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Title:

Neurons from senescence-accelerated SAMP8 mice are protected against frailty by the sirtuin 1 promoting agents melatonin and resveratrol

Authors and affiliation:

R Cristòfol¹, D Porquet², R Corpas¹, A Coto-Montes³, J Serret¹, A Camins², M Pallàs², C Sanfeliu¹.

1. Institut d'Investigacions Biomèdiques de Barcelona (IIBB), CSIC, IDIBAPS, Barcelona, Spain.
2. Unitat de Farmacologia i Farmacognòsia, Facultat de Farmàcia, Institut de Biomedicina (IBUB), and Centros de Investigación Biomédica en Red de Enfermedades Neurodegenerativas (CIBERNED), Universitat de Barcelona, Barcelona, Spain.
3. Departamento de Morfología y Biología Celular, Facultad de Medicina, Universidad de Oviedo, Oviedo, Spain.

Running title:

Melatonin and resveratrol protect SAMP8 neurons

Corresponding author:

Coral Sanfeliu
IIBB-CSIC, IDIBAPS
c/Rosselló 161, 6th floor
08036 Barcelona
Spain
Tel: +34 933638338
Fax: +34 933638301
E-mail: coral.sanfeliu@iibb.csic.es

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Abstract

The senescence-accelerated prone 8 (SAMP8) mouse strain shows early cognitive loss that mimics the deterioration of learning and memory in the elderly, and is widely used as an animal model of aging. SAMP8 mouse brain suffers oxidative stress, as well as tau- and amyloid-related pathology. Mitochondrial dysfunction and the subsequent increase in cellular oxidative stress are central to the aging processes of the organism. Here, we examined the mitochondrial status of neocortical neurons cultured from SAMP8 and senescence-accelerated-resistant (SAMR1) mice. SAMP8 mouse mitochondria showed a reduced membrane potential and higher vulnerability to inhibitors and uncouplers than SAMR1 mitochondria. DL-buthionine-[S,R]-sulfoximine (BSO) caused greater oxidative damage in neurons from SAMP8 mice than in those from SAMR1 mice. This increased vulnerability, indicative of frailty-associated senescence, was protected by the anti-aging agents melatonin and resveratrol. The sirtuin 1 inhibitor, sirtinol, demonstrated that the neuroprotection against BSO was partially mediated by increased sirtuin 1 expression. Melatonin, like resveratrol, enhanced sirtuin 1 expression in neuron cultures of SAMR1 and SAMP8 mice. Therefore, a deficiency in the neuroprotection and longevity of the sirtuin 1 pathway in SAMP8 neurons may contribute to the early age-related brain damage in these mice. This supports the therapeutic use of sirtuin 1-enhancing agents against age-related nerve cell dysfunction and brain frailty.

Introduction

The physiological process of aging involves a progressive cognitive loss caused by deteriorating brain function, which includes a decrease in learning and memory skills and slower responses to intellectual stimuli. However, many people maintain their cognitive and intellectual abilities up to an advanced age. Indeed, normal brain aging and pathological aging, such as sporadic Alzheimer's disease (AD), appear to be distinct [1]. Data gathered from diverse studies confirm that AD is not merely an advanced aging process [2]. There are various mechanisms that trigger the change from the natural process into a pathological one in AD. One being related to cellular oxidative stress [3,4]. Oxidative stress and specifically mitochondrial oxidative stress is deeply involved in age-related deleterious changes. The free radical theory of aging by Harman [5] proposed the process is mediated by accumulated macromolecular damage through reactions involving free radicals. This theory was reformulated by Miquel and coworkers [6] into the mitochondrial theory of aging, which, although it has much experimental support, has not been universally vindicated. It proposes that aging is caused by free radical damage to the mitochondria of post-mitotic cells, which have a high rate of oxygen consumption and generate reactive oxygen species (ROS) that cause oxidative stress by overwhelming the antioxidant cellular defense [7]. Therefore, as well as being the source of energy for the cell, mitochondria are also a major site of oxidative damage. Indeed, in the aging mammalian brain, mitochondria show a decline in the respiration rate and an accumulation of oxidized molecules [8]. Thus, correct mitochondrial function might determine brain cognition, whole body resilience and health during aging.

A number of healthy lifestyles and anti-aging therapies reduce or delay cognitive decline and the risk of dementia [9,10]. Antioxidant food, caloric restriction and physical exercise, all converge to improve cell physiology and homeostatic functions. These healthy lifestyles boost neuroprotective pathways that improve brain function. For instance, we have recently reported that voluntary physical exercise improves cognitive and non-cognitive behaviors, brain redox homeostasis, neurotrophic status and synaptic function in a mouse model of AD [11]. One neuroprotective pathway that has recently drawn much attention is mediated by the protein sirtuin 1 (SIRT1 gene) [12]. Sirtuins are a family of nicotinamide adenine dinucleotide (NAD)-dependent deacetylases (class III histone deacetylases) that were named after the founding member, the *Saccharomyces cerevisiae* silent information regulator 2 (sir2) protein. Sirtuins are involved in responses to stress (such as heat or starvation) and are considered longevity genes [13]. The human ortholog of sir2 is sirtuin 1. Pharmacological modulation of SIRT1 may be of potential interest in managing neurodegenerative diseases such as AD [14]. The possibility of using dietary activators of SIRT1 such as resveratrol [15] or melatonin [16] makes sirtuin 1 a promising anti-aging target.

Experimental aging models are useful in uncovering the molecular mechanisms of aging and testing preventive treatments for age-related diseases. Cell cultures can be used to analyze mitochondrial function and changes in expression of specific proteins involved in key survival pathways, as well as testing the influence of different cell environments. For instance, cultured fibroblasts have recently been proposed as a model for comparative biogerontological studies because they maintain key characteristics

of the animal species from which they arise [17]. Cellular models of brain aging are useful for studying neurodegenerative mechanisms and testing anti-aging agents. Cultured brain astrocytes aged *in vitro* (through maintaining in culture for long periods of up to 3 months) show characteristics of senescence such as replicative senescence, oxidative stress and inflammation [18]. However, astrocyte cultures from the senescence-accelerated prone 8 (SAMP8) mouse strain reproduce traits of oxidative stress, inflammation and proteomic alterations in conventional culture conditions [19,20]. SAMP8 mice are widely used as a model of age-related cognitive loss and brain neurodegeneration [21,22]. We found that astrocytes from both SAMP8 mice and a long-term culture of a conventional mouse strain had a reduced neuroprotective capacity, thus demonstrating that aged astrocytes exacerbate neuronal injury in age-related neurodegeneration [18,19]. Neuron cultures from SAMP8 mice can be used to model aging neurons and determine the mechanisms of age-related brain disturbances. In previous studies, we did not see a reduced survival of SAMP8 neurons *in vitro* compared to senescence-accelerated-resistant strain 1 (SAMR1) mouse neurons [19]. Indeed, neuronal loss occurs in advanced stages of AD, but only to a limited extent in aging [23]. Nevertheless, proteomic analysis of cultured SAMP8 neurons [20] shows abnormal expression of proteins associated with mitochondria and other pathways altered in the tissues of aged, AD or SAMP8 brain [24,25].

In this study, we examined the mitochondrial status of neurons cultured from SAMP8 and SAMR1 mouse embryos to show that SAMP8 neurons model age-related dysfunctional neurons *in vitro*. Mitochondria are one of melatonin main targets [26]. Its recently discovered effects on mitochondrial biogenesis mechanisms and its potent antioxidant action make melatonin a promising protective agent against age-related mitochondrial decline. Therefore, we also analyzed the neuroprotective effects of melatonin and another anti-aging agent, resveratrol, as well as their underlying mechanisms involving the sirtuin 1 pathway. Resveratrol was chosen as a reference agent because of its established effects on mitochondrial function preservation, which mimick some of the molecular and functional effects of dietary restriction. [27].

Materials and Methods

Neuron cultures

Neuron cultures were obtained from SAMR1 and SAMP8 mouse E17 embryos. Mice were bred in the University of Barcelona Animal House (UB, Barcelona, Spain). First breeding pairs were obtained from the Council for SAM Research, Kyoto, Japan, through Harlan (Barcelona, Spain). All experimental procedures were approved by the local animal experimentation ethics committee (CEEa, UB). The culture procedure is described elsewhere [28]. Briefly, pregnant mice were decapitated and embryos were quickly removed in aseptic conditions. The neocortex was dissected out in Krebs buffer, cut into small dices, trypsinized and mechanically triturated into a single cell suspension. The cell suspension was centrifuged and resuspended in Dulbecco's modified Eagle's medium (DMEM) (Biochrom, Berlin, Germany) supplemented with 0.2 mM glutamine, 100 mU/L insulin B, 7 μ M p-aminobenzoic acid and 10% fetal bovine serum (Gibco-BRL, Invitrogen, Paisley, UK). Cells were seeded at a density of 3 x

$10^5/\text{cm}^2$ in 96-well plates and 25-cm^2 T-flasks (NUNC, Roskilde, Denmark) precoated with poly-D-lysine, and maintained in a humidified 37°C and 5% CO_2 incubator. $10\text{ }\mu\text{M}$ cytosine arabinoside was added after 2 days *in vitro* (DIV) to avoid proliferation of non-neuronal cells. Neuron cultures were used 7 to 8 DIV. Test agents were directly added to the culture medium in the plates or flasks from concentrated working solutions. All reagents used in the study were from Sigma (St. Louis, MO, USA), unless otherwise stated.

Mitochondrial membrane potential

Changes in mitochondrial membrane potential were measured with rhodamine 123 (Molecular Probes, Leiden, the Netherlands). Cultures in 96-well plates were washed twice with HEPES buffered saline solution (HBSS) and incubated with $13\text{ }\mu\text{M}$ rhodamine 123 at 37°C for 1 h. The cells were washed again to discard non-loaded rhodamine 123 and fluorescence was determined at 507-nm excitation/529-nm emission in a fluorescence plate reader (Spectramax Gemini XS, Molecular Devices, Wokingham, UK). Hydrogen peroxide treatment was performed by adding the agent immediately after rhodamine 123. Treatment with uncouplers and inhibitors was performed for 1 h after rhodamine 123 incubation. Results were calculated as fluorescence units per μg of protein. Proteins per well were determined by the Bradford method after digestion with 2N NaOH.

Mitochondrial mass

MitoTracker Green FM (mitotracker, Molecular Probes) was used to estimate the mitochondrial mass content in the neuron cultures. Cultures in 96-well plates were washed twice in HBSS and incubated with 50 nM mitotracker at 37°C for 15 min. Fluorescence was determined at 490-nm excitation/516-nm emission. Next, protein concentration per well were determined and the results were expressed as fluorescence units per μg of protein.

Reactive oxygen species generation

Intracellular generation of ROS was determined with dihydroethidium (Molecular Probes). Dihydroethidium oxidation to ethidium measures superoxide anion, a cellular ROS that is mainly generated in the mitochondria. Cultures in 96-well plates were washed with HBSS and loaded with $4.8\text{ }\mu\text{M}$ dihydroethidium. Basal ethidium fluorescence was measured at 485-nm excitation/590-nm emission after 10 min of loading. Incubation with the probe alone or with added hydrogen peroxide was then performed for 1 h at 37°C and then, final measurement recorded. Proteins per well were determined and the results were expressed as fluorescence units per μg of protein.

Protein damage

Oxidative protein damage was analyzed by determining carbonylated protein levels, as described elsewhere [29]. Cultures in T-flasks were washed with cold phosphate buffered saline (PBS), pelleted and frozen at -80°C until analysis. Chromogen 2,4-dinitrophenylhydrazine reacts with the carbonyl groups of damaged proteins. Protein carbonyls were determined at 366 nm.

Glutathione content

Reduced glutathione (GSH) content was measured by staining the neuron cultures in 96-well plates with the specific fluorescent probe monochlorobimane (mBCl). Fluorescence was determined after 30 min of incubation with 40 μ M mBCl at 360-nm excitation / 460-nm emission. Effects of the GSH-depleting agent DL-buthionine-[S,R]-sulfoximine (BSO) were analyzed by measuring GSH after a 24-h exposure. Proteins per well were determined and results were expressed as fluorescence units per μ g of protein.

Survival measurement

Culture viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay. Cultures in 96-well plates were loaded with 0.5 mg/mL MTT to detect any decrease in cell metabolic activity. The assay was performed following standard procedures.

Neuron survival results were confirmed in selected experiments by sequential staining of: (i) dead cells with propidium iodide; (ii) neurons with anti-NeuN antibody; and (iii) all cells with bisbenzamide, as previously described [19]. Results were expressed as the number of living neurons per microscopic field.

Western blot analysis

Expression levels of sirtuin 1 and its substrate acetylated p53 were determined in neuron cultures after a 2-h and 24-h exposure to BSO, melatonin and resveratrol. Western blotting was performed as previously described [19]. Briefly, cultures in T-flasks were washed with cold PBS, lysed with RIPA buffer containing a protease inhibitor cocktail and frozen at -20°C until analysis. 20 μ g of denatured protein extract were electrophoresed by SDS-PAGE (10 %) and transferred onto a polyvinylidene difluoride membrane. This was incubated overnight at 4°C with primary antibodies against: sirtuin 1 (1:1000; 110 kDa, Millipore, Temecula, CA), acetylated p53 (1:1000; ~53 kDa, acetylated at Lys379, Cell Signaling, Danvers, MA), p53 (1:1000; 53 kDa, Santa Cruz Biotechnology, Santa Cruz, CA) and β -actin (1:20,000; 42 kDa, Santa Cruz Biotechnology, Santa Cruz, CA). Horseradish-conjugated secondary antibodies (Amersham, Arlington Heights, IL) were added for 1 h at room temperature. Proteins were detected with a chemiluminescence detection system based on the luminol reaction (ECL kit; Amersham) and band intensities were quantified by densitometric analysis with ChemiDoc (Bio-Rad, Hercules, CA). Treatments did not modify total p53 expression levels (not shown). For all proteins, the levels of immunoreactivity were normalized to that of β -actin.

Statistical analysis

Experiments were performed with neurons from three to nine independent primary cultures for each mouse strain. Data were pooled and the results given as mean $\pm$ SEM; number of samples (n) for each experiment is indicated in the corresponding figure legend. Statistical significance was determined by two-way ANOVA followed by Bonferroni's multiple comparison test or one-way ANOVA followed by Newman-Keules or Student's *t*-test.

Results

Cultured neurons from SAMP8 mice showed normal morphology and survival compared to those from SAMR1 mice (Fig. 1). The number of surviving neurons per microscopic field was 231 ± 24 and 223 ± 21 for SAMR1 and SAMP8 cultures, respectively.

SAMP8 neurons had a lower mitochondrial membrane potential than SAMR1 neurons (Fig. 2A). The uptake of the fluorescent probe rhodamine 123, which is sensitive to mitochondrial membrane potential, was 50% lower in SAMP8 neurons. This was not caused by a smaller number of mitochondria in SAMP8 than SAMR1 neurons because the measurement of mitochondrial mass by the mitotracker probe did not show differences between the two strain cultures (Fig. 2B).

Oxidative injury with hydrogen peroxide increased rhodamine 123 fluorescence, indicating inhibition of the mitochondrial membrane potential following fluorescent probe uptake or a leakage of the fluorescent probe because of membrane lesions caused by oxidative chain reactions. The dequenching or release of rhodamine 123 from the mitochondria elicits an increase in fluorescence [30]. Alternatively, hydrogen peroxide damage can induce mitochondrial membrane hyperpolarization similar to that reported for glutamate injury [31]. One hour of hydrogen peroxide exposure caused a greater mitochondrial disturbance in SAMP8 than in SAMR1 neurons (Fig. 2C) [ANOVA two-way; factor neuron strain: $F(1,108)=28.81$, $p<0.0001$; factor hydrogen peroxide concentration: $F(2, 108)=130$, $p<0.0001$; and interaction strain x concentration: $F(1,108)=11.11$, $p<0.0001$].

To characterize mitochondrial vulnerability of SAMP8 neurons, we used selective uncouplers and inhibitors of mitochondrial function: 10 μ M carbonyl cyanide 4-(trifluoro-methoxy)phenylhydrazone (FCCP), an uncoupler that increases proton permeability and disconnects the electron transport chain from ATP formation; 8 μ M rotenone, a respiratory chain inhibitor specific for complex I; 20 μ M thenoyl-trifluoroacetone (t-trifluoroacetone), an inhibitor of complex II; 4 μ M antimycin A, an inhibitor of complex III; 20 μ M sodium azide, an inhibitor of complex IV; and 20 μ M oligomycin, an inhibitor of phosphorylation. One hour of exposure to these agents induced significant rhodamine 123 dequenching or release from the mitochondria in both types of neuron cultures. However, FCCP, antimycin A, sodium azide and oligomycin showed a higher effect on SAMP8 than on SAMR1 neurons (Fig. 2D) [ANOVA factor neuron strain: $F(1,595)=60.29$, $p<0.0001$; factor mitochondrial agent: $F(6,595)=78.09$, $p<0.0001$; and interaction strain x agent: $F(6,595)=7.261$, $p<0.0001$]. Therefore, complexes II and IV were more vulnerable to mitochondrial damage in SAMP8 than in SAMR1 neurons. Furthermore, SAMP8 neuronal mitochondria were more vulnerable to uncoupling and ATP-synthesis inhibition than SAMR1 ones.

Proteins from SAMP8 and SAMR1 neurons had similar contents of carbonylated proteins (Fig. 3A) and the amount of ROS generated was also similar for both types of neurons (Fig. 3B). Therefore, SAMP8 neurons did not show an increase in oxidative stress in the culture conditions. However, when the neurons were challenged with hydrogen peroxide for 1 h, SAMP8 neurons displayed a higher generation of ROS than SAMR1 ones (Fig. 3C) [ANOVA factor neuron strain: $F(1,108)=25.98$, $p<0.0001$; factor hydrogen peroxide concentration: $F(2,108)=31.58$, $p<0.0001$; and interaction strain x concentration: $F(7,519)=7.519$, $p=0.0009$].

To examine whether this pro-oxidative status was caused by a decreased antioxidant capacity, we analyzed GSH content as the main cell antioxidant. SAMP8 neurons had a lower content of GSH than

SAMR1 neurons and were also more vulnerable to GSH depletion induced by a 24-h exposure to BSO (Fig. 4A) [ANOVA factor neuron strain: $F(1,217)=64.63$, $p<0.0001$; factor BSO concentration: $F(3,217)=17.26$, $p<0.0001$]. Depletion of GSH caused greater toxicity in SAMP8 than SAMR1 neurons (Fig. 4B) [ANOVA factor neuron strain: $F(1,27)=26.86$, $p<0.0001$; factor BSO concentration: $F(3,27)=86.55$, $p<0.0001$; and interaction strain x concentration: $F(3,27)=4.509$, $p=0.0109$]. SAMP8, but not SAMR1, neurons exhibited a marked decrease in sirtuin 1 protein levels after only 2 hours of BSO exposure (Fig. 4C) [ANOVA, factor neuron strain: $F(1,10)=5.640$, $p=0.0390$].

Cell death induced by GSH depletion after 24 h exposure to BSO was ameliorated by melatonin (Fig. 5A and B) and resveratrol (Fig. 5C and D) in both cell types. Melatonin was moderately neuroprotective and resveratrol was slightly effective in SAMR1 neurons (Fig. 5A and C). Both neuroprotective treatments were highly effective in SAMP8 mouse neurons, with a survival of up to 80 to 85% compared to untreated neurons (Fig. 5B and D). We used 5 μ M sirtinol, a sirtuin 1 inhibitor, to see if increased sirtuin 1 mediated the neuroprotective effects. Sirtinol nearly blocked the protection obtained in SAMR1 neurons by melatonin and resveratrol (Fig. 5A and C). In SAMP8 neurons, there was a partial decrease in neuroprotection by melatonin and resveratrol in the presence of sirtinol (Fig. 5 B and D). [ANOVA two-way for the 4 graphs showed an effect of factor treatment with significance $p=0.0007$ for SAMR1 in the resveratrol graph, while $p<0.0001$ for all the other graphs; factor concentration of BSO was $p<0.0001$ for all graphs and the significance of interaction between both factors was $p=0.034$ and $p=0.0035$ for melatonin and resveratrol graphs, respectively, in SAMR1 neurons and $p<0.0001$ in SAMP8 graphs]. Sirtinol alone slightly decreased survival of untreated neurons, but it did not increase cell death induced by BSO (not shown).

Two-hour exposure of either melatonin or resveratrol increased sirtuin 1 protein expression in SAMR1 and SAMP8 neurons (Fig. 6A and B) [ANOVA factor treatment: $F(2,15)=6.727$, $p=0.0090$; factor strain: not significant], while levels of its substrate acetylated p53 decreased accordingly [ANOVA factor treatment: $F(2,17)=4.050$, $p=0.0364$; factor neuron strain: not significant]. After 24 h, the increased sirtuin 1 expression still persisted (not shown). effectively

Discussion

Neuron cultures from SAMP8 mice had functional characteristics of aged neurons. Under their apparently healthy state, they showed a lower defense against oxidative injury, decreased mitochondrial membrane potential and higher vulnerability to mitochondrial damaging agents than neurons of SAMR1 mice. However, SAMP8 neuroprotective pathways were upregulated by certain pro-survival stimuli, indicating that SAMP8 neurons 7 DIV were in a vulnerable but not an irreversible neurodegenerative phase. Therefore, they might mimic a senescence-associated frailty stage [32]

SAMP8 is one of nine senescence-prone strains of senescence-accelerated mice (SAM), which was originally generated from AKR/J mice through phenotype selection [21]. SAMP8 mice display an early onset of cognitive loss with learning and memory deficits in several behavioral tests (passive and active avoidance task [33], spatial learning [34] and object recognition test [35]). Age-related cognitive

loss is not attributed to neuronal loss because the latter is not significant in aged human brains [2]. Similarly, aged monkeys [36] do not show a reduced number of neurons in the hippocampus. There is no evidence of accelerated neuronal death in SAMP8 brains, although there is a reduction in neuronal numbers in several brain areas compared to SAMR1 mice [37,38]. Moreover, no changes in pro- and anti-apoptotic proteins or an increase in terminal dUTP nick-end-labeled cells have been detected in SAMP8 hippocampus [39; unpublished observation from the authors] or whole-brains [40]. However a decrease has been observed in dendritic spines of the pyramidal neurons in SAMP8 hippocampi as well as cortical atrophy [41], probably due to a loss of synapses rather than neuronal death. Thus, the SAMP8 mouse phenotype might be closer to the aging process than to overt AD neurodegeneration [23]. On the other hand, SAMP8 mice show reduced cholinergic markers [38], tau hyperphosphorylation [42] and amyloid- β deposition [43] with an age-related pattern, features that suggest that this mouse model could help to understand the basis of pathological aging and sporadic AD [44].

Diverse neuronal cultures aged *in vitro* have been used as a model of aging neurons. After a long-term culture of 30 to 60 DIV, cerebral cortical or hippocampal neurons from conventional strains of rats or mice show mitochondrial dysfunction [45], decreased Ca^{2+} signaling [46], protein oxidation [47], or amyloidogenesis [48]. However, culture conditions exert some stress on the cells and long-term cultures may induce some changes different from those in aging *in vivo*. In fact, in our previous studies with astrocytes, we found SAMP8 astrocytes cultured for 3 weeks (standard period of time for astrocyte cultures) more reliable as a model of aging [19] than those from a conventional strain cultured for 90 DIV [18]. Neocortical cultures of SAMP8 neurons 7 DIV are viable, but undergo age- and AD-related pathological changes in its proteins that are involved in energy metabolism, biosynthesis, signaling and stress-response pathways [20].

SAMP8 neurons showed much lower mitochondrial function than SAMR1 ones, indicated by lower membrane potential, consistent with previous results in SAMP8 platelets [49] and cultured astrocytes [19], and higher vulnerability than SAMR1 neurons to uncoupler ionophores, membrane damaging agents and inhibitors of the electron transport chain. Using specific inhibitors in SAMP8 neurons, we found dysfunctions or vulnerability to disruptions in the mitochondrial complex III (ubiquinol-cytochrome c reductase), complex IV (cytochrome c oxidase) and the final step of oxidative phosphorylation (complex V, ATP synthase). Impaired activity of mitochondrial complex III and, to a lesser extent, of complex I have been described in SAMP8 brains [50]. Regarding other tissues, enzymatic activities of complex I and IV in liver mitochondria [51] and complex III and complex IV in heart mitochondria [52] are significantly reduced in SAMP8 mice compared to SAMR1. Accordingly, there is an age-related decrease in the ATP content in SAMP8 mice the hippocampi compared to SAMR1 mice [49]. Furthermore, SAMP8 mouse livers and hearts show age-related decrease in oxidative phosphorylation [53]. Progressive failure of mitochondrial respiration might cause the age-associated neurodegeneration [49] and shorter life span in SAMP8 mice [53] compared to SAMR1 ones.

SAMP8 neurons showed a pro-oxidative status, with a lower defense capacity against oxidative damage by either hydrogen peroxide or GSH depletion, and were therefore prone to oxidative stress. The abovementioned mitochondrial electron transport failure could be the cause of oxidative stress and accelerated aging in the SAMP8 brain [50], although longitudinal studies that simultaneously analyze

mitochondrial function and redox homeostasis are needed to establish the sequence of events. Accordingly, an age-related decrease in mitochondrial manganese superoxide dismutase, the main mitochondrial antioxidant enzyme, and a parallel increase in lipid peroxidation have been reported in the cerebral cortex of SAMP8 mice [54]. Several studies have reported enhanced oxidative stress markers in the SAMP8 brain at early [29,55] and mature ages [56], while similar oxidative stress also occurs in SAMP8 mouse peripheral organs [57]. We have previously reported an increase in lipoperoxidation and carbonyl proteins in cultured astrocytes from SAMP8 mice compared to those from SAMR1 mice [19], but SAMP8 neurons do not show increased oxidative stress markers in basal conditions. Thus, the specific contribution of neuronal and glial cells to brain oxidative stress responses needs to be investigated.

The neuroprotective agents melatonin and resveratrol improved SAMP8 and SAMR1 neuron survival after oxidative injury, particularly that induced by GSH depletion. SAMP8 neurons responded strongly to the beneficial effects of both agents, since they overcame a much higher basal damage than that suffered by SAMR1 neurons, while rescued SAMP8 neurons showed a survival rate similar to or even higher than that of SAMR1 neurons. Melatonin and resveratrol showed similar neuroprotective capacities. Therefore, these naturally occurring compounds probably exert their neuron protective effects through similar antioxidant and mitochondria up-regulatory mechanisms. Melatonin and resveratrol, despite their different chemical structure, both converge in several key cell pathways through pleiotropic regulatory mechanisms (see below).

Melatonin, the pineal gland indolamine that regulates the circadian rhythm, is now a very promising molecule against age-related ailments [58]. Its ubiquitous presence and lack of toxicity indicate its many functions and have prompted research on its cellular and protective mechanisms [for recent reviews see: 26,59]. Melatonin levels drop during aging [60-62], which could contribute to age-related cognitive decline and increased risk of AD. Earlier studies on melatonin administration in SAMP8 mice have shown its ability to ameliorate brain mitochondrial dysfunction [63] and oxidative stress [56]. Melatonin improves mitochondrial function in SAMP8 mouse tissues through recovering respiratory complex activities and ATP synthesis [64,65]. These protective effects on the mitochondria may be due to its potent antioxidative capacity [66]. Indeed, several reports have demonstrated that melatonin efficiently scavenges free radicals within mitochondria and this play a crucial role in the protection against a range of mitochondrial diseases [67]. For instance, melatonin affords protection against mitochondria-mediated apoptotic mechanisms [68,69] and mitochondrial bioenergetic dysfunctions [70], and further enhances antioxidant action of other compounds [71]. Besides, melatonin upregulates the glutathione cycle and restores GSH levels in SAMP8 brain [63] and other tissues [57,72]. It also exhibits anti-inflammatory activity in SAMP8 mice [73]. Recently, it has been reported that melatonin activates SIRT1 in SAMP8 brain [74] and aged cultured neurons [16], although the link is not yet clarified [26]. Furthermore, chronic treatment with melatonin increases the maximal half-life span and longevity of SAMP8 mice [65]. Melatonin also ameliorated the mitochondrial function of the AD mouse models APP/PS1 [75] and 3xTg-AD (unpublished results).

Resveratrol, a red-wine-derived phenolic antioxidant, is attracting much interest because it mimics benefits of caloric restriction such as anti-oxidation, anti-inflammation and anti-age-related

diseases [for reviews see: 27,76]. The specific protective effects of resveratrol on mitochondria have been associated with the regulation of mitochondrial function, induction of mitochondrial anti-oxidant systems and promotion of mitochondrial biogenesis [27]. Although resveratrol effects are mainly attributed to SIRT1 activation, it does not extend mouse life span when treatment starts midlife [77]. There are no studies on resveratrol administered to SAMP8 mice, but it is likely to ameliorate its senescence marker expression, as has been reported in aging hybrid mice [78].

Co-incubation of neuron cultures with melatonin or resveratrol plus sirtinol, a SIRT1 gene expression inhibitor [79], confirmed that the neuroprotective effect of both agents was partially mediated by sirtuin 1 activation. Sirtuin 1 involvement was further demonstrated by its increased protein expression and a decrease in its substrate acetylated p53 in the neurons treated with melatonin or resveratrol. The tumor suppressor factor p53, the transcription factor NF- κ B, the FOXO family of transcription factors and the nuclear receptor peroxisome proliferator-activated receptor- γ (PPAR γ) and its transcriptional co-activator PPAR γ coactivator 1- α (PGC-1 α) are substrates deacetylated by sirtuin 1. Therefore, the sirtuin 1 molecular pathway links environmental stresses to the cellular energy metabolism and transcriptional profiles [80]. Thus, sirtuin 1 activation can help frail and injured cells to respond to the environment and recover homeostasis. Additionally to the sirtuin 1-mediated effects, melatonin might protect SAMP8 and SAMR1 neurons by boosting antioxidant defenses and, at least in SAMP8 neurons, ameliorating mitochondrial function. Similarly, the neuroprotective activities of resveratrol not inhibited by sirtinol are probably mediated by its antioxidative and mitochondrial protective actions. Interestingly, SAMP8 neurons were more vulnerable to BSO-mediated injury than SAMR1 neurons but also more effectively rescued by melatonin and resveratrol. This may indicate that both compounds target frail mitochondria in SAMP8 neurons in addition to activate the sirtuin 1 neuroprotective pathway. On the other hand, SAMR1 neurons were protected by melatonin and resveratrol mainly through a sirtuin 1-mediated effect.

In summary, SAMP8 neuron cultures reproduce cell increased vulnerability and age-related mitochondrial dysfunctions of early aging. They are a good tool to discern mitochondrial mechanisms of aging and test preventive and therapeutic approaches against pathological brain aging. Both melatonin and resveratrol promote sirtuin 1 protein expression. The sirtuin 1 pathway is emerging as a promising mechanism to improve the physiological reserve in the brain and protect against the development of age-related neurodegenerative diseases.

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Figures

Fig. 1. Cerebral cortical neuron cultures of (A) SAMR1 and (B) SAMP8 mice 7 DIV. Morphology and survival were similar in both neuron cultures. Representative images of cultures used in the study are shown. Arrowheads indicate neuronal bodies; arrows indicate neurites. Scale bar = 20 μ m.

(A) SAMR1

(B) SAMP8

Fig. 2. Mitochondrial function was impaired in SAMP8 neurons. (A) Mitochondrial membrane potential measured by rhodamine 123 fluorescence was lower in SAMP8 than SAMR1 neurons. (B) Mitochondrial mass measured by MitoTracker Green FM (mitotracker) was similar in both neuron types. (C) Increased rhodamine 123 fluorescence by oxidative damage and (D) increased rhodamine 123 fluorescence by mitochondrial uncouplers and inhibitors were higher in SAMP8 than SAMR1 neurons. Results are expressed as mean $\pm$ SEM; $n = 15$ to 25 from 3 to 5 independent cultures for each strain. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to SAMR1; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to control treatment.

Fig. 3. SAMP8 neurons showed a pro-oxidative status. (A) Presence of carbonylated proteins and (B) generation of reactive oxygen species (ROS) measured by oxidation of dihydroethidium to ethidium was similar in both cultures. (C) However, 1-h hydrogen peroxide exposure increased ROS generation with a higher potency in SAMP8 than in SAMR1 mouse neurons. Results are expressed as mean $\pm$ SEM; n = 8 to 9 cultures in (A) and (B) and n = 15 to 25 from 3 to 5 cultures in (C). **p<0.01, ***p<0.001 compared to SAMR1; #p<0.05, ###p<0.001 compared to control treatment.

Fig. 4. SAMP8 neurons were highly vulnerable to GSH depletion by D,L-buthionine-[S,R]-sulfoximine (BSO). (A) Monochlorobimane (mBCl) fluorescence showed lower levels of GSH in SAMP8 than SAMR1 neurons both in basal conditions and after 24 h of BSO exposure. (B) Accordingly, neuron viability measured by the MTT reduction method was much lower in SAMP8 neurons than SAMR1 neurons after BSO treatment. (C) A short BSO exposure of 2 h induced a decrease in sirutin 1 protein expression only in SAMP8 neurons. Representative blots (top) and normalized density measures (bottom) are shown in (C). Results are expressed as mean $\pm$ SEM; n = 15 to 35 from 4 to 5 cultures in (A) and (B), n = 3 to 4 cultures in (C). *p<0.05, **p<0.01, ***p<0.001 compared to SAMR1; ##p<0.01, ###p<0.001 compared to control treatment.

Fig. 5. (A,B) Melatonin (MEL) and (C,D) resveratrol (RV) showed a neuroprotective effect against BSO-induced cell death as measured by MTT (see legend to Fig. 4) in SAMR1 and SAMP8 neurons. Neuroprotection of MEL and RV was reduced by co-incubation with the sirtuin 1 inhibitor sirtinol. Results are expressed as mean $\pm$ SEM; n = 15 to 35 from 3 to 4 cultures. **p<0.01, ***p<0.001 compared to control treatment (BSO alone); #p<0.05, ##p<0.01, ###p<0.001 compared to BSO + neuroprotective agent + sirtinol.

Fig. 6. Melatonin and resveratrol (A) enhanced sirtuin 1 protein levels and (B) decreased those of its substrate acetylated p53 (acetyl-p53) protein in SAMR1 and SAMP8 neurons. Representative blots (top) and normalized density measures (bottom) are shown for each protein. Treatment of each blot is indicated in the corresponding density measure displayed below. Results are expressed as mean $\pm$ SEM; n = 3 to 4 cultures. Statistics: #p<0.05, ##p<0.01 compared to control treatment.