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The role of SIRT2 inhibition on the aging process of brain in male rats

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ABSTRACT

Background: Though the exact mechanisms regarding brain aging and its relation to neurodegenerative disorders are not precise, oxidative stress, the key regulators of apoptosis and autophagy, such as bcl-2 and beclin 1, seem to be the potential players in the aging of the cerebral cortex and hippocampus. As a type of nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylases, sirtuin 2 (SIRT2) has been associated to age-related diseases. However, the exact role of SIRT2 in brain aging is not well studied. The objective of the current study was to study the role of SIRT2 inhibition on brain aging through the neuroprotective mechanisms.

Methods: We tested the effects of AGK-2, a SIRT2 inhibitor, on oxidative stress parameters, apoptosis and autophagy regulators including bcl-2, bax, beclin1 in young and old rats. 24 Wistar albino rats (3 months-old and 22 months-old) were divided into four groups; Young-Control (4% DMSO+PBS), Young-AGK-2 (10 μM/bw, ip), Aged-Control, and Aged-AGK-2. Following the 30 days of drug administration period the rats were sacrificed and the cerebral cortex, hippocampus, and cerebellum were isolated. Total antioxidant status (TAS) and total oxidant status (TOS) were measured as oxidative stress parameters in all three brain regions. SIRT2, bcl-2, and bax protein expression levels were measured by western blot and gene expression level of beclin 1, Atg5, and SIRT2 by real-time PCR.

Results: The bcl-2, bcl-2/bax ratio, beclin 1, and TAS in the cerebral cortex of the aged group were significantly decreased; however, the TOS, oxidative stress index (OSI), and SIRT2 expression in the cerebral cortex and hippocampus increased. SIRT2 inhibition by AGK-2 reduced TOS and OSI levels in all brain regions and increased bcl-2, bcl-2/bax ratio. In aged animals, AGK-2 also increased the beclin 1 levels in the cortex and hippocampus.

Conclusion: Our results indicate that SIRT2 has an essential role in brain aging. The inhibition of SIRT2 by AGK-2 may increase cell survival and decrease aging related processes in the cerebral cortex and hippocampus via decreasing oxidative stress, and increasing bcl-2 and beclin 1 expression.

Introduction

Brain aging is often accompanied by chronic and low-grade neurodegeneration and neuroinflammation [58]. Late-onset neurodegenerative diseases such as Alzheimer's (AD), Parkinson's (PD) and Huntington's (HD) affect the

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elderly population by constant neuron and cognitive loss [36]. The prefrontal cortex, with its interconnections with subcortical regions, including the hippocampus, seems highly involved in cognitive functions and might be an essential target for aging and neurodegenerative diseases with cognitive decline [4,24]. Different mechanisms, including free radicals, inflammatory processes, impaired autophagy, and increased apoptosis, may contribute to brain aging like the aging process. Neurodegenerative diseases like AD and PD have increased inflammatory response and reduced response to DNA damage [8,24,28].

The brain uses almost 20% of total daily O_2 and glucose consumption. The high O_2 concentration and the iron-rich content of the cortex, striatum, and hippocampus make those sensitive to oxidative stress. Although the lipid-rich nature of the brain makes it a target for oxidative stress, the polyunsaturated fatty acids, including docosahexaenoic acid, may act as indirect antioxidants to prevent brain aging [12,21]. It has been shown that oxidative stress and apoptosis increase and antioxidant capacity decreases the aging process [2,6]. Sirtuin 2 (SIRT2), a member of the sirtuin family, belongs to class III histone deacetylase and abundant in cortex striatum, hippocampus, and medulla spinalis [40]. SIRT2 regulates a wide variety of physiological processes, including genome stability, mitosis, nutritional metabolism, apoptosis, antioxidant mechanism, and cell motility [5,16,40,43]. SIRT2 levels increase with aging [5,11,25,26,40].

In our previous studies, we have shown that aging increases SIRT2 in the hippocampus and colon mucosa, correlated with increased oxidant damage [3,25,26]. Jiao et al have shown that thioacetamide causes liver damage, increases oxidative stress and decreases the antioxidant system; however, exogenous AGK-2, SIRT2 inhibitor, administration reverses all of those changes [23]. SIRT2 inhibitor is also reported to reduce α-synuclein toxicity in PD and HD models [9,37,39,44].

In the current study, considering pleiotropic effects, we aimed to enlighten the role of SIRT2 on brain aging. The effects of SIRT2 on oxidative stress parameters are controversial [33,56]. SIRT2 mRNA increases in response to a rise in oxidative stress via the application of H_2O_2 to HEK293T cells. This increase has been shown to increase cell death by inducing FOXO3a, bim proteins, activating caspase 3, and inhibiting bcl-2. However, SIRT2 also reduces oxidative stress via increasing MnSOD [56]. Therefore, in the current study, one of our objectives was to study the effects of SIRT2 inhibition on oxidative stress which is the main contributor of brain aging.

Most age-related neurodegenerative diseases are characterized by accumulating abnormal protein aggregates in the affected brain areas. Misfolding proteins are the pathological findings of neurodegenerative diseases such as AD, PD, and HD. During the aging, autophagy decreases in brain cells and the risk of neurodegenerative diseases increases. Genetic factors or aging that cause decreased lysosomal and autophagic degradation are associated with various metabolic and neurodegenerative diseases [29]. The lysosomal degradation of autophagy, which plays a role in the homeostasis of cells, tissues, and organisms, depends on autophagy-related genes (Atg) [30]. Beclin 1 (Atg6), an autophagosome inducer, is expressed in many areas of the mammalian adult brain, such as the cerebellum, hippocampus, and cerebral cortex [43]. Atg5-7, responsible for autophagy in the elderly brain, have been downregulated during the aging process [19,43]. Likewise, neuroprotective factors inducing bcl-2 expression may improve brain aging and memory [41]

Although the protective effects of sirtuins in various age-related neurodegenerative diseases have been demonstrated the role of SIRT2 in the pathogenesis of a neurodegenerative disease is controversial [13]. Sighn et al. have shown that overexpression of SIRT2 is protective in SH-SY5Y cells culture against diquat or rotenone administration [51]. On the contrary, genetic or pharmacological inhibition of SIRT2 is protective against neurotoxicity of alpha-synuclein and Huntington protein in intervertebral animals [9,37,44,51]. SIRT2 reduces basal autophagy and its inhibition increases basal autophagy [22]. Accordingly, SIRT2 deacetylase might decrease cellular proliferation through binding AKT, thus preventing regenerative processes [47,48]. However, the interaction of SIRT2 and apoptosis and autophagy, which are involved in the aging process of the brain, is not clear. Therefore, the second objective of the current study is to determine the effects of SIRT2 inhibition on apoptosis and autophagy regulators including bcl-2, Atg5 and beclin 1.

Methods

Animals

24 Wistar albino male rats used in this study were provided by Gazi University Laboratory Animal Breeding and Experimental Research Center (GUDAM). All the animals were kept at equal light and dark periods (12h light-dark periods under standard laboratory conditions and fed with commercial rat chow and water ad libitum. The rats were treated according to the European Convention ETS 123, and the Animal Experiments Ethics Committee approved all the methods used in the current study of Gazi University (#G.U.ET-16.008).

The treatment groups

Young (3 months, n = 12) and aged (22 months, n = 12) rats were divided into four groups: Young-Control-DMSO (n = 6), Young-AGK-2 (n = 6), Aged-Control-DMSO (n = 6), Aged-AGK-2 (n = 6). AGK-2 effectively inhibits the activity of SIRT2 and is used as a neuroprotective agent at the doses of 10 μM [37,44]. The rats in the AGK-2 groups were treated daily with AGK-2 (13178, Cayman Chemical) at the dose of 10 μM/bw, ip, a specific sirtuin inhibitor dissolved in 4% DMSO-PBS dimethylsulfoxide (DMSO) and control groups (4% DMSO-PBS, ip) were used as described previously for 30 days [2,26]. In our previous study, SIRT2 and oxidative stress parameters of PBS and 4% DMSO + PBS application were similar in tissues [2]. Therefore, 4% DMSO + PBS was applied to the control group. On the 31st day, following the intracardiac blood sampling, under ketamine-xylazine anesthesia, rats were sacrificed. The

seven different brain regions: the thalamus, hippocampus, frontal cortex, parietal cortex, occipital cortex, cerebellum, and amygdala, were sampled according to the atlas of Paxinos and Watson and immediately froze in liquid nitrogen and kept in at −80 °C.

Measurement of total oxidant status (TOS) and total antioxidant status (TAS)

The total cerebral cortex, cerebellum, and hippocampus tissues were used for TOS and TAS measurements. To homogenize the cerebral cortex, cerebellum, and hippocampus tissues, we first sonicated in 140 mM KCl buffer (1:9, w/v, pH 7.4). Following the sonication, tissue homogenates were centrifuged (at +4 °C 3,000 rpm, for 5 min), and the supernatants were stored at −80 °C. According to the manufacturer's recommendations, TOS and TAS levels were assayed by commercial kits (RL0024, RL0017, Rel Assay Diagnostics, TR). TOS levels at 595 nm and TAS at 660 nm were determined by spectrophotometry. The TOS levels were expressed in terms of μmol H_2O_2 Equiv./L as the assay calibrated by 10 μmol/L of H_2O_2. The TAS assay was calibrated with Trolox Equivalent (1mmol/L), a stable antioxidant standard solution, and the results were expressed in terms of mmol Trolox Equiv./L [52].

Oxidative stress index (OSI)

The percent ratio of TOS to TAS level according to the formula [(TOS, μmol H_2O_2 Equiv./L)/(TAS, μmol Trolox Equiv./L) × 100] was defined as oxidative stress index and considered as an indicator of oxidative stress level [52].

Western blot analysis for protein expression

The protein expression of SIRT2, bcl-2, and bax was assayed by western blotting. Briefly, the cerebral cortex, cerebellum, and hippocampus tissue lysates were prepared on ice with 300 μl of RIPA Lysis Buffer per 20 mg of tissue (Santa Cruz Biotechnology, TX). The samples containing 20 μg of total protein were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% or 10%). The protein bands were then transferred to nitrocellulose membranes (Bio-Rad, CA) blocked with Tris-buffered saline with 0.1% Tween 20 (TBST) containing 5% (w/v) nonfat dry milk (Santa Cruz Biotechnology, TX) for overnight at +4 °C. Following the transfer of the proteins, the membranes were incubated with primary antibodies against SIRT2 (1:500, sc-28298; Santa Cruz Biotechnology, TX), bcl-2 (1:1000, MA1-12246), bax (1:500, sc-493), or β-actin (1:125, sc-130657; Santa Cruz Biotechnology, TX) for 2 hours. Then the membranes were incubated for 1 hour with horseradish peroxidase (HRP) conjugated secondary antibodies (anti-rabbit, 1:5000, sc-2004; or anti-mouse, 1:5000, sc-2005; Santa Cruz Biotechnology, TX) in room temperature. Enhanced chemiluminescence detection reagent (Pierce™ Thermo Sci, IL) was used to visualize the specific protein bands on X-ray film (Carestream Health Inc. NY). The bands were quantified by using ImageJ software.

Quantification of Atg5, Beclin 1 and SIRT2 gene expression levels

The mTOR pathway is involved in autophagy, and beclin 1 and Atg5, involved in this pathway, are mainly used as autophagy markers [31]. In the current study, we quantified the mRNA levels of atg5, beclin 1, and SIRT2 by qPCR. RNA was extracted from the cortex, hippocampus, and cerebellum tissues using the commercial kit (Macherey Nagel, DE). A total of 500 ng RNA was used in the cDNA synthesis in line with the manufacturer's instructions (cDNA synthesis Kit, Bio-Rad, CA). Briefly, the cDNA was synthesized using oligo(dT) and random hexamers primers with thermocycling conditions as follows: 5min at 25 °C, 20min at 46 °C, and 1min at 95 °C. Primers and probes for qPCR were as follows: Atg5: forward, 5'-ATGTGTGAAG GAAGCTGACG-3', reversed, 5'-ATGAGTTTCCGGTT GATGGT-3' and probe, 5'-FAM-TGCAGTCCCATCCA GAGCTGC-TAMRA-3', beclin 1: forward, 5'-CAAGATCCTG GACCGAGTGA-3', reversed, 5'-CTTCGAGAGACATCCTG-3' and probe, 5'-FAM-ACTTACCACAGCCCAGGCGAAACCAG-TAMRA-3', SIRT2: forward, 5'-TTCAGACTCGGACACT GAGG-3', reversed, 5'-GCATGTAGCGTGTCACTCCT-3', and probe, 5'-AGACCCTGGGCCTGGGTTCC-3', and β-actin: forward, 5'-TGTCACCAACTGGGACGAT-3', reversed, 5'-GGGG TGTTGAAGGTCTCAAAC-3' and probe, 5'-FAM-CACTTTCTACAATGAGCTGCGTGTGG- TAMRA-3'. The reaction was performed using qPCR Mix (Solis Biodyne 5× Hot FirePol Probe Mix Universal qPCR Mix, EST), as triplicate with thermocycling conditions as follows: 10 min at 95 °C, 20s at 95 °C and 60s at 60 °C. The threshold cycle (Ct) of each target product was normalized against b-actin. For the validation process, the PCR products were separated on 2 % agarose gel and the product sizes were confirmed by visualizing the gel with ethidium bromide staining.

Statistical analysis

All quantitative results are presented as mean ± standard error of the mean (SEM). The ANOVA post-hoc LSD test was performed for multiple comparisons. Pearson's r was also calculated to determine the relationships between the variables. The statistical significance level was accepted as $p < 0.05$.

Results

Aging increases SIRT2 expression in the brain

Aging significantly increased SIRT2 protein expression in the cerebral cortex and hippocampus, while SIRT2 mRNA expression was increased in the hippocampus and cerebellum (Tables 1, 2 and 3; Figs. 1 and 2). In general, the SIRT2 expression was increased in all three brain regions of aged rats, though it was significantly increased in the hippocampus, both in mRNA and protein levels (Table 2; Figs. 1C & 2A, B).

Though it is an enzyme inhibitor, AGK-2 administration in aged rats decreased SIRT2 protein expression of cerebral

Table 1
The oxidative stress parameters and protein expression of the cerebral cortex in the study groups (mean ± standard deviation).

	YOUNG		AGED	
	CONTROL	AGK-2	CONTROL	AGK-2
TOS (μmol H_2O_2 Equiv./L)	12.46 ± 0.74[&]	10.05 ± 1.45[*,&]	24.81 ± 0.46[*,#]	13.81 ± 0.61[#]
TAS (mmol Trolox Equiv./L)	1.22 ± 0.15[&]	1.85 ± 0.18[*,&]	1.31 ± 0.17[#]	1.84 ± 0.19[#]
OSI	1.1 ± 0.21[&]	0.54 ± 0.07[*,&]	1.93 ± 0.29[*,#]	0.76 ± 0.1[#]
SIRT2/β-actin	1.03 ± 0.21	1.18 ± 0.33	1.45 ± 0.29[*,#]	1.08 ± 0.16[#]
Bcl-2/Bax ratio	0.85 ± 0.12	0.88 ± 0.11	0.68 ± 0.05[*,#]	0.88 ± 0.08[#]
Bcl-2/β-actin	1.15 ± 0.08	1.25 ± 0.17	1.02 ± 0.1[*,#]	1.37 ± 0.28[#]
Bax/β-actin	1.35 ± 0.08	1.43 ± 0.19	1.52 ± 0.17	1.54 ± 0.18

[&*#]denote significant difference at the level of P < 0.05 between the marked parameters.

Table 2
The oxidative stress parameters and protein expression of the hippocampus in the study groups (mean ± standard deviation).

	YOUNG		AGED	
	CONTROL	AGK-2	CONTROL	AGK-2
TOS (μmol H_2O_2 Equiv./L)	7.71 ± 0.86[&]	4.92 ± 0.44[*]	12.83 ± 0.69[*,#]	6.7 ± 0.45[#]
TAS (mmol Trolox Equiv./L)	1.94 ± 0.22	2.01 ± 0.27[*]	1.66 ± 0.11[*,#]	3.06 ± 0.34[#]
OSI	0.4 ± 0.08[&]	0.25 ± 0.05[*]	0.73 ± 0.1[*,#]	0.22 ± 0.03[#]
SIRT2/β-actin	0.94 ± 0.21	1.03 ± 0.29	1.28 ± 0.2[*,#]	0.93 ± 0.11[#]
Bcl-2/Bax ratio	0.7 ± 0.13	0.77 ± 0.15	0.46 ± 0.05[*,#]	0.73 ± 0.18[#]
Bcl-2/β-actin	0.8 ± 0.24	1.02 ± 0.32	0.64 ± 0.09	0.91 ± 0.31
Bax/β-actin	1.14 ± 0.24	1.34 ± 0.31	1.6 ± 0.52	1.25 ± 0.29

[&*#]denote significant difference at the level of P < 0.05 between the marked parameters.

Table 3
The oxidative stress parameters and protein expression of the cerebellum in the study groups (mean ± standard deviation).

	YOUNG		AGED	
	CONTROL	AGK-2	CONTROL	AGK-2
TOS (μmol H_2O_2 Equiv./L)	7.05 ± 0.39	6.64 ± 0.39[*]	8.36 ± 0.31[*,#]	6.6 ± 0.32[#]
TAS (mmol Trolox Equiv./L)	1.49 ± 0.14[&]	2.07 ± 0.3[*,&]	1.47 ± 0.2[*,#]	2.01 ± 0.24[#]
OSI	0.48 ± 0.04[&]	0.33 ± 0.04[*,&]	0.58 ± 0.07[*,#]	0.33 ± 0.04[#]
SIRT2/β-actin	0.65 ± 0.15	0.72 ± 0.26	0.74 ± 0.25	0.8 ± 0.27
Bcl-2/Bax ratio	0.66 ± 0.24	0.8 ± 0.26	0.56 ± 0.15	0.65 ± 0.2
Bcl-2/β-actin	0.61 ± 0.15	0.68 ± 0.21	0.58 ± 0.16	0.67 ± 0.16
Bax/β-actin	0.97 ± 0.19	0.91 ± 0.31	1.06 ± 0.24	1.1 ± 0.29

[&*#]Denote significant difference at the level of P < 0.05 between the marked parameters.

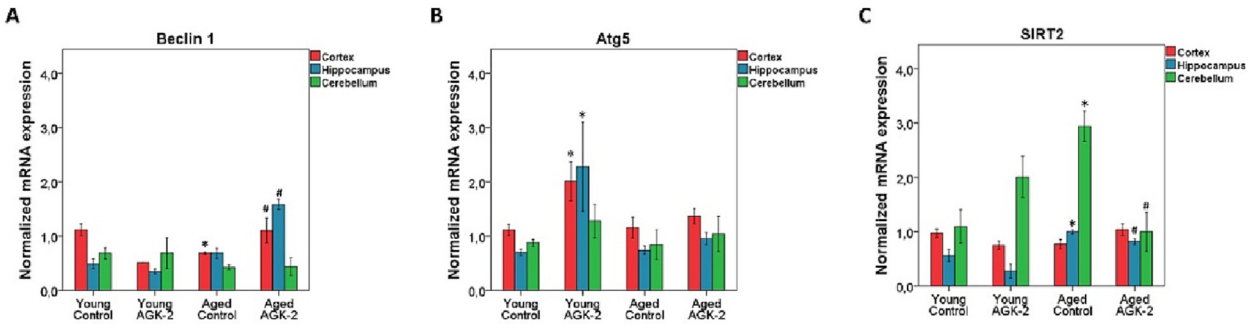

Fig. 1. mRNA expression of Beclin1 (A), Atg5 (B), and SIRT2 (C) in the cerebral cortex, hippocampus, and cerebellum of young and aged groups. The SIRT2 expression was increased in all three brain regions and beclin1 in the cortex of aged rats. For each group, n = 6, and the data were presented as mean ± SEM. [*]refers to significant difference at the level of p < 0.05 compared to the young control group, [#]refers to significant difference at the level of p < 0.05 compared to the aged control group.

cortex and hippocampus but not cerebellum (Tables 1 and 2). However, SIRT2 mRNA expression in aged rats was significantly decreased by AGK-2 administration in the cerebellum (Fig. 1C). AGK-2 did not change the SIRT2 expression of the studied brain regions in young rats (Tables 1, 2 and 3; Fig. 2A, 2B).

AGK2 decreased oxidative stress parameters in the cerebral cortex and hippocampus

We studied the TOS and TAS as the parameters of oxidant and antioxidant status in aged and young rats and calculated OSI as an indicator of oxidative stress. TOS and OSI were significantly increased in all brain regions of aged rats, while TAS remains unchanged in the cerebral cortex and cerebellum compared to young ones ($p < 0.05$) (Tables 1, 2 and 3; Fig. 3A, B, C). TAS was significantly decreased in the hippocampus of aged rats compared to young rats ($p < 0.05$) (Table 2; Fig. 3A).

The TOS levels and OSI of the cerebral cortex ($r = 0.728$ $p < 0.001$ and $r = 0.624$ $p = 0.003$) and hippocampus ($r = 0.714$ $p < 0.001$ and $r = 0.577$ $p = 0.006$) were significantly correlated with SIRT2 levels. No significant correlations between the TOS levels and OSI of the cerebellum and SIRT2 expression were found.

AGK-2 treatment decreased the cortical and hippocampal TOS and OSI in aged and young rats (Tables 1 and 2; Fig. 3B, C). AGK-2 decreased OSI in the cerebellum of young and aged rats and also decreased TOS only in aged rats ($p < 0.05$) (Table 3; Fig.3B, C). AGK-2 application did not affect the levels of cerebellum TOS in young rats (Fig. 3B). The TAS levels were increased significantly in all brain regions of aged rats treated with AGK-2 (Tables 1, 2 and 3). AGK-2 also increased TAS in the cortex and cerebellum but not the hippocampus of young rats (Tables 1, 2 and 3; Fig. 3A).

AGK2 increased Beclin 1 expression in the cerebral cortex and hippocampus in aged rats

While the beclin 1 expression in the cerebral cortex decreased in aged rats ($p < 0.05$) (Fig. 1A), there was no change in the hippocampus and cerebellum. AGK-2 administration increased the beclin 1 levels of the cerebral cortex and hippocampus in aged animals but not in the cerebellum.

The Atg5 levels were similar in all three brain regions of young and aged animals. AGK-2 administration significantly increased Atg5 mRNA expression in the cerebral cortex and hippocampus of young rats. ($p < 0.05$) (Fig. 1B). AGK-2 did not affect the Atg5 expression in the cerebellum of young and old rats and old rats' cerebral cortex.

AGK2 increased bcl-2 expression and bcl-2/bax ratio

Aging reduced bcl-2 expression in the cerebral cortex of aged rats ($p < 0.05$) (Fig. 2C). Bax levels were not changed significantly with aging ($p > 0.05$) (Fig. 2D). The bcl-2/bax ratio was found to be decreased with aging in the cerebral cortex and hippocampus (Fig. 2E).

Bax expression was positively correlated with SIRT2 protein expression in the cerebellum and hippocampus (respectively, $r = 0.609$ $p = 0.002$, $r = 0.704$ $p < 0.001$). In the cerebral cortex, bcl-2 and bcl-2/bax ratio were negatively correlated with SIRT2 expression levels (respectively, $r = -0.542$ $p = 0.011$, $r = -0.744$ $p < 0.001$).

AGK-2 application significantly increased the bcl-2/bax ratio in both cerebral cortex and hippocampus in elderly rats ($p < 0,05$) (Fig. 2E). In young rats, AGK-2 did not significantly affect bcl-2, bax, and bcl-2/bax ratio compared to control ones (Tables 1, 2 and 3; Fig. 2C, 2D, 2E).

Discussion

Aging is a risk factor for neurodegenerative diseases. The changes in the cortex, with its interconnections with subcortical regions, including the hippocampus and other subcortical regions, might be responsible for cognitive decline seen during aging and neurodegenerative diseases [53]. As the free radical theory still maintains the mainstay of aging theory, oxidative stress might be a critical factor for brain aging and an underlying factor leading the neurodegenerative diseases. Accordingly, the increased levels of inflammatory markers like IL-6, CRP, and IL-1β accompanying cognitive impairment and their relation to the diet also support the role of oxidative stress in brain aging [20]. Likewise, apoptosis and autophagy are two critical processes that play a significant role in maintaining cell homeostasis, and dysregulation of those mechanisms might increase the degenerative processes in the brain [15]. The pleiotropic effects of SIRT2 on different pathways make it an attractive target for brain aging studies. However, the role of SIRT2 in the pathogenesis of neurodegenerative diseases is contradictory. In addition to studies showing that SIRT2 plays a role in PD pathogenesis [35]. Through various processes such as alpha-synuclein aggregation, microtubule function, oxidative stress inflammation, and autophagy, the protective role of SIRT2 on dopaminergic neurons has also been demonstrated [11].

There were differences in cerebral cortex, hippocampus, and cerebellum in terms of levels of oxidative stress parameters and apoptosis activity during aging [2,7]. In our study, the hippocampus and cortex were the most sensitive areas to oxidative stress, while the cerebellum was more resistant. The cerebral cortex is more sensitive to oxidant damage than the cerebellum, which is explained by at least two times the cortex's metabolic activity [35]. Hippocampus is the most sensitive brain region against oxidative stress due to insufficient antioxidant function with age [15].

The increase of SIRT2 in aging is accompanied by increased oxidative damage [25,26]. SIRT2 expression has been shown to increase oxidative stress in the presence of hydrogen peroxide and apoptosis through Bim in the presence of stress [56]. Rotenone, an inhibitor of mitochondrial electron transport, increased SIRT2 and MDA levels in subtype nigra in elderly rats and decreased GSH levels, but these effects were age-related and did not occur in young rats [57]. Rotenone did not increase oxidative stress in

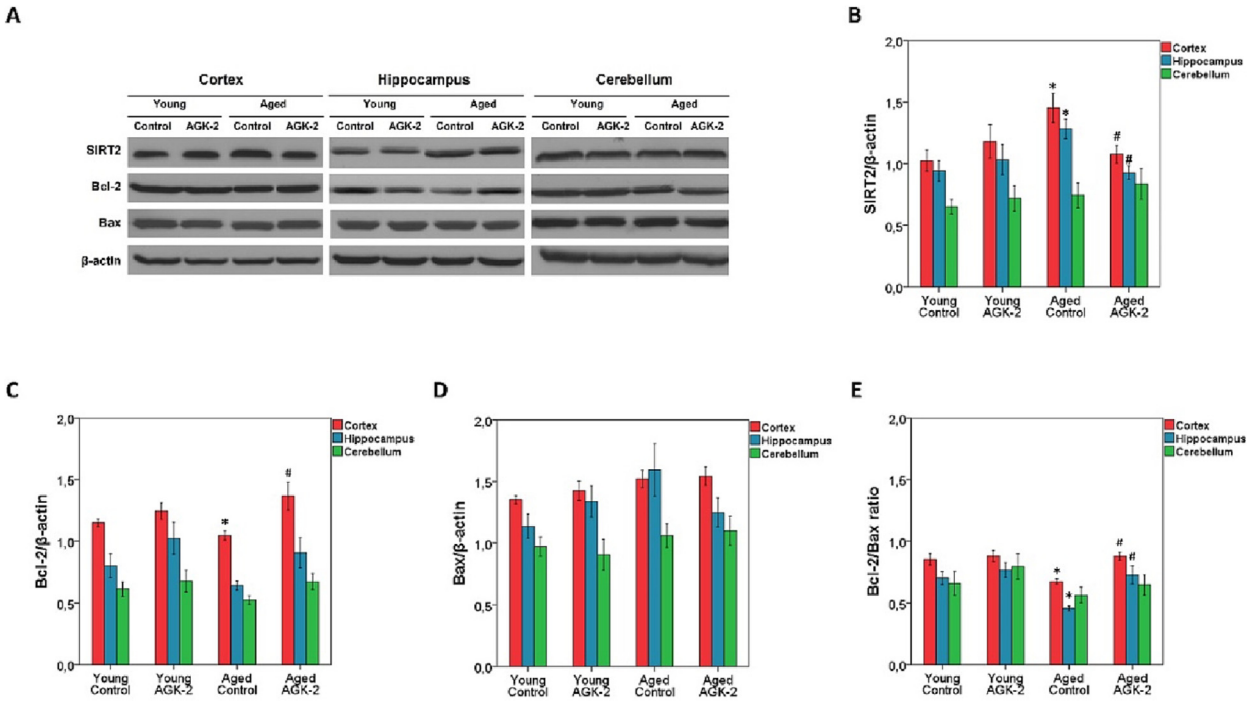

Fig. 2. Representative western blots for SIRT2, bcl-2, Bax and β-actin in the cerebral cortex, hippocampus and cerebellum of young and aged groups (A). SIRT2 protein expression levels (B), bcl-2 protein expression levels (C), Bax protein expression levels (D), bcl-2/Bax ratio (E). The protein expression levels were evaluated by western blotting, and normalized to β-actin expression. AGK administration significantly decreased SIRT2 expression of cerebral cortex and hippocampus and bcl-2 expression of cerebral cortex in aged rats. For each group, n = 6, and the data were presented as mean ± SEM. *refers to significant difference at the level of $p < 0.05$ compared to the young control group, #refers to significant difference at the level of $p < 0.05$ compared to the aged control group.

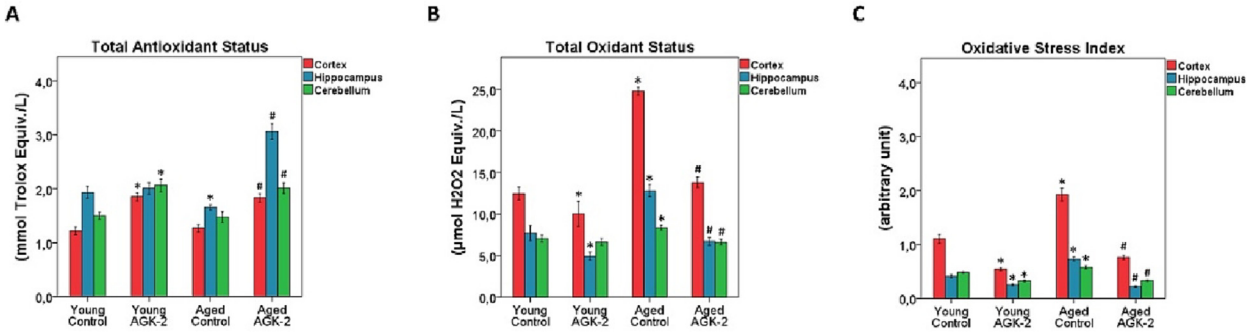

Fig. 3. Total antioxidant status (TAS) (A), total oxidant status (TOS) (B), and oxidative stress index (OSI) (C) changes in the cerebral cortex, hippocampus and cerebellum of young and aged groups. OSI value was calculated according to the following formula: [(TOS, μmol H_2O_2 Equiv./L)/(TAS, μmol Trolox Equiv./L) × 100]. TOS and OSI were significantly increased in all brain regions of aged rats and AGK-2 decreased those levels significantly. For each group, n = 6, and the data were presented as mean ± SEM. *refers to significant difference at the level of $p < 0.05$ compared to the young control group, #refers to significant difference at the level of $p < 0.05$ compared to the aged control group.

the cerebellum in rats. Inhibition of SIRT2 reversed the side effects of rotenone on oxidative stress.

In the current study, we investigated the protective effects of AGK-2, a specific SIRT2 inhibitor, on aging. We aimed to reveal the relationship between SIRT2, bcl-2, bax, oxidative stress parameters, and autophagy regulators of Atg5 and beclin 1 during aging. We found that SIRT2 expression was significantly increased in the brain regions of aged rats (Fig. 1C, Fig. 2A,B). Likewise, the oxidative stress parameters were also significantly increased in all studied brain regions (Fig. 3B,C) and correlated with SIRT2 expression during aging. AGK-2 administration significantly decreased oxidative stress parameters and increased TAS (Fig. 3A). Apart from being an enzyme inhibitor, interestingly we found a decreased expression of SIRT2 in cerebral cortex and hippocampus of aged rats with AGK-2 administration (Fig. 1C and Fig. 2A, B). AGK-2 is a potent and selective inhibitor of SIRT2 with an IC50 of 3.5 μM. SIRT2 contains four binding sites, A, B, C, D, at the large NAD+ binding site [54,57]. In the mechanism of inhibition

of SIRT2 by AGK-2, the ligand-binding site is a C pocket confirmed by low-energy ligand conformation. However, the effects of AGK-2 on protein and gene expression of SIRT2 are not well known. We also studied the effect of AGK-2 on SIRT2 expression. We found a significant decrease in SIRT2 expression of aged rats with AGK-2 administration (Figs. 1C and Fig. 2A,B). Interestingly, AGK2 did not cause any change in SIRT2 expression of the studied brain regions in young rats (Figs. 2A, 2B). However, the effects of AGK-2 on gene and protein expression of SIRT2 need to be further investigated.

Autophagy decreases with age, as the cleaning process of defective proteins through aging decreases with the ubiquitin-proteosome system or autophagy-phagosome route [30]. While autophagy markers decrease with age, [27,32] the loss of Atg5 and 7 genes in the mouse model results in progressive protein aggregation, neuron loss, and neurodegeneration [19,43]. Overexpression of Atg7 increases autophagy and prolongs life, and overexpression of Atg5 in mice activates autophagy and extends lifespan. Beclin1 has been shown to correlate with autophagy activity via binding the antiapoptotic protein bcl-2 [10]. Under basal conditions, the autophagy level is maintained by bcl-2 binding to the beclin 1, while stress conditions such as starvation lead to disruption of the beclin 1/bcl-2 complex. Long-term disruption of the beclin 1/bcl-2 complex prolongs life in mice and is protective against neurodegenerative diseases and cancer [38]. In the SAMP8, genetically modified aging-accelerated model, the mice have similar characteristic changes in older people and AD [38]. Very dense clumps and autophagic vacuoles were found in the hippocampal neuron cytoplasm and axons of 12-month-old SAMP8 mice. LC3–II expression increased between 7-12 months in the hippocampus and cortex and decreased after 12 months. Pickfort et al showed that the level of beclin 1 decreased in the midfrontal cortex gray matter of Alzheimer's and MID patients [46]. Gal et al showed that SIRT2 overexpression leads to the accumulation of autophagosomes, decreased ubiquite aggresome formation, and cytotoxicity with the increase of ubiquite protein aggregates. SIRT2's knockout or pharmacological inhibition increases autophagy [17].

Although autophagic flow shows regional differences in the brain in many studies, the reason is unknown [36]. The regulation of autophagy is not precise but probably depends on tissue and cell.

In the current study, we showed that the beclin 1 is decreased in the cerebral cortex in elderly rats, and the application of AGK-2 increases the beclin 1 in the cerebral cortex and hippocampus (Fig. 1A). The beclin 1 level was not changed with age in the hippocampus and cerebellum and with the administration of AGK-2 in the cerebellum. AGK-2 administration significantly increased Atg5 mRNA expression in the cerebral cortex and hippocampus of young rats ($p < 0.05$) (Fig. 1B). Atg5 did not show age-related changes in all three brain regions. AGK-2 application was not effective in the cerebellum of young and old rats. Our findings suggest a possible relation of SIRT2 with beclin 1 in the cerebral cortex and hippocampus but not in the cerebellum.

The apoptosis rate is increased in many tissues with aging [6,51]. Deregulation of apoptosis plays a role in many diseases, including cancer and neurodegenerative diseases such as AD, PD, HD, and amyotrophic sclerosis [1]. The growth of neuronal damage in the hippocampus has been shown to disrupt bcl-2 upregulation with antioxidant and antiapoptotic properties, and consequently, an increase in neuronal apoptosis parallel to decreased bcl-2 level. With aging, the rate of bcl-2 was decreased in the hippocampus, while the ratio of bax and bax/bcl-2 increased and the level of beclin 1 decreased in 24-month-old rats compared to the young ones [33]. On the other hand, overexpression of SIRT2 in glioma cells upregulates caspase and bax protein and down-regulates bcl-2 [34]. Liu et al showed that caspase 3 activity increased in the 8th hour in the PD model induced by MPTP in SH-SY5Y neuroblastoma cells, whereas silencing of SIRT2 has decreased the level of caspase 3 and SIRT2 overexpression did not affect caspase 3 activity [33]. We found that the bcl-2 level is decreased significantly with age in the cerebral cortex (Fig. 2A, C). Though we could not find a significant change in bax levels, the bcl-2/bax ratio was significantly decreased in the cerebral cortex and hippocampus of the aged rats (Fig. 2A, 2D, 2E).

SIRT2 inhibition corrects neurological and behavioral disorders in elderly mice linked in PD model to MPTP [18]. Genetic or pharmacological inhibition of SIRT2 has been shown to protect against the neurotoxicity of alpha-synuclein and Huntington protein in a primary neuron or intervertebral animal models [9,14,37,44,45]. Loss of SIRT2 improves microtubule stabilization and increases autophagy to ensure cell survival by eliminating toxic A beta oligomers [50]. We found a positive correlation between SIRT2 and bax expression levels and a negative correlation between bcl-2 and bcl-2/bax ratio in the current study. The pharmacological inhibition of SIRT2 with AGK-2 reversed the decreased bcl-2 and bcl-2/bax ratio suggests an inducer role of SIRT2 on apoptosis in the aging process.

In conclusion, our findings provide a rationale for a working hypothesis on the role of SIRT2 in brain aging and the pathogenesis of neurodegenerative diseases. Briefly, SIRT2 increases apoptosis via decreasing bcl-2 expression and bcl-2/bax ratio. Likewise, the increased oxidative stress also increases apoptosis. The SIRT2 might decrease autophagy by decreasing the beclin 1 level. The increased apoptosis and decreased autophagy contribute to brain aging in the elderly and also might trigger neurodegenerative disorders (See Fig. 4).

Autophagy-modulation has already been tested in animal models of neurodegenerative diseases with success [55]. In addition, an mTOR pathway inhibitor, sirolimus, is already in clinical trials to be tested for AD [42]. However, a recent report showed that inhibiting mTOR activity through rapamycin could be detrimental to AD by reducing the plaque clearance in a mice model [49]. Our results suggest that modulators, like SIRT2 inhibitors having multiple effects related to the pathogenesis of aging and age-related diseases, might help treat neurodegenerative diseases.

The lack of the study of mechanistic interaction of SIRT2 and apoptosis and autophagy regulating pathways is a limitation of our study. However, a more detailed study on the effects of SIRT2, including in-vitro studies with SIRT2

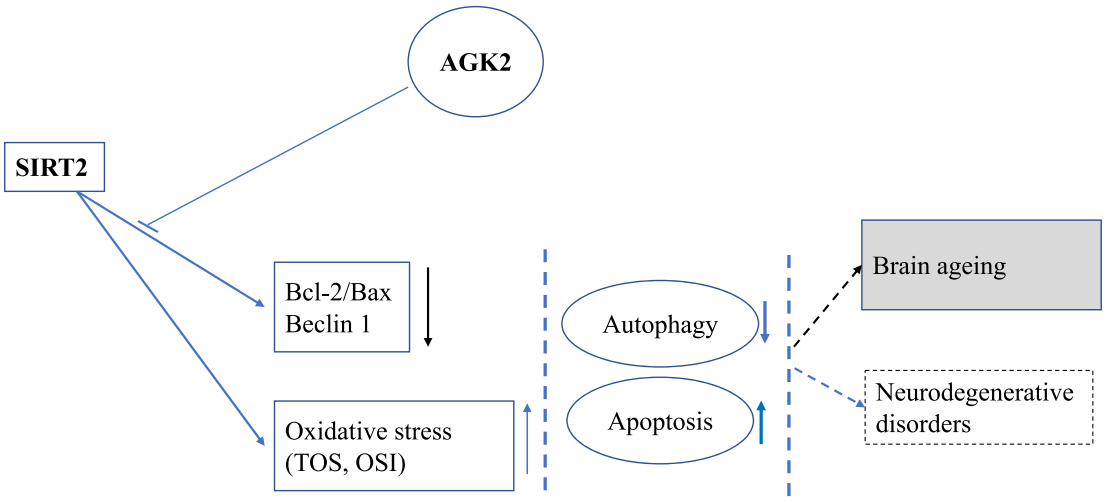

Fig. 4. A working hypothesis on the role of SIRT2 in brain aging and neurodegenerative diseases.

knock-in and -out cells with the interaction of all the regulators of autophagy and apoptosis, will provide more support for the hypothesis.

Limitations and future directions

In our study, oxidative stress was evaluated with TAS, TOS and OSI. However, it would be appropriate to examine the activity of enzymes such as GSH, catalase and SOD. Besides the parameters shoving the initiation and progression level for autophagy, LC3/LC1 would be helpful in evaluating the termination phase on the autophagy process. In the study, the relationshıp of inflammatory parameters and SIRT2 inhibition could have been worked. Studying the neuroprotective effect of SIRT2 inhibition may provide protection from neurodegenerative diseases.

Conclusions

During aging, oxidative stress, apoptosis and SIRT2 increased in different parts of the brain, while autophagy decreased. SIRT2 inhibition with AGK-2 reversed this table.

CRediT authorship contribution statement

K.G. Akbulut: Conceptualization, Methodology, Investigation, Project administration, Supervision, Writing – original draft. **A. Keskin-Aktan:** Methodology, Validation, Investigation, Resources, Formal analysis. **S.A. Abgarmi:** Methodology, Validation. **H. Akbulut:** Validation, Formal analysis, Writing – review & editing.

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.

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