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NEURODEGENERATION IN THE MICE BRAIN: EFFECTS OF CALORIE RESTRICTION ON SIRTUIN PROTEIN LEVELS

DOCTOR OF PHILOSOPHY THESIS

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I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree except where due acknowledgment has been made in the text.

Başar Atalay

DEDICATION

I dedicate to my thesis to the love of my life Ayce, my daughters Ece & Oya, my parents Sevgi & Seyfi and my brother Belir for their endless love and support.

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LIST OF SYMBOLS AND ABBREVIATIONS

6 HD: 6-hydroxydopamine

AG: Angiotensin 2

AD: Alzheimer`s disease

AL: Ad libitum

ALS: Amyotrophic lateral sclerosis

CR: Calorie restriction

CREB: cAMP responsive-element binding

DG: Dentate gyrus

DM: Diabetes type 2

HC: High calorie

Hipp: Hippocampus

IH: Immunohistochemistry

KA: Kainic acid

MO: Months-old

MS: Multiple sclerosis

NAD: Nicotinamide adenine dinucleotide

NC: Normal calorie

ND: Neurodegeneration

NDD: Neurodegenerative disorder

PBEF: Rate limiting enzyme in NAD production-which is a substrate of SIRT

PD: Parkinson`s disease

OSA: Obstructive sleep apnea

SIRT: Sirtuin

SLE: Systemic lupus erythematosis

SOD: Superoxide dismutase

WB: Western blot

Wks: Weeks-old

ZMR: Zitter mutant rats

ABSTRACT

Atalay B. Neurodegeneration in The Mice Brain: Effects of Calorie Restriction on Sirtuin Protein Levels. Yeditepe University, Institute of Health Science, Doctoral Program in Neuroscience. Ph.D. Thesis. Istanbul, 2020.

Neurodegenerative (ND) disorders affect a significant portion of the world's population. Alzheimer's disease and Parkinson's disease are gaining importance as a significant public health problem. Despite many advancements in current medicine and clarification of the molecular pathways involved in neurodegeneration, these diseases are progressive and there is no cure at the moment.

Calorie restriction (CR) is accomplished as a neuroprotective method and have beneficial roles in the metabolism. CR is suggested to activate protective mechanisms in ND and age-related diseases. There are different types of established CR models. It is shown that CR extended the life-span by increasing the activity of the Sirtuins (SIRT). SIRT are a family of proteins which are histone deacetylases bound to nicotinamide adenine dinucleotide. They were identified as genetic silencing factors and were found to prolong life in the yeast. When the histones are deacetylated by SIRT, the chromatin is closed, or tightly and precisely wound, meaning gene expression is stopped, or silenced, protecting the DNA from damage. SIRT have a protective role in ND diseases related to aging. SIRT 1, 3, and 6 have critical roles in the brain.

In this research study we have tested the effect of different types of CR as chronic CR, or intermittent CR on SIRT levels and neurodegeneration in the mice brain. SIRT 1, SIRT 3 and SIRT 6 protein levels were measured via ELISA method. Neurodegeneration was evaluated by fluoro-jade fluorescein staining and image-based quantification method. We found significantly increased mean SIRT values in CR groups compared to Ad-libitum and baseline controls. Fluoro-jade image quantification results revealed significantly increased neurodegeneration in ad-libitum compared to CR groups. We have concluded that different types of CR prevents neurodegeneration in the mice and SIRT are involved in this process. Our findings of significantly increased SIRT expression and decreased neurodegeneration after intermittent CR is a novel contribution which can be a therapeutic strategy in the treatment of ND diseases in the future.

***Key Words*:** Ad libitum, calorie restriction, ELISA, fluoro jade, mice brain, neurodegeneration, sirtuin

ÖZET

Atalay B. Fare Beyninde Nörodejenerasyon: Kalori Kısıtlanmasının Sirtuin Protein Seviyelerine Etkileri. Yeditepe Universitesi, Sağlık Bilimleri Enstitüsü, Sinir Bilim Doktora Programı. PhD Doktora Tezi. İstanbul, 2020.

Nörodejeneratif (ND) hastalıklar Dünya popülasyonunun önemli bir bölümünü etkilemektedir. Alzheimer ve Parkinson hastalıkları önemli bir halk sağlığı problemi olarak önem kazanmaktadır. Güncel tıptaki birçok ilerlemelere ve nörodejenerasyondaki (NDN) moleküler yolların dahi açıkca anlaşılmasına rağmen bu hastalıklar progresyon göstermekte olup kesin bir tedavi yöntemi bulunmamaktadır.

Kalori kısıtlanması (KK) etkinliği gösterilmiş bir nöroprotektif metoddur. Metabolizma üzerinde olumlu etkileri vardır. KK nın ND ve yaşa bağımlı hastalıklarda vücuttaki nöroprotektif mekanizmaları aktive ettiği düşünülmektedir. Değişik tiplerde etkinliği gösterilmiş KK modelleri bulunmaktadır. KK nın sirtuin proteinlerinin (SIRT) aktivasyonu aracılığıyla yaşam süresini uzattığı gösterilmiştir. SIRT histon deasetilaz aktivitesi olan bir grup protein ailesidir ve nikotinamid adenin dinükleotide bağlıdır. SIRT genetik suskunluk faktörleri olarak da adlandırılmaktadır ve mayalarda yaşam süresini uzattıkları gösterilmiştir. Histonlar SIRT ile deasetile olduklarında, kromatin kapalı, sıkıca ve muntazam olarak sarmalanmıştır. Bu durum gen ekspresyonunun durduğu, sessizleştiği ve DNA nın hasar almaktan korunduğu anlamına gelmektedir. SIRT`ın yaşlanmaya bağlı ND hastalıklarda koruyucu etkisi vardır. SIRT 1, SIRT 3 ve SIRT 6`nın beyinde kritik rolleri bulunmaktadır.

Bu çalışmada değişik tipteki kronik ve aralıklı KK metodlarının etkilerini fare beynindeki NDN ve SIRT seviyeleri üzerinde test ettik. SIRT 1, SIRT 3 ve SIRT 6 protein seviyeleri ELISA metodu ile ölçüldü. NDN ise fluoro-jade flörosan boyası ve görüntü kaynaklı kuantifikasyon yöntemi ile gösterildi. Sonuç olarak bu çalışmada KK gruplarında ad libitum ve kontrol gruplarına göre SIRT değerlerinin belirgin olarak arttığını bulduk. Fluoro-jade ve görüntü kuantifikasyon yöntemleri de ad-libitum grubunda KK grubuna göre belirgin olarak NDN artışı gösterdi. Çalışmamızda farklı şekillerdeki KK`nın SIRT proteinleri aracılığıyla fare beyninde NDN`u önlediği sonucuna vardık. Bulgularımızdaki belirgin SIRT artışı ve azalmış NDN özellikle aralıklı KK grubunda daha belirgin olması bu çalışmamızın en yeni katkısıdır. Bu yöntemler gelecekte ND hastalıkların tedavisinde yol gösterici olabilir.

***Anahtar Kelimeler*:** Ad libitum, ELISA, fare beyni, fluoro jade, kalorikısıtlaması, nörodejenerasyon, sirtuin

1. STATEMENT AND PURPOSE

Neurodegenerative diseases (ND) are progressive and debilitating health problems related to aging. There is no definitive cure at the moment. Calorie restriction (CR) has many favorable effects on metabolism and recent research revealed the protective role of CR in neurodegeneration. Reduced caloric intake is related to stimulation of neurogenesis and decreased gene expression concerning inflammation and stress-response. Sirtuin proteins (SIRT) are important regulators of gene transcription. Current research revealed the protective role of SIRT on ND and a direct correlation of SIRT levels with CR.

The protective role of SIRT proteins against age-related neurodegenerative disorders (NDD) are reported in the literature; however, none of them simultaneously explored the level of SIRT proteins and the protective effect of CR versus intermittent calorie restriction (ICR) compared to Ad-libitum (AL) in NDD. Furthermore, different types of CR like chronic calorie restriction (CCR) or ICR and their difference from the baseline or AL were not explored in these studies. Moreover, the consequences of various kinds of long-term CR on SIRT proteins and their neuroprotective effects on ND have also not been explored in the brain.

We have assessed the effect of different types of CR (up to 50 weeks) on SIRT1, SIRT3 and SIRT6 protein levels in mice brain. ND levels in hippocampal areas of 50 weeks old mice fed with different types of CR were compared. Hippocampal Brain slices were evaluated by histopathological analysis and SIRT1, SIRT3 and SIRT6 protein levels were determined in whole brain tissue. The hypothesis of the current study was “SIRT protein levels are modulated by the calorie restriction and the way calorie is consumed has different effects on it.” To stimulate stress, we chose the continuous or intermittent CR models. We compared the CR groups with AL and baseline. We assumed that the number of cells with ND is more in mice on the AL diet than the mice on ICR or CCR diet. We also hypothesized that measurement of SIRT1, SIRT3 and SIRT6 levels by Enzyme-linked immunosorbent assay (ELISA) method would reveal a higher level of expression in mice neural tissue on ICR or CCR diet and, with a lower level of activation of SIRT 1, 3 and 6 levels in mice brain tissue on AL diet.

2. LITERATURE REVIEW

2.1. Neurodegenerative Disorders and Sirtuin Levels

Neurodegenerative disorders are compelling health problems that affect a considerable portion of the society leading to disability, loss of function, and even death for almost thirty million individuals in the world[1]. Today the population of the world is rapidly increasing. Parkinson's disease (PD), Alzheimer's disease (AD), and also Huntington's disease are gaining importance as common neurological disorders. Costs of treatment and taking care of patients with the NDD increase since this rate of older individuals continues to rise[1,2]. Portion of medical care expenses in nations' economy getting larger each year. Despite many advancements in current medicine and clarification of the etiopathogeneses of neurodegeneration related disease, they are progressive and there is no certain treatment for NDD at the moment.

Neurodegeneration is characterized by loss of function, progressive atrophy, and death of neurons, which are the most prevalent features of AD and PD [2]. ND is characterized by loss of previously gained neural functions and, progressive loss of motor and cognitive functions. Neurodegenerative health problems are among the primary causes of fatality in elder people moreover, they are the foremost reason for morbidity, which is related to aging [2]. Pathophysiology of the NDD is complex and multifactorial such as environmental, genetic, diet, and lifestyles which all play essential roles. In the etiology, aging has always been the most important determinant for the onset and prognosis of NDD, thus the factors influencing aging may also affect NDD [2,3]. For example, therapeutic strategies targeting aging may work in the management of NDD. Furthermore, in addition to brain dysfunction, ND could also cause several spinal cord disorders in addition to amyotrophic lateral sclerosis (ALS), and multiple sclerosis (MS) [4–6]. It has been proven that all NDD are age-related and progressive and they lower the quality of life.

Alzheimer's disease is the leading NDD in the elder population. It is the major culprit of neuronal cell death and brain atrophy in human beings [5,7]. Health quality is severely affected and patients become dependent most of the time. Psychologically, widespread neurological damage leads to memory loss, cognitive and functional decline which eventually leads to death [7,8]. On the other hand, anatomically, Hippocampus is severely affected in AD. In this context, the Neuropeptide-Y neuron network in the main hippocampal regions are most severely affected in AD. The number of neurofibrillary tangles and neuritic

plaques is correlated with the severely affected hippocampal regions in AD patients brains [9,10]. Another common NDD that follows AD is the PD. PD is identified principally with the degeneration of dopamine-releasing nerves, and PD is a leading movement disorder [2,3]. Whatever insult that provokes ND, PD pathophysiology involves oxidative phosphorylation stress, mitochondrial malfunction, misfolding, and aggregation of intracellular proteins leading to the expiration of dopamine-releasing nerves [11]. Recent studies have suggested a link between Sirtuin proteins (SIRT) and AD [3,5,6]. SIRT1 was originally determined to be protected from AD in calorie restriction (CR) studies, and in this context, Dauer et al. have found that CR was effective in reducing the plaques in "transgenic AD mice" [12].

The involvement of SIRT in neurological diseases have been reported in numerous studies using different animal models. For example, spinal cord related NDD like ALS are progressive, chronic, and fatal neurodegenerative conditions. Motor nerve cell death in the spinal cord and brain cortex is the characteristic pathology in ALS [13]. A mutated type of superoxide dismutase 1 was synthesized in a study with an animal model for ALS, in which, SIRT1 levels were upregulated in motor neurons.[13] In another study, SIRT1 overexpression is shown to protect neurons from toxicity which is triggered by mutated superoxide dismutase 1 in both neuron cultures and the brains of mice [14]. Another NDD is MS which is a myelin sheath disorder characterized by demyelinating plaques in cerebrum, cerebellum, and the spinal cord. MS probably has an autoimmune etiology, it is an inflammatory disease. On the other hand, MS has also been designated as an NDD by reason of the constant neuronal injury and demise [3]. Experimental autoimmune encephalitis in mouse models of MS, have demonstrated that SIRT1 activation maintains axonal structures, protects against neuronal loss, and improves neural function [15].

There is a huge amount of medical research on every aspect of aging and ND. Aging and ND are complicated issues and related to many different fields of science like genetics, molecular biology, metabolism, environmental and psychosocial factors. This makes the research process difficult and requires the collaboration of many different scientific fields including, medical, genetic, biotechnological, and environmental disciplines.

2.2. Functions of SIRT Proteins

Sirtuins are a family of proteins which are histone deacetylases. **Figure 1** shows the 3D molecular structure model of the SIRT protein. Nicotinamide adenine dinucleotide (NAD) is a very important coenzyme and it exists in every living cell. SIRT exclusively function in the existence of NAD[16,17]. SIRT act on histones in the cell and they regulate some functions of the cellular proteins. SIRT also have mono ADP ribosyltransferase activity [5]. Zhang et al. have explored the role of a rate limiting enzyme in NAD production, which is a substrate of SIRT and called as PBEF. They studied in an ischemia model on 10-month-old mice. They utilized FJB to demonstrate ND, and Western blot for the measurement of PBEF and NAD. In this study, they have found more ND in ischemia and ischemia volume with PBEF knock-out mice than wild type [18] .

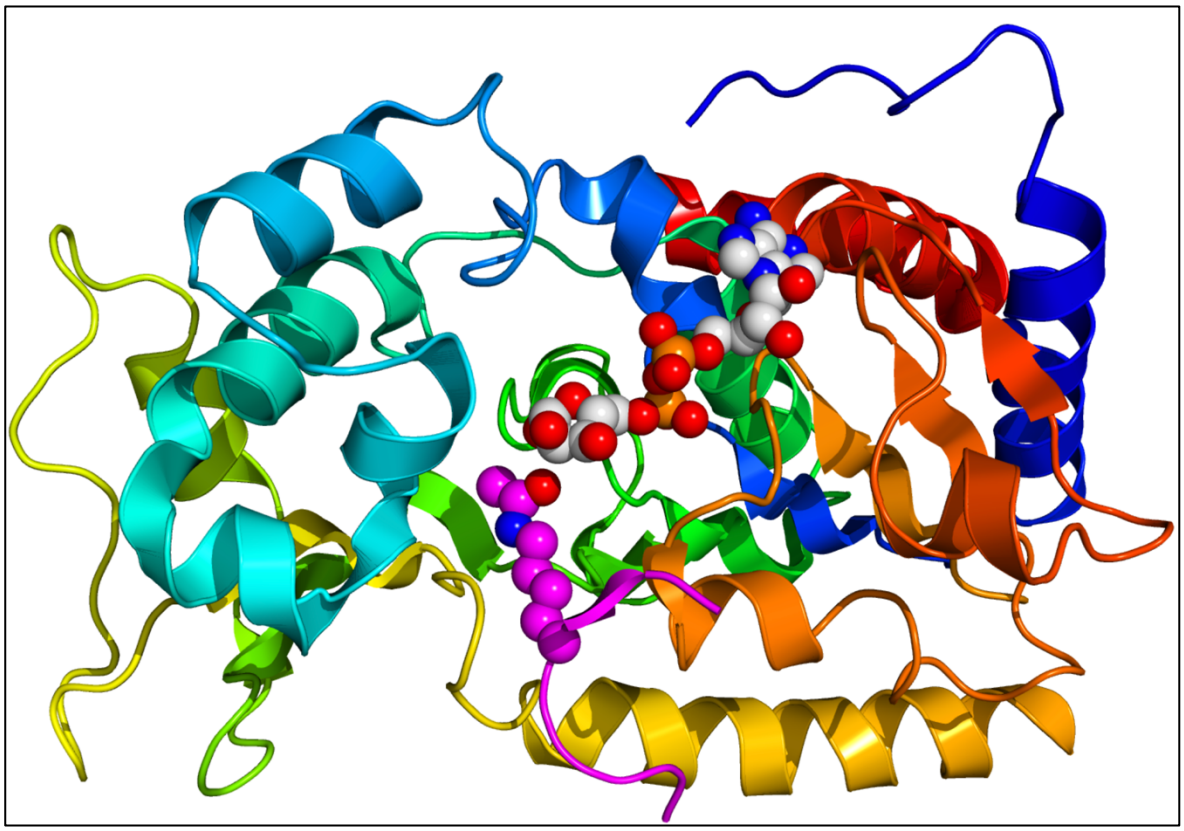


Figure 1. Sirtuin molecule bound to histone and ADP molecules. (Adapted from Wikipedia.)

Sirtuins take part in a chain of cellular and physiological reactions. They are essential proteins in gene silencing, genomic stability, cell longevity, and metabolic regulation through the deacetylation of histones. In this context, SIRT regulate the remodeling of the chromatin, cellular mechanisms, and expression of the gene. Specifically, when the histones are deacetylated by SIRT, the chromatin is closed, or firmly and smartly enveloped, meaning

gene expression is stopped, or silenced, preventing the DNA from injury [4,5]. Studies have reported that SIRT are involved in many different signaling pathways, transcription factor deacetylations, including metabolic cofactors such as p53, E2F1, NF kappa B, FOXO. In addition, SIRT has important functions related to the oxidative stress. They are shown to have antioxidant functions by their control over mitochondrial performance. Therefore, it was concluded that SIRT proteins protect against mitochondrial degradation and neuronal death which was induced by superoxide dismutase enzyme[5]. All these studies have shown that involvement of SIRT in the regulation of DNA damage repair, inflammation, and apoptosis contribute to SIRT`s ability to prolong life span in many species [5,6,19].

Sirtuins play a role in aging by controlling cellular health. They were recognized as suppressors of genetic factors. In the yeast, SIRT were shown to elongate the life span. In the species of Saccharomyces cerevisiae, lifespan is regulated by the SIRT gene: lifespan shortens by null mutations, and on the other hand, longevity extends by an additional copy of SIRT2 [20]. These findings are thought to come from the suppression of chromatin in the ribosomal DNA by SIRT proteins. This suppression abates expression of the gene of ribosomal DNA, and the generation of toxic extrachromosomal ribosomal DNA circles would decline as a consequence [21]. C. elegans species has been found to be very different from the aging of the yeast. A SIRT homolog, sir-2.1, regulates the lifespan in a way, that transgenic worms carrying additional duplicates of sir-2.1 have a longer lifespan [22]. Genetic analysis indicates that SIRT has important roles in the insulin signaling pathway. Molecular changes by genetic mutations in insulin pathways decrease metabolic signaling of this pathway and provides an increased lifespan [23,24]. Cohen et al demonstrated that SIRT1 inhibits stress-induced apoptotic cell death in the brain, liver, and kidney tissues of the rats[25]. Fan et al found that SIRT3 is apoptotic in cancer cells and to inhibit apoptosis in normal cells[26]. Fusco et al found an increased survival in mice with the stimulation of SIRT1 gene induction [27].

Having a protective role in NDD related to aging, SIRT have seven different variants, SIRT1-7 [4–6]. Each variant has more than one function in different cell structures and cellular organelles[2,3]. As appreciated in **Figure 2**, SIRT1, SIRT2, SIRT6 are found mostly in the nucleus, and SIRT7 is in the nucleolus while SIRT3, SIRT4, and SIRT5 are found in the mitochondria [3,5]. SIRT1 and SIRT2 can be localized both in cytoplasm and nucleus[5]. Anatomically, SIRT have been shown to be present in many critical organ systems including the brain and spinal cord. For example, SIRT 1 is highly synthesized in the spinal cord, brain and dorsal root ganglion [3]. SIRT 1, 3 and 6 have very important functions in metabolism,

SIRT 2 is important for tumorigenesis, SIRT 1 and 3 regulates programmed cell death, while SIRT1 and 6 have functions in repairing of DNA, SIRT 1, 2 and 3 regulates the gluconeogenesis, furthermore SIRT1, 3 and 6 prevents the cell from the oxidative stress, on the other hand, SIRT 3, 4 and 5 helps in the preservation of energy, in addition SIRT 1, 4, 5, 6 and 7 have important functions in the secretion of insulin, and SIRT 1-6 regulates inflammation [28–30]. **Table 1** reviews the reported information about localization, function, distribution of SIRT proteins, in addition to their role in possible disorders in the brain. For example, the role of SIRT1, SIRT3, and SIRT6 have been reported in AD [8,31]. SIRT1, SIRT3 and SIRT6 are very active in neurons and astrocytes. SIRT1 regulates neuroplasticity and memory. SIRT3 has a role in long term memory potentiation. SIRT1 and SIRT6 have functions in cell senescence, apoptosis, and inflammation [2,3]. In addition, it is reported that SIRT3 and SIRT5 may be therapeutic targets in some NDD of the medulla spinalis like ALS [32–35]. It is also reported that SIRT are implicated in the mechanism of the aging and they may be associated with the protective mechanisms against NDD [5].

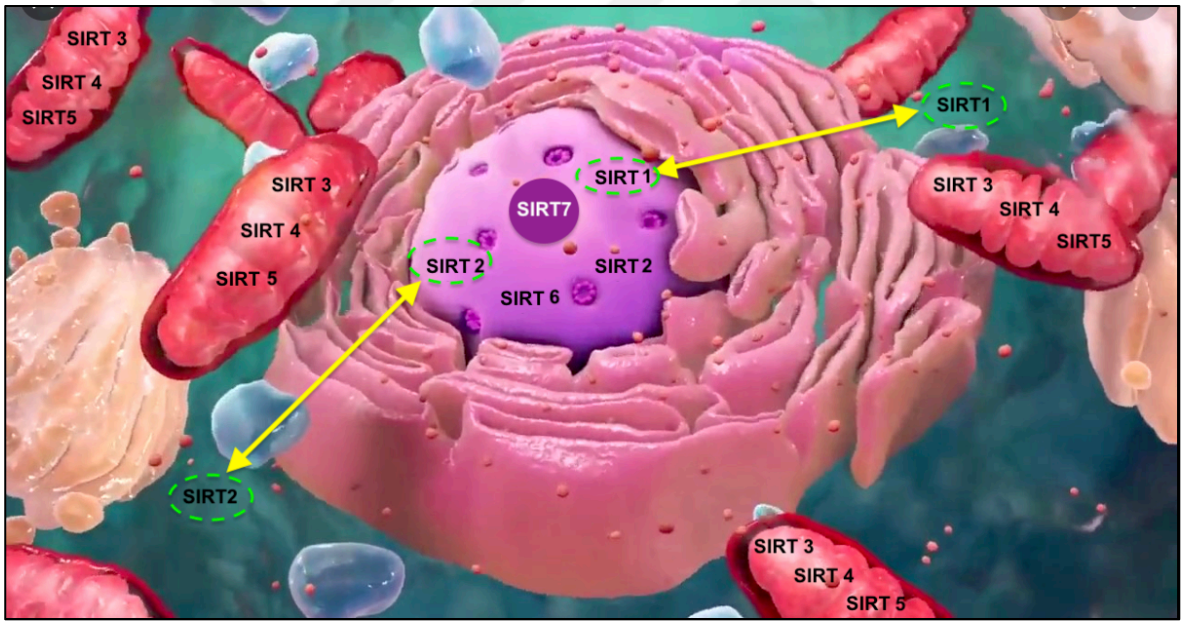


Figure 2. Intracellular localization of the Sirtuins (SIRT). SIRT 1, SIRT 2 are localized in both nucleus and cytoplasm. SIRT 3, SIRT 4, and SIRT 5 are localized in mitochondria, SIRT 6 is localized in the nucleus and SIRT 7 is localized in the nucleolus (Adapted from the free web). **SIRT**: Sirtuin protein

SIRTUINS	CLASS	ENZYME ACTIVITY	SUBCELLULAR LOCATION	ROLE	DISTRIBUTION	Brain disorder
SIRT1	Class 1	Deacetylase	Nucleus, Cytoplasm	DNA repair,DNA damage sensor,glycolysis, gluconeogenesis	Brain-spinal cord (neurons,astrocytes) , dorsal root ganglion	AD, PD, ALS, WD, MS
SIRT 2	Class 1	Deacetylase	Cytoplasm, Nucleus	Tumorigenesis, Myelin formation,gluconeogenesis	Oligodendrocyte, Schwann cells(myelin producing cells) olfactory,hippocampus	AD, PD, HD,low in gliomas
SIRT 3	Class 1	Deacetylase	Mitochondrial	Glycolysis, Apoptosis, ROS production,gluconeogenesis, energy preservation	Neurons	AD, PD, HD, ALS, Stroke, HE
SIRT 4	Class 2	ADP-ribosyltransferase	Mitochondrial	Cell cycle arrest, glutaminolysis,energy preservation	Astrocytes	Not reported
SIRT 5	Class 3	Deacetylase + demalonylase+ desuccinylase	Mitochondrial	Energy preservation	Neurons and astrocytes	Not reported
SIRT 6	Class 4	Weak Deacetylase + ADP-ribosyltransferase	Mitochondrial, Nucleus	DNA repair glycolysis, insulin secretion, inflammation regulation	Neuron and astrocytes	AD
SIRT 7	Class 4	Weak Deacetylase	Nucleolus	Insulin secretion	Neuron and astrocytes	Not reported

Table 1. SIRT have seven different variants, SIRT 1-7. Each variant has more than one function in different cell structures and cellular organelles. The table summarizes SIRT proteins according to their class, enzymatic activity, subcellular localization, their function, anatomic distribution, and related brain disease. "**AD:** Alzheimer`s disease; **PD:** Parkinson`s disease; **HD:** Huntington`s disease; **ALS:** Amyotrophic lateral sclerosis; **HE:** Hepatic encephalopathy; **MS:** Multiple sclerosis"

2.3. Calorie Restriction, SIRT Proteins and NDD

Calorie restriction (CR) is explained as reducing the amount of consumed calories in diet without causing any nutritional deficiency. In laboratory rodents, cutting down the ad libitum food intake by 30–40% is found to improve the animals` longevity by 50% [36]. Two types of CR protocols are commonly utilized in animal studies [1,37–39]. One of these CR is termed as chronic calorie restriction (CCR) and the other is termed as intermittent calorie restriction (ICR) [38,39]. These protocols are suggested to activate protective mechanisms in ND and diseases related to aging [27,40].

Recent research proposed that CR stimulates adult neurogenesis. Furthermore, it suppresses genes of inflammation and stress reactions, and as a consequence, these responses are all related to the anti-aging property of CR [41]. In a study Dong et al. have found a decreased cell density in the hippocampus of the mice fed by high-calorie diet and an increased cell density in the mice fed with a normal diet[42]. Loncarevic et al. explored head trauma on the mice and found a lower level of ND in mice with a CR diet [40]. In a study on the mice, Xu et al. tested the consequence of high calorie and CR on learning and remembering. They have found that mice with a high-calorie diet had impaired learning and memory function[43]. On the other hand, CR mice had improved learning and memory. In addition to that these mice had upregulated SIRT1 levels[43].

The number of high impact scientific research articles, especially on mammals, are currently increasing. We have summarized the pertinent literature on SIRTs, calorie restriction, and ND in **Table 2.** In these studies, mostly the level of SIRT 1 protein in the brain tissue was reported while the studies related to SIRT 3 and 6 are limited. For example, in a neurodegenerative research study, it was found that measured levels of SIRT 1 activation were increased after CR in brain samples [44]. In this research Ma et al. tested consequences of long term CR on mice and measured SIRT1 activity by WB, and they found that SIRT1 levels were higher in mice on high-calorie diet in comparison to CR mice [44]. CR slows aging in many species [39]. There are many studies about the related effects of SIRTs on NDD and the aging process. This topic is very up to date and, it`s one of the high yield fields in neuroscience.

Author, Year	Species, time	Intervention	Method	Results
Dong 2015[42]	10 MO mice CR	NC, CR and HC	Nissl staining-Hipp, Learning-memory evaluated- by Morris water maze	Decreased cell density in the HC group. CR group cell density increased
Ma 2015 [44]	6 MO mice 44 Wks CR	HC, CR	WB	Significantly increased SIRT1 immunoreactive cells in Hipp of CR fed mice compared to NC. CR group SIRT1 mRNA expression higher than the HC group
Natasa Loncare vic-2012 [40]	3 MO rats 50% CR for 3 months	CR	FJ for ND. Hoechst for neuronal death and WB for TNF alfa, caspase	Head trauma-cortical injury Lower level ND in CR in comparison to ad-lib, caspase and TNF-alpha levels lower in CR. SIRT are not explored
Xhang,2 010 [18]	10-13 Wks mice	İschemia model	FJ for ND, WB for level of PBEF, NAD (substrate for SIRT)	Role of PBEF identified. More ND in ischemia and ischemia volume with PBEF knock-out mice than wild type
Xiaoshe ng Fan, 2019 [26]	6 Wks mice	ND, apoptosis, aging	WB-SIRT 3 Apoptosis by TUNNEL method	Effect of AG on Hipp apoptosis, SIRT3 proapoptotic in cancer cells but inhibits apoptosis in cellular DNA damage. SIRT 3 Boosts apoptosis in AG induced HT in mice Hipp neurons.
Cohen, 2004 [25]	12 MO rats Human embryonic kidney cells	CR %60 of ad-lib	SIRT1 protein levels measured by Gel electrophoresis Cell culture model Apoptosis evaluated	SIRT1 inhibits stress induced apoptotic cell death, in brain, fat, liver, kidney
Davari,2 020 [45]	Type 2 DM Human serum	No intervention	SIRT1 levels measured by ELISA	Tested for a change in SIRT1 levels after cinnamon supplementation
Contesta bile, 2004 [41]	Rats 8 Wks 6 MO CR	CR, ad -lib	WB for P75(neurotropic protein)	CR is protective against ND
Fusco 2012 [27]	Mice, 4 Wks CR	HC, CR	WB for SIRT1 Immunofluorescence analysis for CREB1 in cortex and Hipp	SIRT1 and gene induction of neurons and survival in the cortex and Hipp of CR animal were reduced by CREB deficiency. Transcription of the SIRT1 is regulated by CREB the in neurons.
Xu, 2015 [43]	Mice, 6 MO CR	HC, CR	Water maze for learning. PCR for SIRT1 mRNA expression, WB for SIRT,p53, p16, PPAR IH for Hipp neurons	Tested HC and CR on learning, remembering. HC effects worse than CR on learning and remembering SIRT1 increased and p53, p16, and PPARγ decreased by CR
Ehara, 2009 [46]	Rats, ZMR 1,4,12 MO	6-HD Injection for acute ND of dopaminergic neurons, ZMR for chronic ND	FJ for ND and immunolabeling of tyrosine hydroxylase of DA neurons	FJ detects chronic neuronal degeneration. FJ jade C may bind to aggregated materials intracellularly.

Machado, 2020 [47]	C57B56 Mice (same with our species)	KA induced Hipp.injury. Calpastatin inject	FJ for ND and behavioral tests like water maze	Calpain inhibition prevents the impairment in new neuron formation. Cognitive function was preserved by calpain inhibition via calpastatin
Ballok, 2003 [48]	Fas-deficient MRL-1pr mice, 5 MO	SLE model in mice evaluating ND	FJ B method and image quantification. Tunnel method. T cell infiltration by IH	There is a link between autoimmunity and ND. FJ is more specific than Tunnel method. Tunnel method is reliable in apoptosis but it shows the late stages.
Lin 2019 [49]	OSA patients	New Mandibular device	Elisa for SIRT1 levels	Patients with OSA have lower SIRT1 levels compared to controls. Treatment with Mandibular device elevated SIRT levels
Noureldein 2015 [50]	Obese patients with DM	Medications Fenofibrate and pioglitazone	Elisa for SIRT1	SIRT1 levels were lower in obese type 2 DM patients than obese without DM. Fenofibrate and pioglitazone increased SIRT levels

Table 2. Pertinent literature on Sirtuins, calorie restriction, and neurodegeneration are summarized in the table.**6 HD**: 6-hydroxydopamine; **AG**: angiotensin 2; **CREB**: cAMP responsive-element binding; **CR**: Calorie restriction; **DM**: Diabetes type 2; **FJ:** Fluoro-Jade; **HC**: High calorie; **Hipp**: Hippocampus; **IH**: Immunohistochemistry; **KA**: Kainic acid; **MO**: months-old; **ND**: neurodegeneration; **NAD**: Nicotinamide adenine dinucleotide **NC**: Normal calorie; **PBEF**: rate-limiting enzyme in NAD production-which is a substrate of SIRT; **OSA**: Obstructive sleep apnea**; SIRT**: Sirtuins; **SLE**: Systemic lupus erythematosus; **WB**: Western blot; **Wks**: weeks-old; **ZMR**: Zitter mutant rats

3. MATERIALS AND METHODS

3.1. Mice and research setup

Eight-weeks old MMTV-TGFα (C57BL/6) female mice were enrolled. Research started after 2 weeks of adaptation period [38,39]. Mice were kept underneath standard circumstances in cages at a temperature of 21–24℃ and 12 hr dark/light cycle and unrestricted access to tap water and were held separately. Mice were observed daily for any health problem. All measures were performed under the instructions and with the agreement of Yeditepe University Animal Care and Use Committee no: 603-12/5/2017.

Mice were registered into three special dietary allocations randomly at 10 weeks of age. Allocated groups were: Ad-libitum (AL), chronic calorie restriction (CCR), or intermittent calorie restriction (ICR). All mice were fed with Altromin TPF 1414 diets that were supplied from Kobay AS (Ankara, Turkey). Throughout the study mice in the AL group had unrestricted access to nourishment. Mice that were provided 85% of the daily nutrition intake of the age-matched AL group were assigned to the CCR group. This corresponds to a 15% calorie decline in comparison to the AL group. Mice that were fed with 40% of AL fed mice calorie intake for 1 week and then were fed AL for the following 3 weeks were assigned to the ICR group and; this also resulted in an overall calorie reduction of 15% compared to AL mice [38]. Until mice were decapitated at chosen time points, this protocol was applied to ICR mice in cyclical periods. The number of animals in each experimental group is summarized in **Table 3**.

Groups	**10th week (Baseline)**	**17/18th week**	**49/50th week**	**81/82th week**
AL	8	2	7	3
CCR		7	7	3
ICR-R		3	*7*	6
ICR-RF		-	7	-
Total	8	12	28	12

Table 3. Experiment groups are summarized in the table divided into "three different dietary groups: Ad-libitum, chronic calorie restriction, or intermittent calorie restriction". Mice were allocated into different weeks for sacrification. ***SIRT levels were measured in 49/50 week groups.*** "**AL**: Ad-libitum, **CCR**: chronic calorie restriction, **ICR**: intermittent calorie restriction, **ICR-RF**: ICR refeeding, **ICR-R**: ICR calorie restriction".

Food consumptions of all mice were measured after they were nourished daily at 9 a.m. Every Monday at 9 a.m body weights were measured. A veterinarian checked the healthiness of the mice weekly. Animals were fasted during the night and were sacrificed at designated ages. The designated ages were 10 (baseline), 17-18; 49-50; and 81-82 weeks of age. ICR fed mice were further divided into two groups. The ones at the end of 3 weeks of AL feeding (Weeks 17, 49, and 81) were referred to as ICR-refeed (ICR-RF). The ones at the end of the 1 week of CR period (Weeks 18, 50, and 82) were referred to as ICR-restricted (ICR-R). Previous studies indicated that there were no differences for several parameters including body weights of AL and CCR group for the 1-week differences [38,39]. Therefore, the data were combined for 17 and 18, 49 and 50, 81, and 82 at each time point for AL and CCR groups. Experimental design and number of the sacrificed animals were represented in **Figure 3**.

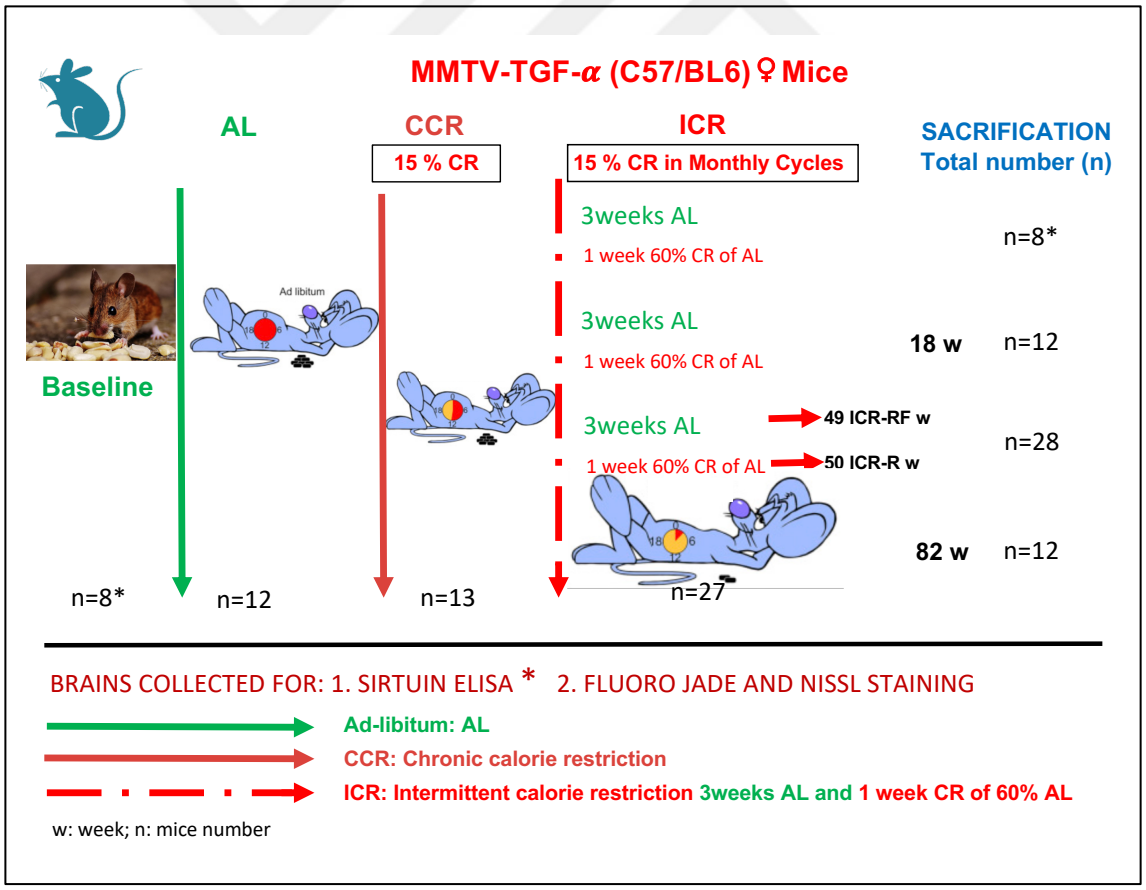


Figure 3. Experiment chart including all groups.

After decapitation half of the brain and spinal cords were placed in formaldehyde for histopathological analysis and the other half were preserved at -80℃ until laboratory analysis. Brain and spinal cord samples were fixated by 10% formalin and embedded into paraffin blocks. Six sections of 20 micrometers from the paraffin blocks were cut by a microtome and the sections were left to dry during night time (16-18 hours) at 37℃.

3.2. Measurement of Sirtuin levels

Sirtuin 1, 3, and 6 levels in whole brain tissue of mice in AL, CCR, ICR-R, and ICR-RF groups at 10 (baseline) and 49/50 weeks old mice were measured using ELISA method. SIRT 1, 3, and 6 ELISA kits were used to measure sirtuin levels according to the protocols [45,50–52]. ELISA kits were purchased from Bioassay Technology Laboratory, SIRT1: Cat.No E1563Mo; SIRT3: Cat.No E2586Mo; SIRT6: Cat.No E2587Mo.

Specimen collection: Brain samples kept in -80°C freezer were removed and washed with PBS for three times. Then, samples were weighed and homogenized with the Bullet Blender at speed 8 for 3 minutes (0,10 gauge beat for each).The supernatant was taken into clean tubes and PBS was added then, each sample was aliquoted total of 100 µl volume.

Assay procedure: SIRT1, Cat.No E1563Mo, Standard Curve Range: 0.05ng/ml – 30ng/ml. Samples were diluted 5 times in PBS. SIRT3, Cat.No E2586Mo, Standard Curve Range: 0.1ng/ml - 40ng/ml. Samples were diluted 15 times in PBS. Standards were between 24ng/ml and 0 ng/ml. SIRT6, Cat.No E2587Mo. Standard Curve Range: 7 ng/ml – 1500 ng/ml. Samples were diluted 15 and 30 times in PBS. Standards were between 800 ng/ml and 0 ng/ml.

ELISA reagents were added into each well after all reagents, samples and standards were prepared. This was incubated for 1 hour at 37°C. After incubation, we washed the plate 5 times and we added substrate solutions A and B. We incubated the solution for 10 minutes at 37°C. After this incubation stop solution was added and color developed. OD value was read within 10 minutes. (https://arquerdx.com/arquer-diagnostics-2017-year-review/elisa-plate-pipetting-blue_preview)

3.3. Cresyl violet (Nissl) Staining

The cresyl violet stain was used to stain Nissl bodies in the neuron. In our cresyl violet staining, we deparaffinized the brain sections and washed them, first by using 100%

and 95% alcohol, and rewashed them by distilled water to remoisturize the brain sections. The brain sections were then incubated with 0.1% cresyl violet stain for 20 minutes and rewashed with distilled water and they were left to dry after cleaned up with xylene. CA1, CA3, and dentate gyrus (DG) sections were picture framed by a fluorescence microscope (Zeiss, fluorescein microscope, Switzerland). Stained cells were counted by an automatic marking software (Image J 1.52q, NIH, USA) separately in blind eye fashion by two different researchers who were unaware of the groups.

3.4. Fluoro Jade B Staining of Tissue Sections

We have stained hippocampal tissue with Fluoro Jade B (FJB) in order to determine neuronal damage in the current Ph.D. thesis. FJB is an anionic fluorescein byproduct used for the histopathological marking of neurons undergoing degeneration like its predecessor Fluoro-Jade [46–48]. Regardless of the type of cell death, the FJB stain has an affinity for the whole degenerating neuron involving the cell body, dendrites, axon, and axon terminals [48]. FJB can be visualized using a fluorescein/FITC filter and it has an "excitation and emission peak of 480 and 525 nanometer" respectively. FJB staining was completed following the guidelines described below. For the staining FJB (Cat. AG310-30MG), Potassium permanganate ($KMnO_4$), Glacial Acetic acid (CH_3CO_2H), Ethanol Absolute, Xylene were used. For preparation of Stock (0.01%) FJB solution: 0.01 gr FJB was diluted in 100 ml of sterile water then kept in dark shelters at -20 °C. For the staining (0.0003%) FJB solution: a total of 3 ml stock solution was added to 96 ml of 0.1% acetic acid in order to obtain 100 ml of staining solution. For 100 ml 0.1% acetic acid, 10 µl glacial acetic acid was added to 99.9 ml of distilled water. The staining solution was prepared within 10 minutes before it was used.

3.4.1. Procedure

Slides were incubated in an incubator at 70°C for 60 minutes. Making sure that paraffin is completely melted. Warm slides were incubated in xylene at room temperature for 20 minutes respectively to remove paraffin. Each set of xylene was used fresh. If there was still paraffin on slides, incubation time lengthened. Dehydration of slides were performed as follows: slides were incubated in 100% Ethanol 3 times for 3 minutes each. Then slides were incubated in 95% Ethanol 2 times for 3 minutes each; slides then were

incubated in 70% ethanol for 3 minutes and then they were incubated in 50% Ethanol for 3 minutes.

Slides were rinsed with distilled water for 5 minutes. All steps after this were made in the dark. Slides were incubated in 0.06% potassium permanganate at room temperature for 5 minutes. Slides were rinsed with distilled water for 3 minutes. Slides were incubated in 0.003% FJB solution at room temperature for 10 minutes and gently shook on orbital shaker. Slides were rinsed with distilled water 3 times for 3 minutes each and, dried slides in the incubator and we covered slip with DPX (non-fluorescent mounting medium) and wait until completely dry at room temperature before microscopic analysis.

3.4.2. Data analysis

Microscopic brain cortex and coronal sections of the hippocampus were assessed with a fluorescent microscope (Zeiss, fluorescein microscope, Switzerland). All the pictures were taken from the same locations in hippocampal slices as shown in the **Figure 4** [53].

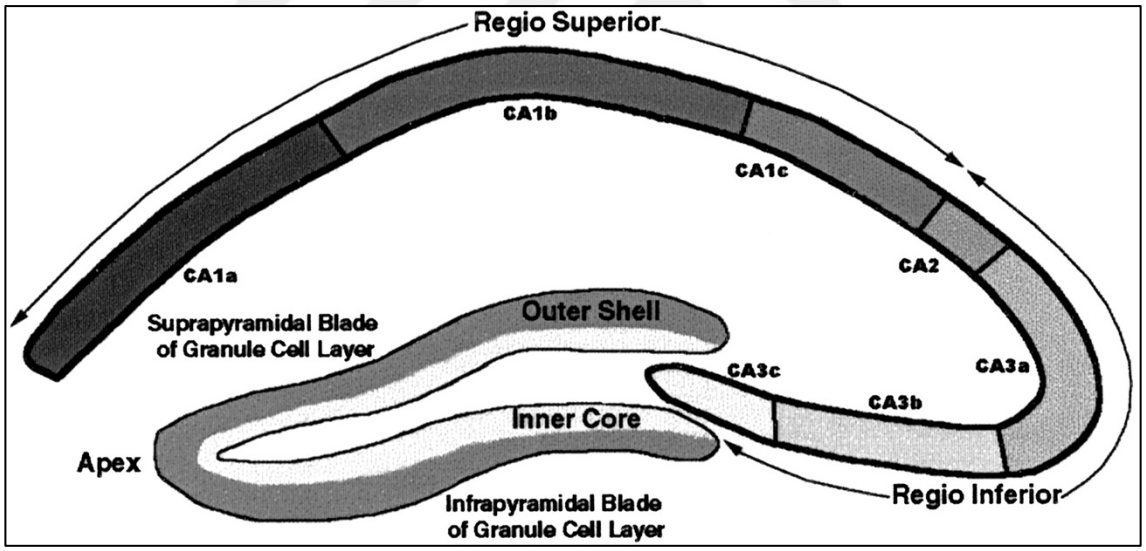


Figure 4. Illustrated section of the hippocampus demonstrating important regions. Regio superior, regio inferior, outer shell, inner core, apex. CA 1, CA 2, CA 3. (Adapted from Martin et. al. J.of.Neuroscience(1))

Images were captured with Zeiss software. Throughout the same analysis, the pictures were documented in the same imaging settings. Image J application (National Institutes of Health, Bethesda, MD, USA) was used to quantify the number of FJB + cells. We used

the Image based tool for counting nuclei (ITCN) plug-in of the Image J software after measuring typical dimensions of the FJB + neurons.[54,55] This plug-in automatically marks and counts the FJB + cells in the region of interest (ROI). (**Figure 5**). Cell dimensions for this plug-in were determined by measuring the sample cell diameters, intercellular distance, and thresholds in Image J software under high magnification. We checked the correct marking of the FJB cells by a magnification tool that is built in the software after counting the cells to review if the correct cells were counted (**Figure 5**). Values obtained from each group were averaged and groups were compared.

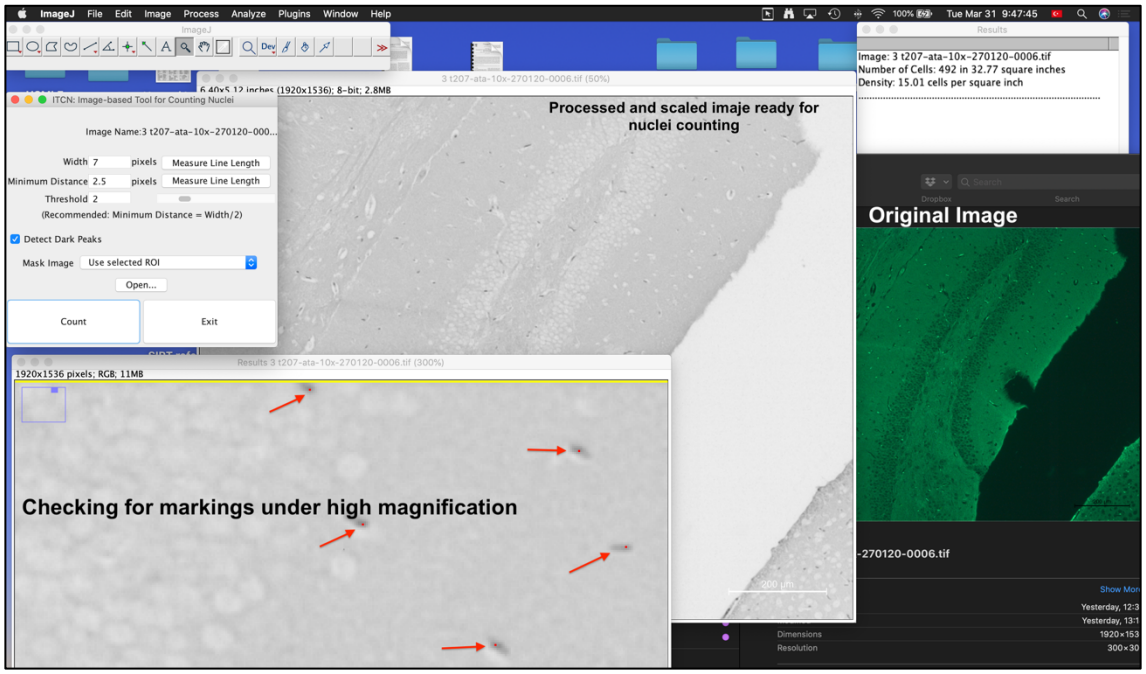


Figure 5. The number of Fluoro-Jade B (FJB) + cells were quantified by using Image J software. Image based tool for counting nuclei plug-in of the Image J software was utilized after measuring typical dimensions of the FJB + neurons. This plug-in automatically marks and counts the FJB + cells in the region of interest (ROI). The figure shows a sequence of image quantification including original image, quantification setting, checking of the counted cells under high magnification whether right cells were marked (red dots of the software were indicated by arrows on the counted cells). **FJB**: Fluoro-Jade B

3.5. Statistical Evaluation

The data were analyzed by a *one-way analysis of variance (ANOVA*) and *Student's t-test* where appropriate. *Fisher test Two-sample for variances* was used to measure equal or unequal variances between the variables before using the *t-test*. Computations were performed using the Excel (version.16.16.20) for.Mac.statistical package, and the accepted level of significance was $P < .05$. Graphs show *means* ± *Standard deviation (Std)*, with $*P < .05$ representing the significant difference between groups.

4. RESULTS

Effects of Different Type of Calorie Restriction on Sirtuin Levels in Brain

4.1. Sirtuin 1 Results

SIRT1 levels were measured in brain samples from 10 (baseline) and, 49-50 weeks old mice in AL, CCR, ICR-R, and ICR-RF groups (**Figure 6**). *One-way Anova test* revealed a significant difference among the groups ($P < .05$). There was no significant difference between the mean values of Baseline, AL group, or ICR-RF group by *student t-test* analyses ($P > .05$). However, the SIRT1 level of ICR-R was significantly higher than the 10 weeks baseline group ($P = .02$) (Figure 6). In addition, SIRT1 levels were also significantly higher in the ICR-R group than that of the AL group at 49-50 weeks ($P = .02$). Moreover, SIRT1 levels of CCR were higher than the baseline group if the *P* values were calculated by using only one-tail *t-test analyses* ($P = .05$) (Figure 6). Again 49-50 weeks CCR group SIRT1 levels were higher than 49-50 weeks of AL SIRT1 levels but the significance was only one-tail significant ($P = .05$). Figure 6 demonstrates SIRT1 levels in groups and levels of significance were demonstrated by Asterix and plus signs.

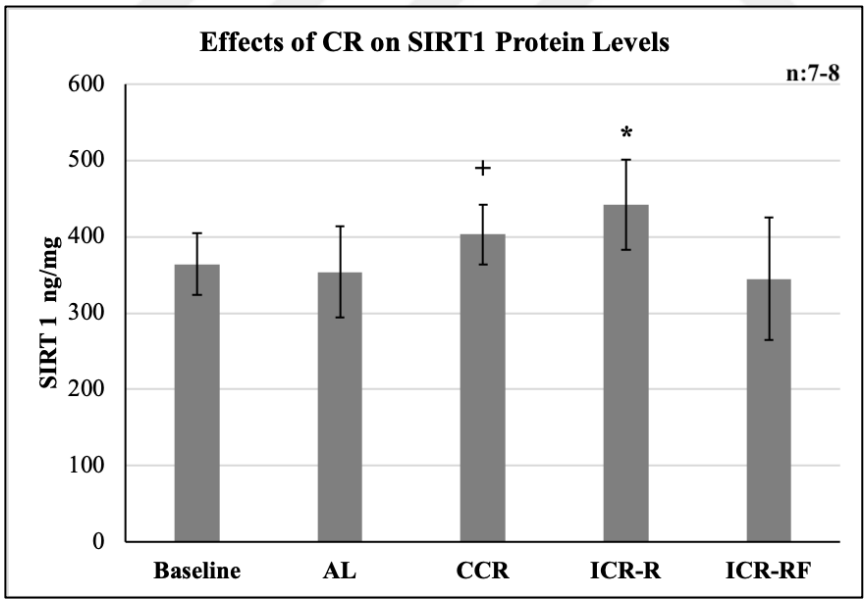


Figure 6. Sirtuin 1 ELISA results demonstrating a significant difference in CCR ($^{+}P< .05$-*one-tailed significance*) and ICR-R groups (*$P< .05$-*two-tailed significance*) from the baseline, Ad-lib, and ICR-RF groups. **AL**: Ad- libitum group; **CCR**: Chronic calorie restriction group, **ICR-R**: intermittent calorie restriction group, **ICR-RF**: intermittent calorie restriction refeeding group

4.2. Sirtuin 3 Results

SIRT3 results revealed a significant difference among the groups ($P < .05$, **Figure 7**). SIRT3 levels in AL and ICR-RF groups at week 49/50 were similar and there was no statistically significant difference between the groups ($P > .05$). There was also no significant difference between baseline and AL or ICR-RF groups. But, SIRT3 levels were significantly higher either in CCR ($P = .04$) or ICR-R groups ($P = .004$) compared to 10 weeks baseline. In addition, SIRT3 levels were significantly higher either in CCR ($P = .02$) or ICR-R groups ($P = .003$) than the AL group at week 49/50 (Figure 7). Figure 7 demonstrates SIRT3 levels in groups and levels of significance were demonstrated by Asterix signs.

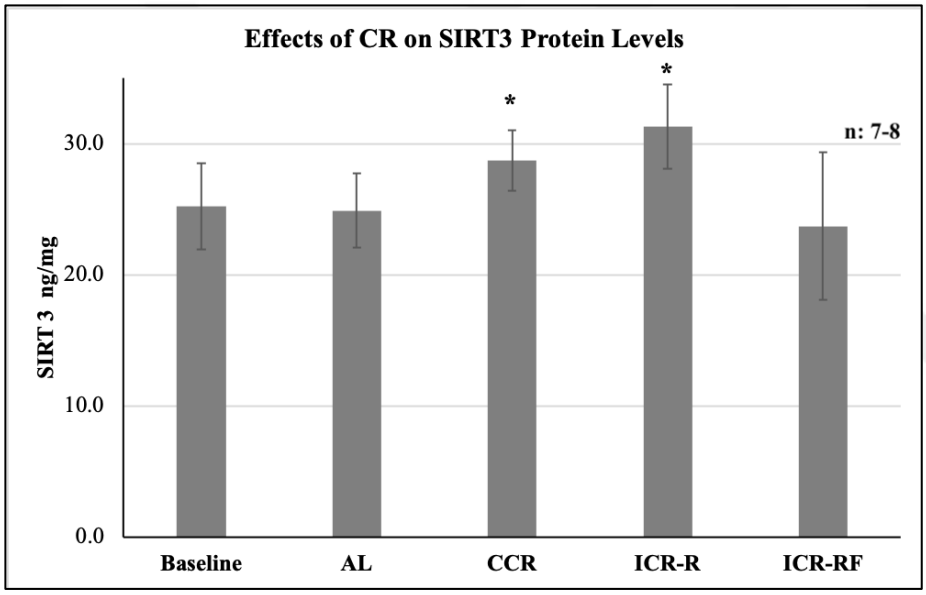


Figure 7. Sirtuin 3 ELISA results demonstrating a significant difference in CCR (*$P< .05$) and ICR-R groups (*$P< .05$) from the baseline, Ad-lib, and ICR-RF groups. **AL**: Ad-libitum group; **CCR**: Chronic calorie restriction group, **ICR-R**: intermittent calorie restriction group, **ICR-RF**: intermittent calorie restriction refeeding group

4.3. Sirtuin 6 Results

Sirtuin 6 results revealed a significant difference among the groups ($P < .05$). In groups of 10 weeks Baseline, and 49-50 weeks AL and ICR-RF mean SIRT6 levels were similar and not significantly different from each other ($P > .05$). But, SIRT6 levels were significantly higher in either CCR ($P = .03$) or ICR-R groups ($P = .002$) compared to

baseline (Figure 8). In addition, SIRT6 levels either in CCR (*P = ,04*) or ICR-R group were significantly higher than that of the AL group at week 49/50. **Figure 8** demonstrates SIRT6 levels in groups and levels of significance were demonstrated by Asterix signs.

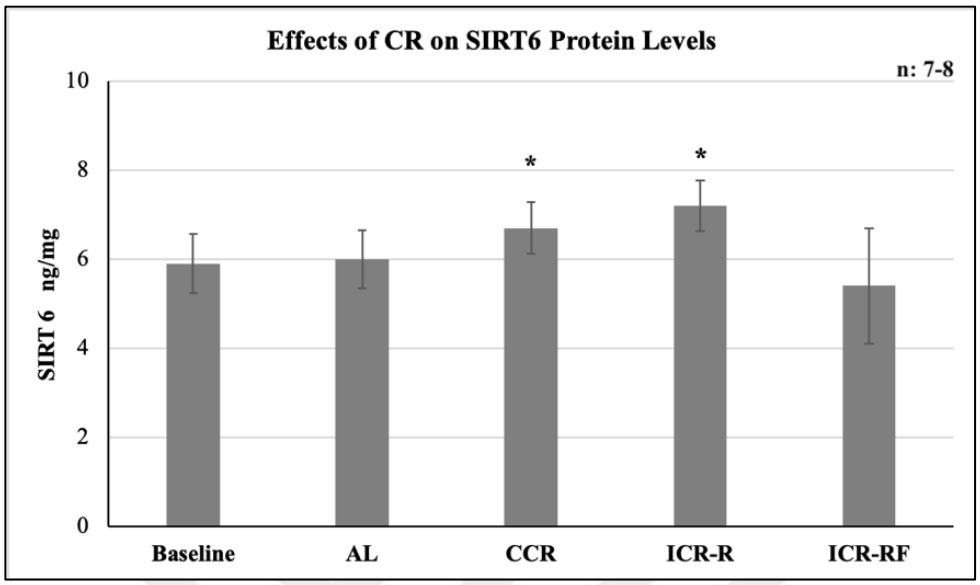
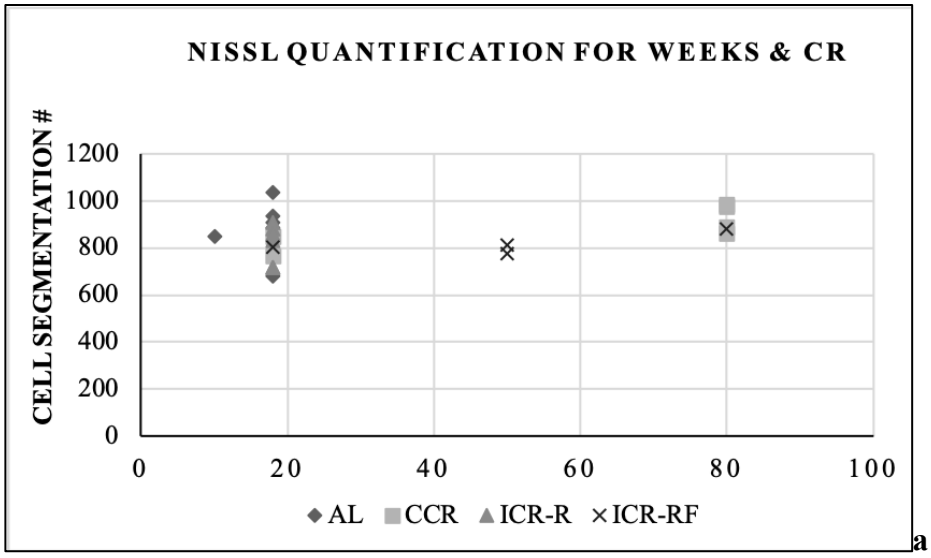


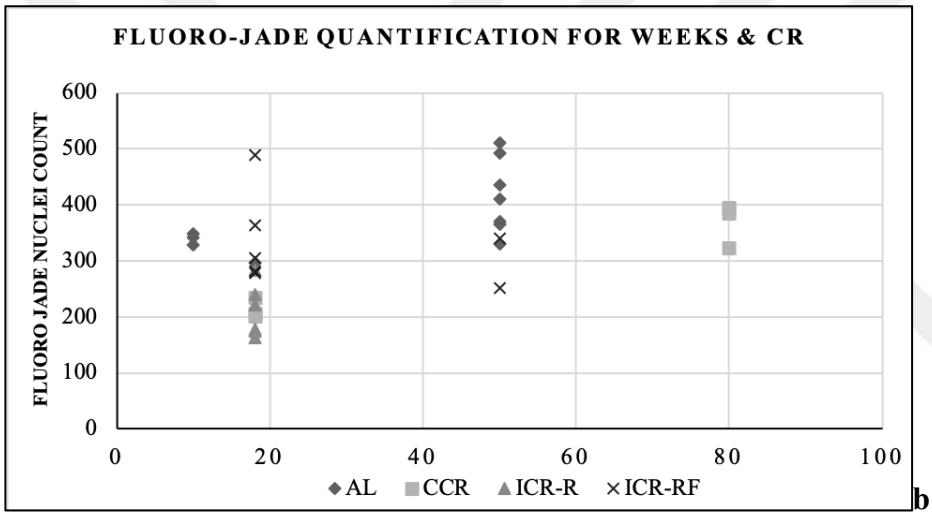
Figure 8. Sirtuin 6 ELISA results demonstrating a significant difference in CCR (*$P< .05$) and ICR-R groups (*$P< .05$) from the baseline, Ad-lib, and ICR-RF groups. **AL**: Ad-libitum group; **CCR**: Chronic calorie restriction group, **ICR-R**: intermittent calorie restriction group, **ICR-RF**: intermittent calorie restriction refeeding group

4.4. Histopathological Evaluation and Image Quantification Results

Histopathological evaluations of the brains were performed by using FJB and cresyl violet stains. All image quantification results were evaluated by scattered point graphs and *Anova*. **Figure 9** demonstrates the image quantification results of scattered point graphs of cresyl violet cell segmentation, and FJB nuclei counts respectively. Results were not significantly different when the mice were allocated and compared according to sacrification weeks and rendering this finding we combined the different sacrification weeks (either 17/18, 49/50 or 81/82) of each AL, CCR, ICR-R and ICR-RF group separately. As a result, we made quantifications only based on the type of CR and we ignored sacrification weeks.



a



b

Figure 9. Image quantification results were evaluated by scattered point graphs of cresyl violet cell segmentation (**a**), and FJB nuclei counts (**b**) respectively.

4.4.1. Effects of Various Calorie Restriction Types on Neurodegeneration in Brain

Cresyl violet-Nissl staining and image quantification results

Cresyl violet stainings of 18th week ICR mice hippocampus, CA1, CA3, and dentate gyrus (DG) sections were demonstrated in **Figure 10**. The analysis of these results is as follows. A dense network of interwoven nerve fibers and their branches were stained purple-blue. Neurons were identified by their basic anatomical structures. Nissl substance appeared dark purple in the figure as a result of staining of the ribosomal RNA and gives the cytoplasm a spotted presentation (Figure 10).

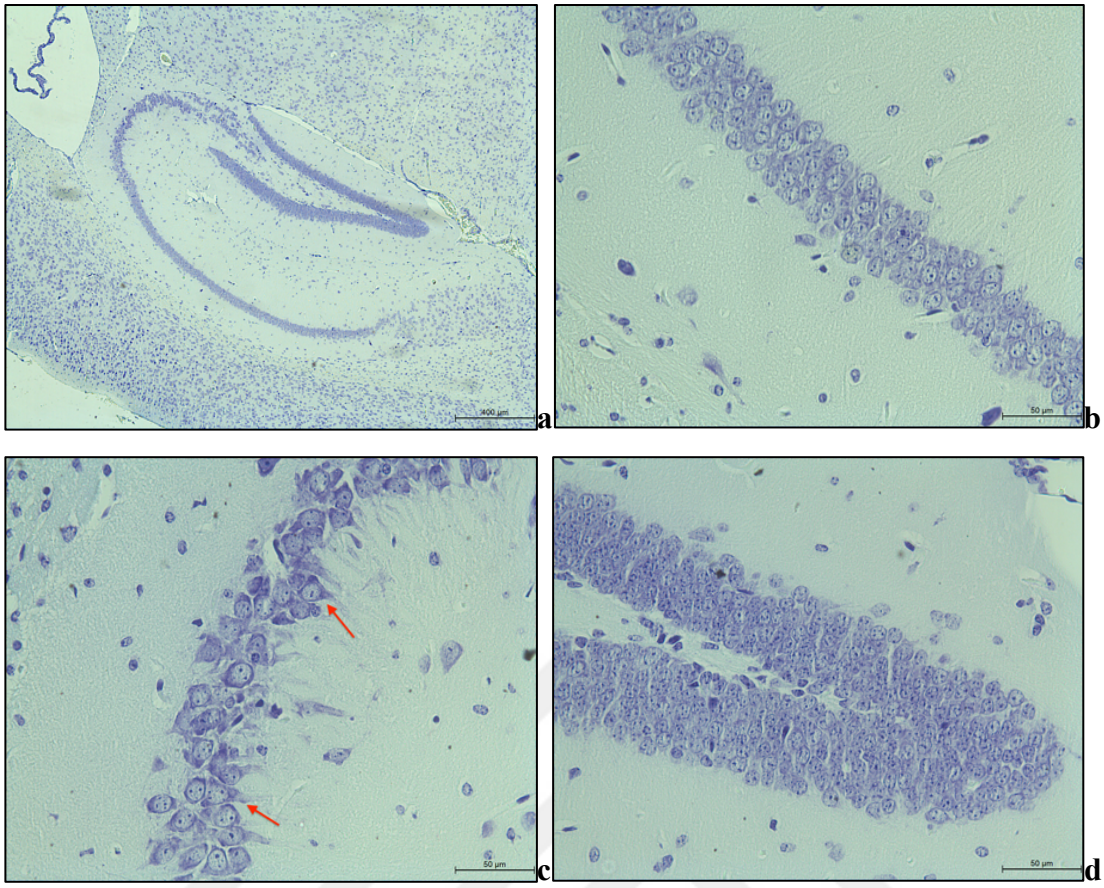
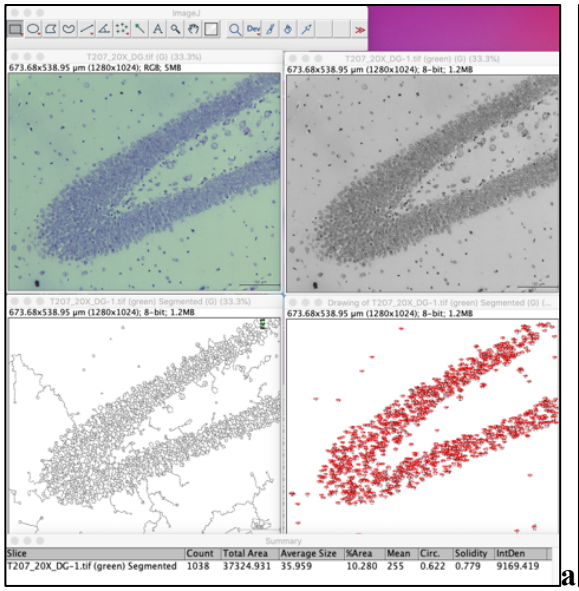


Figure 10. Cresyl violet stainings of 18th week ICR mice brain sections of Hippocampus (5X) (a) CA1 (40X) (b), CA3 (40X) (c), and Dentate gyrus (40X) (DG) (d) areas. A dense network of interwoven nerve fibers and their branches were stained purple-blue. Important structural features of the neurons were identified (arrows). In higher magnification, neuronal structures are more evident (arrows). Fluorescent microscope (Zeiss). (Bars are 400µm and 50µm)

Cresyl Violet (Nissl) image quantifications were made on the hippocampus DG sections of each group with the same magnification (20X). Image J software was used to make the segmentation and neuronal cell counting of the DG. Figure 11 demonstrates the image quantification in AL (Figure 11a), CCR (Figure 11b), ICR-R (Figure 11c), and ICR-RF (Figure 11d) groups. Statistical evaluation revealed no significant difference among the groups. $P > .05$ (Figure 11e).


ImageJ
T207_20X_DG.tif (G) (33.3%)
673.68x538.95 µm (1280x1024); RGB; 5MB
T207_20X_DG-1.tif (green) (G) (33.3%)
673.68x538.95 µm (1280x1024); 8-bit; 1.2MB
T207_20X_DG-1.tif (green) Segmented (G) (33.3%)
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Drawing of T207_20X_DG-1.tif (green) Segmented (G) (...
673.68x538.95 µm (1280x1024); 8-bit; 1.2MB
Summary
Slice Count Total Area Average Size %Area Mean Circ. Solidity IntDen
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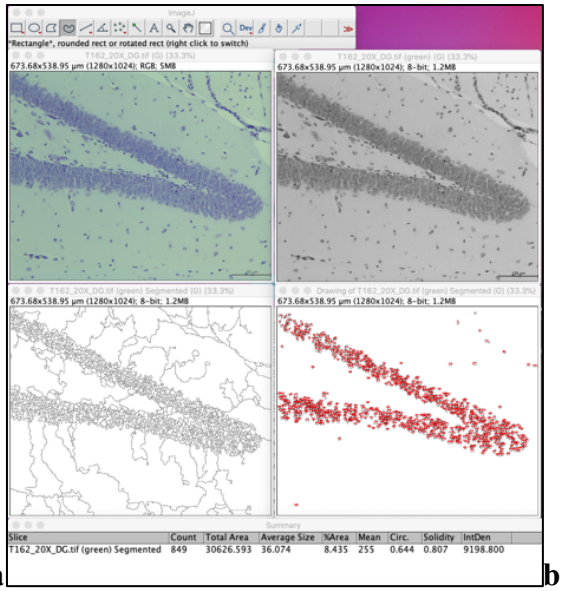
a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"Rectangle", rounded rect or rotated rect (right click to switch)
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T162_20X_DG.tif (green) (G) (33.3%)
673.68x538.95 µm (1280x1024); 8-bit; 1.2MB
T162_20X_DG.tif (green) Segmented (G) (33.3%)
673.68x538.95 µm (1280x1024); 8-bit; 1.2MB
Drawing of T162_20X_DG.tif (green) Segmented (G) (33.3%)
673.68x538.95 µm (1280x1024); 8-bit; 1.2MB
Summary
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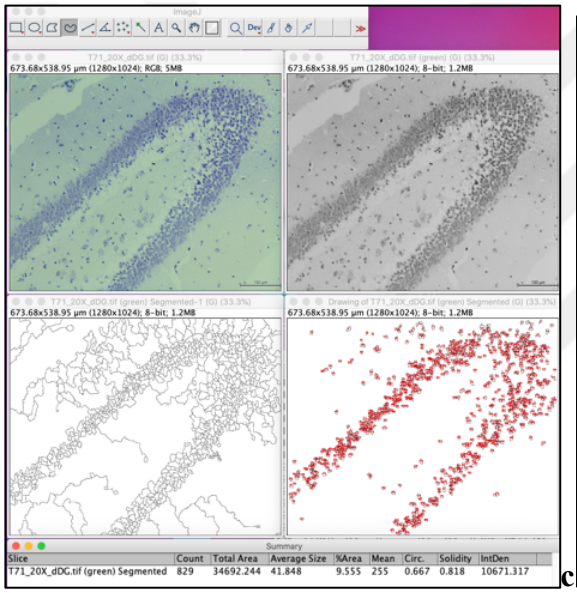
b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T71_20X_dDG.tif (green) Segmented-1 (G) (33.3%)
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Drawing of T71_20X_dDG.tif (green) Segmented (G) (33.3%)
673.68x538.95 µm (1280x1024); 8-bit; 1.2MB
Summary
Slice Count Total Area Average Size %Area Mean Circ. Solidity IntDen
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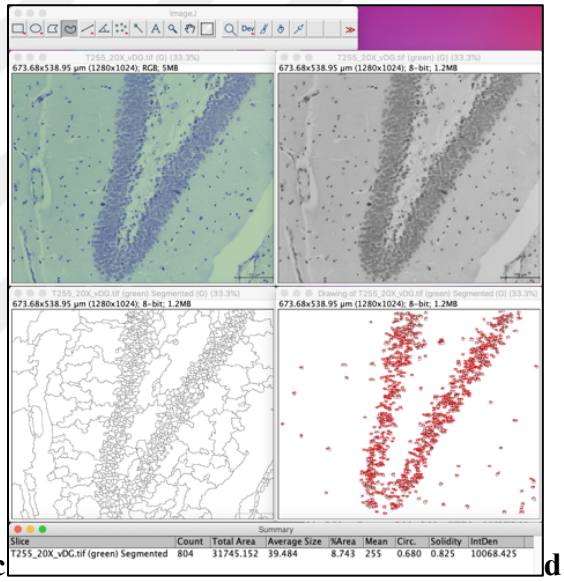
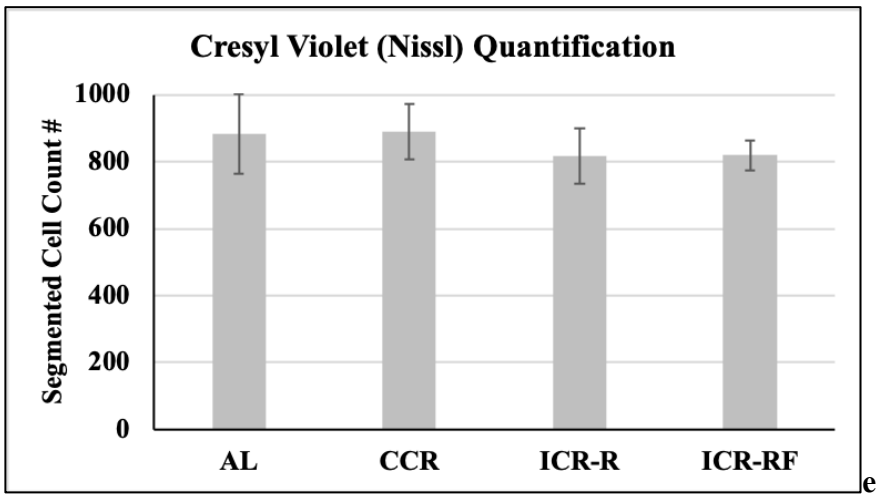
c


ImageJ
T255_20X_vDG.tif (G) (33.3%)
673.68x538.95 µm (1280x1024); RGB; 5MB
T255_20X_vDG.tif (green) (G) (33.3%)
673.68x538.95 µm (1280x1024); 8-bit; 1.2MB
T255_20X_vDG.tif (green) Segmented (G) (33.3%)
673.68x538.95 µm (1280x1024); 8-bit; 1.2MB
Drawing of T255_20X_vDG.tif (green) Segmented (G) (33.3%)
673.68x538.95 µm (1280x1024); 8-bit; 1.2MB
Summary
Slice Count Total Area Average Size %Area Mean Circ. Solidity IntDen
T255_20X_vDG.tif (green) Segmented 804 31745.152 39.484 8.743 255 0.680 0.825 10068.425

d


Cresyl Violet (Nissl) Quantification
Segmented Cell Count #
1000
800
600
400
200
0
AL
CCR
ICR-R
ICR-RF

e

Figure 11. Cresyl Violet (Nissl) image quantifications were made on the hippocampus dentate gyrus sections of each group with the same magnification (20X). Image J software was used to make the segmentation and neuronal cell counting. Image quantification is demonstrated in AL (a), CCR (b), ICR-R (c), and ICR-RF (d) groups. Statistical evaluation revealed no significant difference between groups. $P > .05$ (e).

4.4.2. Effects of Various Calorie Restriction Types on Neurodegeneration in Brain

Fluoro Jade B Staining image quantification results

Neurodegeneration was evaluated by FJB immunofluorescent staining. FJB stain has an affinity for the whole degenerating neuron. Cell bodies and processes of most FJB positive cells were bright green and displayed physiologic neuronal morphologies. They had apparent neuronal features in the cortex and hippocampus. AL groups revealed widespread neurodegeneration and + FJB staining was demonstrated. Comparative results of FJB staining of the AL group demonstrated increased fluorescein markings compared to the CCR group. Results of FJB staining of AL group demonstrated increased fluorescein markings compared to the ICR group, No difference was observed between the results of FJB staining of AL group compared to the ICR-RF group. (**Figure 12**).

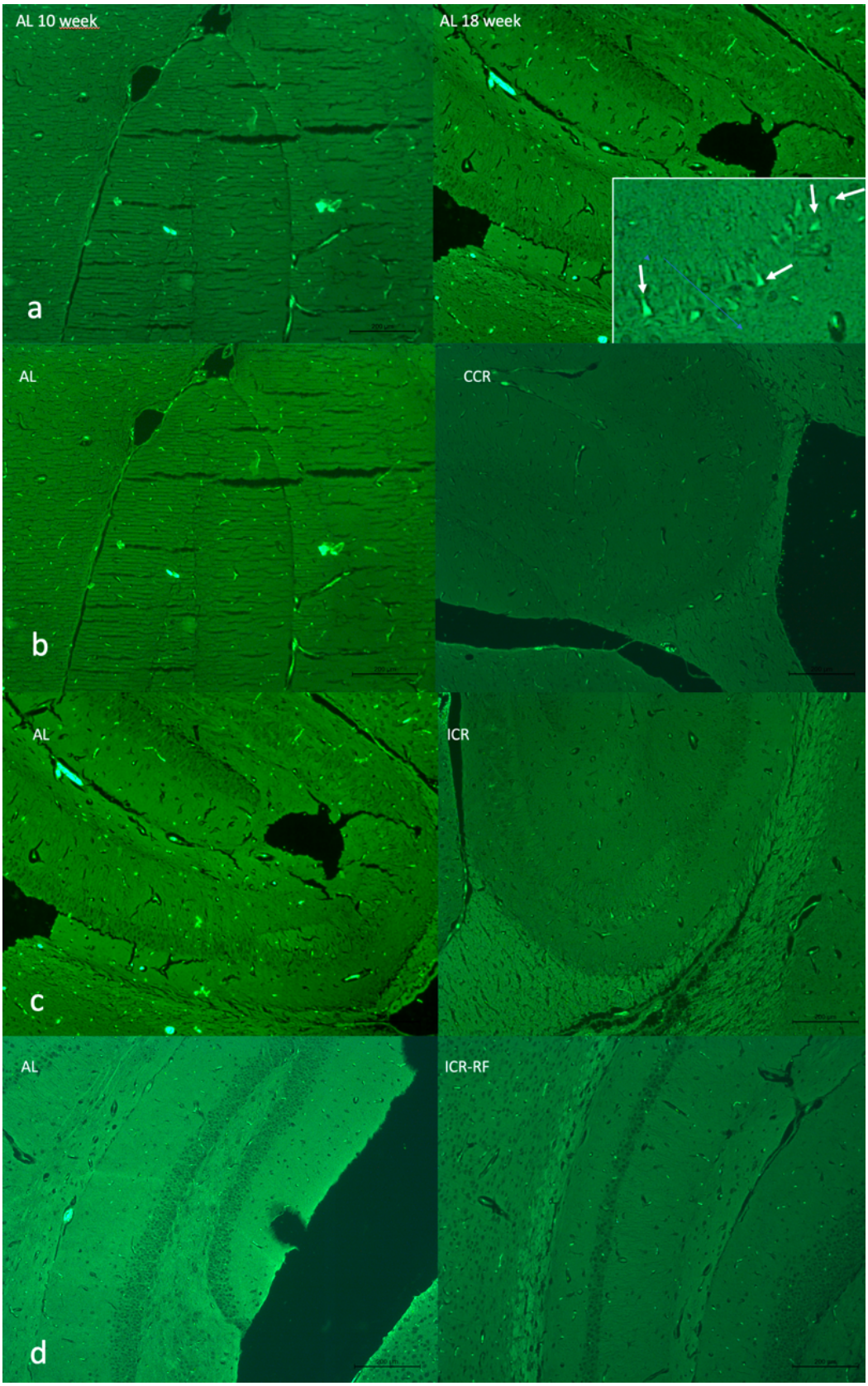


Figure 12. Neurodegeneration was evaluated by Fluoro-Jade B (FJB) immunofluorescent staining. FJB stain has an affinity for the entire degenerating neuron. Cell bodies and

processes of most FJB positive cells were bright green and exhibited neuronal profiles. They were identifiable by their apparent neuronal-like morphology in the cortex and hippocampus. In the figure, AL groups of 10 and 18 weeks with widespread neurodegeneration and + FJB staining were demonstrated (magnified figure inlet with white arrows showing FJB + neuronal bodies) (a). Comparative results of FJB staining of the AL group demonstrated increased fluorescein markings compared to the CCR group (b). Results of FJB staining of AL group demonstrated increased fluorescein markings compared to ICR group (c), no difference was observed between the results of FJB staining of AL group compared to ICR-RF group (d). **AL**: Ad-libitum; **CCR**: Chronic calorie-restricted group; **FJB**: Fluoro-Jade B; **ICR-R**: Intermittent Calorie Restriction; **ICR-RF**: Intermittent calorie restricted-refeeding group (10X magnification) (Bars 200µm)

FJB image quantification results were obtained from the *Image-based tool for counting nuclei plug-in* (ITCN) of the Image J software. Quantification of the FJB fluorescein staining by Image J revealed an elevated number of FJB + degenerated neurons in the AL group compared to either CCR ($P = .04$) or ICR-R groups ($P = .04$). There was not any significant change between AL and ICR-RF groups. **Figure 13** demonstrates the statistical significance among the groups.

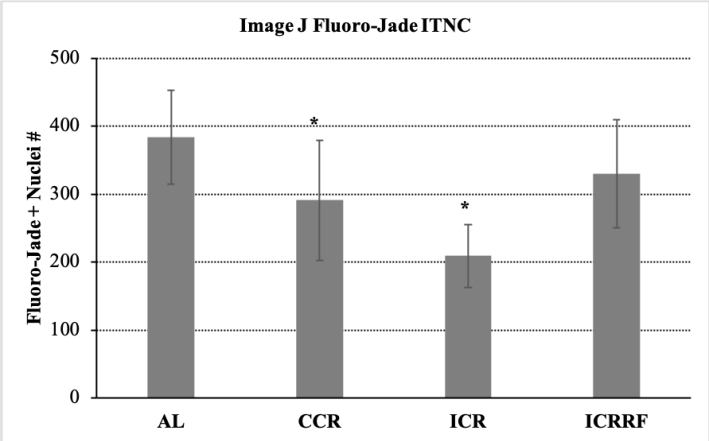


Figure 13. Fluoro-Jade B (FJB) image quantification results obtained from the Image-based tool for counting nuclei plug-in of the Image J software. Quantification of the FJB fluorescein staining by Image J revealed an increased number of FJB + neurodegenerated

cells in the AL group compared to CCR (*$P = .04$) and ICR-R groups (*$P = .04$). **AL**: Ad-libitum group; **CCR**: Chronic calorie restriction group, **ICR-R**: intermittent calorie restriction group, **ICR-RF**: intermittent calorie restriction refeeding group

5. DISCUSSION AND CONCLUSION

In this thesis study, we intended to investigate the complicated interplay concerning CR, ND, and SIRT proteins in the mice brain. The beneficial effect of two types of CR (CCR and ICR) feeding on the ND process in the mice brain and SIRT1, SIRT3 and SIRT6 expression levels were investigated. Thus, we aimed to clarify whether SIRT levels were elevated due to the CR type and whether this change has a correlation to the ND process in the mice brain. For this purpose, we applied 3 different diets as CCR, ICR, and AL (ad-lib, free eating) and measured the SIRT levels at 10 weeks baseline, 49, and 50 weeks in groups of AL, CCR and ICR either ICR-R or ICR-RF of mice brain. Neural cells were marked with Nissl stain and ND was evaluated by immunofluorescence by FJB stain to compare different dietary groups. SIRT1, SIRT3 and SIRT6 protein levels were measured by the ELISA. We have found that brain tissue levels of SIRT 1, SIRT 3, and SIRT 6 in the CCR and ICR-R groups were higher than AL at weeks 50 and baseline control at week 10. In addition, less ND was determined in the CCR and ICR groups compared to other dietary types. These results demonstrated that CR increases the SIRT expression levels in mice brain and leads to less ND. This information, contributed to the literature by the novelty of our study in measuring SIRT levels and evaluating ND by FJB in different types of CR especially by utilizing ICR.

CR was shown to promote survival in organisms ranging from yeast to rodents, and perhaps primates [37,55]. Even in mammals, CR delays many age-related diseases like cancer, ND, atherosclerosis, diabetes mellitus. In addition, many studies are revealing that the CR is neuroprotective especially at old age or cerebral injuries [37]. For example, Loncarevic et al. showed that 3 months of 50% CCR before head trauma and cortical contusion prevented neuronal death which is determined by Hoechst stain. Furthermore in this study suppression of microglial activation, and increase in the level of TNF-α, caspase-3 were observed. Even more interestingly the level of ND was decreased, which was determined by FJB staining during the recovery period of injury in rats [40] . However, they have not measured SIRT activity in this research [40]. In another study Contestabile et al. applied 6 months of CCR on rats and they have made a neurotoxic cerebral injury, by stereotaxically injecting ibotenic acid into the telencephalic area of the nucleus basalis

magnocellularis, which is an ideal model that consistently ends in extensive deterioration of the neurons [41]. When Contestabile et al. measured choline acetyltransferase and glutamate decarboxylase levels by ELISA they have demonstrated lower levels indicating ND in AL group compared to CCR [41]. They have not measured SIRT levels but they measured P75 protein levels to determine ND in hippocampal neurons, and they have also demonstrated decreased ND in CR rats compared to AL [41]. Beneficial and metabolic effects of CR might be mediated by the SIRT gene family [2,20,22,56–58]. Although some studies are demonstrating the link between protective effects of CR and SIRT1 levels, to the best of our knowledge the possible contribution of other SIRT family proteins were not studied extensively and the mechanism of these beneficial effects is not yet clear.

In this study, we have shown that the SIRT1 levels of mice brain were lower in the CCR and ICR-R groups compared to AL at week 50 and baseline control at week 10. In the literature there are several studies in line with our CCR findings however mostly in the literature SIRT1 measurements were performed via non-quantitative measurement techniques such as western blot or immunoreactivity. For example, Ma et al. have found improved memory and learning in mice after CCR, and CCR mice showed upregulation of SIRT1 expression [44]. In their study, they have tested 44 weeks of CCR in mice and have demonstrated that SIRT1 immunoreactivity was significantly increased in the hippocampus compared to controls. They have determined SIRT1 levels by WB and SIRT1 mRNA levels by PCR and reported that CR up-regulates SIRT1 levels and down-regulates mTOR signaling and as a result CR can modify neural cell growth and survival [44]. It is concluded in the same study that protein synthesis, protein degradation, inflammation, and apoptosis, including mTOR-mediated autophagy and other biological activities like aging were all related to SIRT1 protein [44]. Currently, it is getting more evident in mammals that SIRT1 is the main controller of existence in response to strain and cell defense [25]. In this thesis, SIRT1 levels were significantly increased and ND was decreased in CCR and ICR fed groups, which support previous findings of SIRT1 in reaction to metabolic strain. Cells try to defend and repair themselves in response to stress, but if they are not successful they undergo programmed cell death, which is called apoptosis. In addition, Xu et. al. explored the consequences of CR and high calorie on learning and memory in mice [43]. They utilized water maze for learning and memory after 6 months of CR. They measured the levels of SIRT 1, p53, p16 and, PPAR via Western blot. They have evaluated SIRT1 mRNA expression by PCR and examined the

hippocampal neurons by immunohistochemistry. They have discovered that a high-calorie diet compromised learning and memory function. They also showed that CR upregulated SIRT1 expression and down-regulated p53, p16, and PPARγ [43]. These results were in concordance with our findings in CR animals.

Sirtuins are seven protein complexes and they are well preserved in evolution [44]. They have a very critical function in aging, apoptosis, and ND by controlling cellular health. We preferred to investigate SIRT in ND and focused on the levels of SIRT1, SIRT3 and SIRT6 because these SIRT proteins have very important functions in response to CR and multiple physiological regulations, and effects on neurons like cognition and aging. SIRT3, protects the cell from metabolic or oxidative stress by its critical localization in the mitochondria [2,31]. Deficiencies of SIRT have been shown to aggravate ND in toxic neurological diseases [2]. Synaptic plasticity and memory formation are very critical neural functions and it has been cited that SIRT1 modulates these functions [59]. SIRT1 and SIRT3 present the greatest action in the neurons and are stated to control most of the critical brain functions and thus, considered to be involved in the pathogenesis of almost all neurodegenerative brain disorders [31]. Behavior and cognitive functions of SIRT3 knockout mice were examined by Kim et al. and poor remote memory was observed in these mice [59]. Long term memory was also diminished in the SIRT 3-knockout mice [59]. Kim et. al. have observed a decreased number of neurons in the anterior cingulate cortex, and authors have concluded that this finding has contributed to the deficiencies in the memory [59]. The importance of both SIRT1 and SIRT6 comes from their regulatory effect on NF-κB. NF-κB regulates the action of the genes taking part in apoptosis, cell aging, inflammation, and immunity, and its activity increase with age in many mammalian tissues and stem cells [60]. In multiple human and mouse tissues NF-κB has associations in the activation of age-related transcriptional changes and, SIRT1 and SIRT6, inhibit transcription of genes by NF-κB [61]. NF-κB blockade increased the proliferative capacity of the skin and reversed several markers of cellular aging to levels that are observed in young animals [2]. In conclusion blockade of NF-κB by SIRT may prevent pro-inflammatory disorders like NDD. In addition to these findings, SIRT 6 has important functions in the pathophysiology of AD. SIRT 6 has been recognized to take part in telomere preservation, DNA repair, genome integrity, energy metabolism, and inflammation, which eventually control the duration of the life [8]. The latest discoveries

also determined that SIRT 6 is missing in AD patients, suggesting that it can be a new possible curative target in AD [8]. Our findings of increased SIRT1, SIRT3 and SIRT6 levels in CR groups in our research and increased positive staining with FJB in baseline and AL groups supports the findings of NDD in the literature. The positive correlation of increased SIRT levels in CR groups clarified the importance of the SIRT proteins in ND. Our findings of increased SIRT expression in ICR groups is a novel contribution to the pertinent literature which can be a therapeutic strategy in the treatment of NDD like AD or PD in the future.

An important comprehension of regulation of aging in yeast and post-mitotic aging in worms both regulated by SIRT genes comes from a consideration of the biochemical activity of SIRT proteins as NAD-dependent protein deacetylases [58,62,63]. With respect to its inherent nature, SIRT proteins might have the capacity to monitor metabolic rate, reflected by the amount of available NAD, and couple this status to regulatory events, such as the silencing of the chromatin [64]. SIRT genes control aging in various species, even though the molecular events that it controls are different (i.e. ribosomal DNA silencing in yeast and insulin signaling in worms). Thus, even if the molecular causes of aging are different in different organisms, aging and ND related mechanisms might still be regulated by SIRT deacetylation of histones or by other protein substrates in the degeneration pathways [23]. Aging seems to be the major culprit in the ND process and SIRT seems to regulate the ND process. We have not tested the effects of aging and we focused mainly on the effects of CR, however, more complex experimental models including aging and CR modalities may provide a more detailed explanation of SIRT functions in the future. In addition, the measurement of the direct activity level of SIRT proteins in the brain might be important to determine the mechanism of the ND process.

Aging is associated with increased rates of stress-induced accumulated apoptosis, which is one of the mechanisms underlying ND, cardiovascular disease, and debility [65–67]. Apoptosis has a fundamental function in cell development and aging, which is related to a series of diseases, such as ND. We have found significantly increased SIRT3 levels and decreased ND after CCR and ICR in this thesis. In literature, when we explored other SIRT3 research on mice, Fan et al. found that SIRT 3 exerts pro-apoptotic roles in angiotensin II-induced hippocampal apoptosis via changing mitochondrial apoptosis

proteins and mitochondria permeability[26]. In this study, authors have concluded that hippocampal apoptosis is linked with impaired learning ability in hypertensive mice and SIRT3 inhibits apoptosis in cellular DNA damage [26]. Lin et al. observed low leukocyte telomere length and lower SIRT3 protein levels in obstructive sleep apnea patients with oxidative stress and, systemic inflammation as seen with the aging process compared to healthy controls. In this research after three months of effective treatment, they have demonstrated the normalized levels of SIRT3 proteins by ELISA method [68]. Mitochondrial dysfunction is a major issue in apoptosis. In the pathogenesis of several degenerative disorders, SIRT have very critical functions in the intrinsic path of apoptosis located in the mitochondria [69].

According to our current knowledge CR defers brain senility protecting from ND. Other regulators of these critical ND paths other than the NAD-dependent SIRTs are still unknown. Fusco et al. have evaluated ND in 6 months of mice and tested 4 weeks of CR [27]. They have measured SIRT levels by Western blot and made immunofluorescence analysis for cAMP responsive-element binding (CREB1) in the cortex and hippocampus. They have reported an increased CREB in the forebrain [27]. This finding was different from the known NAD-dependent histone deacetylase pathway. Furthermore, the expression of SIRT1 and the induction of genes relevant to neuronal metabolism and survival in the hippocampus and cortex of CR animals were drastically reduced by CREB deficiency. They have concluded that CREB controls the transcription of the SIRT in neurons directly [27]. The critical role of CREB in regulating SIRT levels may be supported by its other additional regulations over injured neurons. For example, melatonin blocks the phosphorylation of CREB and aggravates CREB activity [70]. Yilmaz et al. tested the effect of melatonin on mice cerebral ischemia and they have found a significantly decreased volume of cerebral infarct and the level of apoptosis in mice treated by melatonin compared to vehicle-treated mice [70]. Thus, future studies are necessary to determine the underlying molecular mechanisms of CR protection and SIRT proteins on ND.

Sirtuins are very important and functionally active proteins. It is even possible to change their activities through many different strategies and develop novel medications. In fact, the management of SIRT activity genetically or pharmacologically has been

shown to influence numerous illnesses in rodent models [2,3]. SIRT proteins have been connected with CR and aging by regulating energy metabolism, genomic stability, and stress resistance[5,31]. SIRT enzymes are possible curative targets in multiple human diseases including diabetes, cancer, inflammatory disorders, and NDD. Courses of several aggregate-forming neurodegenerative diseases including AD, PD, Huntington's disease, MS, ALS, and spinal and bulbar muscular atrophy have been shown to be impacted by the modulation of SIRT activity [3,5,31,68,71].

Conclusion

The etiologies and consequences of human aging and ND are not easily deciphered. In an evolutionary perspective, in the post-reproductive phase of life, executed by the aging process ND may lead several fundamental systems to deteriorate. At this degeneration stage therapeutic intervention may be very tough. Currently medical research is introducing a ND course that is automated resembling other developmental sequences. These mechanisms are most probably controlled by specific genes, like SIRT genes, pointing out that many therapeutic interventions including diet modifications and specifically designed calorie restrictions may be conceivable in the future.

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7. APPENDICES

7.1.FORMS

Ethical Approval

YEDİTEPE ÜNİVERSİTESİ

T.C. YEDİTEPE ÜNİVERSİTESİ, DENEY HAYVANLARI ETİK KURULU

(YÜDHEK)

ETİK KURUL KARARI

Toplantı Tarihi	Karar No	İlgi	Proje Yürütücüsü
12.05.2017	603	28.04.2017	Uzm. Dr. Burhan Oral Güdü

'Farklı şekillerde kalori kısıtlaması uygulanan farelerde yaşlanma ile ilişkili SIRT proteinlerinin seviyelerinin belirlenmesi.' adlı bilimsel çalışma etik kurulumuzda görüşülmüş olup, çalışmanın etik kurallara uygun olduğuna oy birliğiyle karar verilmiştir.

Etik Onay Geçerlilik Süresi: 2 Yıl	**Hayvan Türü ve cinsiyeti:**	**Hayvan Sayısı:**

GÖREVİ	ADI SOYADI	
Başkan	Prof. Dr. Bayram YILMAZ	
Başkan Yardımcısı	Prof. Dr. Erdem YEŞİLADA	
Raportör	Vet. Hekim Engin SÜMER	
Üye	Prof. Dr. M. Ece GENÇ	
Üye	Doç. Dr. Rukset ATTAR	**KATILMADI**
Üye	Doç. Dr. Soner DOĞAN	**KATILMADI**
Üye	Doç. Dr. Ediz DENİZ	
Üye	Prof. Dr. Gamze TORUN KÖSE	**KATILMADI**
Üye	Yrd. Doç. Dr. Aylin YABA UÇAR	
Üye	Hakan GÖKSEL	
Üye	Ahmet ŞENKARDEŞLER	

7.2CIRRUCULUM VITAE

Personal Informations

Name	Başar	**Surname**	Atalay
Place of Birth	Çaycuma	**Date of Birth**	13.05.1969
Nationality	T.C.	**ID**	20005936364
Email	drbasaratalay@gmail.com	**Phone**	5357869959

Educational Informations

	Name of Institution	**Year**
Doctorate	Yeditepe University Institute of Health Science	2016-2020
Medical Expertise	Hacettepe University Faculty of Medicine Department of Neurosurgery, Ankara, Turkey	1994 – 2001
University	Hacettepe University Medical Faculty	1987 – 1993

Work Experience

	Responsibilty	**Institution**	**Year**
	Professor	Yeditepe Üniversitesi Hastanesi	2012-2018
	Assoc. Professor	Yeditepe Üniversitesi Hastanesi	2007-2012
	Assoc. Professor	Başkent Üniversitesi Hastanesi	2005-2006
	Asist. Professor	Başkent Üniversitesi Hastanesi	2003-2005
	Specialist	Başkent Üniversitesi Hastanesi	2001-2003
	Residency	Hacettepe Üniversitesi Nöroşirürji ABD	1995-2001
	Government Duty	Turkish M.H. Bartın Saglik Ocagi	1993-1995

Language	**Reading***	**Speaking***	**Writing***	**KPDS/UDS Score**	**(Other) Score**
English	Very Good	Very Good	Very Good	90	

*Very Good, Good, Basic

Computer skills

Program	**Ability to use**
Microsoft Office	Very Good

Publications / Notices Certificates / Awards

HONORS AND AWARDS

2003	**Young Neurosurgeon Research Award**: **Atalay B**, Bolay H, Dalkara T, et al. "Transcorneal stimulation of trigeminal nerve afferents to increase cerebral blood flow in rats with cerebral vasospasm: a noninvasive method to activate the trigeminovascular reflex." *Journal of Neurosurgery*, **97,** 1179-1183 (2002).
2017	**Istanbul international inventions Fair ISIF 2017, Invention, R&D and Innovation**: Silver Medal Certificate, A Spinal Surgery Table, Yeditepe University Technology Transfer Office, 4 March 2017, Istanbul

PATENTS

1.Spine surgery Table (national patent – 7 years) No: TR 2012 13288 B

2. Spine surgery Table (international patent- 20 years) No: TR 2012 11729 B US 9,351,895, B2 & Date of Patent: May 31, 2016

Publications in SCI-SSCI-Pubmed Journals

Number of International Publications: 56

Number of National Publications: 27

Number of National Congress Presentations: 93

Number of International Congress Presentations: 57

Book Chapters: 16

International Book Reviews:2

Number of citations: 881 (2020)

Number of Patents: 2

H index: 18 (2020)