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Resveratrol improves episodic-like memory and motor coordination through modulating neuroinflammation in old rats

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ABSTRACT

Strategies focus on the use of molecules found in food derived from plants as polyphenols, arouse interest for combating aging. The polyphenol resveratrol, due to its antioxidants and anti-inflammatory properties, is increasingly studied, being still unknown their effects on episodic-like memory and motor coordination. This research evaluated the in vivo effect of chronic resveratrol treatment (20 mg/kg/day, i.p, 28 days) in episodic-like memory and motor coordination in non-pathological old rats, comparing with young and old vehicle-treated rats, using the novel object recognition and the rotarod tests. In combination with the analyses of the effect of resveratrol on the immunoreactivity of the main molecular proteins involved in neuroinflammation (SIRT1, SIRT1-75 kDa fragment, NF-κB acetylation) in the hippocampus of these rats. Results suggest a beneficial effect of resveratrol on episodic-like memory and in motor coordination through modulating neuroinflammation, acting on SIRT1 and NF-κB signaling pathway in the hippocampus of old rats.

1. Introduction

Resveratrol is a natural polyphenolic compound present in various plants, and therefore in plant-derived foods including grapes, berry fruits, olives, among others, which is quite famous for its association with several health benefits such as anti-obesity, cardioprotective, neuroprotective, antitumor, antidiabetic, antioxidants, and anti-aging effects (Zhang et al., 2021). The anti-aging and neuroprotective potential exerted by resveratrol are explained in part due to their natural antioxidant and anti-inflammatory properties, together with its ability to preserve the homeostasis and the neuronal survival by modulating autophagy (Stéphane Bastianetto et al., 2015). These properties become this molecule a potential protective agent, found in diet, against neuroinflammation. Neuroinflammation, is present in aging, and many neurological disorders, including neurodegenerative diseases (DiSabato et al., 2016), and is defined as the inflammatory response mediated by the production of cytokines, chemokines, reactive oxygen species, and secondary messengers in the brain or spinal cord. These mediators are produced by microglia and astrocytes, endothelial cells, and peripherally derived immune cells. There are immune, physiological, biochemical, cognitive, motor and psychological consequences of these neuroinflammatory responses, especially in aging, due to the reduction in the antioxidant and anti-inflammatory natural defenses (DiSabato et al., 2016). Therefore, the control of neuroinflammation is essential in the prevention of the cognitive impairments induced in the imbalances, caused by pathological conditions or by normal physiological processes as it is the aging. Regarding the first, there are evidences in animal models, that point to positive effects of resveratrol in case of several brain damages as for example in ischemic cerebral injury (Yu et al., 2021), depression (Ardianto et al., 2021), Alzheimer's disease (Wang et al., 2017; Wang et al., 2020), Parkinson's disease (Su et al., 2021), or aggressive glioblastoma (Arabzadeh et al., 2021), among others brain disturbances (Feng et al., 2016; Lopez et al., 2015; Song et al., 2016; Finnell et al., 2017). Besides, in humans several clinical trials related to this topic have shown positive effects in cardiovascular health (Banez et al., 2020) and brain (Berman et al., 2017; Tomé-Carneiro et al., 2013; Omraninava et al., 2021). In non-pathological aging process, it was

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Nomenclature

List of abbreviations

ANOVA	analysis of variance
BDNF	brain-derived neurotrophic factor
CREB	cAMP response element-binding protein
CRM1	chromosomal maintenance 1 or exportin 1
FLSirT1	full-length SirT1
Glu230	amino acid glutamate 230
IL	interleukin
i.p	intraperitoneally
MAO-A	monoamine oxidase enzyme A
NA	noradrenaline
NF-κB	nuclear factor kappa B
SIRT1	sirtuin 1
5-HT	serotonin
ROS	reactive oxygen species
STAC	sirtuin activating compound
TNF-α	tumoral necrosis factor alfa
TPH	tryptophan hydroxylase enzyme
TH	tyrosine hydroxylase enzyme

demonstrated that a chronic resveratrol treatment improves spatial working memory in old rats, recovering also the monoamines deficit produced by aging, through the increase in the activity of the limiting enzymes of the synthesis of noradrenaline (NA) and serotonin (5-HT) in the hippocampus (Sarubbo et al., 2015). Accordingly, similar effects together with an improvement of the motor coordination were observed after chronic treatments with other polyphenols, such as silymarin, quercetin and naringenin or with a diet enriched with polyphenols (Ramis et al., 2021a) or also with the α-tocopherol (Ramis et al., 2016), being also related to the increased concentration of monoamines and the ability to protect the brain in front of the inflammatory state present in aging (Ramis et al., 2021b; Nasso et al., 2021).

Since now and at molecular level, the cause of these positive effects has been linked to the action of polyphenols on the sirtuin 1 (SIRT1), since resveratrol has been considered as a sirtuin-activating compound (STAC) (Nasso et al., 2021). SIRT1 is the mammalian homolog of yeast Silent information regulator 2 (Sir2) and a NAD-dependent class III histone deacetylase, whose role is crucial in cellular processes; being an essential enzyme in the protection against aging, since is precisely implicated in the maintenance of neural systems and behavior during normal aging (Hubbard et al., 2013; Herskovits & Guarente, 2014). Interestingly, it was described in a model of articular chondrocytes, that SIRT1 has a cell survival mechanism in front of stress signals, which is activated by means of the fragmentation of the SIRT1 native form. Precisely, the increase of inflammatory factors like the tumoral necrosis factor alfa (TNF-α) triggers an intracellular response, which includes a caspase 8 dependent lysosomal permeability, that increases the levels of Cathepsin B in the cell cytoplasm and nucleus. Active Cathepsin B cleaves nuclear full-length SIRT1 (FLSirT1; 110 kDa) to generate an inactive stable 75 kDa SIRT1 fragment, which is exported to the cytoplasm via the Chromosomal Maintenance 1 (CRM1). Once located in the cytoplasm, this SIRT1 fragment interacts with the Cytochrome *C* on the mitochondrial membrane to block downstream apoptosome assembly, so that avoiding apoptosis (Oppenheimer et al., 2012). Despite the importance of this process, there are no studies addressing this process in brain tissue during normal aging. Regarding this, it is noteworthy that, during aging in rats, SIRT1 expression decreases in cognitive key regions, such as the hippocampus and the cerebral cortex progressively (Sidorova-Darmos et al., 2014; Sarubbo et al., 2018b), probably contributing to the detriment of cell defense mechanisms against chronic inflammation, and leading to cognitive and motor deficits (Ng et al., 2015). Furthermore, this deficit is accompanied by several factors, like an increased number of activated astrocytes and microglia, with elevated basal levels of pro-inflammatory cytokines and reduced the levels of anti-inflammatory cytokines, especially in the hippocampus (Campuzano et al., 2009; Lovatel et al., 2013; Flowers et al., 2015). In fact, in aging there is a proinflammatory characteristic state known as inflammaging, triggered by the brain's deregulated response to the accumulated damage due to aging (Franceschi et al., 2000). Precisely, SIRT1 has been reported as a modulator of the inflammaging (Sarubbo et al., 2018; Jiao & Gong, 2020), by deacetylating intracellular targets (Michan & Sinclair, 2007); for example, the nuclear factor kappa B (NF-κB) signaling through deacetylation of the RelA/p65 subunit at lysine 310 (Yeung et al., 2004; Chen & Greene, 2003; Kauppinen et al., 2013). In fact, the attention on the modulation of NF-κB has been centered in their acetylation, because since now it is described that aging does not affect the levels of the NF-κB native (Adler et al., 2007) or phosphorylated form (Meberg et al., 1996). It has also been suggested that the modulation of NF-κB mediates cytokine expression in microglial cells leading to neuroinflammation (Jana et al., 2002; Nakajima et al., 2006; Tian et al., 2016).

Therefore, the aim of this study is to analyze together the effect of resveratrol on SIRT1, their fragmentation and NF-κB signaling pathway in the hippocampus of non-pathological aged rats, in parallel with the evaluation of their relation with specific, brain functions, as it is the episodic-like memory, not described since now, and the motor coordination. The hypothesis proposed is that food components, feasible to introduce or increase in the diet, as it is resveratrol, can modulate the neuroinflammation, specifically by means of their effect on SIRT1, which in turn modulates NF-kB signaling pathway, also favoring cell survival by the formation of a SIRT1 75 kDa fragment; possessing altogether the ability to preserve episodic memory, and motor coordination.

2. Materials and method

2.1. Subjects

Young (3 months) and old (20 months), male Wistar rats (Harlan, Spain) were used. Animal procedures were in accordance with the European Convention for the Protection of Animals used for Scientific Purposes (Directive 2010/63/UE). The Ethics Committee for Animal Experimentation of the University of Balearic Islands revised and approved the protocols (2014/05/AEXP). All efforts were made to minimize the number of animals used and their suffering. The animals were housed in methacrylate cages, following the specifications of the Spanish regulation (RD 53/2013, 1st February, Establishing the Basic Rules Applicable to the Protection of Animals Used for Experimental and Other Scientific Purposes, Including Teaching, 2013), with wood shavings (Ultrasorb, Panlab s.l., Barcelona, Spain) under controlled conditions of temperature (21 °C), humidity (70%) and light/dark cycle (light period: 08:00 to 20:00 h), and with free access to a standard diet (Panlab A04) and tap water. For several days, prior to starting the test, and to reduce the stress, animals were handled.

2.2. Experimental groups and treatments

Old rats were chronically treated with resveratrol (20 mg/kg/day, i.p, 28 days, n = 6) or vehicle (corn oil, 1 ml/kg/day, i.p, 28 days, n = 6), following previous studies (Sarubbo et al., 2015; Sarubbo et al., 2018b; Ramis et al., 2021b; Ramis et al., 2020). Young rats, that received vehicle, were included for aging effects comparison (n = 8). The animals were slaughtered by decapitation 24 h after the last dose of treatment. The brains were rapidly removed and the hippocampus dissected on ice, frozen in liquid nitrogen and then stored at −80 °C until determinations.

2.3. Study of the episodic-like memory through the novel object recognition test

The novel object recognition test is a procedure to evaluate episodic-like memory in rodents (Ennaceur & Delacour, 1988; Ennaceur, 2010). This methodology assesses the natural ability of rodents to explore the novel object as their natural propensity to the novelty, without rewards, external motivation or punishment (Antunes & Biala, 2012). The test was performed in the open field apparatus, as described previously (Ramis et al., 2013). The procedure consists of three phases: habituation, familiarization, and test phase. In the habituation phase, each animal is freely allowed to explore the open field in the absence of objects for 10 min, daily, during four consecutive days. On day 5, each animal was placed in the device and allowed to explore for 1 min for re-habituation. During the familiarization phase, two identical objects were placed 16 cm away from the walls, and the animal was placed in the center of the apparatus and allowed to explore both objects until 10 min had elapsed. At this phase the animals show no preference for an object. Object exploration was considered when the animal's nose or mouth was in contact with the object; while running around the object, sitting or climbing on it, was not recorded as exploration. After 10 min, in the test phase, the animals were placed back in the open field with one object familiar (same as the previous phase) and one novel object (different from the previous phase). The time spent exploring each object was recorded until 10 min had elapsed. The objects were always placed in the same location. In addition, the objects had no natural significance for rats, made of plastic in different shapes, and had never been associated with reinforcement. The objects and the apparatus were cleaned with ethanol solution (95%) between trials. Discrimination index allows discrimination between the novel and familiar objects (Antunes & Biala, 2012), and was calculated as the difference in the exploration time for novel versus familiar items relative to the time spend exploring both objects (total objects investigation).

2.4. Study of motor coordination through rotarod test

To assess the motor ability and balance of the animals, the rotarod treadmill device (Panlab®, Barcelona, Spain) was used. Four training sessions, one per day, were done on the rotating wheel in order to familiarize rats with the equipment. For the test phase, rats were placed on the rotarod to record the latency to fall in an acceleration mode (from 4 to 40 rpm during a period of 60 s). Five trials were performed by each rat, separated by a 10 min for the physical recovery of the animals, and competency was defined as the average of time recorded. The apparatus was cleaned with ethanol solution (95%) between trials and rats.

2.5. Study of SIRT1 and NF-kB signaling pathway in the hippocampus

After thawing, each hippocampus was homogenized (1:15, wt/vol) in cold 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA; 2 % SDS and protease inhibitor cocktail (Pierce). Homogenates were sonicated (3 × 5 s) and mixed with an equal volume of electrophoresis loading buffer (50 mM Tris-HCl, pH 6.8, 1.5 % SDS, 10 % glycerol, 2.5 % ß-mercaptoethanol and 0.1 % bromophenol blue), which was then denatured at 95 °C for 5 min and stored at −20 °C until use. Protein concentration in total homogenates was determined with bicinchoninic acid (BCA) assay kit (Pierce Biotechnology, Rockford, IL, USA). Hippocampal samples (20 µg) were resolved by gel electrophoresis on 10% sodium dodecyl sulphate SDS-polyacrilamide minigels (Bio-Rad Laboratories, Hercules, CA, USA), transferred to nitrocellulose membranes (Bio-Rad Laboratories), and blocked at room temperature for 1 h with phosphate-buffered saline solution, containing 5% non-fat dry milk, 0.5% bovine serum albumin and 0.2% Tween 20 (blocking solution). Then, the membranes were incubated overnight at 4 °C in blocking solution containing the appropriate primary antibody (see Table 1, for details). The secondary antibody, horseradish peroxidase-linked anti-rabbit or anti-mouse IgG, was incubated at 1:5,000 dilution in the blocking solution at room temperature for 2 h. Immunoreactivity of target proteins was detected with the Enhanced Chemiluminiscence (ECL) Western Blot Detection system (Amersham International, Buckinghamshire, UK). The chemiluminescence bans were digitalized with GeneGnome XRQ (Syngene, USA) and analyzed with the public domain software ImageJ (Rasband, 1997–2016). Drugs and reagents were purchased from Sigma chemical company (USA).

2.6. Statistics

A descriptive analysis of all variables was done, together with the Shapiro-Wilk normality test. Variables were expressed as mean values ± standard error of the mean (SEM). For inference the following parametric tests were performed: Student's two-tailed *t*-test, one and two-way analysis of variance (ANOVA) followed by the Bonferroni's post hoc test for multiple comparison. In relevant cases one or two-way ANOVA with repeated measures was used to evaluate evolution through the treatment within each group. The level of significance was set up at $p \leq 0.05$. All data were analyzed and represented with GraphPad PrismTM, version 6.0 (GraphPad Software, Inc., San Diego, CA, USA).

3. Results

3.1. Effects of chronic resveratrol treatment on the episodic-like memory

For assessing the effect of chronic resveratrol treatment on the episodic-like memory of old rats and to corroborated the effect of age, the object recognition test was performed. Fig. 1 shows the results of the effects of the chronic resveratrol treatment in old rats (20 mg/kg/day, i.p) when compared with young and old vehicle-treated rats (corn oil 1 ml/kg/day, i.p). First, and to then analyzed properly the effect of resveratrol, there was checked the effect of the age by observing the difference in time of exploration of the new object respect the familiar one in young and old vehicle groups at basal time. In this regard, a significant difference in the time of exploration of the new object respect the familiar one was found and only detected in the group of young rats ($p < 0.001$, *t*-test), indicating that old vehicle animals did not remember the familiar object, being the outcome of the deterioration of the episodic-like memory in old animals. At middle and end treatment periods, in young rats and old-vehicle rats the same effect that in basal measure was found, with the same statistic significance in young rats ($p < 0.001$, *t*-test). In the case of resveratrol-treated rats, it was observed a significance difference in the time spend exploring the new object respect the familiar one, at the end of the treatment ($p < 0.01$, *t*-test), indicating an improvement of episodic-like memory due to the chronic resveratrol treatment ($p < 0.01$, *t*-test) **(**Fig. 1**a)**. Accordingly, the discrimination index between objects confirmed the significant loss of episodic-like memory in old vehicle rats respect to young ones, from the basal up to the end of treatment; and that at the end of the treatment period resveratrol administration, in old rats, induced a significant improvement, respect to the old vehicle rats ($F(2, 20) = 6.43$, $p < 0.05$, two-way ANOVA followed by Bonferroni post hoc test for multiple comparisons) **(**Fig. 1**b)**.

3.2. Effects of chronic resveratrol treatment on the motor coordination

For assessing the effect of chronic resveratrol treatment on motor coordination of old rats and to corroborate the effect of age results, rotarod test was performed. Fig. 2 shows the performance time in the rotarod device of young rats, old control rats, and old chronic resveratrol treated rats (20 mg/kg/day, i.p) at basal time, middle and end of the treatment. The effect of aging was corroborated, since from the basal measure young rats remain in the apparatus an average of 77% more than the old rats ($F(2, 19) = 39.83$, $p < 0.001$, one-way ANOVA followed

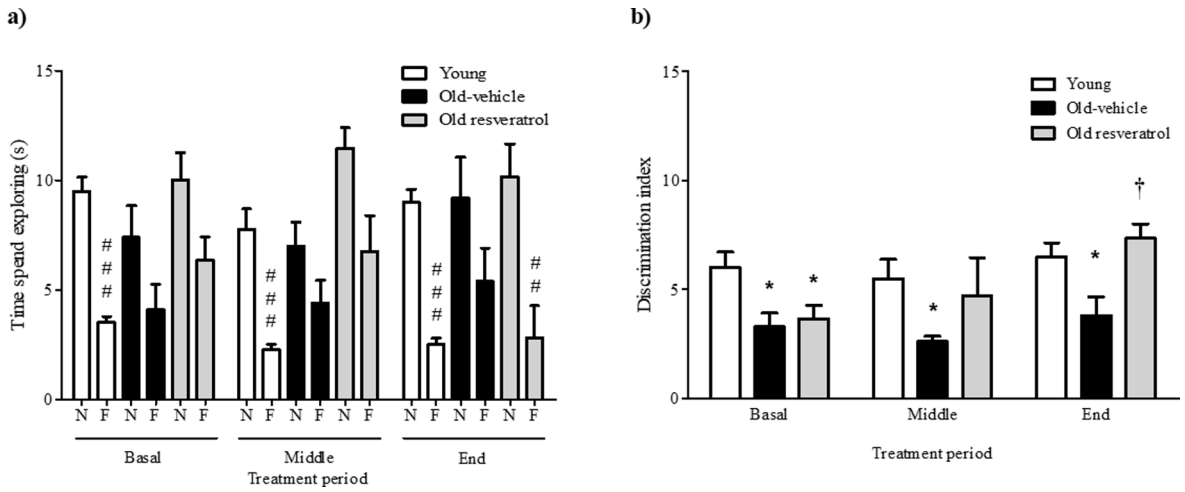


Fig. 1. Effects of chronic resveratrol treatment (20 mg/kg/day, i.p., for 28 days; n = 6) on the novel object recognition test in old rats at basal time, middle and the end of treatments. Results are compared with young (n = 8) and old (n = 6) vehicle-treated rats. (a) Time exploring the novel (N) and familiar (F) objects. Columns represent mean ± standard error of the mean (SEM) of the time spend exploring the objects. $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ (*t*-test analyses) when compared time spend exploring the familiar object (F) with the corresponding time exploring the novel object (N), at each observed time and for each experimental group. (b) Discrimination index, i.e. ratio of the difference in the time exploring the novel and the familiar objects with respect to the total time of exploration. Columns represent mean ± SEM of the individual discrimination indexes. $^{*}p < 0.05$ when compared with the young vehicle-treated group and $^{\dagger}p < 0.05$ when compared with the old vehicle treated group; two-way ANOVA followed by Bonferroni post hoc test for multiple comparisons ($F(2, 20) = 6.43$, $p < 0.05$).

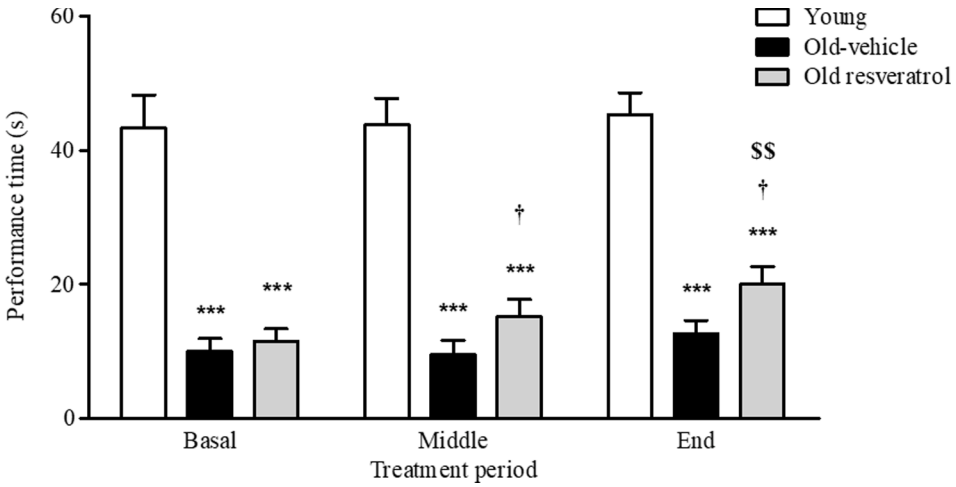


Fig. 2. Effects of chronic resveratrol treatment (20 mg/kg/day, i.p., for 28 days; n = 6) on the rotarod test in old rats at basal time, middle and end of the treatment. Results are compared with young (n = 8) and old (n = 6) vehicle-treated rats. Columns represent mean ± SEM of the performance time on the apparatus. $^{***}p < 0.001$ when compared with the young vehicle-treated group; $^{\dagger}p < 0.05$ when compared with the old vehicle treated group (one-way ANOVA followed by Bonferroni post hoc test); $\$\$p < 0.01$ when compared with their same group (old resveratrol) at the beginning of the treatment (one-way ANOVA repeated measures followed by Bonferroni post hoc test). Two-way ANOVA, followed by Bonferroni post hoc test, confirmed that the differences were due to the factor groups of animals ($F(2, 36) = 113.2$, $p < 0.001$).

by Bonferroni). After 15 days of treatment, the animals treated with resveratrol increased their performance time by 58.65%, compared to the old vehicle group ($F(2, 19) = 39.46$, $p < 0.05$, one-way ANOVA followed by Bonferroni). At the end of the treatment, this difference was maintained, since the animals treated with resveratrol endured 58.72% more than the old control group on the wheel ($F(2, 19) = 41.14$, $p < 0.05$, one-way ANOVA followed by Bonferroni). Additionally, at the end of the treatment, an improvement was observed within the group of animals treated with resveratrol compared to their basal measure, increasing the time spent on the wheel by 74.12% ($F(2, 18) = 7.56$, $p < 0.01$, one-way ANOVA repeated measures followed by Bonferroni). Finally, throughout the treatment, there was a tendency to increase the performance time, due to the training factor in the three groups studied, which was not significant in the case of young animals and old controls (young: $F(2, 24) = 0.06$, $p = 0.93$; old: $F(2, 15) = 0.56$, $p = 0.59$, one-way ANOVA repeated measures followed by Bonferroni). Two-way ANOVA followed by Bonferroni post hoc test, confirmed that the differences were due to the factor groups of animals ($F(2, 36) = 113.2$, $p < 0.001$).

3.3. Effects of chronic resveratrol treatment on SIRT1 and NF-κB in the hippocampus

The immunoreactivity of SIRT1 (110 kDa), their fragment (75 kDa) and the different forms of NF-κB were studied in order to assess the effects of aging and the chronic resveratrol treatment on the leading proteins involve in brain aging and inflammation, in the hippocampus of old rats.

Fig. 3 shows the effect of aging and resveratrol treatment on the hippocampal immunoreactivity levels of **a)** SIRT1 (110 kDa), **b)** 75 kDa fragment, and **c)** SIRT1 fragmentation index (ratio SIRT1 75 kDa/ SIRT1 110 kDa). First, it was corroborated the effect of aging. In the hippocampus of old vehicle-treated rats a significant reduction in the immunoreactivity of SIRT1 (110 kDa) (36%, $p < 0.001$) (Fig. 3**a**), and in their 75 kDa fragment (14%, $p < 0.01$) was observed when compared with young rats (Fig. 3**b**). On the other hand, it was also detected an effect of the chronic resveratrol treatment, since an increased in the immunoreactivity of SIRT1 (110 kDa) (41% increase, $p < 0.001$) (Fig. 3**a**) and in their 75 kDa fragment (20% increase, $p < 0.01$) (Fig. 3**b**) in the hippocampus of old resveratrol-treated rats were observed when compared

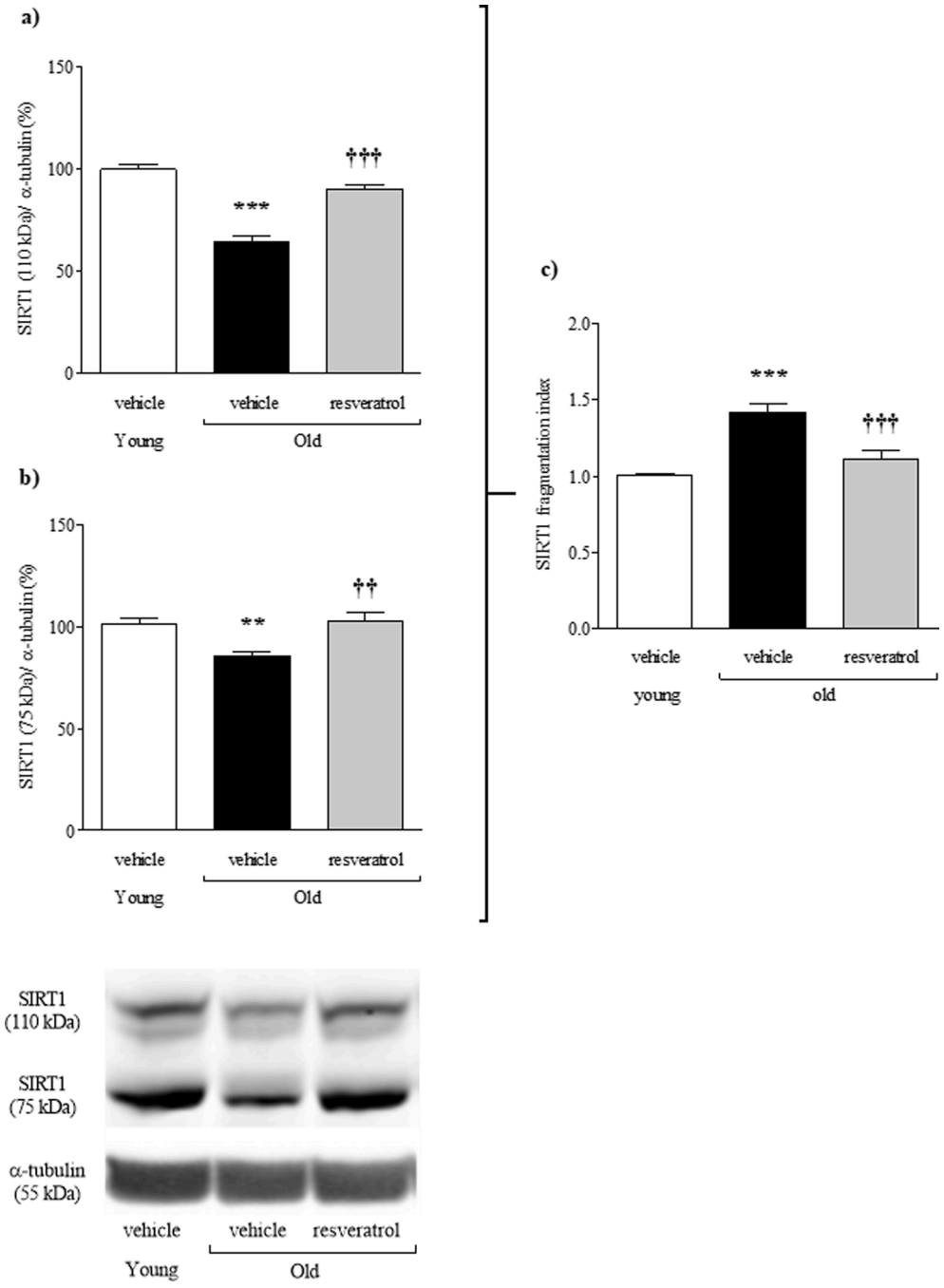
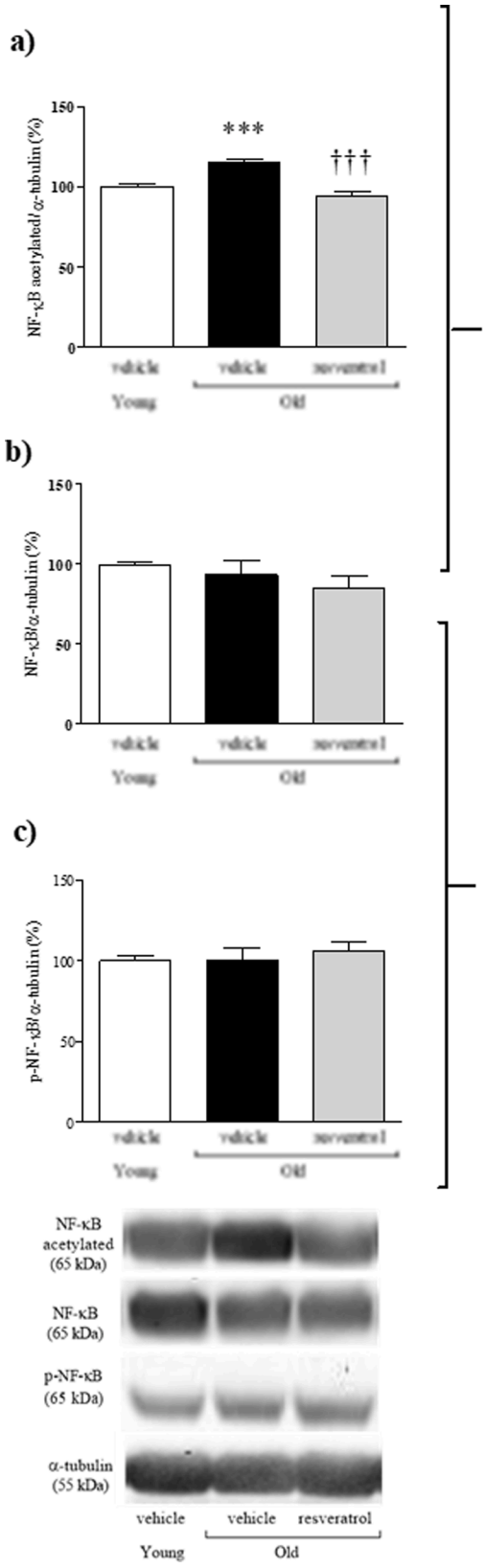


Fig. 3. Effects of chronic resveratrol treatment (20 mg/kg/day, i.p., for 28 days; n = 6) on (a) SIRT1 (110 kDa), (b) SIRT1 (75 kDa), and (c) SIRT1 fragmentation index (ratio SIRT1 75 kDa/ SIRT1 110 kDa) in the hippocampus of old rats. Results are compared with young (n = 8) and old (n = 6) vehicle treated rats. Columns represent mean ± SEM. One-way ANOVA followed by Bonferroni post hoc test detected significant differences between groups in SIRT1 (110 kDa) ($F(2,12) = 53.28$, $p < 0.001$), SIRT1 (75 kDa) ($F(2,12) = 11.01$, $p < 0.01$) and the SIRT1 fragmentation index ($F(2,20) = 28.31$, $p < 0.001$). **p < 0.01, ***p < 0.001 when compared with young vehicle-treated rats; ††p < 0.01, †††p < 0.001 when compared with old vehicle-treated rats. Bottom panels: representative immunoblots of SIRT1 (110 kDa), SIRT1 (75 kDa) and α-tubulin (loading control) of each set of experiments. The molecular masses of target proteins were estimated from referenced standards.

with old vehicle-treated rats. The statistical analyses with one-way ANOVA followed by Bonferroni post hoc test detected significant differences between groups for SIRT1 (110 kDa) ($F(2, 12) = 53.28$, $p < 0.001$) (Fig. 3**a**) and SIRT1 (75 kDa) ($F(2, 12) = 11.01$, $p < 0.01$) (Fig. 3**b**). Accordingly, the SIRT1 fragmentation index in old vehicle-treated group was higher than in young rats (Fig. 3**c**). Conversely, the SIRT1 fragmentation index after resveratrol treatment dropped to values close to those of young rats ($F(2, 20) = 28.31$, $p < 0.001$, one-way ANOVA follow by Bonferroni post hoc test). These results indicate that in old rats there was a substantial decrease in SIRT1 native form, accompanied by a higher rate of fragmentation. This effect was reversed by chronic resveratrol treatment.

Concomitantly, it was analyzed the NF-κB signaling pathway, due to the fact that it has been pointed out as the pathway most involved in the triggering of inflammation, being also modulated by SIRT1. Fig. 4 shows the effect of aging and chronic resveratrol treatment on the hippocampal immunoreactivity levels of **a)** acetylated NF-κB, **b)** NF-κB native form, **c)** p-NF-κB, **d)** NF-κB acetylation ratio (ratio NF-κB acetylated/ NF-κB native form) and **e)** p-NF-κB ratio (ratio p-NF-κB/ NF-κB native form). First, it was observed the effect of aging, by detecting in the hippocampus of old vehicle-treated rats the following changes when compared with young rats: a significant increase in the immunoreactivity of acetylated form of NF-κB (15%, $p < 0.01$) (Fig. 4**a**), without alterations in the levels of NF-κB native (Fig. 4**b**) and phosphorylated form of the protein (Fig. 4**c**). It was accompanied by a significant increase in the NF-κB acetylation ratio (Fig. 4**d**) and no differences in the p-NF-κB ratio (Fig. 4**e**). On the contrary, after chronic resveratrol treatment (20 mg/kg/day, i.p), it was observed a reduction in the acetylated form of NF-κB (18% reduction, $p < 0.001$) when compared with the old-vehicle group, with levels tended to resemble those of the



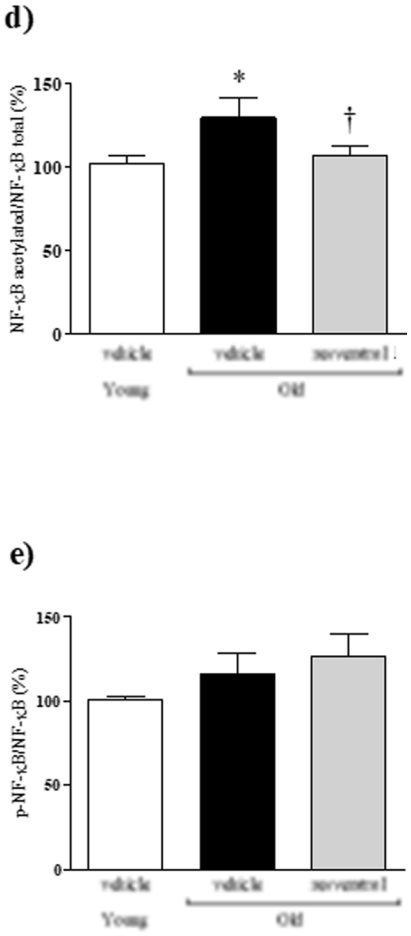


Fig. 4. Effects of chronic resveratrol treatment (20 mg/kg/day, i.p., for 28 days; n = 6) on (a) NF-κB acetylated, (b) NF-κB native form, (c) p-NF-κB, (d) NF-κB acetylation ratio (NF-κB acetylated/ NF-κB native form) and (e) p-NF-κB ratio (p-NF-κB/ NF-κB native form) in the hippocampus of old rats. Results are compared with young (n = 8) and old (n = 6) vehicle-treated rats. Columns represent mean ± SEM. One-way ANOVA follow by the Bonferroni post hoc test detected significant differences between groups for NF-κB acetylated ($F(2,12) = 20.30$, $p < 0.001$), but not for NF-κB native form ($F(2,12) = 1.026$, $p = 0.38$) and p-NF-κB ($F(2,20) = 2.53$, $p = 0.1$). Regarding, ratios there were found significant differences in NF-κB acetylation ratio ($F(2,20) = 4.45$, $p < 0.05$) but not in p-NF-κB ratio ($F(2, 12) = 2.53$, $p = 0.1$). *$p < 0.05$, ***$p < 0.001$ when compared with young vehicle-treated; †$p < 0.05$, †††$p < 0.001$ and when compared with old vehicle-treated rats. Bottom panels: representative immunoblots of NF-κB acetylated (acetyl K310), NF-κB native form, p-NF-κB and α-tubulin (loading control) of each set of experiments. The molecular masses of target proteins were estimated from referenced standards.

young rats (Fig. 4**a).** This is accompanied by no changes in the levels of the NF-κB native (Fig. 4**b)** or phosphorylated form respect young and old-vehicle treated rats (Fig. 4**c).** The statistical analyses with one-way ANOVA follow by Bonferroni post hoc test detected significant differences between groups only for the acetylated form of NF-κB ($F(2,12) = 20.30$, $p < 0.001$) (Fig. 4**a**), not for NF-kB native ($F(2,12) = 1.026$, $p = 0.38$) (Fig. 4 **b)** or phosphorylated form ($F(2,20) = 2.53$, $p = 0.1$) (Fig. 4**c).** Consistent with all these results, in old resveratrol treated rats the NF-κB acetylation ratio decreased respect old vehicle rats, achieving levels close to those of young rats ($F(2,20) = 4.45$, $p < 0.05$, one-way

ANOVA follow by Bonferroni post hoc test) (Fig. 4**d).** No changes in the compared groups of animals were detected for the NF-κB phosphorylated ratio ($F(2,12) = 2.53$, $p > 0.05$, one-way ANOVA follow by Bonferroni post hoc test) (Fig. 4**e).**

4. Discussion

The demographic increase of elderly people and the concomitant escalation in the frequency of age-related neurological deterioration symptoms justify the search for treatments that may help to prevent their causes. This is an issue of great importance because it has a direct impact on the health of the population and on health systems. Therefore, the results in basic research are essential in addressing this topic. Feasible strategies to combat neurodegeneration are being studied more and more. Nutrition, including the nutrients of diet and the use of natural molecules present in diet is among these strategies. Polyphenols and specially resveratrol has attracted attention due to its beneficial properties as an antioxidant and anti-inflammatory molecule, which is in turn easily found in many consumed fruit and vegetables (Zhang et al., 2021). Regarding, this topic the present work has corroborated the negative effects of aging in the loss of episodic-like memory and motor coordination, while has demonstrated the resveratrol's ability to counteract these aging symptoms, by modulating SIRT1 protein and NF-κB signaling pathway in hippocampus of aged rats.

The worsening of cognition and motor coordination in aging is cause in part due to the accumulation in the brain of molecules derived from an oxidative stress situation such as the reactive oxygen species (ROS) and derived from an inflammatory status, which produces among other cytokines and interleukins (IL), which altogether generates neuroinflammation (DiSabato et al., 2016), and if this happens during aging it is called as inflammaging (Franceschi et al., 2000). The result of the neuroinflammation is the brain damage that generates cognitive and motor disfunction, due to the lack of a sufficient physiological response to counteract it, because of the neural wear caused over the years (Wang et al., 2020; Barrientos et al., 2015). Accordingly, the present work corroborates the loss in episodic-like memory and motor coordination in non-pathological aging in rats. The hippocampus is one of the brain regions more implicated in memory (Thompson & Kim, 1996; Nyberg, 2017), being also involved in the spatial navigation (Eichenbaum, 2017) and motor sequence memory (Albouy et al., 2008), altogether affecting movement. Although the leading brain areas involve in motor control are cerebellum, motor cortex, supplementary motor area, and pre-motor cortex; both hippocampus and striatum, but also prefrontal cortex, seem to be important in consolidation of motor sequence memory (Gann et al., 2021). All these regions are needed for the integration and execution of the motor responses generated (Kerr et al., 2017). Besides, hippocampus, together with the associative cortices, are essential during movement perturbations; where, after an unexpected disturbance, previous plan must be inhibit and a new movement plan must be created to compensate (Kerr et al., 2017). These movement perturbation compensations are essential for an appropriate performance in rotarod test. Besides, the whole hippocampus is one of the most worn brain areas due to the processes triggered in aging, suffering from a pro-inflammatory environment in which basal levels of pro-inflammatory cytokines, like TNFα and IL-1ß, are elevated, whereas those of the anti-inflammatory cytokines, and IL like IL-4 and IL-10 are reduced (Flowers et al., 2015; Lovatel et al., 2013; Marques Orellana et al., 2015). Consequently, the neutralization of the responsible mechanisms of damage in hippocampus, constitutes a clear target for the prevention of aging symptoms.

For counteracting neuroinflammation there have been pointed out two mechanisms as the most related and close to the modulation of neutralization of oxidative stress and inflammation. These are the modulation of SIRT1 protein and their connection with the NF-κB transcription factor. NF-κB is held quiescent in the cytoplasm when in complex with IκBα. In response to a proinflammatory stimulus (e.g. TNFα, or IL-1) via Toll-like receptors or cytokine receptors, IκBα is phosphorylated by the protein kinase IKK and subject to ubiquitin-dependent proteasomal degradation, thereby allowing NF-κB to translocate to the nucleus and activate the transcription of a cascade of proinflammatory cytokines and chemokines to induce inflammatory responses (Goh & Midwood, 2012; Viatour et al., 2005). Activation of NF-κB-regulated gene expression is also modulated by post-transcriptional modifications, such as methylation phosphorylation or acetylation, which can be altered upon stimulation (Viatour et al., 2005; Yang et al., 2009; Ghizzoni et al., 2011). Of particular interest is the acetylation of p65/RelA, a subunit of the heterodimeric NF-κB protein, which can either potentiate or diminish NF-κB signaling depending on the particular acetylated lysine residue (Chen et al., 2002). Among the seven lysines (lysine 122, 123, 218, 221, 310, 314, 315) that are acetylated, acetylation of lysine 310 is critical for full activation of NF-κB transcription potential (Chen et al., 2002), and this can be deacetylated by SIRT1 (Yeung et al., 2004). Therefore, SIRT1 protein, which is a cell survival enzyme, could prevent inflammation by the deacetylation of the NF-κB protein (Yang et al., 2012). The acetylated form of NF-κB has been described to be increased in hippocampus during aging (Sarubbo et al., 2018b; Flowers et al., 2015; Franceschi et al., 2000). In connection with this information and in line with another related work, the results of this study corroborated that the immunoreactivity levels of SIRT1 in hippocampus of old rats are reduced compared to young rats, and interestingly, this is accompanied with an increase in the levels of the acetylated form of NF-kB. These results glimpsed that in aging it is produced a lack of the well described inhibitory effect of SIRT1 against the NF-κB signaling, through the deacetylation of the p65 component of the NF-κB complex (Chen et al., 2005; Yeung et al., 2004; Kauppinen et al., 2013). Altogether agreeing with other authors that have confirmed that the down-regulation of SIRT1 due to aging contributes to the stimulation of the NF-κB-induced inflammatory responses in the brain (Salminen et al., 2013), and with the described key role of SIRT1 as a mediator of cognitive decline in normal aging (Cho et al., 2015). No changes in NF-kB native or phosphorylated form were detected in the present work. These results coincide with previous studies that indicate that aging did not cause alterations in the levels of the different components of NF-kB in the hippocampus of old rats (Korhonen et al., 1997), and in mice (Adler et al., 2007); describing that the hippocampus is the brain region with the highest levels of NF-kB (Meberg et al., 1996). Regarding phosphorylation, these results also showed an effect similar to the observed in lymphocytes of old rats, where it was detected that age did not produce an increase in phosphorylation of NF-kB (Meberg et al., 1996). Therefore, aging does not seem to modulate or activate NF-kB by the mechanism of phosphorylation. However, this does not exclude that aging can enhance other NF-kB modulating mechanisms.

Due to the above information, SIRT1 constitutes an ideal concrete target in the search of anti-neuroinflammation and antiaging treatments. In this sense, some SIRT1 activators compounds were studied, like the SRT3657, which can increase the expression and activity of SIRT1 in mouse hippocampus, delaying the onset of neurodegeneration, by preserving structural and functional synaptic plasticity (Gräff & Tsai, 2013). Polyphenols by their antioxidant and anti-inflammatory capacity are described also as good candidates for the modulation of the previous mentioned targets and therefore for the prevention of brain functionality decline. For example, silymarin, quercetin, naringenin (Sarubbo et al., 2018b), chatechin and a diet enriched with polyphenols have been described as modulators of the SIRT1 and the activation of NF-κB signaling pathway, with a positive impact in the recovery of working memory (Sarubbo et al., 2018b; Ramis et al., 2015), episodic-like memory (Burke et al., 2010; Oliveros et al., 2016) and motor coordination (Ramis et al., 2015). Quercetin, catechin and naringenin are flavonoids with anti-inflammatory and anti-aging properties (for more information see the review (Calis et al., 2019). Regarding resveratrol, it was demonstrated an improvement of working memory performance in aged rats (Witte et al., 2014), by recovering the monoamines levels in hippocampus and striatum (Sarubbo et al., 2018b). In accordance, the

results of the present work revealed a new result, which is that in the hippocampus of aged rats, resveratrol also modulated SIRT1, increasing their immunoreactivity levels, which is accompanied by a decrease in the acetylated form of NF-κB in the hippocampus of the same rats, while the levels of phosphorylated NF-κB did not show differences. It was also observed that after chronic resveratrol treatment, the levels of SIRT1 and acetylated NF-κB were similar to the corresponding young vehicle-treated rats. In this line, the present work demonstrated that the NF-κB acetylated ratio in hippocampus was increased due to aging, while reverted by the chronic resveratrol treatment. Similar results have been described in experiments using models of experimental traumatic brain injury (Feng et al., 2016), lipopolysaccharide-induced neuro-inflammation (Ge et al., 2015) or after global cerebral ischemia (Simão et al., 2012). These findings, are in agreement with other studies using in vitro models, that also shown that resveratrol decreases acetylated NF-κB by a SIRT1 dependent mechanism (Zhu et al., 2011). These suggests that this acetylation of the NF-κB signaling pathway is the target of the SIRT1 protein. Other studies reinforces this affirmation since SIRT1 activators suppressed the inflammatory responses through promotion of p65 deacetylation and the inhibition of NF-κB Activity (Yang et al., 2012). The consequence is that resveratrol could attenuate the inflammatory response by inhibiting NF-κB signaling pathway. Thus, the decrease of acetylated NF-κB as a consequence of resveratrol treatment, could contribute to the neuroprotection by reducing the pro-inflammatory state in the hippocampus of aged rats. In line with this result, it has been described that the age-related increases in the expression of inflammatory cytokines (e.g. TNFα, IL-6 and IL-1ß) in mice were attenuated by resveratrol treatment in peripheral tissues like liver, muscle, adipose and heart; being consistent with the reduction in NF-κB-responsive genes expression in the same tissues (Ghowsi et al., 2018). Similarly, it has also been described that resveratrol treatment attenuated the release of inflammatory cytokines (TNFα and IL-1β) in spinal cord injury (Xu et al., 2018; Liu et al., 2011) and traumatic brain injury of rat models (Feng et al., 2016).

Interestingly, the protective action of SIRT1 is not limited to the modulation of NF-kB acetylation, since although poorly studied it has been described that in front of stress molecules, such as TNFα and IL-1β, SIRT1 could avoid apoptosis trough the activation of their fragmentation, generating an inactive but stable 75 kDa fragment. The first mention to this mechanism was when studying inflammation in osteoarthritic chondrocytes (Dvir-Ginzberg et al., 2011; Oppenheimer et al., 2012) and then in draining of lymph node cells (Gardner et al., 2015), describing that in these cells after the exposition to TNFα, it is triggered an intracellular response, which includes a release of cathepsin B that, in turn, induces the inactivation and cleavage of the SIRT1. Later, other studies described that the cleavage of SIRT1 protein mediated by cathepsin also occurs in different situations, concretely, after stress in endothelial progenitor cells promoting senescence (Chen et al., 2012), high-fat diet in adipose tissue by the inflammation-activated caspase-1, providing a link between dietary stress and predisposition to metabolic dysfunction (Chattopadhyay et al., 2018) or after the liberation of toxic bacterial substances in infection in macrophages (Meka et al., 2021). Furthermore, these previous published results confirmed that the fragment of 75 kDa belongs to the protein SIRT1. The proteolytic cleavage of the full-length 110 kDa SIRT1 generates a stable and enzymatically inactive form of 75 kDa, which joins the apoptosome avoiding apoptosis and favoring cell survival (Oppenheimer et al., 2012). Regarding to this aspect of SIRT1, when analyzing the SIRT1 levels in the current work, two immunoreactive bands appeared in the western blot membranes, one at 110 kDa corresponding to the native SIRT1 protein and the other at 75 kDa which was modulated by the different experimental conditions. It was observed a reduction of SIRT1 (110 kDa) in aging accompanied by the reduction of the levels of the 75 kDa fragment, which presented the same N-terminal polyclonal antibody than in the previous mentioned studies (see coincidence in the Table 1 and in the supplementary material of the following articles (Dvir-Ginzberg et al., 2011; Gardner et al., 2015; Oppenheimer et al., 2012), so confirming the same 75 kDa fragment of SIRT1. It should be taken into account that the modulation of the two forms is differential (Dvir-Ginzberg et al., 2011; Oppenheimer et al., 2012); in fact, there is a greater decrease in the native protein than in the 75 kDa fragment during aging. This determines that the fragmentation index (SIRT1-75 kDa/SIRT1-110 kDa) was higher in old rats than in young ones. As it has been described, this fragment is related to inflammatory processes (Dvir-Ginzberg et al., 2011) and a marker of aging (Kumar et al., 2018; Oppenheimer et al., 2012; Hubbard et al., 2013), so that these results would be consistent with the presence of a pro-inflammatory state during aging, where cells could possibly be protected by the mechanism of fragmenting SIRT1, preserving the 75 kDa fragment. Similarly, an increase in the SIRT1 cleavage and therefore in the SIRT1 fragmentation index in the case of cartilage degeneration due to osteoarthritis were described (Batshon et al., 2019).This result is important in cognitive involved regions as the hippocampus. The results of this work also demonstrated an effect of resveratrol in the fragmentation of SIRT1, since the levels of the 75 kDa fragment of old rats treated with resveratrol were similar to those of young rats. However, in this case, the fragmentation index is lower than in old rats, indicating less fragmentation of the SIRT1 protein after the treatments. This could be related to the fact, also mentioned in other studies (Deng et al., 2019), that polyphenols could reduce the inflammatory state of cells, determining a decrease in SIRT1 fragmentation. This could indicate that the SIRT1 modulation by resveratrol may be secondary to a reduction effect of the general inflammatory state, which in turn contributes to reducing the inflammatory response mediated by NF-κB.

Table 1
Primary antibodies used for the detection and quantification of target proteins in the rat hippocampus.

Protein	Antigen	Host	Catalog	Batch	Company
SIRT1	Mouse SIRT1 (1-131 residues)	Rabbit	07-131	2,428,681	Millipore
NF-κB p65 (acetyl K310)	Human NF-kB p65 (peptide containing acetyl K310)	Rabbit	ab52175	GR45081-2S	Abcam
NF-κB p65	Human NF-κB p65 (C-terminal residues)	Rabbit	sc-372	L1610	Santa Cruz
pNF-κB p65 (Ser 275)	Human NF-κB p65 (peptide containing p-Ser 275)	Rabbit	sc-101749	C3012	Santa Cruz
α-tubulin	Sperm axonemes from sea urchin (clone B-5-1-2)	Mouse	T5168	051K4820	Sigma

Therefore, the results obtained in the present work indicate that resveratrol exhibit their beneficial properties through a set of mechanisms, including the potential to modulate the triggering of neuro-inflammation associated with aging, by reducing the acetylation of NF-κB which in turn could be due to the rise in SIRT1 levels in key regions for cognitive processes such as the hippocampus. The increase in SIRT1 implies not only a decrease in neuroinflammation but also a relation to the regulation of the functionality of cognitive processes, since SIRT1 regulates the expression of neurotrophins involved in the morphology and functionality of synapses, therefore regulating synaptic plasticity, and adult hippocampal neurogenesis (Kodali et al., 2015) which affects cognitive functions (Gao et al., 2010; Zocchi & Sassone-Corsi, 2012; Ng et al., 2015). This is accompanied by the antioxidant effects of polyphenols in brain (Oppenheimer et al., 2012; S Bastianetto et al., 2000), since they prevent the oxidation of enzymes such as tryptophan hydroxylase enzyme (TPH) and the tyrosine hydroxylase enzyme (TH); and

the inhibition of the monoamine oxidase enzyme A (MAO-A) enzyme, which together favors the increase in the synthesis and accumulation of monoamines (Sarubbo et al., 2015). For example, resveratrol had anti-oxidant activity and enhanced activation of SIRT1 in a diabetic aged female rat model (Akgun-Unal et al., 2023); and in a rat model of renal ischemia, resveratrol treatment normalized malondialdehyde and glutathione, which are anti-oxidant enzymes, counteracting lipid oxidation (Baltaci et al., 2019). Moreover, it has been demonstrated that resveratrol enhanced the cholinergic system and brain-derived neurotrophic factor (BDNF) and cAMP response element-binding protein (CREB) signaling pathways in the prefrontal cortex in Alzheimer's disease mouse model. This can also improve physical strength (Chung et al., 2016). Although it cannot be ruled out other effects described after chronic resveratrol treatments involve in the contribution of the cognitive and motor improvement observed here, it can be postulated that the modulation of SIRT1 and the NF-kB transcription factor would be part of this improvement.

One limitation of this kind of study is the difficulty to transfer these results to humans, because there are some questions that should be addressed, as for example regarding the extrapolation of the doses used in animals to humans. In this case, it is needed a conversion factor that takes body surface area into account (Reagan-Shaw et al., 2008). Thus, the dose of polyphenols in human equivalent to the 20 mg/kg administered in rats is 3.24 mg/kg; which would mean 194.4 mg of these polyphenols for a person weighing 60 kg. To obtain it from the diet, we would have to consider the concentration of resveratrol present in the different types of food, and also the amount that we would ingest if these foods are mixed. Considering that generally the concentration of polyphenols in foods is not high, probably great amounts of foods enriched with polyphenols are needed to achieve these levels. However, these quantities could be achieved by the orally administration of pills with the extract for example of resveratrol. In this sense, clinical trials should be necessary to assess the kinetics and bioavailability of these kind of compounds in humans.

Therefore, in this field and for combating neuroinflammation and aging through the strategy of the use of the properties of polyphenols presented in the diet, future investigations are required, since some questions are still unanswered. For instance, if the effects described are due to an indirect mechanism of action of resveratrol on SIRT1, or to a direct action of this polyphenol on this enzyme, as it occurs in the in vitro experiments described in (Gertz et al., 2012; Hubbard et al., 2013). In this regard, another point to be answered is if the protective effect on sirtuin is linked to the modulation of inflammation. They demonstrated that sirtuin activating compounds (STACs), such as resveratrol, regulate the activity of SIRT1, through an allosteric activation by their binding to the amino acid Glu230 present at the N-terminal end of SIRT1. However, the action of STACs on SIRT1 in vivo is still unknown. Therefore, it would be interesting to determine whether the behavioral and biochemical effects of resveratrol treatment, observed in this work, are due to a direct mechanism on the SIRT1 enzyme or to effects on other molecules that activate SIRT1. Thus, the knowledge of the mechanism of action of polyphenols would help in the design of new drugs and more specific and effective anti-aging therapies.

5. Conclusion

Results demonstrate the positive effect in episodic-like memory and in motor coordination of resveratrol, a polyphenol found in plant-derived food. The molecular analysis presented suggests that these cognitive and motor effects could be related, in part, through modulating SIRT1 protein, in their both native and fragmented form, and also the acetylated form of NF-κB signaling pathway, in the hippocampus of aged rats. Indicating that, resveratrol in old rats may exhibit their beneficial properties through a set of mechanisms, including the potential for reducing the overactivation of neuroinflammation associated with aging, decreasing the acetylation level of NF-kB by increasing the levels of SIRT1, in the hippocampus, a key region for cognitive processes.

Conflict of Interest: The authors declare that they have no conflicts of interest. They were informed and consent the manuscript publication. The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethic statement: The Ethics Committee for Animal Experimentation of the University of Balearic Islands revised and approved the protocols (2014/05/AEXP).

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CRediT authorship contribution statement

Sarubbo F: Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Ramis MR:** Investigation. **Tejada S:** Writing – review & editing. **Jimenez M:** Writing – review & editing. **Esteban S:** Conceptualization, Methodology, Validation, Formal analysis, Resources, Data curation, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Miralles A:** Conceptualization, Methodology, Validation, Formal analysis, Resources, Data curation, Supervision, Project administration. **Moranta D:** Methodology, Validation, Formal analysis, Resources, Data curation, Writing – original draft, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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