Normalization of hippocampal retinoic acid level corrects age-related memory deficits in rats

Fabien Dumetz, Corinne Bure, Serge Alfos, Marc Bonneu, Emmanuel Richard, Katia Touyarot, Anaïs Marie, Jean-Marie Schmitter, Clémentine Bosch-Bouju, Véronique Pallet

Normalization of hippocampal retinoic acid level corrects age-related memory deficits in rats

Fabien Dumetz[1], Corinne Buré[2], Serge Alfos[1], Marc Bonneu[2], Emmanuel Richard[3], Katia Touyarot[1], Anaïs Marie[1], Jean-Marie Schmitter[2], Clémentine Bosch-Bouju[1], Véronique Pallet[1]

[1]Univ. Bordeaux, INRA, Bordeaux INP, NutriNeuro, UMR 1286, F-33000, Bordeaux, France

[2]Univ. Bordeaux, CNRS, Bordeaux INP, CBMN, UMR 5248, F-33600, Pessac, France

[3]Univ. Bordeaux, INSERM, U1035, CHU Bordeaux, F-33000, Bordeaux, France

Corresponding author: Véronique Pallet (veronique.pallet@enscbp.fr)

ABSTRACT

Dietary micronutrients constitute a major environmental factor influencing aging processes. Vitamin A (vit. A) is the precursor of retinoic acid, a bioactive molecule that controls the expression of several genes involved in brain function. Evidence suggest a reduction of vit. A bioavailability with aging, but its impact on neuronal network is poorly understood. We investigated the mechanisms linking memory impairments with specific alterations of retinoic acid metabolism in the hippocampus. We compared young (10 weeks) and aged (16 months) rats, supplemented or not with dietary vitamin A (20 IU retinol/g) for 4 weeks. Our study reveals that aging induced dysregulation of gene expression involved in vit. A and retinoic acid metabolism in the liver. Furthermore, vit. A supplementation restored the integrity of the hippocampal neuronal morphology altered by aging. Importantly, we found a high correlation between hippocampal levels of retinoic acid and memory performance. The present work establishes the link between collapse of retinoid metabolism and age-related cognitive decline, highlighting the role of vit. A in maintaining memory through aging.

KEY WORDS: aging, retinol, retinoic acid, memory, neuronal morphology, hippocampus

ABBREVIATIONS: vit.A, vitamin A; RA, retinoic acid; IU : International Unit ; RBPR2, Retinol Binding Protein Receptor 2; LRAT, Lecithin:Retinol AcylTransferase; REH, Retinyl Ester Hydrolase; RALDH2, retinaldehyde dehydrogenase 2; CYP26A1, cytochrome P26A1; LC-ESI-MS/MS, liquid chromatography electrospray ionization tandem mass spectrometry; RAR, retinoic acid receptor; RXR, retinoid X receptor;

INTRODUCTION

Mechanisms of aging and age-related cognitive decline are currently a matter of intense research aiming at defining strategies for maintaining a good cognitive state and quality of life of the elderly. At the cellular and network levels, it is now well established that age-related cognitive impairments are paralleled by a loss of synaptic plasticity in the hippocampus, a key structure for memory processes (Burke and Barnes, 2006; Rosenzweig and Barnes, 2003; Shetty et al., 2017).

Optimal nutrition appears as a promising way to prevent and slow down age-related cognitive decline, without heavy pharmacological intervention. Indeed, it is now recognized that dietary factors, and micronutrients in particular, can act as signaling molecules to maintain brain functions (Gómez-Pinilla, 2008). Among them, vitamin A is of particular interest to maintain cognition along aging (Feart et al., 2005; Touyarot et al., 2013). Vitamin A (vit. A) is a micronutrient provided by animal foods, such as meat and dairy products. It can also be synthesized from carotenoids present in vegetables. In the organism, retinol is metabolized in retinoic acid (RA), its main bioactive derivative, which is implicated in the regulation of a large panel of genes (Blomhoff and Blomhoff, 2006). RA plays crucial roles during development but is also essential at adult age (Olson and Mello, 2010; Shearer et al., 2012; Stoney and McCaffery, 2016). In the brain, RA is notably involved in synaptic plasticity in the hippocampus (Aoto et al., 2008; Arendt et al., 2015a; Chen et al., 2014; Misner et al., 2001).

With aging, vit. A seems to accumulate in the liver, its storage organ (Pallet et al., 1997; van der Loo et al., 2004; Azaïs-Braesco et al., 1995); however, its bioavailability for target tissues appears altered. Supporting this, we previously demonstrated that receptors, transporters and molecules involved in the signaling pathway of RA in the brain are dysregulated with aging (Enderlin et al., 1997; Pallet et al., 1997; Touyarot et al., 2013). The resulting decrease in RA signaling is associated with impairments of memory performance. In particular, RA signaling in the brain is crucial for spatial and episodic-like memory (Cocco et al., 2002; Etchamendy et al., 2003; Mingaud et al., 2008; Olson and Mello, 2010; Touyarot et al., 2013). Recently, an association between low level of circulating retinol and age-related cognitive decline has also been found in humans (Huang et al., 2018).

As a clue of the causality between RA deficiency and memory impairments, vit. A supplementation in aged rodents was shown to reduce memory impairments (Mingaud et al., 2008; Touyarot et al., 2013). However, mechanisms linking alteration of vit.A metabolism in the liver and hyposignaling of RA pathway in the brain have not been elucidated yet. Additionally, the impact on neuronal networks of a reduced brain concentration of RA is poorly understood. Thus, there is no direct

evidence for the relationship between the collapse of vit. A metabolism /RA brain bioavailability and age-related cognitive impairment.

In this study we investigated the mechanisms linking specific alterations of RA metabolism in the hippocampus to memory impairments in aged rats supplemented or not with vit. A. Aging was associated with reduced RA availability and dysregulation of vit. A and RA metabolism in the liver. Importantly, we found a high correlation between hippocampal levels of RA and memory performance. Vit. A supplementation led to a concomitant restoration of the neuronal morphology of pyramidal cells in the hippocampus and of memory capacities altered with aging.

The present work establishes the link between retinoid metabolic collapse and age-related cognitive decline. It highlights the potential capacity of vit. A supplementation to restore memory capacities. This study brings new and useful knowledge on the importance of RA signaling for cognitive processes during aging.

1. MATERIAL AND METHODS

2.1. Animals and diet

All experiments were performed in accordance to criteria of the European Communities Council Directive 2010/63/UE and the French National Committee (4184-2016022209565094). Experiments were performed on male Wistar rats obtained from Janvier Labs (France). Young (10 weeks) and old (16 months) rats 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 hours), 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 arrival for 4 weeks. Control rats were fed control diet, with 5 IU retinol/g (INRA, Jouy-en-Josas), while rats enriched with vit. A received a diet containing 20 IU retinol/g (INRA, Jouy-en-Josas). The two diets were isocaloric and identical except the amount of retinol.

2.2. Behavioral assessment (Y-maze test)

Spatial memory was assessed in rats after 4 weeks of dietary protocol. The Y-maze paradigm was used to assess spatial memory as previously described (Delpech et al., 2015). Rats were handled daily for 1 week 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 made of dark grey plastic. Each arm was 34-cm long, 8-cm wide and 14-cm high. The floor of the maze was covered with used 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 2h of inter-trial interval, rats were placed back in the start arm and allowed 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.

2.3. Vitamin A concentration measurement

Blood collected during euthanasia was spun at 1,500 g for 15 min, the supernatant was removed and stored at -20°C until used. Aliquots of liver collected during euthanasia were stored at -80°C. Before retinol extraction they were homogenized in sodium phosphate-EDTA (0.05 M, pH 7.8) buffer. Retinol was extracted with hexane/BHT and assayed by HPLC according to a previously described method (Biesalski et al., 1983).

2.4. RA levels measurements

Tissues samples were collected and weighed under red light and retinoids were handled with glass single-use containers and pipettes. RA was extracted by a two-step acid-base extraction as described previously by Kane et Napoli (Kane and Napoli, 2010). 5 µM Acitretin (Sigma-Aldrich) was added as internal standard into samples to normalize extraction efficiency. LC-ESI-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. Analyses were achieved in the positive mode; nitrogen was used for the curtain gas (set to 20), gas1 (set to 35), gas2 (set to 0) and as collision gas. Needle voltage was at +5,500 V without needle heating; the declustering potential was set at +101 V and +236 V and the collision energy at +27 eV and +35 eV for RA and acitretin, respectively. 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). One transition is used for quantitation and the other for confirmation. The area of LC peaks was determined using MultiQuant software

(v2.1, Sciex). Reversed phase separations were carried out on an Ascentis RP Amide 150×1 mm column, with 3 µm particles (Supelco, Sigma Aldrich, St Quentin Fallavier, France). Eluent A was H_2O+0.1 % formic acid and eluent B was CH_3CN+0.1 % formic acid. The gradient elution program was: 0 min, 30 % B; 8 min, 30 % B; 10 min, 70 % B; 35–36 min, 87 % B; 37 min, 30 % B. The flow rate was 50 µL/min; 10µL sample volumes were injected. This protocol allows All-trans RA separation from its isomers. 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 correlate LC peaks area onto RA concentrations.

2.5. Neuronal morphology analysis

Golgi-Cox staining. Rats were deeply anesthetized with isoflurane and then decapitated. 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 (Janthakin et al., 2017). Briefly, one hemisphere per brain was immersed in the Golgi-Cox solution for 8 days before deep freezing in isopentane. 100 µm coronal sections containing the dorsal hippocampus were collected at −24 °C using a cryostat (Leica, Solms, Germany) and mounted on 3% gelatin-coated slides. When slides were totally dry (3 days after), they were stained and coverslipped with Depex. Exposure to light was limited during 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. Dendritic arbor complexity was evaluated in pyramidal neurons of the hippocampal CA1 subfield. The following criteria were used to select pyramidal neurons: (a) full impregnation of the neurons along the entire length of the dendritic tree; (b) dendrites without significant truncation of branches; and (c) relative isolation from neighboring impregnated neurons, astrocytes or blood vessels. For each rat, 4-6 neurons fulfilling the criteria were finally selected.

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 (http://imagej.nih.gov.gate2.inist.fr/ij/) and NDPI tools plugin (Deroulers et al., 2013). For each selected neuron, all branches of the dendritic tree were semi-automatically reconstructed in 3D using the Imaris software (Oxford Instruments, Zürich, Switzerland).

2.6. mRNA expression analysis by RT-qPCR

Aliquots of liver stored at -80°C were homogenized in 1ml of Trizol reagent (Invitrogen, France) and total RNAs were extracted according to the manufacturer's instructions. The quality and the concentration of the purified RNA was measured by using a Nanodrop One (Ozyme, France). The integrity of RNA samples was assessed using the RNA 6000 Nano LabChip kit in combination with the 2100 Bioanalyzer (Agilent Technologies, France). Using oligo dT and random primers, cDNA was synthesized with ImProm II reverse transcriptase (Promega, France) according to the manufacturer's instructions. The real-time PCR was performed using the LightCycler 480 system (Roche Diagnostics, Mannheim, Germany) with a 384-well format, in a final volume of 10 µl, using the SYBR Green I Master kit, as previously described (Touyarot et al., 2013). The forward and reverse primer sequences used are the following: *Rbpr2*, 5'-TGCTTCTCATCGGAGGCATG-3' and 5'-AGTAACCACAAACCAGGTCAGG-3'; *Lrat* 5'-AGTGTCACGGACCCATTTTACC-3' and 5'-ACCTTCTGAGTGCGTTCCTTG-3'; *Reh* 5'-TGAAGTTCTGGGCCAACTTTGC-3' and 5'-TGGCACCAATCTGCAAATACCC-3'; *Raldh2* 5'-AAGCTTGCAGACTTGGTGGAAC-3' and 5'-AAGCTTGCAGGAATGGCTTACC-3'; *Cyp26a1* 5'-AAGCGCAGGAAATACGGCTTC-3' and 5'-AAGATGCGCCGCACATTATCC-3'; *Bmg* 5'-GCCCAACTTCCTCAACTGCTACG-3' and 5'-GCATATACATCGGTCTCGGTGGG-3'. The specificity of the amplified products was verified by the melting curve analysis showing a single melting peak after amplification. Data analysis was performed using the Roche's E-method of relative quantification, which uses standard curve derived efficiencies, of the LightCycler 480 1.5 version software. In this study we used the b2-microglobulin (BMG) housekeeping gene as the reference gene since its expression level was unaffected by our experimental conditions.

2.7. Statistical analysis

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 ± SEM. Statistical analyses performed for each experiment are summarized in each figure legend with the chosen statistical test, n and p-values. Normality of data were first attested with D'agostino & Pearson normality test. As appropriate, we used RM 1-way ANOVA with Tukey's multiple comparison test or 2-way ANOVA followed by the Bonferroni's multiple comparison test when interaction has a p value < 0.05. Correlations were calculated with Pearson test or non-parametric Spearman test when appropriate. Calculated F and p values are summarized in Supplemental Table 1.

3. RESULTS

3.1. RA levels are restored by dietary vit. A in aged rats

Young and 16-month old rats were fed with either control (5 IU retinol/g) or vit. A supplemented diet (20 IU retinol/g) for 4 weeks. We first assessed the levels of retinol and RA in the serum. Quantitative measurements relied on a purposely designed LC-ESI-MS/MS method using Multiple Reaction Monitoring (MRM). In accordance with previous reports (Chevalier et al., 1999; Touyarot et al., 2013), a lower retinol concentration was found in the serum of aged rats compared to control rats (young: 1.87 ± 0.01 µM *vs* aged: 0.72 ± 0.02 µM, Figure 1A). Here, we found additionally that RA concentration was also significantly reduced in the serum of aged rats compared to young rats (young: 11.88 ± 3.42 pmol/ml *vs* aged: 0.92 ± 0.17 pmol/ml, Figure 1B). These results support a decreased bioavailability of retinol and RA with aging. Of note, individual variability within a group was high for RA concentration, while it was lower for retinol. Four weeks of dietary supplementation with vit. A corrected level of both retinol and RA in the serum of aged rats, but did not alter concentrations in young rats (Figure 1 A, B).

We then quantified levels of RA in the hippocampus, a brain structure highly involved in spatial memory in rats. We found that hippocampal concentration of RA in aged animals was highly decreased compared to young rats (young: 113.9 ± 17.6 pmol/g *vs* aged: 23.2 ± 5.5 pmol/g, Figure 1C). Furthermore, 4 weeks of vit. A supplementation efficiently normalized RA concentration in their hippocampus (Figure 1C). Of note, hippocampus level of RA correlated with RA level in the serum in aged animals, but not with retinol level (Figure 1D, RA: Spearman r = 0.7614, p = 0.0002; retinol: Spearman r = 0.3973, p = 0.1025). This suggests that blood RA is more likely the source of hippocampal RA.

3.2. Metabolism of retinol and RA is altered with aging and restored by supplementation

We hypothesized that decreased levels of retinol and RA observed upon aging originated from a metabolism dysfunction. To investigate this, we compared retinol and RA concentrations in the liver of young and aged rats. We found that retinol and RA concentrations were massively increased in the liver of aged rats compared to young rats (retinol: young: 0.98 ± 0.01 pmol/g *vs* aged: 5.27 ± 0.47 pmol/g; RA: young: 142.0 ± 9.5 pmol/g *vs* aged: 422.6 ± 104.1 pmol/g, Figure 2A, B). Notably, Vit. A supplementation for 4 weeks further increased retinol levels in the liver in both

young and aged rats (Figure 2A), while RA levels were not affected by vit. A supplementation (Figure 2B).

The discrepancy between decreased levels of retinol / RA in the serum and elevated levels in the liver suggests that aging induces a dysregulation of retinol / RA release from the liver to the serum. To better understand this, we analyzed the mRNA expression level of genes coding for proteins involved in the metabolism of retinol and RA. In aged rats, the gene expression for the protein responsible of retinol entry in the liver, RBPR2 (Retinol Binding Protein Receptor 2) was increased (young: 0.51 ± 0.04 *vs* aged: 1.02 ± 0.10, Figure 2C). Meanwhile, we observed a decreased mRNA expression for *Lrat* (Lecithin:Retinol AcylTransferase) (young: 0.83 ± 0.08 *vs* aged: 0.14 ± 0.05) and a trend to decrease for *Reh* (Retinyl Ester Hydrolase) (young: 0.33 ± 0.01 *vs* aged: 0.25 ± 0.03) in the liver of aged rats, suggesting a reduced retinol esterification (Figure 2 D, E). This is in accordance with high levels of retinol in the liver of aged rats. Expression levels of *Rbpr2*, *Lrat* and *Reh* mRNAs were normalized by vit. A supplementation in aged rats. Additionally, the mRNA expression of *Raldh2*, the enzyme of RA synthesis, was slightly decreased in aged rats compared to young rats, and was significantly increased by vit. A supplementation (young: 0.30 ± 0.03*;* aged: 0.17 ± 0.06; young + vit. A: 0.23 ± 0.02*;* aged + vit. A: 0.46 ± 0.05; Figure 2F). Finally, the gene expression of *Cyp26a1*, coding for the degradation enzyme of RA, was significantly decreased in aged rats and normalized after 4 weeks of vit. A supplementation, similarly to serum levels of RA and retinol (young: 0.40 ± 0.07*;* aged: 0.14 ± 0.02; young + vit. A: 0.35 ± 0.04*;* aged + vit. A: 0.40 ± 0.06; Figure 2G).

Together, these data are coherent with a decreased ability of the liver to release retinol and RA with aging. In addition, the regulation of enzymes by the supplement of Vit. A brought by the enriched diet, may contribute to reactivate the release of retinol and RA from liver, and to normalize RA levels in the serum.

3.3. Neuronal morphology in aged rats is normalized by vit. A supplementation

Aging is associated to a reduction of hippocampal volume and neuronal arborization in both humans and rodents that is thought to participate to age-related cognitive decline (Bartsch and Wulff, 2015; Burke and Barnes, 2006; de Flores et al., 2015). To decipher the impact of vitamin A on neuronal network, we analyzed the neuronal morphology of pyramidal cells in the CA1 region of the dorsal hippocampus (Figure 3A). Golgi staining revealed that the total dendrite length of pyramidal neurons was significantly lowered in aged animals compared to young rats; Figure 3 A-C). This reduction in dendrite length was present at both basal (young: 3358 ± 291 µm *vs* aged:

2079 ± 174 µm) and apical (young: 2577 ± 156 µm *vs* aged: 1738 ± 114 µm) poles of CA1 pyramidal neurons. In details, dendritic lengths between aged and young rats were similar for primary and secondary dendrites, but were reduced for aged rats at higher levels of dendrites (Figure 3 D, E). This result indicates an atrophy of neurons at more distal dendrites, for both basal and apical dendrite trees. The number of branches was also significantly reduced in aged rats (Apical: young: 41.2 ± 2.7 µm *vs* aged: 24.9 ± 1.7 µm; basal: young: 53.5 ± 4.9 µm *vs* aged: 27.4 ± 2.6 µm; Figure 3F, G), indicating a reduction in dendrite ramifications. Sholl intersections were significantly fewer in aged rats at a distance of 40-160 µm from the soma for apical dendrites (Figure 3H) and 60-220 µm from the soma for basal dendrites (Figure 3I).

For aged rats, a diet supplemented in Vit. A ameliorates all measured parameters of both apical and basal dendrite morphology. Total dendrite length of CA1 pyramidal neurons in vit. A supplemented aged-rats was similar to young rats (Figure 3 A-E), as well as the number of branches (Figure 3F, G) and Sholl intersections (Figure 3 H, I). In parallel, Vit. A supplementation in young rats did not alter the measured morphological parameters. Thus, vit. A supplementation abolished alterations of neuronal morphology due to aging.

3.4. Spatial memory performance of aged rats match with hippocampal RA levels

It is commonly assumed that dendritic tree structure shapes brain networks and thus influences memory performance. To understand the consequence of RA bioavailability on age-related cognitive decline, we evaluated memory performance with the Y-maze paradigm. The total distance traveled in the Y-maze was not different between the 4 groups (Figure 4A). In the Y-maze task, young rats spent significantly more time in the new arm than in the familiar arm (70.4 ± 2.7 % of time in the new arm; Figure 4B). Conversely, aged rats did not discriminate the familiar arm from new one (42.0 ± 4.7 % of time in the new arm; Figure 4B). Four weeks of vit. A supplementation efficiently corrected impairment of spatial memory observed in aged rats and had no impact on spatial memory of young rats (young + vit. A: 81.5 ± 2.4 % of time in the new arm *vs* aged + vit. A: 64.1 ± 6.4 % of time in the new arm; Figure 4C).

To clarify the link between hippocampal RA levels and memory, we plotted the time spent in the new arm as a factor of hippocampus RA concentration (Figure 4D). We observed a significant correlation between memory performance and hippocampus RA concentration when collating all groups (Pearson $r = 0.6719$, $p < 0.0001$). Conversely, memory performance correlated poorly with serum levels of RA (Spearman $r = 0.3172$, $p = 0.0523$).

When groups were considered independently, correlation between memory performance and RA level in the hippocampus was significant only for aged rats (Pearson r = 0.6448, p = 0.0441), and not for young rats (Pearson r = 0.0.3671, p = 0.1340) or aged rats submitted to a diet enriched with vit. A (Pearson r = 0.5242, p = 0.1199) (Figure 4D). While RA hippocampus level seems to become a limiting factor in aged rats, enriched aged rats and young +/- vit. A supplementation exhibited a concentration sufficient to enable good memory performance. We used a non-linear regression to fit optimally the data between hippocampus RA concentration and memory performance in the Y-maze for the four groups. We observed that hippocampus RA level and memory were closely related for RA concentration values below 34 pmol/g, while plots over this value were distributed along a plateau (Figure 4D). This final result underlines the need for a minimal level of RA in the hippocampus to function correctly and permit memory performance.

4. DISCUSSION

In summary, our work showed that a vit. A enriched diet constitutes a powerful strategy for improving hippocampus neuronal network and memory performance in aged rats. In addition, we showed for the first time that memory improvement is directly related to hippocampus RA concentration increase induced by vit. A supplementation.

4.1. Retinoids availability reduces with aging

The impact of aging on retinoid signaling has been previously studied indirectly by measuring expression levels of related enzymes and receptors, in both humans and rodent models (Azaïs-Braesco et al., 1995; Enderlin et al., 1997; Etchamendy et al., 2003; Feart et al., 2005; Touyarot et al., 2013). Here, on the basis of a previously described method (Kane and Napoli, 2010), we have designed a highly sensitive analytical method with LC-ESI-MS/MS to directly measure RA levels into the hippocampus. The small quantities of tissue required (> 15mg) enable regional specificity. Remarkably, our results revealed that levels of RA in the hippocampus of young rats are within the same range than those found in the liver. These results are in accordance with a previous report (Kane and Napoli, 2010), highlighting the importance of RA levels in the hippocampus.

Here, we demonstrated that both retinol and RA concentrations were reduced in the serum of aged rats. The observation of a decreased retinol level in the serum was in accordance with previous reports (Chevalier et al., 1999; Touyarot et al., 2013). Additionally, we revealed that vit A dietary supplementation for 4 weeks is sufficient to significantly increase serum levels of retinol,

as well as RA in aged rats. Our data showed that levels of RA in the serum and in the hippocampus were variable between rats. This variability was reliable for a same animal between the serum and the hippocampus, indicating that the variability was not due to technical aspects of RA measurement. This suggests a high inter-individual variability, however we did not find a factor explaining these differences (e.g. no correlation with weight or weight gain). Our data also highlighted the very low level of RA in the hippocampus of aged rats. Considering that RA directly activates the expression of its own receptors, this confirms previous results showing an age-related decreased expression of RA receptors (Enderlin et al., 1997; Etchamendy et al., 2001).

The present study demonstrates that a reduced RA concentration in the hippocampus is an important trait in the physiopathology of aging. Considering that RA is critical for many processes in the adult brain (Olson and Mello, 2010; Shearer et al., 2012; Stoney and McCaffery, 2016), maintaining sufficient RA levels through aging might constitute an important issue to prevent aged-related brain dysfunctions in humans. Indeed, since hypoactivity of retinoid signaling has also been demonstrated in the serum of aged humans (Feart et al., 2005), we can suppose that, as in rodents, RA concentration might be decreased in aged humans. Our experiment demonstrates that vit A. supplementation of aged rats allows restoration of hippocampus RA to the level of young rats. This level of RA seems to be well regulated when vit. A is provided to aged rats through the diet, with no risk of overloading the brain with RA, as seen with stable levels in supplemented young rats. Conversely, direct injection of RA in aged rats induced an increase of hippocampus RA level well over the one of young rats (data not shown). Dietary vit. A supplementation thus appears as a promising way to maintain RA levels through aging. However, while our data clearly demonstrate the advantages of enriching vit. A in the diet, it should be called to mind that excess of vitamin A can be toxic (Adams, 2010). Therefore, toxicity studies will be needed before dietary vit. A supplementation is envisaged. Here, we found that RA concentration in the hippocampus of aged rats significantly correlates with RA but not retinol level in the serum, which suggests that part of brain RA directly comes from serum RA. This result enhances previous knowledge about sources of RA in the hippocampus (serum and local synthesis) (Arendt et al., 2015b; Goodman et al., 2012; Kurlandsky et al., 1995; Lane and Bailey, 2005). In addition, our data suggests that RA level in the serum could be relevant as a marker of brain RA levels, and might serve to identify aged people at risk of RA deficiency.

4.2. Retinoids metabolism in the liver is dysregulated with aging

In this paper, we addressed the question of the origin of retinol and RA collapse occurring with aging. We showed that in the liver of aged rats, retinol and RA were present in remarkably high concentrations compared to young animals. While it was already known that hepatic retinol stock increases with age (Chevalier et al., 1999; Pallet et al., 1997; van der Loo et al., 2004), increased RA level in the liver of aged rats constitutes an original result of importance. It is noteworthy that the hepatic variations of retinol and RA level with aging are diametrically opposed to their variations in the serum. This discrepancy suggests that aging induces an important dysregulation of retinol / RA release from the liver to the serum. Thus, aging leads to hepatic trapping not only of vit.A, but also of RA, which is no longer captured by the bloodstream. Importantly, dietary vit.A supplementation reverses these processes and restores circulating levels of RA.

The mechanism for RA export from the liver to the blood is unknown, as well as the nature of proteins responsible for RA transport in the blood, other than albumin (Sani et al., 1978; Smith et al., 1973). This study does not give additional clue to understand mechanisms of RA export from the liver to the blood, and its deregulation occurring with age. However, we can postulate that age-related accumulation of retinol and RA in the liver is due to a dysregulation of several enzymes involved in retinol and RA metabolism. Indeed, retinol entry *via* RBPR2 transporter is increased, concomitantly with decreased metabolism, i.e. esterification and dehydrogenation through LRAT and RALDH2 enzymes respectively. In parallel, RA increase is amplified by a lack of its degradation due to CYP26A1 decreased expression (Figure 5).

Remarkably, vit. A supplementation restored the levels of mRNA expression of all of these metabolism enzymes together with RA levels in the blood and in the hippocampus. This nutritional supplementation, by increasing retinol serum levels, may regulate retinoid metabolism enzymes in the liver and thus, create a virtuous circle that may release RA to the serum, and consequently to the hippocampus. Moreover, the restoration by the supplementation of the retinoid mobilization from the liver may be explained by the bypass pathway (Goodman et al., 1965; Li et al., 2014). This pathway processes dietary retinol without passing by the liver. A more recent study found that in neonates under vitamin A-marginal condition, supplementation with vitamin A increases the part of retinol in the brain that is coming from this bypass (Hodges et al., 2016). Further investigations with radiolabeled retinol and metabolic models, (Hodges et al., 2017, 2016) are needed to determine the precursory events that initiate the dysregulation occurring with aging and how precisely dietary vit. A supplementation arrests it.

4.3. Normalization of hippocampal retinoic acid level corrects hippocampal network and age-related memory deficits in rats

Dendritic arborization of neurons determine their capacity to adequately process in brain networks (Jan and Jan, 2010). Quality and complexity of dendritic branching of hippocampal neurons is thus crucial for cognitive and memory processes (Kulkarni and Firestein, 2012). Aging is characterized by a shrinkage of dendritic arborization, particularly in the hippocampus (Bartsch and Wulff, 2015; Burke and Barnes, 2006), which is presumably an important determinant for age-related cognitive decline (Bartsch and Wulff, 2015; Kulkarni and Firestein, 2012). In our study, we focused on the CA1 region, a subfield of the hippocampus that is highly involved spatial memory as tested in the Y-maze test. Furthermore, CA1 region expresses RARα, RARγ and RXRβ (Goodman et al., 2012; Krezel et al., 1999), which makes it sensitive to modulation by dietary vitamin A. We confirmed that aged rats exhibit a strong reduction of dendritic arborization, in terms of total length as well as branching density, in the CA1 region of the dorsal hippocampus. We further demonstrated that dietary vit. A supplementation totally normalized dendritic arborization of aged rats to the levels of young rats, in terms of length and branching. The mechanisms by which vit. A supplementation restores dendritic arborization in CA1 region may be multifactorial, which would need further investigation. However, we can assume that these mechanisms involve directly and/or indirectly RA, the active metabolite of vit. A in the brain. As a matter of fact, it is established that RA *via* its nuclear receptors is a developmental morphogen responsible for early patterning of the brain (Maden, 2007). At adult age, RA is also described as a key player in plasticity. Indeed, retinoic acid and its nuclear receptors (RAR, RXR) are involved in dendritic spines formation (Chen and Napoli, 2008) as well as in synaptic plasticity, notably in the hippocampus (Aoto et al., 2008; Chen et al., 2014; Chiang et al., 1998). In accordance with this, we previously showed that RA in aged rodents improves synaptic plasticity, i.e. long term potentiation and expression of genes involved in synaptic plasticity (Etchamendy et al., 2001; Féart et al., 2005). RA also facilitates neuronal survival and neurite outgrowth and induces neuronal differentiation (Chen and Napoli, 2008; Christie et al., 2010; Takahashi et al., 1999). At the molecular level, RA binds to nuclear receptors which modulates gene transcription. Thus, future investigation with transcriptomic approaches may identify all target genes of RA in relation to plasticity of dendritic arborization through aging.

Finally, our study reveals that restoration of dendritic arborization and RA level in the hippocampus by dietary vit. A supplementation is concomitant with a reinstatement of memory

performance in aged rats. The direct role of hippocampal RA in the modulation of memory performances along the life has already been argued (Etchamendy et al., 2003; Touyarot et al., 2013) but without evidence for a direct link. Our present study reveals a non-linear correlation between RA levels in the hippocampus and memory performance of aged rats. Importantly, our results are in accordance with a recent study showing a relation between blood levels of retinol and age-related cognitive decline in humans (Huang et al., 2018). In the present work, we assessed episodic-like memory since it is particularly sensitive to aging processes. In non-supplemented aged rats, the correlation between memory performance and RA levels in the hippocampus was highly significant, with the best performing rats exhibiting the highest levels of RA in the hippocampus. Interestingly, in supplemented aged rats, the correlation becomes low (no significant), similarly to young rats. This reflects high memory performance enabled by a sufficient amount of hippocampal RA. The non-linear fit of the data reveals that most of rats with high memory performance (at the level of young rats) exhibit a concentration of RA on the hippocampus higher than 34 pmol/g. This threshold might represent the minimal and necessary concentration of RA in the rat hippocampus for the elaboration of neuronal enriched circuits, required for optimal memory processes.

In conclusion, these original results underpinned the importance of RA signaling for hippocampus functionality. This brings new knowledge on the mechanisms linking alteration of vit. A metabolism, RA signaling in the brain and aged-related cognitive impairments.

COMPETING INTEREST

No competing interest is declared by the authors.

FUNDING

This work was supported by Institut CARNOT LISA (ANR) and the Région Nouvelle Aquitaine. Mass spectrometry equipment was supported by the Région Nouvelle Aquitaine and the Platform Proteome (https://proteome.cgfb.u-bordeaux.fr).

ACKNOWLEDGMENTS

The authors would like to thank staff from NutriNeuro animal facility and office. The microscopy was done in the Bordeaux Imaging Center a service unit of the CNRS-INSERM and Bordeaux University, member of the national infrastructure France BioImaging supported by the French

National Research Agency (ANR-10-INBS-04). The help of Christel Poujol and Sébastien Marais is acknowledged.

AUTHORS CONTRIBUTION

Conceptualization and project administration: VP

Funding and supervision of staff: VP

Methods development: FD, CB, KT

Experiments: FD, CB, SA, AM, ER, CB-B

Statistical analyses: CB-B, FD, SA

Visualization: CB-B

Writing – original draft: CB-B, VP

Writing – Review & Editing: CB-B, VP, CB, FD, SA, MB, KT, AM, J-MS

REFERENCES

Adams, J., 2010. The neurobehavioral teratology of retinoids: a 50-year history. Birt. Defects Res. A. Clin. Mol. Teratol. 88, 895–905. https://doi.org/10.1002/bdra.20721

Aoto, J., Nam, C.I., Poon, M.M., Ting, P., Chen, L., 2008. Synaptic signaling by all-trans retinoic acid in homeostatic synaptic plasticity. Neuron 60, 308–320. https://doi.org/10.1016/j.neuron.2008.08.012

Arendt, K.L., Zhang, Y., Jurado, S., Malenka, R.C., Südhof, T.C., Chen, L., 2015a. Retinoic Acid and LTP Recruit Postsynaptic AMPA Receptors Using Distinct SNARE-Dependent Mechanisms. Neuron 86, 442–456. https://doi.org/10.1016/j.neuron.2015.03.009

Arendt, K.L., Zhang, Z., Ganesan, S., Hintze, M., Shin, M.M., Tang, Y., Cho, A., Graef, I.A., Chen, L., 2015b. Calcineurin mediates homeostatic synaptic plasticity by regulating retinoic acid synthesis. Proc. Natl. Acad. Sci. 112, E5744–E5752. https://doi.org/10.1073/pnas.1510239112

Azaïs-Braesco, V., Morinière, C., Guesne, B., Partier, A., Bellenand, P., Baguelin, D., Grolier, P., Alix, E., 1995. Vitamin A status in the institutionalized elderly. Critical analysis of four evaluation criteria: dietary vitamin A intake, serum retinol, relative dose-response test (RDR) and impression cytology with transfer (ICT). Int. J. Vitam. Nutr. Res. Int. Z. Vitam.-Ernahrungsforschung J. Int. Vitaminol. Nutr. 65, 151–161.

Bartsch, T., Wulff, P., 2015. The hippocampus in aging and disease: From plasticity to vulnerability. Neuroscience 309, 1–16. https://doi.org/10.1016/j.neuroscience.2015.07.084

Biesalski, H.K., Ehrenthal, W., Gross, M., Hafner, G., Harth, O., 1983. Rapid determination of retinol (vitamin A) in serum by high pressure liquid chromatography (HPLC). Int. J. Vitam. Nutr. Res. Int. Z. Vitam.- Ernahrungsforschung J. Int. Vitaminol. Nutr. 53, 130–137.

Blomhoff, R., Blomhoff, H.K., 2006. Overview of retinoid metabolism and function. J. Neurobiol. 66, 606–630. https://doi.org/10.1002/neu.20242

Burke, S.N., Barnes, C.A., 2006. Neural plasticity in the ageing brain. Nat. Rev. Neurosci. 7, 30–40. https://doi.org/10.1038/nrn1809

Chen, L., Lau, A.G., Sarti, F., 2014. Synaptic retinoic acid signaling and homeostatic synaptic plasticity. Neuropharmacology 78, 3–12. https://doi.org/10.1016/j.neuropharm.2012.12.004

Chen, N., Napoli, J.L., 2008. All-trans-retinoic acid stimulates translation and induces spine formation in hippocampal neurons through a membrane-associated RARalpha. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. 22, 236–245. https://doi.org/10.1096/fj.07-8739com

Chevalier, S., Blaner, W.S., Azais-Braesco, V., Tuchweber, B., 1999. Dietary restriction alters retinol and retinol-binding protein metabolism in aging rats. J. Gerontol. A. Biol. Sci. Med. Sci. 54, B384-392.

Chiang, M.Y., Misner, D., Kempermann, G., Schikorski, T., Giguère, V., Sucov, H.M., Gage, F.H., Stevens, C.F., Evans, R.M., 1998. An essential role for retinoid receptors RARbeta and RXRgamma in long-term potentiation and depression. Neuron 21, 1353–1361.

Christie, V.B., Maltman, D.J., Henderson, A.P., Whiting, A., Marder, T.B., Lako, M., Przyborski, S.A., 2010. Retinoid supplementation of differentiating human neural progenitors and embryonic stem cells leads to enhanced neurogenesis in vitro. J. Neurosci. Methods 193, 239–245. https://doi.org/10.1016/j.jneumeth.2010.08.022

Cocco, S., Diaz, G., Stancampiano, R., Diana, A., Carta, M., Curreli, R., Sarais, L., Fadda, F., 2002. Vitamin A deficiency produces spatial learning and memory impairment in rats. Neuroscience 115, 475–482.

de Flores, R., La Joie, R., Chételat, G., 2015. Structural imaging of hippocampal subfields in healthy aging and Alzheimer's disease. Neuroscience 309, 29–50. https://doi.org/10.1016/j.neuroscience.2015.08.033

Delpech, J.-C., Madore, C., Joffre, C., Aubert, A., Kang, J.X., Nadjar, A., Layé, S., 2015. Transgenic increase in n-3/n-6 fatty acid ratio protects against cognitive deficits induced by an immune challenge through decrease of neuroinflammation. Neuropsychopharmacol. Off. Publ. Am. Coll. Neuropsychopharmacol. 40, 525–536. https://doi.org/10.1038/npp.2014.196

Deroulers, C., Ameisen, D., Badoual, M., Gerin, C., Granier, A., Lartaud, M., 2013. Analyzing huge pathology images with open source software. Diagn. Pathol. 8, 92. https://doi.org/10.1186/1746-1596-8-92

Enderlin, V., Pallet, V., Alfos, S., Dargelos, E., Jaffard, R., Garcin, H., Higueret, P., 1997. Age-related decreases in mRNA for brain nuclear receptors and target genes are reversed by retinoic acid treatment. Neurosci. Lett. 229, 125–129.

Etchamendy, N., Enderlin, V., Marighetto, A., Pallet, V., Higueret, P., Jaffard, R., 2003. Vitamin A deficiency and relational memory deficit in adult mice: relationships with changes in brain retinoid signalling. Behav. Brain Res. 145, 37–49.

Etchamendy, N., Enderlin, V., Marighetto, A., Vouimba, R.M., Pallet, V., Jaffard, R., Higueret, P., 2001. Alleviation of a selective age-related relational memory deficit in mice by pharmacologically induced normalization of brain retinoid signaling. J. Neurosci. Off. J. Soc. Neurosci. 21, 6423–6429.

Féart, C., Mingaud, F., Enderlin, V., Husson, M., Alfos, S., Higueret, P., Pallet, V., 2005. Differential effect of retinoic acid and triiodothyronine on the age-related hypo-expression of

neurogranin in rat. Neurobiol. Aging 26, 729–738. https://doi.org/10.1016/j.neurobiolaging.2004.06.004
Feart, C., Pallet, V., Boucheron, C., Higueret, D., Alfos, S., Letenneur, L., Dartigues, J.F., Higueret, P., 2005. Aging affects the retinoic acid and the triiodothyronine nuclear receptor mRNA expression in human peripheral blood mononuclear cells. Eur. J. Endocrinol. 152, 449–458. https://doi.org/10.1530/eje.1.01858
Gómez-Pinilla, F., 2008. Brain foods: the effects of nutrients on brain function. Nat. Rev. Neurosci. 9, 568–578. https://doi.org/10.1038/nrn2421
Goodman, D.S., Huang, H.S., Shiratori, T., 1965. Tissue distribution and metabolism of newly absorbed vitamin A in the rat 7.
Goodman, T., Crandall, J.E., Nanescu, S.E., Quadro, L., Shearer, K., Ross, A., McCaffery, P., 2012. Patterning of retinoic acid signaling and cell proliferation in the hippocampus. Hippocampus 22, 2171–2183. https://doi.org/10.1002/hipo.22037
Hodges, J.K., Tan, L., Green, M.H., Ross, A.C., 2017. Vitamin A supplementation redirects the flow of retinyl esters from peripheral to central organs of neonatal rats raised under vitamin A-marginal conditions. Am. J. Clin. Nutr. 105, 1110–1121. https://doi.org/10.3945/ajcn.116.149039
Hodges, J.K., Tan, L., Green, M.H., Ross, A.C., 2016. Vitamin A Supplementation Increases the Uptake of Chylomicron Retinyl Esters into the Brain of Neonatal Rats Raised under Vitamin A-Marginal Conditions. J. Nutr. 146, 1677–1683. https://doi.org/10.3945/jn.116.233692
Huang, X., Zhang, H., Zhen, J., Dong, S., Guo, Y., Van Halm-Lutterodt, N., Yuan, L., 2018. Diminished circulating retinol and elevated α-TOH/retinol ratio predict an increased risk of cognitive decline in aging Chinese adults, especially in subjects with ApoE2 or ApoE4 genotype. Aging 10, 4066–4083. https://doi.org/10.18632/aging.101694
Jan, Y.-N., Jan, L.Y., 2010. Branching out: mechanisms of dendritic arborization. Nat. Rev. Neurosci. 11, 316–328. https://doi.org/10.1038/nrn2836
Kane, M.A., Napoli, J.L., 2010. Quantification of endogenous retinoids. Methods Mol. Biol. Clifton NJ 652, 1–54. https://doi.org/10.1007/978-1-60327-325-1_1
Krezel, W., Kastner, P., Chambon, P., 1999. Differential expression of retinoid receptors in the adult mouse central nervous system. Neuroscience 89, 1291–1300.
Kulkarni, V.A., Firestein, B.L., 2012. The dendritic tree and brain disorders. Mol. Cell. Neurosci. 50, 10–20. https://doi.org/10.1016/j.mcn.2012.03.005
Kurlandsky, S.B., Gamble, M.V., Ramakrishnan, R., Blaner, W.S., 1995. Plasma delivery of retinoic acid to tissues in the rat. J. Biol. Chem. 270, 17850–17857. https://doi.org/10.1074/jbc.270.30.17850
Lane, M.A., Bailey, S.J., 2005. Role of retinoid signalling in the adult brain. Prog. Neurobiol. 75, 275–293. https://doi.org/10.1016/j.pneurobio.2005.03.002
Li, Y., Wongsiriroj, N., Blaner, W.S., 2014. The multifaceted nature of retinoid transport and metabolism. Hepatobiliary Surg. Nutr. 3, 126–139. https://doi.org/10.3978/j.issn.2304-3881.2014.05.04
Maden, M., 2007. Retinoic acid in the development, regeneration and maintenance of the nervous system. Nat. Rev. Neurosci. 8, 755–765. https://doi.org/10.1038/nrn2212
Mingaud, F., Mormede, C., Etchamendy, N., Mons, N., Niedergang, B., Wietrzych, M., Pallet, V., Jaffard, R., Krezel, W., Higueret, P., Marighetto, A., 2008. Retinoid hyposignaling contributes to aging-related decline in hippocampal function in short-term/working memory organization and long-term declarative memory encoding in mice. J. Neurosci. Off. J. Soc. Neurosci. 28, 279–291. https://doi.org/10.1523/JNEUROSCI.4065-07.2008
Misner, D.L., Jacobs, S., Shimizu, Y., de Urquiza, A.M., Solomin, L., Perlmann, T., De Luca, L.M., Stevens, C.F., Evans, R.M., 2001. Vitamin A deprivation results in reversible loss of

hippocampal long-term synaptic plasticity. Proc. Natl. Acad. Sci. U. S. A. 98, 11714–11719. https://doi.org/10.1073/pnas.191369798
Olson, C.R., Mello, C.V., 2010. Significance of vitamin A to brain function, behavior and learning. Mol. Nutr. Food Res. 54, 489–495. https://doi.org/10.1002/mnfr.200900246
Pallet, V., Azaïs-Braesco, V., Enderlin, V., Grolier, P., Noël-Suberville, C., Garcin, H., Higueret, P., 1997. Aging decreases retinoic acid and triiodothyronine nuclear expression in rat liver: exogenous retinol and retinoic acid differentially modulate this decreased expression. Mech. Ageing Dev. 99, 123–136.
Rosenzweig, E.S., Barnes, C.A., 2003. Impact of aging on hippocampal function: plasticity, network dynamics, and cognition. Prog. Neurobiol. 69, 143–179.
Sani, B.P., Titus, B.C., Banerjee, C.K., 1978. Determination of binding affinities of retinoids to retinoic acid-binding protein and serum albumin. Biochem. J. 171, 711–717. https://doi.org/10.1042/bj1710711
Shearer, K.D., Stoney, P.N., Morgan, P.J., McCaffery, P.J., 2012. A vitamin for the brain. Trends Neurosci. 35, 733–741. https://doi.org/10.1016/j.tins.2012.08.005
Shetty, M.S., Sharma, M., Sajikumar, S., 2017. Chelation of hippocampal zinc enhances long-term potentiation and synaptic tagging/capture in CA1 pyramidal neurons of aged rats: implications to aging and memory. Aging Cell 16, 136–148. https://doi.org/10.1111/acel.12537
Smith, J.E., Milch, P.O., Muto, Y., Goodman, D.S., 1973. The plasma transport and metabolism of retinoic acid in the rat. Biochem. J. 132, 821–827. https://doi.org/10.1042/bj1320821
Stoney, P.N., McCaffery, P., 2016. A Vitamin on the Mind: New Discoveries on Control of the Brain by Vitamin A. World Rev. Nutr. Diet. 115, 98–108. https://doi.org/10.1159/000442076
Takahashi, J., Palmer, T.D., Gage, F.H., 1999. Retinoic acid and neurotrophins collaborate to regulate neurogenesis in adult-derived neural stem cell cultures. J. Neurobiol. 38, 65–81.
Touyarot, K., Bonhomme, D., Roux, P., Alfos, S., Lafenêtre, P., Richard, E., Higueret, P., Pallet, V., 2013. A mid-life vitamin A supplementation prevents age-related spatial memory deficits and hippocampal neurogenesis alterations through CRABP-I. PloS One 8, e72101. https://doi.org/10.1371/journal.pone.0072101
van der Loo, B., Labugger, R., Aebischer, C.P., Bachschmid, M., Spitzer, V., Kilo, J., Altwegg, L., Ullrich, V., Lüscher, T.F., 2004. Age-related changes of vitamin A status. J. Cardiovasc. Pharmacol. 43, 26–30.

FIGURE LEGENDS

Figure 1: RA and retinol levels in young and aged rats, supplemented or not with dietary vit. A. ***(A)*** levels of retinol (µM) in the serum for young and aged rats, supplemented or not with dietary vit. A (n=8 per group). Two-way ANOVA: Age effect, p<0.0001; vit. A effect: p=0.0005; interaction: p=0.0015; Bonferroni posthoc comparison with the group young rats: young + vit. A p>0.9999; aged p<0.0001; aged + vit. A p<0.0001; comparison between aged and aged + vit. A, p<0.0001. Bonferroni posthoc comparison between aged and aged + vit. A groups: p<0.0001. **(B)** levels of RA (pmol/ml) in the serum for young and aged rats, supplemented or not with dietary vit. A (n=10 per group except for aged rats, n=9). Two-way ANOVA: Age effect, p=0.1555; vit. A effect: p=0.0484; interaction: p=0.0086; Bonferroni posthoc comparison with the group young rats: young + vit. A p>0.9999; aged p=0.0335; aged +vit. A p>0.9999 comparison between aged and aged + vit. A: p=0.0111. ***(C)*** Levels of RA (pmol/g) in the hippocampus (HPC) for the 4 groups (n=10 per group except for young rats, n=9). Two-way ANOVA: Age effect, p=0.0056; vit. A effect: p=0.0074; interaction: p=0.0398. Bonferroni post-hoc comparison with the group young rats: young + vit. A p>0.9999; aged p=0.0067; aged +vit. A p>0.9999. Comparison between aged and aged + vit. A: p=0.0064. ***(D)*** Plot of RA level in the hippocampus as a function of RA level in the serum for aged rats, supplemented or not with dietary vit. A. Histogram are represented as mean ± SEM with individual data points. Young rats: white bars; young + vit. A supplementation: light orange bars; aged rats: grey bars; aged rats + vit. A supplementation: dark orange bars. a-c: values significantly different.

Figure 2: Analysis of retinol and RA metabolism in the liver. The illustration (top right) draws the involvement of RBPR2, LRAT, REH, RALDH2 and CYP26A1 proteins in retinol metabolism in the liver. atRA: *all-trans* retinoic acid. ***(A)*** levels of retinol (µmol/g) in the liver for young and aged rats, supplemented or not with dietary vit. A (n=8 per group). Two-way ANOVA: Age effect, p<0.0001; vit. A effect: p<0.0001; interaction: p=0.0008. Bonferroni post-hoc comparison with the group young rats: young + vit. A p<0.0001; aged p<0.0001; aged +vit. A p<0.0001. Bonferroni post-hoc comparison between aged and aged + vit. A groups: p=0.4542. Bonferroni post-hoc comparison between aged and young + vit. A groups: p=0.0069. ***(B)*** levels of RA (pmol/g) in the liver for young and aged rats, supplemented or not with dietary vit. A (n=10 per group, except for aged + vit.A, n=9). Two-way ANOVA: Age effect, p<0.0001; vit. A effect: p=0.7052; interaction: p=0.8226. ***(C-G)*** mRNA level expression of genes coding for RBPR2 *(C)*, LRAT *(D)*, REH *(E)*, RALDH2 *(F)* and CYP26A1 *(G)*. ***(C)*** *Rbpr2*: n=10 per group. Two-way ANOVA: Age effect, p=0.0001; vit. A effect: p=0.0286;

interaction: p=0.0020. Bonferroni post-hoc comparison with the group young rats: young + vit. A p>0.9999; aged p<0.0001; aged +vit. A p=0.4736. ***(D)*** *Lrat*: n=10 per group. Two-way ANOVA: Age effect, p=0.0093; vit. A effect: p<0.0001; interaction: p=0.0004. Bonferroni post-hoc comparison with the group young rats: young + vit. A p>0.9999; aged p=0.0001; aged +vit. A p=0.4315. ***(E)*** *Reh*: n=10 per group. Two-way ANOVA: Age effect, p=0.5429; vit. A effect: p=0.0187; interaction: p=0.0241. Bonferroni post-hoc comparison with the group young rats: young + vit. A p>0.9999; aged p=0.1285; aged +vit. A p=0.5983. ***(F)*** *Raldh2*: young, n=7; young + vit., A n=7; aged, n=5; aged + vit.A, n=7. Two-way ANOVA: Age effect, p=0.2384; vit. A effect: p=0.0174; interaction: p=0.0003. Bonferroni post-hoc comparison with the group young rats: young + vit. A p=0.6761; aged p=0.1545; aged + vit. A p=0.0309. ***(G)*** *Cyp26a1*: young, n=6; young + vit. A, n=10; aged, n=7; aged + vit.A, n=8. Two-way ANOVA: Age effect, p=0.0335; vit. A effect: p=0.0274; interaction: p=0.0028. Bonferroni post-hoc comparison with the group young rats: young + vit. A p>0.9999; aged p=0.0035; aged +vit. A p>0.9999. Histogram are represented as mean ± SEM with individual data points. Young rats: white bars; young + vit. A supplementation: light orange bars; aged rats: grey bars; aged rats + vit. A supplementation: dark orange bars. a-c: values significantly different.

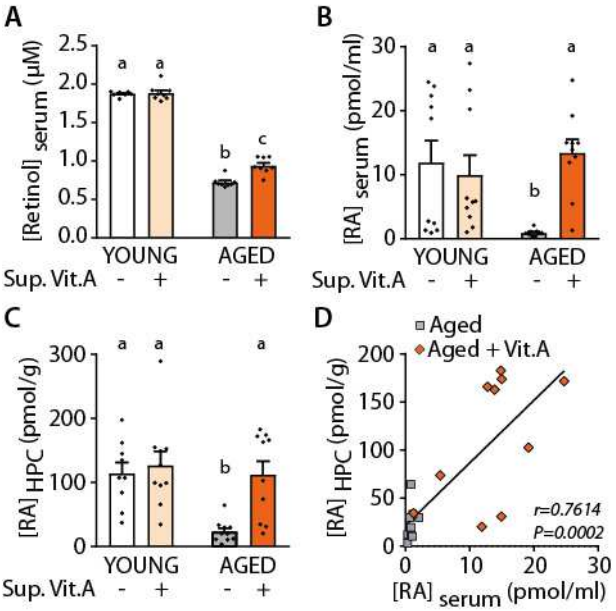
Figure 3: Dendritic morphology of pyramidal neurons in the CA region of the dorsal hippocampus. ***(A)*** Representative neurons from young rat (first left), young rat supplemented (second left), aged rat (third left) and aged rat supplemented (right), following Golgi staining and Imaris 3D reconstruction. Apical pole: young, n=24; young + vit. A, n=21; aged, n=20; aged + vit.A, n=20. Basal pole: young, n=20; young + vit. A, n=21; aged, n=20; aged + vit.A, n=20. ***(B, C)*** Total length (µm) of dendritic trees for the apical *(B)* and basal *(C)* poles of CA1 pyramidal neurons. Neurons from (A) are represented with blue diamond. ***(B)*** Apical pole: Two-way ANOVA: Age effect, p=0.0246; vit. A effect: p=0.1375; interaction: p=0.00. Bonferroni post-hoc comparison with the group young rats: young + vit. A p=0.5933; aged p=0.0004; aged +vit. A p>0.9999. ***(C)*** Basal pole: Two-way ANOVA: Age effect, p=0.0989; vit. A effect: p=0.0407; interaction: p=0.0003. Bonferroni post-hoc comparison with the group young rats: young + vit. A p=0.6869; aged p=0.0008; aged +vit. A p>0.9999. ***(D, E)*** Dendritic length of apical *(D)* and basal *(E)* poles of CA1 neurons classified by dendrite level. ***(D)*** Apical pole: two-way ANOVA: group effect, p=0.0012; dendritic level effect: p<0.0001; interaction: p=0.7527. ***(E)*** Basal pole: two-way ANOVA: group effect, p=0.0005; dendritic level effect: p<0.0001; interaction: p=0.0169. Bonferroni post-hoc comparison between young and aged rats significant for levels 3 (p=0.0054), 4 (p=0.0003) and 5 (p=0.0159). ***(F, G)*** Number (#) of branches for each apical and basal poles of CA1 neurons in the 4

groups. *(F)* Apical pole: Two-way ANOVA: Age effect, p=0.0017; vit. A effect: p=0.0793; interaction: p=0.0014. Bonferroni post-hoc comparison with the group young rats: young + vit. A p=0.7951; aged p<0.0001; aged +vit. A p=0.8786. ***(G)*** Basal pole: Two-way ANOVA: Age effect, p=0.0204; vit. A effect: p=0.0029; interaction: p<0.0001. Bonferroni post-hoc comparison with the group young rats: young + vit. A p=0.8351; aged p<0.0001; aged +vit. A p>0.9999. ***(H, I)*** Diagram of the number of Sholl intersections by cumulative distance from soma for apical *(H)* and basal *(I)* poles of CA1 neurons. *(H)* Apical pole: RM two-way ANOVA: group effect, p=0.0007; dendritic level effect: p<0.0001; interaction: p<0.0001. Bonferroni pos-thoc comparison: between young and aged rats significant for distance from the soma of 60 µm-160µm (p values 0.0012 to <0.0001); between young and aged + vit.A rats significant for distance from the soma of 40 µm (p=0.0322), 60 µm (p=0.0487) and 120 µm (p=0.0112). Histogram are represented as mean ± SEM with individual data points. Young rats: white bars; young + vit. A supplementation: light orange bars; aged rats: grey bars; aged rats + vit. A supplementation: dark orange bars. a-c: values significantly different.

Figure 4: Spatial memory performance of aged rats match with hippocampal RA levels. *(A, B)* Total distance and time spent in individuals Y-maze arms for the 4 groups: young, n=9; young + vit. A, n=10; aged, n=10; aged+ vit. A, n=10. (A) Total distance (cm) in Y-maze test. Two-way ANOVA, Age effect: p=0.5666, vit. A effect: p=0.2347, interaction: p=0.4901. *(B)* Absolute time (s) spent in start (empty bar), familiar (dots pattern) and new arms (square pattern) of the Y-maze. Separate analysis for each group with one-way ANOVA. Young: p=0.0052; familiar vs. new arm: p=0.0004. Young + vit. A: p<0.0001; familiar vs. new arm: p<0.0001; start vs new arm: p=0.0011. Aged: p=0.0844; Aged + vit.A: p=0.0223; familiar vs. new arm: p=0.1556; start vs new arm: p=0.0279. *(C)* Proportion (%) of time spent in the new arm, comparatively to the familiar arm (chance level = 50 %). Two-way ANOVA: Age effect: p<0.0001; vit. A effect: p=0.0007; interaction: p=0.2215. **(D)** Plot of percent time spent in the new arm of the Y-maze (memory performance) as a factor of RA levels in the hippocampus. Dashed horizontal line at 50 % represents chance level (absence of memorization). Light-orange straight line represents linear regression for young + young+vit. A rats. Grey straight line represents linear regression for aged rats. Orange straight line represents linear regression for aged rats supplemented with dietary vit. A. Curved black line represents non-linear regression of aged rats, supplemented and not supplemented (equation: $y=(85.3\pm5.3*x)/(17.8\pm5.1 + x)$, goodness of fit: $r^2 = 0.514$). Histogram are represented as mean ± SEM with individual data points. Young rats: white bars; young + vit. A supplementation: light

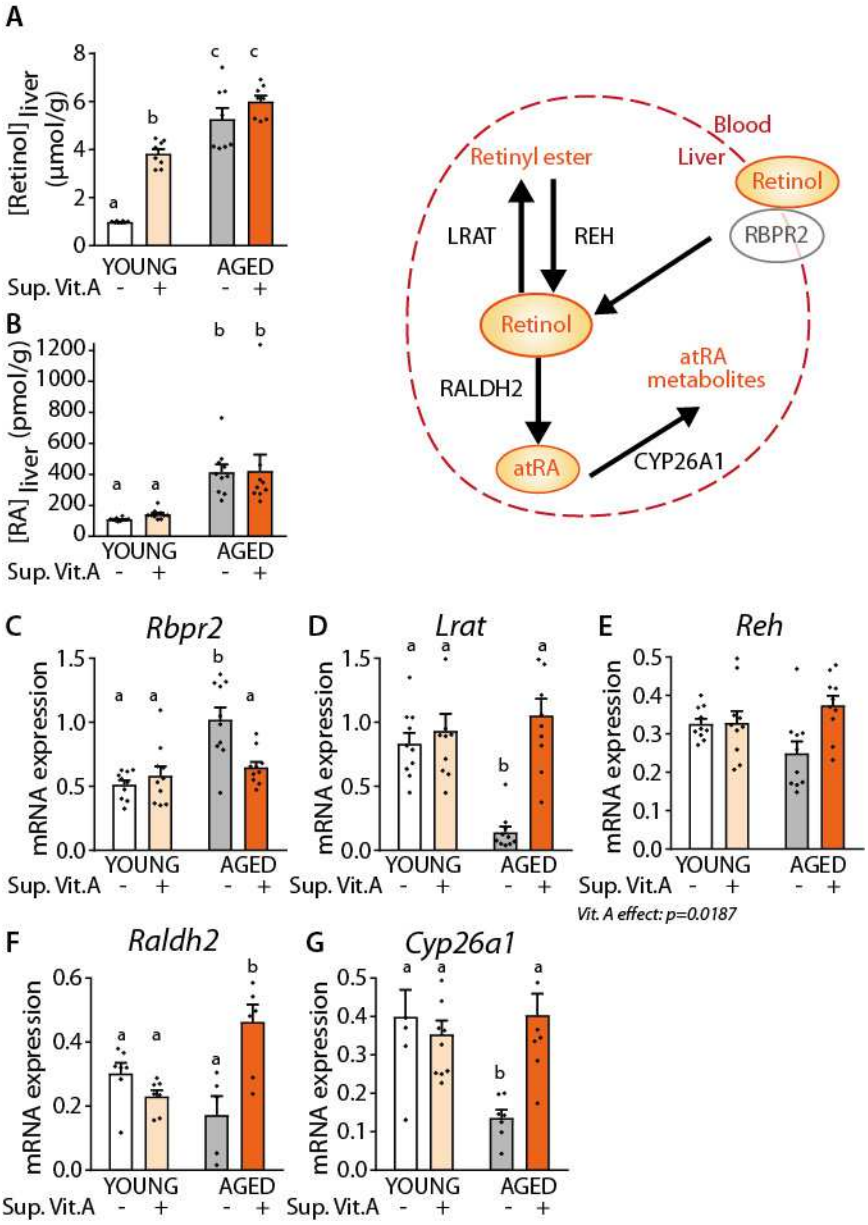
715 orange bars; aged rats: grey bars; aged rats + vit. A supplementation: dark orange bars. a-c: values
716 significantly different.
717
718 Figure 5: **Recapitulative illustration.** RA is synthetized in the liver and circulate through the blood
719 stream to access the hippocampus. Hippocampal RA is necessary for neuronal circuits that enable
720 memory performance.
721

Figure 1


A
[Retinol] serum (µM)
2.5
2.0
1.5
1.0
0.5
0.0
a
a
b
c
YOUNG
AGED
Sup. Vit.A - + - +
B
[RA] serum (pmol/ml)
30
20
10
0
a
a
b
a
YOUNG
AGED
Sup. Vit.A - + - +
C
[RA] HPC (pmol/g)
300
200
100
0
a
a
b
a
YOUNG
AGED
Sup. Vit.A - + - +
D
Aged
Aged + Vit.A
[RA] HPC (pmol/g)
200
150
100
50
0
r=0.7614
P=0.0002
0
10
20
30
[RA] serum (pmol/ml)

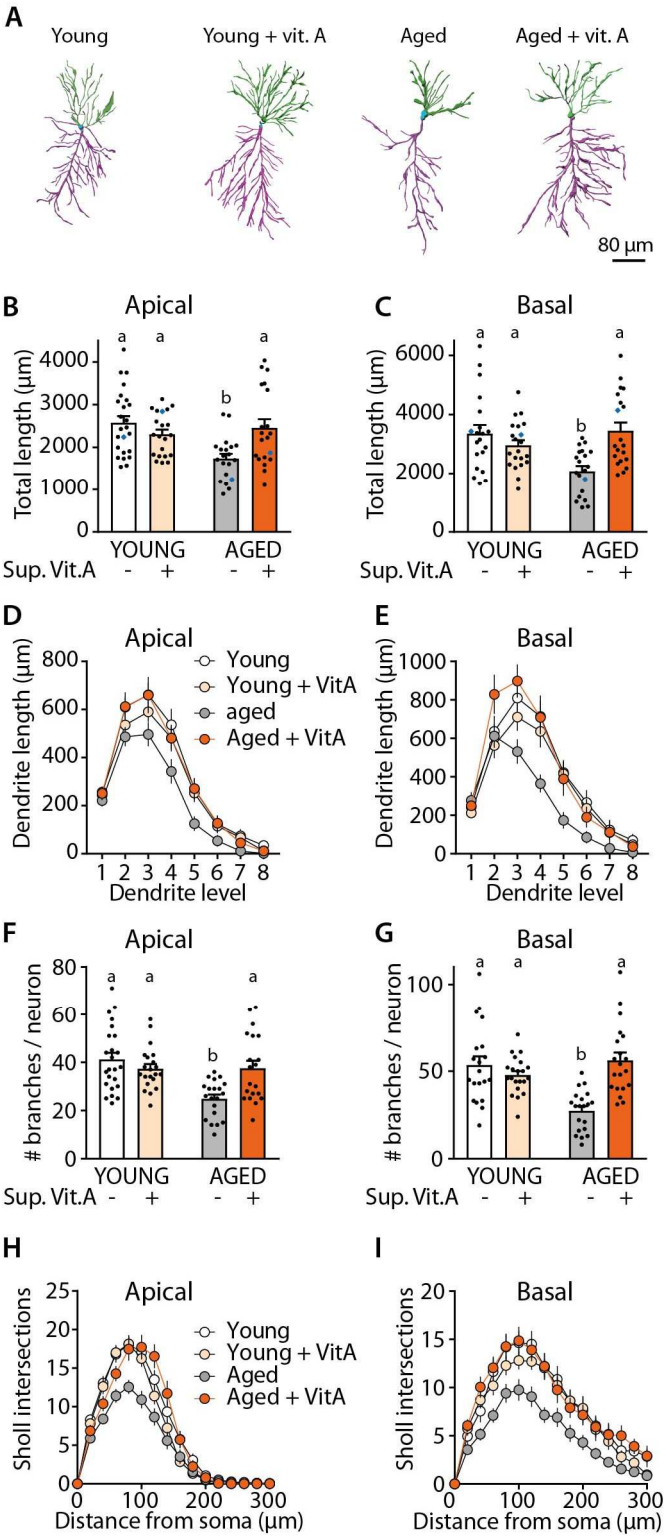
724

Figure 2



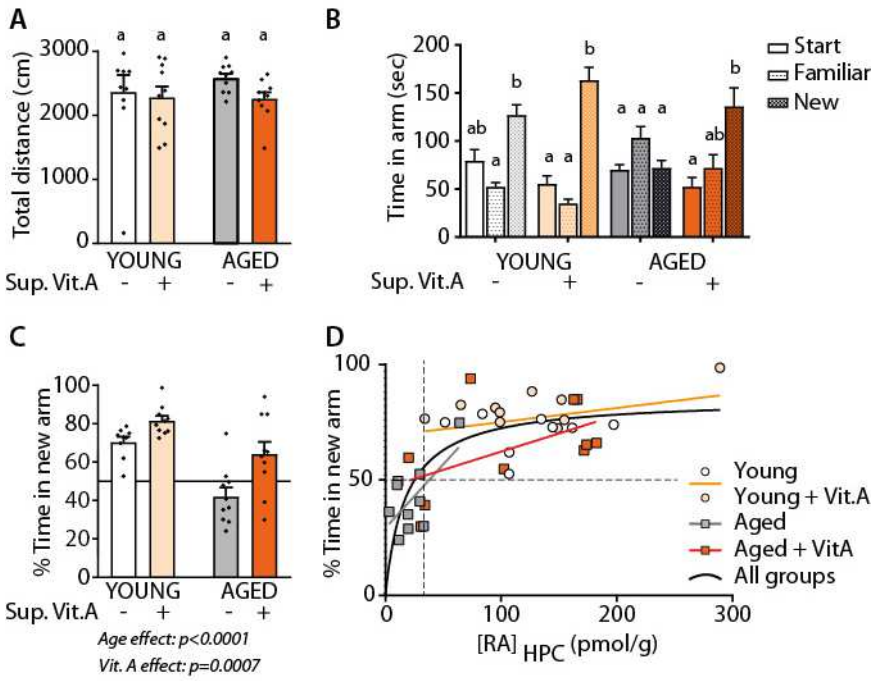
725

Figure 3


A
Young
Young + vit. A
Aged
Aged + vit. A
80 µm
B
Apical
Total length (µm)
YOUNG
AGED
Sup. Vit.A
C
Basal
Total length (µm)
YOUNG
AGED
Sup. Vit.A
D
Apical
Dendrite length (µm)
Young
Young + VitA
aged
Aged + VitA
Dendrite level
E
Basal
Dendrite length (µm)
Dendrite level
F
Apical
branches / neuron
YOUNG
AGED
Sup. Vit.A
G
Basal
branches / neuron
YOUNG
AGED
Sup. Vit.A
H
Apical
Sholl intersections
Young
Young + VitA
Aged
Aged + VitA
Distance from soma (µm)
I
Basal
Sholl intersections
Distance from soma (µm)

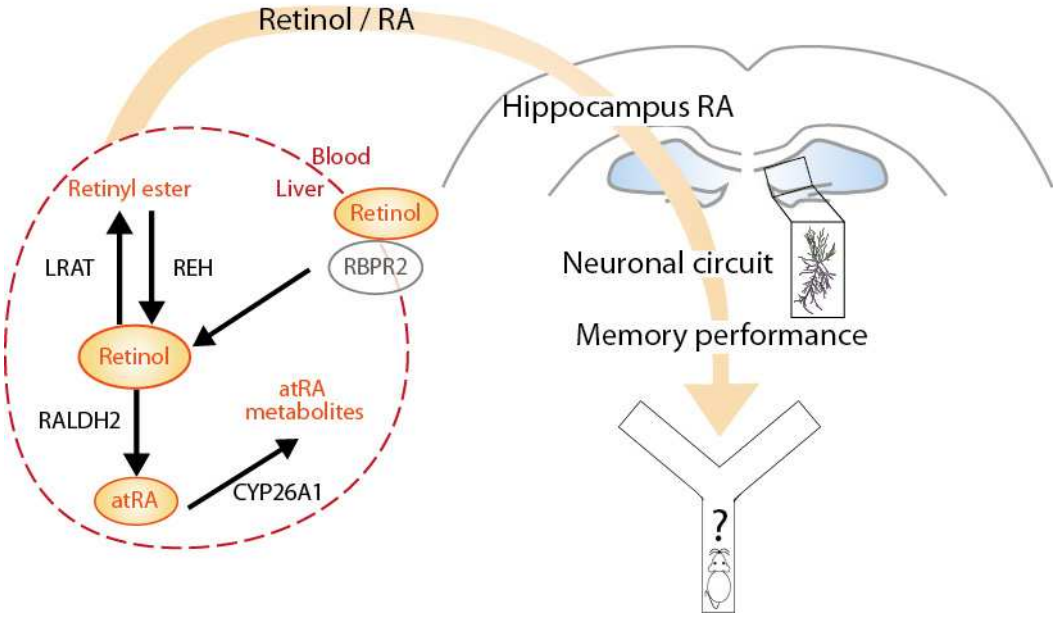
728

Figure 4

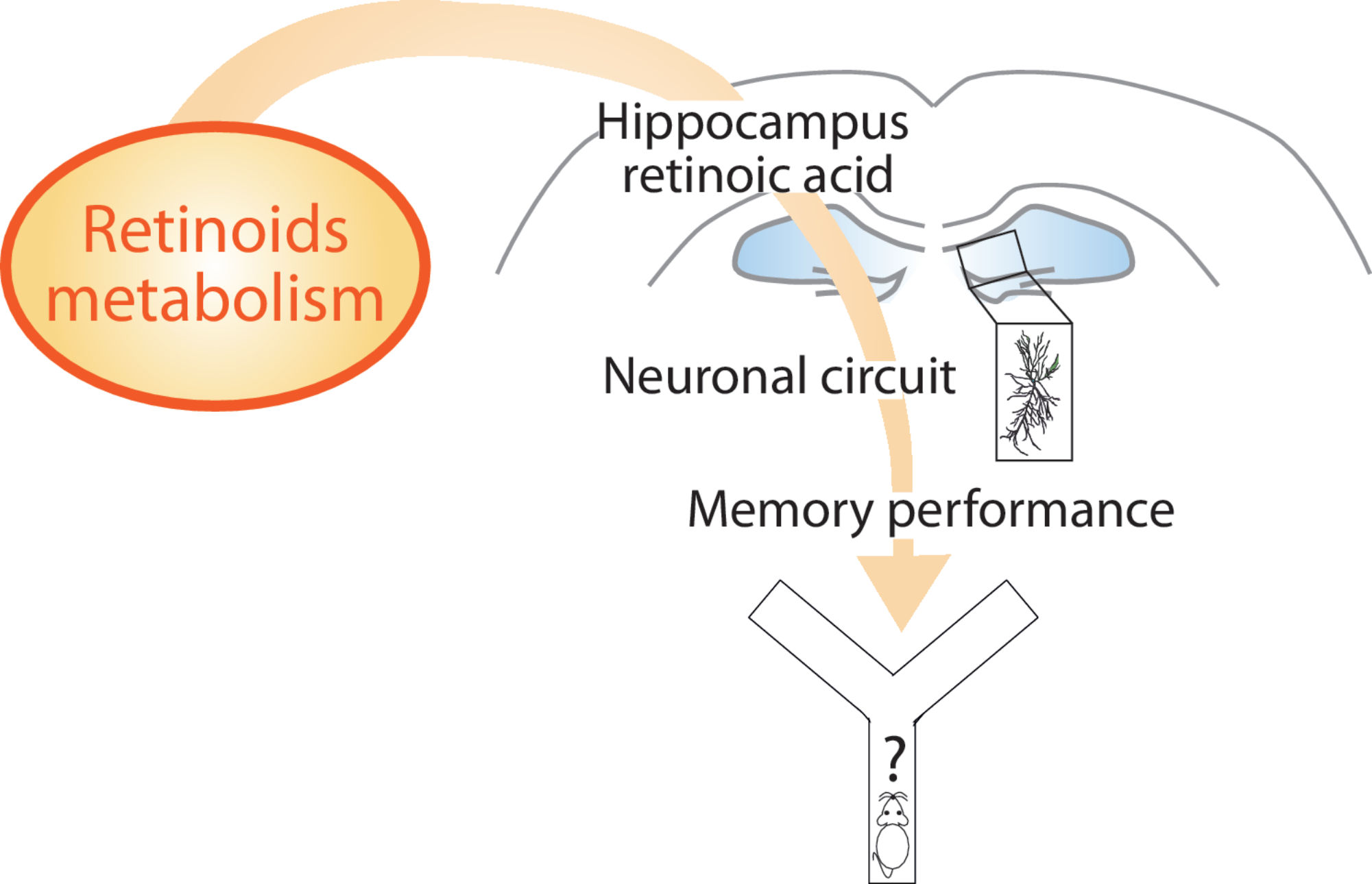


729

Figure 5


Retinol / RA
Hippocampus RA
Blood
Liver
Retinyl ester
Retinol
LRAT
REH
RBPR2
Retinol
atRA
metabolites
RALDH2
atRA
CYP26A1
Neuronal circuit
Memory performance
?

730


Retinoids metabolism
Hippocampus retinoic acid
Neuronal circuit
Memory performance
?