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Short communication

FMR protein: Evidence of an emerging role in retinal aging?

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ABSTRACT

Aging is a multifactorial process that affects the entire organism by cumulative alterations. Visual function impairments that go along with aging are commonly observed, causing lower visual acuity, lower contrast sensitivity, and impaired dark adaptation. Electroretinogram analysis revealed that the amplitudes of rod- and cone-mediated responses are reduced in aged mice and humans. Reports suggested that age-related changes observed in both rod and cone photoreceptor functionality were linked to oxidative stress regulation or free radical production homeostasis. Interestingly, several recent reports linked the fragile X mental retardation protein (FMRP) cellular activity with oxidative stress regulation in several tissue including brain tissue where FMRP participates to the response to stress via protein translation in neurite or is involved in free radical production and abnormal glutathione homeostasis. Based on these recent literatures, we raised the question about the effect of FMRP absence in the aging retina of *Fmr1*$^{-/y}$ compared to their WT littermates. Indeed, up to now, only young or adult mice (<6 months) were investigated and have shown a specific retinal phenotype. Herein, we demonstrated that *Fmr1*$^{-/y}$ mice do not present the aging effect on retinal function observed in WT littermates since ERG a- and b-waves amplitudes as well as oscillatory potentials amplitudes were not collapsed with age (12/18 months old). Absence of FMRP and its consequences seem to protect the retina against aging effect, rising a pivotal role of FMRP in retinal aging process.

1. Short communication

FMRP, the fragile X mental retardation protein, is an RNA-binding protein mainly expressed in cerebral neuronal dendrites where it assists transport, stabilization and translational regulation of specific synaptic proteins mRNA (Brown et al., 2001; Bassell and Warren, 2008; Sethna et al., 2014). FMRP is a well-known key regulator of synaptic plasticity since this protein is found in dendritic spines (Ferrari et al., 2007; Sidorov et al., 2013), an important postsynaptic site of plasticity induction and maintenance, and had been shown to regulate dendritic mRNAs translation (Bassell and Warren, 2008) required for multiple forms of plasticity (Sutton and Schuman, 2006). As a consequence, the loss of FMRP induces alterations of synapses in their structure and maturity as in their functions (Irwin et al., 2000; Nimchinsky et al., 2001). This absence leads to the well-known neurodevelopmental disorder known as the Fragile X Syndrome (FXS). Reduced FMRP expression has also been identified in other neurodevelopmental human disorders such as autism, schizophrenia, and bipolar disorder (Fatemi et al., 2010, 2011, 2011, 2013, 2013; Fatemi et al., 2011; Fatemi et al., 2013a,b; Fernandez et al., 2013; Kelemen et al., 2013; Kovacs et al., 2013; Jacquemont et al., 2014). Interestingly, all FMRP deficits were associated with retinal function alteration characterized by electroretinogram (ERG) wave modifications (Constable et al., 2016, 2020; Hebert et al., 2017, 2020; Perche et al., 2021). Indeed, under Light-Adapted (LA) conditions, the absence of FMRP (Perche et al., 2021) or its downregulation (Hebert et al., 2015, 2017, 2020; Constable et al., 2016, 2020) was linked to a decreased b-wave amplitude and prolonged latencies whereas delayed LA ERG response were described in specific

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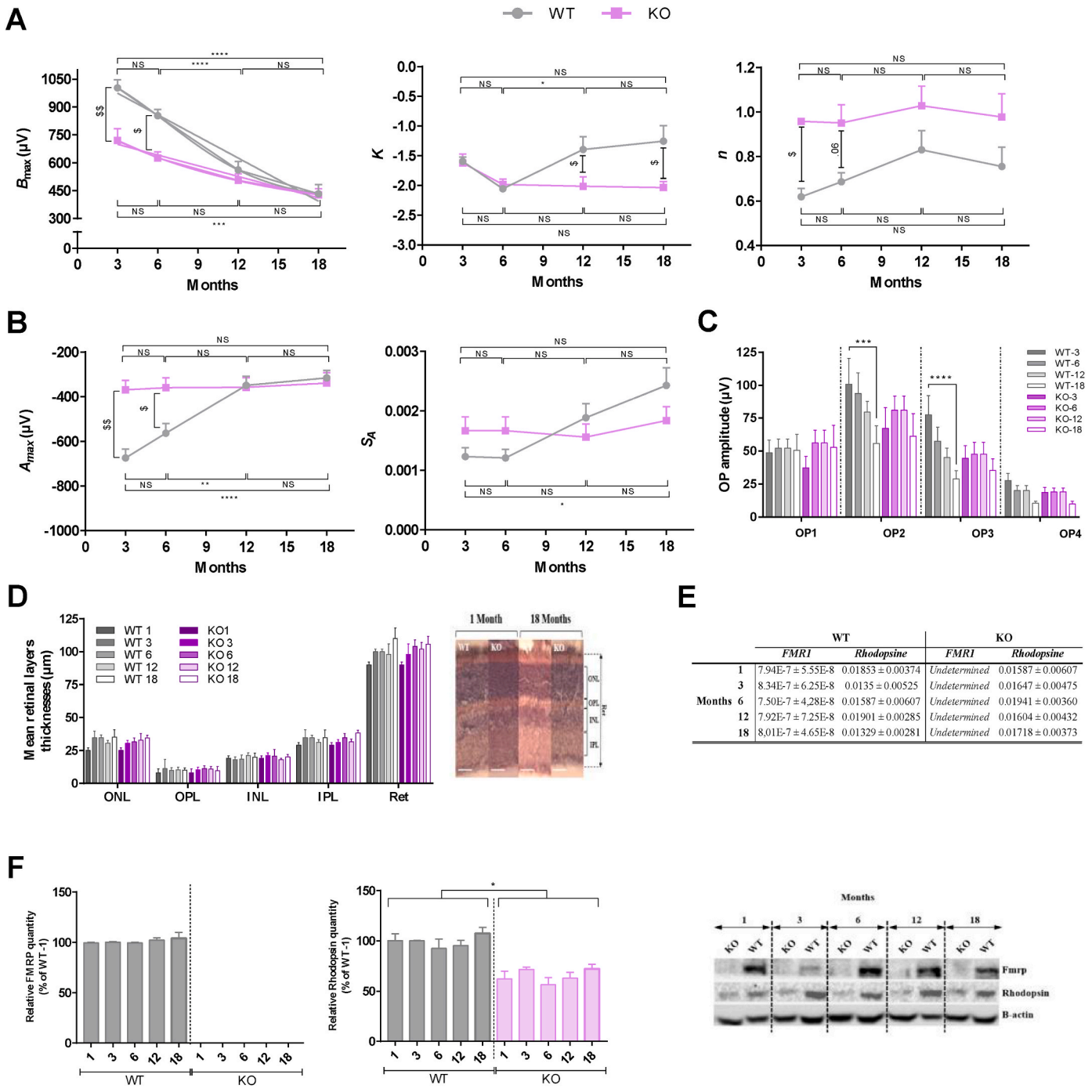
cases. In addition, few studies focusing on Dark-Adapted (DA) conditions have also shown alteration in the b-wave amplitude (Constable et al., 2016). In parallel of these clinical works, preclinical data demonstrated that FMRP is expressed in all retinal layers (Rossignol et al., 2014; Guimaraes-Souza et al., 2016; Zhang et al., 2020) and that its total absence in young and adult *Fmr1* KO mice (murine model of FXS) is associated to several protein deficits, neuronal immaturity, and thus to retinal functional alterations (Rossignol et al., 2014; Perche et al., 2018) as observed in human conditions (Perche et al., 2021). Moreover, retinal Fmrp content is dependent of light exposure, since studies showed higher levels of Fmrp RNA in retinas exposed to light when compared to DA retinas (Guimaraes-Souza et al., 2016). These results were lately confirmed by others on drosophila's retinas (Wang et al., 2016) and completed by outstanding observations documenting that Fmrp plays a key role in Rhodopsin cycle regulation in the retina (Wang et al., 2016). All these reports established a pivotal role of FMRP in retinal function, and in the overall visual pathway.

Aging is a multifactorial process that affects the entire organism by cumulative alterations (Tacutu et al., 2011). Visual function impairments that go along with aging are commonly observed, causing lower visual acuity, lower contrast sensitivity, and impaired dark adaptation (Rubin et al., 1997; Salvi et al., 2006; Pani, 2011; Stefanatos and Sanz, 2011; Blagosklonny, 2012; Calkins, 2013). In the aged retina, mislocalization of synapses, where the photoreceptor cell terminus makes contact with the dendritic terminus of bipolar and horizontal cells, was observed in both mice and humans (Liets et al., 2006; Eliasieh et al., 2007; Samuel et al., 2011). ERG analyses revealed that the amplitudes of rod- and cone-mediated responses are reduced in aged mice and humans (Gresh et al., 2003; Williams and Jacobs, 2007; Kolesnikov et al., 2010). These reports suggested that age-related changes were observed in both rod and cone photoreceptor systems. Interestingly, several recent reports linked FMRP cellular activity with oxidative stress regulation in several tissues including brain tissue (Feoktistova et al., 2011; Zhang et al., 2020; Taha et al., 2021). Indeed, under conditions of oxidative stress, Fmrp participates to the response to stress via protein translation in neurite (Dolzhanskaya et al., 2006) or is involved in free radical production and abnormal glutathione homeostasis (el Bekay et al., 2007; de Diego-Otero et al., 2009). At the cellular level, Fmrp depletion or low expression had been shown to sensitize cells to oxidative stress situation (Basu et al., 2022) by regulating the expression of Superoxide Dismutase (Bechara et al., 2009) or the mitochondrial activity (Shen et al., 2019). Furthermore, *Fmr1*$^{-/y}$ mice treated with antioxidant showed improvement in anxiety behaviour and learning deficits, highlighting the contribution of oxidative stress to clinical features associated with FXS (de Diego-Otero et al., 2009; Romero-Zerbo et al., 2009).

Based on these recent literatures, we raised the question about the effect of Fmrp absence in the aging retina of *Fmr1*$^{-/y}$ compared to their WT littermates. Indeed, up to now, only young or adult mice (<6 months) were investigated and the status of old animals can be questioned. Herein, we demonstrated that *Fmr1*$^{-/y}$ mice present few aging effect on retinal function compared to WT littermates since ERG a-wave amplitude as well as oscillatory potentials (OPs) amplitudes were not collapsed with age (12/18 months of age). Absence of Fmrp and its consequences seem to protect the retina against aging effect, raising a pivotal role of Fmrp in retinal aging process.

For our investigation we used *Fmr1*$^{-/y}$ male and their wild-type (WT) littermates (Consortium, 1994), generated by breeding heterozygous *Fmr1*$^{+/-}$ females with C57BL/6J background WT males as previously described (Rossignol et al., 2014). Animals were maintained under controlled temperature (22 °C) and humidity (55%) conditions with a 12:12 h dim light–dark cycle (25 lux, lights on at 7 a.m.). All experimental protocols received full review and approval by the regional animal care and use committee (Comité Régional d'Ethique à l'Expérimentation Animale – CREEA - CREEA—TSA-DM Therapie1100) prior to conducting the experiments. Regarding the assessments, ERGs were recorded using flash intensities ranging from −3.47 to +0.46 log cd s/m^2 as previously described by our team (Rossignol et al., 2014; Perche et al., 2018) on dark-adapted (DA) WT and *Fmr1*$^{-/y}$ mice littermates (n = 12 for each genotype) at 3, 6, 12 and 18-month of age. For ERG analysis, the leading edge of the a-wave obtained in response to high-intensity stimuli was analyzed with a modified form of the Lamb–Pugh model of rod phototransduction (Granit, 1933, Lamb and Pugh, 1992, Lamb and Pugh, 1992) equation: $P3 = \{1-\exp[-iS_A(t-td)^2]\}A_{max}$ (1) where P3 represents the massed response of the rod photoreceptors and is analogous to the PIII component of Granit (1933). The amplitude of P3 is expressed as a function of flash energy (i) and time (t) after flash onset. S_A is the gain of phototransduction, A_{max} is the maximum response, and td is a brief delay. The amplitude of the b-wave is calculated from the minimum of the a-wave to the maximum of the b-wave. Intensity–response function of the b-wave amplitude was fitted with the Naka–Rushton equation: $B/B_{max} = I^n/(I^n + K^n)$ (2) where I is the stimulus luminance of the flash (2.88 cd s.m^{-2}), B is the b-wave amplitude of ERG at I luminance, B_{max} is the asymptotic b-wave amplitude, K is the half-saturation constant corresponding to retinal sensitivity and n is a dimensionless constant controlling the slope of the function. The latency is the time interval between the stimulation and the peak of the b-wave or the a-wave. Oscillatory Potentials (OPs) are recorded by switching the amplifier to 100–300 Hz. Retinal histology and molecular investigation were performed as previously described by our team on DA mice aged of 1, 3, 6, 12, and 18 months (n = 8 WT and n = 8 *Fmr1*$^{-/y}$ at each age, for each technique) (Perche et al., 2007, 2018; Rossignol et al., 2014). SOD investigation was performed on n = 8 WT and n = 8 *Fmr1*$^{-/y}$ at each age, for each technique. The western-blot specific antibody (anti-SOD, 1:500) was purchased from AbCam (Paris, France) and the activity kit (Superoxide Dismutase, SOD, Activity Assay Kit, CS0009) was purchased from Sigma-Aldrich (Illkirch, France). For fatty acid composition, experiments were performed as described previously (Ardourel et al., 2021) using frozen DA retinas. Data analysis was performed using GraphPad Prism 7.00 and all results are expressed as mean ± SEM. Statistical comparisons among groups were conducted using one-Way ANOVA followed by the post-hoc Tukey test. Statistical significance was defined as $p < 0.05$ and significant differences between groups are noted by *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. Significant differences between ages among a genotype are noted by $^{\$}p < 0.05$; $^{\$\$}p < 0.01$; $^{\$\$\$}p < 0.001$; $^{\$\$\$\$}p < 0.0001$.

We first assessed whether retinal function changes with age by recording ERG b and a-waves in scotopic condition. In WT mice, the B_{max} progressively decreased by 53% ($p < 0.0001$) over time (from 3 to 18 months) with a major collapse between 6 and 12 months of age ($p < 0.0001$) (Fig. 1A). K absolute value was significantly decreased between 6 and 12 months of age whereas n tended to increase from 3 to 12 months (Fig. 1A). Regarding the photoreceptor's cell response to light, A_{max} was progressively reduced by 53% ($p < 0.0001$) over time (from 3 to 18 months) with a major collapse between 6 and 12 months of age ($p = 0.0095$) (Fig. 1B) whereas S_A significantly increased by 97% between 3- and 18-month-old mice (Fig. 1B). Oscillatory potential, especially OP2 and OP3, progressively decreased by 44–46% and 62 - 62% respectively from 3 to 18 months of age (Fig. 1C). Aging has no effect neither on a- and b-waves latencies nor on OPs latencies (data not shown). These first results demonstrate that physiologic aging of the WT retinas (C57BL/6J) are associated to negative consequences on retinal function in a gradual process but with a more important acceleration between 6 and 12 months. Interestingly our data are in total accordance with a recent report investigating the age effect on retinal function of the C57BL/6J strain and demonstrating that the a- and b-waves amplitudes was reduced over time (up to 2 years old) with a main decrease (around 40–50%) between Postnatal Day (PD) 60–180 and PD 365–544 (Ferdous et al., 2021). Ferdous et al. data are parallel to ours in terms of timing and strength of the age effect in WT littermates (C57BL/6J), but also with healthy human data (Weleber, 1981, Birch and Anderson, 1992) or other WT animal models (Li et al., 2001). Interestingly, all the WT electrophysiological changes were not associated with gross retinal



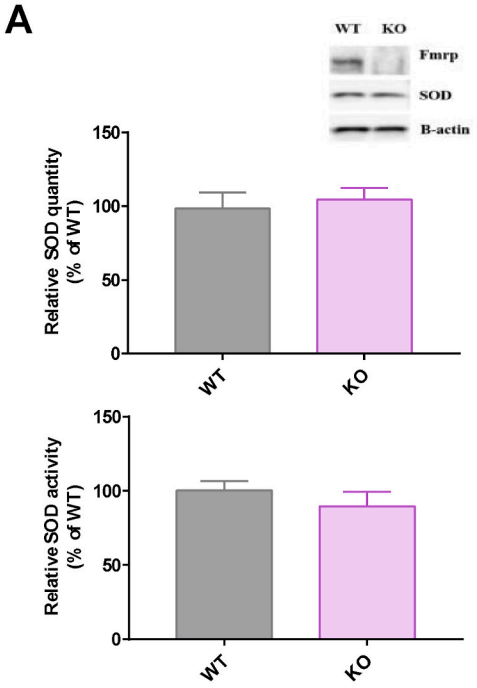
		WT		KO	
		FMR1	*Rhodopsine*	*FMR1*	*Rhodopsine*
Months	1	7.94E-7 ± 5.55E-8	0.01853 ± 0.00374	*Undetermined*	0.01587 ± 0.00607
	3	8.34E-7 ± 6.25E-8	0.0135 ± 0.00525	*Undetermined*	0.01647 ± 0.00475
	6	7.50E-7 ± 4,28E-8	0.01587 ± 0.00607	*Undetermined*	0.01941 ± 0.00360
	12	7.92E-7 ± 7.25E-8	0.01901 ± 0.00285	*Undetermined*	0.01604 ± 0.00432
	18	8,01E-7 ± 4.65E-8	0.01329 ± 0.00281	*Undetermined*	0.01718 ± 0.00373

Fig. 1. Retinal function, structure and protein content in WT and ***Fmr1***$^{-/y}$ (KO) littermates at 1, 3, 6, 12 and 18 months old. Retinal function was evaluated from 3 to 18 months by recording ElectroRetinoGrams (ERGs) (n = 12 WT and n = 12 *KO* for each age). (A) From the fitted b-wave sensitivity curve obtained by serial responses to increasing flash stimuli (−3.47 log(cd.s.m^{-2}) to 0.6 log(cd.s.m^{-2})) we calculated the saturated b-wave amplitude (B_{max}), the *K* parameter (intensity providing half saturation) and the *n* parameter (representing the b-wave sensitivity curves slope). (B) For each typical ERG obtained at a light intensity of −2.88 log (cd.s.m^{-2}), the decreasing part of the a-wave is fitted to calculate the extrapolated maximal a-wave amplitude (A_{max}) and S_A parameter reflecting the photoreceptor sensitivity. (C) OPs were recorded by using a band-pass ranging from 30 Hz to 300 Hz. For each OP, the amplitude was calculated from the baseline to the peak. Retinal structure was evaluated by histology from 1 to 18 months (n = 8 WT and n = 8 KO for each age). (D) ONL (Outer Nuclear Layer), OPL (Outer Plexiform Layer), INL (Inner Nuclear Layer), IPL (Inner Plexiform Layer) and Total Retina thickness were evaluated in both genotypes over the different ages. FMRP and Rhodospin content were evaluated from 1 to 18 months using RT-PCR and Western-blot (n = 8 WT and n = 8 KO for each age). (E) mRNA expressions are expressed as $2^{-\Delta Ct}$ values and normalized to 18S RNA internal control. (F) Fmrp and Rhodopsin protein expression amounts were normalized to β-actin expression. Three independent experiments were performed with similar results. A representative experiment is presented. Data are presented as Mean ± SEM. Significant differences between WT and KO are noted by *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. Significant differences between ages among a genotype are noted by $^{\$}$p < 0.05; $^{\$\$}$p < 0.01; $^{\$\$\$}$p < 0.001; $^{\$\$\$\$}$p < 0.0001.

structure modification since no variation of ONL (Outer Nuclear Layer), OPL (Outer Plexiform Layer), INL (Inner Nuclear Layer), IPL (Inner Plexiform Layer) or Total Retinal thicknesses was observed whatever the age of the mice (Fig. 1D). In addition, no modification of Fmrp and rhodopsin protein expressions was observed over time for WT (Fig. 1E and F). These data are in accordance with previous data demonstrating that aging effect on retinal function was not associated neither with any change in retinal histology, cellular density, nor with modification of total rhodopsin content (Curcio et al., 1993; Li et al., 2001; Harazny et al., 2009).

Regarding *Fmr1*$^{-/y}$ mice, we could expect to have a similar age-dependent kinetics on ERG or even a worse effect because of an additive effect of aging and the *Fmr1*$^{-/y}$ phenotype but starting from lower values since the absence of FMRP had been demonstrated to collapse the retinal function from early stage of development (Perche et al., 2018). Unexpectedly, in absence of Fmrp, no age-dependent effect was observed in ERG since K, n, A_{max}, S_A and OPs were stable over all experimental time points (Fig. 1A, B, 1C). Surprisingly in *Fmr1*$^{-/y}$, B_{max} was decreased between 3 and 18 months by 39% compared to the 53% in WT. Nevertheless, the aging kinetics was drastically slower in *Fmr1*$^{-/y}$ (slope: 19.18 ± 2.28) compared to WT (slope: 38.69 ± 4.96) retinas with a two-fold factor between both genotypes (Fig. 1A). Aging had no effect neither on a- and b-waves latencies, nor on OPs latencies in *Fmr1*$^{-/y}$ retinas (data not shown). Thus, the difference observed between WT and *Fmr1*$^{-/y}$ in ERG parameters at 3 and 6 months of age was reduced over time to reach a non-significant difference at 12 and 18 months-old animals excepted for K parameter. These evolutions over older ages are exclusively due to the age-related variation of electrophysiological response in WT mice. Similar retinal structure was observed in *Fmr1*$^{-/y}$ and WT whatever the age without any changes over time (Fig. 1D). In *Fmr1*$^{-/y}$ retinas, Rhodopsin protein content was stable over time, but always lower by 50% compared to WT animals (Fig. 1E and F). Overall, these data suggest that the absence of FMRP drastically reduces aging effect on retinal functionality raising the question of FMRP role in the

A



B

	WT		KO	
	Mean	SEM	Mean	SEM
C14:0	0,09	0,01	0,08	0,01
C15:0	0,07	0,00	0,07	0,01
DMA16:0	1,59	0,00	1,55	0,04
C16:0	17,53	0,42	16,41	0,48
C16:1n-9	0,25	0,01	0,23	0,01
C16:1n-7	0,33	0,02	0,27	0,01
C17:0	0,13	0,01	0,13	0,01
DMA18:0	2,11	0,01	2,19	0,02
DMA18:1n-9	0,23	0,00	0,24	0,01
DMA18:1n-7	0,19	0,01	0,18	0,01
C18:0	20,48	0,08	20,91	0,10
C18:1trans	0,18	0,01	0,18	0,01
C18:1n-9	8,93	0,10	9,11	0,06
C18:1n-7	2,36	0,04	2,23	0,03
C18:2n-6	1,23	0,04	1,17	0,02
C20:0	0,17	0,01	0,18	0,02
C20:1n-9	0,34	0,01	0,37	0,02
C18:3n-3	0,08	0,00	0,09	0,01
C20:2n-6	0,35	0,01	0,35	0,01
C20:3n-9	0,08	0,00	0,08	0,01
C22:0	0,16	0,01	0,20	0,02
C20:3n-6	0,64	0,01	0,59	0,02
C20:4n-7	8,70	0,07	8,91	0,10
C20:5n-3	0,28	0,01	0,32	0,01
C22:4n-6	1,01	0,01	1,01	0,03
C22:5n-6	0,18	0,01	0,17	0,01
C22:5n-3	0,17	0,01	0,08	0,02
C22:6n-3	31,59	0,47	31,99	0,46
Sum Saturated	38,63	0,38	37,98	0,45
Sum Mono-unsaturated	12,38	0,10	12,37	0,08
Sum Poly-unsaturated	44,87	0,41	45,49	0,48
Sum DMA	4,12	0,04	4,16	0,05
Sum Omega-6	12,11	0,09	12,20	0,12
Sum Omega-3	32,69	0,45	33,20	0,48
Omega-6 / Omega-3 Ratio	0,37	0,01	0,37	0,01
Cholesterol	9,30	0,50	10,30	0,40
Phospholipids	90,70	0,50	89,70	0,40

Fig. 2. (A) SOD and lipids status of the WT and *Fmr1*$^{-/y}$ (KO) littermates at 6 months old. SOD protein expression and activity were assessed at 6 months of age using Western-blot and enzymatic dosage (n = 8 WT and n = 8 KO for each age). SOD protein expression was normalized to β-actin expression. Three independent experiments were performed with similar results. A representative experiment is presented. **(B)** Lipid composition of whole retinas. Data are presented as Mean ± SEM.

aging process.

Aging had been associated to cumulative damage of reactive oxygen species (ROS) due to imbalance between defence mechanism and ROS production leading to consequences on electrophysiological function of neuronal tissue (Johnson et al., 1999) (Harazny et al., 2009). By its intrinsic function of light sensoring, the retina is particularly exposed to water photolysis and thus ROS production (Organisciak and Vaughan, 2010). Among retinal biochemical parameters influencing the sensitivity of the retina to ROS, membrane lipid composition is playing a major role. Indeed, ROS was shown to react with PolyUnsaturated Fatty Acids (PUFAs) and generate lipid peroxides (Apel and Hirt, 2004) which promotes aging processes (Yefimova et al., 2002; Hadziahmetovic et al., 2011; Hamano et al., 2021). In the disc membrane of retinal photoreceptors, the long-chain PUFA docosahexaenoic acid (DHA), particularly susceptible to ROS attacks, represents about 50% of the total fatty acid content of phospholipids (Johansson et al., 2015). This high concentrations in PUFA confers to the retina a high sensitivity to oxidation damage, and thus to lipid peroxidation (Zhao et al., 2021). Therefore, the reduced retinal aging in *Fmr1*$^{-/y}$ could be link to a change in oxidative stress sensitivity of the retina either through an increase in defence mechanism or a decrease in sensitising biomolecules. Therefore, we checked the status of one of the defence enzyme SOD as well as the lipid composition of whole retinas at 6 months of age. As shown on Fig. 2, no difference in SOD expression or activity was observed between genotypes, nor in whole retinal membrane fatty acid composition (Fig. 2A and B). Although further experiments are needed to better understand the origin of the *Fmr1*$^{-/y}$ phenotype, SOD status and membrane lipid composition did not seem to be at the origin of the protective effect of the absence of Fmrp against retinal aging. Interestingly, another major factor influencing susceptibility to oxidative stress is the amount of rhodopsin into the retina and its regeneration after bleaching. Indeed, it was shown that light induces retinal damage through ROS (Ranchon et al., 2001, 2003) and is depending on rhodopsin absorption (Grimm et al., 2000, 2001). Indeed, mouse strains with slow metabolic rhodopsin regeneration are more resistant to ROS than the ones with fast regeneration kinetics (Wenzel et al., 2001). These data suggested that a higher content of rhodopsin in WT could lead to cumulative light induce damage over time (Samardzija et al., 2019). Herein, *Fmr1*$^{-/y}$ retinas present a 50%-decrease in rhodopsin protein expression (Fig. 1E and F), associated to an outer segment destabilisation confirmed by Sa increase as already observed at younger age (Rossignol et al., 2014). During aging process of wild-type retinas, it had been shown that photoreceptors cell response (a-wave) was progressively decreased over time (Ferdous et al., 2021) and that no variation of rhodopsin content was described over ages (Curcio et al., 1993; Fulton et al., 1999). These studies suggest that light absorption via rhodopsin lead to oxidative stress accumulation in the retina over time and thus to the decreased retinal function performance. In our case, the stability of A_{max} parameters could be link to the FMRP-related decrease of Rhodopsin expression leading to a reduced light efficiency, to less light absorption, to less oxidative stress in the retina, and so to a lower impact of aging. Nevertheless, in *Fmr1*$^{-/y}$ retinas decreased rhodopsin content could not explain the lower impact of age on B_{max} or the stability of the OP2 and OP3 amplitude compared to WT response. It suggests that absence of FMRP is impacting the aging manifestations in the retina via others pathways. Since lack of FMRP is known to be related to deficits of several synaptic marker expression in the retina (i.e SYT1a, PSD95, GS etc …) (Rossignol et al., 2014; Perche et al., 2018; Ardourel et al., 2022), we could hypothesize a pivotal role of FMRP-related pathway in slowing down the aging processes. Our opinion is that FMRP absence have broadly consequences on the retinal function and retinal aging process.

Further experiments are needed to better understand the link between FMRP absence or deregulation and the aging processes. However, these experiments provide the first evidence of a reduced aging at least at retinal level in absence of FMRP. It confirms the essential role of light absorption and so light damage in retinal alteration over age, and present FMRP as an import actor of the processes. This pivotal role of Fmrp in the protection against retinal aging opens the way for the discovery of new preventive or therapeutic strategies in retinal aging.

Authorship contributions

OP and IRC conceptualized the study. AM, AP, CF, NA, FL and IRC conducted the experiments and MA, IRC, OP analyzed the data. OP, FL and MA wrote the manuscript. MA, FL, OP, SB, IRC, NA and CF discussed and reviewed the manuscript.

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Declaration of competing interest

Authors declare no conflict of interest.

Data availability

Data will be made available on request.

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