

A Mid-Life Vitamin A Supplementation Prevents Age-Related Spatial Memory Deficits and Hippocampal Neurogenesis Alterations through CRABP-I

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Abstract

Age-related memory decline including spatial reference memory is considered to begin at middle-age and coincides with reduced adult hippocampal neurogenesis. Moreover, a dysfunction of vitamin A hippocampal signalling pathway has been involved in the appearance of age-related memory deficits but also in adult hippocampal neurogenesis alterations. The present study aims at testing the hypothesis that a mid-life vitamin A supplementation would be a successful strategy to prevent age-related memory deficits. Thus, middle-aged Wistar rats were submitted to a vitamin A enriched diet and were tested 4 months later in a spatial memory task. In order to better understand the potential mechanisms mediating the effects of vitamin A supplementation on hippocampal functions, we studied different aspects of hippocampal adult neurogenesis and evaluated hippocampal CRABP-I expression, known to modulate differentiation processes. Here, we show that vitamin A supplementation from middle-age enhances spatial memory and improves the dendritic arborisation of newborn immature neurons probably resulting in a better survival and neuronal differentiation in aged rats. Moreover, our results suggest that hippocampal CRABP-I expression which controls the intracellular availability of retinoic acid (RA), may be an important regulator of neuronal differentiation processes in the aged hippocampus. Thus, vitamin A supplementation from middle-age could be a good strategy to maintain hippocampal plasticity and functions.

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Introduction

The vitamin A, through its main metabolite retinoic acid (RA), plays a key role in brain development by regulating neuronal differentiation, neurite outgrowth and the anteroposterior axis of the neural tube [1,2,3]. It is now established that retinoids are required for cognitive functions in the adulthood [4,5,6,7] and that retinoid hyp signalling contributes to the deterioration of hippocampal synaptic plasticity and functions [8,9,10]. In aged rodents, the naturally occurring hypoactivity of the retinoid signalling pathway has been associated with the reduction of hippocampal synaptic plasticity [11,12], known to underlie at least in part relational memory processing [13,14]. Indeed, pharmacological activation of retinoid signalling by short-term RA treatment in aged mice restored their impaired hippocampal long-term potentiation as well as their long-term relational memory deficits [7]. Moreover, it has recently been shown that a lifelong nutritional vitamin A supplementation induced similar (even more) beneficial effects on hippocampal plasticity and memory processes [15].

One hippocampal plasticity that has mainly been studied in the recent decades is adult neurogenesis. Indeed, new neurons can be generated and survive in the adult dentate gyrus (cell proliferation,

survival mechanisms and subsequent differentiation processes) but the neurogenic rate declines precipitously from middle-age [16,17,18,19,20]. These newly born neurons have been shown to be preferentially recruited into circuits supporting various types of learning and memory [21,22,23,24,25,26], providing evidence for a critical role of adult neurogenesis in hippocampus-dependent memory including spatial memory in the Morris Water Maze [27,28,29,30]. It has been reported that decreased neurogenesis correlates with aging-associated impairments in learning and memory [31,32] but some controversial studies have shown that poorer performance was associated to a better neuronal differentiation and survival [33,34].

Vitamin A and its derivatives, as RA, act on memory processes by modulating different aspects of hippocampal plasticity including synaptic plasticity [7,12] but also adult neurogenesis in the dentate gyrus (DG) [35,36,37,38]. Indeed, it has recently been demonstrated that RA treatment can restore normal level of neurogenesis in vitamin A deficient rats [36]. Moreover, a gradient of RA, generated in the meninges, would differentially modulate adult neurogenesis between the two pyramidal blades of the rodent DG [38]. However, no studies have explored the possibility to stimulate adult hippocampal neurogenesis by acting on the retinoid signalling pathway during senescence. It is now accepted,

that in rodents and humans, aging induces an alteration of retinol metabolism that leads to a reduction of cellular RA bioavailability in target tissues [12,39,40]. In the brain, the bioavailability of RA is controlled, from circulating retinol, by local RA anabolizing and catabolizing enzymes, but also by RA binding proteins such as CRABP-I (cellular retinoic acid binding protein I) that is highly expressed in the DG [38,41]. The crucial role of CRABP-I in neurogenesis has previously been revealed during development [42]. Interestingly, a surexpression of CRABP-I has been associated with a reduction of differentiation in human neuroblastoma cells suggesting that the regulation of RA bioavailability by CRABP-I could be determinant in the modulation of neurogenesis [43]. However, its potential role in adult hippocampal neurogenesis has not been elucidated yet.

In the present study, we consequently investigated the ability of a vitamin A supplementation started at middle age, to counteract age-related spatial reference memory decline. In order to better understand the potential mechanisms mediating the effects of vitamin A supplementation on hippocampal functions, we studied different aspects of hippocampal neurogenesis (cell proliferation, cell survival and neuronal differentiation processes). Besides, we measured plasma retinol levels and hippocampal CRABP-I mRNA expression. In this study, we demonstrated for the first time that a mid-life vitamin A supplementation would be a successful strategy to prevent age-related spatial memory deficits by stimulating the differentiation of newly formed neurons and cell survival in aged rats. Moreover, our results suggest that the control of the cellular bioavailability of RA by CRABP-I could be an essential mechanism in the modulation of hippocampal adult neurogenesis by RA.

Materials and Methods

Animals

Male Wistar rats were purchased from Janvier (Le Genest Saint-Isle, France) at the age of weaning (3 weeks) and 13 months (middle-aged). They were housed two per cage in a room with a constant airflow system, controlled temperature (21–23°C), and a 12 h light/dark cycle. They were randomly divided into three experimental groups designed as young and aged groups receiving a control diet (5 month-old and 17 month-old respectively), and enriched-aged group receiving a vitamin A enriched diet (17 month-old). All animals were individually housed from 3 weeks prior to the beginning of the behavioral test until sacrifice. All experiments were performed in accordance with the European Communities Council Directives (86/609/EEC) and the French national Committee (87/848) recommendations. All animal procedures were approved by the Animal Care and Use Committee of Bordeaux.

Diet

The weaned rats (n=10) and half of the middle-aged rats (n=10) were fed with a control diet containing 5 IU retinol/g during 4 months (control condition): they are referred to as the 5-month-old young rats and the 17-month-old aged rats, respectively. The second half of the middle-aged group (n=10) received the vitamin A-enriched diet (45 IU retinol/g) for the same duration: they are referred to as the 17-month-old enriched-aged rats. The composition of the control diet was the same as the vitamin A-enriched diet, excepted for retinol content. The 45 IU diet was chosen on the basis of literature as a moderate supplementation to avoid potential toxicity of hypervitaminosis (for review, see [44]). Moreover, this vitamin A supplemented diet

was shown to counteract declarative-like memory deficits in aged mice when started at the age of 2 months [15].

Experimental Design

We have studied the effects of a mid-life vitamin A supplementation on a spatial reference memory task and hippocampal neurogenesis, known to be altered during aging (Fig. 1). Thus, the weaned rats (3-week-old) and middle-aged rats (13-month-old) received a control diet or a vitamin A enriched diet during 4 months. 3 months after the beginning of the diets, the three groups (young n=10, aged n=10, enriched-aged n=10) were injected with BrdU for 5 days. Rats were allowed to survive for another three weeks after the last injection of BrdU and continued diet in their respective experimental conditions. All groups were tested in a spatial reference memory task in the water maze and sacrificed one week after the completion of behavioral testing, to analyze hippocampal neurogenesis, serum retinol concentration and mRNA expression of CRABP-I.

5-Bromo-2'-deoxyuridine (BrdU) Injections

In order to label the newly born cells and examine hippocampal cell survival and differentiation, BrdU, a thymidine analogue incorporated into genetic material during synthetic DNA phase of mitotic division, was used. Rats received a daily intraperitoneal injection of BrdU (50 mg/kg, Sigma), dissolved in phosphate buffer (0.1 M, pH 8.4), during the 5 consecutive days beginning 3 months after the different diets.

Behavioral Testing

Rats were tested in a Morris water maze (180 cm in diameter, 60 cm high) filled with water (22°C) made opaque by addition of white paint. An escape platform was hidden 2 cm below the surface of the water in a fixed location in one of four quadrants halfway between the wall and the middle of the pool. Before the start of the training, animals were habituated to the pool without a platform for 1 min/day for 2 days. During training, animals were required to locate the submerged platform by using distal extramaze cues. They were trained for four trials per day (90 s with an intertrial interval of 60 s, starting from three different start points randomized every day) for 9 consecutive days. The swim speed, the time to reach the platform (latency) and the distance covered to find the platform were measured with a computerized tracking system (Videotrack, Viewpoint). On day 10, animals were tested for 60 s in the pool without the platform (probe test). Performance was evaluated by the percentage of time spent in the quadrant where the platform was located during training (target quadrant). Finally, in order to control for visual acuity deficits, the hidden platform was replaced by a visible platform located in the opposite quadrant, and animals were tested for three trials (90 s) over one day. Two aged rats (receiving the control diet and the enriched diet) were excluded from the experiment due to failure to search for the platform during the acquisition phase (floating) leading to a number of 9 animals in the aged group and in the enriched-aged group. For the statistical analyses, results from blocks of trials for each day were averaged for every rat.

Immunohistochemistry

Rats were euthanized with isoflurane and the brain quickly removed. Half of the dissected brains were washed with 0.9% sodium chloride, followed by 4% paraformaldehyde. After a 3-week postfixation period in paraformaldehyde, 50 µm frontal sections were cut on a vibratome (Leica). Free-floating sections were processed with a standard immunohistochemical procedure

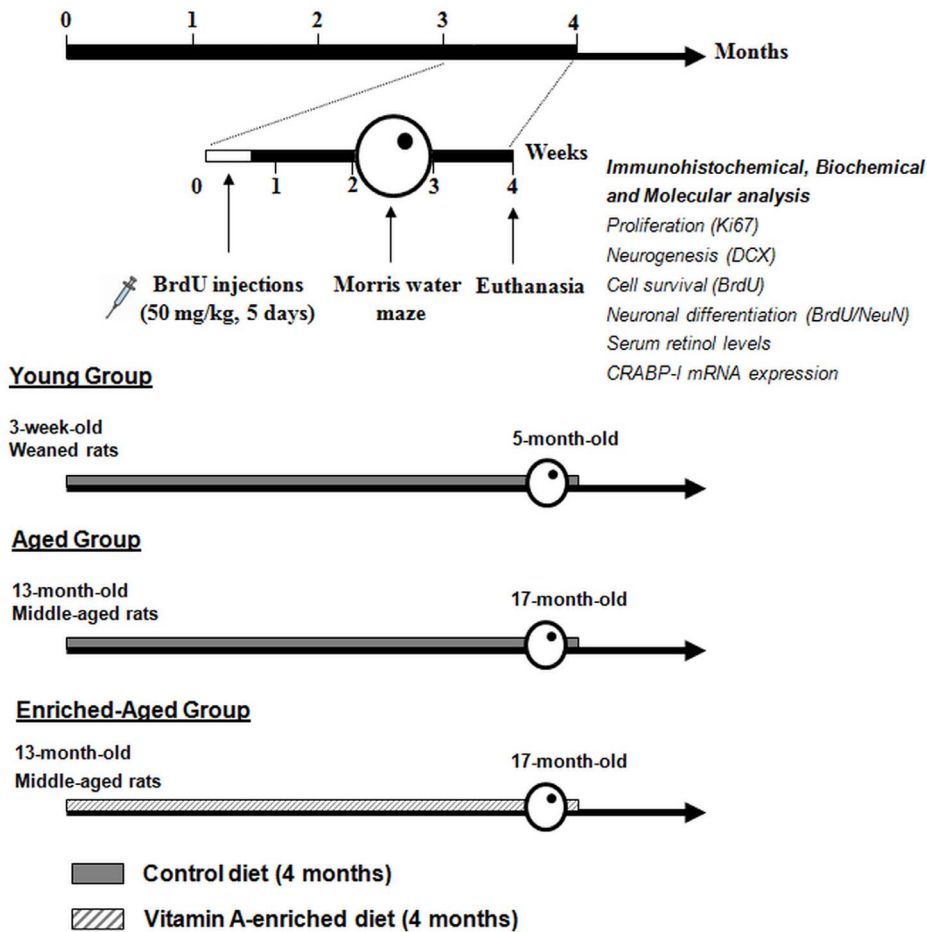


Figure 1. Experimental protocol. Effects of 4 months of vitamin A supplementation started at mid-age on spatial memory, hippocampal neurogenesis and retinoid status in rats. The weaned rats and half of the middle-aged rats were fed with a control diet containing 5 IU retinol/g during 4 months (control condition): they are referred to as the 5-month-old young rats and the 17-month-old aged rats, respectively. The second half of the middle-aged group received the vitamin A-enriched diet (45 IU retinol/g) for the same duration: they are referred to as the 17-month-old enriched-aged rats.

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[45]. A one-in-ten section was treated for Ki-67 immunoreactivity using a mouse anti-Ki-67 monoclonal antibody (1:200, Novocastra) or for doublecortin (DCX) immunoreactivity using a goat polyclonal antibody (1:1000, Santa Cruz Biotechnology). The sections were then incubated with biotinylated secondary horse anti-mouse or donkey anti-goat antibodies respectively (1:200, AbCys; 1:200, Amersham). For BrdU labeling, adjacent sections were treated with 2N HCl to denature DNA (30 min at 37°C) and then washed in phosphate buffer. Sections were incubated with a mouse monoclonal anti-BrdU antibody (1:200, Dako) followed by the biotinylated horse anti-mouse antibody (1:200, AbCys). Sections were processed in parallel, and immunoreactivities were visualized by the biotin-streptavidin technique (ABC kit, Dako) by using 3,3'-diaminobenzidine as chromogen.

The number of immunoreactive (IR) cells in the left DG was estimated by using a modified version of the optical fractionator method with a systematic random sampling of every 10 sections along the rostrocaudal axis of the DG. On each section, IR cells in the granular and subgranular layers of the DG were counted with a 100x microscope objective. All results are expressed as the total number of cells in the whole DG. The fraction of DCX-IR new neurons with large vertical dendrites (mature new neurons) corresponding to a higher level of differentiation was also counted.

To analyze the phenotype of BrdU labeled cells, 4 rats per group were randomly selected. One in-ten section was incubated with a rat anti-BrdU monoclonal antibody (1:500, Servibio), which were revealed by using goat cy3-labeled anti-rat IgG antibodies (1:1000, Interchim). Sections were then incubated with mouse monoclonal anti-NeuN antibodies (1:1000, Millipore), and bound anti-NeuN monoclonal antibodies were visualized with an Alexa 488 goat anti-rabbit IgG (1:1000, Interchim). The percentage of BrdU-labeled cells that expressed NeuN was determined throughout the DG by using a fluorescence microscope (Nikon eclipse E400) equipped with Hamamatsu digital camera and piezoelectric z-axis controller. All BrdU double labeled cells were examined, and sections were optically sliced in the Z plane by using a 1 µm interval. Cells were rotated in orthogonal planes to verify double labeling and images were analyzed by using a Nikon NIS-elements acquisition software.

Real-Time PCR Analysis of CRABP-I Expression

After the removal of the brain, the other half of the hippocampus was rapidly removed and stored at -80°C in order to measure CRABP-I expression. Extraction of RNA was conducted using an extraction kit (TRIzol reagent, Invitrogen) according to the manufacturer's instructions. The quality and the

concentration of RNA were determined by using a nanodrop (ND-1000) (Labtech). Then, the integrity of the purified RNA was verified using the RNA 6000 Nano LabChip kit in combination with the 2100 bioanalyser (Agilent Technologies). Using OligodT and random primers (Promega), cDNA was synthesized with ImPromII reverse transcriptase (Promega). Briefly, 1 µg of total RNA mixed with RNasin (Promega) and DNase (Roche) was incubated at 37°C. Then, OligodT and random primers were added for incubation at 70°C. The reverse transcriptase reaction was performed at 42°C for 60 min in a final volume of 20 µl.

The real-time PCR was performed using the LightCycler 480 system with a 96-well format (Roche Diagnostics) in a final volume of 20 µl, containing 1×LightCycler 480 SYBR Green I Master solution, 0.5 µM of each primer and 6 µl of cDNA. The forward and reverse primer sequences for CRABP-I and Ppib that was used as a house-keeping gene are given in Table 1. The following program started with an initial denaturation step for 10 min at 95°C, then an amplification for 40 cycles (10 s denaturation at 95°C, 6 s annealing at 62°C, and 10 s extension at 72°C), finally a melting curve analysis was run. In order to verify the specificity and the identity of the amplified products: (1) the melting curve analysis showed a single melting peak after amplification, and (2) amplified products for each gene were verified by sequencing with the Big Dye Terminator v1.1. (Applied Biosystems) and analyzed on a ABI3130 sequencer (Applied Biosystems).

The peptidylprolyl isomerase B (Ppib) housekeeping gene was used as the reference gene for relative quantification as its expression level was unaffected by our experimental conditions. Quantification data were analyzed using the LightCycler 480 Relative Quantification software (version 1.5). In order to compensate for differences in target and reference gene amplification efficiency, either within or between experiments, this software provides a calibrator-normalized relative quantification including a PCR efficiency correction. Therefore, the results are expressed as the target/reference ratio divided by the target/reference ratio of the calibrator. In our case, the calibrator was chosen among the young rats.

Measurement of Serum Retinol Concentration

Blood was collected at the sacrifice and spun at 1500 g for 15 minutes. The supernatant was removed and stored at -20°C. Serum retinol was assayed by HPLC according to a previously described method [46].

Statistical Analysis

Spatial learning and swim speed data were analyzed using a 2-way ANOVA (3 groups) followed by a post-hoc Bonferroni test. Probe test, immunohistochemical (Ki-67 and DCX only) and PCR

data were analyzed using a 1-way ANOVA followed by a post-hoc Bonferroni test. Two pairwise comparisons were performed between young and aged rats (age effect) but also between aged and enriched-aged rats (supplementation effect) using Bonferroni adjusted significance levels of 0.025. Probe test comparisons to chance level (25%) were performed in each group using one sample test. Results are expressed as mean and standard error of the mean, and they were considered significant when $p < 0.025$ for ANOVAs and Bonferroni tests or $p < 0.05$ for one sample test.

Results

Effects of a Mid-life Vitamin A Supplementation on Spatial Reference Learning and Memory in Aged Rats

The aim of this study was to determine whether a mid-life vitamin A supplementation would be efficient to prevent the age-related spatial memory deficits in rats.

Spatial learning. Results from blocks of trials for each training day were averaged for every rat. The latency to reach the hidden platform, the classical parameter used in the Morris water maze task, may reflect differences in the swimming speed of young versus aged rats. Even though we could not really put them on the fore, we rather used the distance covered to reach the platform as a more appropriate measure and as a good index of the acquisition rate for spatial learning. Indeed, the swimming speed displayed by rats over the nine training sessions has been controlled (young rats: 22.6 ± 0.36 cm/s, aged rats: 19.1 ± 0.5 cm/s, enriched-aged rats: 20.9 ± 0.38 cm/s) and the ANOVA revealed no significant difference between groups [$F(2,25) = 2.64$, $p = 0.09$] indicating that aged rats did not exhibit motor impairments.

Young and aged animals learned this task as shown by the progressive decrease in the distance covered to reach the hidden platform over the nine days of training [$F(8,200) = 19.49$, $p < 0.001$]. A significant group effect [$F(2,25) = 4.52$, $p = 0.02$] but without interaction group×days [$F(8,200) = 1.54$, n.s.] was observed in the distance covered by rats to reach the platform (Fig. 2A). Indeed, aged rats travelled significantly longer distance to find the hidden platform than young rats, putting on the fore learning impairments in aged rats (Bonferroni test between aged rats: 10.26 ± 0.4 m and young rats: 7.5 ± 0.5 m, $p < 0.01$). However, this spatial learning deficit was not reversed in aged rats receiving vitamin A enriched diet even if supplemented rats tended to travel a smaller distance to find the platform over the nine training sessions compared to aged rats receiving a control diet (Bonferroni test between aged rats: 10.26 ± 0.4 m and enriched-aged rats: 8.5 ± 0.39 m, $p = 0.08$, n.s.).

Spatial reference memory. One day later, spatial memory for the platform location was tested using a probe test i.e. in the absence of the platform. ANOVA was performed on the percentage of time spent in the target quadrant in which the platform was placed at training and revealed a group effect [$F(2,25) = 8.9$, $p < 0.01$]. During the probe test, young rats spent $38.6 \pm 2.9\%$ of their time in the target quadrant suggesting that they do remember where the platform was (Fig. 2B). Indeed, one sample test analysis revealed that this percentage is significantly different from the chance level (vs 25%, $p = 0.001$). On the contrary, aged rats failed to display a memory for the platform location, as indicated by a percentage of time in the target quadrant around the chance level ($21.2 \pm 2.6\%$ vs 25%, $p = 0.2$) showing spatial reference memory deficits (Bonferroni test, between aged and young rats, $p < 0.001$). Interestingly, supplemented aged rats spent significantly more time ($33.8 \pm 3.3\%$, which is different from the chance level, $p = 0.029$) than aged rats looking

Table 1. Primers used for Light Cycler RT-PCR.

Gene name	Nucleotide sequence 5'-3'	Product length (bp)
Ppib	F : GTTCTGGAAGGCATGGATGT	153
	R : TCCCCGAGGCTCTCTACT	
CRABP-I	F : TCTTCTCCACACACCTCTCC	124
	R : TCATGCAGATGCCAAACAG	

Sequences are shown for forward (F) and reverse (R) primers. Ppib: peptidylprolyl isomerase B (cyclophilin B); CRABP I: cellular retinoic acid binding protein I.

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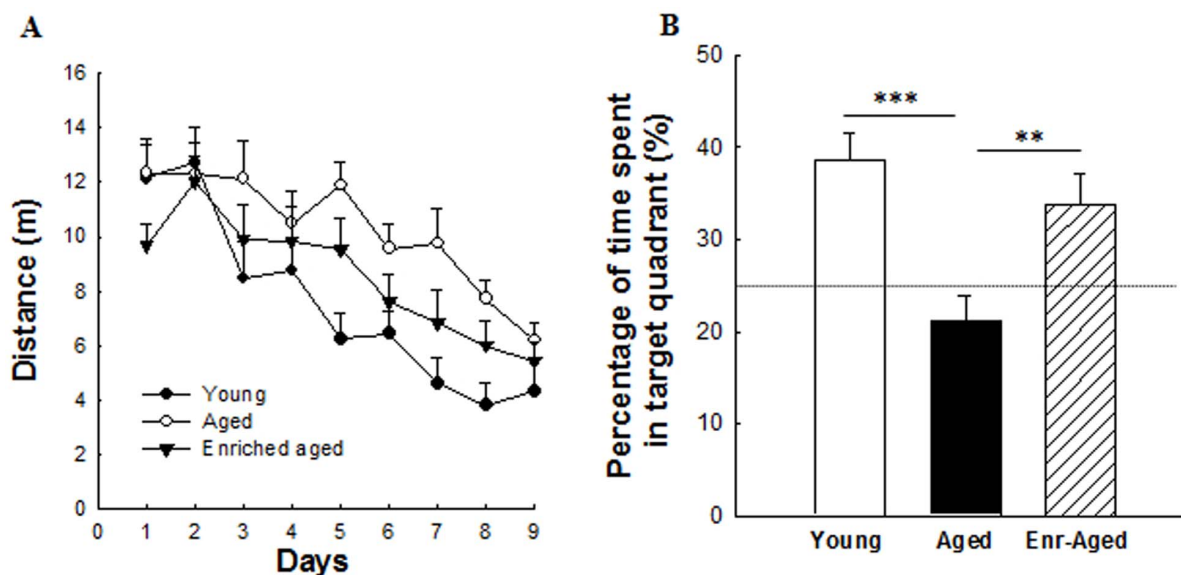


Figure 2. Effects of aging and a mid-life vitamin A supplementation on spatial learning and memory abilities in the Morris water maze. (A) Distance covered by rats to find the hidden platform along nine consecutive days (*Training*; blocks of trials for each training day are averaged) and (B) the recall of the platform location in the target quadrant (probe test) were evaluated in all groups. The dotted line corresponds to chance level (25%). Delayed rate of acquisition and reduced percentage of time in the target quadrant were observed in aged rats. Enriched-aged rats showed a clear spatial reference memory improvement. Significant *p* values after Bonferroni correction for pairwise comparisons (level $\alpha=0.025$; ***p*<0.01; ****p*<0.001).

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after the platform in the correct quadrant (Bonferroni test, between aged and enriched-aged rats, $p<0.01$).

Visible platform. After the probe trial, on day 11, rats were trained to swim towards a visible platform over three trials for a day. The distance covered to locate the visible platform was similar for all groups [$F(2,25)=1.44$, $p=0.25$] without interaction group \times trials [$F(2,50)=0.82$, $p=0.5$] indicating that the old subjects did not have any potential visual impairments (data not shown).

Effects of a Mid-life Vitamin A Supplementation on Hippocampal Neurogenesis in Aged Rats

The effects of retinoids on spatial memory have been proposed to be mediated, at least in part, by a modulation of hippocampal neurogenesis [36]. The aim of this study was thus to determine for the first time, the ability of a mid-life vitamin A supplementation to modulate the three different aspects of hippocampal neurogenesis (cell proliferation, survival of newborn cells, neuronal differentiation processes) during aging.

Cell proliferation. Cell proliferation was measured using an endogenous marker of cell cycle, Ki-67. Ki-67-labeled cells were located within the subgranular zone and were isolated or grouped in clusters (Fig. 3A). A quantitative analysis revealed a group effect [$F(2,25)=73.6$, $p<0.001$]. Aging is associated with a significant reduction (-86%) in cell proliferation (Fig. 3B, Bonferroni test, number of Ki-67-IR cells in young rats : 6368 ± 523.7 vs in aged rats : 897.7 ± 224.6 , $p<0.0001$). We tested whether the modulation of retinoid signalling in aged rats could be accompanied by a beneficial effect on cell proliferation. No effect of a mid-life vitamin A supplementation was observed on cell proliferation during aging (Bonferroni test, number of Ki-67-IR cells in aged rats 897.7 ± 224.6 vs in enriched-aged rats : 1153.3 ± 199.2 , n.s.).

Number of immature neurons. The strong reduction in cell proliferation in aged rats suggested a decrease in hippocampal neurogenesis. To test this hypothesis, we quantified the number of

immature neurons. We used doublecortin (DCX), a microtubule-associated phosphoprotein, as a marker of neurogenesis. DCX-IR cells were located in the deepest region of granule cell layer (GCL) at the interface with the hilus. In young animals, we observed many DCX-IR cells by contrast to old animals exhibiting a strong reduction in the number of DCX-IR cells (Fig. 3B). Indeed, a quantitative analysis revealed a group effect [$F(2,25)=191.7$, $p<0.001$] and Bonferroni test ($p<0.0001$) confirmed that aging is associated with a strong reduction (-90%) in the number of immature neurons in the rat hippocampus [Fig. 3B, number of immature neurons in young rats : 22090 ± 1205 vs in aged rats : 2368.8 ± 279.3]. However, vitamin A supplementation had no effect on the number of newly generated immature neurons in aged rats (Bonferroni test, n.s, number of immature neurons in enriched-aged rats : 3175.5 ± 530.4).

Dendritic arborisation of immature neurons, cell survival and neuronal differentiation. Immunohistochemical images revealed that DCX-positive immature neurons in aged rats were not only fewer in number but also characterized by a decrease in dendritic arborisation, potentially reflecting retarded maturation (Fig. 4A). Therefore, we investigated the degree of maturation of the newly born neurons by differentiating more mature DCX-IR cells with vertical dendrites emanating from the soma and extending into the dentate molecular layer through the GCL (presumably more mature new neurons) from DCX-IR cells with horizontally orientated soma and dendrites in the SGZ or without dendritic projections [47]. We studied the ability of vitamin A supplementation to promote dendritic growth and neuronal maturation during aging. Quantitative analysis revealed that aged rats receiving a vitamin A enriched diet had significantly higher proportion of immature DCX-IR neurons with vertical dendrites, reflecting better maturation, compared to control aged rats [$F(1,16)=10.5$, $+12\%$ between aged rats : $53.2 \pm 1.9\%$ and enriched aged rats : $65.1 \pm 2.8\%$, $p<0.01$].

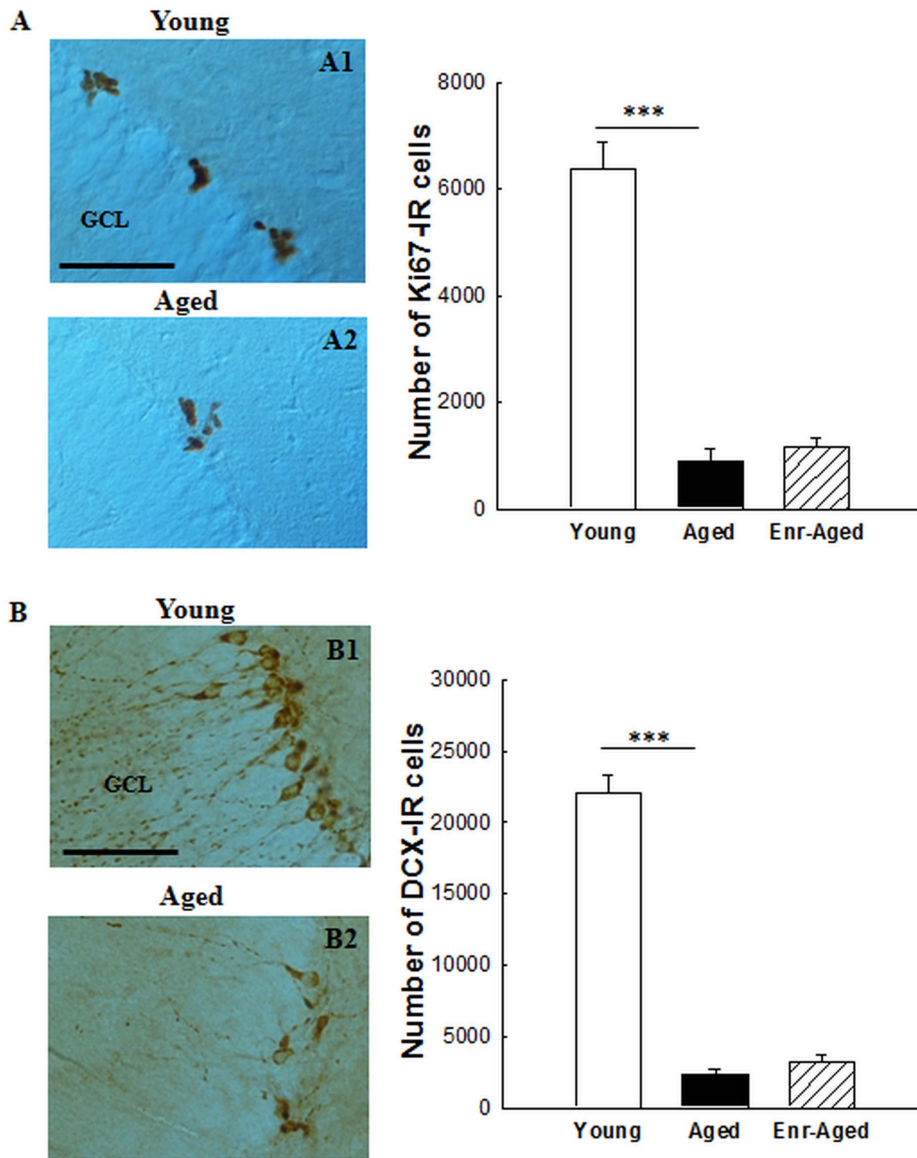


Figure 3. Effects of aging and a mid-life vitamin A supplementation on the number of Ki67-IR and DCX-IR cells. Images show the immunoperoxidase staining of (A) Ki67-IR and (B) DCX-IR cells in the DG granule cell layer of young and aged rats. The graphs indicate the strong reduction of the number of Ki67-IR and DCX-IR cells in aged rats. Vitamin A supplementation does not affect cell proliferation nor the number of immature neurons in aged rats. Significant p values after Bonferroni correction for pairwise comparisons (level $\alpha=0.025$; $***p<0.001$). Scale bar : 50 μm . GCL=granule cell layer.
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To investigate whether the higher degree of maturation of new born cells in enriched rats could be associated with a more pronounced survival of these cells, we analyzed cell survival using a 3-week interval between injection of BrdU and sacrifice of the animals. We found that most of the 3-week-old surviving BrdU-IR cells were isolated, round, large and located within the GCL (Fig. 4B). As shown in Fig. 4B, a vitamin A enriched diet from mid-life stimulated the survival BrdU-IR cells as they were more numerous in enriched-aged rats (+26%) than in their aged control group [$F(1,16)=8.5$, number of BrdU-IR cells in aged rats : 793.3 ± 65.7 vs in enriched aged rats : 1078 ± 65.8 , $p<0.01$].

The phenotype of newly born cells labeled with BrdU was determined using NeuN, a mature neuronal marker. The percentage of BrdU/NeuN double stained cells located in the GCL (Fig. 4C) was increased in enriched-aged rats [$F(1,6)=51.88$,

$p<0.001$, between aged rats: $14.4\pm 1.7\%$ and enriched-aged rats : $35.2\pm 2.25\%$]. Thus, a more developed dendritic arborisation of immature neurons in enriched aged rats would result in a selective and better survival and neuronal differentiation of this particular population of newly born cells.

Effects of a Mid-life vitamin A Supplementation on Retinoid Status in Aged Rats

Assay of serum retinol. The analysis of serum retinol levels was performed after the behavioral studies in order to confirm the retinoid status of aged rats (Fig. 5A). Quantitative analysis revealed differences between groups [$F(2,25)=28.72$; $p<0.001$]. A significant reduction (- 41%) in serum retinol concentration was observed in aged rats relative to young rats (Bonferroni test, $p<0.001$, between young and aged rats; $1.033\pm 0.06 \mu\text{mol/l}$

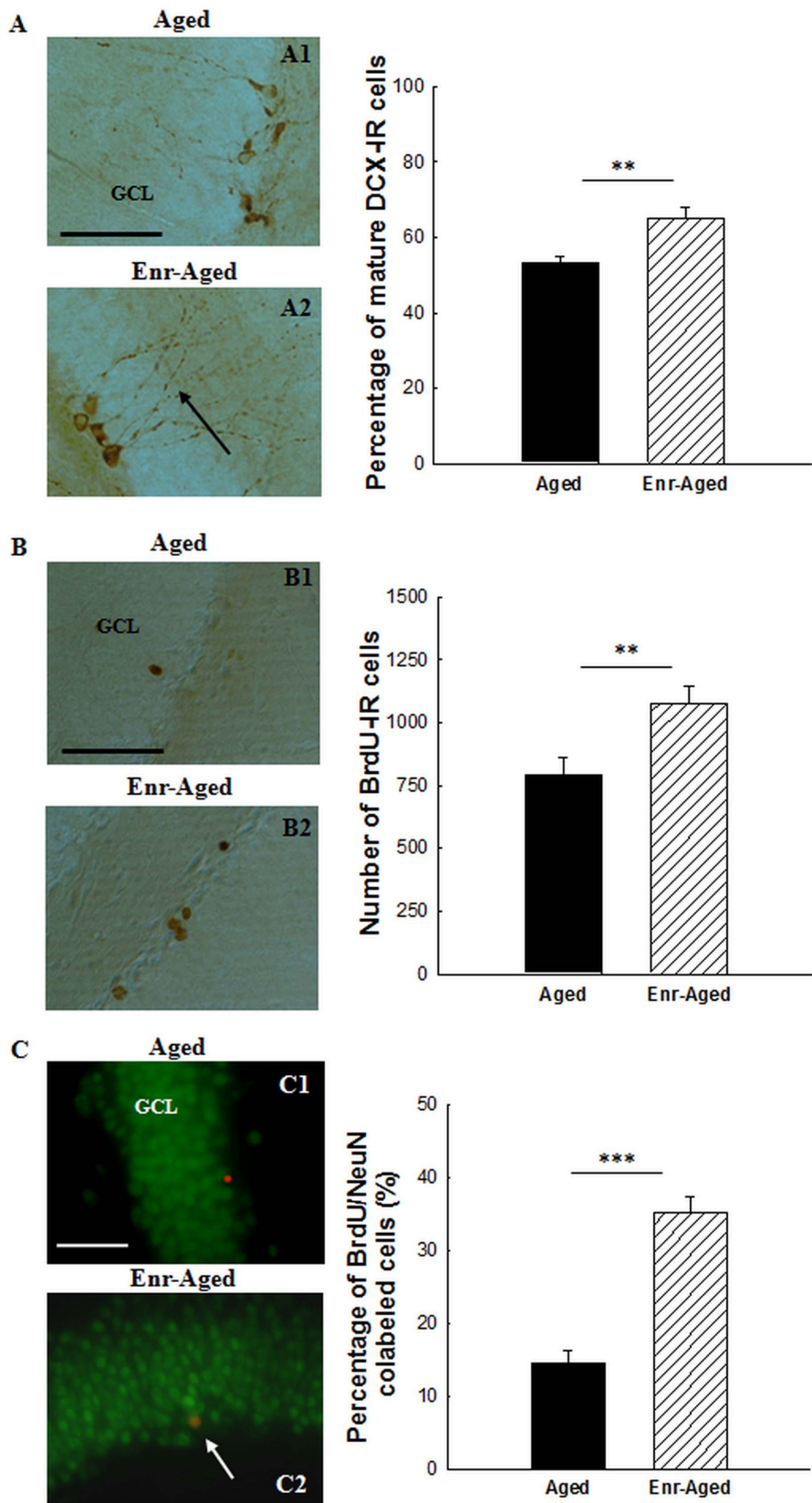


Figure 4. Effects of a mid-life vitamin A supplementation on maturation of DCX-IR cells, survival of BrdU-IR cells and neuronal differentiation in aged rats. Images show immunostaining of (A) DCX-IR cells (B) BrdU-IR cells (C) BrdU/NeuN colabeled cells in the DG granule cell layer of aged and enriched-aged rats. Enriched-aged rats showed (A) an increased number of mature DCX-IR cells, (B) an increased number of BrdU-IR cells and (C) a higher percentage of BrdU/NeuN colabeled cells. Significant p values after ANOVA (** $p < 0.01$, *** $p < 0.001$). The black arrow in A2 points

to DCX-IR cells with vertical dendritic ramifications and the white arrow in C2 to a BrdU/NeuN colabeled cell in enriched aged rats. Scale bar : 50 μm . GCL = granule cell layer.
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versus $0.6 \pm 0.04 \mu\text{mol/l}$). However, a vitamin A supplementation diet did not increase serum retinol concentration in aged rats (Bonferroni test, n.s. between aged and enriched-aged rats; $0.6 \pm 0.04 \mu\text{mol/l}$ versus $0.465 \pm 0.05 \mu\text{mol/l}$).

We examined the existence of linear correlations between the degree of maturation of newly born neurons and serum retinol levels for individual rats. A positive correlation was observed between the percentage of DCX-IR cells with vertical dendrites and serum retinol levels in young and aged rats (Fig. 5B; $r = 0.64$, $p < 0.002$, $n = 19$): rats with high level of serum retinol exhibited a higher percentage of DCX-IR cells with a more mature

phenotype. Moreover, we have also observed a positive correlation between serum retinol levels and spatial memory in young and aged rats (Fig. 5C probe test and serum retinol level, $r = 0.67$, $p = 0.001$). No correlation has been observed if we integrate the enriched-aged group to the regression analysis (data not shown).

mRNA expression of CRABP-I in the hippocampus. The ability of vitamin A supplementation to promote the maturation of newly generated neurons and improve memory abilities in aged rats could be in part mediated by the intracellular availability of RA which is determined by many regulatory proteins such as retinoic acid binding proteins I (CRABP-I), known to play an

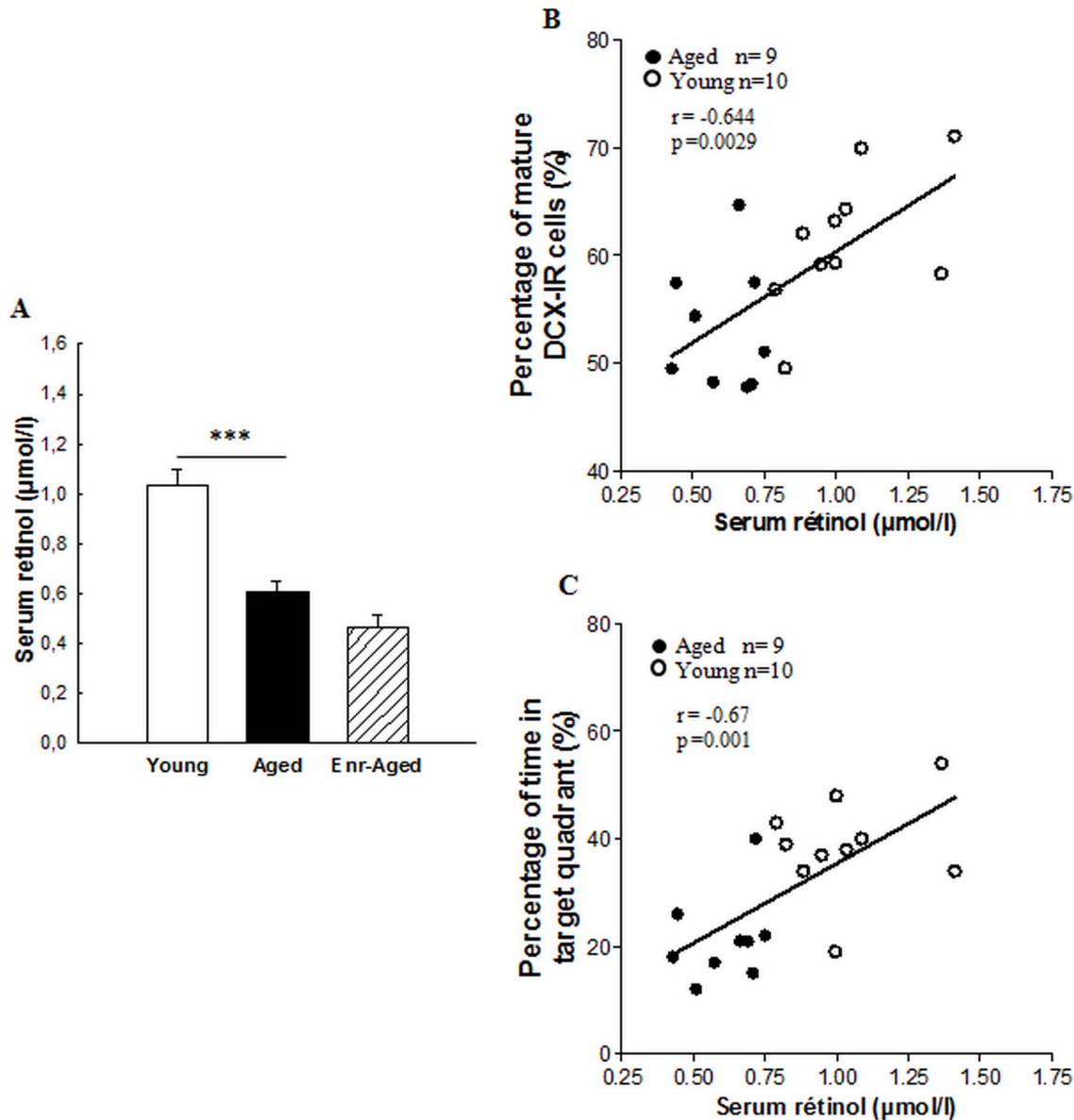


Figure 5. Effects of aging and a mid-life vitamin A supplementation on serum retinol levels. (A) Aging decreases serum retinol levels. Significant p values after Bonferroni correction for pairwise comparisons (level $\alpha = 0.025$; $***p < 0.001$). (B)(C) Correlation analyses. (B) Percentage of mature DCX-IR cells and (C) spatial memory correlate with serum retinol levels in young and aged rats ($r = 0.644$, $p < 0.01$ and $r = 0.67$, $p < 0.001$ respectively).
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important role in RA-mediated differentiation processes [42]. As seen in Fig. 6A, quantitative analysis of hippocampal CRABP-I mRNA expression indicated differences between groups [$F(2,25)=4.95$; $p<0.025$]. Indeed, we observed that aging up-regulates CRABP-I mRNA expression (+70%, Bonferroni test, $p<0.01$, between young rats : 1 ± 0.09 a.u. and aged rats : 1.7 ± 0.25 a.u.). By contrast, vitamin A supplementation reduced hippocampal CRABP-I mRNA expression in aged rats (Bonferroni test, $p<0.025$, between aged rats : 1.7 ± 0.25 a.u. and enriched-aged rats : 1.1 ± 0.13 a.u.).

We examined the existence of linear correlations between the degree of maturation of newly born neurons and hippocampal CRABP-I mRNA expression for the three groups of rats. A negative correlation was observed between CRABP-I mRNA expression levels and the percentage of DCX-IR cells with vertical dendrites ($r=0.432$, $p=0.02$, $n=28$, Fig. 6B) : rats with lower level of hippocampal CRABP-I mRNA expression exhibited a high percentage of DCX-IR cells with vertical dendrites, reflecting a more mature phenotype. This correlation was also observed if we only integrated young and aged groups to the regression analysis ($r=0.476$, $p=0.03$, $n=19$, data not shown).

Discussion

In the present study, we demonstrate for the first time that 4 months of nutritional vitamin A supplementation started at mid-life improve the spatial memory and the level of differentiation of newborn neurons in aged Wistar rats. Moreover, our results suggest that hippocampal CRABP-I expression which controls the intracellular availability of RA, may be an important regulator of neuronal differentiation processes in the aged hippocampus.

In agreement with a previous study showing that a life-long vitamin A supplementation can prevent age-related short term working memory deterioration [15], our data confirm the idea that acting on the retinoid signalling pathway by a nutritional approach could be of great interest to alleviate age-induced memory deficits. Some memory deficits during aging can be partly explained by

alterations of cerebral plasticity including adult hippocampal neurogenesis [48]. Moreover, age-related memory loss begins to appear at middle-age and coincides with reduced adult hippocampal neurogenesis [19,49]. Thus, our study confirms the beneficial effects of vitamin A on age-induced spatial memory deficits but also demonstrates for the first time that supplementation started at mid-life is efficient to improve hippocampal neurogenesis and functions in aged rats.

Consistent with the age-related decline in hippocampal neurogenesis [19,33,50,51], our study shows a strong decrease in cell proliferation and in the number of immature neurons in aging rats. Interestingly, we report that the spatial memory impairments in aged rats were accompanied by an abnormal differentiation of immature neurons. Here, we demonstrate that aged rats had significant lower mature DCX positive cells with vertical dendritic ramifications that are usually observed in young rats. This finding is in line with a recent study showing that age-related spatial memory impairments are associated with alterations in the differentiation of immature neurons [32]. Indeed, Nyffeler et al. have shown a higher proportion of Nestin-DCX double labelled cells in learning-impaired aged rats, suggesting a less mature stage of this pool of neuronal progenitors. This hypothesis is also in line with data showing a significant retardation of dendritic growth during the DCX expression phase of newborn neurons in aged rats [18].

Our experiments also suggest that promotion of maturation of newborn neurons in supplemented aged rats could be one plausible mechanism by which vitamin A supplementation might exert promnesic effects. Indeed, we have shown that vitamin A supplementation started at mid-life does not improve cell proliferation or the number of immature neurons in aged rats, but this preventive nutritional strategy is very potent to increase dendritic arborisation of DCX-positive cells, probably resulting in a better survival and neuronal differentiation. This is partly confirmed by the higher percentage of BrdU/NeuN cells observed in enriched-aged rats compared to aged rats. Thus, the integration

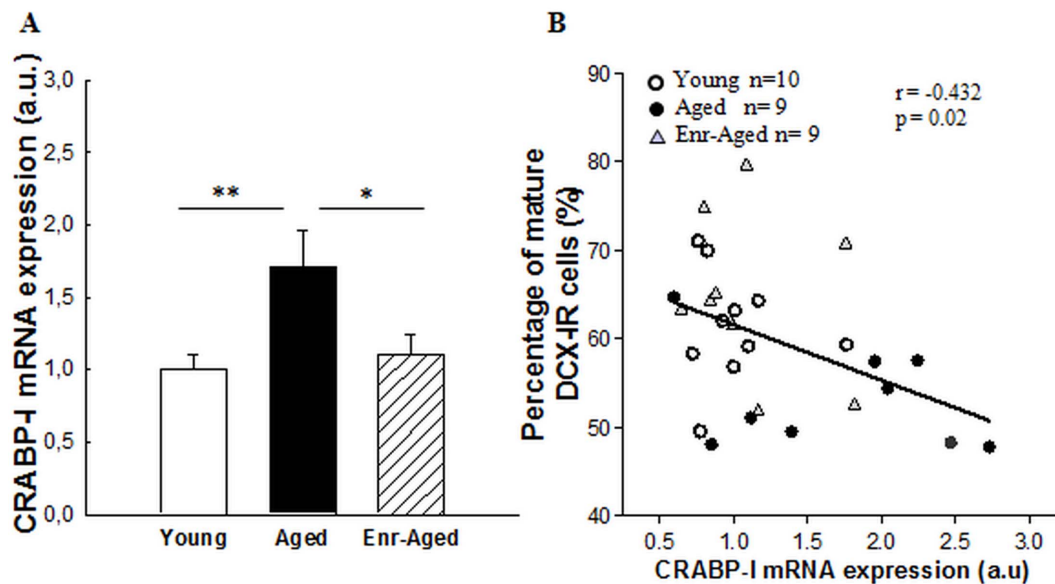


Figure 6. Effects of aging and a mid-life vitamin A supplementation on mRNA expression of CRABP-I in the hippocampus. (A) CRABP-I mRNA expression as quantified by Real Time-PCR. Vitamin A supplementation normalized aging-induced up-regulation of CRABP-I mRNA expression. Significant p values after Bonferroni correction for pairwise comparisons (level $\alpha = 0.025$; * $p<0.025$). (B) Correlation analyses. Percentage of mature DCX-IR cells correlates with hippocampal CRABP-I mRNA expression in all groups ($r=0.43$, $p<0.05$). doi:10.1371/journal.pone.0072101.g006

of these newborn neurons into the hippocampal circuitry could facilitate spatial memory performances in the enriched-aged rats. Consistent with the present results, it has been shown that aged rats with preserved spatial memory exhibited a better survival and differentiation examined 3 weeks after learning (under basal conditions) compared to aged rats displaying spatial memory impairments [31]. Moreover, the ability to perform hippocampus-related functions could also result from changing the dynamics of hippocampal neurogenesis in the course of learning. Indeed, spatial learning has been reported to promote the survival of newborn cells generated before learning [52,53,54], to accelerate the differentiation of selected newborn cells toward a neuronal phenotype and to increase the dendritic development [55]. Thus, although our results might suggest that vitamin A supplementation could directly increase maturation of new neurons in aged rats showing better spatial memory performances, we cannot exclude the potential influence of spatial learning on hippocampal neurogenesis in the different groups.

Similarly, several studies using vitamin A deficiency (VAD) models, have shown that this vitamin is necessary for neuronal differentiation and survival [35] but also for cell proliferation in the DG of the hippocampus [36]. Indeed, it has been demonstrated that the effect of vitamin A deficient diet on the level of hippocampal neurogenesis is reversible and that RA treatment can restore hippocampal neurogenesis and correct spatial memory deficits in vitamin A deficient rats [36]. Nutrition is an important extrinsic factor which modulates adult hippocampal neurogenesis [56], but other factors such as environmental enrichment or physical activity starting from middle-age, have also been shown to be efficient in the prevention of hippocampal neurogenesis and memory alterations [17,47]. Indeed, comparable increases in BrdU-positive cells double-labeled for the neuronal marker NeuN have been described for aged mice stimulated with an enriched environment and showing preserved spatial learning performances [51].

Such effects on hippocampal neurogenesis may probably act in concert with stimulation of synaptic plasticity. Indeed, the suppression of neurogenesis by irradiation or genetic ablation has been shown to disrupt LTP in the dentate gyrus [57] and impairs some forms of hippocampus-dependent learning and memory [58,59]. Conversely, conditions that stimulate adult neurogenesis in mice also increase LTP [60,61]. Similarly, the activation of the RA signalling pathway can improve hippocampal plasticity either by enhancing synaptic strength as measured by LTP or by synaptic remodelling, known to modulate hippocampal-dependent memory processes [7,8,15,62].

In order to better understand some potential mechanisms involved in the regulation of hippocampal neurogenesis and spatial learning by vitamin A supplementation, we have evaluated serum retinol concentrations and hippocampal CRABP-I expression, indicators of vitamin A status and intracellular availability of RA. Vitamin A is mobilized from liver stores and transported into the plasma bound to a carrier protein [63]. At the target cell level, retinol is oxidised to RA which binds to cellular retinoic acid-binding proteins (CRABPs). This binding is known to regulate the nuclear activity of RA [63,64]. In the present study, aged rats exhibited a significant decrease in the serum retinol concentration compared to young rats, but vitamin A enriched diet failed to normalize this level. This reduction has been earlier reported in aged rats and humans [39,40,65] and could be explained during senescence by a loss of the capacity to mobilize vitamin A from the liver and thereby to regulate serum retinol levels, without affecting its ability of storage [66,67]. Thus, these age-induced alterations of retinol metabolism would explain why the newly absorbed vitamin

A from the diet was unable to correct serum retinol levels in aged rats. It has been shown that reduction in vitamin A metabolism during aging resulted in RA deficit at the cellular level [40]. However, in vitamin A deficiency, target tissues can directly use newly absorbed vitamin A from diet, in order to rapidly synthesize RA [41]. Thus, in enriched-aged rats which failed to normalize the serum retinol level, this newly absorbed vitamin A would be immediately used by target tissues such as the brain, to increase local synthesis of RA. In the rodent hippocampus, RA is synthesized from the meninges by anabolic enzymes (RALDHs) and would diffuse from this source to the blades of the DG [38]. It is probably one of the mechanisms by which vitamin A enriched diet would increase the levels of intracellular RA in the aged hippocampus without mobilization of vitamin A from the liver.

Moreover, the CRABPs (CRABP I and CRABP II) are also important regulators of the cellular bioavailability of RA in the brain [68,69]. Only CRABP-I is expressed in the hippocampus and more particularly in the dentate gyrus [38]. The precise role of CRABPs in RA signalling is still unclear, but some data provide evidence that CRABP-I buffers the free intracellular RA, and regulates by this way, the amount accessible to its nuclear receptors and therefore, its activity [70,71]. Here, we show that aging induced an up-regulation of hippocampal CRABP-I mRNA expression, normalized by vitamin A supplementation. This result suggests that vitamin A enriched-diet could normalize the level of free cellular RA in aged rats by a negative regulation of CRABP-I expression. This hypothesis is also sustained by a negative correlation evidenced between serum retinol levels and CRABP-I expression in young and aged groups (data not shown).

Together, the decreased hippocampal CRABP-I expression and the increased amount of intracellular RA synthesized from newly absorbed vitamin A in enriched aged group would lead to an over saturation of the binding capacities of CRABP-I. Therefore, the remaining free RA could bind to their nuclear receptors and modulate transcription of genes involved in hippocampal plasticity such as neurogenesis.

In the present study, we observed that inadequate vitamin A metabolism in aged rats could have some consequences on hippocampal neurogenesis and functions. Indeed, we evidenced a positive correlation between level of serum retinol, maturation of DCX positive cells and also spatial memory in young and aged groups. This result is coherent with other studies showing that demented patients exhibited low serum retinol concentration [72,73]. In enriched aged rats, the newly absorbed vitamin A from diet would be used to increase local synthesis of RA which would stimulate hippocampal neurogenesis and spatial memory. This hypothesis is supported by the detection of a gradient of RA which diffuses from the meninges across the infrapyramidal and suprapyramidal blades of the DG to differentially regulate hippocampal neurogenesis between these two blades [38].

Moreover, it has been shown that CRABP-I could play a crucial role in differentiation processes *in vitro* : (i) the up-regulation of CRABP-I in human neuroblastoma cells reduced their differentiation potential [43], (ii) the overexpression of CRABP I in F9 cells decreased their RA-induced differentiation and high concentration of RA become required for this cells to recover the responsiveness to RA, (iii) a reduced level of CRABP I led to a higher RA sensitivity of the cells to differentiate [74,75]. Accordingly to these studies, we have shown negative correlations between hippocampal CRABP-I expression and maturation of DCX positive cells suggesting that age-related up-regulation of hippocampal CRABP-I could contribute to hippocampal neurogenesis alterations. Moreover, this correlation is maintained if we integrate the enriched-aged group to the regression analysis. On

the contrary, no significant correlations were found between serum retinol and hippocampal neurogenesis in all groups (young, aged and enriched-aged). Thus, even if during senescence there is a loss of the capacity to regulate serum retinol levels by the diet, the newly absorbed vitamin A would be immediately used by target tissues such as the hippocampus leading to the normalization of the level of CRABP-I gene expression which could be determinant for hippocampal differentiation processes and thereby for hippocampal functions.

Thus, the expression of CRABP-I gene must be tightly controlled in order to maintain a homeostatic supply of free intracellular RA but this bioavailability can also be evaluated by measuring the expression of retinoid receptors. Several studies have demonstrated age-related decreases in mRNA expression or protein levels of retinoid receptors in mice and rat brain [7,11,12,15,50] indicating a decreased bioavailability of the ligand, which can be normalized by RA treatment or vitamin A supplementation. Interestingly, it has recently been demonstrated that the dysfunctioning of retinoid signalling induced by VAD decreased immunoreactivity of some retinoid receptors (RAR α and β) in the hippocampus, whereas immunoreactivity of RXR α and β was increased in the same area [76,77]. Similarly, an hippocampal over-expression of RXR α has been found in the aged rats of the present study and this effect can be restored by vitamin A supplementation (data not shown). Thus, although the newly absorbed vitamin A from the diet was unable to correct serum retinol levels in aged rats, as previously discussed, vitamin A supplementation can modulate hippocampal expression of some

retinoid receptors in aged rats suggesting that our findings on CRABP-I gene expression, neurogenesis and cognitive functions could be mediated, at least in part, through these retinoid receptors.

Altogether, the present study demonstrates for the first time that vitamin A supplementation from middle-age improves the level of differentiation of newly born neurons and enhances spatial memory during senescence. The normalization of the hippocampal expression of CRABP-I and thereby the increased bioavailability of intracellular RA could be a possible mechanism by which vitamin A supplementation acts on neuronal differentiation processes and exerts its promnesic effects during aging. As the dysfunctioning of retinoid signalling has been suggested to be involved in the aetiology of some neurodegenerative diseases such as Alzheimer's Disease (AD) [78,79,80,81], vitamin A supplementation from middle-age could be widely proposed for alleviating cognitive decline but also for delaying neurodegenerative processes.

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Author Contributions

Conceived and designed the experiments: KT PH. Performed the experiments: KT DB PR ER. Analyzed the data: KT. Contributed reagents/materials/analysis tools: ER. Wrote the paper: KT SA PL VP.

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