

Title Impact of dietary vitamin A on striatal function in adult rats

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Title: Impact of dietary vitamin A on striatal function in adult rats

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ABSTRACT

The striatum is a brain structure involved in the control of voluntary movement. Striatum contains high amounts of retinoic acid, the active metabolite of vitamin A, as well as retinoid receptors, RAR β and RXR γ . Previous studies revealed that disruption of retinoid signaling initiated during development is deleterious for striatal physiology and related motor functions. However, the alteration of retinoid signaling, and the importance of vitamin A supply during adulthood on striatal physiology and function has never been established.

In the present study, we investigated the impact of vitamin A supply on striatal function. Adult Sprague Dawley rats were fed with three specific diets, either sub-deficient, sufficient, or enriched in vitamin A (0.4, 5 and 20 international units (IU) of retinol per g of diet, respectively) for 6 months. We first validated that vitamin A sub-deficient diet in adult rats constitutes a physiological model of retinoid signaling reduction in the striatum. We then revealed subtle alterations of fine motor skills in sub-deficient rats using a new behavioral apparatus specifically designed to test forepaw reach-and-grasp skills relying on striatal function. Finally, we showed using qPCR analysis and immunofluorescence that the striatal dopaminergic system *per se* was not affected by vitamin A sub-deficiency at adult age. Rather, cholinergic synthesis in the striatum and μ -opioid receptor expression in striosomes sub-territories were the most affected by vitamin A sub-deficiency starting at adulthood.

Taken together these results revealed that retinoid signaling alteration at adulthood is associated with motor learning deficits together with discrete neurobiological alterations in the striatum.

51 **KEY WORDS:** Vitamin A; retinoic acid; striatum; motor function; acetylcholine; striosomes

52

53 **SHORT TITLE:** Vitamin A & adult striatal function

54

55 **ABBREVIATIONS**

56 ALDH1A1: aldehyde dehydrogenase 1 subtype
57 A1

58 B2M: B2-microglobulin

59 ChAT: choline acetyl-transferase

60 CRABP2: Cellular Retinoic Acid Binding Protein 2

61 CYP26 a1: cytochrome P26 a1

62 DA: dopamine

63 DRD1: dopamine D1 receptor

64 DRD2: dopamine D2 receptor

65 GAPDH: Glyceraldehyde 3-phosphate
66 dehydrogenase

67 HPLC: high performance liquid chromatography

68 IU: international unit

69 LRAT: lecithin:retinol acyltransferase

70 MOR: μ -opioid receptor

71 NOS1: neuronal nitric oxide synthase

72 PBS: phosphate buffer saline

73 PD: Parkinson's disease

74 RA: *all-trans*-retinoic acid

75 RAR: retinoic acid receptor

76 RBP4: retinol binding protein 4

77 REH: retinyl ester hydrolase

78 ROI: region of interest

79 RPM: rotation per minute

80 RT-qPCR: Real-Time quantitative Polymerase
81 Chain Reaction

82 RXR: retinoid X receptor

83 TH: tyrosine hydroxylase

84 SNc: substantia nigra *pars compacta*

85 STRA6: Retinoic Acid gene 6

86

INTRODUCTION

Vitamin A (retinol) is an essential micronutrient provided through dietary intake¹. Vitamin A signaling, mediated by its active metabolite, *all-trans*-retinoic acid (RA), is essential throughout life for the development, plasticity and maintenance of the central nervous system²⁻⁵. RA binds to heterodimeric nuclear receptors, namely retinoic acid receptor (RAR) and retinoid X receptor (RXR), to modulate the expression of target genes⁶⁻⁸.

Dysfunction of RA signaling is investigated in various diseases, including neurological disorders⁵. In particular, retinoid signaling is suggested to participate in Parkinson's disease (PD) pathophysiology^{9,10}. PD is a degenerative disorder characterized by motor **disability** symptoms, such as tremor at rest, akinesia and bradykinesia¹¹. The main neurobiological landmark of PD is the degeneration of dopaminergic (DA) neurons of the substantia nigra *pars compacta* (SNc) that innervate the striatum. Striatal output that is critical for the control of voluntary movement, is tightly shaped by DA transmission that is in close interaction with other local modulatory systems, such as cholinergic, opioidergic and serotonergic systems¹². Yet, these neuromodulatory systems and more broadly, striatal physiology, are modulated by RA signaling^{10,13}.

In fact, the striatum contains high quantities of RA and retinoid **receptor isotypes** RAR β and RXR γ ¹⁴⁻¹⁶, and **knockout mice** for these **isotypes** exhibit significant impairments in locomotion and motor coordination, suggesting an involvement of retinoid signaling in striatal motor function¹⁷. Moreover, total vitamin A deficiency initiated at weaning and maintained during adolescence induces motor impairments, associated with decrease in acetylcholine release in the striatum^{18,19}. Finally, aldehyde dehydrogenase 1 subtype A1 (ALDH1A1), the RA synthesis enzyme of RA, is specifically expressed in a sub-population of SNc DA neurons that innervate the striatum^{20,21}. **ALDH1A1^{-/-} mice do not exhibit DA neurodegeneration, while ALDH1A1^{-/-}/ALDH2^{-/-} do, and ALDH1A1^{-/-}/A53T mice have enhanced DA neurodegeneration compared to A53T mice model of Parkinson's disease^{13,21-23}. Furthermore, ALDH1A1^{-/-} mice exhibit a reduction of μ -opioid receptors in the striosomes, forming sub-territories in**

the striatum, along with increased dopamine release in striosomes and reduced modulation of dopamine release by acetylcholine^{13,24}.

Yet, the majority of the studies investigating retinoid signaling and striatal function were based on models where retinoid signaling disruption occurred coincidentally during the development of the DA system²⁵. Therefore, the consequences of retinoid signaling deficiency on striatal function and its neuromodulation by DA have never been clearly investigated at adulthood. In the present study, we used specific diets, either sub-deficient, sufficient or enriched in vitamin A, starting at adulthood and lasting for 6 months, to assess the impact of adult vitamin A deficiency on striatal physiology and motor behaviors. With this nutritional approach, we found that alteration of retinoid signaling at adulthood impairs striatal function in a unique way, and differently than when occurring during developmental periods.

MATERIAL AND METHODS

Animals and diet

Six-week-old (weight 180-200 g) Sprague-Dawley male rats were obtained from Janvier Labs (France). Animals were housed in pairs with a 12-h light/dark cycle (light on at 7 a.m.) in enriched and controlled environment (22 °C ± 2°C, 40 % of humidity). Two cohorts of rats were used for this study. For the first cohort, a batch of 32 rats was randomly divided into four experimental groups of 8 upon their arrival. The first group received a diet with a retinol content considered sufficient to cover an adult animal body's need (5 IU retinol/g of diet, INRAE, Jouy-en-Josas, 1 IU = 0.3 µg retinol), the second group received a mild supplementation of vitamin A (20 IU retinol/g of diet, INRAE, Jouy-en-Josas). The third group received a diet with retinol-deficient content (0.4 IU retinol/g of diet, INRAE, Jouy-en-Josas). The three groups received the same diet for 26 weeks. A fourth group received a diet with retinol-deficient content (0.4 IU/g) for 20 weeks and then a diet with mild supplementation (20 IU / g) for 6 weeks

before euthanasia. A second cohort was used for the operant reach-and-grasp lever task. For this cohort, a batch of 24 rats was randomly divided into two experimental groups upon their arrival: group 1 (n=6 rats) received diet with 20 IU/g and group 2 (n=18 rats) received diet with 0.4 IU/g. Following 20 weeks of dietary treatment, the diet was changed for 8 rats of group 2 randomly chosen, switching for a diet with 20 IU/g, until the end of the experiment. The other rats remained with the same dietary treatment. Rats had *ad libitum* access to their customs diets, which were isocaloric and differed only by the amount of retinol provided under the form of retinyl palmitate. Rats were weighted weekly and no body weight difference existed between the four groups at experiment onset. All experiments were performed according to criteria of the European Communities Council Directive (2010/63/UE) and the French National Committee (authorization 16476-2018052314372190). All efforts were made to reduce the number of animals used and to minimize suffering.

Stepping test

Postural stability of rats was assessed using the stepping test paradigm started from week-4 after the specific diet regime started and lasting until the end of week-26²⁶. The test was performed every two or three weeks. Animals were first habituated to the hand restraint for two consecutive days. Rats were held by a trained experimenter, in a way to count the number of adjusting steps made by the rat with each forelimb in response to experimenter-induced lateral movement. Rats were slowly moved over a 90 cm-long bench (right to left and reverse). The procedure was performed 3 times for each paw, in both directions. For analysis we used the average number of steps in 3 trials for both forelimbs. Results were represented as the percentage of total number of adjusting steps made by the rat in the first test for each experimental group.

Rotarod test

Rotarod test was used to assess motor coordination and balance of rats. Rotarod test was performed with rotarod apparatus (Rota-Rod ENV-578(R), MedAssociates) as previously described²⁷. Briefly, rats were placed on a rotative axis and their latency to fall was measured. Each session had a maximum duration of 5 min. Three days before the first test, training sessions were performed on three consecutive days, with one session per day at constant speed (day 1 at 12 rotations per min (rpm), day 2 at 10 rpm, day 3 morning at 12 rpm and day 3 afternoon 14 rpm) and one session per day at progressive speed (2 to 20 rpm). For test sessions, the rotative axis followed a linear acceleration from 5 to 45 rpm. Data were represented as latency to fall (s) for each animal, as automatically registered by the apparatus.

Actimetry

Spontaneous locomotor activity of rats after six months of diet was assessed by recording rat's activity in cages over a 24-hour period. To do so, actimetry cages (20 x 33 x 18 cm; Imetronic, Pessac, France) were used, with *ad libitum* access to water and customs diets. The cages were provided with two infrared light beams associated with sensors and connected to a computer for recording activity. After 2h of habituation in cages, the total activity of rats, corresponding to the horizontal activity, was recording during 24h. Blocks of 2 hours were considered for the statistical analysis.

Cylinder test

Cylinder test was used to assess spontaneous forelimb use during exploration of a confined and unfamiliar environment. Cylinder test was performed as previously described²⁷. In a clear plexiglass cylinder (diameter: 16 cm; height: 31 cm), the number of touches with both forepaws on the cylinder wall while the rat was rearing was quantified during 3 min-sessions²⁸. Results were represented as the total number of touches made with both forepaws.

185

186 **Operant reach-and-grasp lever task**

187 Fine motor learning and execution were tested using an operant motor task based on water-restricted
188 rats trained to press a lever following a 50-ms 'Go' cue tone to obtain a liquid reward. To execute this
189 aim, we used a new operant box apparatus with a new behavioral paradigm, that has been conceived
190 to specifically test motor skills relying on striatal function. All rats (n=24) started the operant training
191 at 6 weeks of age (weighing 120-150 g) concomitantly with the beginning of enriched diet (n=6 rats) or
192 the vitamin A sub-deficient diet (n=18 rats). Water restriction was established 5 days / week (with a
193 minimum liquid intake of 10 ml / rat / day) with *ad libitum* access to water the remaining 2 days /
194 week. Body weight was monitored daily to avoid weight losses superior to 20 % of body weight in 1
195 week. The operant apparatus (Imetronic, Bordeaux, France) was composed of 2 levers (a left L1 vs. a
196 right L2) mounted either side of a sucrose-dispensing cup, one speaker, three LEDs, and a CCTV camera
197 to supervise the animals' behavior during training. A small metal ball (0.5 mm) was soldered on top of
198 each lever to facilitate the reach-and-grasp lever press motor behavior. In addition, two metallic pads
199 located on the ground below each lever were used as a capacitive circuit to detect the rat's forelimb
200 position based on touch sensing. These metallic pads ensured that the starting position of the forelimb
201 was similar across trials and allowed precise measurement of the reaction time (RT; time from 'Go' cue
202 to pad off) and the movement time (MT; time from pad off to lever press) on each trial. The operant
203 box was programmed through a software interface (POLY, Imetronic) and training sessions lasted
204 between 30 to 60 min. Motor training consisted of teaching the rats with different versions of the task
205 of increasing complexity (from level 1 to 7). Briefly, the first level of training consisted in the
206 reinforcement of each press on either lever with liquid reward. Level 2 was identical but lever pressing
207 triggered reward delivery together with the activation of a cue tone (50 ms duration) of either 1kHz
208 (i.e. low frequency) following a left lever press or 6kHz (i.e. high frequency) after a right lever press.
209 For level 3, the two cue tones previously associated with left/right lever pressing were randomly played

as a 'Go' signal. Here, rats would receive a reward only when they pressed the lever associated with the corresponding cue tone. On level 3, pressing the wrong lever was devaluated by the absence of reward delivery, but was not recorded as an error *per se*. Level 4 was identical to level 3 but here, pressing any lever before the 'Go' cue tone was considered as an 'anticipatory' error trial. All errors were associated with a 6s 'time out' period. Level 5 had the same structure but this time pressing the wrong lever after the 'Go' cue tone lead to a 'wrong lever press' error trial. In level 6, the rats had to place their forepaws onto the metallic starting pads in order to initiate a trial. Animals had to press the cued-lever within less than 3s after the 'Go' signal. As such, 'reaction out' and 'movements out' errors were counted as over reaction time (RT) or movement time (MT) epochs, respectively. In the final stage of training, level 7, lever press movements triggered by 'Go' signals had to be initiated with the appropriate forelimb (as measured by the first paw lifting the metallic pads). Moving the wrong paw after the 'Go' signal was counted as a 'wrong paw' error.

By the end of the training, rats learned to press the left or the right lever in response to a low frequency (i.e. 1 kHz) or a high frequency (i.e. 6 kHz) tone, respectively. The correct positioning of both forelimbs onto the metallic starting pads was signaled by the central LED being turned on, then the Go left or Go right cue tones were played at random (50-50%), after a variable delay (300 - 1200 ms). After each Go signal, rats had a limited amount of time to reach and grasp the metal ball, which induced the pressing of the lever (3000 ms for both RT and MT). Correct trials were rewarded with 40 μ L of 5 % sucrose water, incorrect trials were signaled by lighting up the 3 LEDs and a time-out penalty lasting 6 s during which the rat could neither initiate a new trial nor gain reward. The possible errors included lever presses before Go cue (i.e. anticipatory responses), pressing the wrong lever after Go cue (i.e. bad left or bad right lever), reacting or moving too slowly (i.e. cutoff RT or MT). Overall, to pass from one level to the next one, rats were required to reach performance level of at least 70 % or more during one session.

Sample collection

In order to assess the effect of dietary treatment, blood was collected from the tip of the rat's tail at 7 and 16 weeks of diet (**Fig. 1A**). A cut at the end of the tail was made, in the least innervated part, which healed quickly following the procedure. Following the 26-week feeding period, all animals were euthanized for subsequent analyzes (**Fig. 1A**). Rats were euthanized with intraperitoneal injection of pentobarbital (Exagon 150 mg/Kg)/lidocaine (230 mg/Kg). Blood was collected *via* an intracardiac puncture and rats were then transcardially perfused with a solution of phosphate buffer saline (PBS) in order to remove blood from tissues. After decapitation, brains were quickly extracted and divided into 2 parts. The right hemisphere was immediately frozen in isopentane and stored at -80 °C. The left hemisphere was post-fixed in 4 % paraformaldehyde for 24 h and plunged in 30 % sucrose solution for 48h before storage at -80 °C. Samples of liver were also collected and stored at -80 °C for retinol assay and RT-qPCR analysis. All Blood samples were centrifuged (10 min, 10 000 x g, 4 °C) and plasma was collected and stored at -80 °C until used.

Retinol assays

Serum and liver retinol levels were determined by high performance liquid chromatography (HPLC) as previously described ²⁹. Liver is the storage organ for vitamin A in the body. Liver extracts were homogenized in sodium phosphate-EDTA (0.05 M, pH 7.8) buffer. Briefly, 100 µL of liver homogenates or plasma were added to 150 µL of tocopherol acetate (1.057 mmol/L, T3376, Sigma St. Louis, MO, USA) solution, used as internal standard. After addition of 600 µL of hexane and butyl-hydroxy-toluene solution (20 mg/mL, ref B1378, Sigma St. Louis, MO, USA), 200 µL of homogenates were centrifuged (2 min, 20 °C, 16,000 × g) and the supernatant containing retinol was recovered and evaporated under nitrogen. The resulting residues containing retinol were treated with methanol before HPLC analysis. Two vitamin A calibration standards (Chromsystems 34004) were processed in parallel with samples. Results are given in µmol/g and µmol / l for the liver and plasma samples, respectively.

260

261 **Real-Time quantitative Polymerase Chain Reaction (RT-qPCR)**

262 The expressions of the different genes of interest in the striatum and in the liver were evaluated by
263 RT-qPCR. Samples of striatum were obtained from 1 mm-punches performed in the right hemisphere
264 of brain frozen in isopentane and stored at -80 °C at the time of euthanasia. To do so, 200 µm-coronal
265 brain sections were performed using a cryostat tissue slicer (Leica Biosystems, Germany). Based on a
266 rat brain atlas ³⁰, punches of the striatum were taken between 2.20 and -1.20-mm from bregma and
267 immediately stored at -80 °C for subsequent RT-qPCR.

268 Prior to the qPCR, total RNA was extracted from aliquots of the liver stored at -80 °C and punches of
269 striatum using TRIzol® reagent (Invitrogen, Fisher Scientific, France) as previously described ³¹. The
270 purity and quantity of RNAs obtained were measured by using a Nanodrop One Spectrophotometer
271 (Life technologies, France), and their quality was assessed using the RNA 6000 Nano LabChip kit in
272 combination with the 2100 Bioanalyzer (Agilent Technologies, France). Next, 500 ng of RNA from the
273 striatum and 1 µg of RNA from the liver, were reverse-transcribed into complementary DNA using the
274 enzyme ImProm-II reverse transcriptase (ref A3802, Promega, France) in a final volume of 20 µL. Then,
275 the RT-qPCR was performed in duplicate for each sample, with 1:50 cDNA diluted for striatum and
276 1:100 cDNA diluted for liver in a final volume of 10 µL, deposited in 384-well plates, and using the
277 LightCycler 480 II system (Roche Diagnostics, Meylan, France). Gene amplification was performed using
278 a SYBR Green I Master kit (ref RR420L, TAKARA BIO Europe, France) and appropriate primers. The
279 forward and reverse primer sequences used to amplify genes of interest are summarized in (Table 1).
280 Data were analyzed using the comparative threshold cycle method performed with GenEx version 7
281 software (MultiD Analyses AB, Göteborg Sweden). Results were normalized with 2 house-keeping
282 genes, B2-microglobulin (B2M) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Table 1)
283 and expressed as relative expression of mRNA compared to mean result for 5 IU rats.

284

285 **Immunofluorescence staining**

Right hemispheres of frozen brain post-fixed in 4 %-paraformaldehyde were used to cut 40- μ m thick coronal sections using a cryostat tissue slicer (Leica Biosystems, Germany). According to ³², we targeted 3 levels of interest of the striatum, designated as anterior (bregma + 2.2), intermediate (bregma + 1.6) and posterior (bregma + 0.7). Sections were washed with phosphate buffer saline (PBS) solution and then incubated in blocking solution for 1-2 h at room temperature. Sections were then incubated with primary antibodies diluted in blocking solution over night at 4 °C. The next day, sections were washed 3-4 times with blocking solution and incubated for 2 h at room temperature with secondary antibodies conjugated to a fluorochrome diluted in blocking solution. From the incubation with secondary antibodies until the end of the experiment, sections were protected from light. Finally, sections were washed with PBS before being mounted in mounting medium with DAPI (sc-359850, CliniSciences, France).

For double-fluorescent immunohistochemistry targeting tyrosine hydroxylase (TH) and ALDH1A1, blocking solution containing 3 % bovine serum albumin and 0.3 % Triton X-100 in PBS was used. For immunostaining targeting choline acetyl-transferase (ChAT), blocking solution containing 10 % fetal calf serum and 0.2 % Triton X-100 in PBS was used. For μ -opioid receptor (MOR) staining, blocking solution containing 10 % donkey serum, 1 % BSA serum and 0.3 % Triton X-100 in PBS was used.

Primary antibodies and dilutions used in this study were: mouse anti-TH (1:1000, mab 318, Merck); rabbit anti-ALDH1A1 (1:1000, HPA002123, Sigma); goat anti-ChAT (1:500, AB144P, Merck Millipore) and rabbit anti-MOR (1:1000, #24216, Immunostar). Secondary antibodies and dilutions used in this study were: donkey anti-mouse IgG-Alexa 488 (1:1000, ab150105, Abcam); donkey anti-rabbit IgG-Alexa 568 (1:1000, ab175470, Abcam) and donkey anti-goat IgG-Alexa 488 (1:1000, A32814, Abcam).

Image analysis

Immunofluorescence staining and image acquisition were performed on half-brain sections (right hemisphere). All striatum sections were scanned using a widefield microscope (Hamamatsu Nanosizer 2.0 HT) with 20X (20X, NA 0.75) objective to visualize the whole half striatum. Setting

parameters for acquisition, such as laser power or photomultipliers gain, were kept constant between all animals for a given labelling. Digital images obtained were processed with QuPath 0.3.2 (University of Edinburgh, UK)³³ or Image J software 1.53C (NIH, USA)³⁴ with semi-automated quantification to limit analysis bias.

For TH and ALDH1A1 staining in the striatum, intensity of staining was quantified. Green and magenta fluorescence channels were assigned to TH and ALDH1A1 associated signals respectively. From TH channel, we selected the region corresponding to the whole striatum using the “wand” set tool in Image J, which defines the region of interest (ROI). Area and staining intensity for TH and ALDH1A1 were measured based on this ROI.

For MOR and ChAT staining, QuPath software was used. For each slice, dorsal striatum boundaries were delineated by hand with polygon tool. MOR-positive (MOR⁺) areas were automatically defined with ‘pixel classifier tool’, with resolution at 3.63 $\mu\text{m}/\text{px}$, Gaussian prefilter, smoothing sigma of 0.5, and adjusted threshold. MOR⁺ area was expressed as the percent of total striatum area, as defined by boundaries.

ChAT-positive (ChAT⁺) neurons in the striatum were detected with ‘Cell detection’ tool with request pixel size at 2 μm , background radius at 30 μm , sigma at 1.5 μm and threshold at 10. The number of ChAT⁺ neurons was normalized by the total striatum area, as defined by boundaries.

Statistical analyzes

Statistical analyzes were performed with GraphPad prism 7.0 (Graphpad software) and details are summarized in (**Table 2**). The effect of the three dietary treatments was assessed with Krustal-Wallis test. A post hoc test was performed, based on the two-stage step-up method of Benjamini, in case of significant interaction ($p < 0.05$). When we compared only two experimental groups we performed Mann-Whitney tests, or unpaired t-test when the data were normal. When appropriate, we used two-way ANOVA with repeated measures (time effect or training level effect) or not (striatum level).

For operant lever press task, Chi-square test was used to compare the distribution of errors between groups. Distribution of action times were fitted with lognormal fits and comparison of fits was performed. Furthermore, data analyzed with 2-way ANOVAs were bootstrapped to dampen different n values between groups (20 IU: n=6 vs. 0.4 IU: n=18). Repeated measures bootstrapping was performed in R Studio version 4.0.3³⁵ using the *nmle* package³⁶ with 100 bootstraps. Briefly, experimental data was input and bootstrapped (100 resamples), returning bootstrapped p-values in the form of a 2-way repeated measures ANOVA table.

RESULTS

3.1 Characterization of the vitamin A sub-deficiency nutritional model

Adult male rats (n=24) were subjected to three different doses of dietary vitamin A, which correspond to an enriched diet (20 IU retinol/g of diet, n = 8), a standard diet (5 IU retinol/g of diet, n = 8) or a sub-deficient diet (0.4 IU retinol/g of diet, n = 8), referred thereafter to as 20 IU, 5 IU and 0.4 IU, respectively. In order to assess the long-term effects of the dietary dose of vitamin A, the nutritional model was carried out for 6 months (26 weeks) from adult age (**Fig. 1A**).

We first **noted** that dietary vitamin A dose did not induce **a** body weight difference in rats (diet effect: p=0.842) (**Fig. 1B**). Therefore, 26 weeks of nutritional vitamin A sub-deficiency at **an** adult age did not induce growth deficit, contrary to total vitamin A deficiency from weaning as shown in the literature^{37–39}.

We then evaluated vitamin A status through retinol assays from the plasma (circulating retinol) and the liver (retinol storage). In physiological conditions, the concentration of plasma retinol is **tightly** regulated by the body and maintained at 2 µmol/l in rodents⁴⁰. In our study, plasma retinol was maintained around 2 µmol/l for the three groups after 7 weeks **on the diets**. However, this rate decreased **by 16 weeks** (**Fig. 1C**). Indeed, plasma retinol level of 0.4 IU rats was drastically decreased at week 16 of dietary treatment, and further decreased after 26 weeks of deprivation, compared with

5 IU and 20 IU groups ($p < 0.001$ for both) (**Fig. 1C**). Of note, plasma retinol levels were comparable between rats fed with 5 IU or 20 IU diets. However, levels significantly decreased over the 26-week feeding period to reach 1.5 $\mu\text{mol/l}$ for both groups (time effect: $p < 0.001$) (**Fig. 1C**). In the liver, retinol levels at the end of the experiment were significantly lower in rats fed with 5 IU diet, compared to rats fed with 20 IU diets ($p = 0.048$) (**Fig. 1D**). In addition, retinol was barely detectable in rats fed with 0.4 IU diet (**Fig. 1D**). Altogether, these results confirmed the impact of dietary vitamin A dose on retinol metabolism and validated the use of 0.4 IU diet as a model for altered retinoid signaling in adult rats, without alteration of growth.

3.2 Impact of dietary vitamin A on retinol metabolism in the liver

In order to further characterize the metabolic impact of dietary vitamin A dose, we measured the mRNA expression level of genes coding for proteins involved in retinol metabolism and storage in the liver (**Fig. 2A**). Gene expression of retinyl ester hydrolase (REH) and retinol binding protein 4 (RBP4), both involved in the mobilization of hepatic retinol storage to its target tissues, was not impacted by dietary vitamin A dose ($p = 0.474$ and $p = 0.517$, respectively) (**Fig. 2B, C**). By contrast, gene expression of lecithin:retinol acyltransferase (LRAT), the enzyme for retinol esterification, was strongly decreased in 0.4 IU rats compared to rats fed with 5 IU ($p < 0.001$) and 20 IU ($p = 0.003$) diets (**Fig. 2D**). Gene expression of cytochrome P26 a1 (CYP26 a1), the enzyme for RA degradation, was also significantly decreased in rats under 0.4 IU diet, compared to 5 IU ($p = 0.044$) and 20 IU ($p = 0.002$) diets (**Fig. 2E**). Of note, no significant difference was observed between rats fed with 5 or 20 IU diets for gene expression of REH, RBP4, LRAT and CYP26 a1.

These results demonstrated a clear decrease in liver retinol storage and catabolism of RA in rats with vitamin A sub-deficiency. This compensatory mechanism aims at reducing the lack of vitamin A bioavailability for other target organs, such as the brain ⁴¹.

3.3 Impact of dietary vitamin A on retinol metabolism in the striatum

We next assessed whether vitamin A sub-deficiency altered retinoid signaling in the striatum by quantifying expression of genes coding for proteins involved in retinol metabolism and signaling (**Fig. 3A**). Vitamin A diet did not alter gene expression for striatal RBP4 ($p=0.474$), CYP26b1 ($p=0.683$) and the Cellular Retinoic Acid Binding Protein 2 (CRABP2, $p=0.335$) (**Fig. 3B-D**). By contrast, a significant decrease of gene expression for Stimulated by Retinoic Acid gene 6 (STRA6) was observed in vitamin A sub-deficient rats, compared to 5 IU and 20 IU rats ($p<0.001$, **Fig. 3E**), confirming a decrease of retinol entry in the brain. Moreover, mRNA expression of RAR β and RXR γ , the 2 **isotypes** of retinoids receptors highly expressed in the striatum¹⁷, was significantly decreased in 0.4 IU rats compared to 20 IU rats ($p<0.001$ and $p=0.037$, respectively) (**Fig. 3F, G**). Of note, level of mRNA expression for RAR β in 5 IU rats was in between levels for 20 IU and 0.4 IU rats (5 IU vs 20 IU: $p=0.062$, 5 IU vs 0.4 IU: $p=0.058$) (**Fig. 3F**).

Furthermore, we measured the expression level of genes coding for proteins implicated in neuroinflammation and oxidative stress since vitamin A can participate to both processes¹⁰. In our conditions, results did not reveal a significant effect of vitamin A sub-deficiency on mRNA expression of protein linked to neuroinflammation in the striatum such as the purinergic P2X7 receptor (P2X7R) and NLRP3 inflammasome ($p=0.1692$ and $p=0.7984$ respectively) (**Sup Fig. 1**). By contrast, mRNA expression of neuronal nitric oxide synthase (NOS1) was significantly decreased in 0.4 IU rats ($p=0.009$), without a significant effect on SOD1, SOD2 and NOS3 ($p=0.078$, $p=0.645$ and $p=0.458$, respectively) (**Sup Fig. 1**).

Altogether, these results confirmed the reduction of retinoid signaling in the striatum induced by vitamin A sub-deficiency at adulthood.

3.4 Impact of dietary vitamin A on motor abilities

Alteration of retinoid signaling during development or at weaning is associated with motor disabilities^{18,21–23,42}. Here, we investigated whether reduced retinoid signaling induced by adult vitamin A sub-deficiency also induces motor alteration. We first performed behavioral tests to analyze gross motor abilities and coordination.

In 24h-long actimetry and in cylinder test, no difference was observed between groups (diet effect: for actimetry $p=0.620$, for cylinder test, $p=0.776$, **Fig. 4A, B**). In rotarod test, the training phase did not significantly differ between groups, even though variability was observed for the three groups (**Fig. 4C**). following training sessions, vitamin A sub-deficient rats exhibit a clear reduction of the latency to fall, compared to rats under 5 IU and 20 IU diets, at 3, 5 and 7 weeks after the beginning of diet exposure (diet effect: $p=0.024$, **Fig. 4C**). However, with repetition of rotarod sessions, the latency to fall was significantly reduced for all groups. Of note, rats fed the 5 IU diet displayed performance higher than sub-deficient rats, but lower than supplemented rats (see **Table 2** for detailed statistics).

In the stepping test, sub-deficient rats showed a progressive reduction of the number of steps performed, compared to supplemented rats, which was significantly different from 6 weeks of diet (diet effect: $p<0.001$, **Fig. 4D**). Moreover, rats fed the 5 IU diet had significantly lower performance than enriched rats and exhibited performance similar to sub-deficient rats (see **Table 2** for detailed statistics).

These results suggested that vitamin A sub-deficient rats exhibited lower performance with time in task involving constrained movements. Furthermore, rats fed the 5 IU diet exhibit poor motor performance, compared to enriched rats. Consequently, we compared for the rest of the study the sub-deficient rats (*i.e.* 0.4 IU/g) to the supplemented rats (*i.e.* 20 IU/g).

3.5 Impact of dietary vitamin A on fine movement learning and execution

To further investigate the potential impact of vitamin A sub-deficiency on finer motor abilities, we trained a new cohort of rats to perform a cued reach-and-grasp task. This forelimb motor task is a combination of operant responding, reach-and-grasp, as well as response withholding and it was specifically implemented because it relied on the integrity of DA transmission in the striatum^{43–46}. The motor task was performed using an operant box apparatus with metal balls attached to 2 levers (right and left) and 2 auditory cues (**Fig. 5A**). The final experimental paradigm required rats to maintain their forepaws on pads to initiate a new trial in a reproducible starting position, and to reach-and-grasp the metal ball attached on the right or left lever, depending on the auditory Go cue signal, in a precise timing (limited time of 3000 ms for both the RT and MT), in order to press the lever (**Fig. 5A**). Reaction time (RT) and movement time (MT) were recorded and successful trial completion was rewarded with a drop of sweet water. The motor vigor was defined as the action time, which corresponds to the sum of RT + MT, as classically done in the field⁴⁷. Operant training started simultaneously with dietary treatment and lasted around 10 weeks during which rats were subjected to tasks with increasing complexity (**Fig. 5A**). Accordingly, different error types were implemented starting from level 4 of training, where only anticipation errors (lever press before cue tone) were taken into account (**Fig. 5B**, see methods). Errors types were then added cumulatively: press on the wrong lever (level 5), RT+MT < 3 s (level 6) and pressing the lever with the ipsilateral paw (level 7) (**Fig. 5B**, see methods).

At first, comparison of action vigor in fully-trained rats ('last sessions' in **Fig. 5B**) revealed a significant difference between groups, the sub-deficient rats being slower in average than the supplemented rats to perform the lever press movements ($p < 0.001$, **Fig. 5C**). Moreover, when looking at the progression of rats across difficulty levels, we observed that sub-deficient rats required a few more sessions to go through levels 6 and 7 (diet effect: $p = 0.116$, **Fig. 5D**). By contrast, rats did a similar proportion of successful trials and errors types between the sub-deficient and supplemented rats ($p = 0.753$, **Fig. 5E**). Indeed, successful trials represented $70.1 \pm 2.7\%$ and $66.0 \pm 5.0\%$ in supplemented and sub-deficient rats, respectively. Furthermore, the number of trials / min did not differ between groups across levels ($p = 0.483$, data not shown).

Task levels 6 and 7 represent the implementation of a cutOff penalty time (level 6), and the necessity to use the ipsilateral paw to press the correctly-cued lever (level 7). We observed that sub-deficient rats tend to produce less successful trials compared to supplemented rats, especially in task levels 6 and 7 (diet effect: $p=0.08$, **Fig. 5F**). This lower proportion of success rate was neither due to increased number of anticipations (diet effect: $p=0.65$, **Fig. 5G**), nor to an increase of cutOff times (diet effect: $p=0.51$ for reactions out of time and $p=0.34$ for movements out of time, **Fig. 5H, I**). When focusing on correct lever-pressed trials, the difference in performance between supplemented and sub-deficient rats was stronger when trials involved the right lever, in accordance with most rats being left-handed (**Fig. 5J**). Indeed, successful trials with right lever were lower in sub-deficient rats, compared to supplemented rats (diet effect: $p=0.03$, **Fig. 5J**). This was partly due to more incorrect lever presses during task levels 5 and 6 (diet effect: $p=0.07$, **Fig. 5K**), and possibly more incorrect paw use during level 7 ($p=0.177$, **Fig. 5L**).

Altogether, these results showed that sub-deficient rats exhibited alteration in motor execution, in addition to the need of more time to learn a complex motor task, that could involve striatal dysfunction.

3.6 Impact of vitamin A sub-deficiency on dopamine signaling in the striatum

In order to decipher how vitamin A sub-deficiency at adult age participates to motor learning deficits, we investigated whether a neurobiological signature could be identified in the striatum.

We first investigated the expression of genes coding for the main proteins of the striatal dopaminergic system. Indeed, it is established that $RAR\beta$ and $RXR\gamma$ modulate the expression of genes involved in dopamine transmission during striatal development^{48–52}. Yet, expression of genes coding for $RAR\beta$ and $RXR\gamma$ is decreased in vitamin A sub-deficient rats of the present study (**Fig. 3F, G**). We quantified genes coding for AADC (aromatic l-amino acid decarboxylase), MAOA (monoamine

oxidase A), DARPP-32 (Dopamine- and cAMP-regulated phosphoprotein, Mr 32 kDa), and the DA receptors DRD1, DRD2 and DRD3. No significant difference was observed for any of the mRNA that were investigated between rats fed with 0.4 IU or 20 IU diets from adult age (**Fig. 6A**).

Since the striatum is highly heterogeneous and can be divided in sub-territories, we further analyzed the DA system in the striatum with Tyrosine hydroxylase (TH) and ALDH1A1 immunostaining (**Fig. 6B-E**). Fluorescent intensities were measured for both markers independently, in three antero-posterior levels of the striatum (named anterior, intermediate and posterior), and for each level, the striatum was further sub-divided in 6 areas (medial/lateral and dorsal/medial/ventral). Despite this high level of segmentation, no significant difference between 0.4 IU and 20 IU rats was found, for both TH and ALDH1A1 staining (TH: Anterior $p=0.804$; Intermediate $p=0.754$; Posterior $p=0.694$; ALDH1A1: Anterior $p=0.804$; Intermediate $p=0.662$; Posterior $p=0.231$) (**Fig. 6B-E**).

Altogether, these results suggested that the nigro-striatal dopaminergic system is not sensitive to vitamin A sub-deficiency started at adulthood.

3.7 Impact of vitamin A sub-deficiency on striatal neuromodulation

We next hypothesized that motor impairments in sub-deficient rats were related to alterations of neuromodulation in the striatum, other than the DA system. Indeed, other neuromodulatory systems in the striatum are known to be modulated by retinoid signaling, such as the cholinergic system, cannabinoid receptors, opioid receptors or adenosine receptors^{18,24,48}. No significant difference was observed between 0.4 IU and 20 IU rats for expression of genes coding for cannabinoid receptor CB1, or adenosine receptor ADORA2A ($p=0.536$ and $p=0.591$, respectively, **Fig. 7A**).

By contrast, the expression of gene coding for choline acetyltransferase (ChAT) was consistently reduced in 0.4 IU rats ($p=0.007$, **Fig. 7A**). However, expression of **other proteins** involved in cholinergic transmission were not significantly changed between groups (acetylcholinesterase (AChE): $p=0.235$ and muscarinic receptors 1 and 4, (ChRM1, ChRM4), $p=0.634$ and $p=0.4855$, respectively). Similarly,

quantification of ChAT-positive (ChAT⁺) cholinergic interneurons in the striatum revealed no difference between groups ($p=0.668$, **Fig. 7B, C**).

Finally, the expression of gene coding for μ -opioid receptor (MOR), a marker of anterior striosomes in the striatum⁵³, was significantly reduced ($p=0.030$). In addition, quantification of MOR-positive (MOR⁺) striosomes with immunofluorescence revealed a significant reduction of MOR⁺ striosome surface in 0.4 IU rats ($p=0.0372$, **Fig. 7D, E**), confirming MOR gene expression analysis. Of note, gene expression of Nur77, an orphan nuclear receptor that can dimerize with RXR and that is enriched in striosomes, was not different between groups ($p=0.662$, **Fig. 7A**).

3.8 Reversibility of nutritional vitamin A deficiency

Finally, we investigated the reversibility of retinoid signaling alteration induced by vitamin A sub-deficiency at adulthood. To do so, an experimental group of rats was fed with a sub-deficient diet for 20 weeks, followed by an enriched diet for the last 6 weeks of the experiment. Only parameters found as significantly altered earlier were analyzed. Statistical analysis was performed on raw data but results were normalized by the median of both 0.4 and 20 IU groups (min to max normalization), in order to better visualize the effect of the dietary switch (**Fig. 8**). This analysis revealed that plasma retinol level was completely rescued with 6 weeks of enriched diet, while liver retinol was only partially recovered (**Fig. 8A**). In the liver, the mRNA expression level of LRAT was improved by enriched diet, however, the mRNA expression level of CYP26 a1 remained different from the results obtained in the enriched group (**Fig. 8B, Table 2**). In the striatum, mRNA expression level of STRA6, as well as RAR β and RXR γ were not different from 20 IU group, suggesting that a 6-week period of enriched diet was enough to rescue impaired retinoids signaling induced by vitamin A sub-deficiency (**Fig. 8B, Table 2**). Furthermore, mRNA expression level of MOR and ChAT, as well as MOR⁺ striosomes area were not improved by 6 weeks of enriched diet, (**Fig. 8C, Table 2**).

Finally, motor function measured with the stepping test was significantly improved by enriched diet, even though the deficit was not totally reversed (20 IU vs 0.4 IU: $p<0.001$, 5 IU vs 0.4 IU: $p<0.001$, **Fig.**

8D). However, re-supplementation was not sufficient to restore normal motor vigor as measured in the cued-lever pressing task (distribution of error types: $p=0.261$, distribution of action times: $p<0.001$, Fig. 8E, F).

DISCUSSION

In this study, we examined the impact of a 6-month vitamin A sub-deficiency on striatal neurobiology and associated motor functions in adult Sprague-Dawley male rats. We showed that striatal alteration of retinoid signaling at adulthood was associated with an impairment of learning and execution skills in a complex reach-and-grasp lever pressing task. In the striatum, these motor impairments were not associated to a dysfunction of dopamine-dependent signaling *per se*, as initially expected from previous studies based on perturbations of retinoid signaling during developmental periods^{42,22,18,21}. Rather, low vitamin A supply at adult age modified cholinergic and opioidergic neuromodulatory systems in the striatum. These main results are further discussed below.

First, we studied vitamin A deficiency with a new dietary paradigm, with a sub-deficient diet (0.4 IU/g) starting at adulthood for 6 months. Nutritional vitamin A deficiency is particularly challenging to induce in rodents, given the hepatic stocks of retinol that are formed during embryogenesis and that persist throughout life^{8,54}. Between mice and rats, mice are particularly resistant to vitamin A deficiency, for this reason we chose to perform the study on rats. Previous studies have investigated the effect of vitamin A deficiency with 0 IU/g of diet from weaning (50 to 100 g of bodyweight), however, it induced growth deficits^{18,37–39,55}. In our conditions, the initiation of vitamin A sub-deficiency at 6 weeks of age (180-200 g of bodyweight) with a dose of 0.4 IU/g did not induce body weight decrease, which indicated that this model did not induce growth retardation. Yet, the drastic decrease in plasma retinol levels after 16 weeks of diet confirmed the deficit in vitamin A bioavailability, similar to results obtained in previous studies with total deficiency³⁹. Moreover, the decrease of gene expression for

560 LRAT and CYP26a1 in the liver, as well as for STRA6, RAR β and RXR γ in the striatum of sub-deficient
561 rats, demonstrated the alteration of retinoid signaling in this rat model.

562 Of note, a non-negligible decrease in plasma retinol level was also observed in sufficient and
563 supplemented rats along the 6-month period of diet. This highlighted a collapse of plasma retinol
564 content with age, as found in humans ^{56–59} and aged rats ^{31,60}. Besides, liver retinol contents correlated
565 better with dietary dose of vitamin A and STRA6 expression in the striatum than plasma retinol content.
566 Indeed, retinol content is highly regulated in the plasma, while the liver is the major storage organ,
567 participating in buffering retinol level in the plasma. This suggested that measuring retinol in the liver
568 may more accurately reflect vitamin A bioavailability than plasma retinol level.

569 Taken together, these results validated the sub-deficiency performed with 0.4 IU/g at adulthood as a
570 model of non-developmental alteration of retinoid signaling.

571

572 Second, we investigated in this study the motor performance of rats under vitamin A diets using
573 multiple motor tests. When rats were free to move in a close environment, no difference was detected
574 between dietary treatments. This suggested that low vitamin A supply at adult age did not affect the
575 rat's basic motion. By contrast, when rats were forced to produce motor actions (rotarod and stepping
576 tests), which involved a stronger proprioceptive feedback ⁶¹, a clear decline along dietary treatment
577 was observed for rats with sub-deficient diet. However, these results need to be interpreted cautiously
578 because rats fed with 20 IU/g also decreased their performance with time. This may be due to the
579 repetition of the motor tests and/or the weight of the animals being over 600 g after 4 months of age.

580 This latter factor may impact the general motion abilities of rats for all groups. Furthermore, these
581 motor investigations revealed that rats fed with 5 IU/g exhibited motor performance in between
582 supplemented and sub-deficient rats. Gene expression of RAR β in the striatum for 5 IU rats was also in
583 between 20 IU and 0.4 IU rats. This raises the question of a dose-effect of dietary vitamin A, between
584 very low supply, sufficient supply and mild supplementation, for some parameters such as retinoid
585 signaling and motor performance. Furthermore, results in rotarod and step tests revealed motor

impairments starting 3 weeks after initiation of dietary treatment, while decrease of retinol in the plasma was not yet significant. However, plasma retinol concentration is highly regulated and maintained at homeostatic concentrations², compared to liver and brain. Therefore, early onset of motor impairment in vitamin A sub-deficient rats may be explained by reduced vitamin A supply in the brain.

To go deeper in the understanding of vitamin A sub-deficiency impact on striatum-related motor behaviors, we trained sub-deficient and supplemented rats to learn and execute a fine motor task, with cued- and rewarded- grasping movements. This task has been specifically developed to investigate motor abilities relying on proper striatal function. Using this tailor-made behavioral paradigm, we showed that sub-deficient rats were generally able to learn and perform the task, but they exhibit discrete motor alterations. In particular, rats tended to require more time to learn the last phases of the training, to perform the action with slower vigor, and showed more errors with the non-preferred paw at final levels of training. This demonstrates that alteration of retinoid signaling at adulthood can induce mild disabilities in fine motor execution. Here, we chose to start the training of the rats from the beginning of the deficiency. Going deeper in the understanding of these motor learning alterations may require exposing rats to this complex task once the deficiency is fully established, which is equivalent to 4 months of sub-deficient diet.

Next, we explored the neurobiological processes in the striatum that could underlie the motor alterations observed in vitamin A sub-deficient rats. We initially hypothesized that sub-deficient rats would exhibit alterations in DA transmission in the striatum. Indeed, constitutive knock-out of RAR β and RXR γ in mice from gestation result in a strong decrease in the expression of DA receptors (DRD1 and DRD2) and their transcripts in the striatum at adult age, associated with motor deficits ¹⁷. Moreover, retinoid signaling is established as being necessary for the proper wiring of the nigro-striatal pathway and output striatal neurons during development ^{4,50,62}. Here, our study revealed that vitamin A deficiency at adulthood did not significantly alter striatal DA transmission *per se*. This unexpected

result suggested that vitamin A signaling for DA transmission in the striatum is especially required during the establishment of the nigro-striatal pathway, while this system can exhibit resiliency with a reduced retinoid supply at adulthood. A longer vitamin A deficiency would help to determine more precisely the robustness of the striatal dopaminoceptive system to retinoid signaling failure.

By contrast, the present study revealed that striatal cholinergic and opioid systems are highly sensitive to retinoid signaling at adulthood. The decrease of gene expression for ChAT in this study is coherent with the decrease of ChAT expression in the striatum of RXR γ ^{-/-} mice⁶³. In addition, the study of Carta and al. in 2006¹⁸ showed a reduction of striatal content of acetylcholine in 24-week vitamin A deficient rats associated with impairment of motor behavior in the rotarod test. Furthermore, comparable to our study, a significant decrease of MOR expression is observed in aldh1a1^{-/-} mice¹³. Since retinoids modulates gene transcription of ChAT and MOR^{62,64}, we can presume that in our study, the decrease of gene expression for RAR β and RXR γ in the striatum following 6 months of vitamin A sub-deficiency decreased expression of these genes. Remarkably, these alterations are partially reversed by 6 weeks of vitamin A dietary enrichment. From previous studies, the crosstalk between cholinergic transmission and MOR⁺ striosomes is established^{53,65,66}. However, the role of these modulatory systems in motor learning and motor actions in physiological and pathological conditions is not investigated as extensively as is the DA system. This is partly due to the lack, until recently, of specific tools allowing the precise monitoring and manipulation of these microcircuits. Here, our study reveals that adult vitamin A deficiency constitutes an original model to specifically interrogate these striatal circuits. Therefore, deeper investigation in vitamin A sub-deficient rats may help to determine how alteration of cholinergic and opioid systems contributes to motor execution and motor learning impairments.

Finally, our study demonstrated the importance of retinoid signaling for the functional neuromodulation of the striatum at adulthood in rats. Yet, it is established that vitamin A signaling collapses with age^{31,67–69}, and age remains the first risk factor for PD¹¹. This suggested that in the

pathological context of PD, **defects** in vitamin A signaling may participate to the pathophysiology of the disease through an alteration of striatal neuromodulation. In PD, the role of these neuromodulatory systems in the striatum is important to focus on, since they exhibit plasticity processes in the absence of DA, likely to compensate for the dysfunction of the network^{70–72,53,73}. However, **these compensatory mechanisms also participate in side effects of L-dopa based treatments**, it is thus important to include the other neuromodulators in the equation of the pharmacological **treatments** proposed to patients. In this context, the management of vitamin A signaling in PD may contribute to refine PD treatment. Yet, vitamin A supplementation can improve vitamin A bioavailability for the brain^{31,60,74}. Moreover, nutritional approaches are easier to implement in human than pharmacological administration, with reduced risk of side effects^{75–78}. In our study, we showed the relative reversibility of deficits induced by vitamin A deprivation, which encourages the necessity of maintaining adequate vitamin A intakes, and the identification of reliable biomarkers to assess the vitamin A status of individuals.

DATA AVAILABLE STATEMENT

The data that support the findings of this study are available on request to the corresponding author.

CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to report.

AUTHOR CONTRIBUTION

Conceptualization and project administration: CB-B, NM; Funding and supervision of staff: CB-B, NM; Experiments: AM, RK, JCH, MD, PC, SA, ER; Statistical analyzes: AM, MDM, RK, NM, AF, CB-B; Visualization: AM, NM, CB-B; Writing – original draft: AM, CB-B, NM, PT; Writing – Review & Editing: all authors.

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Figure and LEGENDS

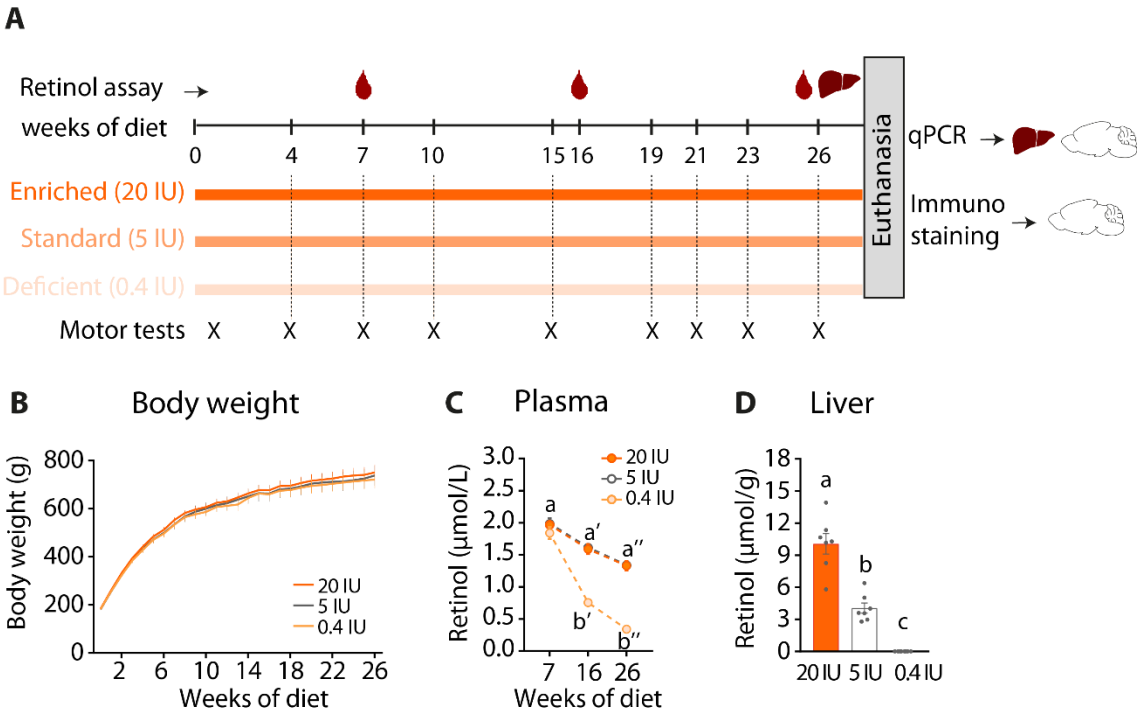


Figure 1. Characterization of the nutritional model with vitamin A diets

(A) Schematic overview of experimental design. 0.4 IU, 5 IU and 20 IU stand for 0.4 IU/g of diet, 5 IU/g of diet and 20 IU /g of diet, respectively. Blood samples were collected after 7, 16 and 26 weeks of diet. Behavioral tests were performed at different times (X). Liver and brains were collected at the time of euthanasia. (B) Changes in rats' body weight (g) throughout experiment. (C, D) Levels of retinol in the plasma ($\mu\text{mol} / \text{l}$) (C) and in the liver ($\mu\text{mol/g}$) (D). Results are expressed as means $\pm$ SEM with individual data points. a, b and c: values significantly different ($p < 0.05$). Details of the statistical analysis are summarized in (Table. 2).

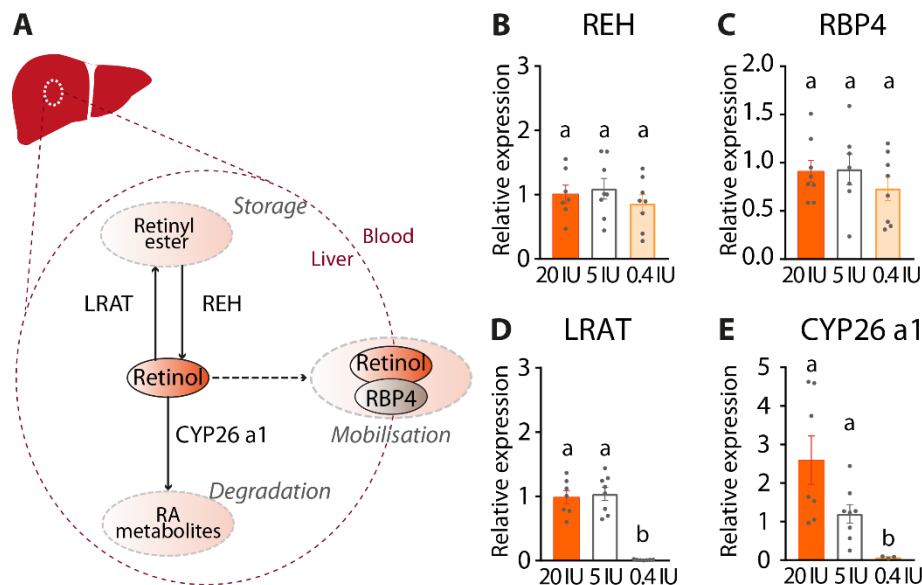


Figure 2. Impact of dietary vitamin A on liver retinol metabolism

(A) The diagram illustrates a simplified model of vitamin A metabolism in the liver. (B-E) mRNA relative expression of genes coding for REH (B), RBP4 (C), LRAT (D) and CYP26 a1 (E). Results are expressed as means $\pm$ SEM with individual data points. a-b: values significantly different ($p < 0.05$). Details of the statistical analysis are summarized in (Table. 2). RA, retinoic acid; REH, retinyl ester hydrolase; RBP4, retinol binding protein 4; LRAT, lecithin:retinol acyltransferase; CYP26a1, cytochrome P26a1.

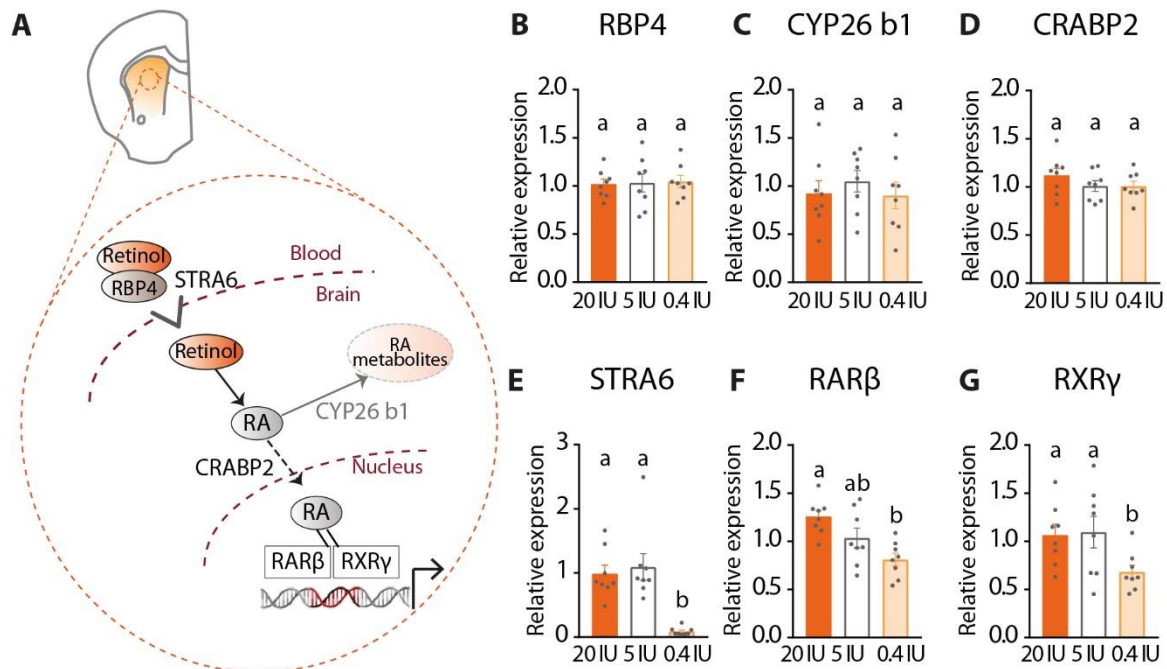


Figure 3. Impact of vitamin A sub-deficiency on retinol metabolism in the striatum

(A) The illustration shows a simplified model of vitamin A signaling in the striatum. **(B-G)** mRNA relative expression of genes coding for RBP4 (**B**), CYP26 b1 (**C**), CRABP2 (**D**), STRA6 (**E**), RARβ (**F**) and RXRγ (**G**) in the striatum. Results are expressed as means $\pm$ SEM with individual data points. a-b: values significantly different ($p < 0.05$). Details of the statistical analysis are summarized in **(Table. 2)**. RA, retinoic acid; RBP4, retinol binding protein 4; STRA6, stimulated by retinoic acid gene 6; CYP26 b1, cytochrome P26b1; CRABP2, cellular retinoic acid binding protein 2; RARβ, retinoic acid receptor isoform β; RXRγ, Retinoid X receptor isoform γ.

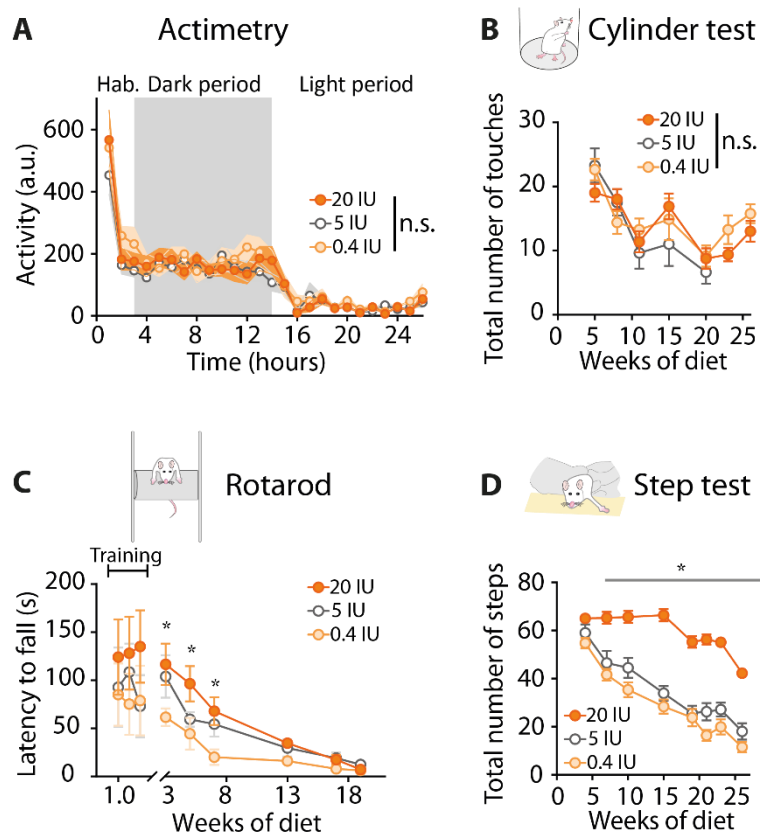


Figure 4. Impact of dietary vitamin A on motor abilities

(A) General horizontal activity of rats in actimetry cages for 26h, including 2h of habituation, as measured by the number of infrared light beams breaking. (B) Total number of touches made by forepaws on cylinder walls over 3-min sessions. (C) Latency to fall (s) from the rotarod apparatus was measured for each rats. Training sessions and test sessions were performed with increasing speed, as detailed in methods. (D) Total number of steps made by both forepaws during the stepping test protocol (see methods for details of the procedure). Results are expressed as means $\pm$ SEM. *: $p < 0.05$. Details of the statistical analysis are summarized in (Table. 2).

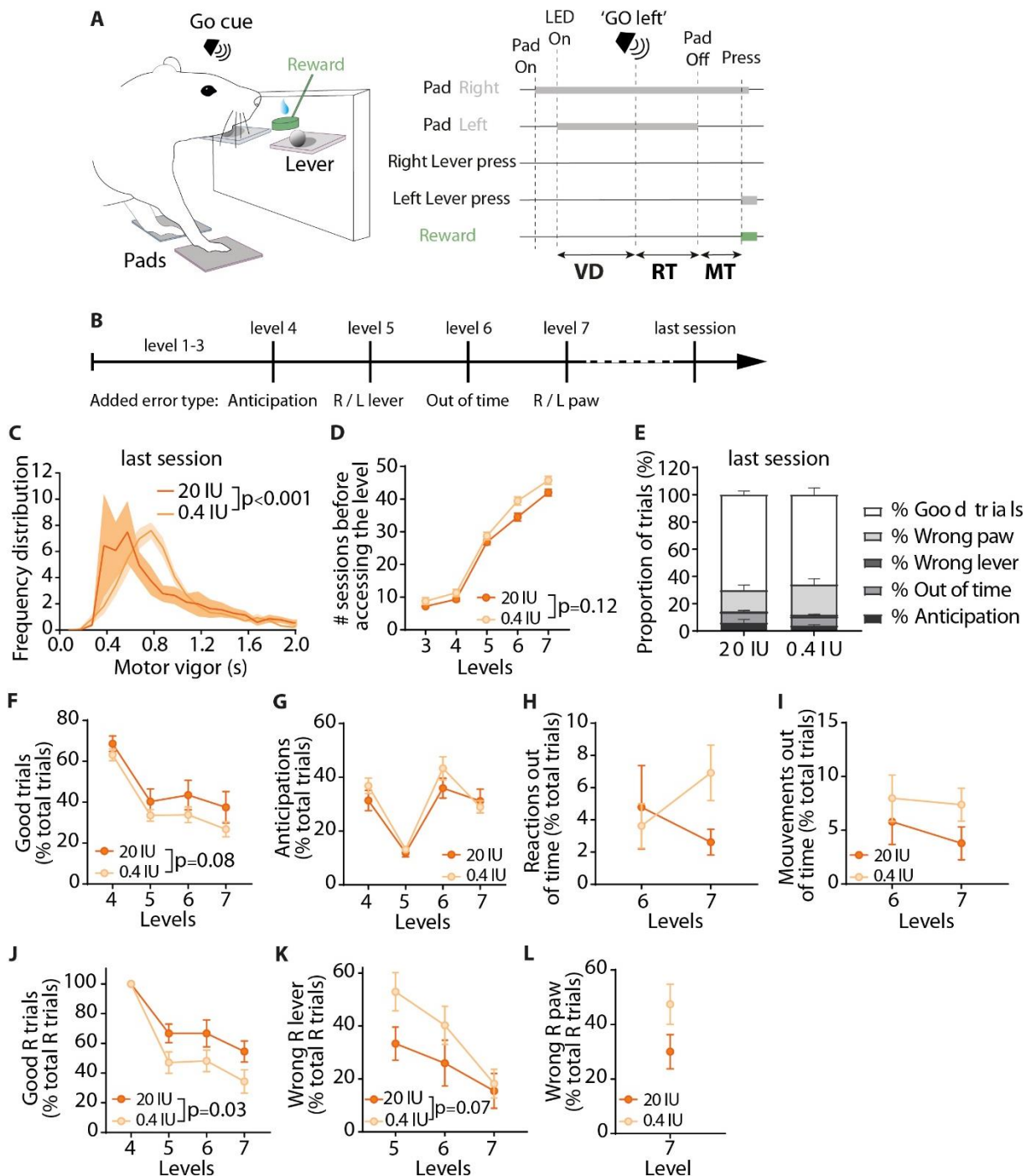


Figure 5. Impact of vitamin A sub-deficiency on fine movement

(A) Schematic of the behavioral paradigm for the cued- and skilled-motor task. At the end of the training, the complete task for the rat consists of placing the right and left forepaws on right and left pads respectively. With the LED light ON, the rat must wait 300-1200 ms (variable delay, VD). Then an auditory cue is emitted and the rat has to use the right or left forepaw, depending on the auditory cue, to press the right or left lever, respectively. The action time (reaction time (RT) + movement time (MT)) has to be completed in less than 3 seconds. If the trial is successful, then the rat receives a sweet

reward. **(B)** Time line of error types successively recorded during the training phase of the cued- and skilled-motor task. Each new error type increments the level of training. **(C)** Frequency distribution of action times of successful trials of rats during the last session, when the task is fully acquired by all rats. **(D)** Average number of sessions across rats needed to pass to the next level for 0.4 IU and 20 IU groups. **(E)** Distribution of all trials made by rats during the last session, depending on their success or the type of error displayed. **(F-I)** Mean proportion of good trials (F), trials with anticipation (G), trials with reaction out of limit time (H) or movement out of limit time (I) for 0.4 IU and 20 IU groups across levels. **(J-K)** Mean proportion of good trials (J), trials pressing the wrong lever (K) or trials using the wrong paw (L), focusing on the right paw, the less preferred paw for all rats, across levels. Results are expressed as means $\pm$ SEM. Details of the statistical analysis are summarized in **(Table. 2)**.

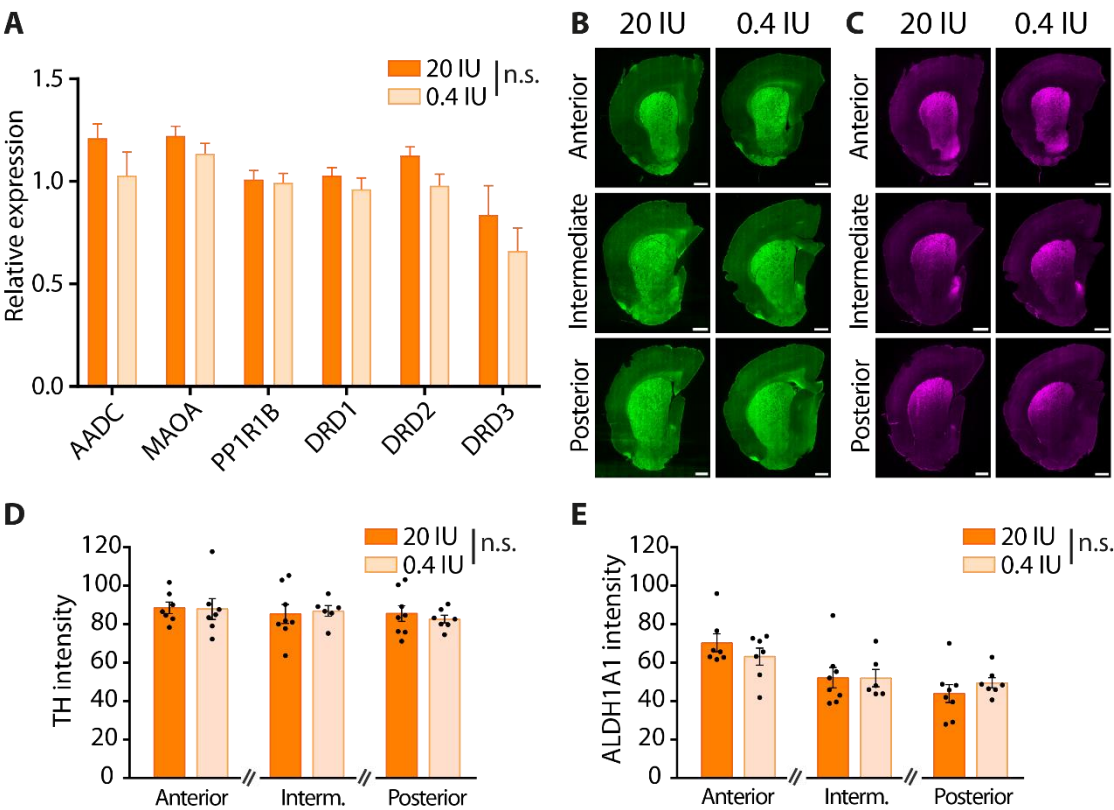


Figure 6. Impact of vitamin A sub-deficiency on the dopaminergic system in the striatum

(A) mRNA relative expression of genes coding for AADC, MAOA, PP1R1B, DRD1, DRD2 and DRD3. (B, C) Representative images for 0.4 IU and 20 IU groups of anterior, intermediate and posterior striatum immunostained for TH (B, green) and ALDH1A1(C, magenta). Scale bars: 1 mm. (D, E) Quantification of fluorescence intensity of TH (D) and ALDH1A1 (E) staining for three analyzed antero-posterior levels of striatum in the coronal plan. Results are expressed as means $\pm$ SEM (A) with individual data points (C, E). Details of the statistical analysis are summarized in (Table. 2). AADC, aromatic l-amino acid decarboxylase; MAOA, monoamine oxidase A; PP1R1B, Protein Phosphatase 1 Regulatory Inhibitor Subunit 1B; DRD1, dopamine receptor subtype 1; DRD2, dopamine receptor subtype 2; DRD3, dopamine receptor subtype 3.

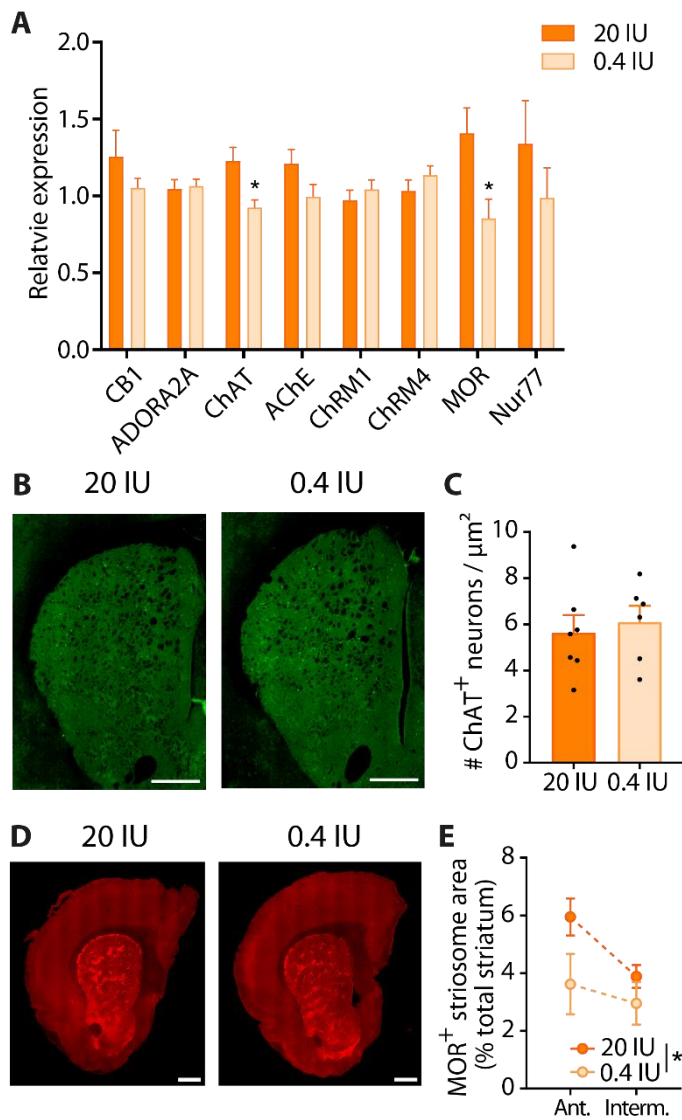


Figure 7. Impact of vitamin A sub-deficiency on striatal neuromodulation

(A) mRNA relative expression of genes coding for CB1, ADORA2A, ChAT, AChE, ChRM1, ChRM4, MOR and Nurr77. (B) Representative images for 0.4 IU and 20 IU groups of intermediate striatum immunostained for ChAT (green). Scale bar: 1 mm. (C) Quantification of ChAT⁺ neurons normalized to striatum area. (D) Representative images for 0.4 IU and 20 IU groups of anterior striatum immunostained for MOR (red). Scale bar: 1 mm. (E) Quantification of MOR⁺ striosome areas as a percent of total striatum area. Results are expressed as means $\pm$ SEM (A) with individual data points (C, E). *: values significantly different between 20 IU and 0.4 IU group ($p < 0.05$). Details of the statistical analysis are summarized in (Table. 2). Abbreviations: CB1, cannabinoid receptor 1; ADORA2A, adenosine receptor 2A; ChAT, choline acetyltransferase; AChE, acetylcholinesterase; ChRM1, cholinergic muscarinic receptor 1; ChRM4, cholinergic muscarinic receptor 4; MOR, μ -opioid receptor; Nur77, orphan nuclear receptor 77.

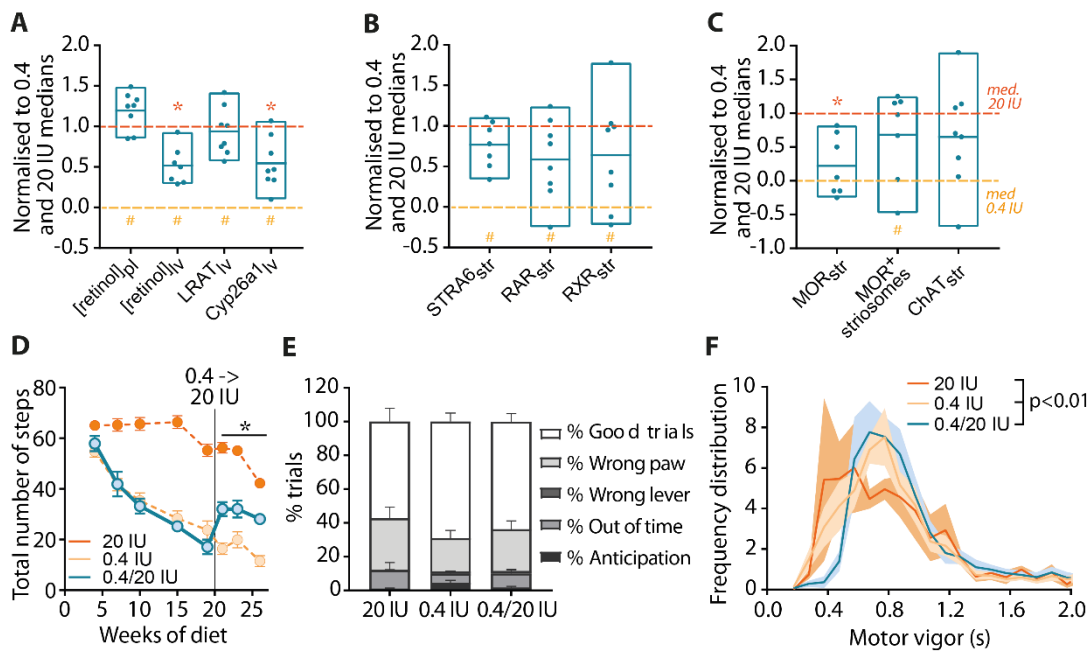
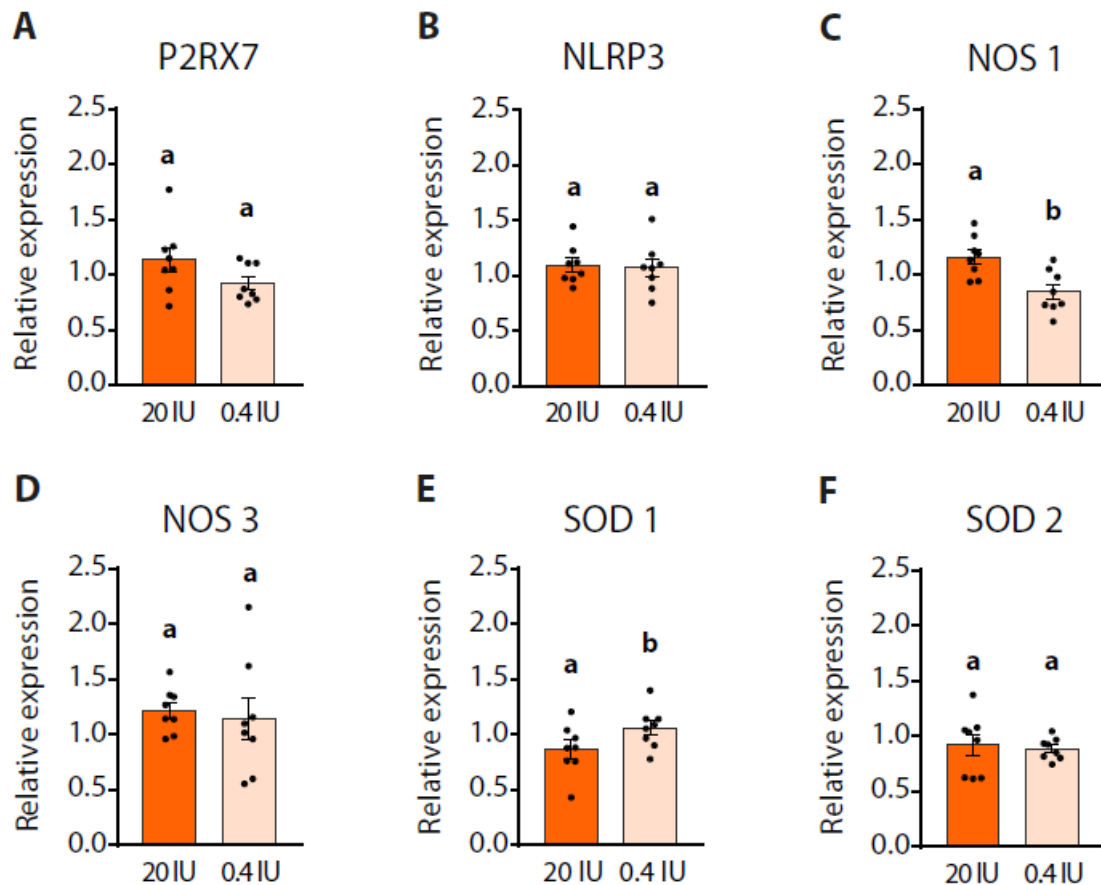


Figure 8. Reversibility of vitamin A sub-deficiency with vitamin A enriched diet

(A-C) Normalized values of parameters that were different between 0.4 and 20 IU rats, for rats fed with sub-deficient diet for 20 weeks and enriched diet for 6 weeks. (A) Plasma ([retinol]_{pl}) and liver ([retinol]_{liv}) retinol levels and mRNA relative expression of genes coding for LRAT (LRAT_{liv}) and CYP26a1 (Cyp26a1_{liv}) in the liver. (B) mRNA relative expression of genes coding for STRA6 (STRA6_{str}), RAR β (RAR_{str}) and RXR γ (RXR_{str}) in the striatum. (C) mRNA relative expression of genes coding for MOR (MOR_{str}), ChAT (ChAT_{str}) and MOR⁺ striosomes relative areas. Results are normalized to median values of sub-deficient rats (med 0.4 IU, dotted lines light orange at 0) and enriched rats (med 20 IU, dotted lines dark orange at 1). Results are expressed as floating bars (min to max) with plot of individual data points and median. Statistical analyses were performed on raw data with 1-way ANOVA: #p<0.05 between 0.4 IU and 0.4/20 IU; *p<0.05 between 20 IU and 0.4/20 IU. (D) Measure of the number of steps made by both forepaw over time with the stepping test protocol. (E) Distribution of all trials made by rats, depending on their success or the type of error displayed. (F) Frequency distribution of action times of **successful** trials of rats at the end of the experiment, 6 weeks after 20 IU supplementation for the 0.4/20 IU group. Details of the statistical analysis are summarized in (Table. 2).



Supp. Fig. 1. Impact of vitamin A intake on neuroinflammation and oxidative stress

(A-F) mRNA relative expression of genes coding for P2RX7 (A), NLRP3 (B), NOS 1 (C), NOS 3 (D), SOD 1 (E), SOD 2 (F). Results are expressed as means ± SEM with individual data points. a-b: values significantly different ($p < 0.005$). Details of the statistical analysis are summarized in **Supp.Table. 1**. P2RX7, purinergic P2X7 receptor; NLRP3, NLR family pyrin domain containing 3; NOS 1, nitric oxide synthase neuronal; NOS 3, nitric oxide synthase endothelial; SOD1, superoxide dismutase 1; SOD2, superoxide dismutase 2.

TABLES

Table 1: Primer sequences for RT-qPCR analyses. Genes in bold are housekeeping genes.

Gene	Sequence Forward primer (5'- 3')	Sequence reverse primer (5'- 3')
AADC	CAGCTATGCTTGCGGACATG	AGCCCAGGAGAAGCCAATG
AChE	AGTATCTTCCGGTTTTCTTCGT	TGCAGGTCTTGAAAATCTCCAGTA
ALDH1A1	TGGCCTTCACGGGATCAA	GCTTTTTCTGCAGCTTCCTT
ChAT	TCCCAGAAGCCTAAGAATGCA	TCCCTTGGCAGCTATGTGAGA
CRABP2	TGTGGATGGGAGACCCTGTAA	CGCACACCATTTTGTTCCTT
CYP26 a1	AAGCGCAGGAAATACGGCTTC	AAGATGCGCCGCACATTATCC
CYP26 b1	ACTCCATTGGCGACATCCA	GGTAGCTCTCGAGTGCCTCATG
D1R	GCCCCGAGGCTCCATCT	ACGGCATGAGGGATCAGGTA
D2R	TTATCATGAAGTCTAATGGGAGTTTCC	CTTGACAGCATCTCCATTTCCA
LRAT	ACCTTCTGAGTGCGTTCCTTG	AGTGTACGGACCCATTTTACC
MAOA	GCTGGAGAACGAGCAGCTAGA	TCCTTCTTCGCGACTTTTCC
MOR1	CATGCTATCGGGCTCCAAAG	CAGCACCATCCGGGTGAT
NLRP3	GTGCGTGGGACTGAAGCAT	GACAACACGCAGATGTGAGACA
NOS 1	CAACTATAGACTCACCCCGTCCTT	GCCAATTTCTTAAAGCCGATAGC
NOS 3	GACCGATTACACGACATTGAGATC	CCGGTAGAGATGGTCCAGTTG
P2RX7	CACCATCAAGTGGATCTTGCA	GCTTGTCGCTCATCAAAGCA
RAR β	AGTGGGCATGTCCAAAGAGTCT	TCTGTGCACTCCTGCTTTGAA
RBP4	TCCAGCGAGGAAACGATGAC	TGCAGAGCGAAGGTGTCGTA
REH	TGAAGTTCTGGGCCAACTTTGC	TGGCACCAATCTGCAATACCC
RXR γ	TTGGAGGACCAGGTCATTCTG	AGAAGGAGGCAATCAGCAATTC
SOD 1	CCATCATTGGCCGTACTATGG	TCCACCTTTGCCCAAGTCA
SOD 2	GCCAAGGGAGATGTTACAACTCA	CCCCGCCATTGAACTTCA
STRA6	TCTACACTCCAGAGCCAGGATTC	CAGGGTGGCCGAGATCAC
B2M	GCGTGGGAGGAGCATCAG	TGTGCTGTAGGCCCAACAGA
GAPDH	GCGTGGGAGGAGCATCAG	TGTGCTGTAGGCCCAACAGA

1047 **Table 2: Detailed statistical analyses.**
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Figure 1	Statistical test	n per group	Outcome measure	Diet effect	Time effect	Interaction	PostHoc test
Figure 1B	2-way RM ANOVA	8	Body weight (g)	F (2, 21) = 0.1735 p=0.842	F (13, 273) = 514.5 p<0.001***	F (26, 273) = 0.08736 P>0.999	/
Figure 1C	2-way ANOVA	5-8	[retinol] _{plasma} (μmol/l)	F (2, 58) = 88.68 p<0.001***	F (2, 58) = 142.1 p<0.001***	F (4, 58) = 14.95 p<0.001***	5IU vs 0.4 and 20 IU, weeks 16 and 26: p<0.001***
Figure 1D	Kruskal-Wallis 1-way ANOVA	7-8	[retinol] _{liver} (μmol/g)	p<0.001***	/	/	20 IU vs 5 IU p=0.048*; 20 IU vs 0.4 IU p<0.001***; 5 IU vs 0.4 IU p=0.032*
Figure 2	Statistical test	n per group	Outcome measure	Diet effect	Time effect	Interaction	PostHoc test
Figure 2B		8	REH _{liver}	p=0.474	/	/	/
Figure 2C	Kruskal-Wallis 1-way ANOVA	7-8	RBP4 _{liver}	p=0.517	/	/	/
Figure 2D	2-way ANOVAs	7-8	LRAT _{liver}	p<0.001***	20 IU vs 0.4 IU p=0.003**	5 IU vs 0.4 IU p<0.001**	/
Figure 2E		3-8	CYP26a1 _{liver}	p=0.004**	20 IU vs 0.4 IU p=0.002**	5 IU vs 0.4 IU p=0.044*	/
Figure 3	Statistical test	n per group	Outcome measure	Diet effect	Time effect	Interaction	PostHoc test
Figure 3B	1-way ANOVA	8	RBP4 _{striatum}	p=0.969	/	/	/
Figure 3C	1-way ANOVA	8	CYP26b1 _{striatum}	p=0.683	/	/	/
Figure 3D	1-way ANOVA	8	CRABP2 _{striatum}	p=0.335	/	/	/
Figure 3E	Kruskal-Wallis 1-way ANOVA	8	STRA6 _{striatum}	p<0.001***	20 IU vs 0.4 IU p<0.001**	5 IU vs 0.4 IU p<0.001**	/
Figure 3F	1-way ANOVA	8	RARβ _{striatum}	p=0.003**	20 IU vs 0.4 IU p<0.001**	5 IU vs 0.4 IU p=0.058	/
Figure 3G	1-way ANOVA	8	RXRγ _{striatum}	p=0.046*	20 IU vs 0.4 IU p=0.037*	5 IU vs 0.4 IU p<0.026*	/
Figure 4	Statistical test	n per group	Outcome measure	Diet effect	Time effect	Interaction	PostHoc test
Figure 4A		8	activity (a.u.)	F (4, 35) = 0.666 p=0.620	F (25, 875) = 62.31 p<0.001***	F (100, 875) = 0.751 p=0.964	/
Figure 4B		8	# touches	F (2, 21) = 0.563 p=0.776	F (6, 126) = 20.62 p<0.001***	F (12, 126) = 1.77 p=0.060 [†]	/
Figure 4C	2-way RM ANOVAs	8	Latency to fall (s)	F (2, 21) = 4.486 p=0.024*	F (5, 105) = 35.2 p<0.001***	F (10, 105) = 1.8 p=0.070 [†]	week3: 5 IU vs 0.4 IU p=0.02, 20 IU vs 0.4 IU p=0.003**
Figure 4D		8	# steps	F (2, 21) = 60.65 p<0.001***	F (7, 147) = 87.14 p<0.001***	F (14, 147) = 7.303 p<0.001***	week 5: 20 IU vs 5 IU p=0.048*, 20 IU vs 0.4 IU p=0.005**
							week 7-26: 20 IU vs 5 IU p<0.001***, 20 IU vs 0.4 IU p<0.001***
							week 10: 5 IU vs 0.4 IU p=0.028*, week 21: 5 IU vs 0.4 IU p=0.007**
Figure 5	Statistical test	n per group	Outcome measure	Diet effect	Training level effect	Interaction	PostHoc test
Figure 5C	Comparison of lognormal fits		Action times (s)	F(3, 714) = 19.68 p<0.001***	/	/	/
Figure 5D	2-way RM ANOVA		# sessions before next level distribution of errors (% trials)	F (1, 22) = 2.68 p=0.116	F (4, 88) = 578.4 p<0.001***	F (4, 88) = 1.191 p=0.321	/
Figure 5E	Chi-square test		% Good trials	X ² = 1.909, Df = 4 p=0.753	/	/	/
Figure 5F		0.4 IU: n = 18; 20 IU: n = 6	% anticipations	F (1,22) = 3.23 p=0.08 [†]	F (3,66) = 61.33 p<0.001***	F (3,66) = 0.37 p=0.59	/
Figure 5G			% reactions of out time	F (1,22) = 0.65 p=0.42	F (4,88) = 0.58 p=0.30	F (4,88) = 0.26 p=0.54	/
Figure 5H	2-way RM ANOVA		% movements out of time	F (1,22) = 0.51 p=0.51	F (1,22) = 1.32 p=0.23	F (1,22) = 1.97 p=0.23	/
Figure 5I	bootstrapped with R = 100		% Good right trials	F (1,22) = 0.87 p=0.34	F (1,22) = 0.53 p=0.49	F (1,22) = 0.22 p=0.64	/
Figure 5J			% press of wrong levers (right trials)	F (1,22) = 5.14 p=0.03*	F (3,66) = 53.01 p<0.001***	F (3,66) = 1.07 p=0.32	/
Figure 5K			% wrong paw (right trials)	F (1,22) = 2.87 p=0.07 [†]	F (2,44) = 16.94 p<0.001***	F (2,44) = 0.97 p=0.36	/
Figure 5L	Mann-Whitney test		% wrong paw (right trials)	p=0.177	/	/	/
Figure 6	Statistical test	n per group	Outcome measure	Diet effect	Time effect	Interaction	PostHoc test
Figure 6A		8	AADC; MAOA; PP1R1B; DRD1; DRD2; DRD3	p=0.083*; 0.328; 0.859; 0.505; 0.083*; 0.426	/	/	/
Figure 6D	Mann-Whitney tests	6-8	TH intensity in striatum anterior ; intermediate; posterior	p=0.804; 0.754; 0.694	/	/	/
Figure 6E		6-8	ALDH1 intensity in striatum anterior ; intermediate; posterior	p=0.804; 0.662; 0.231	/	/	/
Figure 7	Statistical test	n per group	Outcome measure	Diet effect	striatum level	Interaction	PostHoc test
Figure 7A	Mann-Whitney tests	6-8	CB1; ADORA2A; ChAT; AChE; ChRM1; ChRM4; MOR; Nur77;	p=0.536; 0.591; 0.007**	/	/	/
Figure 7C	Unpaired t-test	7-6	#ChAT ⁺ neurons / μm ²	t=0.441 df=11 p=0.668	/	/	/
Figure 7E	2-way ANOVA	4-8	MOR ⁺ area (% total striatum)	F (1, 19) = 5.306 p=0.033*	F (1, 19) = 3.703 p=0.069 [†]	F (1, 19) = 0.9704 p=0.337	/
Figure 8	Statistical test	n per group	Outcome measure	0.4/20 IU vs. 0.4 IU	0.4/20 IU vs. 20 IU	Interaction	PostHoc test
Figure 8A		7-8	[retinol] _{pl} ; [retinol] _{lv} ; LRAT _{liver} ; CYP26a1 _{liver}	p=0.001***; 0.031*; 0.003**	p=0.999; 0.140; 0.999; 0.640	/	/
Figure 8B	Kruskal-Wallis ANOVA, Dunn's multiple comparison	8	STRA6 _{str} ; RARβ _{str} ; RXRγ _{str}	p=0.005**	p=0.959; 0.358; 0.516	/	/
Figure 8C		4-8	MOR _{str} ; MOR ⁺ area; ChAT _{str}	p=0.931; 0.350; 0.1217	p=0.246; 0.999; 0.772	/	/
Figure 8	Statistical test	n per group	Outcome measure	Diet effect	Time effect	Interaction	PostHoc test
Figure 8D	2-way RM ANOVA	8	# steps	F (2, 14) = 79.85 p<0.001***	F (7,49) = 67.06 p<0.001***	F (14,98) = 10.87 p<0.001***	weeks 4-19: 20 IU vs 0.4 IU and 20 IU vs 0.4/20 IU; p<0.011*
Figure 8E	Chi-square test	6-10	distribution of errors (% trials)	X ² = 10.04, Df = 8 p=0.2621	/	/	weeks 21-26: 20 IU vs 0.4 IU and 0.4/20 IU p<0.001***
Figure 8F	Comparison of lognormal fits	6-10	Action times (s)	F(6, 687) = 8.825 p<0.001***	/	/	0.4 vs 0.4/20 IU p<0.001***

Supplemental Table 1: Detailed analysis for supplemental Figure 1.

Supplemental Figure 1	Statistical test	n	Outcome measure	Diet effect
Supp. Fig.1A: P2RX7 Striatum expression	Mann-Whitney test	8	Realtive quantity	p=0.1692
Supp. Fig.1B: NLRP3 Striatum expression	Mann-Whitney test	8	Realtive quantity	p=0.7984
Supp. Fig.1C: NOS 1 Striatum expression	Mann-Whitney test	8	Realtive quantity	p=0.009 **
Supp. Fig.1D: NOS 3 Striatum expression	Mann-Whitney test	8	Realtive quantity	p=0.458
Supp. Fig.1E: SOD 1 Striatum expression	Mann-Whitney test	8	Realtive quantity	p=0.078 #
Supp. Fig.1F: SOD 2 Striatum expression	Mann-Whitney test	8	Realtive quantity	p=0.645