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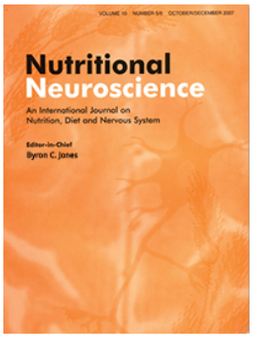

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Neuronal morphology and synaptic plasticity in the hippocampus of vitamin A deficient rats

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ABSTRACT

Vitamin A (retinol) and related retinoids are micronutrients provided by food. Retinol derivatives are growth factors important for development, cell differentiation and tissue homeostasis, especially in the brain.

Objective: The hippocampus is a pivotal brain structure for learning and memory and hippocampal-dependent memory is highly sensitive to retinoids action. However, the underlying mechanisms are still unclear. In this study, we characterized the impact of vitamin A deficiency on memory and neuronal plasticity, focusing on the CA1 region of the hippocampus in rats.

Methods: Weaned male Wistar rats were fed a control (5 UI/g) or deficient vitamin A diet (0 UI/g) for 10 weeks. The effect of vitamin A supplementation (20 UI/g) for 3 weeks was also tested. Memory performances were assessed in the Y-maze ($n = 24–30$/group), retinoic acid levels were measured (LC-MS/MS) in the serum and in the hippocampus ($n = 5$/group), CA1 neuronal architecture was analyzed with Golgi staining ($n = 17–20$ neurons/group) and electrophysiological patch-clamp recordings were performed on hippocampal brain slices ($n = 6–11$/group).

Results: Vitamin A deficiency from weaning significantly lowered hippocampal levels of retinoic acid, reduced dendritic length and branching of CA1 pyramidal neurons and decreased spontaneous glutamatergic synaptic events and synaptic plasticity. When replenishment with moderate dose of dietary vitamin A for 3 weeks was done, most of the synaptic and morphological alterations were absent.

Conclusion: This study provides new mechanistic insight to understand the critical role of retinoic acid in hippocampal function.

KEYWORDS

Vitamin A; retinoic acid; hippocampus; memory; neuron morphology; synaptic network

Abbreviation list

ACSF	artificial cerebral spinal fluid
EPSC	excitatory post-synaptic current
fEPSP	field excitatory post-synaptic potential
HFS	high-frequency stimulation
LC-ESI-MS/MS	liquid chromatography Electrospray Ionization Tandem Mass Spectrometric
MRM	multiple reaction monitoring
RA	retinoic acid
VAD	vitamin A deficient
Vit. A	vitamin A

1. Introduction

Vitamin A (retinol) and its precursors are micronutrients provided by food, such as dairy products, meat and vegetables. The retinol derivative, retinoic acid (RA), is a transcription factor that regulates expression of hundreds of genes for normal embryogenic development and cell differentiation [1,2]. At adulthood, retinoids participate in the homeostasis of several functions, such as vision, immunity and reproduction, and they are also critical for brain functions [3–6].

The hippocampus is a brain structure playing a pivotal role in spatial and relational learning and memory and, in the adult brain, is highly sensitive to retinoid

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action. This region is equipped with the molecular machinery for retinoid metabolism and signaling [4,7–9], and it contains high levels of RA [10–13]. In particular, the sensitivity of hippocampus to RA action at adulthood has been investigated through post-weaning dietary vitamin A deficiency, which led to strong impairment of diverse hippocampal-dependent memory [14–20]. Importantly, replenishment with vitamin A or its derivatives, delivered through the diet or injected, can reverse these behavioral effects [14–18,20].

The ability of the hippocampus to enable learning and memory is determined by the plasticity of its neuronal network, including synaptic efficiency, neuronal morphology and neurogenesis [4]. Despite evidence that vitamin A deficiency at adulthood lowers hippocampal neurogenesis [14,15], characterization of the cellular impact of vitamin A deficiency at adulthood on hippocampus remains unclear. As retinoids are crucial for neuronal morphology during perinatal development [21,22], we hypothesize that vitamin A deficiency at adulthood alters hippocampal function and synaptic plasticity [18,23,24] through changes of neuronal morphology.

In this study, we investigated the impact of vitamin A deficiency on hippocampal levels of retinoic acid, neuronal morphology and synaptic plasticity in CA1 region of the hippocampus as well as hippocampal-dependent spatial memory. This study provides new mechanistic insight to understand the critical role of retinoic acid in hippocampal function.

2. Methods

2.1. Animals and diet

All experiments were performed in accordance to criteria of the European Communities Council Directive and the French National Committee (4184-2016022209565094). The vitamin A deficiency model was performed as previously described [14]. Experiments were performed on male Wistar rats obtained from Janvier Labs (France). Weaned rats (55 g ± 10%) were maintained under standard housing conditions in a temperature – (23 ± 1°C) and humidity – (40%) controlled animal room with a 12-h light/dark cycle (0700–1900 h), with ad libitum access to food and water. Rats were housed 2 per cage and were weighted weekly. All rats received a custom diet upon weaning (Figure 1(A)). Group 1 and 2 were fed with control diet, containing 5 IU retinol/g (INRA, Jouy-en-Josas), while group 3 and 4 received a vitamin A deficient diet with 0 IU retinol/g (Laboratorio Piccionni, Italy). At week 10, groups 2 and 4 were switched to an enriched diet containing 20 IU retinol/g (INRA, Jouy-en-Josas). The three diets were isocaloric and identical, except the amount of retinol. All animals were sacrificed 13-4 weeks after weaning for subsequent analyses.

2.2. Behavioral assessment (Y-maze test)

Spatial memory was assessed at week 13 after weaning. The Y-maze paradigm was used to assess spatial memory as previously described [10,25]. Rats were handled every day for 5 days before the test. All tests were conducted in a sound-attenuated separate experimental room. Behavioral sessions were recorded with a ceiling-mounted video camera and analyzed using tracking software (ViewPoint Behavioral Technology). The apparatus was a Y-shaped maze 34-cm long, 8-cm wide and 14-cm high. The floor of the maze was covered with litter from the home cages of all animals and was mixed between sessions to scramble olfactory cues. Visual cues were placed in the testing room and kept constant during the whole test. In the first trial of the test, one arm of the Y-maze was closed with a guillotine door and rats were allowed to visit two arms of the Y-maze for 5 min. Closed arms were randomly assigned for each rat. After 2 h of inter-trial interval, rats were placed back in the start arm and allowed for free access to the three arms for 5 min. Data are presented as the time spent exploring the novel arm during the 5 min of the second trial compared to familiar and start arms. This memory test was performed a single time, 13 weeks after weaning, due to the fact that repetitions of the Y-maze test can interfere with behavioral performances of the animals.

2.3. RA levels measurements

Measurements were performed as previously described [10]. Briefly, RA was extracted by a two-step acid–base extraction and acitretin (5 µM, Sigma-Aldrich) was added as internal standard into samples to quantify extraction efficiency. LC-MS/MS (MRM mode) analyses were performed with a mass spectrometer model QTRAP® 5500 (Sciex, Villebon sur Yvette, France) coupled to a LC system (LC-20AD XR pump (Shimatzu, Marne-la-Vallée, France) and PAL HTC-xt Autosampler (CTC Analytics, Zwingen, Switzerland)). Extracts were dissolved in 35 µL of CH_3CN/H_2O 80/20. MS/MS experiments were performed by following two MRM transitions for RA (301.1/123.2 and 301.1/161.1) and acitretin (327.2/131.0 and 327.2/159.1). The area of LC peaks was quantified with MultiQuant software (v2.1, Sciex). Reversed phase separations were carried out on an Ascentis RP Amide 150 × 1 mm

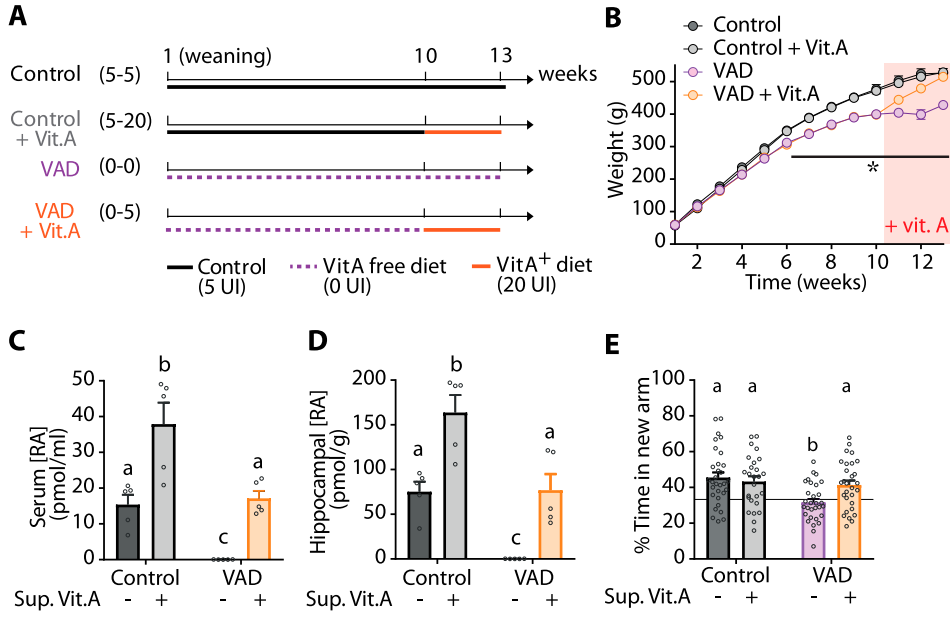

Figure 1. Impact of dietary vitamin A on spatial memory. A. Experimental design. B. Weight gain curve for control (dark grey), control supplemented (Control + Vit.A, light grey), deficient (VAD, purple) and deficient/supplemented (VAD + Vit.A, orange). Orange shadowing from week 10 represents duration of supplementation. Repeated measures 2-way ANOVA with Holm-Sidak post-hoc test, $n = 14$ / group, $^*p < 0.05$. C, D, Concentration of RA measured by LC-MS/MS in the serum (C) and in the hippocampus (D). Two-way ANOVA with Holm-Sidak post-hoc test, $n = 5$ / group, a–c: values significantly different. E. Proportion of time spent in the new arm, compared to the two other arms (familiar and start arms). Two-way ANOVA with Holm-Sidak post-hoc test, $n = 26–31$/group, a–c: values significantly different. Histogram are represented as mean ± SEM with individual data points. Points and connecting lines are represented as mean ± SEM. Sup. Vit.A: vitamin A supplementation.

column, with 3 µm particles (Supelco, Sigma Aldrich, St Quentin Fallavier, France), with a gradient elution program at a flow rate of 50 µL/min (10 µL injected/sample). The gradient elution program was as follows: 0 minutes, 30% B; 8 minutes, 30% B; 10 minutes, 70% B; 35–36 minutes, 87% B; 37 minutes, 30% B, with eluent A being H_2O + 0.1% formic acid and eluent B CH_3CN + 0.1% formic acid. For each experiment, a standard curve consisting of triplicated extracted all-trans RA (Sigma-Aldrich) samples of known concentrations (0.07–2.5 µM) was used to convert LC peaks area onto RA concentrations.

2.4. Neuronal morphology analysis

Golgi-Cox staining. Rats ($n = 4$/group) were deeply anesthetized with isoflurane and brain was removed after decapitation. Four animals per group were used. Brains were processed according to the Golgi-Cox kit guidelines (PK401 FD Rapid Golgi Stain KIT, Neurotechnologies INC, Paris, France) as previously described [26]. Briefly, one hemisphere per brain was immersed in Golgi-Cox A + B solution for 10 days, then in solution C for 2 days before deep frozen in isopentane at −70°C. Brains were stored at −80°C for 6 days. Twelve coronal sections of 200 µm thickness containing the dorsal hippocampus were collected for each rat at −22°C using a cryostat (Leica, Solms, Germany). Two days later, dried slices were stained with solutions D and E, dehydrated and coverslipped with Depex. Light exposure was limited throughout the whole process.

Structural analysis. Images were obtained at the Bordeaux Imaging Center (CNRS-INSERM and Bordeaux University, France BioImaging) with Nanozoomer slide scanner (Hamamatsu Nanozoomer 2.0 HT) and analyzed using Imaris software (Bitplane, Oxford Instrument Compagny). The experimenter remained blind to the treatment conditions throughout the procedure. Complexity of dendritic tree was analyzed in pyramidal neurons of the CA1 hippocampus. For each rat, 4–7 neurons were analyzed. Therefore, a total of 17–20 neurons were analyzed in each group.

Dendritic morphology. Briefly, NDPI images at X 20 magnification (one image every 2 µm in the z axis) obtained with Nanozoomer were converted into TIFF format using the ImageJ software (https://imagej.nih.gov/ij/) and NDPI tools plugin [27]. For each selected neuron, all branches of the dendritic tree were semi-automatically reconstructed and manually completed in 3D using the Imaris software. Analyzed parameters were dendrite length, number of dendrite branches and sholl analysis.

2.5. Electrophysiological recordings

Hippocampus slice preparation. Coronal hippocampal slices (350 µm) were prepared as previously described [28] with a vibrating blade microtome (VT1000S, Leica Microsystems) in an oxygenated artificial cerebrospinal fluid (ACSF) containing in (mM): 25 $NaHCO_3$, 87 NaCl, 75 Sucrose, 25 Glucose, 4 $MgCl_2$, 2.5 KCl, 0.5 $CaCl_2$, 1.25 NaH_2PO_4. Slices were stored at 34°C for 30 min, then at room temperature and recordings were started after at least 1 hour of rest. ACSF solution for storage and recording contained (in mM): 25 $NaHCO_3$, 125 NaCl, 25 Glucose, 1 $MgCl_2$, 2.5 KCl, 2 $CaCl_2$, 1.25 NaH_2PO_4.

Electrophysiological recordings. Recordings were performed at 30°C using a temperature control system (TC-344B, Warner Instrument Corporation) and slices were continuously superfused at 1–2 ml/min with recording ACSF. Recordings were performed with glass borosilicate glass pipettes of 4–6 MΩ resistance filled with recording ACSF. For whole-cell patch-clamp recordings, CA1 pyramidal neurons were visualized using an infrared microscope (BX-51WI, Olympus). Spontaneous excitatory post-synaptic potentials (sEPSCs) were recorded at −70 mV with pipettes filled with balanced intracellular solutions (in mM): 128 cesium methane-sulfonate (CH_3O_3SCs), 20 NaCl, 1 $MgCl_2$, 1 EGTA, 0.3 $CaCl_2$, 2 Na^{2+}-ATP, 0.3 Na^{+}-GTP, 10 glucose buffered with 10 HEPES, pH 7.3, osmolarity 290 mOsm. Extracellular field excitatory postsynaptic potentials (fEPSPs) were recorded in the CA1 stratum radiatum. Synaptic events were evoked by stimulation (150 µs, 0.1 Hz) of the schaffer collateral pathway afferents in CA3 region of the hippocampus bipolar concentric electrode from Phymep (France), and stimulator A365 from WPI (USA).

Data acquisition and analysis. Signals were amplified using Multiclamp 700B amplifier (Molecular Devices) controlled with pClamp 10.7 software via a Digidata 1440A interface (Molecular Devices). Data were digitized at 20 kHz for field recordings and 200 kHz for voltage-clamp recordings and filtered at 5 kHz. For spontaneous excitatory activities, sEPSCs were measured for 1 min at −70 mV. Amplitude and inter-event interval time were analyzed with Clampfit 10.7 using the template search function. The threshold of amplitude detection was set at −10 pA. For field recordings, stable synaptic responses were recorded for 10 min at 0.1 Hz, with a stimulus intensity of 40–50% of the maximum fEPSP. Two different High frequency stimulation (HFS) protocols were used to elicit potentiation: 2 stimulation trains at 100 Hz for 1 sec with 20 s interval between the 2 stimulation trains in presence of picrotoxin (50 µM, Sigma-Aldrich) or 3 stimulation trains at 100 Hz for 1 sec with 20 s interval, without picrotoxin. Comparison revealed no difference between the two protocols (Mann Whitney tests, control: U = 8, $p = 0.2468$; control + vitamin A: U = 14, $p = 0.8081$; vitamin A deficient: U = 26, $p = 0.8665$; deficient / supplemented: U = 22, $p = 0.3282$), therefore data were pooled. After HFS protocol, synaptic events were recorded for up to 40 min at 0.1 Hz. For each experiment, fEPSP amplitude was normalized to baseline (10 min before HFS protocol).

2.6. Statistics

Statistical tests were performed with GraphPad Prism 7.0 (GraphPad software, San Diego, CA, USA) using a critical probability of $p < 0.05$. All values are given as mean ± s.e.m. Normality of data was tested with D'Agostino & Pearson normality tests. As appropriate, we used 2-way ANOVA or repeated measures 2-way ANOVA, with Holm-Sidak's multiple comparison test as post hoc tests. Correlation was performed with Pearson test (parametric data).

3. Results

3.1. Impact of dietary vitamin A on weight gain

To investigate the role of dietary vitamin A and its derivative retinoic acid (RA) on hippocampal function, we used the established vitamin A deficient rat model. At weaning, rats were divided into 4 groups. Two groups received a diet deficient for vitamin A (0 IU/g) while the two other groups received a control diet (5 IU/g of vitamin A). At week 10, half of the rats in each group received a diet enriched in vitamin A (20 IU/g) for 3 weeks (Figure 1(A)).

Figure 1(B) shows that weight gain in vitamin A deficient rats decreased from week 4 of diet, compared to control groups, while supplementation rapidly rescued it. Repeated-measures ANOVA revealed a significant effect of the diet ($F(3, 39) = 15.89$, $p < 0.0001$) with a significant interaction with time factor ($F(33, 429) = 13.26$, $p < 0.0001$). Multiple comparison further showed that the weight of VAD rats was significantly lower from week 4 of dietary treatment (Holm-Sidak's multiple comparison test: DF = 429, $t = 2.905$, $p = 0.226$; Figure 1(B)). Three weeks of vitamin A supplementation from week 10 significantly increased weight gain in deficient animals ($t = 10.36$, $p < 0.0001$), but not to the levels of control animals ($t = 5.784$, $p < 0.0001$; Figure 1(B)). Vitamin A supplementation in control animals did not affect weight gain ($t = 1.144$, p=0.2533; Figure 1(B)).

3.2. Modulation of serum and hippocampal retinoic acid concentrations by dietary vitamin A

RA levels in serum and hippocampus were measured with designed LC-ESI-MS/MS method using Multiple Reaction Monitoring (MRM). As expected, RA levels were totally abolished in the serum after 13 weeks of deficient diet ($F(1, 16) = 27.32$, $P < 0.0001$, Figure 1(C)). Conversely, vitamin A supplementation for 3 weeks increased serum levels of RA in both control and deficient rats ($F(1, 16) = 32.88$, $p < 0.0001$, Figure 1(C)), without interaction between factors ($F(1, 16) = 0.6021$, $p = 0.4491$).

In the hippocampus, RA was nearly undetectable in deficient rats, which was reversed by vitamin A supplementation (Figure 1(D), deficiency factor: $F(1, 16) = 31.89$, $p < 0.0001$; supplementation factor: $F(1, 16) = 33{,}14$, $p < 0.0001$; interaction: $F(1, 16) = 0.1657$, $p = 0.6894$). Vitamin A supplementation also increased levels of RA in control rats (Figure 1(D)).

3.3. Modulation of memory performance in Y-maze test by dietary vitamin A

Rats were allowed to explore two arms in a Y-maze for 5 min. Two hours later, rats were placed for 5 min in the Y-maze with free access to the three arms. In this test, rats with intact spatial memory explored longer the newly opened arm. In proportion, 90% of control rats ($n = 29$ rats) and supplemented control rats ($n = 24$ rats) visited the new arm first. By comparison, 47% of deficient rats ($n = 30$ rats) chose the new arm at first, against 83% of deficient-supplemented rats ($n = 29$ rats). In proportion of time spent in the Y-maze, control rats and controls supplemented rats explored significantly more the new arm, compared to the two other arms (control: $t = 4.338$, df = 30, $p = 0.0001$, control + vitamin A: $t = 3.487$, df = 25, $p = 0.0018$, Figure 1(E)). Conversely, vitamin A deficient rats did not discriminate the newly opened arm and spent similar time in all arms ($t = 0.8639$, df = 29, $p = 0.3947$, Figure 1(E)). Deficient-supplemented rats were able to significantly discriminate the new arm ($t = 3.174$, df = 28, $p = 0.0036$, Figure 1(E)). Two-way ANOVA analysis revealed that memory performance in the Y-maze was significantly different for deficient rats, compared to the three other groups (interaction: $F(1, 112) = 5.412$, $p = 0.0218$; deficiency factor: $F(1, 112) = 9.612$, $p = 0.0024$; supplementation factor: $F(1, 112) = 2.138$, $p = 0.1464$; Holm-Sidak's multiple comparison test: DF = 112, *vs* control: $t = 3.943$, $p = 0.0008$, *vs* control + vitamin A: $t = 3.169$, $p = 0.0098$, *vs* VAD + vitamin A: $t = 2.708$, $p = 0.0310$).

3.4. Modulation of neuronal morphology by dietary vitamin A

Here we hypothesize that brain availability for RA modulates memory performance through dendritic tree modeling. To test our hypothesis, we analyzed three-dimension morphology of pyramidal neurons in the CA1 region of the hippocampus with Golgi-Cox staining. Total length of cumulated dendrites was different between control and vitamin A deficient rats (deficiency factor: $F(1, 73) = 4.067$, $p = 0.0474$, Figure 2(A)). Additionally, total dendritic length was higher with vitamin A supplementation, for both control and deficient rats (supplementation factor: $F(1, 73) = 4.067$, $p = 0.0134$), without interaction between factors (interaction: $F(1, 73) = 0.6863$, $p = 0.4101$, Figure 2(A)).

To more deeply analyze the morphology of dendritic tree and segregate proximal from distal dendrites, dendrite lengths were measured independently for each dendrite level. Figure 2(B) shows that deficient rats, supplemented or not, had shorter length for secondary and tertiary dendrites, compared to control groups. Repeated measures ANOVA revealed a dendrite level effect and a group effect, without interaction (dendrite level effect: $F(7, 511) = 105.3$, $p < 0.0001$; group effect: $F(3, 73) = 3.928$, $p = 0.0117$; interaction: $F(21, 511) = 0.5379$, $p = 0.9549$).

Additionally, we analyzed the complexity of dendrite ramifications by measuring the number of branch points for each neuron. Figure 2(C) shows a reduction in the number of branch points for deficient rats that is not observed for deficient-supplemented rats. Two-way ANOVA showed a significant effect of both factors, without interaction (deficiency effect: $F(1, 73) = 9.077$ $p < 0.0036$; supplementation effect: $F(1, 73) = 5.19$, $p = 0.0256$; interaction: $F(1, 73) = 0.5827$, $p = 0.5827$). Multiple comparisons showed a significance difference between deficient and control-supplemented rats (Holm-Sidak's multiple comparison test: DF = 73, *vs* control: $t = 2.576$, $p = 0.0587$, *vs* control + vitamin A: $t = 3.665$, $p = 0.0028$, *vs* VAD + vitamin A: $t = 2.045$, $p = 0.1664$).

Finally, Sholl analysis was applied, with radius increment of 20 µm, up to 800 µm distance from the soma of CA pyramidal neurons. Figure 2(D) showed a clear reduction in the number of Sholl intersections for deficient rats, at distance between 100 and 240 µm from the soma, compared to other groups. Repeated measures ANOVA revealed a radius effect and a significant interaction, without group effect (radius effect: $F(40, 2920) = 209.2$, $p < 0.0001$; group effect: $F(3, 73) = 1.807$, $p = 0.1534$; interaction: $F(120, 2920) = 1.335$, $p = 0.0098$). Multiple comparisons explained that deficient rats had significantly less Sholl intersections from 80-

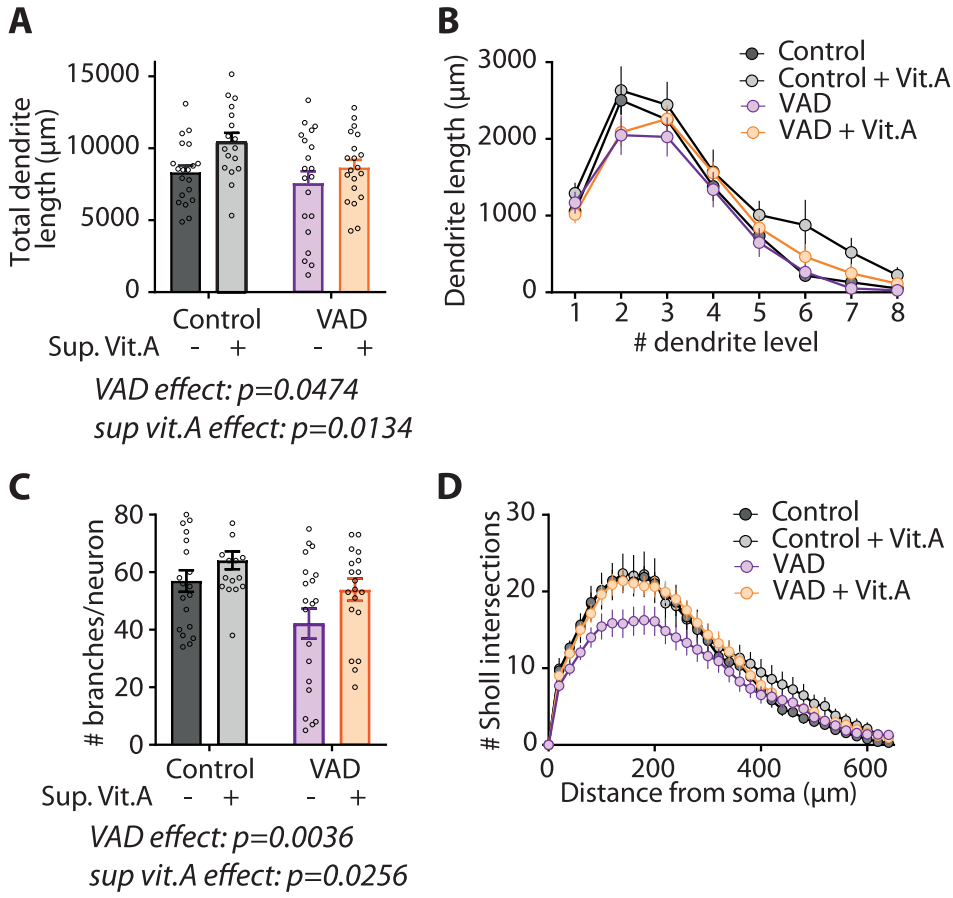

Figure 2. Impact of dietary vitamin A on dendrite tree of CA1 pyramidal neurons. A. Total dendrite length measured for CA1 pyramidal neurons, two-way ANOVA, $n = 17–20$/group. B. Dendrite length per dendrite level, repeated measures two-way ANOVA, $n = 17–20$/group. C. Total number of branches per neuron, two-way ANOVA, $n = 17–20$/group. D. Distribution of the number of Sholl intersections along the dendrite tree, repeated measures two-way ANOVA, $n = 17–20$/group. Histograms are represented as mean ± SEM with individual data points. Points and connecting lines are represented as mean ± SEM. Sup. Vit.A: vitamin A supplementation. Control, dark grey, control supplemented (Control + Vit.A), light grey, deficient (VAD), purple, and deficient/supplemented (VAD + Vit.A), orange.

µm radius to 240-µm radius, compared to control rats (Holm-Sidak's multiple comparison test: DF = 2993, $t = 2.73–3.353$ $p < 0.0377–0.0040$). Deficient rats differed significantly from control-supplemented rats between 100 and 180 µm (Holm-Sidak's multiple comparison test: DF = 2993, $t = 2.622–3.727$ $p < 0.0432–0.0012$). Deficient rats were also significantly different from deficient-supplemented rats for radius between 120 and 240 µm (Holm-Sidak's multiple comparison test: DF = 2993, $t = 2.641–3.293$ $p < 0.0329–0.0040$), but deficient-supplemented rats were not different from control groups.

3.5. Modulation of excitatory current in CA1 neurons by dietary vitamin A

Dendrite tree determines the number of synapses that can be formed onto neurons. To better understand the functional implication of dendrite remodeling induced by vitamin A deficiency, we patch-clamp recorded spontaneous excitatory synaptic events (sEPSCs) in CA1 hippocampal brain slices (Figure 3). As shown in Figure 3(A–C), frequency of sEPSCs was reduced in deficient rats, supplemented or not. Two-way ANOVA analysis showed a significant effect of deficiency, without effect of supplementation factor nor interaction (deficiency factor: $F(1, 32) = 5.316$, $p = 0.0278$; supplementation factor: $F(1, 32) = 0.01955$, $p = 0.8897$; interaction: $F(1, 32) = 0.4728$, $p = 0.4966$).

Cumulative distribution of inter-event intervals (Figure 3(C)) confirms that vitamin A deficient rats, with or without supplementation, displayed a decreased occurrence in sEPSCs. Repeated measures ANOVA revealed an inter-event intervals effect and a significant interaction, without group effect (inter-event intervals effect: $F(40, 1280) = 304.3$, $p < 0.0001$; group effect: $F(3, 32) = 2.775$, $p = 0.0572$; interaction: $F(120, 1280) = 2.064$, $p < 0.0001$). Multiple comparisons showed that vitamin A deficient rats were different from control rats for inter-event intervals comprised between 750

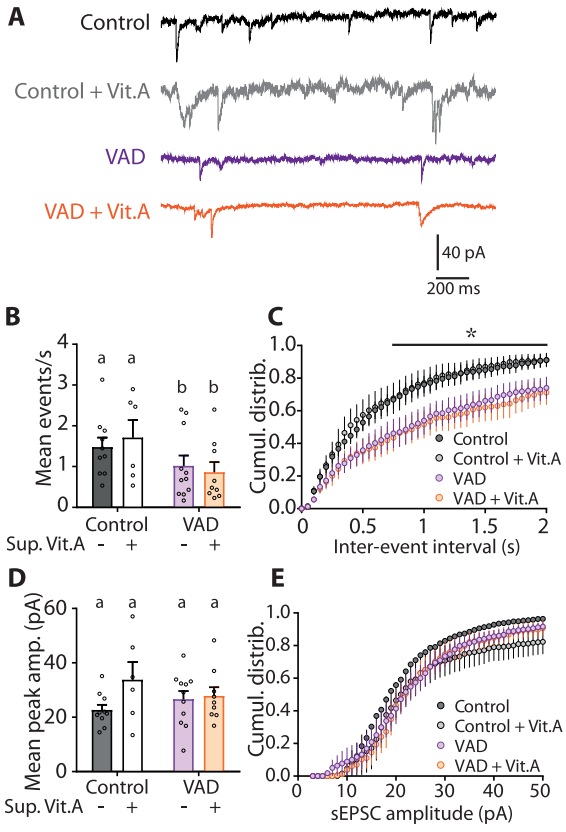

Figure 3. Impact of dietary vitamin A on spontaneous synaptic activity in CA1 pyramidal neurons. A. Representative traces of spontaneous synaptic activity in CA1 neurons. Scale bar: 40 pA, 200 ms. Control, dark grey, control supplemented (Control + Vit.A), light grey, deficient (VAD), purple, and deficient/supplemented (VAD + Vit.A), orange. B. Mean frequency (events/s) of sEPSCs recorded for 1 min. Two-way ANOVA with Holm-Sidak post-hoc test, $n = 6$–11/group a–c: values significantly different. C. Cumulative distribution of inter-event intervals. Repeated measures two-way ANOVA with Holm-Sidak post-hoc test, $n = 6$-11 / group, $^*p < 0.05$ D. Mean amplitude of sEPSCs (pA), Two-way ANOVA, $n = 6$–11/group. E. Cumulative distribution of sEPSC amplitude. Repeated measures two-way ANOVA, $n = 6$–11/group. Histogram are represented as mean ± SEM with individual data points. Points and connecting lines are represented as mean ± SEM. Sup. Vit.A: vitamin A supplementation. Control, dark grey, control supplemented (Control + Vit.A), light grey, deficient (VAD), purple, and deficient/supplemented (VAD + Vit.A), orange.

and 1350 ms (Holm-Sidak's multiple comparisons test: DF = 1312, $t = 2.221$–2.625, $p < 0.0494$–0.0261).

Peak amplitudes of sEPSCs recorded in CA1 pyramidal neurons were not different between groups, as shown in Figure 3(A, D, E). Two-way ANOVA analysis revealed no effect of both factors and no interaction (deficiency factor: $F(1, 32) = 0.08136$, $p = 07773$; supplementation factor: $F(1, 32) = 3.093$, $p = 0.0882$; interaction: $F(1, 32) = 2.031$, $p = 0.1638$). Cumulative distribution of peak amplitudes (Figure 3(E)) further showed that peak amplitude of sEPSCs recorded in CA1 pyramidal neurons displayed similar distributions between groups (repeated measures ANOVA, peak amplitude effect: $F(77, 2464) = 277.5$, $p < 0.0001$; group effect: $F(3, 32) = 1.01$, $p = 0.9125$; interaction: $F(231, 2464) = 0.6334$, $p > 0.9999$).

3.6. Modulation of long-term plasticity at CA3-CA1 synapses by dietary vitamin A and spatial memory

Finally, we measured potentiation of field excitatory post-synaptic potentials (fEPSPs) in CA1 region following high-frequency stimulation (HFS) protocol (Figure 4). In control rats, HFS induced a long-lasting potentiation of fEPSPs in CA1 (one sample t-test with theoretical mean of 100, $t = 3.628$, df = 10, $p = 0.0046$), but not in deficient rats ($t = 1.983$, df = 14, $p = 0.0673$; Figure 4(A–D)). This stimulation protocol also induced a potentiation of fEPSP in control-supplemented ($t = 4.456$, df = 11, $p = 0.0010$) and deficient-supplemented rats ($t = 2.409$, df = 15, $p = 0.0293$; Figure 4(A–D)).

We then analyzed fEPSP plasticity in animals that were firstly tested in the Y-maze apparatus (Figure 4 (E–G)). Figure 4(E) showed that control rats did not exhibit potentiation, while deficient rats displayed more potentiation. Statistical analysis confirmed the absence of potentiation in control rats following Y-maze (one sample t-test with theoretical mean of 100, $t = 0.1963$, df = 9, $p = 0.8487$; Figure 4(G)). However, statistics revealed only a trend for potentiation in deficient rats ($t = 2.2589$, df = 5, $p = 0.0736$; Figure 4 (G)). In supplemented rats firstly tested in the Y-maze, potentiation was significant in both control-supplemented ($t = 3.028$, df = 8, $p = 0.0164$) and deficient-supplemented ($t = 2.869$, df = 5, $p = 0.0350$; Figure 4(F, G)).

When we compared potentiation following Y-maze test or not, we found a significant interaction between control and vitamin A deficient groups ($F(1, 38) = 9.346$, $p = 0.0041$, Figure 4(H)) that was absent for supplemented rats $F(1, 39) = 0.6697$, $p = 0.4181$; Figure 4 (I)). Finally, we found a significant correlation between the time spent in the new arm of the y-maze test, and the normalized value of fEPSP amplitude following HFS stimulation (Pearson $r = -0.6691$, $p = 0.0046$).

4. Discussion

This study shows that vitamin A deficiency in rats alters morphological and electrophysiological synaptic plasticity in the CA1 region of the hippocampus. Indeed, dendrite branching of pyramidal neurons was reduced, synaptic network onto these neurons was decreased,

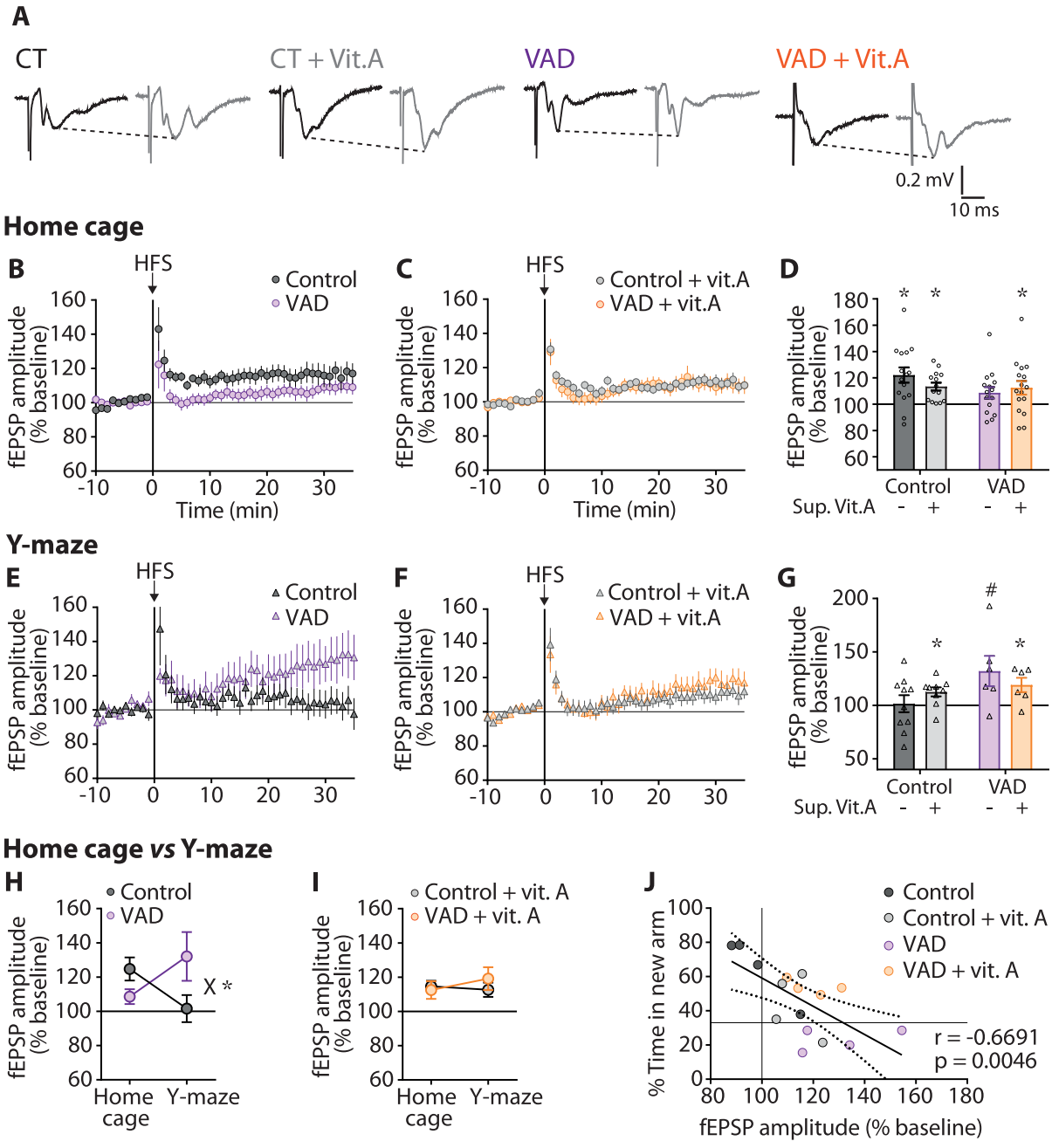

Figure 4. Impact of dietary vitamin A on field potential potentiation at CA3-CA1 synapse. A. Representative fEPSP recordings before (black) and after HFS protocol (grey). Scale bar: 0.2 mV, 10 ms. B-D. Results obtained in 'home cage' rats, that were not assessed in Y-maze test. B, C. Time course of mean fEPSP amplitude, normalized to baseline for control (dark grey) and deficient (VAD, purple) groups (B), and for supplemented control (Control + Vit.A, light grey) and supplemented deficient (VAD + Vit.A, orange) groups (C). Each dot represents mean ± SEM for 1 min. D. Mean fEPSP amplitude normalized to baseline. One sample *t*-test with the theoretical mean of 100, *n*=11-16 / group, $*p < 0.05$. E-G. Results obtained in 'Y-maze' rats, that were assessed in Y-maze before plasticity experiments. E, F. Time course of mean fEPSP amplitude, normalized to baseline for control (dark grey) and deficient (VAD, purple) groups (E), and for supplemented control (Control + Vit.A, light grey) and supplemented deficient (VAD + Vit.A, orange) groups (F). Each dot represents mean ± s.e.m for 1 min. G. Mean fEPSP amplitude normalized to baseline. One sample *t*-test with the theoretical mean of 100, *n*=6-10 / group, $*p < 0.05$, # = 0.0736. H, I, comparison of fEPSP normalized amplitude following HFS protocol in 'home cage' *vs* 'Y-maze' rats, for control (dark grey) and deficient (VAD, purple) groups (H), and for supplemented control (Control + Vit.A, light grey) and supplemented deficient (VAD + Vit.A, orange) groups (I). Two-way ANOVA with Holm-Sidak post-hoc test. J. Percentage of time spent in the new arm of the Y-maze plotted against normalized fEPSP amplitude after Y maze task and HFS protocol. 1–3 HFS experiments were averaged for each rat. Linear regression (dark straight line) with 95% confident interval (dark dashed curved lines) and Pearson correlation. Histogram are represented as mean ± SEM with individual data points. Sup. Vit.A: vitamin A supplementation.

and plasticity was not induced by HFS stimulation. Most of these alterations were reversible, since they were unobserved following vitamin A supplementation in deficient rats. These results provide new mechanistic insight to understand the critical role of dietary vitamin A, and its derivative RA, in the structure and function of hippocampus.

4.1. Vitamin A deficient rats are depleted for RA in the hippocampus

In the adult brain, it is established that hippocampus is highly sensitive to RA action. Metabolism experiments following injection of radioactive retinol in vitamin A deficient rats showed that hippocampus contains high levels of RA, compared to other brain regions [13].

Detection methods for RA have been challenging, due to its sensitivity to light and its lipophilic nature. Here, in order to optimize detection of RA in hippocampus, we used a method based on LC-ESI-MS/MS with Multiple Reaction Monitoring (MRM) [10]. This method was derived from a previously validated method [4,11,12] and we calculated a yield of 85.5% in our experiments (data not shown). Here, we showed that vitamin A deficiency started at weaning significantly lowered levels of RA in the serum at adulthood, which was restored by vitamin A supplementation. RA levels in the hippocampus paralleled RA levels in the serum, with a strong decrease in deficient rats that was reversed by supplementation. These results are in accordance with a previous report of decreased expression of retinoic acid receptors (RARα, RARγ and RXRβ) in the hippocampus of vitamin A deficient mice [18]. Hippocampus RA levels measured in this study was on the same range than previously observed [10–12,29]. More precisely, RA levels was slightly higher than previously measured in mice [11,12], which may be due to differences in species, diet, and improvement of the extraction / detection method.

4.2. Dietary vitamin A models neuronal architecture in the hippocampus

RA is known to model neuronal network during development and neuronal differentiation [4,21,22]. However, its role at adulthood, where remodeling is necessary for plasticity and thus behavior adaptation, is less characterized. Here, we demonstrated that CA1 neurons in deficient rats had smaller dendrites on average. This decrease in length was not specific to distal or proximal dendrites, but rather affected all levels of dendrites. The strongest effect of vitamin A deficiency on neuronal morphology was on dendrite branching, as seen with Sholl analysis. This indicates that a lack of RA in the hippocampus following 13 weeks of post-weaning deficiency was sufficient to decrease the complexity of CA1 neurons. Of note, this rearrangement of neuronal morphology was confirmed by a reduced frequency of spontaneous synaptic activity in CA1 neurons of deficient rats, denoting a lower number of active synapses [30].

Further, we showed that the complexity of dendrites in deficient rats that were supplemented for 3 weeks was overall similar to controls. This powerful effect with short duration and moderate quantity of vitamin A is encouraging, since supplementation with high doses of retinol or 13-*cis* RA, can be deleterious [31,32]. Remarkably, the frequency of spontaneous activity was not modified by supplementation in deficient rats. This indicates that even though the complexity of the dendrite tree is restored, synapses were not functional. This does not preclude the hypothesis that vitamin A supplementation can restore synapses, but that these synapses are silent and need a delay or an event to become active. It would be of interest to investigate this further, to understand what makes synapses functional following diet-induced neuronal remodeling.

The lack of total restoration of neuronal architecture and network may also be due to weight gain retardation in vitamin A deficient animals. Indeed, as previously observed [14,17,33], vitamin A deficient rats exhibited lower weight gain from week 6 of diet exposure (9 weeks of age), which could impair the late development of the hippocampus. However, correlative analysis (data not shown) revealed that weight gain did not correlate with neuron architecture nor Y-maze performance for vitamin A deficient rats. This suggests that, even though slowed weight gain may contribute to altered cognitive performance in vitamin A deficient rats, it is not a major factor for alteration of hippocampus structure and function.

Altogether, these results show that the neuronal network in hippocampus is highly plastic and sensitive to dietary vitamin A, with modification of dendrite complexity within few weeks.

4.3. Dietary vitamin A affects spatial memory and synaptic plasticity

It is now well established that disruption of RA signaling induces alterations of hippocampus-dependent memory in rodents. This has been observed in mice and rats, with dietary and transgenic approaches and behavioral paradigms addressing spatial, social and working memory [14–20]. In this study, we tested spatial memory with a simple Y-maze test. We confirmed alteration of spatial

memory in deficient rats. Additionally, we showed that vitamin A supplementation for 3 weeks in vitamin A deficient rats induced spatial memory similar to control level. The rescue of memory in deficient rodents has already been observed, either with RA injection [15,18] or vitamin A dietary supplementation [14,17]. Restoration with short dietary supplementation appears safer than RA injection, since deleterious effects on hippocampus-dependent memory have been observed with prolonged 13-*cis* AR injection [34,35].

To investigate the mechanism leading to memory deficit following RA signaling alteration, some studies have analyzed synaptic plasticity in parallel of behavioral assessment. Thus, it has been shown that vitamin A deficiency or disruption of RA receptors can reduce synaptic plasticity in the hippocampus [16,19,24]. In the case of dietary deficiency, altered plasticity was nicely restored by short vitamin A replenishment [24]. However, none of these studies have correlated both measures of memory and synaptic plasticity. Here, we demonstrated that HFS-induced potentiation at CA3-CA1 synapse was decreased by vitamin A deficiency and was restored by supplementation. Furthermore, we found that testing spatial memory in control and deficient rats upstream of *ex vivo* HFS protocol, switched the ability of the hippocampal network to display potentiation. Therefore, following behavioral assessment, potentiation was absent in control rats, while it tended to increase in deficient rats. This effect of behavior on plasticity induction in control is designated as plasticity occlusion. This has been well described in the hippocampus [36,37], but also in stress-related structures [38,39] and motor learning brain networks [40–42]. This suggests that behavioral test has induced synaptic plasticity [43] that preclude further plasticity induction when elicited electrically, due to ceiling effect. Accordingly, we found a significant correlation between memory performance and post-behavior synapse potentiation. In deficient rats supplemented with vitamin A, plasticity occlusion was absent, which corroborates the hypothesis that supplementation can remodel the network and create new silent synapses that can be subsequently activated by stimulations such as behavioral test or electrical stimulation (HFS). Intriguingly, plasticity occlusion was also absent in control supplemented rats, since this group exhibit both behavioral performance and fEPSP potentiation. Deeper investigations will help understanding the complex impact of dietary vitamin A on hippocampal network and function.

5. Conclusion

In conclusion, our study reveals that vitamin A deficiency in rats alters plasticity of neuronal architecture and synaptic network, which points out a critical role for RA in this brain structure. Importantly, vitamin A deficiency in our conditions is reversible, since a mild vitamin A supplementation is sufficient to restore the majority of the phenotype. Knowing that vitamin A deficiency is a model for accelerated aging and cognitive decline [18,23,44], our results support a positive impact of vitamin A supplementation to prevent age-related memory alteration.

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