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Role of Retinoid X Receptors (RXRs) and dietary vitamin A in Alzheimer's disease: Evidence from clinicopathological and preclinical studies

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ABSTRACT

Background: Vitamin A (VitA), via its active metabolite retinoic acid (RA), is critical for the maintenance of memory function with advancing age. Although its role in Alzheimer's disease (AD) is not well understood, data suggest that impaired brain VitA signaling is associated with the accumulation of β-amyloid peptides (Aβ), and could thus contribute to the onset of AD.

Methods: We evaluated the protective action of a six-month-long dietary VitA-supplementation (20 IU/g), starting at 8 months of age, on the memory and the neuropathology of the 3xTg-AD mouse model of AD (n = 11-14/group; including 4–6 females and 7–8 males). We also measured protein levels of Retinoic Acid Receptor β (RARβ) and Retinoid X Receptor γ (RXRγ) in homogenates from the inferior parietal cortex of 60 participants of the Religious Orders study (ROS) divided in three groups: no cognitive impairment (NCI) (n = 20), mild cognitive impairment (MCI) (n = 20) and AD (n = 20).

Results: The VitA-enriched diet preserved spatial memory of 3xTg-AD mice in the Y maze. VitA-supplementation affected hippocampal RXR expression in an opposite way according to sex by tending to increase in males and decrease in females their mRNA expression. VitA-enriched diet also reduced the amount of hippocampal $A\beta_{40}$ and $A\beta_{42}$, as well as the phosphorylation of tau protein at sites Ser396/Ser404 (PHF-1) in males. VitA-supplementation had no effect on tau phosphorylation in females but worsened their hippocampal Aβ load.

Abbreviations: Aβ, β-amyloid; AD, Alzheimer's disease; ADAM10, A Disintegrin And Metalloprotease 10; ANOVA, Analysis of variances; APP, Amyloid Precursor Protein; BACE, Beta-Amyloid Cleaving Enzyme; BHT, Butylated hydroxylotuene; BSA, Bovine Serum Albumine; CDK5, Cyclin Dependent Kinase type 5; CERAD, Consortium to Establish a Registry for Alzheimer's Disease; CSF, Cerebrospinal fluid; DR, Discrimination ratio; ELISA, Enzyme-linked immunosorbent assay; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GFAP, Glial Fibrillary Acidic Protein; HRP, Horseradish Peroxidase; HPLC, High performance liquid chromatography; IBA-1, Ionized calcium Binding Adaptor molecule 1; IDE, Insulin Degrading Enzyme; IDPs/IDRs, Intrinsically Disordered Proteins/Regions; IU, International unit; kDa, kilodalton; MCI, Mild cognitive impairment; MMSE, Mini Mental State Examination; NCI, No cognitive impairment; NIA-AA, National Institute of Aging – Alzheimer's association; NTg, Non-transgenic; OKR, Opto-Kinetic Response; PBS, Phosphate-buffered saline; PDVF, Polyvinylidene fluoride; PHF-1, Paired-helical filaments type 1; PS1, Presenilin 1; PTM, Post-translational modifications; RA, retinoic acid; RALDH, Retinaldehyde Dehydrogenase; RAR, Retinoic Acid Receptor; RNA, Ribonucleic acid; ROS, Religious Orders study; RT-PCR, Real-time polymerase chain reaction; RXR, Retinoid X Receptor; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; SEM, Standard error of the mean; TBS, Tris-buffered saline; Tg, transgenic (3xTg-AD); UPAE, Unité de préparation des aliments expérimentaux; VitA, vitamin A; μM, micromolar; 3xTg-AD, triple-transgenic mouse model of Alzheimer's disease..

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However, the expression of *Rxr-β* in the hippocampus was negatively correlated with the amount of both soluble and insoluble Aβ in both males and females.
Western immunoblotting in the human cortical samples of the ROS study did not reveal differences in RARβ levels. However, it evidenced a switch from a 60-kDa-RXRγ to a 55-kDa-RXRγ in AD, correlating with *ante mortem* cognitive decline and the accumulation of neuritic plaques in the brain cortex.
Conclusion: Our data suggest that (i) an altered expression of RXRs receptors is a contributor to β-amyloid pathology in both humans and 3xTg-AD mice, (ii) a chronic exposure of 3xTg-AD mice to a VitA-enriched diet may be protective in males, but not in females.

1. Introduction

Alzheimer's disease (AD) is currently affecting over 27 million individuals worldwide (Silva et al., 2019), and this number will continue to rise until after 2050 (Winblad et al., 2016), constituting a massive social and economic burden. AD leads to numerous cognitive alterations ranging from memory loss to language and executive function impairments (Scheltens et al., 2016; Silva et al., 2019; Skaper, 2012). The final AD diagnosis is made *post mortem* (Ballard et al., 2011), with the observation of (i) insoluble β-amyloid peptides (Aβ) in the extracellular compartment, referred to as amyloid pathology, and (ii) neurofibrillary tangles, consecutive to excessive and abnormal phosphorylation of tau protein inside the neurons, referred to as Tau pathology (Montine et al., 2012; Tremblay et al., 2017).

The risk of developing AD strongly increases with age, concurrently with the decrease of vitamin A (VitA) signaling, a crucial vitamin for the adult brain function (Lane and Bailey, 2005; Mey and McCaffery, 2004; Ransom et al., 2014; Stoney and McCaffery, 2016). VitA (*all-trans*-retinol) is exclusively obtained in food and its circulating concentration is maintained around 2 μM (Harrison, 2012; Li et al., 2014). When needed by target organs, such as the brain, retinol is taken up from the circulation by target cells and metabolized into retinoic acid (RA), its active metabolite. RA binds to ligand-inducible receptors in the nucleus, Retinoic Acid Receptors (RARs) and Retinoid X Receptors (RXRs), belonging to the nuclear receptor superfamily (Blomhoff and Blomhoff, 2006; Lane and Bailey, 2005; Ransom et al., 2014). Due to the presence of retinoic acid responsive elements (RARE) in promoters of their target genes, RARs and RXRs regulate the transcription of genes involved in cognition, particularly in learning and memory processes (Lane and Bailey, 2005; Olson and Mello, 2010; Shearer et al., 2012).

Reduced serum concentrations of retinol in the elderly (Leotsinidis et al., 2000; Succari et al., 1991) have been associated with cognitive decline assessed with clinical tests, such as the Mini-Mental State Examination (MMSE) (Huang et al., 2018; Zeng et al., 2017). Lower expression of RARα and RXRβ in blood peripheral mononuclear cells is also reported in old (65 years old) versus young (25 years old) individuals (Brtko et al., 2007; Feart et al., 2005b). Consistently, a decrease in serum retinol and brain RARs and RXRs expression is observed in aging rodents and is associated with memory alterations (Enderlin et al., 1997a, 1997b; Etchamendy et al., 2001; Mingaud et al., 2008; Touyarot et al., 2013). Interestingly, in preclinical intervention studies, the administration of pharmacological RA or nutritional VitA enhances synaptic plasticity, improves hippocampal neurogenesis and rescues multiple memory deficits in aged rats and mice (Bonhomme et al., 2014b; Etchamendy et al., 2001; Feart et al., 2005a; Mingaud et al., 2008; Touyarot et al., 2013).

In parallel, there is a growing body of literature strengthening the idea that retinoids could be a therapeutic option in AD (Goodman, 2006; Lee et al., 2009; Lerner et al., 2012; Ono and Yamada, 2012), as the stimulation of retinoid signaling regulates multiple targets involved in Alzheimer neuropathology, including Aβ-producing enzymes (Ding et al., 2008; Koryakina et al., 2009; Tippmann et al., 2009). Furthermore, lower levels of serum retinol have been reported in AD patients compared to age-matched controls (Bourdel-Marchasson et al., 2001; Lopes da Silva et al., 2014; Rinaldi et al., 2003), along with decreased expression of RARs and retinal dehydrogenase (RALDH, the RA-synthesis enzyme), in the neocortex of AD patients (Corcoran et al., 2004). Finally, VitA deficiency induces Aβ accumulation *in vivo* (Corcoran et al., 2004), and the presence of Aβ peptides shuts down RA synthesis *in vitro* (Goncalves et al., 2013), suggesting a vicious cycle between a reduced retinoid signaling activity and the onset of amyloid pathology in the brain. More interestingly, the specific stimulation of RXR is proposed to be a promising therapeutic strategy against AD (Chakrabarti et al., 2016; Muñoz-Cabrera et al., 2019; Tousi, 2015).

We therefore hypothesized that a dietary VitA-supplementation would have a disease-alleviating effect in the 3xTg-AD mouse model of AD neuropathology, possibly via regulating the expression of RXRs. We evaluated the preventive effect of a VitA-enriched diet on memory abilities and neuropathology of the 3xTg-AD mouse, which progressively develops both hallmarks characteristic of AD: β-amyloid- and Tau pathologies. Second, we assessed the protein levels of RARβ, which expression reflects an activation by RA (Etchamendy et al., 2001; Idres et al., 2002; Mingaud et al., 2008) and RXRγ, involved in regenerative processes in the central nervous system (Huang et al., 2011; Sołtys and Ożyhar, 2020; Yang et al., 2017), in the inferior parietal cortex of subjects from the Religious Orders Study (ROS) to question their relationship with both cognitive decline and AD neuropathology.

2. Materials & methods

2.1. Animals and diet

Male and female 3xTg-AD (Tg) mice were used (APP_{swe}, $PS1_{M146V}$, Tau_{P301L}) to model AD neuropathology and were compared to Non-transgenic (NTg) mice on the same genetic background (C57BL6/129SvJ), all of which were produced concomitantly in our animal facility (Tournissac et al., 2019). They were housed in cages of 1 to 5 animals in a 12 h/12 h light/dark cycle and had *ad libitum* access to food and water. They were split into experimental groups differing by the diet received; either a VitA-control diet providing a basal VitA supply of 5 IU/g incorporated into the pellets or a VitA-enriched diet containing 20 IU/g of VitA (UPAE, INRA, Jouy-en-Josas, France) (Dumetz et al., 2019). Both diets were isocaloric and distinguishable only on the basis of the amount of VitA they contained. They provided 3.3 kcal/g (14% lipids from vegetable oils, 60% carbohydrates, mostly from starch). The composition of the diet is detailed in Table 1. Dietary exposure started

Table 1
Composition of the diet.

Diet composition	Quantity (g / 100 g of diet)
Cellulose	2
Hydrochloric casein	18
Minerals (# 102)	1
Vitamins (except vitamin A)	1
Vitamin A	500 IU (control diet) / 100 g 2000 IU (enriched diet) / 100 g
Methionine	0.1
Corn starch	45
Sucrose	24.9
Peanut oil	2.5
Rapeseed oil	2.5

when animals were 8-month-old, before the occurrence of massive AD neuropathology and its associated cognitive alterations. Diet exposure lasted 6 months, until 14 months of age. Food intake was determined by weighing the food every two weeks in collective cages and averaged on the number of mice present in each cage. The four experimental groups are referred to as NTg, NTg +vitA, Tg, Tg +vitA.

2.2. Behavioral assessment

The behavioral assessment was started when the mice were 13.5 months old.

2.2.1. Y-maze

The protocol is based on the two-trial spatial recognition task proposed by Dellu et al., 2000 (Dellu et al., 2000). For acquisition, animals were placed in a starting arm of the apparatus, randomly assigned for each mouse. They were allowed a free exploration of only two arms of the maze for 5 min before returning to their home cages for one hour. For the test, they were placed in the same starting arm and allowed to freely explore the whole maze for 5 min. Light intensity in the center of the maze was 25 lux. Animals that spent more than 90% of the time in the starting arm during acquisition or test phases were excluded from the statistical analysis. This criterion led to the exclusion of 14 mice (8 females of which 5 are of the 3xTg-AD genotype and 6 males, of which 3 are of the 3xTg-AD genotype, regardless of the diet received). The discrimination ratio (DR) was calculated as follows: DR = (distance traveled or time spent in: novel arm – familiar arm) / (distance travelled or time spent in: novel arm + familiar arm) x 100.

2.2.2. Light-dark box

To assess anxiety-like behavior, animals were placed in the dark compartment of the box and had 5 min to dare enter and explore the anxiogenic light compartment (235 lux in the center). The dimensions of each compartment were 20 × 20 × 20 cm (Arsenault et al., 2013; St-Amour et al., 2014).

2.2.3. Open field

Locomotor activity of mice was assessed individually in a 40 × 40 cm plexiglass box and recorded by an automated recording of photobeam breaks over a period of 30 min (Bories et al., 2012; St-Amour et al., 2014).

2.2.4. Opto-kinetic-response (OKR)

Visual acuity of each eye of twelve-month-old non-transgenic (NTg) and 3xTg-AD (Tg) mice was measured for three consecutive days before being exposed to the VitA-enriched diet (20 IU/g) using the OKR evaluation (Mdzomba et al., 2018). Briefly, mice were introduced in a box surrounded by four computer screens and placed on an elevated platform. Moving black and white bands of varying frequencies were displayed on the screens and allowed the recording of the reflexive tracking movement of the mouse's head (Mdzomba et al., 2018). Their visual acuity was retested over three days, after four weeks of VitA-enriched diet, a duration known to be sufficient to increase RA concentration in the brain (Dumetz et al., 2019). As female 3xTg-AD mice have a similar visual acuity than males (King et al., 2018), the assessment was made only in males.

2.3. Tissue collection

Animals received a dose of a lethal ketamine-xylazine mix (300 mg/kg of ketamine and 30 mg/kg of xylazine) and were maintained on a heating pad to maintain body temperature, until they were deeply anesthetized. Then, blood was collected intracardially and animals were perfused with 50 ml of cold phosphate-buffered saline (PBS) containing protease and phosphatase inhibitors, delivered at a rate of 8 ml/min (Bourassa et al., 2019a; Tournissac et al., 2019). Hippocampi were collected and snap-frozen on dry ice.

2.4. Real-time PCR (RT-PCR)

Total hippocampal RNAs were extracted using TRIzol Reagent™ (Invitrogen, Saint Aubin, France). RNA integrity, concentration and purity were obtained by the use of both RNA 6000 Nano LabChip kit for Nanodrop spectrophotometer (NanoDrop™ technologies, Fisher Scientific) and 2100 Bioanalyzer (Agilent Technologies). Then, 1 μg of RNA was reverse transcribed into cDNAs using oligodT, random primers and ImProm II reverse transcriptase (Promega, Charbonnières-les-Bains, France) according to the manufacturer's instructions. The real-time PCR was operated with the LightCycler 480 system (Roche Diagnostics) and both GAPDH and actin served as housekeeping genes as their expression was equivalent in all experimental groups. mRNA relative quantification was obtained with the GenEx™ MultiD software (Göteborg, Sweden), using the comparative threshold method described elsewhere (Pfaffl, 2001).

2.5. Plasmatic retinol

Plasma samples containing retinol were solubilized in hexane added with butylated hydroxytoluene (BHT) as an antioxidant, after the addition of internal standard solution (α-tocopherol). Following thorough vortex, samples were centrifuged for 2 min at 12000 rpm at room temperature. Then, samples were submitted to nitrogen evaporation and dry residue containing retinol was resuspended in methanol. The amount of retinol was measured by high-performance liquid chromatography (HPLC) (Marissal-Arvy et al., 2013).

2.6. Subjects from the Religious Orders Study (ROS)

Participants did not present any known dementia upon inclusion in the study and were subjected to uniform structured evaluations until death. The study was approved by an Institutional Review Board of Rush University Medical Center. All participants signed an informed consent, an Anatomic Gift Act, and a repository consent that allowed their data and biospecimens to be repurposed. Briefly, dementia and AD diagnoses were given when at least two domains of cognitive function were impaired, including episodic memory, based on the results of 21 cognitive performance tests every year, reviewed by a clinical neuropsychologist and an expert clinician (Bennett et al., 2006a). Participants with Mild-Cognitive Impairment (MCI) presented a cognitive impairment assessed by the neuropsychologist, but not dementia evaluated by the clinician (Bennett et al., 2002). Participants without cognitive impairment constitute the No-Cognitive Impairment group (NCI) (Bennett et al., 2012b). A global cognitive score was generated from 19 cognitive performance tests covering multiple cognitive domains including episodic, semantic, working memories and perceptual speed (Wilson et al., 2002). The average interval from last evaluation to brain autopsy was 9 months and did not exceed 12 months. Participants received their final clinical diagnosis of NCI ($n = 20$), MCI (n = 20) and AD (n = 20) at death by a neurologist blinded to all pathologic data, as previously described (Bennett et al., 2006b). At death, inferior parietal cortex samples were collected considering it is a region known to be impaired in the early steps of AD development (Desikan et al., 2009; Desikan et al., 2008). Besides, the parietal cortex is relevant for neuropathological detection of Aβ and Tau pathologies (Markesbery et al., 2006; Nelson et al., 2009). The samples were submitted to a neuropathological diagnosis using the ABC scoring method according to the National Institute of Aging-Alzheimer's Association (NIA-AA) guidelines (Montine et al., 2012). This score combines the evaluation of three parameters: (A) Thal score assessing phases of Aβ plaque accumulation (Thal et al., 2002), (B) Braak score assessing neurofibrillary tangle pathology (Braak and Braak, 1991), and (C) CERAD score assessing neuritic plaque pathology (Mirra et al., 1991). The ABC score was given

for each sample by examiners blinded to clinical data (Bennett et al., 2005). Following the NIA-AA guidelines, ABC scores were converted to four levels of AD neuropathology: not, low, intermediate or high (Montine et al., 2012). In the present study, the AD group includes the participants with intermediate or high levels of AD neuropathology, and the control group contains the participants with no or low level of AD neuropathology. A description of the cohort is presented in Table 2. Previous publications contain further details on tissue handling (Bourassa et al., 2020; Bourassa et al., 2019b; Tremblay et al., 2017; Tremblay et al., 2007) and on the ROS study (Bennett et al., 2018; Bennett et al., 2012a).

2.7. Protein extraction

For protein extraction, mouse hippocampi and human inferior parietal cortices were homogenized in Tris-buffered saline (TBS) and sequentially centrifuged in order to allow a separation between a TBS-soluble cytosolic fraction, a detergent-soluble membrane fraction and a detergent-insoluble fraction (formic acid extracts), as described (Bourassa et al., 2019a; Tournissac et al., 2019). After the first centrifugation, the supernatant was the TBS-soluble protein fraction and was collected and stored at −80 °C. The remaining pellet was dissolved in a lysis buffer containing detergents (150 mmol/L NaCl, 10 mmol/L NaH_2PO_4, 0.5% sodium deoxycholate, 0.5% sodium dodecyl sulfate, 1% Triton X-100) with protease and phosphatase inhibitors and centrifuged (100,000 g, 20 min, 4 °C). The supernatants corresponding to the detergent-soluble membrane protein fraction were collected and stored at −80 °C. The remaining pellet was solubilized in formic acid (99%) and centrifuged (100,000 g, 20 min, 4°C). The supernatants corresponding to the detergent-insoluble protein fraction were evaporated at room temperature for three days. The dry residue was split in two, one was resuspended in guanidine 50 mM and the other in laemmli buffer 1× (Vandal et al., 2015). After protein extraction, the quantification of protein content in each sample was determined for the cytosolic soluble and membrane protein fractions only, by using a BCA Protein Kit Assay (Pierce™, ThermoFisher Scientific, USA).

2.8. $A\beta_{40}$ and $A\beta_{42}$ quantification

The amount of human $A\beta_{40}$ and $A\beta_{42}$ produced by the expression of the transgenes inserted in 3xTg-AD mice was determined in the TBS-soluble and detergent-insoluble (formic acid extracts) fractions generated from the hippocampus, using Human $A\beta_{40}$ ELISA kit and Human $A\beta_{42}$ Ultrasensitive ELISA kit from Invitrogen, ThermoFisher Scientific, according to the manufacturer's instructions. Soluble amount of amyloid was related to the total quantity of proteins found in the cytosolic fraction. Insoluble amount of amyloid was related to the weight of hippocampus before homogenization. Absorbance was read at 450 nm.

2.9. Western Immunoblotting

In mouse hippocampal samples, Western blot analyses were performed on both cytosolic and membrane protein fractions. In human inferior parietal cortex samples, Western blot analysis was made on cytosolic protein fraction only. The protocol used is described elsewhere (Bourassa et al., 2019a). Briefly, 20 μg of proteins of each murine sample and 12 μg of proteins of each human sample were loaded on an acrylamide gel (10%) and separated by SDS-PAGE Electrophoresis. Then, samples were transferred on a PDVF membrane, which was blocked with a solution of milk (milk 5%, BSA (bovine serum albumin) 0.5%) for one hour. The membrane was then incubated with primary antibodies overnight at 4°C followed by an incubation with secondary antibodies for one hour. The luminescence produced by the addition of chemiluminescent reagents (Immobilon Forte Western HRP substrate; Millipore) on the membrane was recorded with MyECLimager system (ThermoFisher Scientific). The quantification was performed with ImageLab software (Biorad). All samples were run on the same gel. Antibodies used are provided in Table 3. Optical density data from experiments performed with human brain samples underwent normalization to the total amount of proteins assessed with a Ponceau staining.

Table 2
ROS study cohort description.

Characteristics	Clinical diagnosis				Neuropathological diagnosis		
	NCI	MCI	AD	Statistical analysis	Controls	AD	Statistical analysis
n	20	20	20	n/a	21	39	n/a
Men (%)	20	45	35	n/a	43	28	n/a
Age at death	87.10 (5.81)	87.10 (5.20)	87.33 (4.85)	ANOVA: $p = 0.987$	86.36 (4.13)	87.62 (5.71)	Welch's test: $p = 0.33$
Post mortem interval	7.45 (5.52)	7.82 (5.22)	7.78 (4.81)	ANOVA: $p = 0.971$	8.20 (5.08)	7.39 (5.15)	Welch's test: $p = 0.56$
Global cognitive score	−0.027 (0.72)	−0.430 (0.66)	−1.657 (0.89)****,$ $$	K-W: p < 0.0001	−0.318 (0.83)	−0.926 (0.92)	Welch's test: $p = 0.0126$*
Episodic memory score	0.266 (0.48)	−0.431 (0.73)	−1.968 (1.19)****,$ $	K-W: p < 0.0001	−0.226 (1.17)	−0.890 (1.25)	Welch's test: $p = 0.0244$*
Semantic memory score	−0.210 (0.63)	−0.349 (0.61)	−1.496 (1.23)***,$$	K-W: $p = 0.0004$	−0.329 (0.98)	−0.890 (1.03)	Welch's test: $p = 0.044$*
Working memory score	−0.245 (0.46)	−0.428 (0.67)	−0.963 (0.85)*	K-W: $p = 0.0113$	−0.316 (0.48)	−0.675 (0.82)	Mann-Whitney test: $p = 0.0991$
Perceptual speed score	−0.362 (0.80)	−0.890 (0.90)	−2.170 (0.85)****,$ $	K-W: p < 0.0001	−0.863 (1.16)	−1.301 (1.10)	Welch's test: $p = 0.1664$
Senile plaques count	6.300 (8.93)	6.950 (7.69)	17.950 (15.45)**,$	K-W: $p = 0.0027$	1.333 (3.28)	15.282 (12.58)	Mann-Whitney test: p < 0.0001****
Neurofibrillary tangles count	0.150 (0.49)	0.250 (0.72)	5.250 (11.10)*,$	K-W: $p = 0.0083$	0.095 (0.44)	2.846 (8.25)	Mann-Whitney test: $p = 0.0141$*

Data are expressed as mean (SD). The clinical diagnosis refers to the assignment of subjects to NCI, MCI and AD groups based on the scores obtained by the participants ante-mortem in multiple clinical tests evaluating the state of their cognitive function in multiple domains. The neuropathological diagnosis was done on the same subjects, *post-mortem* after an evaluation of their Alzheimer neuropathology namely amyloid- and tau-pathologies in parietal cortex samples using the ABC scoring method. Statistics: Clinical diagnosis, when variances were equal between groups, ANOVA was performed, otherwise the Kruskal-Wallis (K-W) test and upon significance, followed by Dunn's post hoc test: * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 versus NCI; $ p < 0.05, $$ p < 0.01, $$$ p < 0.001 versus MCI. Neuropathological diagnosis, when variances were equal between groups, Welch's unpaired *t*-test was performed, otherwise the non parametric Mann-Whitney test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ versus Controls.

Table 3
Summary of antibodies used for Western blot.

Antibody	Host	Dilution	Supplier
Primary antibodies			
β-Actin	Mouse	1:10000	ABM
ADAM10	Rabbit	1:1000	Millipore
APP (clone 6E10)	Mouse	1:500	BioLegend
BACE	Rabbit	1:500	Abcam
CDK5	Rabbit	1:4000	Santa-Cruz Technologies
GFAP	Rabbit	1:10000	Abcam
IBA-1	Rabbit	1:1000	Wako
IDE	Rabbit	1:4000	Abcam
RARβ	Rabbit	1:1000	Sigma
RXRγ	Rabbit	1:1000	Abcam
Tau PHF1 (phospho Ser_{396} and Ser_{404})	Mouse	1:1000	Generous gift from Peter Davies
Tau C-terminal (total)	Rabbit	1:20000	Dako
Secondary antibodies			
Anti-mouse(HRP)	Goat	1:60000	Jackson
Anti-rabbit(HRP)	Goat	1:60000	Jackson

2.10. Statistical analysis

Data are expressed as mean +/− standard error of the mean (SEM). Statistical significance was set at $p \leq 0.05$. At the beginning of the dietary treatment, there were 14 NTg (6 females and 8 males), 15 NTg + vitA (7 females and 8 males), 14 Tg (6 females and 8 males) and 15 Tg +vitA (7 females and 8 males). Within the 6-month-long diet exposure, 6 mice died of old age: 3 NTg (2 females and 1 male), 1 NTg + vitA (female), 1 Tg (male) and 1 Tg +vitA (female).

For intergroup comparison, equality of variances between groups was assessed using Bartlett's test for each measurement. When variances were unequal, the nonparametric Kruskal-Wallis test was used to compare groups, and upon significance, followed by a Dunn's post-hoc test. In the case of equal variances, three-way-ANOVA (or Repeated-Measurements when appropriate) was performed to assess the effects of the following independent factors: genotype, VitA-supplementation and sex in mice, and diagnosis and sex in humans. When the effect of sex was significant, the statistical analysis was performed separately in males and females (Two-way ANOVA), which importantly reduced the size of groups. The effect of sex was not significant in human data. A Tukey's post-hoc test was done to compare groups when appropriate. The comparison of only 2 groups was done using unpaired Student *t*-tests when variances were equal; otherwise, a nonparametric Mann-Whitney test was performed. Two-tailed Student *t*-tests were used to compare a group's mean with a theoretical value (Y-maze). The correlation between observations was obtained using linear regression to determine coefficients of determination. All measurements were submitted to the Grubb's test to allow the detection of a significant outlier ($p < 0.05$). A significant outlier was removed from the statistical analysis. All statistical analyses were performed on Prism 7 (GraphPad software, San Diego, CA, USA) or JMP (version 14.0.0; SAS Institute Inc., Cary, IL, USA).

3. Results

3.1. VitA-supplementation preserved short-term memory of 3xTg-AD mice

As hippocampus-dependent memories, including episodic and spatial memories, are affected in early stages of AD (Moodley et al., 2015; Tromp et al., 2015; Zanco et al., 2018), we used the two-trial recognition task in the Y-maze known to assess short-term spatial memory in mice (Dellu et al., 2000; Wahl et al., 2017). The protocol used is schematized in Fig. 1A. During the test phase, the Tg group did not differentiate the novel and familiar arms, as indicated by the discrimination ratio not differing from zero (Fig. 1B). However, the administration of a VitA-enriched diet (Tg +vitA) allowed Tg animals to recognize the novel arm, based on the distance travelled but not on the time spent in each arm (Fig. 1B). In contrast, VitA-supplementation in NTg group (NTg +vitA) impaired their ability to perform in the test (Fig. 1B). As many females did not meet inclusion criteria, the effect of dietary VitA could not be analyzed in a sex-dependent manner. The total locomotor activity in the open field over a 30-min period was equivalent between groups (Fig. 1C), suggesting that the effect of VitA in the Y-maze was not explained by change in motor behavior. In the light-dark box, the transgenic mice (Tg and Tg +vitA groups) displayed significantly more nose pokes in the light compartment compared to non-

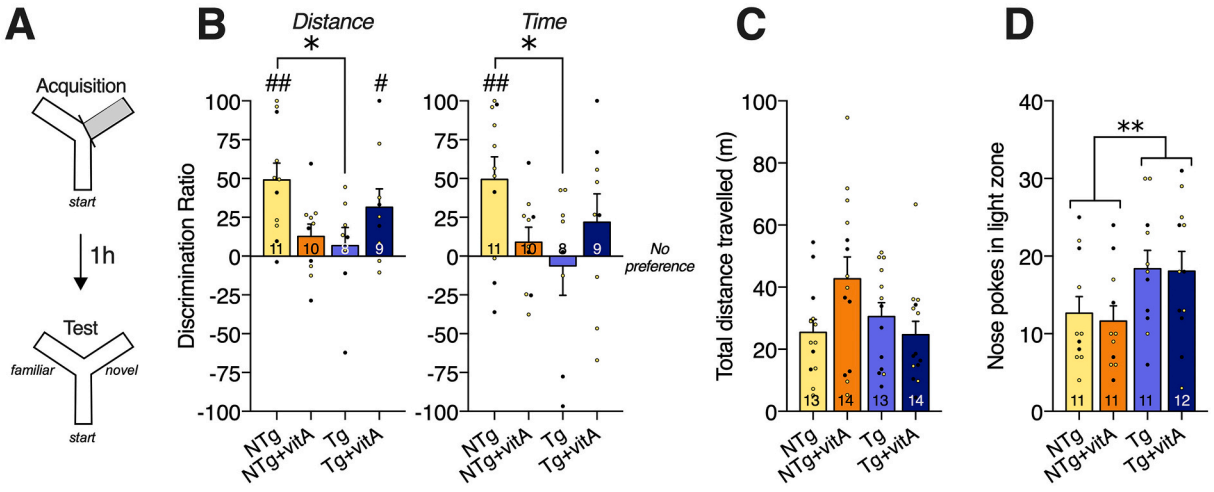

Fig. 1. VitA supplementation preserved short-term memory of 3xTg-AD mice. Data are expressed as mean ± SEM. Black dots: females, yellow dots: males. **A**: Protocol used for short-term spatial recognition memory assessment in the Y-maze. Animals were allowed to freely explore only two arms of the apparatus for 5 min during the acquisition phase. An hour later, access was open to the third arm for free exploration of the apparatus during the 5-min-lasting test phase. **B**: Discrimination ratio (DR) between the distance travelled and the time spent in the familiar and novel arms of the Y-maze. A positive DR indicates a higher exploration of the novel arm, whereas a null or negative DR means no preference or a higher exploration of the familiar arm respectively. **C**: Total distance travelled in the open field for 30 min. **D**: Number of nose-pokes in the light zone of the Light-Dark box.
Statistics: B, One-sample *t*-test comparison to chance level: # $p < 0.05$, ## $p < 0.01$; Two-way ANOVA, genotype x VitA-supplementation interaction significant (Distance-DR: $p = 0.007$; Time-DR: $p = 0.006$) followed by Tukey's post hoc test: * $p < 0.05$. C, Kruskal-Wallis test, p = NS. D, Two-way ANOVA, genotype effect: ** $p < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

transgenic mice (NTg and NTg + vitA groups), suggesting that they were not subjected to anxiety (Fig. 1D). Finally, VitA-supplementation did not affect weight gain, neither the average food intake during the diet exposure (Supplemental Fig. 1A, B).

VitA is known to be necessary for vision (Saari, 2016). Thus, we probed whether the effects observed in the Y-maze in response to VitA-supplementation could be the result of an enhancement of visual acuity, instead of memory. However, we did not observe significant effect of 4-week-VitA-supplementation on visual acuity (Supplemental Fig. 1C).

Overall, results from the behavior tests indicate that dietary VitA-supplementation preserved hippocampal-dependent short-term memory of 3xTg-AD mice.

3.2. VitA status and expression of RXRs

To determine VitA status following dietary treatment, we next analyzed plasma concentrations of retinol in groups of mice and found very different patterns according to the sex of the animals (Fig. 2), which led to a separate analysis in males and females.

Female 3xTg-AD mice had lower levels of plasmatic retinol compared to female NTg mice, a difference not observed between male 3xTg-AD and NTg mice (Fig. 2A). VitA-supplementation did not alter plasma retinol in females, whereas it induced an increase in plasma retinol in male non-transgenic mice (NTg +vitA group), but not in male 3xTg-AD mice (Fig. 2A). This suggests a complex relationship between plasma retinol, sex and transgenes.

As our aim was to understand the action of dietary VitA on AD-type neuropathology, which develops only in 3xTg-AD mice, we focused our analyses on transgenic mice in the rest of the study.

RARs and RXRs are ligand-inducible receptors and some of them display a RARE in their own promoter (Asson-Batres and Rochette-Egly, 2014; Blomhoff and Blomhoff, 2006; Lane and Bailey, 2005; Navigatore Fonzo et al., 2013). Therefore, the level of their expression can be considered as a reflection of retinoid bioactivity in tissues (Brtko et al., 2007; Enderlin et al., 1997a; Etchamendy et al., 2001, 2003). As RXRs are considered a promising therapeutic strategy against AD (Chakrabarti et al., 2016; Muñoz-Cabrera et al., 2019; Tousi, 2015), including by improving β-amyloid peptide clearance (Bachmeier et al., 2013), we measured the mRNA expression of RXRs in the hippocampus of 3xTg-AD mice. We observed that VitA-supplementation decreased the expression of *Rxr-α* in female transgenic mice (Tg + vitA group) but rather tended to increase its expression in males (Fig. 2B). Similar expression patterns were seen for *Rxr-β* and *Rxr-γ* without reaching statistical significance (Fig. 2C, D). No change was observed on the expression of *Raldh* mRNA,

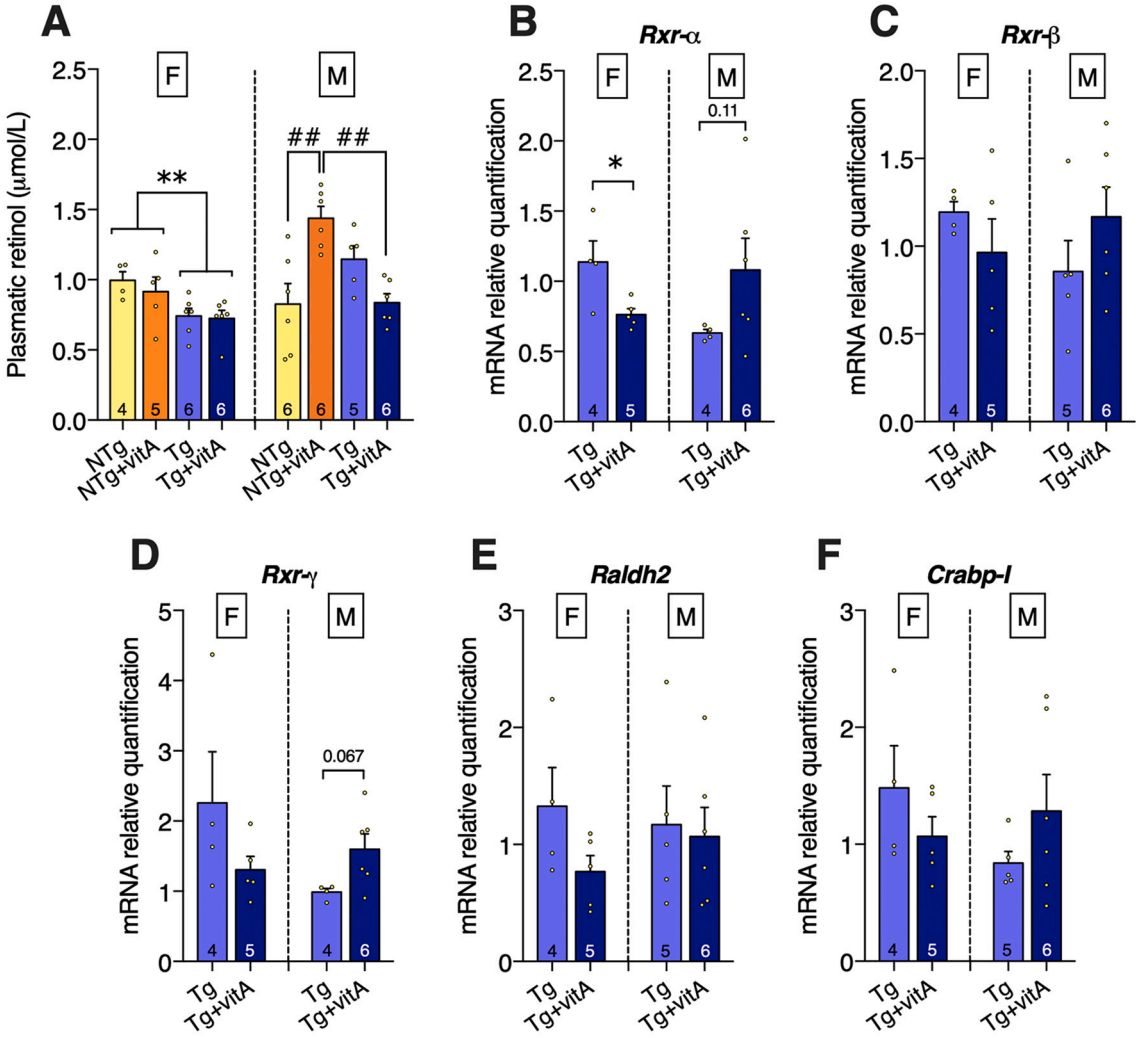

Fig. 2. VitA status and hippocampal expression of RXRs, Raldh2 and Crapb-I. Data are expressed as mean ± SEM. **A**: Plasmatic concentration of retinol. **B-F**: Hippocampal expression of genes coding for the Retinoid X Receptors (RXRs); *Rxr-α* (B), *Rxr-β* (C), *Rxr-γ* (D), the enzyme responsible for retinoic acid synthesis, *Raldh2* (E) and the Cellular Retinoic Acid Binding protein type 1, *Crabp-I* (F) obtained by RT-PCR in 3xTg-AD females, F, and males, M.
Statistics: A, Two-way ANOVA, genotype effect, for females: ** $p < 0.01$, genotype x VitA-supplementation interaction significant ($p = 0.0003$) followed by Tukey's post-hoc test for males: ## $p < 0.01$. B-F, Unpaired *t*-test or Mann-Whitney comparison test were used: Tg vs. Tg + vitA, *$p < 0.05$.

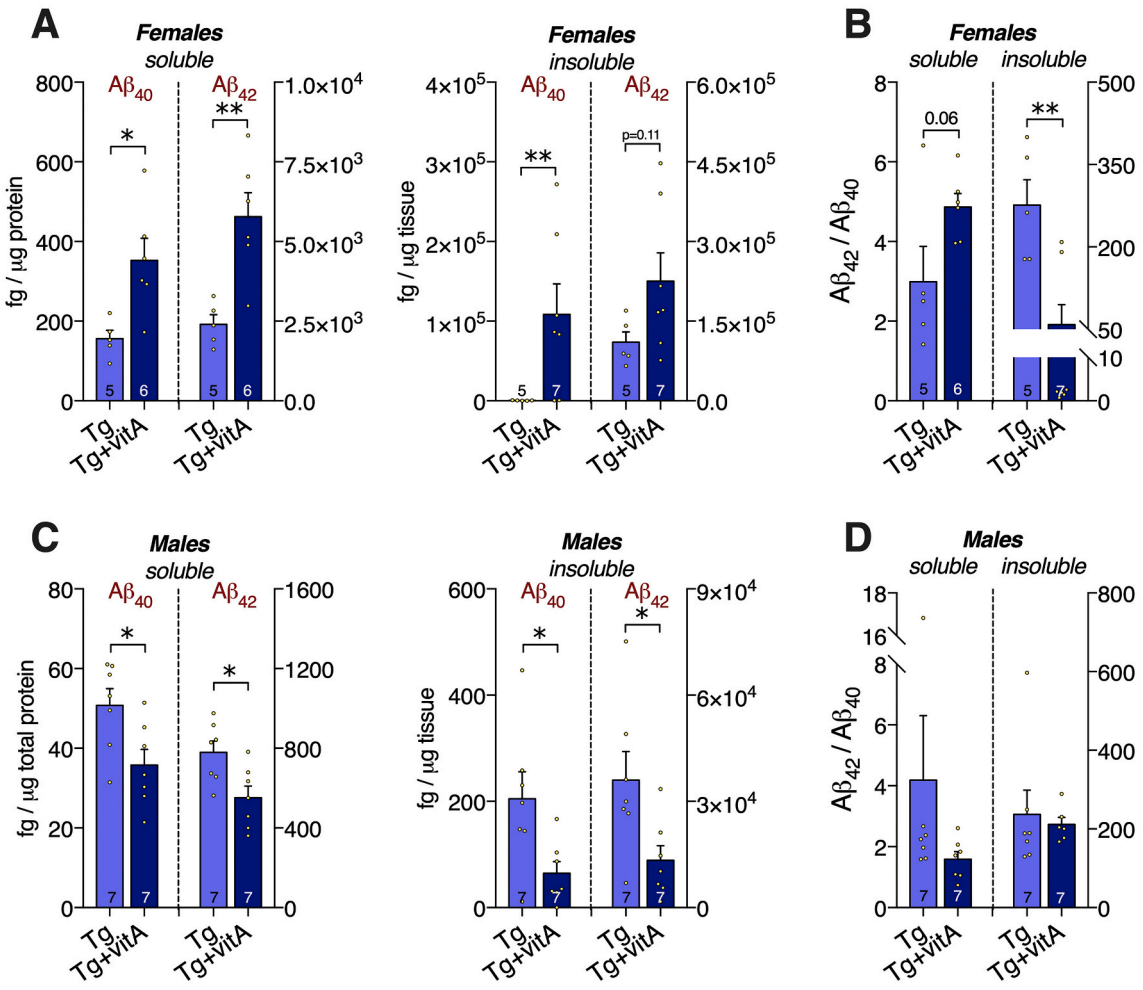

Fig. 3. VitA-enriched-diet reduced β-amyloid peptide levels in male 3xTg-AD mice only. Data are expressed as mean ± SEM. **A**: Amount of both soluble and insoluble $A\beta_{40}$ and $A\beta_{42}$ in the hippocampus of female 3xTg-AD mice with corresponding $A\beta_{42}$ / $A\beta_{40}$ ratios in **B**. **C**: Amount of both soluble and insoluble $A\beta_{40}$ and $A\beta_{42}$ in the hippocampus of male 3xTg-AD mice with corresponding $A\beta_{42}$ / $A\beta_{40}$ ratios in **D**.
Statistics: A-D, Unpaired *t*-test or Mann-Whitney test: Tg vs. Tg + vitA, *p < 0.05, **p < 0.01.

the limiting enzyme for RA synthesis (Fig. 2E), nor on the Cellular Retinoic Acid Binding Protein type I (Crapb-I) mRNA, which is a RXR-target gene (Li et al., 2015).

3.3. VitA-enriched diet reduced Aβ peptide production and Tau phosphorylation in male 3xTg-AD mice but not in females

We then assessed AD-type neuropathology in the hippocampus of mice and observed a strong influence of sex on the concentrations of Aβ and their modulation following VitA intake. Aβ in 3xTg-AD mice were found at up to ten-fold higher levels in females compared to males, consistent with previous studies in the 3xTg-AD model (Bories et al., 2012; Hirata-Fukae et al., 2008; Vandal et al., 2015; Yang et al., 2018). More interestingly, the levels of soluble and insoluble $A\beta_{40}$ and $A\beta_{42}$ were increased in the hippocampus of females but decreased in males, following VitA treatment (Fig. 3A, C). Additionally, in female 3xTg-AD mice, VitA-supplementation tended to increase the soluble $A\beta_{42}/A\beta_{40}$ ratio but significantly decreased the insoluble $A\beta_{42}/A\beta_{40}$ ratio (Fig. 3B). In male 3xTg-AD mice, the soluble and insoluble $A\beta_{42}/A\beta_{40}$ ratios were not affected by VitA supplementation (Fig. 3D).

The higher Aβ levels in females were associated with increased Amyloid Precursor Protein (APP) concentrations following VitA supplementation, whereas no such association was observed in males (Fig. 4A). Hippocampus content in A Disintegrin And Metalloprotease 10 (ADAM10), involved in non-amyloidogenic processing of APP, β-Amyloid Cleaving Enzyme (BACE), an enzyme promoting the production of Aβ from APP, or Insulin-Degrading Enzyme (IDE), an enzyme capable of degrading Aβ peptides, were not affected by VitA enrichment in both sexes (Fig. 4B, C, D).

Extensive accumulation of the protein Tau, particularly in its hyperphosphorylated form, is closely linked to AD symptoms (Tremblay et al., 2017). The total amount of Tau in the hippocampus of mice was equivalent in both males and females, with no impact of VitA-enriched diet (Fig. 4E). However, the diet significantly decreased the phosphorylation of Ser_{396}/Ser_{404} only in males (Fig. 4F). This reduced Tau phosphorylation was associated with decreased levels of Cyclin-Dependent Kinase type 5 (CDK5) (Fig. 4G), a kinase involved in Tau phosphorylation, including the PHF1 epitope (Cuadrado-Tejedor et al., 2011) and which expression and activity are known to be regulated by RA (Brossaud et al., 2017; Ding et al., 2008; Watamura et al., 2016).

The protein levels of the glial fibrillary acidic protein (GFAP) (Fig. 4H), and the ionized calcium binding adaptor molecule 1 (IBA-1) (Fig. 4I) were similar between groups, suggesting that the hippocampal inflammatory environment was unaffected by VitA supplementation in males and females. Fig. 4J shows examples of bands obtained by Western blot for the assessment of protein levels in VitA-control-fed (Tg) and VitA-enriched-fed (Tg +vitA) mice. The Western Blots are presented in Supplemental Fig. 2.

Overall, VitA-supplementation alleviated both β-amyloid and Tau pathologies in male 3xTg-AD mice, but not in females.

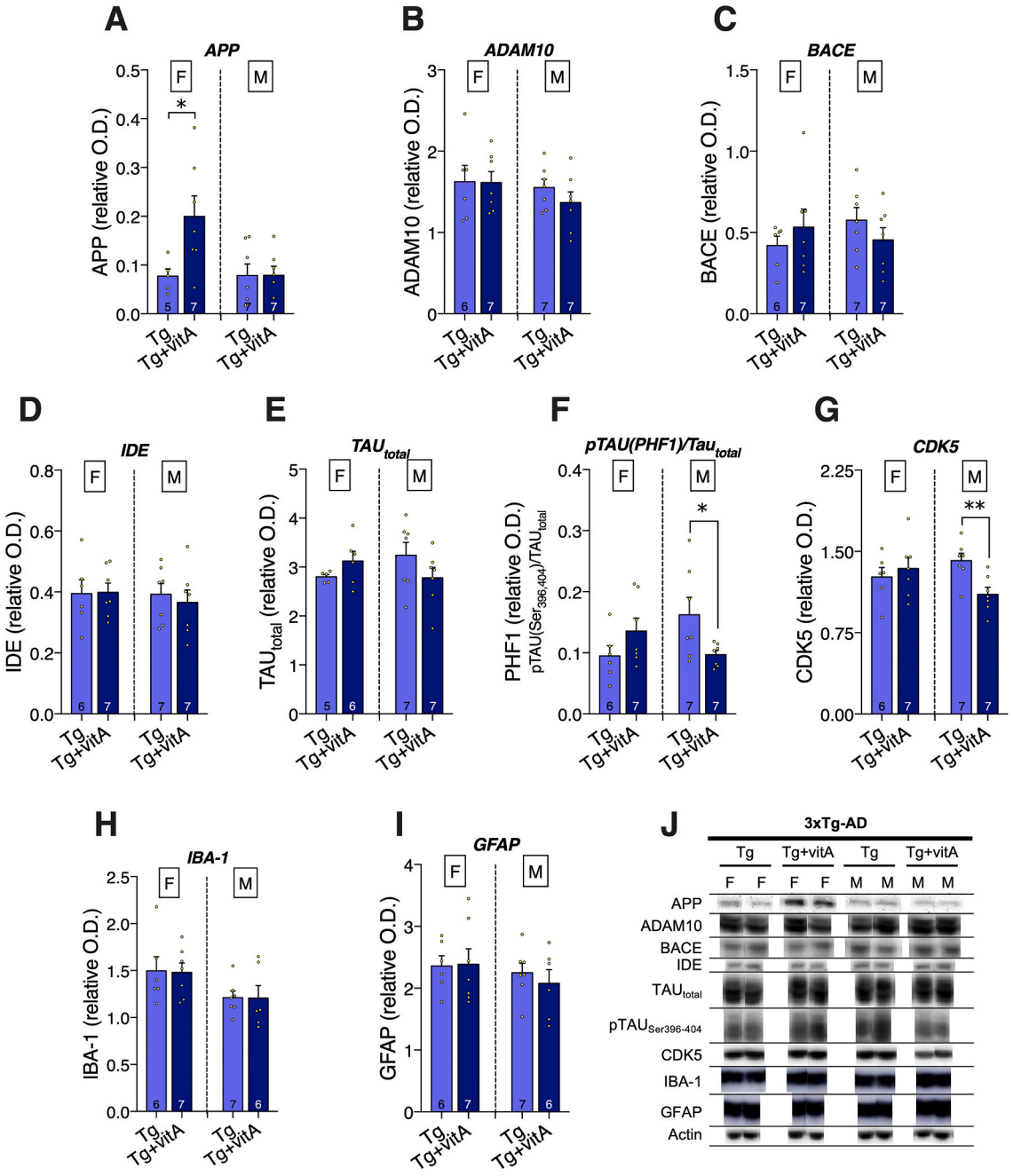

Fig. 4. VitA-enriched-diet reduced Tau phosphorylation in male 3xTg-AD mice only.
A-D: Levels of proteins involved in Aβ-peptide production: Amyloid Precursor Protein, APP (A), ADAM10 (B), BACE (C) and IDE (D). **E-G**: Protein levels of soluble total Tau protein (E), phospho-Tau on PHF1 epitope (F), CDK5, a major kinase for Tau phosphorylation (G). **H-I**: Protein levels of two markers of gliosis: Iba-1 (H) and GFAP (I). All protein levels were obtained by Western blot in the hippocampus of female, F, and male, M, 3xTg-AD mice. **J**: Examples of Western blots assessing the protein levels of 3xTg-AD mice under VitA-control (Tg) and VitA-enriched diets (Tg + vitA).
Statistics: A-G, Unpaired *t*-test or Mann-Whitney test: Tg vs. Tg + vitA, *p < 0.05, ** p < 0.01.

3.4. Higher RXR expression is associated with reduced Aβ peptide production in 3xTg-AD mice

Considering the therapeutic potential of RXR activation against AD (Chakrabarti et al., 2016; Muñoz-Cabrera et al., 2019; Ono and Yamada, 2012; Tousi, 2015), we then investigated whether sex differences in Aβ pathology in response to VitA supplementation were related to the expression of RXRs. First, the hippocampal expression of *Rxr-β* was negatively correlated to hippocampal soluble $A\beta_{40}$ in both male and female transgenic mice (Fig. 5A), and also with soluble $A\beta_{42}$ (Fig. 5B, Tg and Tg + vitA groups pooled). However, the higher expression of *Rxr-γ* was associated with a lower accumulation of soluble $A\beta_{42}$ (Fig. 5C), as well as insoluble $A\beta_{40}$ (Fig. 5D) in the hippocampus of males only. These results support the idea that impaired RXR expression in the hippocampus is associated with the extent of Aβ pathology in 3xTg-AD mice of both sexes, but more strongly in males.

3.5. Relationship between ante mortem cognitive score and post mortem RXR protein levels in human parietal cortex samples

To supplement the data obtained in 3xTg-AD mice and further investigate the link between retinoid receptors and AD, we measured the

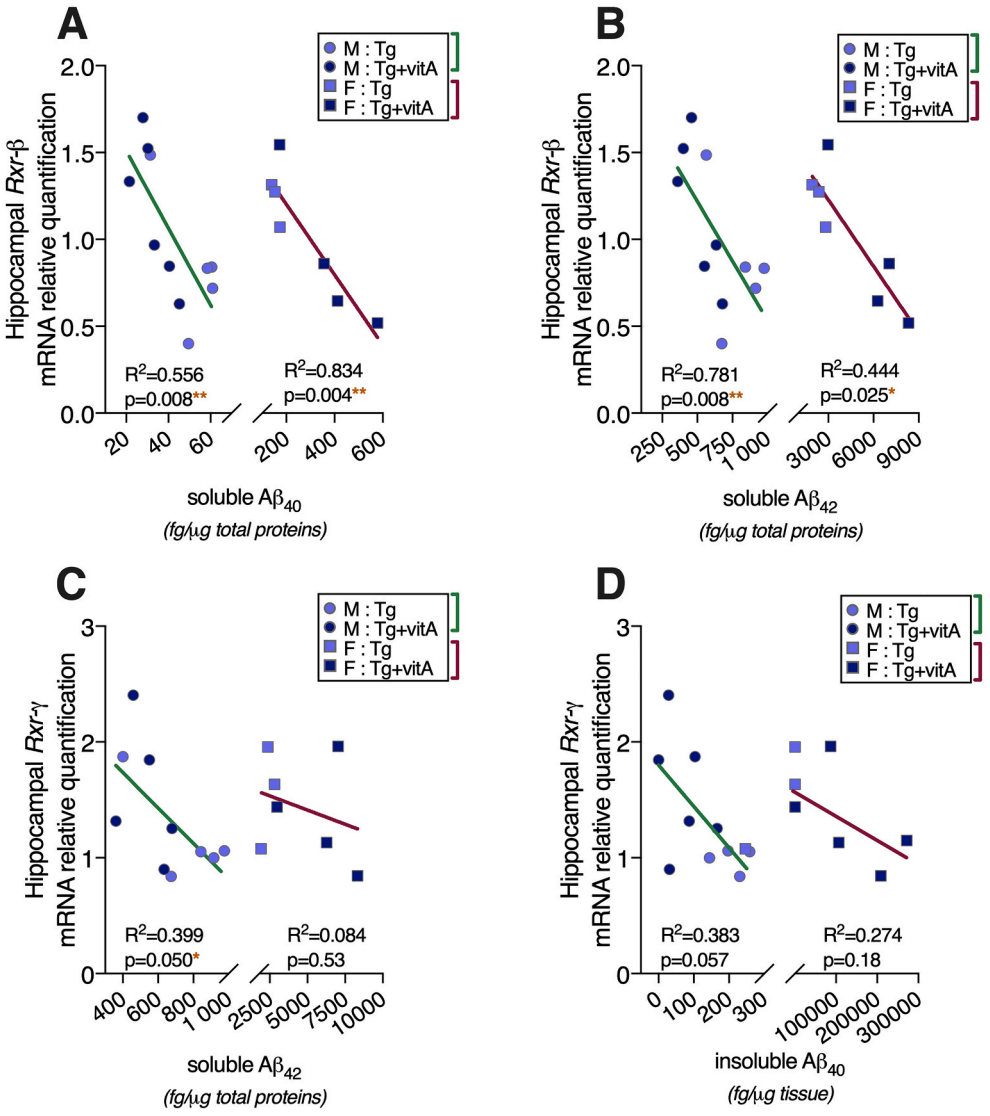

Fig. 5. Relationship between *RXR* sexpression and Aβ peptides in all 3xTg-AD mice.

A-B: Correlations between hippocampal *Rxr-β* mRNA expression and the amount of soluble $A\beta_{40}$ (A) and $A\beta_{42}$ (B) produced in the hippocampus of female and male 3xTg-AD mice (Tg and Tg + vitA pooled). **C-D**: Correlation between hippocampal *Rxr-γ* mRNA expression and the amount of soluble $A\beta_{42}$ (C) and insoluble $A\beta_{40}$ (D) produced in the hippocampus of female and male 3xTg-AD mice (Tg and Tg + vitA pooled).

Statistics: A-D, The correlations include all male (under VitA-control and VitA-enriched diets, green line) and all female 3xTg-AD mice (pink line). The lilac and blue dots are illustrated to indicate how the animals of each group are distributed in the correlations. Pearson's correlation coefficient was determined with a linear regression analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

protein concentrations of RARβ and RXRγ in the inferior parietal cortex of 60 autopsied ROS participants (Bennett et al., 2018; Bennett et al., 2012a) clinically classified as NCI ($n = 20$), MCI (n = 20) or AD (n = 20) (Table 2 and Supplemental Fig. 3). We first observed no difference of cortical levels of RARβ between groups based on the clinical diagnosis (Fig. 6A). However, the Western blot analysis revealed two distinct bands of RXRγ with approximate molecular weights of 55 kDa and 60 kDa, with the former increasing and the latter decreasing along with cognitive symptoms (Fig. 7 and Supplemental Fig. 4). This observation suggests a modification of the receptor that affects its migration on the gel.

Quantification of the 55-kDa-RXRγ revealed a higher concentration in AD participants compared to the NCI group (Fig. 6B). The levels of the 60-kDa-RXRγ were not different between the three groups (Fig. 6C). Consistently, smaller 60/55-kDa-RXRγ ratios were found in the AD group compared to the NCI group (Fig. 6D). Examples of the bands obtained by Western blot are provided in Fig. 6E and in more details in Supplemental Fig. 4.

When comparing groups based on the neuropathological diagnosis using the ABC score (Bourassa et al., 2020; Montine et al., 2012) in cortical samples (Supplemental Fig. 3B), we observed, again, no difference between groups for the RARβ protein levels (Fig. 6F), higher levels of 55-kDa-RXRγ in AD subjects compared to controls (Fig. 6G), similar 60-kDa RXRγ levels in both groups (Fig. 6H), but a lower 60-kDa/55-kDa RXRγ ratio in individuals with AD (Fig. 6I). Examples of the bands obtained by Western blot are provided in Fig. 6J and in more details in Supplemental Fig. 4.

No significant effect of sex on RXRγ levels was detected in the parietal cortex samples investigated.

We next evaluated the correlation between *ante mortem* cognitive function and *post mortem* levels of RXRγ in the parietal cortex. The levels of the 55-kDa-RXRγ were inversely correlated with global cognitive scores, including episodic memory, semantic memory, working memory and perceptual speed (Fig. 7A). In contrast, the 60-kDa-RXRγ was positively correlated with episodic memory, perceptual speed and with global cognitive scores (Supplemental Fig. 5A). Accordingly, the 60-kDa/55-kDa-RXRγ ratio was positively correlated with these cognitive scores (Fig. 7B), suggesting that the transition from a 60-kDa-RXRγ to a 55-kDa-RXRγ is related to AD symptoms.

These RXRγ protein levels were also associated with AD neuropathology. Both 55-kDa-RXRγ and its ratio relative to the 60-kDa-RXRγ showed an association with insoluble $A\beta_{42}$ and the number of neuritic plaques found in the inferior parietal cortex of the same subjects (Fig. 7C, D). This positive relationship between the 55-kDa-RXRγ and plaques was particularly strong in men ($R^2 = 0.3442$, $p = 0.0065$), but not in women ($R^2 = 0.0598$, $p = 0.1389$) (Fig. 7D), although the global

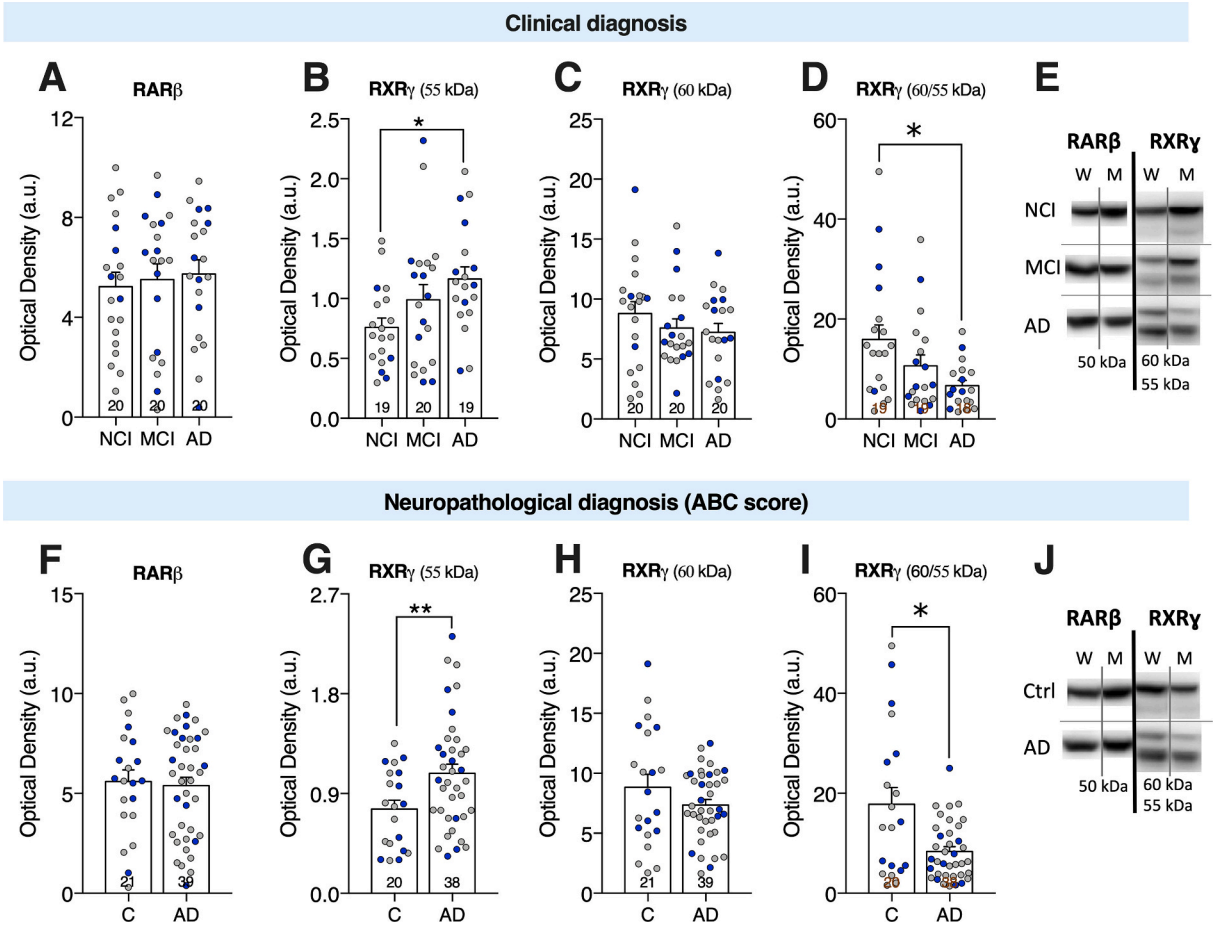

Fig. 6. Protein levels of RARβ and RXRγ in the parietal cortex of MCI and AD subjects. Data are expressed as mean ± SEM. The grey and blue dots correspond to women and men, respectively. **A-D**: Volunteers have been assigned to No-Cognitive Impairment (NCI), Mild-Cognitive Impairment (MCI) or Alzheimer's Disease (AD) groups according to a clinical diagnosis based on multiple clinical tests assessing cognitive function. Protein levels of the RARβ (A); the 55-kDa-RXRγ (B); the 60-kDa-RXRγ (C) and the ratio between the 60-kDa-RXRγ and the 55-kDa-RXRγ (D) obtained by Western blot in the inferior parietal cortex of the subjects. **E**: Examples of bands obtained in Western blots when assessing the protein levels of RARβ and RXRγ in human inferior parietal cortex samples according to the clinical diagnosis. (W, woman; M, man). **F-I**: The same subjects have been assigned to control (C) or Alzheimer's disease (AD) groups according to a neuropathological diagnosis based on the ABC score evaluating the *post mortem* amyloidopathy and the tauopathy in the parietal cortex of the subjects. Protein levels of the RARβ (F); the 55-kDa-RXRγ (G); the 60-kDa-RXRγ (H) and the ratio between the 60-kDa-RXRγ and the 55-kDa-RXRγ (I) obtained by Western blot in the parietal cortex of the subjects. **J**: Examples of bands obtained in Western blots when assessing the protein levels of RARβ and RXRγ in human inferior parietal cortex samples according to the neuropathological diagnosis. (W, woman; M, man).
Statistics: A-C, One-way ANOVA followed by Tukey's post-hoc test: * $p < 0.05$. D, Kruskal-Wallis test ($p < 0.05$) followed by Dunn's post-hoc test: * $p < 0.05$. F-G, Unpaired *t*-test: ** $p < 0.01$. H-I, Mann-Whitney comparison test, * $p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

effect of sex was not significant. By contrast, the levels of 60-kDa-RXRγ were not related to insoluble $A\beta_{42}$ but there was a statistical tendency to correlate negatively with the number of neuritic plaques found in the inferior parietal cortex of the subjects (Supplemental Fig. 5B, C). Finally, the 55-kDa-RXRγ was associated with higher counts of neurofibrillary tangles in the inferior parietal cortex (Supplemental Fig. 6A), which was not the case for the ratio (Supplemental Fig. 6B), or the 60-kDa-RXRγ (Supplemental Fig. 6C). Biological sex did not significantly influence the correlations between protein levels of RXRγ and cognitive function or AD neuropathology.

Altogether, these data suggest that a modification affecting RXRγ protein may be associated with cognitive function and β-amyloid pathology in aging subjects.

4. Discussion

We evaluated whether a chronic VitA-enriched diet could limit the development of AD-type neuropathology and consequent behavior impairment in the 3xTg-AD mouse model. We show that six months of VitA-enriched diet (20 IU/g) preserved the short-term spatial memory of 14-month-old 3xTg-AD mice. This protective effect on cognition was accompanied by a decrease in both Aβ and Tau pathologies in the hippocampus of males. In females, VitA supplementation increased the Aβ-peptide load instead, and had no effect on Tau. VitA supplementation would thus affect hippocampal RA signaling in an opposite way in aged males and females. Still, the expression of *Rxr-β* negatively correlated with Aβ peptides in the hippocampus of both sexes.

The additional Western immunoblot investigations in brains from subjects (NCI, MCI and AD) evidenced that a transition of the RXRγ protein from a band migrating at 60 kDa to a band migrating at 55 kDa was associated with *ante mortem* cognitive scores, insoluble $A\beta_{42}$ and the number of neuritic plaques in the cortex. This suggests that a modified RXRγ protein is associated with AD symptoms.

Overall, the present human *post mortem* data support the hypothesis that altering RXRs is a contributor to the cognitive deficits and the neuropathology associated with AD, while the animal study suggests that a dietary VitA supplementation could be a strategy to consider for the prevention of AD neuropathology in males, but not in females.

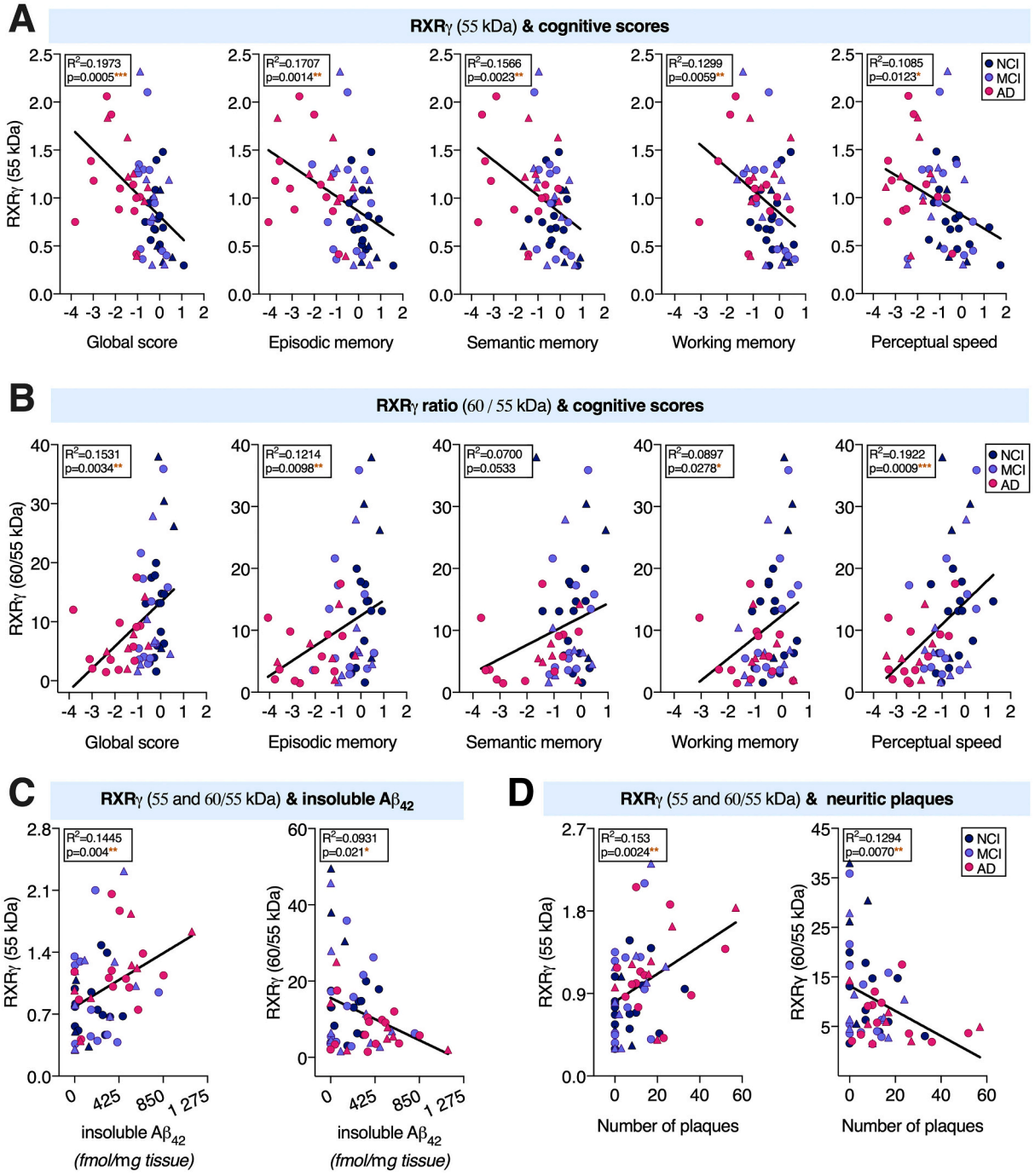

Fig. 7. Relationship between RXRγ protein, cognitive scores, insoluble $A\beta_{42}$ and neuritic plaques in ROS subjects. The circles and triangles correspond to women and men, respectively. Participants have been assigned to No-Cognitive Impairment (NCI), Mild-Cognitive Impairment (MCI) or Alzheimer's Disease (AD) groups according to a clinical diagnosis based on multiple clinical tests assessing cognitive function. **A-B**: Correlation between RXRγ protein levels and cognitive scores: 55-kDa-RXRγ (A); ratio between the 60-kDa-RXRγ and the 55-kDa-RXRγ (B). **C-D**: Correlation between RXRγ protein levels (the 55-kDa-RXRγ or the ratio between the 60-kDa-RXRγ and the 55-kDa-RXRγ) and insoluble $A\beta_{42}$ (C) and neuritic plaques (D) found in the parietal cortex of the subjects.
Statistics: A-D, Pearson correlation coefficient (R^2) was determined with linear regression analysis.

4.1. VitA supplementation preserves hippocampus-dependent short-term memory of 3xTg-AD mice

The preserved spatial recognition memory of the supplemented 3xTg-AD mice suggests that VitA consumed for 6 months limited the development of alterations in the hippocampus with aging. Moreover, the assessment of VitA supplementation effect in the 3xTg-AD mouse model has barely been studied. To our knowledge, only one study previously highlighted a beneficial effect of chronic retinoic acid (RA) treatment (the active form of VitA) on spatial memory of the 3xTg-AD mice (Lerner et al., 2012), although it has been reported in other murine models of AD (Behairi et al., 2016; Ding et al., 2008; Goncalves et al., 2013).

Our results are in line with the literature supporting the advantages of RA signaling stimulation on age-related hippocampal memory deficits, using either dietary or pharmacological VitA administrations (Bonhomme et al., 2014b; Dumetz et al., 2019; Etchamendy et al., 2001; Mingaud et al., 2008; Touyarot et al., 2013). On the other hand,

considering the fine regulation of plasmatic retinol (Blomhoff and Blomhoff, 2006; D'Ambrosio et al., 2011; Li et al., 2014; O'Byrne and Blaner, 2013), an excessive VitA supply (*i.e.* in the absence of VitA deficiency or hyposignaling) can be deleterious and impair memory (Adams, 2010; Crandall et al., 2004; McCaffery et al., 2003; de Oliveira, 2015). This could explain why VitA-supplementation was detrimental on the performances of NTg + vitA animals in the Y-maze.

4.2. VitA-enriched diet has an equivocal effect on VitA status and hippocampal RXR expression according to biological sex

The effect of the transgenes and VitA treatment on serum retinol levels was strongly modulated by biological sex, leading us to consider both sexes separately in subsequent analyses.

The lower plasmatic retinol status observed in female 3xTg-AD mice is consistent with the data collected in elderly and AD patients (Bourdel-Marchasson et al., 2001; Huang et al., 2018; Rinaldi et al., 2003; Zeng et al., 2017), for which the question of sex differences has not been addressed. The absence of VitA-supplementation-induced changes in plasmatic retinol levels has already been reported (Bonhomme et al., 2014a; Letondor et al., 2016; Touyarot et al., 2013) and circulating levels do not necessarily predict the hippocampal RA signaling activity, as shown by the work of Touyarot et al., 2013). Similarly, VitA-supplementation did not increase plasmatic retinol concentrations in male 3xTg-AD mice. Yet, the supplemented 3xTg-AD mice (males and females pooled) could recognize the novel arm, as opposed to the non-supplemented 3xTg-AD mice. Thus, circulating retinol may not always be an appropriate marker of the cellular action of RA in target tissues, as shown by previous studies (Feart et al., 2005b; Lopez-Teros et al., 2014; Tanumihardjo, 2014).

We took advantage of the 3xTg-AD model to investigate the interaction between dietary VitA supplementation, *Rxr* expression and AD neuropathology. The gene expression profiles of *Rxr-α* and *Rxr-γ* in the hippocampus of 3xTg-AD mice in response to VitA supplementation suggest that VitA enrichment would activate RXR signaling in males but do the opposite in females. These observations are consistent with our results in the Y-maze, where males were more abundant, and with data in the literature indicating that the stimulation of RA hyposignaling can improve hippocampus-dependent memory performance (Bonhomme et al., 2014b; Etchamendy et al., 2001; Touyarot et al., 2013). A further investigation of retinoid signaling activity (dimerization or trans-repression processes for instance) (Krezel et al., 2019; Moutinho and Landreth, 2017), would allow the identification of the mechanisms at stake in our conditions.

Nevertheless, to our knowledge, sex-specific differences in retinoid metabolism and signaling at adulthood have not been described. Thus, we cannot provide a compelling explanation for the sex-associated discrepancies in the RXR expression induced by VitA in our AD model.

4.3. VitA enrichment exerts a beneficial effect on AD neuropathology in males only

The higher brain amyloid load in 3xTg-AD females compared to males has consistently been observed in previous studies (Bories et al., 2012; Hirata-Fukae et al., 2008; Vandal et al., 2015; Yang et al., 2018). However, the aggravating action of VitA on Aβ accumulation was surprising and unexpected. This would indicate that the VitA nutritional approach could be inappropriate for females. However, the negative relationship between hippocampal expression of *Rxr-β* and β-amyloid species in both sexes is consistent with the beneficial effect of RXR signaling on amyloid pathology in females as well.

The sex-related difference in Aβ concentrations after dietary VitA supplementation could not be explained by changes in APP or in the enzymes involved in APP processing. This is inconsistent with previous data reporting the capacity of retinoids to regulate the expression of these enzymes, whether it is the pro- or the non-amyloidogenic pathway (Holthoewer et al., 2012; Jarvis et al., 2010; Tippmann et al., 2009; Wang et al., 2015). However, protein levels of enzymes assessed by Western blots may not fully capture changes in their catalytic activity.

Biological sex also influenced the effect of VitA treatment on the $A\beta_{42}/A\beta_{40}$ ratios. Soluble and insoluble $A\beta_{42}/A\beta_{40}$ ratios were increased and decreased respectively by VitA supplementation in females while it was rather the concentrations of each Aβ species that was lowered by VitA-supplementation in males, suggesting different mechanisms are involved in each sex. Female 3xTg-AD mice of the present study (14-month-old) likely had entered menopause, a transition state suspected to increase the risk of developing AD (Scheyer et al., 2018). During menopause in rodents, the ovarian estrogen production decreases progressively (Finch, 2014). This production is regulated by retinoic acid (RA), the active form of VitA (Damdimopoulou et al., 2019), which is in favor of the age-related decrease in retinoid signaling, including in the ovary. Thus, the VitA-enriched supply may be preferentially transported to the ovaries as a compensatory mechanism, to the detriment of the hippocampus. Furthermore, the ovaries host an enzyme that inactivates estrogens (17β-HSD2) also regulated by RA (Cheng et al., 2008; Yamagata et al., 2015; Yilmaz and Bulun, 2019), which would aggravate the menopause-induced decrease of estrogen signaling. Interestingly, VitA cell-entry receptor (*Stra6*) and RA synthesis enzyme (Raldh) are upregulated by estrogens in the hippocampus (Sárvári et al., 2015). Thus, a VitA-induced reduction in circulating active estrogens could have led to intensified RA hyposignaling in the hippocampus, suggested by lower *Rxr-α* expression in female 3xTg-AD mice. As this receptor reduces Aβ accumulation (Kapoor et al., 2013; You et al., 2009), its lower expression in the hippocampus would be contributing to the aggravated amyloid-pathology of female 3xTg-AD mice. This hypothesis would need to be tested.

The beneficial effect of VitA supplementation observed on the hyperphosphorylated Tau protein of male 3xTg-AD mice is likely to involve the regulation of CDK5, consistently with the work of Ding et al., 2008 (Ding et al., 2008).

Our results in males are consistent with our initial mechanistic hypothesis about the protective effects of retinoid treatments on β-amyloid accumulation, supported by *in vitro* and *in vivo* studies in AD models (Ding et al., 2008; Goncalves et al., 2013; Holthoewer et al., 2012; Jarvis et al., 2010; Kitaoka et al., 2013; Tippmann et al., 2009; Wang et al., 2015), most of which are conducted in males. In contrast, the deleterious effect of VitA in females underscores the presence of a previously unexpected complex interplay between central RXR expression, Aβ pathology and changes in sex hormones. The different response of males and females to VitA enrichment could also be the result of a sex-driven RXR regulation, as their expression has been shown to be influenced by sex (Arfaoui et al., 2013). In any case, our results highlight the importance for preclinical studies not to assume that males and females will react similarly to a given treatment.

4.4. Altering RXRγ is associated with AD neuropathology and cognitive impairment

There is very little research conducted on the state of retinoid signaling in early or late stages of AD, despite the large amount of literature proposing retinoids as putative therapeutic molecules against the disease (Chakrabarti et al., 2016; Sodhi and Singh, 2014). For instance, in small double-blind placebo-controlled interventional studies, the oral administration of acitretin (30 mg per day, $n = 11$) or bexarotene (increasing doses, total 300 mg, $n = 16$) for four weeks enhanced the non-amyloidogenic processing of APP (suggested by increased sAPPα fragments in the cerebrospinal fluid (CSF) and increased the brain clearance of $A\beta_{42}$ respectively, in individuals clinically diagnosed with AD and presenting at least one pathological CSF biomarker (Cummings et al., 2016; Endres et al., 2014). However, these studies did not provide data about the VitA status or signaling in the brain of the subjects or the mechanisms underlying the beneficial effects

of the treatments.

The use of samples from the ROS cohort allowed us to include individuals with MCI and without cognitive impairment in order to investigate relationships between RARβ and RXRγ and *ante mortem* cognitive function measured proximate to death. RARβ and RXRγ are key retinoid receptors mediating VitA signaling. RARβ mRNA expression is upregulated by VitA supplementation (Etchamendy et al., 2001; Idres et al., 2002; Mingaud et al., 2008), whereas RXRγ expression is induced in acutely lesioned brain and its signaling has been shown to stimulate remyelination in the central nervous system (Huang et al., 2011; Sołtys and Ożyhar, 2020; Yang et al., 2017). This is coherent with an involvement of the latter in regenerative processes relevant to AD.

The present analysis unveiled a pattern of differences in RXRγ in which a band migrating at 55 kDa increases along the progression of AD, while the band at 60 kDa recedes. This pattern was associated with both clinical and neuropathological diagnosis, but the latter was more strongly related to Aβ than Tau pathology. This suggests the presence of a pathological switch from full-length RXRγ towards a modified form in AD. The very small size difference between both bands is suggestive of a protein modification rather than the existence of two distinct isoforms of RXRγ. As far as we know, no distinct mRNA transcripts of RXRγ have been identified in humans, in contrast to mice (Liu and Linney, 1993), rats (Georgiades and Brickell, 1998) or chickens (Seleiro et al., 1994). So far, post-translational modifications (PTM) of the RXRγ isoform have barely been described in the literature. The two observed bands could likely be the result of cleavage of the 60-kDa RXRγ by an undefined protease. In addition, PTM could also have affected the migration of the RXRγ protein on the Western Blot gel. Interestingly, the human RXRγ has been shown to belong to intrinsically disordered proteins/regions (IDPs/IDRs) (Sołtys and Ożyhar, 2020), that are, by definition, submitted to a wide variety of PTM, including phosphorylation, sumoylation or ubiquitination (Bah and Forman-Kay, 2016). More specifically, the sequence of the A/B domain of human RXRγ is particularly susceptible to degradation and increased electrophoretic mobility (Sołtys and Ożyhar, 2020). Such an alteration of the A/B domain of human RXRγ could occur in the early stages of AD and would explain the occurrence of the two bands we observed.

There is evidence for a retinoid signaling alteration in normal aging, in the first manifestations of cognitive decline and in AD (Bourdel-Marchasson et al., 2001; Corcoran et al., 2004; Huang et al., 2018; Lopes da Silva et al., 2014; Rinaldi et al., 2003; Zeng et al., 2017). Our results are in line with these data from the literature and suggest that a modification of RXRγ protein integrity, that remains to be identified, is associated with cognitive symptoms and neuropathological signs of AD.

5. Limitations

Although the present study provides clues about the relevance of VitA activity in the etiology of AD in both humans and mice, the exact mechanisms linking RXR expression and function to Aβ pathology remain to be elucidated. Secondly, the sex-induced differences observed in the 3xTg-AD mouse model in response to a VitA-enriched diet were unexpected as, to our knowledge, no differences according to sex have yet been reported in VitA metabolism. The consideration of males and females separately lead to a reduced statistical power for most analyses (especially for group comparisons of RXRs and Raldh2 mRNA expression in the hippocampus, Fig. 2). Consequently, the conclusions made about VitA-enriched diet-induced changes in RXR expression should be taken with caution. Furthermore, ROS participants included unequal numbers of women and men in each group. Thus, sex discrepancies observed in 3xTg-AD mice could not be properly investigated in human samples. Finally, measurements were done in the hippocampus of mice and in the parietal cortex of humans (due to hippocampus sample unavailability). Thus, we cannot extrapolate that the links between RXRs and Aβ pathology in the hippocampus of mice are identical in the hippocampus of humans. Nevertheless, RXRs and Aβ pathology were significantly related in the brain of both 3xTg-AD mice (hippocampus) and ROS participants (parietal cortex), suggesting that their relationship is robust.

6. Conclusion

The present study provides insight into the effects of chronic dietary VitA-supplementation on spatial memory and β-amyloid and Tau pathologies in 3xTg-AD mice. While VitA intake significantly improved memory performance in all 3xTg-AD animals investigated, it decreased hippocampus Aβ and Tau levels in males, but rather aggravated Aβ pathology in females. On the other hand, cerebral alterations of RXR expression were associated with worsened neuropathology in both mice and humans (decrease in mice, integrity change in humans), as well as cognitive function in humans, without clear distinction of sex. This suggests that the maintenance of RXR signaling is a critical factor in the development of the disease. Our results support the hypothesis that dietary VitA-supplementation could be considered as a strategy for the prevention of AD neuropathology, but with an efficacy more likely to be present in males than in females. Finally, our results highlight the absolute necessity of conducting research by systematically integrating females and males into preclinical and clinical research paradigms, especially for diseases such as AD, which are affecting more women.

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Credit author statement

E.F.B., V.P. and F.C. designed the study. V.P. and F.C. secured funding. E.F.B. performed the behavioral assessment. C.T. performed the quantification of Aβ peptides in both murine and human samples and RXRγ Western Blots. E.F.B. realized all the other Western blots. S.A. assessed plasmatic retinol concentrations. J.C.H was a major contributor in the gene expression measurements and analysis. D.A.B. provided the human samples. L.R. and V.P. performed the visual acuity experiments. E.F.B. wrote the original draft of the manuscript, which was edited and reviewed by co-authors. All authors read and approved the final manuscript.

Ethics approval

All experiments performed on the animals were approved by the Laval University ethical committee of animal protection (CPAUL), in accordance with the Canadian Council of Animal Care (CCAC).

All procedures performed with volunteers included in this study were in accordance with the ethical standards of the institutional ethics committees. Informed consent was obtained from all individual participants included in the study.

Availability of data and materials

The datasets used and/or analyzed in the current study are available from the corresponding authors on reasonable request. ROS data can be requested at https://www.radc.rush.edu.

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Declaration of Competing Interest

The authors declare that they have no competing interests.

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