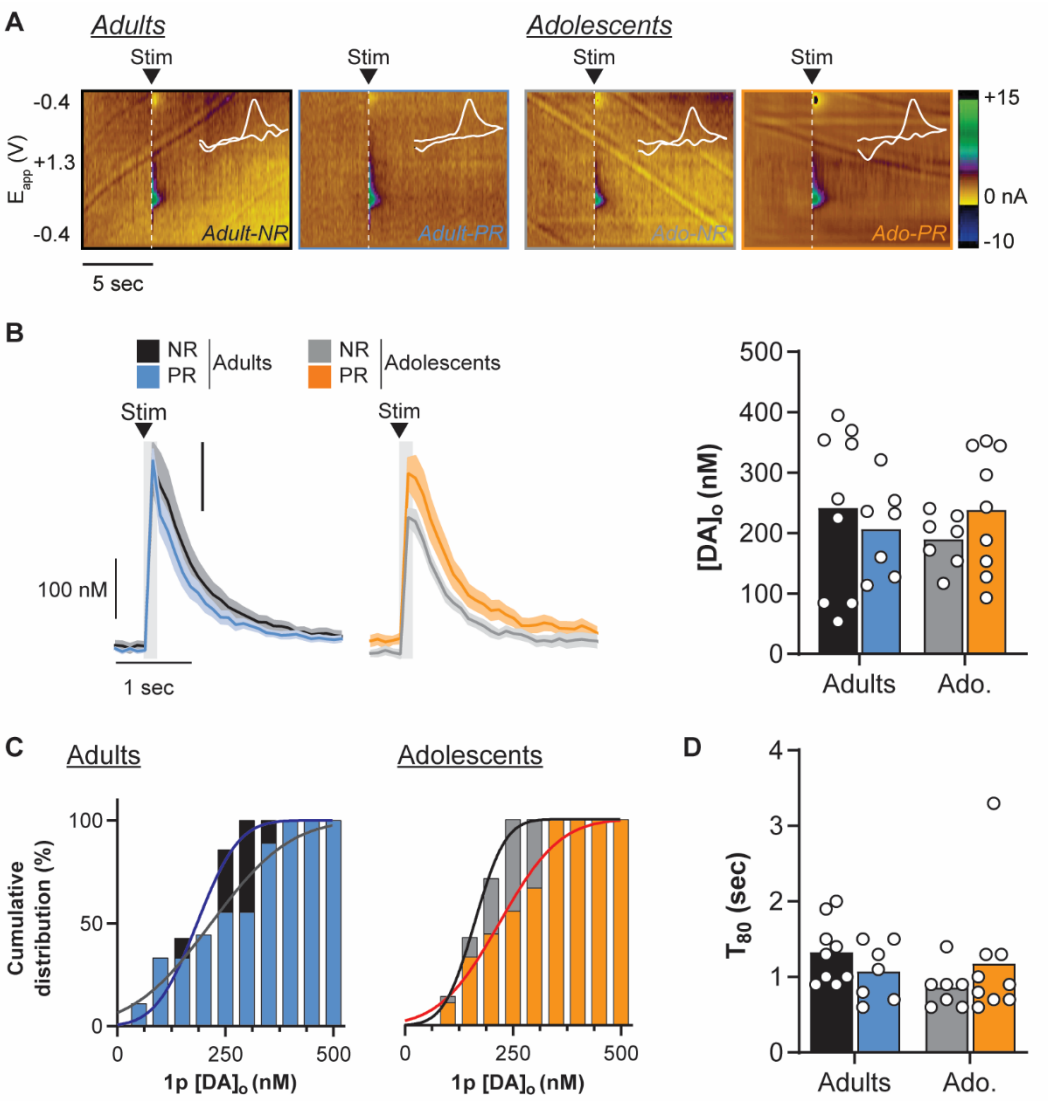

Figure 4. Age-dependent impact of protein restriction on dorsal striatum dopamine release evoked by single pulse. (A) Representative FSCV color plots for each diet group (non-restricted, NR; protein-restricted, PR) depicting current changes (color) over time (x-axis; in sec) as a function of the recording electrode holding potential (y-axis; -0.4 to +1.3 V and back) in response to single pulse electrical stimulation (0.7 mA, 0.2 ms; vertical white dashed lines). White line insets represent voltammogram for each color plot. (B) *Left*: Dorsal striatum $[DA]_o$ (in nM; mean ± SEM) aligned to the single pulse electrical stimulation (black arrow); *Right*: $[DA]_o$ peaks evoked by single pulse stimulation in the dorsal striatum. (C) Cumulative distribution of single pulse evoked dorsal striatum $[DA]_o$ peaks in Adult (*left*) and Adolescent (*right*) groups. (D) Average T_{80} (time for 80% decay from $[DA]_o$ peak) in the dorsal striatum. Adults-NR (black, n = 9), Adults-PR (blue, n = 7), Adolescents-NR (grey, n = 7) and Adolescents-PR (orange, n = 9). Bars show means and circles show individual (e.g. recording site) data points.

Frequency-dependent striatal dopamine release

As previously observed in the NAc, striatal dopamine release increased as a function of the stimulation frequency (**Figure 5A-B**; three-way repeated measures ANOVA: Frequency, $F(3,84) = 81.6$, $p < 0.001$) although this effect was visually smaller than that observed in the NAc (see **Figure 3**). On average, we observed that during adolescence, striatal evoked dopamine release was lower than in adult animals (three-way repeated measures ANOVA:

Age, $F(1,28) = 5.8$, $p < 0.05$) and less sensitive to stimulation frequencies (Frequency x Age, $F(3, 84) = 16.9$, $p < 0.001$). Interestingly, protein restriction altered the response to the different stimulation frequencies (Frequency x Diet, $F(3,84) = 6.7$, $p < 0.001$; Frequency x Age x Diet, $F(3,84) = 0.8$, $p = 0.5$), without significantly changing the average peaks of evoked $[DA]_o$ in different age groups (Diet, $F(1,28) = 2.2$, $p = 0.1$; Diet x Age, $F(1,28) = 0.4$, $p = 0.5$). Separate analyses for each age group confirmed that protein restriction has no significant effect on frequency-dependent striatal dopamine release in adults (two-way repeated measures ANOVA: Diet, $F(1,14) = 0.2$, $p = 0.6$; Frequency, $F(3,42) = 63.8$, $p < 0.001$; Diet x Frequency, $F(3, 42) = 4.2$, $p < 0.05$; Sidak's post hoc tests: all $p > 0.7$). In contrast, protein restriction in adolescent rats significantly increased stimulation-evoked striatal dopamine release (two-way repeated measures ANOVA: Diet, $F(1,14) = 4.8$, $p < 0.05$; Frequency, $F(3,42) = 19.2$, $p < 0.001$; Diet x Frequency, $F(3, 42) = 2.7$, $p = 0.05$) especially at high stimulation frequencies (Sidak's post hoc tests: 1-10 Hz all $p > 0.1$; 20 Hz $p < 0.05$). Furthermore, Dunnett's *post hoc* tests showed that only high stimulation frequencies increased striatal release in Adolescent non restricted control group (5 Hz, $p = 0.3$; 10 Hz, $p < 0.01$; 20 Hz, $p < 0.001$ versus 1 Hz stimulation), whereas all frequencies increased dopamine release in the protein-restricted group (5 Hz, $p < 0.01$; 10-20 Hz, $p < 0.001$ versus 1 Hz stimulation). This last result suggests that the nigrostriatal dopamine system may be sensitized by protein restriction during adolescence, despite an overall decrease in evoked release of dopamine.

Similar to what we observed in the NAc, the 'phasic/tonic' ratio of striatal dopamine release was lower in adolescent slices (**Figure 5C**; Two-way ANOVA: Age, $F(1,28) = 11.7$, $p < 0.01$) but was not altered by protein restriction (Diet, $F(1,28) = 1.8$, $p = 0.2$; Age x Diet, $F(1,28) = 0.001$, $p = 1.0$).

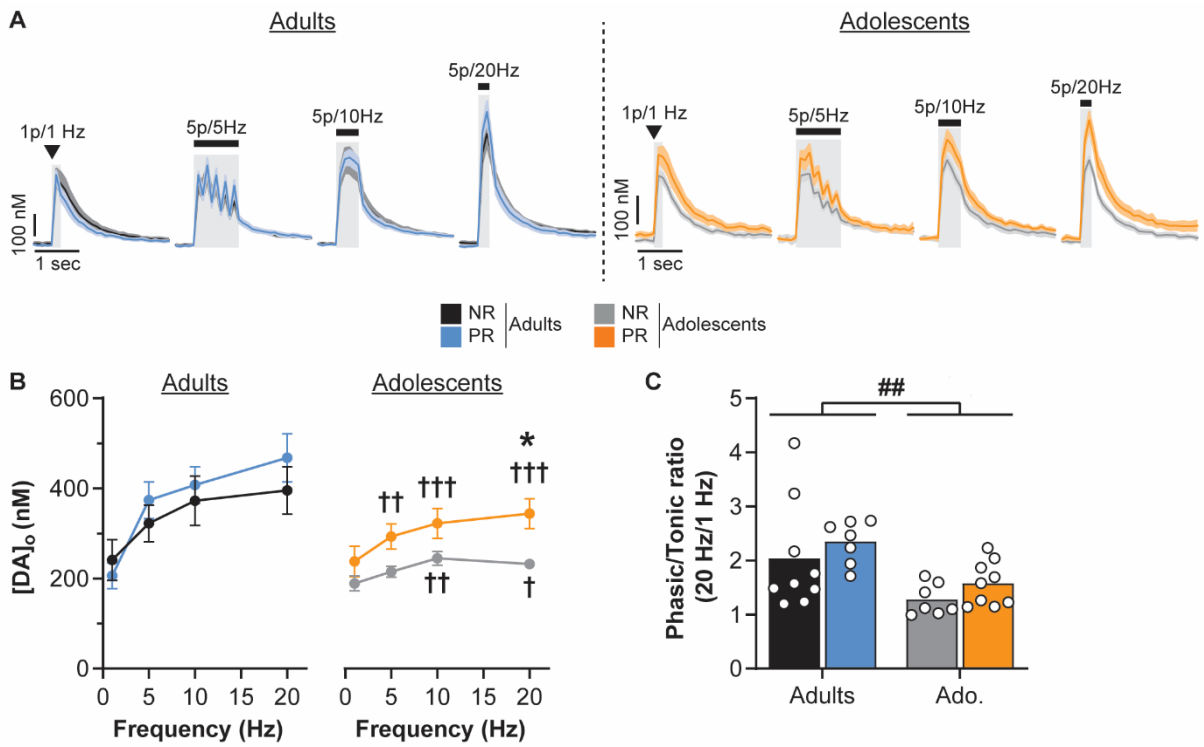

Figure 5. Age-dependent impact of protein restriction on frequency-dependent dorsal striatum dopamine release. (A) Dorsal striatum $[DA]_o$ (in nM, mean ± SEM) for each diet group (non-restricted, NR; protein-restricted, PR) aligned to the electrical stimulation (black symbol) at 1Hz (single pulse), 5, 10 or 20 Hz (5 pulses; 0.7 mA, 0.2 ms). (B) Protein restriction at adulthood did not affect dorsal striatum DA release (*left*) but increased it in Adolescent rats (*right*; mean $[DA]_o$ peaks ± SEM). (C) Protein restriction has no impact on $[DA]_o$ Phasic/Tonic ratios. Adults-NR (black, n = 9), Adults-PR (blue, n = 7), Adolescents-NR (grey, n = 7) and Adolescents-PR (orange, n = 9). Bars show means and circles show individual (e.g. recording site) data points. * $p < 0.05$ Diet effect (two-way ANOVA followed by Sidak's *post hoc* tests), †† and ††† $p < 0.01$ and $p < 0.001$ Frequency effect (two-way ANOVA followed by Dunnett's *post hoc* tests versus 1 Hz), ## $p < 0.01$ Age effect (two-way ANOVA).

DISCUSSION

Protein homeostasis is a crucial physiological function for almost all species throughout the lifespan. Despite the deleterious consequences of protein restriction on a multitude of physiological functions, the neurobiological impact of such a diet at different ages remains largely unexplored. The present study reveals that protein restriction affects the function of the mesolimbic and nigrostriatal dopamine pathways. More importantly, our results demonstrate that these effects are dependent on the age at which protein restriction is experienced, highlighting adolescence as a vulnerability window for the deleterious effects of an unbalanced diet.

When performed at adulthood, protein restriction did not affect rats' weight, consistent with our previous results [11]. Adult animals have lower protein requirements compared to adolescents, who are rapidly growing and developing. Previous studies have shown that the impact of protein restriction on weight is highly dependent on the degree of restriction and the physiological state of the animal [9,10,53]. Accordingly, we previously reported a slight increase in food intake in these diet conditions [11], which may explain the absence of effect

on animals' weight as they attempted to compensate protein deficiency with a general hyperphagia. An alternative explanation is that low protein diet may change energy expenditure, as previously observed [64]. In contrast, protein restriction during adolescence significantly limited animals' normal trajectory of weight gain. In our study, the low protein diet (5% protein from casein) was the only source of nutrients available for growing animals that require elevated daily protein intake. We may then hypothesize that in these conditions the regulation of food intake or energy expenditure were not sufficient to maintain normal growth, generating a greater protein deficiency than in adult rats.

The two main dopamine projections to the NAc and the dorsal striatum are involved in various food-related processes including incentive salience [16] and prediction error [63] using taste and nutritional (post-ingestive) values of food to learn about food availability and food-predictive stimuli [23–28]. Here, we observed that protein restriction differentially affected dopamine release depending on age of diet exposure. At adulthood, protein restriction increased NAc dopamine release but had no effect on dorsal striatum dopamine release. Diet impact on the function of the mesolimbic pathway was especially noticeable in response to multiple pulse trains of stimulation at higher frequencies (10-20 Hz), even if we noticed a slight increase in response to single pulse or low 'tonic' stimulation (5 Hz). These results suggest that in protein restricted animals, 'phasic' bursting activity of dopamine mesolimbic neurons evokes greater dopamine release, relative to control animals. As phasic responses of mesolimbic dopamine neurons are well known to convey important information about primary reinforcers like food and associated stimuli [16,19,23,63,65], an increase in NAc dopamine levels in response to salient events may alter motivated behaviors like food preferences [11,13] and increase the rewarding properties of protein-enriched food in restricted/deprived animals [12]. Interestingly, protein restriction did not alter the phasic/tonic ratio either in NAc or dorsal striatum, suggesting that protein restriction at adulthood may induce a more general increase in capacity of terminals for dopamine release rather than by enhancing the contrast between different dopamine signaling modes [57,66]. Furthermore, there are multiple sites of modulation for the control of terminal dopamine release involving not only different somatic firing patterns but also striatal presynaptic regulation by dopamine D2 autoreceptors, dopamine reuptake and neuromodulatory inputs such as cholinergic interneurons [66]. Although this microcircuit regulation is beyond the scope of the present study, we did not observe an impact of diet on the length of T_{80} suggesting similar reuptake processes in protein-restricted rats.

Protein restriction during adolescence had a broader impact on the function of dopamine terminals. In contrast to what we observed at adulthood, protein restriction in adolescents decreased NAc dopamine release both in response to single pulse stimulation and low

frequency pulse trains (5-10 Hz). Interestingly, in animals restricted during adolescence, dopamine levels in response to burst-like stimulation (5 p at 20 Hz) appeared similar between control and protein restricted groups but represented a higher relative increase compared to single pulse dopamine release. Dopamine neurons exhibit an elevated firing rate during adolescence [49,52] associated with changes in dopamine availability in dopamine projection targets [47,48,50]. Based on this and our first results showing an effect of protein restriction at adulthood on NAc dopamine release, we might have expected an enhancement of the diet effect during adolescence. One way to reconcile these opposite findings is to consider that the degree of protein restriction in adolescent rats may be more profound than in adults. As discussed earlier, we observed a substantial impact of protein restriction on weight gain in protein-restricted adolescents (and not in adults) suggesting a more severe level of restriction. As dietary protein is a major source of amino acids (e.g. tyrosine) required for catecholaminergic metabolism (synthesis, release, enzymatic activity), one hypothesis is that a greater protein deficiency in adolescent rats than adults will affect average dopamine levels and the ability to synthesize and release dopamine. Accordingly, previous studies have reported a decrease in dopamine in several brain regions in response to pre- or perinatal protein malnutrition as well as an hypo-responsivity to psychostimulants (see [5] for review).

In the dorsal striatum in adolescents, we observed an opposite pattern compared to the NAc. As such, evoked dopamine release was increased after protein restriction, especially at high stimulation frequencies. Such an effect partially rules out the hypothesis of a global amino acid deficiency. However, the nigrostriatal dopamine pathway matures earlier than other dopamine pathways [47] and may then be less sensitive to protein restriction. Striatal and NAc dopamine pathways are involved in different aspects of food-related processes and recent advances demonstrated that striatal, but not NAc, dopamine signaling is involved in encoding the nutritional value of food [67]. The increase in evoked dopamine release in striatal areas only seen in adolescent-exposed rats reported in the present study may support a nutrition-seeking response to the elevated protein requirement at this age. The effect of protein restriction may also involve a broader alteration of other neurotransmitter systems known to control either dopamine cell firing patterns and terminal dopamine release [62,66]. Both prefrontal glutamatergic inputs to ventral and dorsal striatal areas [68] and the local cholinergic system [69] mature during adolescence. Diet-induced alteration of these local circuits may ultimately support complex and region-specific changes to the regulation of dopamine release [66]. These issues and the behavioral consequences of such alterations of the dopamine system remain to be investigated to understand the short and long-term consequences of developmental protein restriction.

The direct influence of protein or amino acids levels on dopamine neurons is still unexplored, however, these neurons receive input from hypothalamic regions which are able to detect amino acids [10,14]. Protein restriction also induces a broad metabolic response involving peripheral food-related signals to which dopamine neurons are directly sensitive [31–35]. Dopamine release is especially sensitive to insulin through its actions at specific receptors located both directly on dopamine neurons [70] and on striatal cholinergic neurons [37]. The effects of insulin on the dopamine system and dopamine-related behaviors are complex and depend on insulin concentration, brain region, cell type and the current physiological state [71]. Protein restriction is known to increase insulin sensitivity and glucose metabolism [13,72], which may then modulate dopamine's neurobiological and behavioral functions. The interaction of the dopamine and insulin systems in response to different context of protein diet warrants further *ex vivo* and *in vivo* investigation.

In conclusion, our study provides evidence that prolonged protein restriction has an important impact on function of dopamine terminals in the NAc and striatum. More importantly we highlight the increased sensitivity of the dopamine system during adolescence to the deleterious effects of a diet that is inadequate in protein. Adolescence is characterized by important maturation events within dopamine circuitry and dopamine-related processes [47–52] and numerous studies have now demonstrated that adolescence is an important vulnerability window for diet-related alterations of cognitive and neurobiological functions [42–46]. How protein restriction during adolescence may have different, and potentially long-term, impacts on dopamine-related behaviors considering its opposite effects on the mesolimbic and nigrostriatal pathways remains to be investigated. Given the role of malnutrition and inadequate protein intake on neurodevelopmental psychiatric disorders [5,6] involving alterations of the dopamine system [17,73,74] and having their onset during adolescence [36,75], our current findings also represent a step towards a better understanding of the mechanisms regulating protein appetite, protein malnutrition, and the emergence of dopamine-related disorders.

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AUTHOR CONTRIBUTIONS

FN, KZP and JEM designed research; FN performed research, FN, KZP, AMJY and JEM analyzed data; FN, KZP, AMJY and JEM wrote the manuscript.

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CAPTIONS

Figure 1. (A) Schematic representation of the experimental design. Rats had access to either control chow diet (18% protein; non-restricted, NR) or protein-restricted diet (5% protein; PR) during either adolescence (post-natal day 28 to 42) or adulthood (post-natal-day 70 to 82). At the end of the diet exposure, brains were extracted to perform FSCV recordings of electrically evoked dopamine (DA) release in the NAc and dorsal striatum. (B) Coronal brain sections (modified from [76]) representing the recording sites in the NAc (left) and the dorsal striatum (right) for NR (Adults: black circles; Adolescents: grey circles) and PR groups (Adults: blue circles; Adolescents: orange circles). Numbers indicate the distance from Bregma. (C) Protein restriction during adolescence but not during adulthood altered weight (*left and middle*) and weight gain (*right*). Adults-NR, n = 8, black symbols; Adults-PR, n = 7, blue symbols; Adolescents-NR, n = 6, grey symbols; Adolescents-PR, n = 7, orange symbols. Data are mean ± SEM (weight curves) or mean with individual data points (weight gain). *** $p < 0.001$ Diet effect (two-way ANOVA or Student's unpaired t-test).

Figure 2. Age-dependent impact of protein restriction on NAc dopamine release evoked by single pulse. (A) Representative FSCV color plots for each diet group (non-restricted, NR; protein-restricted, PR) depicting current changes (color) over time (x-axis; in sec) as a function of the recording electrode holding potential (y-axis; -0.4 to +1.3 V and back) in response to single pulse electrical stimulation (0.7 mA, 0.2 ms; vertical white dashed lines). White line insets represent voltammograms for each color plot. (B) *Left:* NAc $[DA]_o$ versus time (in nM; mean ± SEM) in slices from Adult and Adolescent PR and NR rats, aligned to the single pulse electrical stimulation (black arrow); *Right:* Mean $[DA]_o$ peaks evoked by single pulse stimulation in the NAc. (C) Cumulative distribution of single pulse evoked NAc $[DA]_o$ peaks in Adult (*left*) and Adolescent (*right*) groups. (D) Average T_{80} (time for 80% decay from $[DA]_o$ peak) in the NAc. Adults-NR (black, n = 8), Adults-PR (blue, n = 7), Adolescents-NR (grey, n = 9) and Adolescents-PR (orange, n = 5). Bars show means and circles show individual (*e.g.* recording site) data points. * $p < 0.05$ Diet effect (Student's unpaired t-test), # $p < 0.05$ Age effect (two-way ANOVA).

Figure 3. Age-dependent impact of protein restriction on frequency-dependent NAc dopamine release. (A) NAc $[DA]_o$ versus time (in nM, mean ± SEM) for each diet group (non-restricted, NR; protein-restricted, PR) aligned to the electrical stimulation (black symbol) at 1 Hz (single pulse), 5, 10 or 20 Hz (5 pulses; 0.7 mA, 0.2 ms). (B) Protein restriction increased frequency dependent NAc DA release in Adult rats (*left*) but decreased it in Adolescent rats (*right*; mean $[DA]_o$ peaks ± SEM). (C) Protein restriction has no impact on $[DA]_o$ Phasic/Tonic ratios. Adults-NR (black, n = 8), Adults-PR (blue, n = 7), Adolescents-NR (grey, n = 9) and Adolescents-PR (orange, n = 5). Bars show means and circles show individual (*e.g.* recording site) data points. * $p < 0.05$ Diet effect (two-way ANOVA followed by Sidak's *post hoc* tests), ## Age effect (two-way ANOVA).

Figure 4. Age-dependent impact of protein restriction on dorsal striatum dopamine release evoked by single pulse. (A) Representative FSCV color plots for each diet group (non-restricted, NR; protein-restricted, PR) depicting current changes (color) over time (x-axis; in sec) as a function of the recording electrode holding potential (y-axis; -0.4 to +1.3 V and back) in response to single pulse electrical stimulation (0.7 mA, 0.2 ms; vertical white dashed lines). White line insets represent voltammogram for each color plot. (B) *Left:* Dorsal striatum $[DA]_o$ (in nM; mean ± SEM) aligned to the single pulse electrical stimulation (black arrow); *Right:* $[DA]_o$ peaks evoked by single pulse stimulation in the dorsal striatum. (C) Cumulative distribution of single pulse evoked dorsal striatum $[DA]_o$ peaks in Adult (*left*) and Adolescent (*right*) groups. (D) Average T_{80} (time for 80% decay from $[DA]_o$ peak) in the dorsal striatum. Adults-NR (black, n = 9), Adults-PR (blue, n = 7), Adolescents-NR (grey, n = 7) and Adolescents-PR (orange, n = 9). Bars show means and circles show individual (*e.g.* recording site) data points.

Figure 5. Age-dependent impact of protein restriction on frequency-dependent dorsal striatum dopamine release. (A) Dorsal striatum $[DA]_o$ (in nM, mean ± SEM) for each diet group (non-restricted, NR; protein-restricted, PR) aligned to the electrical stimulation (black symbol) at 1Hz (single pulse), 5, 10 or 20 Hz (5 pulses; 0.7 mA, 0.2 ms). (B) Protein restriction at adulthood did not affect dorsal striatum DA release (*left*) but increased it in Adolescent rats (*right*; mean $[DA]_o$ peaks ± SEM). (C) Protein restriction has no impact on $[DA]_o$ Phasic/Tonic ratios. Adults-NR (black, n = 9), Adults-PR (blue, n = 7), Adolescents-NR (grey, n = 7) and Adolescents-PR (orange, n = 9). Bars show means and circles show individual (*e.g.* recording site) data points. * $p < 0.05$ Diet effect (two-way ANOVA followed by Sidak's *post hoc* tests), †† and ††† $p < 0.01$ and $p < 0.001$ Frequency effect (two-way ANOVA followed by Dunnett's *post hoc* tests versus 1 Hz), ## $p < 0.01$ Age effect (two-way ANOVA).