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

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10

11 **ABSTRACT**

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

25

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

27

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

33

34 INTRODUCTION

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

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

52 With aging, vit. A seems to accumulate in the liver, its storage organ (Pallet et al., 1997; van der
53 Loo et al., 2004; Azaïs-Braesco et al., 1995); however, its bioavailability for target tissues appears
54 altered. Supporting this, we previously demonstrated that receptors, transporters and molecules
55 involved in the signaling pathway of RA in the brain are dysregulated with aging (Enderlin et al.,
56 1997; Pallet et al., 1997; Touyarot et al., 2013). The resulting decrease in RA signaling is associated
57 with impairments of memory performance. In particular, RA signaling in the brain is crucial for
58 spatial and episodic-like memory (Cocco et al., 2002; Etchamendy et al., 2003; Mingaud et al.,
59 2008; Olson and Mello, 2010; Touyarot et al., 2013). Recently, an association between low level of
60 circulating retinol and age-related cognitive decline has also been found in humans (Huang et al.,
61 2018).

62 As a clue of the causality between RA deficiency and memory impairments, vit. A supplementation
63 in aged rodents was shown to reduce memory impairments (Mingaud et al., 2008; Touyarot et al.,
64 2013). However, mechanisms linking alteration of vit.A metabolism in the liver and hyposignaling
65 of RA pathway in the brain have not been elucidated yet. Additionally, the impact on neuronal
66 networks of a reduced brain concentration of RA is poorly understood. Thus, there is no direct

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

69 In this study we investigated the mechanisms linking specific alterations of RA metabolism in the
70 hippocampus to memory impairments in aged rats supplemented or not with vit. A. Aging was
71 associated with reduced RA availability and dysregulation of vit. A and RA metabolism in the liver.
72 Importantly, we found a high correlation between hippocampal levels of RA and memory
73 performance. Vit. A supplementation led to a concomitant restoration of the neuronal
74 morphology of pyramidal cells in the hippocampus and of memory capacities altered with aging.
75 The present work establishes the link between retinoid metabolic collapse and age-related
76 cognitive decline. It highlights the potential capacity of vit. A supplementation to restore memory
77 capacities. This study brings new and useful knowledge on the importance of RA signaling for
78 cognitive processes during aging.

79

80 **1. MATERIAL AND METHODS**

81 **2.1. Animals and diet**

82 All experiments were performed in accordance to criteria of the European Communities Council
83 Directive 2010/63/UE and the French National Committee (4184-2016022209565094).
84 Experiments were performed on male Wistar rats obtained from Janvier Labs (France). Young (10
85 weeks) and old (16 months) rats were maintained under standard housing conditions in a
86 temperature- (23 ± 1 °C) and humidity- (40 %) controlled animal room with a 12-h light/dark cycle
87 (0700–1900 hours), with ad libitum access to food and water. Rats were housed 2 per cage and
88 were weighted weekly.

89 All rats received a custom diet upon arrival for 4 weeks. Control rats were fed control diet, with 5
90 IU retinol/g (INRA, Jouy-en-Josas), while rats enriched with vit. A received a diet containing 20 IU
91 retinol/g (INRA, Jouy-en-Josas). The two diets were isocaloric and identical except the amount of
92 retinol.

93

94 **2.2. Behavioral assessment (Y-maze test)**

95 Spatial memory was assessed in rats after 4 weeks of dietary protocol. The Y-maze paradigm was
96 used to assess spatial memory as previously described (Delpech et al., 2015). Rats were handled
97 daily for 1 week before the test. All tests were conducted in a sound-attenuated separate
98 experimental room. Behavioral sessions were recorded with a ceiling-mounted video camera and

99 analyzed using tracking software (ViewPoint Behavioral Technology). The apparatus was a Y-
100 shaped maze made of dark grey plastic. Each arm was 34-cm long, 8-cm wide and 14-cm high. The
101 floor of the maze was covered with used litter from the home cages of all animals and was mixed
102 between sessions to scramble olfactory cues. Visual cues were placed in the testing room and kept
103 constant during the whole test. In the first trial of the test, one arm of the Y-maze was closed with
104 a guillotine door and rats were allowed to visit two arms of the Y-maze for 5 min. Closed arms
105 were randomly assigned for each rat. After 2h of inter-trial interval, rats were placed back in the
106 start arm and allowed free access to the three arms for 5 min. Data are presented as the time
107 spent exploring the novel arm during the 5 min of the second trial compared to familiar and start
108 arms.

109

110 **2.3. Vitamin A concentration measurement**

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

116

117 **2.4. RA levels measurements**

118 Tissues samples were collected and weighed under red light and retinoids were handled with glass
119 single-use containers and pipettes. RA was extracted by a two-step acid-base extraction as
120 described previously by Kane et Napoli (Kane and Napoli, 2010). 5 µM Acitretin (Sigma-Aldrich)
121 was added as internal standard into samples to normalize extraction efficiency. LC-ESI-MS/MS
122 (MRM mode) analyses were performed with a mass spectrometer model QTRAP® 5500 (Sciex,
123 Villebon sur Yvette, France) coupled to a LC system (LC-20AD XR pump (Shimatzu, Marne-la-Vallée,
124 France) and PAL HTC-xt Autosampler (CTC Analytics, Zwingen, Switzerland)). Extracts were
125 dissolved in 35 µL of CH₃CN/H₂O 80/20. Analyses were achieved in the positive mode; nitrogen
126 was used for the curtain gas (set to 20), gas1 (set to 35), gas2 (set to 0) and as collision gas. Needle
127 voltage was at +5,500 V without needle heating; the declustering potential was set at +101 V and
128 +236 V and the collision energy at +27 eV and +35 eV for RA and acitretin, respectively. MS/MS
129 experiments were performed by following two MRM transitions for RA (301.1/123.2 and
130 301.1/161.1) and acitretin (327.2/131.0 and 327.2/159.1). One transition is used for quantitation
131 and the other for confirmation. The area of LC peaks was determined using MultiQuant software

132 (v2.1, Sciex). Reversed phase separations were carried out on an Ascentis RP Amide 150×1 mm
133 column, with 3 µm particles (Supelco, Sigma Aldrich, St Quentin Fallavier, France). Eluent A was
134 H₂O+0.1 % formic acid and eluent B was CH₃CN+0.1 % formic acid. The gradient elution program
135 was: 0 min, 30 % B; 8 min, 30 % B; 10 min, 70 % B; 35–36 min, 87 % B; 37 min, 30 % B. The flow
136 rate was 50 µL/min; 10µL sample volumes were injected. This protocol allows All-trans RA
137 separation from its isomers. For each experiment, a standard curve consisting of triplicated
138 extracted all-Trans RA (Sigma-Aldrich) samples of known concentrations (0.07-2.5 µM) was used to
139 correlate LC peaks area onto RA concentrations.

140

141 **2.5. Neuronal morphology analysis**

142 **Golgi-Cox staining.** Rats were deeply anesthetized with isoflurane and then decapitated. Four
143 animals per group were used. Brains were processed according to the Golgi-Cox kit guidelines
144 (PK401 FD Rapid Golgi Stain KIT, Neurotechnologies INC, Paris, France) as previously described
145 (Janthakin et al., 2017). Briefly, one hemisphere per brain was immersed in the Golgi-Cox solution
146 for 8 days before deep freezing in isopentane. 100 µm coronal sections containing the dorsal
147 hippocampus were collected at –24 °C using a cryostat (Leica, Solms, Germany) and mounted on
148 3% gelatin-coated slides. When slides were totally dry (3 days after), they were stained and
149 coverslipped with Depex. Exposure to light was limited during the whole process.

150 **Structural analysis.** Images were obtained at the Bordeaux Imaging Center (CNRS-INSERM and
151 Bordeaux University, France BiImaging) with Nanozoomer slide scanner (Hamamatsu
152 Nanozoomer 2.0 HT) and analyzed using Imaris software (Bitplane, Oxford Instrument Compagny).
153 The experimenter remained blind to the treatment conditions throughout the procedure.
154 Dendritic arbor complexity was evaluated in pyramidal neurons of the hippocampal CA1 subfield.
155 The following criteria were used to select pyramidal neurons: (a) full impregnation of the neurons
156 along the entire length of the dendritic tree; (b) dendrites without significant truncation of
157 branches; and (c) relative isolation from neighboring impregnated neurons, astrocytes or blood
158 vessels. For each rat, 4-6 neurons fulfilling the criteria were finally selected.

159 **Dendritic morphology.** Briefly, NDPI images at X 20 magnification (one image every 2 µm in the z
160 axis) obtained with Nanozoomer were converted into TIFF format using the ImageJ software
161 (<http://imagej.nih.gov/gate2.inist.fr/ij/>) and NDPI tools plugin (Deroulers et al., 2013). For each
162 selected neuron, all branches of the dendritic tree were semi-automatically reconstructed in 3D
163 using the Imaris software (Oxford Instruments, Zürich, Switzerland).

164

165 **2.6. mRNA expression analysis by RT-qPCR**

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

187

188 **2.7. Statistical analysis**

189 Statistical tests were performed with GraphPad Prism 7.0 (GraphPad software, San Diego, CA,
190 USA) using a critical probability of $p < 0.05$. All values are given as mean \pm SEM. Statistical analyses
191 performed for each experiment are summarized in each figure legend with the chosen statistical
192 test, n and p-values. Normality of data were first attested with D'agostino & Pearson normality
193 test. As appropriate, we used RM 1-way ANOVA with Tukey's multiple comparison test or 2-way
194 ANOVA followed by the Bonferroni's multiple comparison test when interaction has a p value $<$
195 0.05. Correlations were calculated with Pearson test or non-parametric Spearman test when
196 appropriate. Calculated F and p values are summarized in Supplemental Table 1.

197

198

199 **3. RESULTS**

200 **3.1. RA levels are restored by dietary vit. A in aged rats**

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

213 We then quantified levels of RA in the hippocampus, a brain structure highly involved in spatial
214 memory in rats. We found that hippocampal concentration of RA in aged animals was highly
215 decreased compared to young rats (young: $113.9 \pm 17.6 \text{ pmol/g}$ vs aged: $23.2 \pm 5.5 \text{ pmol/g}$, Figure
216 1C). Furthermore, 4 weeks of vit. A supplementation efficiently normalized RA concentration in
217 their hippocampus (Figure 1C). Of note, hippocampus level of RA correlated with RA level in the
218 serum in aged animals, but not with retinol level (Figure 1D, RA: Spearman $r = 0.7614$, $p = 0.0002$;
219 retinol: Spearman $r = 0.3973$, $p = 0.1025$). This suggests that blood RA is more likely the source of
220 hippocampal RA.

221

222 **3.2. Metabolism of retinol and RA is altered with aging and restored by supplementation**

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

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

231 The discrepancy between decreased levels of retinol / RA in the serum and elevated levels in the
232 liver suggests that aging induces a dysregulation of retinol / RA release from the liver to the
233 serum. To better understand this, we analyzed the mRNA expression level of genes coding for
234 proteins involved in the metabolism of retinol and RA. In aged rats, the gene expression for the
235 protein responsible of retinol entry in the liver, RBPR2 (Retinol Binding Protein Receptor 2) was
236 increased (young: 0.51 ± 0.04 vs aged: 1.02 ± 0.10 , Figure 2C). Meanwhile, we observed a
237 decreased mRNA expression for **Lrat** (Lecithin:Retinol AcylTransferase) (young: 0.83 ± 0.08 vs
238 aged: 0.14 ± 0.05) and a trend to decrease for **Reh** (Retinyl Ester Hydrolase) (young: 0.33 ± 0.01 vs
239 aged: 0.25 ± 0.03) in the liver of aged rats, suggesting a reduced retinol esterification (Figure 2 D,
240 E). This is in accordance with high levels of retinol in the liver of aged rats. Expression levels of
241 **Rbpr2**, **Lrat** and **Reh** mRNAs were normalized by vit. A supplementation in aged rats. Additionally,
242 the mRNA expression of **Raldh2**, the enzyme of RA synthesis, was slightly decreased in aged rats
243 compared to young rats, and was significantly increased by vit. A supplementation (young: $0.30 \pm$
244 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
245 gene expression of **Cyp26a1**, coding for the degradation enzyme of RA, was significantly decreased
246 in aged rats and normalized after 4 weeks of vit. A supplementation, similarly to serum levels of
247 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
248 ± 0.06 ; Figure 2G).

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

253

254 **3.3. Neuronal morphology in aged rats is normalized by vit. A supplementation**

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

262 2079 ± 174 μm) and apical (young: 2577 ± 156 μm vs aged: 1738 ± 114 μm) poles of CA1 pyramidal
263 neurons. In details, dendritic lengths between aged and young rats were similar for primary and
264 secondary dendrites, but were reduced for aged rats at higher levels of dendrites (Figure 3 D, E).
265 This result indicates an atrophy of neurons at more distal dendrites, for both basal and apical
266 dendrite trees. The number of branches was also significantly reduced in aged rats (Apical: young:
267 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
268 3F, G), indicating a reduction in dendrite ramifications. Sholl intersections were significantly fewer
269 in aged rats at a distance of 40-160 μm from the soma for apical dendrites (Figure 3H) and 60-220
270 μm from the soma for basal dendrites (Figure 3I).

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

277

278 **3.4. Spatial memory performance of aged rats match with hippocampal RA levels**

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

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

294 When groups were considered independently, correlation between memory performance and RA
295 level in the hippocampus was significant only for aged rats (Pearson $r = 0.6448$, $p = 0.0441$), and
296 not for young rats (Pearson $r = 0.03671$, $p = 0.1340$) or aged rats submitted to a diet enriched with
297 vit. A (Pearson $r = 0.5242$, $p = 0.1199$) (Figure 4D). While RA hippocampus level seems to become a
298 limiting factor in aged rats, enriched aged rats and young +/- vit. A supplementation exhibited a
299 concentration sufficient to enable good memory performance. We used a non-linear regression to
300 fit optimally the data between hippocampus RA concentration and memory performance in the Y-
301 maze for the four groups. We observed that hippocampus RA level and memory were closely
302 related for RA concentration values below 34 pmol/g, while plots over this value were distributed
303 along a plateau (Figure 4D). This final result underlines the need for a minimal level of RA in the
304 hippocampus to function correctly and permit memory performance.

305

306 4. DISCUSSION

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

311

312 4.1. Retinoids availability reduces with aging

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

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

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

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

356 **4.2. Retinoids metabolism in the liver is dysregulated with aging**

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

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

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

388

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

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

417
418
419
420 Finally, our study reveals that restoration of dendritic arborization and RA level in the
421 hippocampus by dietary vit. A supplementation is concomitant with a reinstatement of memory

422 performance in aged rats. The direct role of hippocampal RA in the modulation of memory
423 performances along the life has already been argued (Etchamendy et al., 2003; Touyarot et al.,
424 2013) but without evidence for a direct link. Our present study reveals a non-linear correlation
425 between RA levels in the hippocampus and memory performance of aged rats. Importantly, our
426 results are in accordance with a recent study showing a relation between blood levels of retinol
427 and age-related cognitive decline in humans (Huang et al., 2018). In the present work, we assessed
428 episodic-like memory since it is particularly sensitive to aging processes. In non-supplemented
429 aged rats, the correlation between memory performance and RA levels in the hippocampus was
430 highly significant, with the best performing rats exhibiting the highest levels of RA in the
431 hippocampus. Interestingly, in supplemented aged rats, the correlation becomes low (no
432 significant), similarly to young rats. This reflects high memory performance enabled by a sufficient
433 amount of hippocampal RA. The non-linear fit of the data reveals that most of rats with high
434 memory performance (at the level of young rats) exhibit a concentration of RA on the
435 hippocampus higher than 34 pmol/g. This threshold might represent the minimal and necessary
436 concentration of RA in the rat hippocampus for the elaboration of neuronal enriched circuits,
437 required for optimal memory processes.

438

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

442

443 **COMPETING INTEREST**

444 No competing interest is declared by the authors.

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456 **AUTHORS CONTRIBUTION**

457 Conceptualization and project administration: VP

458 Funding and supervision of staff: VP

459 Methods development: FD, CB, KT

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

461 Statistical analyses: CB-B, FD, SA

462 Visualization: CB-B

463 Writing – original draft: CB-B, VP

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

465

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616

617 **FIGURE LEGENDS**

618 **Figure 1: RA and retinol levels in young and aged rats, supplemented or not with dietary vit. A.**

619 **(A)** levels of retinol (μM) in the serum for young and aged rats, supplemented or not with dietary
620 vit. A (n=8 per group). Two-way ANOVA: Age effect, $p<0.0001$; vit. A effect: $p=0.0005$; interaction:
621 $p=0.0015$; Bonferroni posthoc comparison with the group young rats: young + vit. A $p>0.9999$;
622 aged $p<0.0001$; aged + vit. A $p<0.0001$; comparison between aged and aged + vit. A, $p<0.0001$.
623 Bonferroni posthoc comparison between aged and aged + vit. A groups: $p<0.0001$. **(B)** levels of RA
624 (pmol/ml) in the serum for young and aged rats, supplemented or not with dietary vit. A (n=10 per
625 group except for aged rats, n=9). Two-way ANOVA: Age effect, $p=0.1555$; vit. A effect: $p=0.0484$;
626 interaction: $p=0.0086$; Bonferroni posthoc comparison with the group young rats: young + vit. A
627 $p>0.9999$; aged $p=0.0335$; aged +vit. A $p>0.9999$ comparison between aged and aged + vit. A:
628 $p=0.0111$. **(C)** Levels of RA (pmol/g) in the hippocampus (HPC) for the 4 groups (n=10 per group
629 except for young rats, n=9). Two-way ANOVA: Age effect, $p=0.0056$; vit. A effect: $p=0.0074$;
630 interaction: $p=0.0398$. Bonferroni post-hoc comparison with the group young rats: young + vit. A
631 $p>0.9999$; aged $p=0.0067$; aged +vit. A $p>0.9999$. Comparison between aged and aged + vit. A:
632 $p=0.0064$. **(D)** Plot of RA level in the hippocampus as a function of RA level in the serum for aged
633 rats, supplemented or not with dietary vit. A. Histogram are represented as mean \pm SEM with
634 individual data points. Young rats: white bars; young + vit. A supplementation: light orange bars;
635 aged rats: grey bars; aged rats + vit. A supplementation: dark orange bars. a-c: values significantly
636 different.

637

638 **Figure 2: Analysis of retinol and RA metabolism in the liver.** The illustration (top right) draws the

639 involvement of RBPR2, LRAT, REH, RALDH2 and CYP26A1 proteins in retinol metabolism in the
640 liver. atRA: *all-trans* retinoic acid. **(A)** levels of retinol ($\mu\text{mol/g}$) in the liver for young and aged rats,
641 supplemented or not with dietary vit. A (n=8 per group). Two-way ANOVA: Age effect, $p<0.0001$;
642 vit. A effect: $p<0.0001$; interaction: $p=0.0008$. Bonferroni post-hoc comparison with the group
643 young rats: young + vit. A $p<0.0001$; aged $p<0.0001$; aged +vit. A $p<0.0001$. Bonferroni post-hoc
644 comparison between aged and aged + vit. A groups: $p=0.4542$. Bonferroni post-hoc comparison
645 between aged and young + vit. A groups: $p=0.0069$. **(B)** levels of RA (pmol/g) in the liver for young
646 and aged rats, supplemented or not with dietary vit. A (n=10 per group, except for aged + vit.A,
647 n=9). Two-way ANOVA: Age effect, $p<0.0001$; vit. A effect: $p=0.7052$; interaction: $p=0.8226$. **(C-G)**
648 mRNA level expression of genes coding for RBPR2 (C), LRAT (D), REH (E), RALDH2 (F) and CYP26A1
649 (G). **(C) Rbpr2**: n=10 per group. Two-way ANOVA: Age effect, $p=0.0001$; vit. A effect: $p=0.0286$;

650 interaction: $p=0.0020$. Bonferroni post-hoc comparison with the group young rats: young + vit. A
651 $p>0.9999$; aged $p<0.0001$; aged +vit. A $p=0.4736$. **(D) Lrat**: $n=10$ per group. Two-way ANOVA: Age
652 effect, $p=0.0093$; vit. A effect: $p<0.0001$; interaction: $p=0.0004$. Bonferroni post-hoc comparison
653 with the group young rats: young + vit. A $p>0.9999$; aged $p=0.0001$; aged +vit. A $p=0.4315$. **(E) Reh**:
654 $n=10$ per group. Two-way ANOVA: Age effect, $p=0.5429$; vit. A effect: $p=0.0187$; interaction:
655 $p=0.0241$. Bonferroni post-hoc comparison with the group young rats: young + vit. A $p>0.9999$;
656 aged $p=0.1285$; aged +vit. A $p=0.5983$. **(F) Raldh2**: young, $n=7$; young + vit., A $n=7$; aged, $n=5$; aged
657 + vit.A, $n=7$. Two-way ANOVA: Age effect, $p=0.2384$; vit. A effect: $p=0.0174$; interaction: $p=0.0003$.
658 Bonferroni post-hoc comparison with the group young rats: young + vit. A $p=0.6761$; aged
659 $p=0.1545$; aged + vit. A $p=0.0309$. **(G) Cyp26a1**: young, $n=6$; young + vit. A, $n=10$; aged, $n=7$; aged +
660 vit.A, $n=8$. Two-way ANOVA: Age effect, $p=0.0335$; vit. A effect: $p=0.0274$; interaction: $p=0.0028$.
661 Bonferroni post-hoc comparison with the group young rats: young + vit. A $p>0.9999$; aged
662 $p=0.0035$; aged +vit. A $p>0.9999$. Histogram are represented as mean \pm SEM with individual data
663 points. Young rats: white bars; young + vit. A supplementation: light orange bars; aged rats: grey
664 bars; aged rats + vit. A supplementation: dark orange bars. a-c: values significantly different.

665

666 **Figure 3: Dendritic morphology of pyramidal neurons in the CA region of the dorsal**
667 **hippocampus. (A)** Representative neurons from young rat (first left), young rat supplemented
668 (second left), aged rat (third left) and aged rat supplemented (right), following Golgi staining and
669 Imaris 3D reconstruction. Apical pole: young, $n=24$; young + vit. A, $n=21$; aged, $n=20$; aged + vit.A,
670 $n=20$. Basal pole: young, $n=20$; young + vit. A, $n=21$; aged, $n=20$; aged + vit.A, $n=20$. **(B, C)** Total
671 length (μm) of dendritic trees for the apical **(B)** and basal **(C)** poles of CA1 pyramidal neurons.
672 Neurons from **(A)** are represented with blue diamond. **(B)** Apical pole: Two-way ANOVA: Age
673 effect, $p=0.0246$; vit. A effect: $p=0.1375$; interaction: $p=0.00$. Bonferroni post-hoc comparison with
674 the group young rats: young + vit. A $p=0.5933$; aged $p=0.0004$; aged +vit. A $p>0.9999$. **(C)** Basal
675 pole: Two-way ANOVA: Age effect, $p=0.0989$; vit. A effect: $p=0.0407$; interaction: $p=0.0003$.
676 Bonferroni post-hoc comparison with the group young rats: young + vit. A $p=0.6869$; aged
677 $p=0.0008$; aged +vit. A $p>0.9999$. **(D, E)** Dendritic length of apical **(D)** and basal **(E)** poles of CA1
678 neurons classified by dendrite level. **(D)** Apical pole: two-way ANOVA: group effect, $p=0.0012$;
679 dendritic level effect: $p<0.0001$; interaction: $p=0.7527$. **(E)** Basal pole: two-way ANOVA: group
680 effect, $p=0.0005$; dendritic level effect: $p<0.0001$; interaction: $p=0.0169$. Bonferroni post-hoc
681 comparison between young and aged rats significant for levels 3 ($p=0.0054$), 4 ($p=0.0003$) and 5
682 ($p=0.0159$). **(F, G)** Number (#) of branches for each apical and basal poles of CA1 neurons in the 4

683 groups. (F) Apical pole: Two-way ANOVA: Age effect, $p=0.0017$; vit. A effect: $p=0.0793$; interaction:
 684 $p=0.0014$. Bonferroni post-hoc comparison with the group young rats: young + vit. A $p=0.7951$;
 685 aged $p<0.0001$; aged +vit. A $p=0.8786$. (G) Basal pole: Two-way ANOVA: Age effect, $p=0.0204$; vit.
 686 A effect: $p=0.0029$; interaction: $p<0.0001$. Bonferroni post-hoc comparison with the group young
 687 rats: young + vit. A $p=0.8351$; aged $p<0.0001$; aged +vit. A $p>0.9999$. (H, I) Diagram of the number
 688 of Sholl intersections by cumulative distance from soma for apical (H) and basal (I) poles of CA1
 689 neurons. (H) Apical pole: RM two-way ANOVA: group effect, $p=0.0007$; dendritic level effect:
 690 $p<0.0001$; interaction: $p<0.0001$. Bonferroni pos-thoc comparison: between young and aged rats
 691 significant for distance from the soma of 60 μm -160 μm (p values 0.0012 to <0.0001); between
 692 young and aged + vit.A rats significant for distance from the soma of 40 μm ($p=0.0322$), 60 μm
 693 ($p=0.0487$) and 120 μm ($p=0.0112$). Histogram are represented as mean \pm SEM with individual
 694 data points. Young rats: white bars; young + vit. A supplementation: light orange bars; aged rats:
 695 grey bars; aged rats + vit. A supplementation: dark orange bars. a-c: values significantly different.
 696

697 **Figure 4: Spatial memory performance of aged rats match with hippocampal RA levels. (A, B)**
 698 Total distance and time spent in individuals Y-maze arms for the 4 groups: young, $n=9$; young + vit.
 699 A, $n=10$; aged, $n=10$; aged+ vit. A, $n=10$. (A) Total distance (cm) in Y-maze test. Two-way ANOVA,
 700 Age effect: $p=0.5666$, vit. A effect: $p=0.2347$, interaction: $p=0.4901$. (B) Absolute time (s) spent in
 701 start (empty bar), familiar (dots pattern) and new arms (square pattern) of the Y-maze. Separate
 702 analysis for each group with one-way ANOVA. Young: $p=0.0052$; familiar vs. new arm: $p=0.0004$.
 703 Young + vit. A: $p<0.0001$; familiar vs. new arm: $p<0.0001$; start vs new arm: $p=0.0011$. Aged:
 704 $p=0.0844$; Aged + vit.A: $p=0.0223$; familiar vs. new arm: $p=0.1556$; start vs new arm: $p=0.0279$. (C)
 705 Proportion (%) of time spent in the new arm, comparatively to the familiar arm (chance level = 50
 706 %). Two-way ANOVA: Age effect: $p<0.0001$; vit. A effect: $p=0.0007$; interaction: $p=0.2215$. (D) Plot
 707 of percent time spent in the new arm of the Y-maze (memory performance) as a factor of RA levels
 708 in the hippocampus. Dashed horizontal line at 50 % represents chance level (absence of
 709 memorization). Light-orange straight line represents linear regression for young + young+vit. A
 710 rats. Grey straight line represents linear regression for aged rats. Orange straight line represents
 711 linear regression for aged rats supplemented with dietary vit. A. Curved black line represents non-
 712 linear regression of aged rats, supplemented and not supplemented (equation:
 713 $y=(85.3\pm 5.3*x)/(17.8\pm 5.1 + x)$, goodness of fit: $r^2 = 0.514$). Histogram are represented as mean \pm
 714 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 synthesized 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

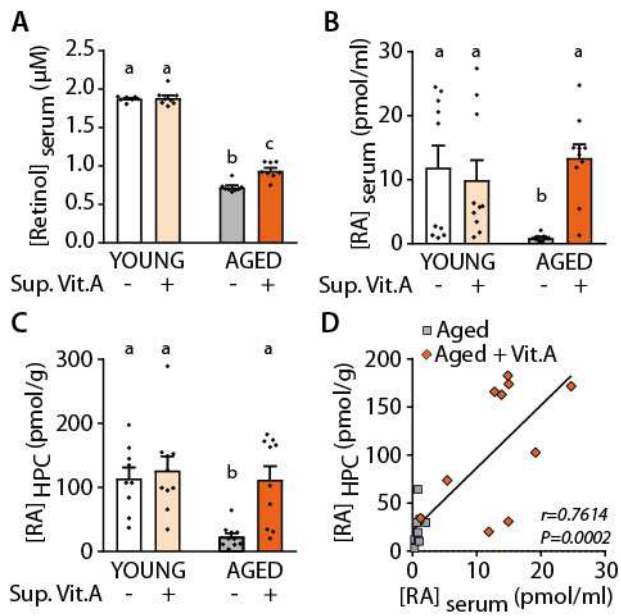


Figure 2

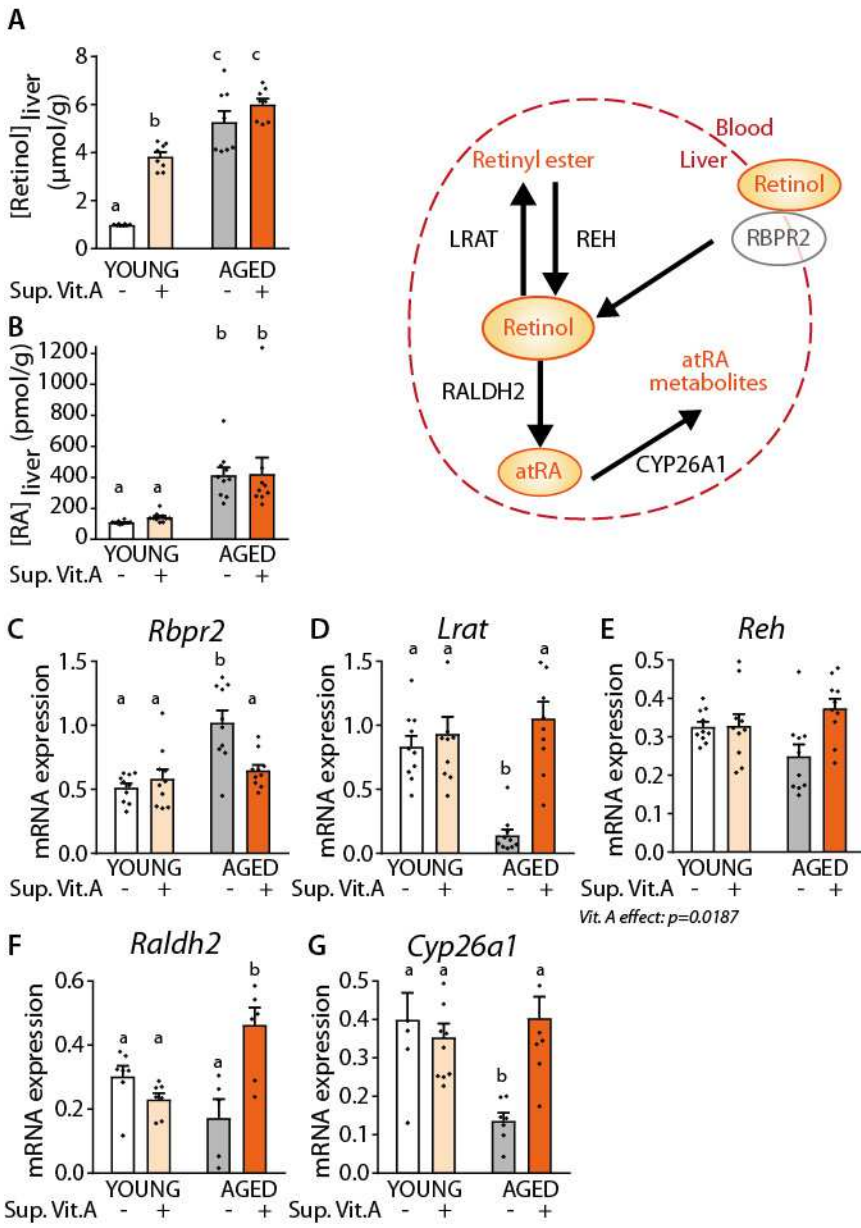
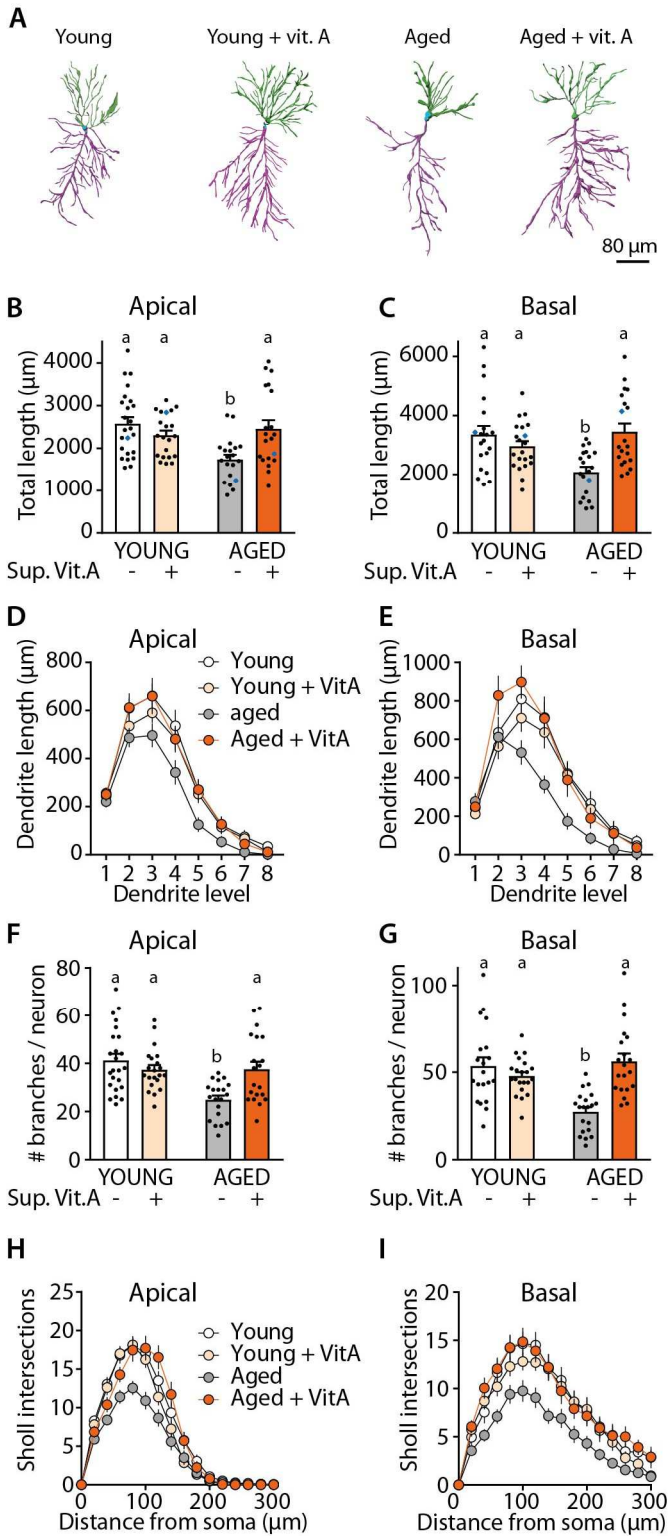


Figure 3



726

727

Figure 4

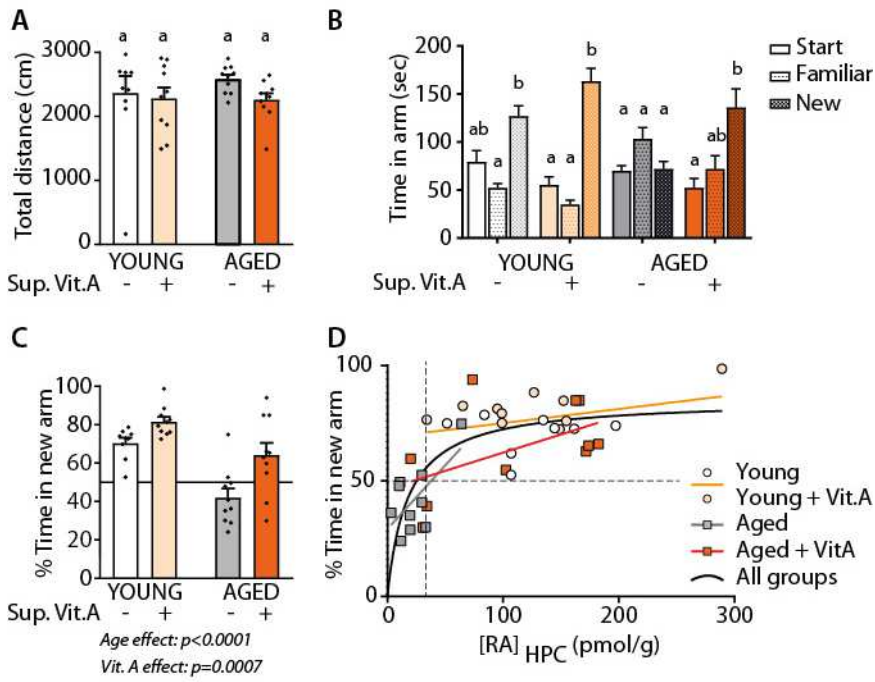
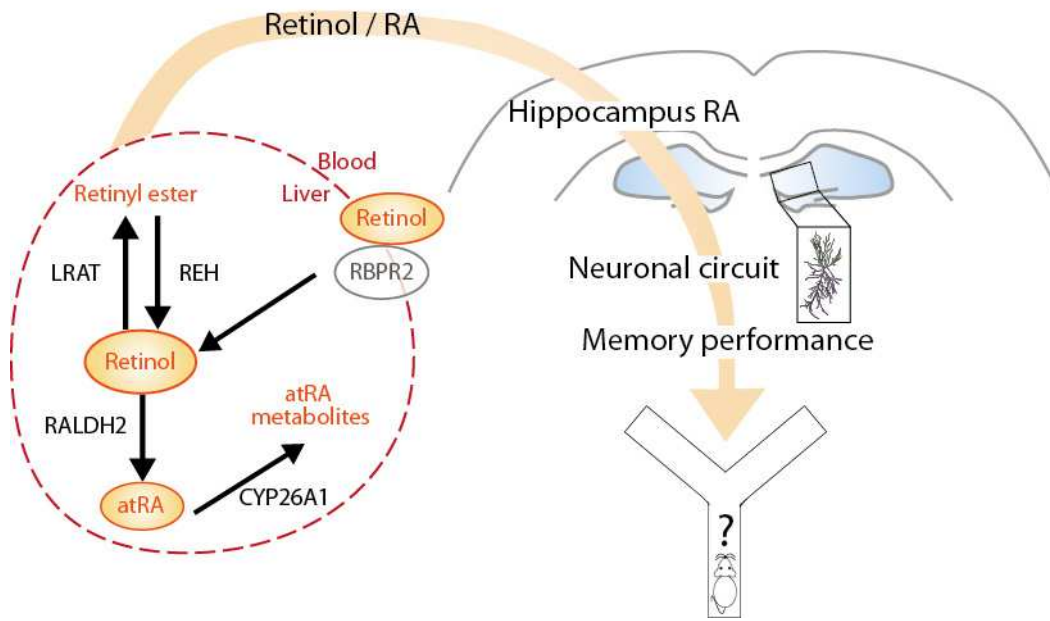


Figure 5



730

Retinoids
metabolism

Hippocampus
retinoic acid

Neuronal circuit

Memory performance

?

