Patterns of histone acetylation as targets for novel therapeutic approaches in neurological diseases

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Date Signature

Dedicated to my parents
for their love, support
and encouragement.

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ACh	Acetylcholine
AChE	Acetycholinesterase
AChEI	Acetycholinesterase inhibitor
AD	Alzheimer's disease
AICD	Amyloid precursor protein C-terminal peptide
Akt/PKB	Akt/Protein kinase B
ALS	Amyotrophic lateral sclerosis
APP	Amyloid precursor protein
ARNT	Aryl hydrocarbon receptor nuclear translocation
Aβ	Amyloid beta
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
CBP	CREB binding protein
ChAT	Choline acetyltransferase
CHO	Chinese hamster ovary cells
CNS	Central nervous system
Cox-2	Cyclooxygenase-2
CREB	cAMP response element binding protein
CXCR2	Macrophage inflammatory protein 2/C-X-C chemokine receptor type 2
DDT	Dichlorodiphenyl-trichloroethane
DNAMT	DNA methyltransferases
DPPH	2, 2'-diphenyl-1-picrylhydrazyl
EAE	Experimental autoimmune encephalomyelitis
EGCG	Epigallocatechingallat
ERK	Extracellular-signal-regulated kinases
fAβ	Amyloid beta
GNAT	Gcn5-related N-acetyltransferase
GSE	Grape seed polyphenol extract
GSH	Glutathion

H3K9	Histone 3 lysine 9
H3K9Ac	Acetyl-histone 3 lysine 9
HATs	Histone acetyl transferases
HD	Huntington’s disease
HDACIs	Histone deacetylase inhibitors
HDACs	Histone deacetylases
HIF-1	Hypoxia-inducible transcription factor-1
HMTs	histone lysine methyltransferases
HMW	High molecular weight
HO-1	Heme oxygenase-1
HTT	Huntingtin
IL	Interleukin
JNK	c-jun amino-terminal kinase
LPS	Lipopolysaccaride
mAChR	Muscarinic acetylcholine receptor
MAPK	Mitogen activated protein kinase
MDA	Malondialdehyde
MPP (+)	1-methyl-4-phenyl pyridinium
MPTP	1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine
nAChR	Nicotinic acetylcholine receptor
NF-κB	Nuclear factor kappa B
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NS	Nitrosative stress
PD	Parkinson’s disease
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PrP	Prion protein
PS	Presenilin
RCM kappa-CN	Reduced and carboxymethylated kappa-casein
RGCs	Retina ganglion cells
ROS	Reactive oxygen species

SCA	Spinocerebellar ataxia
sEPSCs	Spontaneous excitatory postsynaptic currents
SIRT	Sirtuin
SOD	Superoxide dismutase
TNF	Tumor necrosis factor
TSA	Trichostatin A
VEGF	Vascular endothelial growth factor
VEH	Vehicle
VPA	Valproic acid
WHO	World health organization

Summary

Neurological diseases, in particular brain tumors and neurodegenerative disorders, cause significant socio-economic burdens on societies. Exploring epigenetic mechanisms in neurological disorders in recent decades has been an emerging tool for describing the pathogenesis of neurological diseases as well as developing new therapeutics.

Global histone acetylation is an epigenetic entity whose alternating patterns in various neurological diseases have recently raised special attention concerning its potency for therapeutic development. I investigated patterns of global histone 3 lysine 9 acetylation (H3K9Ac) in various brain tumors and neurodegenerative animal models, such as APPPS1-21 mice model of Alzheimer's disease (AD), in order to find out the relevance of this target to clinical outcome of the disease and it potency as a target for therapeutic development. I also tried to find out whether natural products with promising neuroprotective effects in preclinical studies affect H3K9Ac status of the nuclei in the studied models.

In the present study, it was shown that H3K9Ac levels change variably in different brain diseases including benign and malignant tumors as well as neurodegenerative conditions such as AD. In brain tumors, the global H3K9Ac alterations were correlated to the degree of malignancy and disease aggressive behavior. Regarding neurodegenerative conditions, an aberration of H3K9Ac profile in APPPS1-21 transgenic mice was reported, which was reversed after MS-275 treatment. Likewise, therapeutic effect of valproic acid on experimental autoimmune encephalomyelitis rats was associated with increased H3K9Ac of brain cells. According to the results of in vitro study, typical histone deacetylator enzyme (HDAC) modifiers, either selective or non-selective, did not change H3K9Ac patterns despite revealing antioxidative effects, suggesting alternative mechanisms, such as alteration of acetylation in non-histone proteins or modification of other pathways. Similarly, icariin, a natural substance with SIRT-1 modifying and neuroprotective activity, could not change the H3K9Ac profile of APPPS1-21 mice brain although it revealed anti-amyloid pathology effects.

We conclude that H3K9 acetylation is a relevant target in the studied brain diseases and alternative mechanisms might be involved in neuroprotective action of both polyphenol and non-polyphenol HDAC modifiers.

Chapter I: Introduction

1. Epigenetic-based therapeutics in neurological disorders

Despite the significant advancement in our knowledge of nervous system and its related diseases, neurological disorders, and especially neurodegenerative diseases still cause enormous burden on societies. Within recent decades, introduction of new concepts into the etiopathology of complex neurological diseases, such as malignant brain tumors, Alzheimer's diseases (AD), multiple sclerosis (MS), etc., has brought new hopes to the treatment of such maladies. Among these new concepts, epigenetics was a genuine one, not only to describe the pathological aspects of neurological disorders which were not explicable with the concepts in hand, but also to introduce novel therapeutics based on the new definitions [1]. The so-called epigenetic-based therapeutics has been tried in various neurological disorders either in preclinical studies or in routine clinical managements [2]. However, the success of the new approach strongly depends on full understanding of the epigenetic variations in pathological states (Figure 1).

2. Epigenetics

Epigenetics traditionally used to be defined as anything which is not contained in DNA and can still affect the DNA and gene expression [3]. Epigenetic machinery allows the genetically identical cells in multi-cellular organisms to present different phenotypes. This machinery also provides a non-genetic memory for organisms which records and preserves the environmental prompts, to which the cells are being exposed during their life [4]. The primary motive for defining epigenetic systems were the lack of genetic determinants, capable of fully explaining the heritability of complex traits, and inability to find related genetic defects in some heritable complex diseases [4]. Nowadays, the definition of epigenetics is a matter of debate [5]. The confusing nature of labeling any non-genetic system as epigenetic has resulted in various definitions for epigenetic systems [5]. Some of epigenetics definitions are as follows:

- "the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence" [6]
- "the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states" [7]

- "a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence" [8]
- "the inheritance of variation (-genetics) above and beyond (epi-) changes in the DNA sequence" [5]

Afterward, based on more precise definitions [5], epigenetic signals were divided into two classes:

1. "Trans epigenetic signals", transmitted by partitioning of the cytosol during cell division and maintained after division by chromosome-cytosol feedback loops.
2. "Cis epigenetic signals", physically associated with the DNA and inherited through chromosome segregation during cell division.

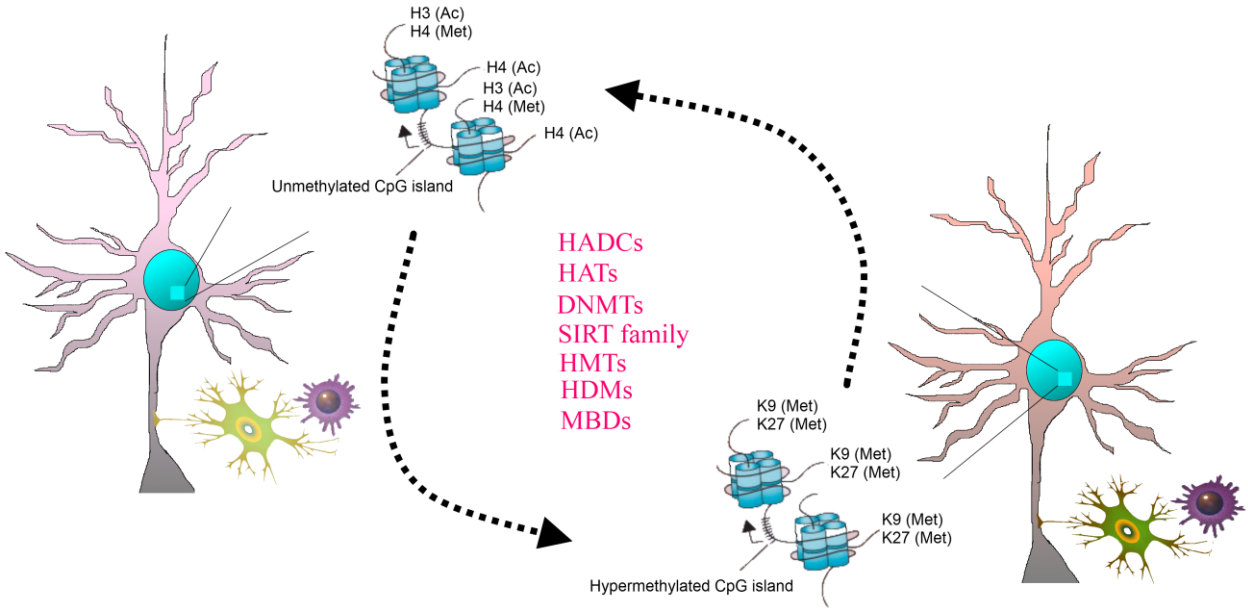

Figure 1: Epigenetic mechanisms in neurological disorders

In a healthy brain cell (left), transcription of a gene occurs in the presence of a combination of epigenetic modifications associated with healthy state "chromatin" conformation (e.g., hyperacetylation and methylation of lysine 4 of histone H3, unmethylated CpG islands). Targeted or generalized disruption of epigenetic healthy setting reverses the circumstances in the cell toward "pathology associated" chromatin conformation (right). The new epigenetic combination might be a combination of several pathology-associated modifications (e.g. dense hypermethylation of the CpG island promoter, methylation of lysine 9 and 27 of histone H3). This pathologic mechanism could involve different brain cell types, such as neurons, astrocytes or microglias. Epigenetic-based therapeutics such as DNA-demethylators, HDAC inhibitors or HAT activators can partially reverse the distorted epigenetic processes and restore the dysregulated gene expression. Ac= acetylation, DNMT = DNA methyltransferase. HAT=histone acetyltransferase. HDAC=histone deacetylase. HDM = histone demethylase, HMT = histone methyltransferase, MBD = methyl-CpG binding domain protein. Met-K4 = methylation of lysine 4, Met-K9 = methylation of lysine 9, Met-K27 = methylation of lysine 27.

An epigenetic system comprises:

1. “Epigenators” including environmental prompts and subsequent signaling pathways, which happen upstream of the first event on the chromatin and lead to the initiator of epigenetic event [8].
2. “Epigenetic initiators” including DNA-binding proteins, non-coding RNAs, or any other entities that define the chromatin location, on which the epigenetic state is going to be established [8, 9].
3. “Epigenetic maintainers” including enzymes that maintain entities such as DNA methylation and histone modifications, histone variants, nucleosomal positioning, and others that sustain the epigenetic state of chromatin [8, 9].

In fact, these three components of epigenetic machinery could be involved in brain pathological states. The role of environmental factors in different maladies has been known for long. In many neurological disorders and tumors, the expression pattern of epigenetic initiators, such as non-coding RNAs, has been changed [2]. Epigenetic maintainers such as DNA methylator enzymes, histone modifier enzymes, etc., are also attractive targets for development of epigenetic-based therapeutics [2].

2.1. Histone modification and histone code

Epigenetic maintainers play essential roles in gene regulation and chromatin-based processes. Among epigenetic maintainers, enzymes that determine histone modifications are important determinants of epigenetic state. Histones undergo several post-translational modifications, such as acetylation and methylation of lysines and arginines as well as phosphorylation, ubiquitylation, glycosylation, sumoylation, ADP ribosylation and carbonylation [10]. The type and position of modification over histone residue determines the active or inactive state of chromatin as euchromatin or heterochromatin. The ‘histone code’ hypothesis states that a combination of various modifications over histone residues allows the gene-expression status to interchangeably switch between on and off, in a cooperative manner, so that a given modification of a specific histone residue is a prerequisite for a modification of other residues on the same histone or adjacent histones [11]. For example, phosphorylation of H3S10 along with acetylation of H3K14 prevents H3K9 methylation [12]. Histone modifications are established by several enzymes, including

histone acetylases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs), and possibly, histone demethylases [13].

2.2. Histone acetylation

The acetylation and deacetylation of histones represent regulatory epigenetic pathways for gene expression [14-17]. There are three to five lysine residues per histone tail and these lysine residues could be acetylated at the amino-terminal regions of histones H2A, H2B, H3 and H4. The level of histone acetylation is maintained by two subclasses of enzymes: HATs which transfer an acetyl group to ε-amino group of the N-terminal of histone tales, and HDACs with reverse action [18]. Adding acetyl groups to the histone tails is mediated by HATs, comprising three super families: GNAT (Gcn5-related N-acetyltransferase), MYST (MOZ, Ybf2/Sas3, Sas2 and TIP60) and p300/CBP [19]. The reverse reaction is mediated by three classes of HDACs including class I HDACs (HDAC1, HDAC2, HDAC3 and HDAC8) localized to the nucleus, class II HDACs (HDAC4, HDAC5, HDAC6, HDAC7, HDAC9 and HDAC10) moving between the nucleus and the cytoplasm and the third class of HDACs, human sirtuin enzymes (SIRT1-7) with various cellular localizations [20].

Histone acetylation can be studied at two levels [21]; local to a specific gene, in which individual residues within a given histone are studied through targeted recruitment by sequence-specific transcription factors (also called candidate gene approach) [22, 23]; and globally all over the DNA at the individual cell level in which the histones are studied throughout the whole genome of a specific gene [24]. H3K9 is an epigenetic marker whose acetylation is known to be associated with active chromatin state. H3K9 acetylation at promoter regions is a hallmark of active gene transcription [21, 25-27]. Even the levels of H3K9 acetylation are positively correlated with transcription rates [26-29]. Based on the current theories, aberrant changes in acetylation of H3K9 in promoter region of genes disrupt the normal gene expression pattern and chromatin architecture, and therefore, could lead up to the pathological states [30-32]. This theory applies not only to histone acetylation of gene-specific promoter regions, but also to global histone acetylation levels of the whole DNA [10, 33]. This is also in accordance with the evidence that changes in global levels of histone modifications correlate with their levels at individual promoters and repetitive DNA elements too [33, 34].

2.3. Histone acetylation in neuroinflammation

Inflammatory cells and specifically microglail cells play a critical role in immune responses in central nervous system (CNS). During pathological processes, the resident CNS immune cells become activated, in order to preserve the balance through various mechanisms [35]. The chronic activation of microglia is thought to induce neuronal damage through activating signaling pathways and releasing cytotoxic molecules such as pro-inflammatory cytokines, reactive oxygen species, proteinases and complement factors [35]. Neuroinflammation plays an important role in the pathogenesis of various neurological disorders, such as stroke, Parkinson's disease (PD), AD, prion diseases, MS and HIV-dementia [36-38]. Epigenetic alterations in association with inflammatory responses have been frequently reported in neuropathological conditions; decreased hippocampal CA3 global H3 acetylation in association with immune response, lasting hours to days after injury, was reported in experimental pediatric traumatic brain injury [39]. Treatment of astrocytes with interleukin (IL)-17, an important mediator of neuro-inflammation, resulted in recruitment of histone acetyltransferases, CREB-binding protein and p300, to the IL-6 promoter and enhanced acetylation of histones H3 and H4 on the IL-6 promoter [40]. In another study, increased H3 acetylation at promoter region of the macrophage inflammatory protein 2/C-X-C chemokine receptor type 2 (CXCR2) elicited neuropathic pain in injured peripheral nerves [41]. Altered histone acetylation mainly functions through changes in expression of inflammatory mediators such as IL-1, 5, 8, 12, and anti-inflammatory genes such as IL-10 [42, 43]. COX-2 expression is augmented via histone acetylation of the promoter region and is suppressed via deacetylation of promoter region by Sirt1, an HDAC [44, 45]. Calorie restriction, a new approach to reduce neuro-inflammation, has been shown to function via reduction of SIRT1 expression that subsequently regulates p300 HAT activity and reduces the expression of inflammatory genes such as NF-κB, AP1, COX-2, and iNOS [46-48].

2.4. Histone acetylation in neurodegeneration

Alterations of epigenetic modifications have recently been shown to play an important role in aging and neurodegeneration [49, 50]. Various neurodegenerative disorders have been reported in association with histone acetylation alterations (Table1) [50, 51]. Recent evidence has shown that loss of histone acetylation is involved in the pathology of brain aging and AD [52].

Table 1: **Patterns of histone acetylation in various neurodegenerative disorders**

Neurodegenerative disorder	Histone acetylation pattern	Mechanism	Affected genes and pathways
Adrenoleukodystrophy	Hypoacetylation	Unknown	Peroxisome proliferation
Amyotrophic lateral sclerosis	Hypoacetylation	FUS inhibition of HAT activity from CBP	CBP-regulated genes
Epilepsy	Hypoacetylation Hyperacetylation	Histone acetylation changes induced by seizures	CREB-regulated and BDNF-regulated genes
Friedreich's ataxia	Hypoacetylation	Reduction of FXN expression and general gene expression impairment	Mitochondrial function and oxidative damage
Huntington's disease	Hypoacetylation	Interactions of mutated HTT and CBP	CBP-regulated genes
Multiple sclerosis	Hypoacetylation	Unknown	Unknown
Parkinson's disease	Hypoacetylation	Inhibition of histone acetylation	TNFα-regulated genes
Rubinstein-Taybi syndrome	Hypoacetylation	Loss of CBP/p300 HAT activity	CREB-regulated genes
Spinal muscular atrophy	Hypoacetylation	Unknown	Unknown

AICD = amyloid precursor protein C-terminal peptide, BDNF = brain-derived neurotrophic factor. CBP = CREB binding protein, CREB = cAMP response element binding protein, FXN = frataxin, FUS = fusion, H2AX = DNA strand break marker, HAT = histone acetyl transferase, HTT = huntingtin. Tip60 = a histone acetyltransferase, TNF = tumor necrosis factor [50, 51].

Studies have shown that neurodegeneration is accompanied with a global decrease in HAT activity, resulting in global histone deacetylation [53, 54]. In a recent study, it was reported that altered global histone acetylation of several lysine residues are associated with age dependent memory impairment in mice [55]. In a study of APPPS1 mice model of AD, levels of hippocampal H4 acetylation in CA3-CA1 hippocampal regions were influenced [56]. In another study of APPPS1-21 mice, severe amyloid pathology of brain was associated with marked shift of histone acetylation in the forebrain [57]. Another example of involvement of

altered histone acetylation in pathology of neurodegeneration is amyotrophic lateral sclerosis (ALS), in which the protein FUS aggregates in cytoplasmic deposits of misfolded proteins. FUS binds to CBP, strongly inhibit its HAT activity and negatively regulate specific CREB target genes that induces histone hypoacetylation [51]. Global decrease in histone acetylation has also been noticed in PD and Huntington's disease (HD) and Friedreich's ataxia [51, 58]. Neprilysin (also known as discoidin domain receptor tyrosine kinase 1; DDR1), the major Aβ-degrading enzyme, is decreased in the hippocampus and mid-temporal gyrus of AD patients [59, 60]. The enzyme is shown to be regulated by histone acetylation in NB7 and SH-SY5Y cells [61].

2.5. Histone modification patterns as brain tumor prognostics

Aberrations of global histone modifications have frequently been reported in various brain tumors. The most prominent alteration in histone modification in cancer cells is thought to be global H4K16 hypoacetylation [34]. H4K16 hypoacetylation has reported to be associated with worse prognosis in meduloblastoma [62]. Alterations of H4K16 acetylation have been noticed in other non-brain tumors as well [18]. However, altered histone modification is not limited to this motif and other motifs can be involved as well [18]. Several histone modifications have shown to be altered in glioblastomas [31, 63-65]. Increased histone H3K9 methylation, loss of H3K9 acetylation, reduced H3K27 methylation, and increased H3K4 methylation are some of altered histone modifications that have been reported in glioblastomas [66]. These alterations have also been accompanied with distorted activity and expression of histone-modifying enzymes in glioblastomas [64]. Rubenstein-Taybi syndrome is a developmental disorder associated with higher risk of cancer; abolished histone acetyltransferase activity and altered histone acetylation level has been reported in this syndrome [67-69]. In our study of global histone acetylation patterns in pituitary adenomas, enhanced global H3K9 acetylation was noticed in pituitary adenomas compared to normal pituitary [70]. Depleted H3K9-dimethylation was detected in 41% of medulloblastomas, according to the results of a tissue microarray study [71]. Moreover, patterns of global histone modifications are correlated with the outcome of various tumor types [63, 72, 73]. In a cohort study of glioblastoma patients, it was noticed that lower levels of H3K18 acetylation was associated with greater survival [63]. Alteration of H3 phosphorylation has shown to be a mitosis-specific marker for meningioma grading [74, 75]. In ependymomas, expression of

Metallothionein 3, the most frequently down-regulated gene in recurrent ependymomas, is shown to be regulated by the levels of histone acetylation [76].

3. Epigenetic-based therapeutics

Epigenetic maintainers and specifically epigenetic modifying enzymes are interesting targets for development of epigenetic-based therapeutics (Table 2) [13].

Table 2: List of some histone modulating compounds

Main class	Subclass	Substance
HAT activators	Small molecule activators	CTPB, Nemorosone, Pentadecylidenemalonate (LoCAM)
Histone-acetylase inhibitors	Natural products	Anacardic acid, Curcumin, Garcinol, Plumbagin, EGCG, Gambogic acid
	Endogenous HAT inhibitors	Heparin, Spermidine
	Acetyl-CoA derived bi-substrate inhibitors	Lys-CoA, H3-CoA-20, H4K16-CoA, Boc-C5-CoA, Spd(N1)-CoA
	Synthetic compounds	Isogarcinol, CTK7A, Quinoline derivatives, a-methylene-g-butyrolactone
DNA-methylation inhibitors	Nucleoside analogues	5-Azacytidine, 5-Aza-2'-deoxycytidine, 5-Fluoro-2'-deoxycytidine, 5,6-Dihydro-5-azacytidine, Zebularine
	Non-nucleoside analogues	Hydralazine, Procainamide, EGCG, Psammaplin A, MG98, RG108
Histone-deacetylase inhibitors	Short-chain fatty acids	Butyrate, Valproic acid
	Hydroxamic acids	m-Carboxy cinnamic acid bishydroxamic acid (CBHA), Oxamflatin, PDX 101, Pyroxamide, Scriptaid, Suberoylanilide hydroxamic acid (SAHA), Trichostatin A (TSA), LBH589, NVP-LAQ824
	Cyclic tetrapeptides	Apicidin, Depsipeptide (FK-228, FR901228), TPX-HA analogue (CHAP), Trapoxin
	Benzamides	CI-994 (N-acetyl dinaline), MS-275

The therapeutic properties of some epigenetic modifying drugs were even known before their epigenetic modifying activities were identified [77]. For instance, valproic acid was known and approved for a long time as the standard treatment of manic episodes associated with bipolar disorder, adjunctive therapy in multiple seizure types (including epilepsy), and prophylaxis of migraine headaches. It was first in 2001 when HDAC inhibitory activity of valproic acid was detected [78]. Since identification of the first HDAC inhibitory compound in 1996, efforts have been focused on the development and discovery of new potent histone modulatory compounds to be used as therapeutics for various diseases [79]. Natural products are one of the most important and attractive resources for drug development. Natural products with pharmaceutical properties has long been used as models for chemical therapeutic development [80].

4. Natural polyphenols: Emerging cancer and neurotherapeutics

Natural polyphenols are the most commonly found chemical compounds in consumed herbal beverages and foods worldwide [81, 82]. They constitute a large group of phytochemicals with more than 8000 identified compounds (Table 3). The primary function of these compounds is to protect the plants against reactive oxygen species (ROS), produced during photosynthesis, and consumption by herbivores [82]. Within the last decades, most of the studies on polyphenols have been focused on anti-oxidant properties of these chemical compounds as their most prominent effect [83]. Along with introducing resveratrol, as a potential cancer therapeutic as well as anti-aging agent, much focus has been placed on protective effects of various polyphenols against aging and cancer [84, 85]. Polyphenols have been shown to increase the life span and improved brain function [86-88]. Various dietary polyphenols interfere with tumor growth and development [89]. Polyphenols can act through different pathways and target various molecules within the pathways.

4.1. Polyphenols as epigenetic modulators

Epigenetic modifications, induced by natural polyphenols, have been evidenced by various studies (Table 4) [90]. The presence of numerous evidences on epigenetic modulating effects of dietary polyphenols has even given the title of “epigenetic diet” to these compounds [91].

Table 3: A simplified classification of natural polyphenols

Polyphenols	**Falvonoids**	Anthocyanins		e.g. Aurantinidin, Cyanidin, Delphinidin, Europinidin, Luteolinidin, Pelargonidin, Malvidin, Peonidin, Petunidin, Rosinidin, etc.
		Flavonols		e.g. 3-hydroxyflavone, Azaleatin, Fisetin, Galangin, Gossypetin, Kaempferide, Kaempferol, Isorhamnetin, Morin, Myricetin, Natsudaidain, Pachypodol, Quercetin, Rhamnazin, Rhamnetin, etc.
		Flavones		e.g. Apigenin , Luteolin, Tangeritin, Chrysin, 6-hydroxyflavone, Baicalein, Scutellarein, Wogonin, Diosmin, Flavoxate, etc.
		Flavanones		e.g. Butin, Eriodictyol, Hesperetin, Hesperidin, Homoeriodictyol, Isosakuranetin, Naringenin, Naringin, Pinocembrin, Poncirin, Sakuranetin, Sakuranin, Sterubin, etc.
		Isoflavonoids	Isoflavones	e.g. Genistein, Daidzein, lonchocarpane, Laxiflorane, etc.
			Isoflavanes	e.g. Equol, etc.
		Flavanols	Monomers	e.g. Catechin, Epicatechin (EC), Epigallocatechin (EGC), Epicatechin gallate (ECG), Epigallocatechin gallate (EGCG), Epiafzelechin, Fisetinidol, Guibourtinidol, Mesquitol, Robinetinidol, etc.
			Oligomers and polymers	e.g. Theaflavins, Thearubigins, Condensed Tannins, Proanthocyanidins, etc.
	Non-flavonoids	Phenolic acids	Derivatives of cinnamic acid	e.g. P-Coumaric, Caffeic, Chlorogenic acid , Ferulic, Sinapic, etc.
			Derivatives of benzoic acid	e.g. Gallic acid, Gentisic acid, Orotocatechuic, Syringic, Vanillic, etc.
		Lignans		e.g. Pinoresinol, Podophyllotoxin, Steganacin, etc.
		Stilbenes		e.g. Resveratrol analogs, etc.

These bioactive components are able to alter the DNA methylation and histone modifications, leading to gene activation or silencing in diseases. We previously mentioned how alterations of epigenetic modifications are involved in the pathology of brain tumors. In tumor cells, polyphenols have shown to mediate epigenetic modifications associated with

the induction of tumor suppressor genes, such as p21WAF1/CIP1, and inhibition of tumor promoting genes, such as the human telomerase reverse transcriptase, during tumorigenesis [89]. Polyphenols can also interfere with growth and apoptosis through changing the epigenetic modification of important mediators within related signaling pathways. Epigallocatechin gallate (EGCG) inhibits EBV-induced B lymphocyte transformation via suppression of RelA acetylation, an important mediator of phosphoinositide 3-kinase (PI3K)/AKT/mTOR pathway [92].

Table 4: Epigenetic effects of natural polyphenols

Polyphenol compound	Epigenetic target	Effect	Reference
Apigentin	DNMT	Inhibition	[93]
Beta-naphtoflavone	HDAC1	Activation	[94]
Biochain	H3, ERK	Inhibition	[95]
	SIRT1	Activation	[95]
Catechin	SIRT1	Activation	[96]
Curcumin	DNMT, HDAC, HAT	Inhibition	[97-103]
	SIRT1	Activation	[96]
DCHC	SIRT1	Activation	[95]
Daidzein	SIRT1	Activation	[95]
	ERK, H3	Inhibition	[95]
EGCG	DNMT, HDAC, SIRT1	Inhibition	[97, 98]
Fisetin	SIRT1	Activation	[104]
Formononetin	SIRT1	Activation	[95]
Genistein	SIRT1, HDAC, DNMT	Inhibition	[105-109]
	HAT	Activation	[110]
Green tea polyphenols	HDAC1	Inhibition	[111]
Icariin	SIRT1, PGC-1 alpha	Activation	[112]
Kaemferol	SIRT3	Activation	[113]
Luteolin	HDAC	Inhibition	[114]
Myricetin	SIRT1(under stabilizing conditions)	Activation	[115, 116]
	SIRT1 (without stabilizing conditions)	Inhibition	[14]
Naringenin	AMPK	Activation	[117]
Phloridzin	SIRT1	Activation	[118]
Piceatannol	SIRT1	Activation	[116]
Persimmon oligomeric proanthocyanidins	SIRT1	Activation	[119, 120]
Procyanidins	SIR-2	Activation	[121]
Quercetin	SIRT1, PCG-1 alpha, H3	Activation	[116, 122-124]
	SIRT1, HDAC1	Inhibition	[14]
Resveratrol	SIRT1, DNAMT	Activation	[125-128]
Silibinin	Bcl-2, SIRT1	Activation	[129]
3,2,3,4,-Tetrahydroxychalcone	SIRT1	Inhibition	[130, 131]
Theophilline	HDAC2	Activation	[130]

Some polyphenols interfere with tumor necrosis factor-related apoptotic pathways and markedly augment programmed death in cancer cells [132]. Green tea polyphenols (EGCG) and soy bean isoflavones (genistein) are also able to affect different pathways of angiogenesis such as vascular endothelial growth factor (VEGF) signal pathway or receptor tyrosine kinases (RTKs) [133].

So far, epigenetic modifying effects of polyphenols have been more punctuated for their anti-cancer properties and less accentuated for its neuroprotective properties. We discussed the potentials of natural polyphenols as neurotherapeutics in our review published in 2012 [134]. Studies on neuroprotective effects of polyphenols can be divided into the following categories: (1) neuroprotective action through antioxidant pathways, (2) interaction with signaling pathways, (3) neuroprotection through modulation of neural mediators and enzymes like acetylcholine (ACh) and acetylcholinesterase (AChE), (4) inhibition of NMDA neurotoxicity and (5) anti-amyloidogenic effects [134].

4.2. Polyphenols as antioxidants

The most prominently discussed effect of polyphenols is their anti-oxidant activity. It is established that oxidative/nitrosative stress (OS/NS) has a pivotal role in pathophysiology of neurodegenerative diseases and many other types of human maladies [83, 135-138]. Oxidative damage to neuronal molecules, accumulation of iron ion species in the brain, and decreased cellular reserve anti-oxidant pool are major pathological aspects of neurodegenerative disorders, like PD, AD or ALS [139-144]. It has been shown, that severe hypoxia or ischemia episodes increase the susceptibility to develop AD [145]. In fact, hypoxia induces amyloid precursor protein (APP) up-regulation at both the mRNA and protein level, and subsequently leads to amyloid beta (Aβ) accumulation [146-148]. NS can damage bio-molecules through reactive species, such as peroxynitrite, and also plays a crucial role in PD by triggering mitochondrial dysfunction [149-152].

Polyphenols exert their anti-oxidant effects through different mechanisms like interacting with the HIF-1 alpha pathway, inducing expression of protective genes against OS, regulating reactive oxygen species (ROS) through interaction with oxidative pathways and scavenging metal ions as pathogenic free radicals [153]. Table 5 summarizes recent studies on anti-oxidant effects of polyphenols in neurodegenerative processes.

Table 5: Anti-oxidative effects of polyphenols in neurotoxicity and neurodegeneration

Substance	Cell line/ animal model	Effect	Ref.
Aloe-emodin	N-methyl-D-aspartate (NMDA)-induced toxicity in retinal ganglion cells (RGCs)	• Elevates levels of RNA and protein expression of superoxide dismutase (SOD) • Attenuates NMDA-induced apoptosis of RGCs	[154]
Curcumin	N27 dopaminergic neurons	• Protects against mitochondrial complex I inhibition (leading to mitochondrial dysfunction) and NS	[149]
	Homocysteine-induced neurotoxicity in rats	• Reduces Malondialdehyde (MDA)[a] and Superoxide anion levels • Reduces lipid peroxidation • Improves learning and memory in rats	[155]
	N27 dopaminergic neuronal cell line	• Increases glutathione[b] (GSH) levels	[156]
	3-nitropropionic acid (3-NP) - induced neurotoxicity in rats	• Improves the 3-NP-induced motor and cognitive impairment • Attenuates 3-NP-induced OS (including lipid peroxidation, reduced GSH and nitrite activity) • Restores the decreased succinate dehydrogenase[c] activity	[157]
EGCG	Glucose oxidase-induced neurotoxicity in H 19-7 (a rat neuronal cell line)	• Enhances cellular resistance to glucose oxidase-mediated oxidative damage • Elevates heme oxygenase-1[d] (HO-1) mRNA and protein expression • Activates transcription factor Nrf2[e]	[158]
	Glutamate-induced toxicity in HT22 mouse hippocampus neuronal cells, Kainic acid-induced neurotoxicity in rats	• Reduces glutamate-induced oxidative cytotoxicity • Inactivates the NFκB signaling pathway[f] • Reduces ROS accumulation and NF-κB transcriptional activity	[159]

Substance	Cell line/ animal model	Effect	Ref.
EGCG	Transient global cerebral ischemia C57BL/6 in mice	• Reduces the development of delayed neuronal death after transient global cerebral ischemia in mouse brain	[160]
	Age-associated oxidative damage in rat brain	• Amplifies the activities of enzymes like SOD, catalase, glutathione peroxidase, glutathione reductase and glucose-6-phosphate dehydrogenase • Improves the activity of antioxidants like tocopherol, ascorbic acid and glutathione • Ameliorates the MDA and protein carbonyl levels	[161]
	Progressive neurotoxic model of long-term serum deprivation in human SH-SY5Y neuroblastoma cells	• Decreases protein levels and mRNA expression of the beta subunit of the enzyme prolyl 4-hydroxylase • Decreases protein levels of two molecular chaperones that are associated with HIF regulation, the immunoglobulin-heavy-chain binding protein and the heat shock protein 90 beta	[162]
	SOD1-G93A transgenic mice (a model of ALS)	• Maintains the normal expression of p85a PI3-K, pAkt, and pGSK-3 (molecular signals of survival) • Reduces activation of NF-kB and the cleaved form of caspase-3 • Reduces microglial activation • Prolongs the life span • Delays the onset of symptoms	[86, 87]
Mangiferin	Glutamate-induced neurotoxicity in rat cerebral cortex neurons	• Prevents neuronal death, oxidative stress and mitochondrial depolarization	[163]
	1-methyl-4-phenyl pyridinium (MPP(+))-induced oxidative stress in the murine neuroblastoma cell line N2A	• Restores the GSH content (to 60% of control levels), and down-regulates both SOD and catalase mRNA expression	[164]

Substance	Cell line/ animal model	Effect	Ref.
		• Quenches reactive oxygen intermediates	
Mangiferin Morin	Glutamate-induced neurotoxicity in rat primary culture of neurons	• Reduces ROS formation • Activates enzymatic anti-oxidant system • Restores mitochondrial membrane potential	[165]
Polyphenol-rich hedeoma multiflorum extract	Biochemical assay on rat brain homogenates	• Inhibits lipid peroxidation • Scavenges 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) radicals	[166]
Polyphenol-rich osmanthus fragrans extract	Glutamate, arachidonic acid, and 6-hydroxydopamine-induced neurotoxicity in rat primary cortical neurons	• Scavenges DPPH and hydroxyl anions • Inhibits lipid peroxidation	[167]
Red wine polyphenols	Rat model of ischemic cerebral stroke	• Prevent the burst of excitatory amino acids in response to ischemia • Reduce brain infarct volumes • Enhance the residual cerebral blood flow during occlusion and reperfusion • Modulate expression of proteins involved in the maintenance of neuronal caliber and axon formation	[168]
Resveratrol	Lipopolysaccaride (LPS)-induced dopaminergic neurodegeneration in rat	• Reduces NADPH^{g} oxidase-mediated generation of ROS • Inhibits microglia activation • Attenuates the activation of MAPK and NF-κB signaling pathways • Implies neuroprotection against LPS-induced dopaminergic neurodegeneration	[169]
	Glutamate-induced toxicity in mice primary culture of neurons	• Induces heme oxygenase 1d (HO-1) in a dose- and time-dependent manner	[170]

Substance	Cell line/ animal model	Effect	Ref.
Resveratrol	Optimized ischemic-reperfusion stroke model in mice	• Protects mouse neurons, subjected to an optimized ischemic-reperfusion stroke model • Protects neurons against excitotoxicity	
	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced Parkinson in mice	• Protects from MPTP-induced motor coordination impairment, hydroxyl radical overloading, and neuronal loss • Scavenges free radicals	[171]
	MPP(+)-induced neurotoxicity in dopaminergic neurons of midbrain slice culture	• Prevents accumulation of ROS, depletion of cellular glutathione, and cellular oxidative damage induced by MPP(+) • Activates sirtuin family of NAD-dependent histone deacetylases	[172]
	A-beta induced toxicity in neurons from a mouse model (Tg2576 line) and mouse neuroblastoma (N2a) cells	• Maintains normal expression of peroxiredoxins and mitochondrial structural genes • Maintains normal mitochondrial function	[173]
Tea polyphenol	NMDA-induced neurotoxicity in mice	• Attenuates the increased production of synaptosomal ROS • Reduces the deteriorative ROS-sensitive Na(+), K(+)-ATPase and Mg(2+)-ATPase activity	[174]

[a]**Malondialdehyde (MDA)** is a highly reactive electrophile species that occurs naturally form degradation of polyunsaturated lipids and is a biomarker for measuring the levels of OS. [b]**Gluthation (GSH)** is an antioxidant, preventing damage to important cellular components caused by ROS such as free radicals and peroxides. [c]**Succinate dehydrogenase** is a Complex II mitochondrial enzyme. [d]**Heme oxygenase-1 (HO-1)** is an enzyme that catalyzes the degradation of heme. [e]**Transcription factor Nrf2** is a master regulator of the anti-oxidant response. [f]**NFκB signaling pathway** is a pathway which is activated in response to cell stresses including OS. [g]**NADPH** is the reduced form of $NADP^+$, a coenzyme used in anabolic reactions, such as lipid and nucleic acid synthesis.

4.2.1. Hypoxia inducible factor 1 (HIF-1) alpha pathway

One strategy of neuroprotection is activation of hypoxia signal transduction pathways through which the hypoxic condition is sensed and appropriate genes are activated and

expressed in order to mediate compensatory survival conditions for the cells. A high percentage of hypoxic responses in cells are controlled by hypoxia-inducible transcription factor-1 [175]. The HIF-1 complex is a heterodimer, consisting of two subunits: The HIF-1α oxygen-regulated subunit and the HIF-1ß subunit, also known as aryl hydrocarbon receptor nuclear translocation (ARNT) [176-180]. Alterations in oxygen levels regulate HIF-1α activity. Under normoxic conditions, HIF-1α is degraded by HIF prolyl-4-hydroxylases [181]. These enzymes require iron as co-factor and oxygen as co-substrate [181, 182]. In contrast, under hypoxic conditions, HIF-1α is translocated to the nucleus where it dimerises with ARNT and subsequently binds to hypoxic binding sites of target genes involved in cell survival, glycolysis, angiogenesis, erythropoiesis, and iron metabolism [183]. Consequently, stabilizing HIF-1α would be a strategy to promote further cytoprotective events. Some natural polyphenols induce HIF-1α protein and lead to a further increase in mRNA levels of HIF-1α target genes [183-188]. Catechins, like EGCG, are a group of polyphenols which exert their neuroprotective effects through induction of the HIF-1α pathway [162, 184]. Resveratrol is another non-flavonoid polyphenol with significant anti-oxidant activity, activating the HIF-1α pathway [189]. However, there is also evidence of definite links between hypoxia, HIF-1 activation and APP/ amyloid beta (Aβ) production [190-192]. The precise role of HIF-1 alpha pathway in neurodegeneration, albeit positive or negative, is in fact a matter of debate [175]. HIF-1α acts like a double-edged sword that can be both beneficial and detrimental to neural cell survival [175].

On the other hand, there are contradictory reports on effects of the same polyphenol on HIF-1α expression and activity. For example, in one study EGCG has been shown to exert anti-oxidant effects through HIF-1 pathway activation, while in another study an inhibitory effect on the same pathway has been reported [162, 184, 193]. Similarly, other polyphenols have been reported to exert anti-oxidant effects through inhibition rather than activation of the HIF-1 pathway [194, 195]. Therefore, claiming neuroprotective effects for polyphenols is still a matter of debate.

4.2.2. ROS regulation

Free oxygen radicals can directly damage cellular micro-organelles and in this regard, mitochondrial damage is of great importance to neurodegenerative diseases [151, 196]. Oxygen radicals can also reduce free metal ions to active radicals, like superoxide anions

which are responsible for reduction of ferric (Fe3+) to ferrous (Fe2+) ions through the Fenton reaction [196]. Polyphenols scavenge superoxide and hydroxyl radicals, as well as the 1,1-diphenyl-3-picrylhydrazyl radical, peroxyl radicals, nitric oxide, carbon-center free radicals, singlet oxygen and lipid free radicals, and peroxynitrite [197-204].

Amongst different polyphenols, EGCG have shown to be the most efficient radical scavenger, even among its other counterparts like ECG, EC and EGC [200, 205, 206]. Strong scavenging properties of EGCG are due to several hydroxyl groups at the side rings of the chemical core [200, 205, 206]. Hydroxyl groups are especially important in biological chemistry because of their tendency to form hydrogen bonds both as donor and acceptor. In fact, polyphenols with hydroxyl groups can act as strong reducing agents and vice versa.

Another important feature of some polyphenols is modulating the activity of enzymes involved in OS. In this regard, previous studies have shown that EGCG can increase the activity of SOD and catalase, two important anti-oxidant enzymes in the mouse striatum [207]. However, ROS generation is the final step in many cell degeneration pathways and act as a non-specific rather than specific neural damage mechanism. Although ROS regulation can be mentioned as an additive mechanism of neuroprotection of polyphenols, claiming a therapeutic effect for polyphenols via ROS regulation as the only mechanism seems to be overdrawn.

4.2.3. Metal ion chelating

Polyphenols are also able to chelate metal ions, like copper (II) and iron (III), to prevent free radical damage [205]. In this regard, some polyphenols have shown to be more efficient than traditional antioxidants like vitamins E and C [200, 201]. Studies have shown that some polyphenols can inhibit lipid peroxidation in the brain [197, 208]. Polyphenols seem to exert this effect, through chelating ferrous ions. For instance, EGCG attenuates paraquat-induced microsomal lipid peroxidation and increases the survival of paraquat-poisoned mice, a PD model, through this mechanism [196, 209].

The ability of polyphenols to chelate metal ions contributes to their neuroprotective activity via inhibition of transition metal catalyzed free radical formation. Two attachment sites for metal ions have been suggested within the molecular structure of flavonols, a subclass of

polyphenols: The o-diphenolic groups in the 30, 40-dihydroxy positions in the B ring, and the keto structure 4-keto, 3-hydroxy or 4-keto and 5-hydroxy in the C ring [210, 211]. These functional groups bind transition metal ions, such as iron or copper [212]. With a closer look at the molecular structures, the same chemical and molecular features can also be found in other polyphenol subclasses [213]. Therefore, we can expect a broad range of polyphenols with the ability to chelate metal ions.

4.2.4. Modulating cell signaling pathways

Several polyphenols have been shown to interact with cellular signaling pathways which are directly or indirectly involved in neurodegeneration. Most of these pathways are, in fact, signaling pathways involved in cell survival and programmed cell death. In this respect, polyphenols have been reported to act at phosphoinositide 3-kinase (PI3K), Akt/protein kinase B (Akt/PKB), tyrosine kinases, protein kinase C (PKC), and mitogen activated protein kinase (MAPK) signaling cascades (Table 6) [214]. They affect cellular function by altering the phosphorylation mode and expression level of targeted molecules within the mentioned pathways. The flavonoid superfamily of polyphenols has the potential to bind to the ATP-binding sites of a large number of proteins of cellular signaling pathways, including mitochondrial ATPase, calcium plasma membrane ATPase, protein kinase A, protein kinase C and topoisomerase [215-222], as well as to benzodiazepine binding sites of GABA-A receptors and adenosine receptors [223, 224]. Resveratrol and the citrus flavanones hesperetin and naringenin inhibit the activity of a number of protein kinases [225-227]. PKC over-expression has been shown to reduce amyloid plaque formation and Aβ levels in human APP transgenic mice [228]. There is strong evidence that PKC signaling pathways regulate important molecular events involved in associative memory storage, and signaling deficits of PKC signaling pathways play an important role in the pathophysiology of neurodegenerative disorders like AD [229, 230]. EGCG, huperzine A and resveratrol have shown to interact with PKC signaling pathway (Table 6). The MAPK signaling pathway is a general target of flavonoids, notably in the nervous system in the context of oxidative insults [214]. ERK1/2 and c-jun amino-terminal kinase (JNK), two important components of MAPK signaling pathway, are involved in various forms of cellular plasticity such as differentiation and apoptosis [231, 232].

Table 6: Neuroprotective polyphenols targeting cellular signaling pathways

Substance	Pathway	Reference
Curcumin	PI-3K/MAPK signaling pathways	[233]
EGCG	NF-κB signaling pathway	[159, 234]
	Akt signaling pathways	[235]
	HIF-1α signaling pathway	[162]
	Erk1/2 signaling pathway	[162, 236]
	PKC signaling pathway	[237, 238]
Fisetin	ERK signaling pathway	[239]
	NF-κB signaling pathway	[240]
GSE	Erk1/2 signaling pathway	[241, 242]
Huperzine A	PKC signaling pathway MAPK signaling pathway	[243, 244]
Naringenin	MAPK signaling pathway	[245]
Resveratrol	ERK signaling pathway	[239]
	NF-κB signaling pathway	[169, 246, 247]
	SIRT1- uncoupling protein 2 pathway	[248-250]
	AMPK signaling pathway	[251]
	Nrf2/ARE anti-oxidant pathway	[246, 252]
	PKC signaling pathway	[253]
	PI3K/AKT signaling pathway	[254]
Mangiferin **Morin**	Erk1/2 signaling pathway Akt signaling pathways NF-κB signaling pathway	[165]

The phosphatidylinositol 3 kinase (PI3K)-Akt/PKB pathway has a pivotal role in neuronal survival [255]. Activation of Akt/PKB in neurons leads to the inhibition of central proteins of the cell death machinery, such as the proapoptotic Bcl-2 family member BAD and members of the caspase family [256-258]. Akt1, an effector molecule of PI3K-Akt/PKB pathway, has also been shown to attenuate the apoptotic effect of $A\beta_{(25-35)}$ [259]. Quercetin has been shown to exert inhibitory activity on PI3K-Akt/PKB pathway through inhibiting PI3K [260].

However, a large number of protein kinases, important mediators of different cell signaling pathways, have been reported as being potential targets of different polyphenols [214]. Therefore, we have an apparent lack of selectivity of action for polyphenols in this respect. This might arise from the fact that many studies, reporting the interactions of polyphenols with cell signaling pathways, have not defined the primary cellular site of action of the studied polyphenol.

4.3. Polyphenols anti-acetycholinesterase activity

The concept of cholinergic system deficit in neurodegeneration and its important role in cognition was proposed nearly 30 years ago [261]. Loss of cholinergic activity, atrophy of the nucleus basalis of Meynert as the major source of acetylcholine, and loss of cortically projecting cholinergic neurons, along with increasing cognitive deficits, are some of notable findings in different neurodegenerative diseases, like AD and PD [262-269]. Cholinergic dysfunction in neurodegenerative diseases can be the result of reduction in ACh synthesis due to reduced choline acetyltransferase (ChAT) or choline uptake, cholinergic neuronal and axonal abnormalities, and degeneration of cholinergic neurons [270]. Accordingly, using acetylcholinesterase inhibitors, which exert their efficacy through stimulation of both muscarinic and nicotinic acetylcholine receptors (mAChR & nAChR), has been a proper therapeutic approach to alleviate the cognitive symptoms of neurodegenerative diseases [265, 268, 271]. There are two distinct receptor subtypes in the brain for ACh: nicotinic (nAChR) and muscarinic (mAChR). nAChRs are mainly ligand-gated ion channels, while mAChRs are metabotropic receptors. Muscarinic receptors include five distinct receptor subtypes (M1-M5). M1 mAChR is the most abundant subtype in cerebral cortex and hippocampus, the most sensitive brain areas to the development of amyloid plaques and neurofibrilary tangles [272, 273].

Several natural polyphenols have shown a cholinesterase inhibitory effect [274]. In most in vivo studies, the anticholinergic activity of polyphenol was accompanied by improvement of cognitive functions, like learning and memory [275-280]. However, the exact mechanism of interaction of polyphenols with the cholinergic system is still not clear. EGCG has shown strong anti-AChE activity [274]. In another study, EGCG has been reported to modulate nAChR signaling pathway through down regulation of α9-nAChR expression as well as inhibition of (3H)-Nic/α9-nAChR binding activity [281]. Resveratrol has shown in a study to block acetylcholine release from adrenal chromaffin cells [282]. Some polyphenols, such as huperzine A, Quercetin, Kuwanon U, E, and C, Kaempferol, tri- and tetrahydroxyflavone, etc., have shown anti-butyrylcholinesterase effects in addition to their anti-cholinesterase activity [241, 242, 274, 280, 283-285]. Huperzine A has shown the most promising effects in this respect [274, 283-285]. Studies have shown that huperzine A is highly specific for AChE [286]. The richest natural source of huperzine A is the plant Huperzia serrata, a fascinating fungal

reservoir of other AChEIs as well [287, 288]. The drug is a potent, reversible and selective inhibitor of acetylcholinesterase and its potency is similar or superior to other AChEIs [286, 289]. Huperzine A has shown a rival potency in AChE inhibition to drugs, like tacrine, galanthamine, and rivastigmine, which are currently being used in the treatment of AD patients [290-296]. Huperzine A has the highest AChEI activity (IC50) after donepezil, while tacrine, physostigmine, galantamine, and rivastigmine were less potent [297]. Compared to the other AChEIs, huperzine A has also shown better penetration through the blood brain barrier, higher oral bioavailability, and longer duration of AChEI activity [294]. The mechanism of action of this drug has still not been clearly defined. In-silico studies have shown a direct binding of huperzine A to acetylcholinesterase [298]. Clinical trials with huperzine A, for treatment of cognitive and functional impairments of AD and schizophrenia and the increase in memory performance of normal individuals, have been promising [299-303]. In China, huperzine A has been studied in phase IV clinical trials and revealed a significant improvement of memory of elderly people, patients with AD and patients with vascular dementia [299, 300, 302]. Several meta-analyses have shown that administration of huperzine A for at least 8 weeks might lead to a significant improvement in cognitive function, mood, behavior and daily activity of patients with AD. Most of its side effects are cholinergic in nature and are generally mild and of brief duration [304-306]. Combinatorial regimens with other selective AChEIs have shown even more promising results [303, 307, 308]. Huperzine A has also butyrylcholinesterase inhibitory activity which is not as promising as the anti-AChE activity [296]. Not all polyphenols have an anti-cholinesterase activity; some of them have a reverse effect. For example, caffeic acid increases the AChE activity and expression [309].

4.4. Polyphenols and protective effects against NMDA neurotoxicity

The role of NMDA neurotoxicity and glutamate excitotoxicity in neurodegenerative diseases like HD, AD and even in cognitive impairment associated with aging has been confirmed many years ago [310-313]. Excessive activation of NMDA receptors induces the production of damaging free radicals (e.g., NO and ROS) and other enzymatic processes that contribute to neuronal damage and cell death [314-319]. Therefore, blocking the NMDA pathway has been a therapeutic strategy for cognitive impairment not only in neurodegenerative diseases but also in psychiatric disorders with cognitive dysfunction [320, 321].

Table 7: Polyphenols protective effects against NMDA neurotoxicity

Substance	Cell line/ animal model	Effect	Ref.
Catechin, Curcumin, Tannic acid	Rat NMDA neurotoxicity model, primary culture of neurons	• Inhibits glutamate-induced excitotoxicity • Inhibits PKC activity, and subsequent phosphorylation of NR1 of the NMDA receptor • Reduces glutamate-mediated Ca^{2+} influx • Inhibits glutamate-induced caspase-3 activation • Reduces glutamate-induced ROS generation	[322]
EGCG	Unilateral cerebral ischemia in gerbils	• Reduces excitotoxin-induced MDA production and neuronal damage • Attenuates the increase in MDA level caused by cerebral ischemia • Reduces the formation of postischemic brain edema and infarct volume	[323]
Honokiol	Mice NMDA toxicity model	• Ameliorates behavioral and neurotoxic effects of NMDA • Reduces seizure occurrence, score, and latency • Decreases ROS production	[282]
Resveratrol	Acute oxygen-glucose deprivation (OGD) model in rat hippocampal slices	• Reduces the frequency and amplitude of 2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl) propanoic acid (AMPA)-mediated sEPSCs in pyramidal neurons • Attenuates OGD-induced neuronal impairment • Reduces the OGD-enhanced AMPA/NMDA receptor mediated neuronal EPSCs	[324]
Tea polyphenol	Mice NMDA toxicity model	• Ameliorates behavioral and neurotoxic effects of NMDA • Reduces seizure occurrence, score, and latency • Decreases ROS production	[282]
Trans-resveratrol	CA1 region of rat hippocampal slices with NMDA toxicity model	• Suppresses glutamate-induced currents in postsynaptic CA1 pyramidal neurons • Inhibits postsynaptic glutamate receptors	[325]
Morin Mangiferin	Glutamate-induced neurotoxicity in rat primary culture of neurons	• Protects cortical neurons from excitotoxic death • Reduces ROS levels and maintains the homeostasis of the enzymatic anti-oxidant system after excitotoxic event • Inhibits glutamate-induced calpain activity • Regulates the release of pro-apoptotic proteins implicated in caspase-dependent and -independent apoptotic neuronal death • Modulates the activity of the Akt and Erk1/2 kinases after excitotoxic events • Prevents the activation of NF-κB and its subsequent translocation to the nucleus	[165]

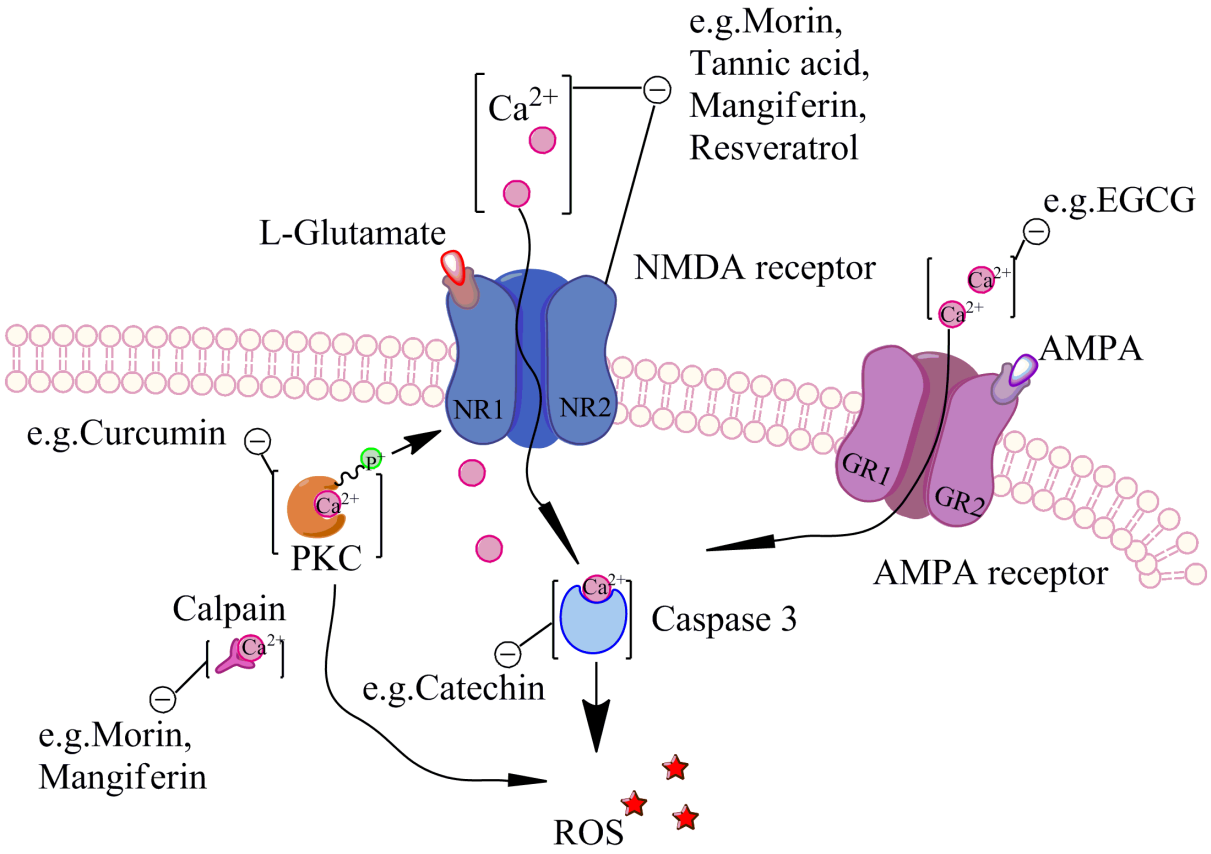

Figure 2: A schematic illustration of NMDA pathway

Polyphenols interact with the NMDA pathway through inhibitory activity on various molecular targets. Catechin has shown to be an effective inhibitor of caspase 3 [322]. NMDA-induced intracellular Ca^{2+} accumulation can activate caspase 3, contributing to ROS generation and neural damage [326]. Catechin can eliminate glutamate-induced toxicity by reducing ROS generation and further neural degeneration. Catechin is also able to inhibit PKC activity [322]. Curcumin and tannic acid are two polyphenols reducing glutamate induced excitotoxicity by inhibiting PKC activity and subsequent phosphorylation of NR1 of the NMDA receptor, thereby reducing glutamate induced Ca^{2+} influx [322]. EGCG has been shown to interact with the NMDA pathway in unilateral cerebral ischemic gerbils by attenuating ischemia-induced MDA elevation [323].

There is strong evidence of protective effects of several natural polyphenols against NMDA neurotoxicity (Table 7) [267, 282, 321, 322, 324].

Polyphenols, act at different locations within the NMDA pathway (Figure 2). In most of the studies, a protective effect against NMDA excitotoxicity has been reported. However, the mechanism of such protection has not clearly been addressed. A few in vitro studies reported a reduction in frequency and amplitude of AMPA/NMDA receptor mediated spontaneous excitatory postsynaptic currents (sEPSCs) in pyramidal neurons. But it remains unclear, whether this is the result of polyphenols anti-oxidant activity or their direct NMDA receptor blocking effect [322, 324, 325].

4.5. Polyphenols and amyloidopathies

Tau hyperphosphorylation and beta amyloid accumulation are believed to be the core pathologies of tauopathies and amyloidopathies [327-329]. Excessive Aβ accumulation can be the result of either increased production or decreased clearance, which in both situations is toxic to the cells [330]. Aβ aggregation leads to the formation of senile plaques (SP) and stimulates a series of biological signaling pathways which leads to an impairment of neuronal synapses and dendrites through oxidative stress and inflammatory responses [331, 332]. Beside SP and Aβ aggregation, neurofibrillary tangles (NFTs), consisting of abnormally hyperphosphorylated tau protein, are other pathologic hallmarks of Alzheimer's disease [333, 334].

For a long time, these two pathological features have been the major therapeutic targets for drug development in tau-amyloidopathies. Polyphenols exert their effect through modulation of α-, β- and γ-secretases, inhibition of Aβ oligomer formation, inhibition of Aβ-induced neurotoxicity and inhibition of Aβ-induced neuroinflammation (Figure 3). Several natural polyphenols have effectively reduced Aβ deposition and Aβ protein concentrations in brain and serum, among which EGCG has shown the most promising anti-amyloidogenic effects (Table 8) [335, 336]. Many studies have shown a direct binding of polyphenols to beta sheet structures [278, 337-339]. ECGC induces α-secretase cleavage activity and inhibit β- and γ-secretases [340, 341]. Several other polyphenols reduce Aβ levels through direct or indirect modulatory effects on α, β, and γ-secretases (Table 8).

Myricetin, quercetin, kaempherol, morin, and apigenin directly inhibit β-secretase activity in a concentration dependent manner [342]. Some of these compounds, like Dihydroguaiaretic acid (NDGA), GSE, tannic acid and wine related polyphenols, affect beta aggregates and destabilize preformed Aβ, while some others affect $A\beta_{(1-40)}$ and $A\beta_{(1-42)}$ and inhibit polymerization and Amyloid beta fibril (fAβ) formation (Table 8, Figure 3).

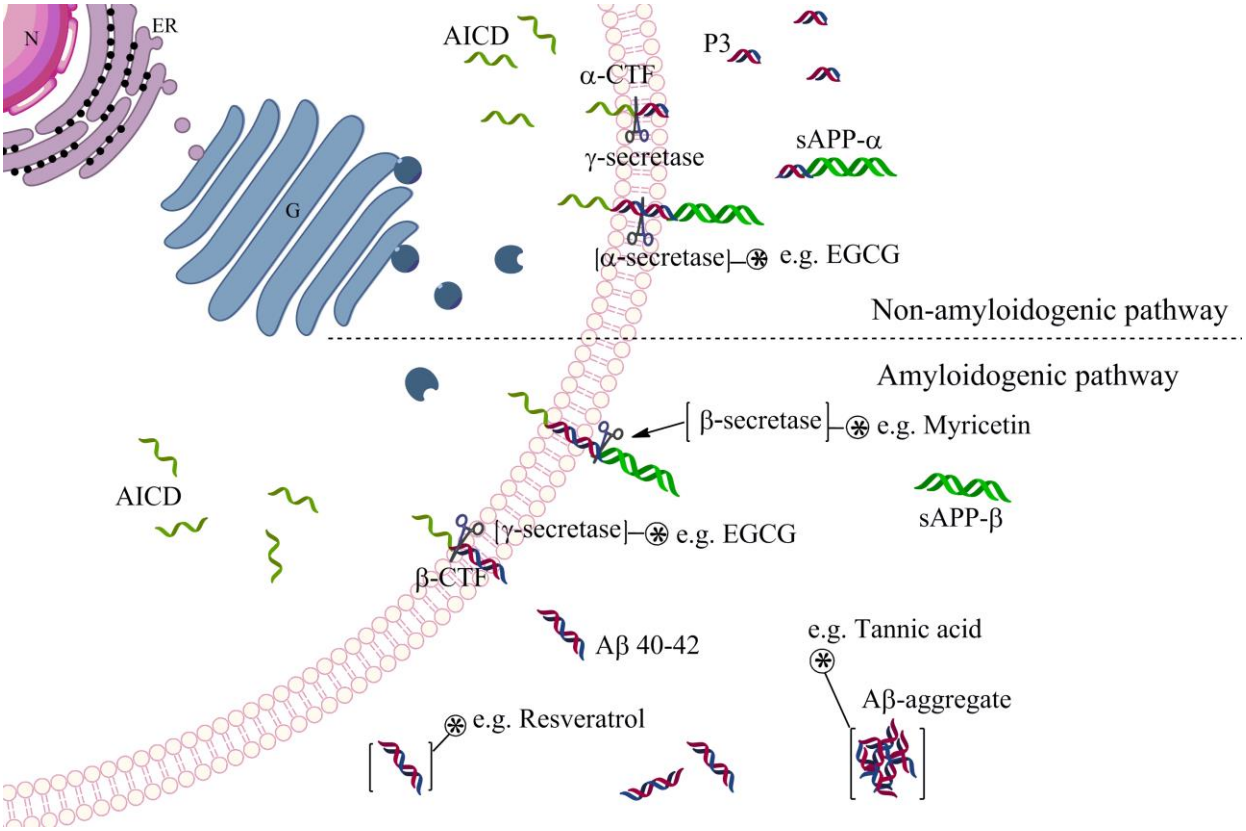

Figure 3: A schematic illustration of amyloidogenic and non-amyloidogenic pathways

Polyphenols exert their effect at different sites within the amyloidogenic pathway. Basically, here are two pathways through which APP can be cleaved. Depending on the enzyme which cleaves the APP, the final product can be amyloidogenic or non-amyloidogenic [343]. In the non-amyloidogenic pathway, APP is cleaved by the membrane-bound enzyme α-secretase within its Aβ domain, resulting in secretion of extracellular soluble sAPPα fragments and a short membrane-bound COOH-terminal fragment, α-CTF or C83, which is cleaved by γ-secretase to p3, a 3-kDa peptide, whereas APP intracellular domain (AICD) is secreted into the cytoplasm [344, 345]. Within the amyloidogenic pathway, APP cleavage by beta-site APP-cleaving enzyme1 (BACE-1, β-secretase) and γ-secretase leads to extracellular sAPPβ fragments and the membrane bound β-C-Terminal Fragment (β-CTF) or C99 fragment, which is subsequently cleaved by γ-secretase at the C-terminal part of the Aβ domain. This leads to the release of APP into the intracellular domain (AICD) and secretion of Aβ into the extracellular spaces [330, 346, 347]. Excessive production of Aβ or decreased Aβ clearance in the later pathway leads to the accumulation and aggregation of Aβ, which is toxic to cells. Therefore, inhibition of either β or γ-secretases or stimulation of α-secretase has been a therapeutic target in amyloidopathies [343]. The polyphenol targets within pathway are indicated by * and a representative compound has been mentioned for each target. For more polyphenols that can affect the pathway, please refer to table 5. N: Nucleus, G: Golgi apparatus, ER: Endoplasmic reticulum.

Table 8: Polyphenols with protective effect against beta-amyloidopathy

Substance	Studied cell line/ animal model	Effect	Ref.
Alvidin	Biochemical assay (UV-visible measurements)	• Implies anti-amyloidogenic effects against whole Aß peptides (1-40 and 1-42)	[348]
Blueberry polyphenolic fractions	Primary hippocampal neurons, LPS, dopamine (DA) or Aβ(42)-induced deficits in Ca^{2+} recovery (CAR)	• Antagonizes LPS, DA- or Aβ (42)-induced deficits in CAR in primary hippocampal neuronal cells	[349]
Curcumin	Brain sections of variant Creutzfeld-Jakob disease cases	• Inhibits in vitro conversion of prion protein (PrP)* and formation of protease resistant PrP • Binds to the alpha-helical intermediate of PrP • Binds to the native form of PrP	[339]
	APP(Swe)/PS1dE9 transgenic mice (a model of AD)	• Reduces Aβ levels in the brain and serum of mice • Reduces amyloid plaques and microgliosis in the brain of Alzheimer's mice • Reduces brain Aβ burden and microglia activation	[350]
Dihydroguaia-retic acid (NDGA)	Biochemical assay (fluorescence spectroscopic analysis)	• Inhibits fAβ* formation and extension from Aβ (1-40) and Aβ (1-42) • Destabilizes preformed fAβ (1-40) and fAβ (1-42)	[351]
EGCG	Yeast prion protein Sup35 Biochemical assay	• Inhibits the formation of aggregating amyloid forms (prions) of Sup35 • Remodels preassembled prions	[352]
	Biochemical assay	• Binds directly to beta-sheet-rich aggregates and mediates the conformational change without their disassembly into monomers or small diffusible oligomers • Converts large, mature alpha-synuclein and Aβ fibrils into smaller, amorphous protein aggregates that are nontoxic to mammalian cells	[337]
	Biochemical assay (A generic model of fibril formation by protein,	• Inhibits in vitro fibril formation by RCM kappa-CN • Inhibits amyloid-fibril formation	[353]

Substance	Studied cell line/ animal model	Effect	Ref.
EGCG	reduced and carboxymethylated kappa-casein (RCM kappa-CN))		
	Biochemical assay	• Inhibits the fibrillogenesis of both alpha-synuclein and Aβ • Binds directly to unfolded polypeptides and preventing their conversion into toxic aggregation intermediates • Promotes the formation of unstructured, nontoxic alpha-synuclein and Aβ oligomers of a new type	[354]
	Inflammatory response induced by interleukin (IL)-1β and Aβ (25-35) in human astrocytoma, U373MG cells	• Inhibits IL-6, IL-8, VEGF and PGE_2 • Attenuates cyclooxygenase-2 expression and activation of NF-κB induced by IL-1β and Aβ (25-35) • Suppresses IL-1β and Aβ (25-35)-induced phosphorylation of the mitogen-activated protein kinase p38 and the c-Jun N-terminal kinase, two protein mediators of MAPK signaling pathway • Induces mitogen-activated protein kinase phosphatase-1 expression	[234]
	Wild type N2 (Bristol), transgenic mutant strain daf-16 (mgDf50), and transgenic Aβ muscle expressing strain of C. Elegans (behavioral and biochemical analysis)	• Attenuates hydrogen peroxide levels • Attenuates age-related behavioral decline • Alleviates an Aβ-induced pathological behavior	[355]
	N2a cells stably transfected with "Swedish" mutant human APP (SweAPP N2a cells)	• Elevates active ADAM10 protein • Increases APPα cleavage • Increases α-secretase cleavage activity • Produces no significant alteration in β-or γ-secretase activities • Elevates soluble sAPPα	[340]
	Human SH-SY5Y neuroblastoma cells, Chinese hamster ovary cells (CHO)	• Shows potent iron-chelating activity • Increases transferrin receptor TfR protein and mRNA levels • Reduces immature and full-length	[356]

Substance	Studied cell line/ animal model	Effect	Ref.
EGCG	overexpressing "Swedish" mutant human APP	cellular holo-APP • Reduces toxic Aβ peptide generation in CHO cells overexpressing the "Swedish" mutant human APP	
	Human SH-SY5Y neuroblastoma, Rat pheochromocytoma PC12 cells, C57/BL mice	• Increases PKCα and PKC in the membrane and the cytosolic fractions of mice hippocampus • Enhances the release of non-amyloidogenic sAPPα • Decreases membrane-bound holo-APP levels, with a concomitant increase in sAPPα levels in the hippocampus	[238]
	Cultured hippocampal neurons exposed to Aβ	• Increases cell survival • Decreases caspase activity • Employs neuroprotection through scavenging ROS	[357]
	MPTP- and DA-induced neurodegeneration in mice and rats	• Prevents the accumulation of iron and α-synuclein in the substantia nigra	[358]
	Yeast model of HD, Transgenic HD flies overexpressing a pathogenic HTTexon 1 protein	• Inhibits mutant htt exon 1 protein aggregation • Modulates misfolding and oligomerization of mutant htt exon 1 protein • Reduces polyQ-mediated htt protein aggregation and cytotoxicity in yeast model of HD • Improves photoreceptor degeneration and motor function in flies	[359]
	Biochemical assay	• Binds to beta-sheet-rich aggregates and mediates the conformational change without their disassembly into monomers or small diffusible oligomers • Converts large, mature alpha-synuclein and Aβ fibrils into smaller, nontoxic amorphous aggregates	[337]
	Human SH-SY5Y neuroblastoma cells(serum deprivation model), CHO cells overexpressing "Swedish"	• Reduces the levels of cellular holo-APP in SH-SY5Y cells • Reduces levels of toxic Aβ peptides in CHO cells over-expressing the APP	[360]

Substance	Studied cell line/ animal model	Effect	Ref.
EGCG	mutant human APP	"Swedish" mutation • Reduces the pro-apoptotic proteins, Bad and Bax • Inhibits the cleavage and activation of caspase-3 • Improves neuronal differentiation	
	SweAPP N2a cells	• Promotes non-amyloidogenic processing of APP • Upregulates α-secretase	[361]
Exifone	Biochemical assay	• Inhibits heparin-induced tau filament formation • Inhibits the formation of Aβ fibrils	[362]
GSE	Tg2576 mice (a transgenic model of AD)	• Inhibits Aβ protein aggregation • Attenuates AD-type cognitive deterioration along with reducing HMW soluble oligomeric Aβ in the brain	[363]
	Transgenic HD PC-12 cells, Transgenic HD model drosophila, R6/2 rodent model of HD	• Inhibits polyQ aggregation • Improves life span • Attenuates motor skill decay	[364]
	TMHT mouse model of tauopathy	• Induces unfolding of tau and diminishes structural stability • Neutralizes phospho-epitopes and disrupts fibrillary conformation leading to disintegration of paired helical filaments (PHFs)* • Attenuates AD type tau neuropathology development in brain • Attenuates extracellular signal-receptor kinase 1/2 signaling in the brain.	[365, 366]
	APP(Swe)/PS1dE9 transgenic mice (a transgenic model of AD)	• Reduces Aβ levels in the brain and serum of the mice • Reduces amyloid plaques and microgliosis in the brain of Alzheimer's mice • Reduces brain Aβ burden and microglia activation	[350]
Green tea polyphenol	Alzheimer's disease like mice induced by D-	• Ameliorates deleterious effects of D-galactose and Aβ (25-35)	[367]

Substance	Studied cell line/ animal model	Effect	Ref.
Green tea polyphenol	galactose and Aβ (25-35) (behavioral study)	• Improves animal's learning and memory • Reduces prolonged latency time and the error numbers • Increases the autonomic activities	
Myricetin	Biochemical assay	• Inhibits fAβ formation from Aβ • Destabilizes preformed fAβ	[368]
Oligonol	Aβ-induced oxidative cell death on rat pheochromocytoma cells (PC12)	• Attenuates Aβ-induced cytotoxicity, apoptotic features, intracellular ROS accumulation, and lipid peroxidation • Increases cellular glutathione pool • Suppresses Aβ-induced activation of NF-κB	[369]
Piceid	Biochemical assay	• Destabilizes Aβ fibrils and oligomers back to monomers • Inhibits Aβ polymerization	[370, 371]
Phenolsulfon -phthalein	Biochemical assay	• Inhibits amyloid fibril formation by islet amyloid polypeptide (IAPP)	[372]
Resveratrol	HEK293 and N2a cells stably transfected with human APP695, APP/PS1 transgenic mice	• Increases cytosolic calcium levels and promotes AMPK activation by the calcium/calmodulin-dependent protein kinase kinase-beta • Lowers extracellular Aβ accumulation • Inhibits AMPK target, mTOR, to trigger autophagy and lysosomal degradation of Aβ • Activates AMPK and reduces cerebral Aβ levels and deposition in cortex	[373]
	Tg19959 transgenic mice model of AD	• Diminishes plaque formation in a region specific manner • Reduces brain glutathione • Increases brain cysteine	[374]
	Biochemical assay	• Inhibits Aβ polymerization	[371]
	HEK293 cells stably transfected with human APP695 (a cell model of AD)	• Shows no inhibition of Aβ production • Shows no effect on β- and ɣ-secretases • Promotes intracellular degradation of Aβ via proteasome system • Reduces secreted and intracellular Aβ levels in different cell lines	[375]

Substance	Studied cell line/ animal model	Effect	Ref.
Resveratrol	Aβ-induced neurotoxicity in SH-SY5Y neuroblastoma cells, Biochemical assay	• Restores GSH content • Employs neuroprotective effects through anti-oxidant activity • Inhibits Aβ (42) fibril formation • Employs no effect on Aβ (42) oligomerization	[376]
Salvianolic acid B	Aβ (25-35) mediated injury of PC-12 cells	• Antagonizes Aβ (25-35)-induced cytotoxicity	[377]
Tannic acid	Biochemical assay	• Inhibits fAβ formation and extension from Aβ (1-40) and Aβ (1-42) • Destabilizes preformed fAβ (1-40) and fAβ (1-42)	[351]
Wine-related polyphenols	Biochemical assay	• Inhibits fAβ formation and extension from Aβ (1-40) and Aβ (1-42) • Destabilizes preformed fAβ (1-40) and fAβ (1-42)	[351]

[a]**Prion protein (PrP)**, in misfolded form, produces transmissible spongiform encephalopathies including bovine spongiform encephalopathy (BSE, also known as "mad cow disease") in cattle and Creutzfeldt-Jakob disease (CJD) in humans. [b]**Poly Q** or polyglutamine is a polypeptide induced by trinucleotide repeat expansion important in trinucleotide repeat disorders like HD. [c]**PHFs** or paired helical filaments are aggregations of hyperphosphorylated tau protein.

Chapter II: Materials and methods

5. Tissue specimens

5.1. Human samples

Tissue material of human samples was retrieved from tumor archives of the Department of Neuropathology, Institute of Pathology and Neuropathology, University of Tuebingen. Tissue handling was performed according to the ethical guidelines of the University of Tuebingen and also in accordance with the principles embodied in the last version of Declaration of Helsinki (Ethics committee reference no. 601/2010BO1- 687/2011BO2). All patients or guardians had given the written informed consent on tissue experiments.

5.1.1. Pituitary adenoma

The samples were diagnosed according to the current World health organization (WHO) criteria for tumors of endocrine organs by at least two senior neuropathologists [378]. Atypical pituitary adenomas were classified into two groups according to their p53 status. One group contained tumors with a MIB-1 index ≥3% and p53 < 10% and the other group contained samples with a MIB-1 index ≥ 3% and p53 ≥ 10%. Specimens were fixed in 4.5% formalin (pH 7) (ready to use solution, Roti®-Histofix 4.5%, Carl Roth, Karlsruhe, Germany) and were embedded in paraffin. The blocks were cut with a microtome (4 µm thickness) and placed on Super Frost Plus slides (Microm International, Walldorf, Germany).

5.1.2. Brain glioma

The tumor samples were diagnosed according to the current WHO criteria for tumors of the nervous system by at least two senior neuropathologists [379]. The human samples consisted of 7 grade II astrocytomas, 8 grade III astrocytomas and 8 grade IV glioblastomas. Specimens were fixed in 4.5% formalin (pH 7) (ready to use solution, Roti®-Histofix 4.5%, Carl Roth, Karlsruhe, Germany) and were embedded in paraffin. The blocks were cut with a microtome (4 µm thickness) and placed on Super Frost Plus slides (Microm International, Walldorf, Germany).

5.1.3. CNS ependymoma

The samples consisted of 85 ependymomas, including 19 WHO grade I (14 myxopapillary and 5 subependymomas), 46 grade II ependymomas and 20 grade III anaplastic ependymomas

diagnosed from 1988 to 2008. 14 cases were recurrent tumors. Clinical data were retrieved from the patients' files. The tumor samples were diagnosed according to the current WHO criteria for tumors of the nervous system by at least two senior neuropathologists.

5.2. Animal models

Animal models were produced by other colleagues as cited. The tissues were retrieved from the animal tissue bank for my experiments.

5.2.1. C6 rat

The C6 tumors were produced by implanting the rapidly proliferating rat C6 glioma cell line into the basal ganglia of Sprague Dawley rats. The rats were sacrificed 2 weeks after tumor cell implantation [380]. C6 samples consisted of 15 brain specimens that were studied in three different tumor regions, including perinecrotic areas, central regions and borders of the tumor. Specimens were fixed in 4.5% formalin (pH 7) (ready to use solution, Roti®-Histofix 4.5%; Carl Roth, Karlsruhe, Germany) and were embedded in paraffin. The blocks were cut with a microtome (4 µm thickness) and placed on Super Frost Plus slides (Microm International, Walldorf, Germany).

5.2.2. APPPS1-21 transgenic mice

APPPS1-21 transgenic mice model of AD was a kind gift from Prof. Mathias Jucker [381]. APPPS1-21 mice overexpress a human APP with Swedish mutation (KM670/671NL) and PS1 with L166P mutation under the control of Thy1 promoter [381]. Brains of the mice were fixed in 4.5% formalin (pH 7) (Ready to use solution, Roti®-Histofix 4.5%, Carl Roth, Karlsruhe, Germany) and were embedded in paraffin. The blocks were cut with a microtome (sections of 4 µm thickness) and placed on Super Frost Plus slides (Microm International, Walldorf, Germany) [382].

5.2.3. Experimental autoimmune encephalomyelitis

Male LEW rats (200-250 g) were immunized with 100 µl of an emulsion of an equal volume of saline and complete Freund's adjuvant (CFA) containing 50 µg of synthetic peptide MBP68–84 (YGSLPQKSQRSQDENPV) and 1 mg/ml mycobacterium tuberculosis [383]. The rats were scored daily for development of Experimental autoimmune encephalomyelitis (EAE) and

neurological signs were scored as follows:

0 – No clinical signs

1 – Loss of tail tone (flaccid tail)

2 – Tail weakness plus hind-limb paresis (ataxia)

3 – Moderate hind-limb paralysis

4 – Tetraparesis

5 – Moribund

EAE rats were housed under a 12 h light, 12 h dark cycle with free access to food and water. All animal procedures were in accordance with a protocol approved by the local administration of district official committee [384].

6. In vivo experiments

The tissues were retrieved from the animal tissue bank for my experiments. Animal treatments were designed and performed in our lab by Zhang et al. and tissue banks were prepared from animal models after each experiment.

6.1. MS-275 treatment

APPPS1-21 mice were divided into two groups. The first group included six APPPS1-21 mice of 5 month old, including 3 males and 3 females. The mice in this group were treated with MS-275 (Alexis Biochemicals, Loerrach, Germany), 5 mg/kg body weight, suspended in 1% carboxymethylcellulose (CMC) (Blanose, Hercules-Aqualon, Düsseldorf, Germany) through daily gavage for 10 days. The second group included six gender and age matched APP/PS1-21 mice, as control, received the same volume of 1% CMC dissolved in water through daily gavage for the same time length [382].

6.2. Valproic acid (VPA) treatment

For preventive treatment, VPA (Sigma–Aldrich Chemie GmbH, Munich, Germany; 250 or 500 mg/kg in phosphate-buffered saline (PBS)) or PBS was given to EAE rats by gavage once daily from Day 0 to Day 18 (6 rats/group). For therapeutic treatment, VPA (500 mg/kg in PBS) or PBS was given by gavage once daily from day 7 to day 18 or from day 9 to day 19 (6 rats/group) [384]. The brains of EAE rats from therapeutic treatment group were used for epigenetic study.

6.3. Icariin treatment

APPPS1-21 mice were divided into two groups. The first group included seven APP/PS1-21 mice of 5 month old, including 3 males and 4 females. The mice in this group were treated with icariin (Alexis Biochemicals, Loerrach, Germany), 100 mg/kg body weight, suspended in 1% carboxymethylcellulose (CMC, Blanose, Hercules-Aqualon, Düsseldorf, Germany) through daily gavage for 10 days. The second group included 7 gender and age matched APP/PS1-21 mice, as control, received the same volume of 1% CMC dissolved in water through daily gavage for the same time length. The experiment was designed and performed in our lab by (Zhang et.al.-unpublished experiment) and tissue sections were used by me for epigenetic study.

7. Cell Culture

Murine N9 microglia cells were grown in RPMI-1640 with 10% heat inactivated fetal calf serum (FCS) with penicillin and streptomycin at 100 U/ml (Gibco, Grand Island, NY) at 37 8C in 5% CO2. 10^5 cells were seeded into 12-well cell culture plates and cultured for 48 h.

8. Lipopolysaccharide induction

Escherichia coli 0111: B4 LPS was purchased from Sigma Aldrich, Munich, Germany. The cells were stimulated with 1 µg/ml LPS, and treated with valproic acid sodium salt (Sigma Aldrich, Munich, Germany) 10, 100 and 1000 µM, or Resveratrol 1, 10, 100 µM for 24 h.

9. Standard Griess assay

The concentration of NO in supernatants was measured by standard Griess assay (Sigma, Munich, Germany). Briefly, 50 µl of supernatants was mixed with an equal volume of Griess reagent (1% sulphanilamide and 0.1% naphthyl-ethylenediamine dihydrochloride in 2.5% phosphoric acid from Sigma Aldrich, Munich, Germany). After 30 min of incubation in the dark at room temperature, the absorbance of the performed chromophore was measured at 560 nm using a multi-plate reader. Nitrite concentration was calculated using a standard calibration curve of sodium nitrite [385]. All assays were performed in triplicate setting.

10. MTT assay

Cell viability of treated N9 cells was detected by MTT assay. After treatments, the cells in 12-well plates were washed with PBS. MTT solution (5 mg/ml, Sigma-Aldrich) was added to each well. After 4 hours incubation at 37°C and 5% CO2, the cells were washed again with PBS and then DMSO was added to each well to thoroughly dissolve the formazan. Thereafter, OD of each well was read at 560 nm and background at 670 nm was subtracted.

11. Resveratrol, valproic acid and curcumin treatment

Murine N9 microglia cells were seeded into 12-well cell culture plates and cultured for 48 h. After reaching the sufficient confluency, the cells were treated with LPS (1µg/ml) for 24 hours as described. The medium supernatants were collected and concentration of nitric oxide (NO) was measured by standard Griess assay. Thereafter, the cells were treated with a wide range of concentrations of curcumin, VPA and resveratrol (1, 10, 100, 1000, and 1500 µM) for 24 hours. MTT assay was performed on treated N9 cells and toxic concentrations were omitted for all compounds. LPS-induced N9 cells were then treated with chosen concentrations of curcumin (1 and 10 µM), VPA (1, 10, 100, 1000 µM) and resveratrol (1, 10, 100 µM) for 24 hours. The supernatants were collected and NO concentration was measured by standard Griess assay after treatment.

12. Cytospin preparation

Cell suspension of 5 x 10^4 cells/ml of medium containing 10% FCS was prepared from each well. Before the slide placement, the cytospin slides were labeled appropriately. The slides were then mounted with the paper pad and the cuvettes were placed in the holder. Each cuvette was loaded with up to 200 µl of the cell suspensions. The slides were spun at 800 rpm for 3 min. The slides were left dried and immediately used for immunohistochemistry. Immunocytochemistry was performed on cytospin preparations of N9 cells treated with VPA 1 mM and resveratrol 100 µM concentrations as previously described.

13. Tissue microarray

TMAs were prepared by Schittenhelm et.al. [386]. Representative tissue microarrays with a sample diameter of 1000 µm were prepared with a TMA machine (Beecher Instruments, Sun

Prairie, 160 WI, USA) [386]. The TMA slides were cut to 4 µm thickness and placed on Super Frost Plus slides (Microm International, Walldorf, Germany). In microarrays from each case 2-4 tissue punches from distinct regions of tumor were available. For the tumors with higher heterogeneity, more tissue punches were prepared.

14. Immunohistochemistry

Immunohistochemistry was performed on consecutive sections of paraffin-embedded tissue samples or TMAs [70]. The slides were de-waxed in chloroform for 30 min, then rehydrated in descending concentration series of ethanol and washed in Tris-buffered saline (TBS). Slides were then boiled in citrate buffer (2.1 g sodium citrate/L, pH 6) in a microwave oven for 15 minutes with 800 W power, and thereafter cooled to the room temperature. Endogenous peroxidase was blocked with 3% H2O2 in methanol for 15 min, and standard swine serum was applied for 15 min in room temperature to prevent nonspecific antibody binding. Sections were incubated with primary antibody overnight at 4°C. After washing in TBS for 5 min, sections were incubated for 30 min with the secondary antibody (1:400) at room temperature. The peroxidase-conjugated avidin-biotin complex (ABC) technique (Dako, Hamburg, Germany) with diaminobenzidine (DAB) (Sigma, Deisenhofen, Germany) as chromogen was used to visualize the antibody binding. All sections were counter-stained with Mayer's hemalum. For negative control, primary antibody was replaced by Immunoglobulin G isotype control antibody in the appropriated concentrations. For positive control proper tissue sections according to the data-sheet of the antibody were used. Following antibodies were used for my experiments:

- H3K9Ac (rabbit polyclonal, Abcam, Cambridge, UK; 1:500) as primary antibody
- Anti-mouse IgG (Rabbit polyclonal, Dako, Hamburg, Germany; 1:400) as secondary antibody

15. Microscopy and Image analysis

Immunolabled sections were visualized using X40 objective lens. All analyses were carried out in comparable areas under the same optical and light conditions. Color images were provided using AxioVision software (Zeiss).

Figure 4: Reference grayscale for analysis of H3K9Ac staining intensity

Optical density spectrum of H3K9Ac staining was illustrated as a 255-grayscale with "0" as the highest optical density (higher H3K9Ac staining intensity) and "255" as the lowest optical density (lower H3K9Ac staining intensity).

Image analysis was performed blindly with respect to all other clinical and histopathological data on tissue sections using software IMAGEJ, version 1.43u (NIH, Bethesda, MD, USA). 200 to 1000 cells were counted within 3-5 different regions based on the cell density of the area and tissue type.

In order to analyze the intensity variation among treatment and control groups we used the histograms of images using ImageJ software and statistically analyzed image parameters widely used in content-based image retrieval systems [387-389]. Staining intensity inside each nucleus was measured as follows; after calibration, image color threshold was adjusted so that the positive nuclei were selected and the rest of the image was ignored during the analysis. The histogram of positive cells was provided. The histogram demonstrates the frequencies of the pixels in a grayscale image and provides the information about the distribution of intensity levels within an image. Our histogram presented the distribution of positive pixels within a 255 gray scale (Figure 4). The optical density (OD) of image background was subtracted from the whole histogram. For each brain region studied, we plotted the cell frequency against these OD values [390].

We quantized our histograms into 26 levels from 0 to 250. The quantization is a process in which the histogram is divided into levels or bins. Every histogram was then quantized into 26 bins as follows:

$$H = \{h(b_0), h(b_{10}), h(b_{20})\ldots h(b_{250})\}$$

where $h(b_0)$ is the frequency of pixel values in bin (b_0) and H is the histogram of 26 bins. The statistical color features, including mean and standard deviation, were then calculated from distribution of intensity levels in histogram bins of H as follows: If μ_j is the mean and σ_j is the standard deviation in a particular bin j, where $j = 0, 10, 20\ldots, 250$, and x_{ji} is the pixel value in

bin *j* and pixel *i* and N is total number of pixels in each bin, then

$$\mu_j = \frac{1}{N}\sum_{i=0}^{N} x_{ji} \qquad \sigma_j = \sqrt{\frac{1}{N}\sum_{i=0}^{N}(x_{ji} - \mu_j)^2}$$

After the calculation of all values, the feature vector FV of these values was constructed as: FV= {μ_1, μ_2 ….. μ_m, σ_1, σ_2 ... σ_m} in both treatment and control group. The values were once compared in each bin, $h(b_j)$, *j = 0, 10, 20…, 250* using independent two-sample T-test *as follows:*

$$T = \frac{\bar{\mu}_j c - \bar{\mu}_j t}{\sigma_{\mu_j c \mu_j t} \cdot \sqrt{2/N}} \qquad \sigma_{\mu_j c \mu_j t} = \sqrt{1/2(\sigma^2_{\mu_j c} + \sigma^2_{\mu_j t})}$$

Where $\bar{\mu}_j c$ is the mean value of (μ_1, μ_2 ….. μ_m) in control group and $\bar{\mu}_j t$ is the mean of (μ_1, μ_2 ….. μ_m) in treatment group and $\sigma_{\mu_j c \mu_j t}$ is the pooled standard deviation. The values were once again compared in the whole histogram this time H = {$h(b_0)$, $h(b_1)$, $h(b_3)$…$h(b_{255})$} of treatment and control group using independent two-sample T-test as follows:

$$T = \frac{\bar{X}_c * f_c - \bar{X}_t * f_t}{S_{X_c X_t} \cdot \sqrt{2/N}} \qquad S_{X_c X_t} = \sqrt{1/2(S^2_{X_c} + S^2_{X_t})}$$

Where $\bar{X}_c$ is the mean of (μ_1, μ_2 ... μ_m) in control group and f_c the mean frequency of acetylated cells in control group and $\bar{X}_t$ is the mean of (μ_1, μ_2 ... μ_m) in treatment group and f_t the mean frequency of acetylated cells in treatment group. $S_{X_c X_t}$ is the pooled standard deviation, c = control group, t = treatment group and the denominator of *T* is the standard error of the difference between two means. For significance testing, the degree of freedom for this test is 2n − 2 where n is the number of mice in each group.

16. Statistical analysis

The arithmetic means were compared by one-way and two-way analyses of variance (ANOVA). For pairwise comparison, paired or unpaired sample T-test was applied where appropriate. Post-hoc analysis was performed where appropriate. Data was tested for the normality of variance. P-value of ≤ 0.05 was considered as the significant difference and

individual P-values were calculated. GraphPad Prism 5.0 (www.graphpad.com) was used for statistical analysis. The graphs were drawn by the same software and data were shown by mean values and standard error of mean (SEM) or standard deviation (SD).

Chapter III: Results

17. Alteration of global H3K9Ac pattern correlates with grade of malignancy in gliomas

Malignant gliomas are devastating brain tumors with no promising treatment. Recent findings about epigenetic mechanisms involved in the pathology of gliomas, along with the development of HDAC inhibitors (HDACIs), have introduced new therapeutic options for gliomas. However, epigenetic-based therapies have not shown the same efficacy for all glioma patients and the target group for such therapeutics needs to be more specified. Any further achievement in glioma therapy hence, requires a deeper knowledge of epigenetic mechanisms and dysregulations in these tumors.

Alteration in acetylation of histone 3 lysine 9 (H3K9Ac), an epigenetic biomarker of active gene transcription, has been associated with many cancers as described in chapter I. Based on this evidence, we studied the acetylation pattern of H3K9 in different grades of human glioma and in rat C6 tumor, a xenograft glioma model. Using immunohistochemistry, we analyzed H3K9 acetylation pattern in 15 cases of C6 tumor as well as 23 human brain glial tumors including 7 astrocytomas WHO grade II, 8 astrocytomas WHO grade III, and 8 glioblastoma multiform (GBM) WHO grade IV.

In C6 tumor samples, the cells were counted in perinecrotic areas, central vital tumor regions and borders of the tumor. Counting was performed once in the whole area of tumor without considering the regions differentially, and another time for each of the three mentioned tumor regions separately. Immune reactive area was localized to the cell nucleus, stained in brown, and non-reactive area of nucleus was counterstained in blue. We used a semi-quantitative scoring method, based on the pattern of nuclear staining, so that the nuclei which were stained totally brown were considered as acetylated or positive, the nuclei which were stained partially in blue and partially in brown were considered as partially acetylated or partially positive and any nucleus that completely lacked brown color was considered as nonacetylated or negative. Up to 1000 cells were counted within 3-5 different fields. In order to better visualize the nuclear staining and discriminate the colors well, we used pseudo-colorization by imageJ software turning blue color to red and brown to green (Figure 5). In C6 tumor, a significantly high proportion of nuclei were H3K9Ac negative (Mean ± SD: non-acetylated 83% ± 17 vs. partially acetylated 14% ± 16 vs. acetylated 1% ± 2; N = 15) ($P< 0.0001$) (Figure 6).

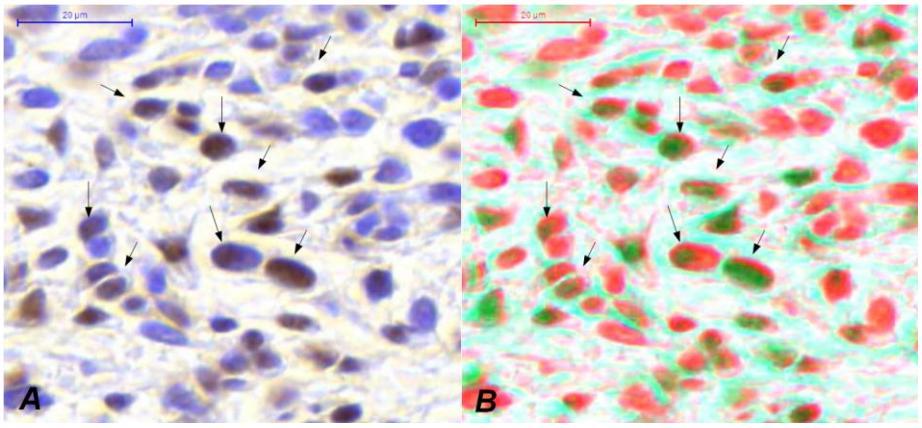

Figure 5: Pseudo-coloring of H3K9Ac nuclear staining using ImageJ software

In order to better visualize the nuclear staining and discriminate the colors well, we performed pseudo-colorization by imageJ software. Blue color was pseudo-colored to red and brown color was pseudo-colored to green. Arrows indicate the partially stained nuclei which are hard to discriminate from totally brown in original image. A: Original H3K9Ac staining pattern in tumor cells, B: Pseudo-colored image of frame A.

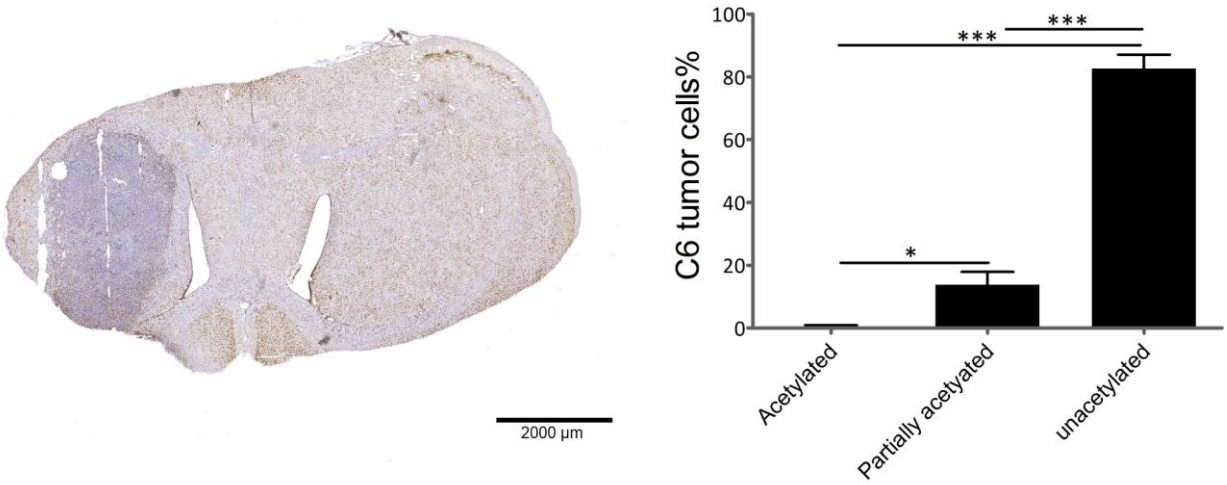

Figure 6: Global H3K9Ac pattern in rat C6 glial tumor

Left: Immunohistochemical demonstration of H3K9Ac in a brain section bearing rat C6 tumor. Color image is provided by AxioVision software (Zeiss). Right: 83% ± 18 of the C6 nuclei were completely non-reactive to H3K9Ac (negative) while 14% ± 16 of the nuclei were partially positive and only 1% ± 2 of the tumor nuclei were strongly positive, *P < 0.05 and ***P < 0.0001.

Interestingly, 3 out of 15 (20%) cases of C6 tumors did not show any H3K9 acetylation in nuclei all over the tumor area; even the nuclei of endothelial cells within the tumor area revealed a complete lack of H3K9Ac staining, while cell nuclei in adjacent brain tissue, surrounding the hypercellular tumor area, were specifically stained for H3K9Ac (Figure 7).

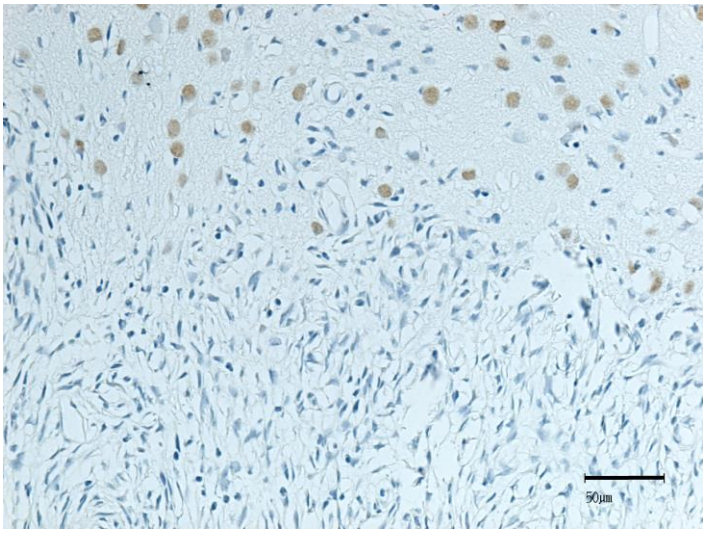

Figure 7: Immunohistochemical demonstration of H3K9Ac in a rat C6 tumor

In 3 out of 15 C6 tumors the nuclei revealed no H3K9Ac immune reactivity. The tumor region was compared with the adjacent brain tissue considering H3K9Ac staining pattern. There was a complete lack of H3K9Ac staining in nuclei of cells within hypercellular tumor region, while adjacent brain tissue reveals H3K9Ac staining in some of the nuclei. Scale bar represents 50 µm.

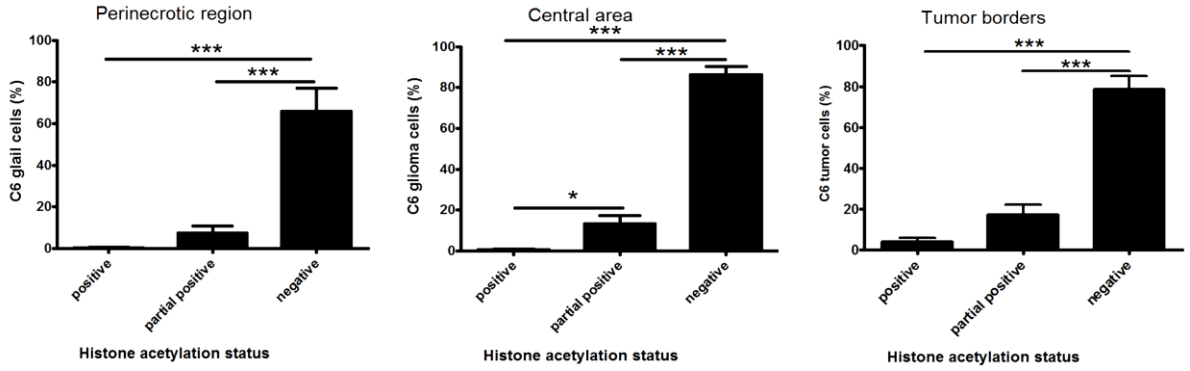

Figure 8: Global H3K9Ac status in different C6 tumor regions

C6 cells were mostly non-acetylated followed by partially acetylated and acetylated nuclei either in infiltrating tumor borders, central intact areas, or perinecrotic areas of tumor. Error bars represent SEM, *P < 0.05 and ***P < 0.0001.

Investigating the global H3K9 acetylation status in different C6 tumor regions, revealed the same pattern in each tumor region so that the highest numbers of C6 cells had non-acetylated nuclei followed by partially acetylated and acetylated nuclei either in infiltrating tumor borders, central intact areas, or perinecrotic areas of tumor (Figure 8). Besides, infiltrating tumor borders had higher number of H3K9 acetylated cells compared to perinecrotic tumor regions, but not to central vital areas of C6 tumor (Mean ± SEM; Infiltrating tumor boarders: 4% ± 2 vs. perinecrotic tumor regions: 0.4% ± 0.2 vs. central

intact areas: 0.6% ± 0.4) (Figure 9). We also analyzed H3K9Ac patterns in human brain glioma (astrocytoma WHO grade II and III and GBM WHO grade IV) using the same method of analysis as for C6 tumors.

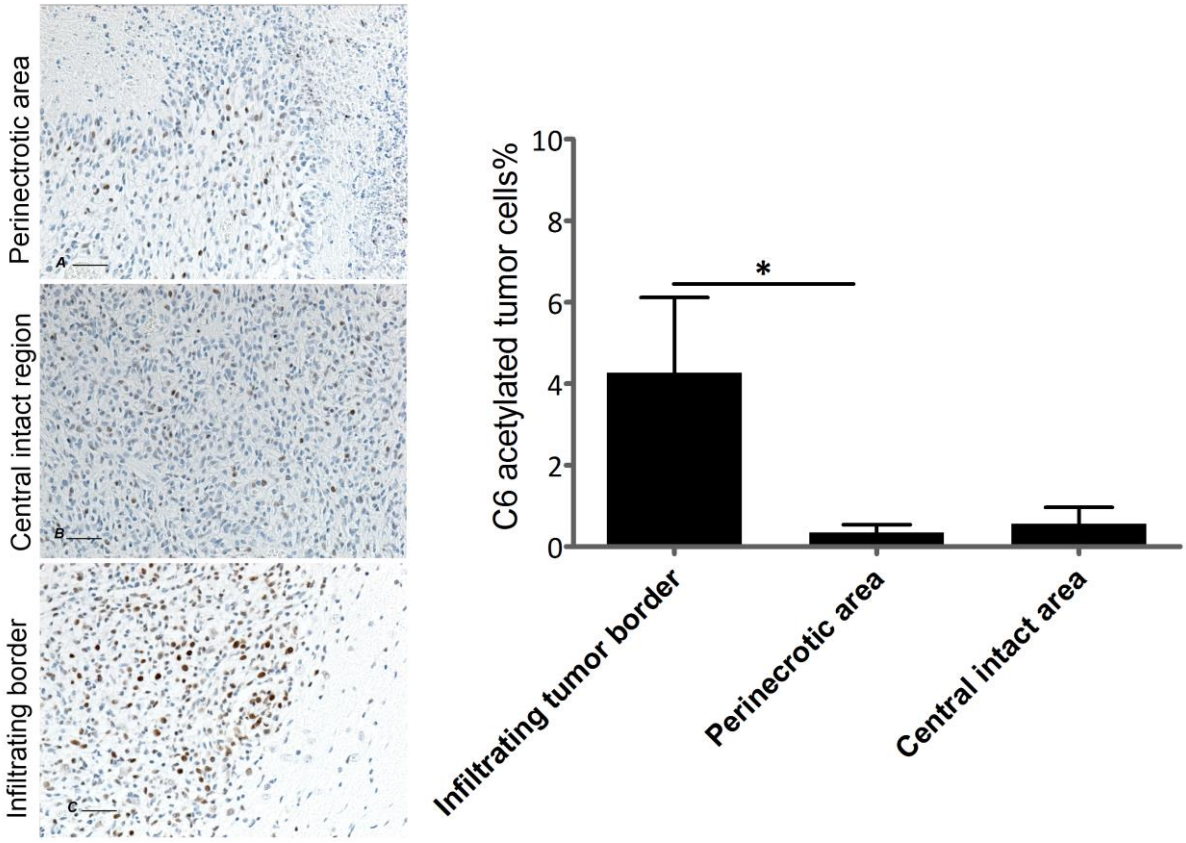

Figure 9: Region specific H3K9Ac patterns in C6 tumor
Infiltrating tumor borders revealed a higher number of acetylated nuclei compared to other tumor regions. In perinecrotic area, the tumor shows serpiginous necrosis with pseudopalisading of tumor cells around necrotic foci. Error bars represent SEM, *P < 0.05.

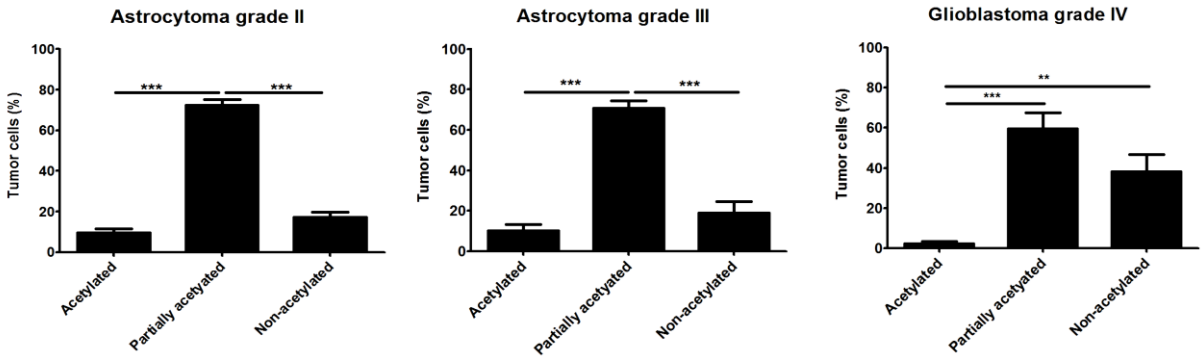

Figure 10: Global H3K9Ac status in diverse WHO grades of human brain glioma (1)
In human samples, tumor nuclei were mostly partially acetylated, either in astrocytoma grade II, III or GBM grade IV. Error bars represent SEM, **P < 0.001 and ***P < 0.0001.

Table 9: Descriptive statistics of H3K9Ac status in various WHO grades of human glioma (1)

Tumor grade	H3K9 acetylation status	Mean	SD	N (tumor samples)
Astrocytoma grade II	Acetylated	10%	2	7
	Partially acetylated	73%	3	7
	Non-acetylated	17%	2	7
Astrocytoma grade III	Acetylated	10%	3	8
	Partially acetylated	71%	4	8
	Non-acetylated	19%	6	8
GBM grade IV	Acetylated	2%	1	8
	Partially acetylated	59%	8	8
	Non-acetylated	38%	9	8

The diagnosis and grading of samples was established by two senior neuropathologists in advance. In human samples, tumor nuclei were mostly partially acetylated, either in astrocytoma grade II, III or GBM grade IV ($P < 0.0001$) (Table 9, figure 10).

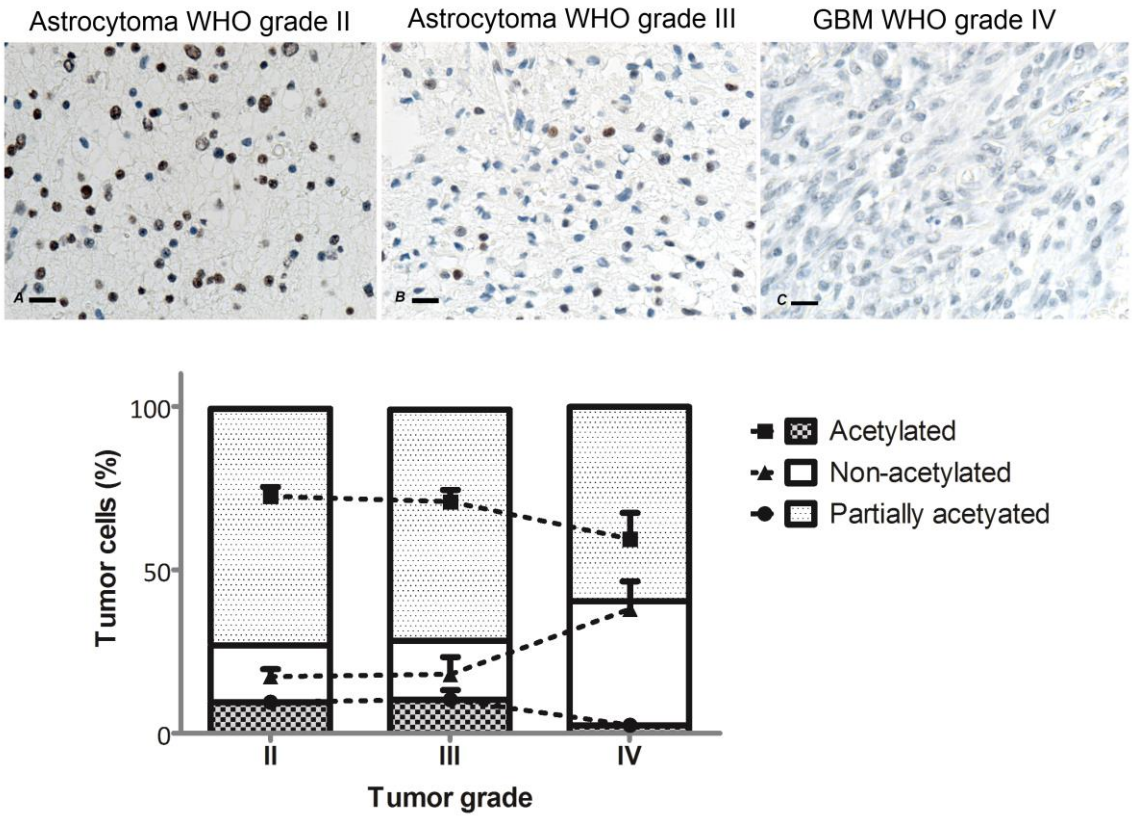

Figure 11: Global H3K9Ac status in diverse WHO grades of human brain glioma (2)

Increased WHO grade of tumors was associated with decreased proportion of acetylated and partially acetylated nuclei, and increased proportion of non-acetylated nuclei, Symbols represent mean and error bars represent SEM.

The difference in H3K9 acetylation between different tumor grades was noticeable so that there were a significantly higher number of non-acetylated nuclei in WHO grade IV tumors compared to grade II and III (P< 0.05) (Figure 11, table 10). Endothelial proliferation is a pathological feature associated with GBM grade IV. Endothelial cell nuclei were acetylated, partially acetylated or non-acetylated in these tumors (Figure 12).

Table 10: Descriptive statistics of H3K9Ac patterns in various WHO grades of human glioma (2)

WHO tumor grade	H3K9 status	Mean difference	Significance	95% CI of difference
II vs. III	Acetylated	0.8	ns	-19.9 to 21.5
	Partially acetylated	-1.7	ns	-22.4 to 18.9
	Non-acetylated	1.6	ns	-19.1 to 22.3
II vs. IV	Acetylated	-7	ns	-27.7 to 13.6
	Partially acetylated	-13	ns	-33.7 to 7.7
	Non-acetylated	20.8	*	0.1 to 41.5
III vs. IV	Acetylated	-7.8	ns	-27.8 to 12.2
	Partially acetylated	-11	ns	-31.3 to 8.7
	Non-acetylated	19.2	*	-0.8 to 39.2

Descriptive statistics of two-way ANOVA analysis of H3K9Ac pattern in various grades of human brain glioma including astrocytoma grade II, III and GBM grade IV. Bonferroni's multiple comparisons were used as post-hoc test. CI: confidence interval, ns: non-significant, *P < 0.05.

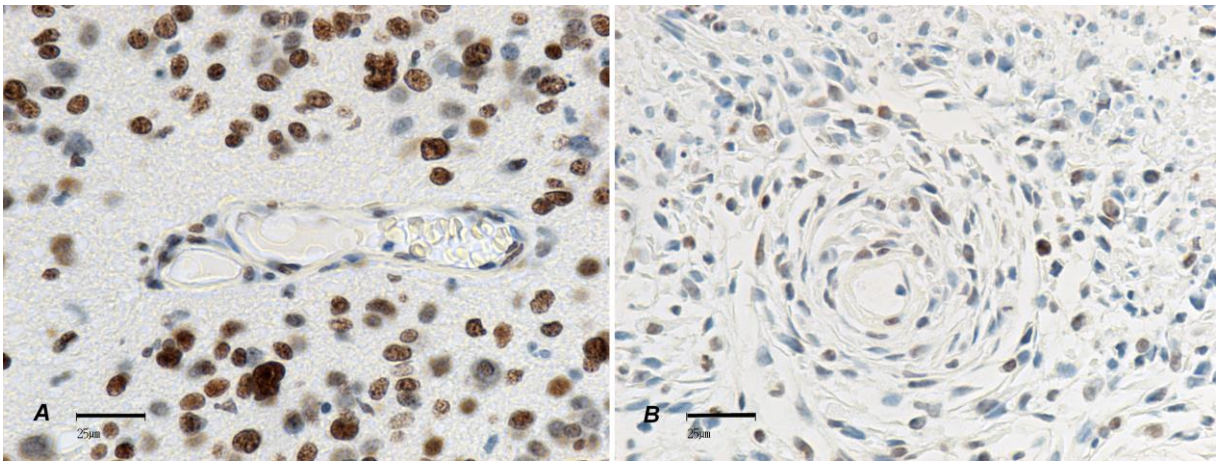

Figure 12: Immunohistochemical demonstration of H3K9Ac in endothelial cells

Left: Non-proliferated endothelial cells of brain vasculature, right: endothelial proliferation in GBM. Endothelial cell nuclei were acetylated, partially acetylated or non-acetylated within tumor region and infiltrating surrounding brain tissue. Scale bars represent 25µm.

18. H3K9Ac patterns of typical and atypical pituitary adenomas indicate epigenetic shift of these tumors

Pituitary adenomas are benign endocrine tumors of anterior pituitary that are subclassified as typical (conventional) or atypical adenomas. Atypical adenomas are associated with an uncertain prognosis based on histopathological features. Clarifying epigenetic alterations of pituitary tumors, and their underlying mechanisms, will hopefully open new windows to treatment and classification of these tumors and might introduce novel markers for survival prediction in these tumors.

In the present study, using immunohistochemistry, we investigated the acetylation pattern of histone 3 lysine 9 (H3K9Ac), in typical and atypical pituitary adenomas and in normal pituitary. A total of 104 cases were investigated, consisting of 19 normal pituitaries, 43 typical pituitary adenomas with a MIB-1 index < 3%, 32 atypical adenomas with a MIB-1 index ≥ 3% and p53 < 10%, and ten atypical adenomas with a MIB-1 index ≥ 3% and p53 ≥ 10% together. The results of this study were published in journal of neuroendocrinology [70].

Cell nuclei of normal pituitary samples were mostly non-acetylated (mean ± SEM; 61% ± 30), although those in typical pituitary adenoma were mostly partially acetylated (mean ± SEM; 61% ± 19), and those in atypical adenoma were mostly acetylated (mean ± SEM; 52% ± 26) (Figure 13).

The proportion of acetylated nuclei therefore increased from normal pituitary to typical adenoma and atypical adenoma ($P < 0.0001$). In typical pituitary adenomas, numbers of partially acetylated nuclei were significantly higher compared to the other two groups ($P < 0.0001$) and, in normal pituitary, numbers of non-acetylated nuclei were also significantly higher compared to any type of pituitary adenoma ($P < 0.0001$) (Figure 14).

We next evaluated the effect of increasing p53 gene expression on acetylation status of atypical adenomas by comparing atypical adenomas, in which the MIB-1 index was ≥ 3% and p53 was < 10%, with atypical adenomas, in which the MIB-1 index was ≥ 3% and p53 was ≥ 10%. In atypical adenomas with a MIB-1 index ≥ 3% and p53 ≥ 10%, we observed a significantly higher proportion of partially acetylated cell nuclei compared to atypical adenomas with a MIB-1 index ≥ 3% and p53 < 10% (Mean ± SEM; 50% ± 21 vs. 33% ± 19, $P < 0.05$) (Figure15).

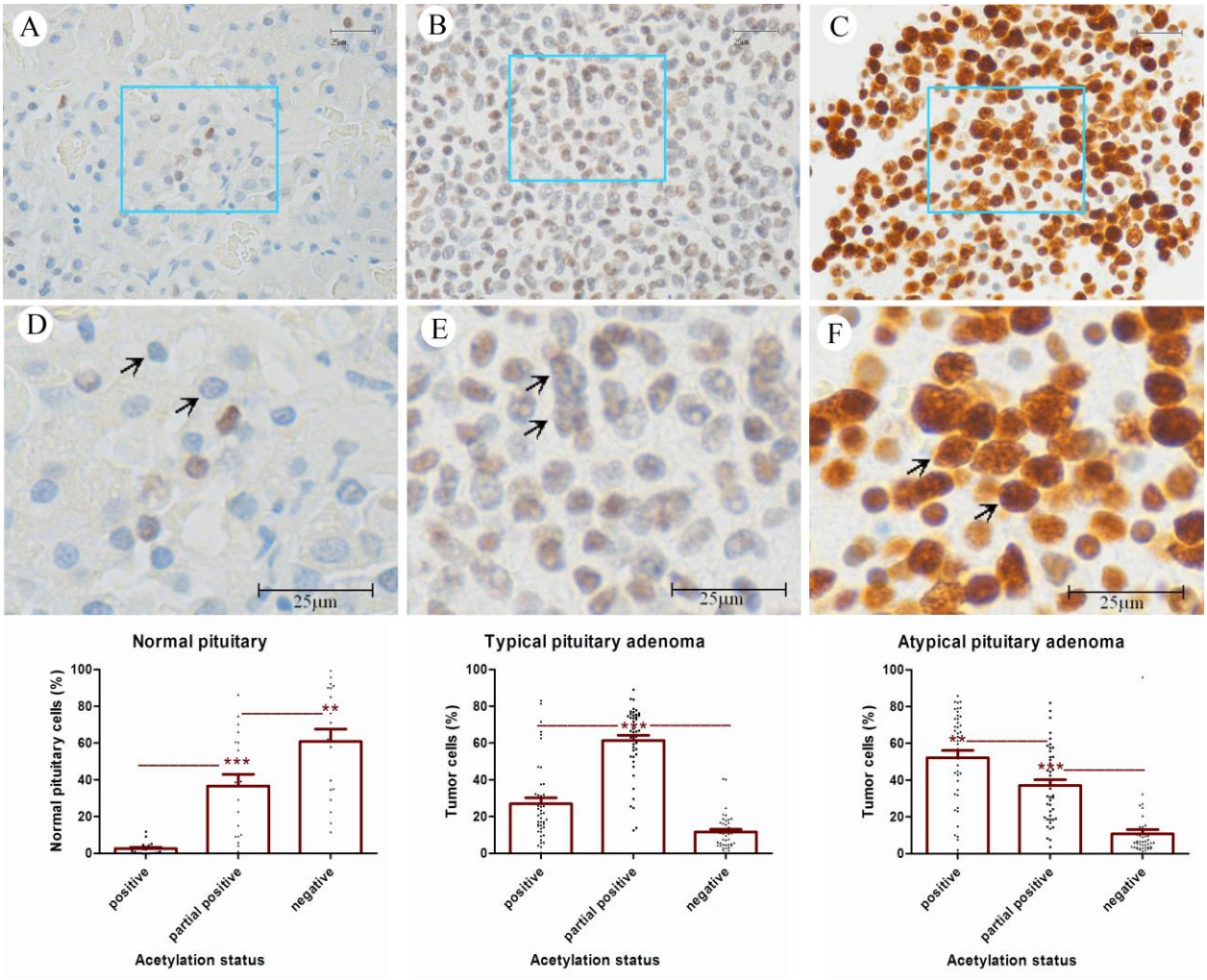

Figure 13: Immunohistochemical demonstration of global H3K9Ac in pituitary tissue

(A) Normal pituitary, (B) typical pituitary adenoma, (C) atypical pituitary adenoma, Arrows in (D) represent negative nuclei, arrows in (E), partial positive nuclei and arrows in (F), positive nuclei. All of the samples were stained in the same way at the same time. **P< 0.001 and ***P< 0.0001.

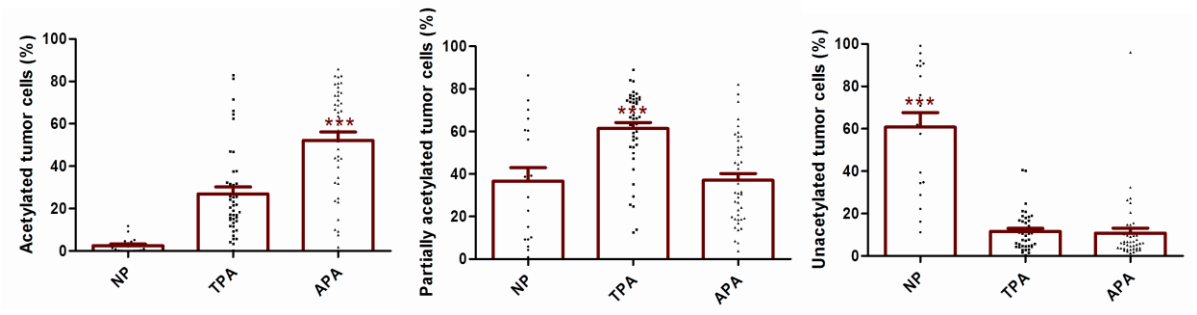

Figure 14: Comparison of global H3K9Ac in normal and pathological pituitary tissue

NP: normal pituitary; TPA: typical pituitary adenoma; APA: atypical pituitary adenoma, ***P < 0.0001.

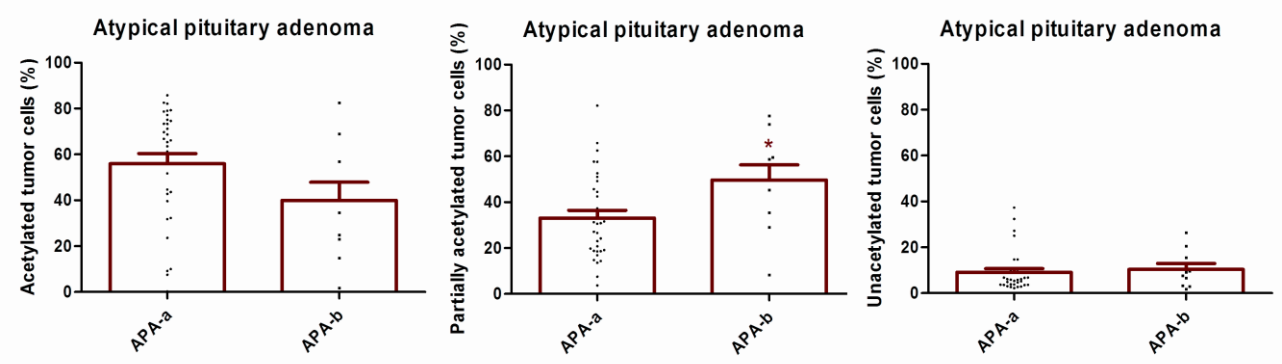

Figure 15: Comparison of global H3K9Ac in atypical adenomas with different p53 expression

(APA)-a) represents atypical pituitary adenomas with MIB-1 ≥ 3% and p53 < 10% and (APA-b) represents atypical pituitary adenomas with MIB-1 ≥ 3% and p53 ≥ 10 (*P< 0.05).

We further compared tumors with a MIB-1 index < 3% versus tumors with a MIB-1 index ≥ 3%, regardless of tumor type, to investigate the effect of increasing MIB-1 index on acetylation pattern of H3K9. Tumors with a higher MIB-1 index ≥ 3%, compared to tumors with a MIB-1 index < 3%, showed significantly higher number of acetylated cells (Mean ± SEM; 51% ± 26 vs. 27% ± 21, P< 0.0001).

19. Global histone 3 lysine 9 acetylation profiling of ependymal tumors indicates localization-specific epigenetic pattern and prognostic relevance in these tumors

Ependymomas are tumors of highly variable clinical and molecular behavior, affecting both children and adults. Due to the paucity of appropriate experimental models, their underlying molecular mechanisms of behavioral variability are poorly understood. Considering increasing evidence of epigenetic changes in various tumors in addition to preclinical success of epigenetic-based therapeutics in tumors of central nervous system, epigenetic study of ependymal tumors is warranted. In this study, using immunohistochemistry, we investigated the patterns of global H3K9Ac, in 85 ependymal tumors of various clinico-pathological characteristics. A total of 85 tumors, from different age groups, diverse CNS localizations and various WHO grades, were evaluated for global H3K9Ac status (Table 11). Similar to previous studies, positive immune reaction for H3K9Ac was localized to the cell nucleus, stained in brown, while non-reactive area of the nucleus was counter-stained in blue in all tumor samples.

Table 11: Clinico-epidemiological and global H3K9Ac profiling of ependymal tumor samples

	WHO Grade I	WHO Grade II	WHO Grade III	Total
Number of patients	19 (22%)	46 (54%)	20 (24%)	85 (100%)
Age (years)				
Median	44	51	29	42
Mean (Range)	41 (17-75)	47 (2-82)	35 (2-73)	43 (2-82)
< 4	0	3	2	5
4-18	1	0	3	4
>18	18	43	15	76
Gender (no. of cases)				
Female	8	25	10	43
Male	11	21	10	42
Localization (no. of cases)				
Supratentorial	5	2	7	14
Parenchymal	1	0	6	7
Ventricular	4	2	1	7
Infratentorial	1	13	10	24
Parenchymal	0	5	4	9
Ventricular	1	8	6	15
Spinal	13	31	3	47
Recurrent cases (no.)	3 (15.8%)	3 (6.5%)	8 (40%)	14 (16.5%)
H3K9Ac status (%)				
Positive cells (Mean ± SD)	34% ± 28	37% ± 26	28% ± 26	34% ± 27
Negative cells (Mean ± SD)	65% ± 28	63% ± 26	72% ± 26	66% ± 27

The nuclei stained in brown, either partially or totally, were considered as positive or acetylated, and the nuclei lacking brown staining (totally blue) were considered as negative or non-acetylated (Figure 16). Cell percentages were used for statistical analysis. In general, most of the nuclei in ependymal tumors were H3K9Ac negative (Mean ± SD; 65.9% ± 26.5 negative vs. 34.1% ± 26.5 positive) and there was no significant difference in global H3K9Ac levels between tumors of grade I, II and III according to WHO grading system (Figure 17).

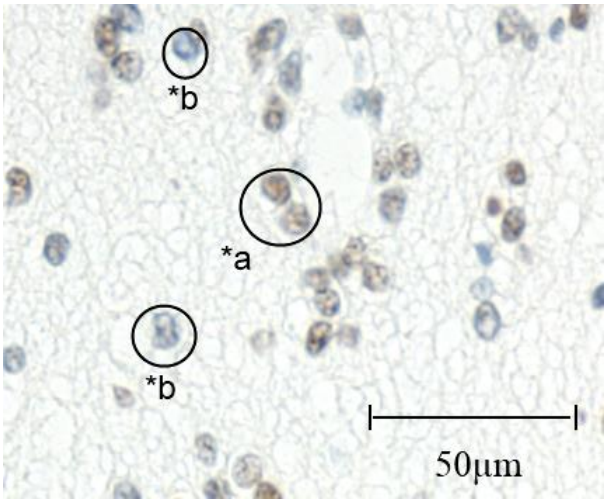

Figure 16: Immunohistochemical demonstration of H3K9Ac in an ependymal tumor

*a: Acetylated nuclei (positive), and *b: non-acetylated nuclei (negative) in an ependymal tumor of WHO grade I.

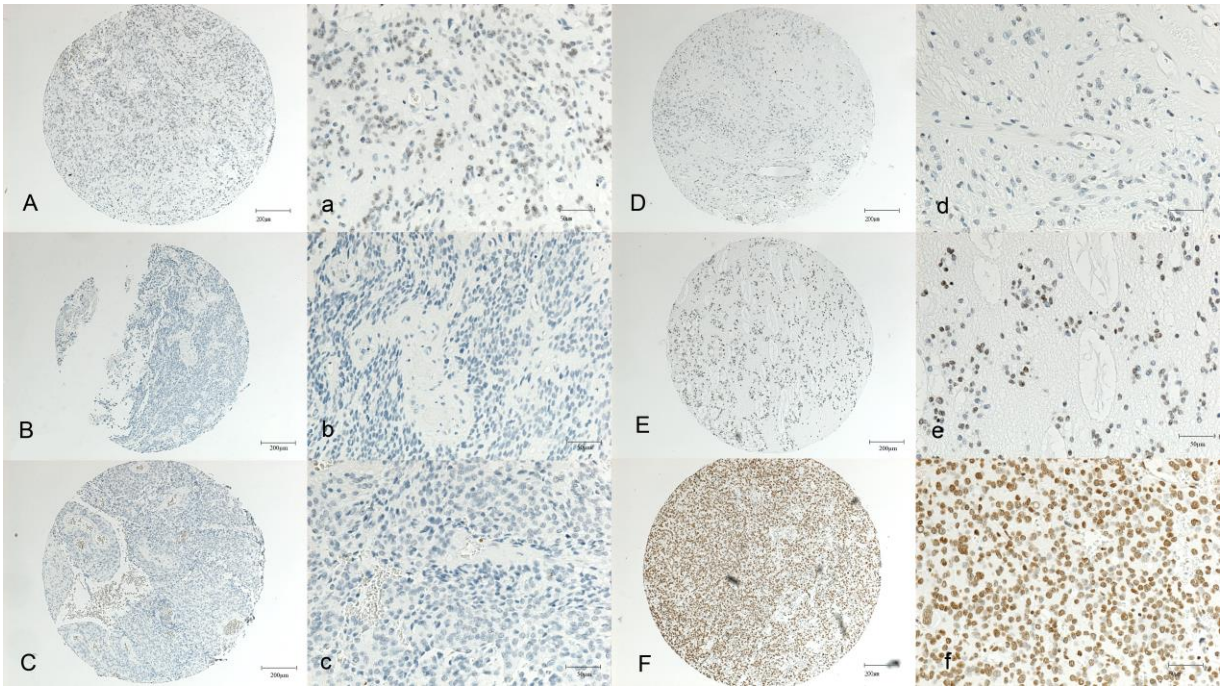

Figure 17: Global H3K9Ac in ependymal tumors of diverse localizations and grades

(A&a) Primary ependymoma of WHO grade II localized to Spinal cord. (B&b) Recurrent ependymal tumor of WHO grade III (anaplastic ependymoma) localized to left fronto-temporal brain parenchyma. (C&c) Primary ependymoma of grade II localized to parenchymal brain of posterior fossa (cerebellum). (D&d) Primary ependymoma of WHO grade II from IVth ventricle. (E&e) Primary ependymal tumor of WHO grade I localized to lateral ventricle. (F&f) Atypical pituitary adenoma, strongly positive for H3K9Ac, as positive control [70].

However, subependymomas had higher number of H3K9Ac positive nuclei (Mean ± SD: 67.2% ± 10.2) than other tumor subtypes including myxopapillary ependymomas (Mean ± SD: 27.5% ± 23.7 positive nuclei), ependymomas (Mean ± SD: 36.8% ± 26.4 positive nuclei) and anaplastic ependymomas (Mean ± SD: 27.6% ± 25.9 positive nuclei) (Figure 18).

Interestingly, global H3K9 acetylation pattern in ependymal tumors was location-specific, so that intracranial parenchymal tumors had lower H3K9Ac positive nuclei (Mean ± SD: 13.1% ± 21.9) compared to tumors of intracranial ventricular (Mean ± SD: 48.3% ± 27.4 positive nuclei; $P < 0.0001$) and spinal localization (Mean ± SD: 36.8% ± 23.9 positive nuclei; $P < 0.001$) (Figure 19). Moreover, supratentorial ventricular tumors had higher H3K9Ac levels (Mean ± SD: 66.4% ± 11.8 positive nuclei) compared to supratentorial parenchymal (Mean ± SD: 12.4% ± 17.2 positive nuclei; $P < 0.0001$), infratetorial parenchymal (Mean ± SD: 13.4% ± 25.2 positive nuclei; $P < 0.0001$), infratentorial ventricular (Mean ± SD: 38.7% ± 28.7 positive nuclei; $P < 0.05$) and spinal tumors (Mean ± SD: 36.8% ± 23.9 positive nuclei; $P < 0.001$) (Figure 19).

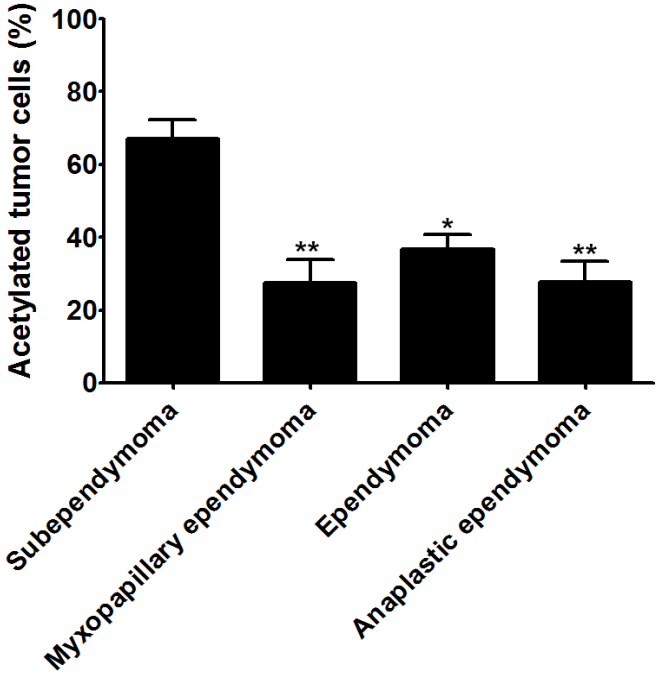

Figure 18: Global H3K9Ac levels in different ependymal tumor subtypes

Error bars indicate SEM, *P < 0.05 & **P < 0.001. P values in each group are shown in comparison to subependymomas.

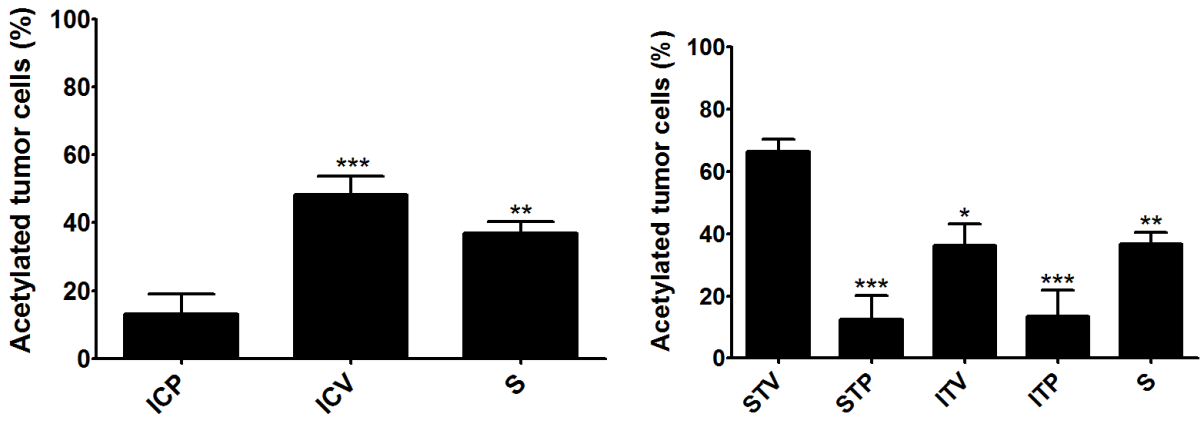

Figure 19: Global H3K9Ac levels in ependymal tumors of diverse localizations

(STV: supratentorial ventricular, STP: supratentorial parenchymal, ITV: infratentorial ventricular, ITP: infratentorial parenchymal, S: spinal, ICP: intracranial parenchymal, ICV: intracranial ventricular). Right: P values in each group are shown as compared to STV. Left: P values in each group are shown as compared to ICP. Error bars represent SEM, ***P < 0.0001, **P < 0.001 & *P < 0.05.

However, there was no significant difference in H3K9Ac levels between supratentorial, infratentorial and spinal tumors when ventricular and parenchymal tumors were considered together. Besides, recurrent tumors had significantly less H3K9Ac positive cells than primary ones (16% ± 22.5 in recurrent (n=14) vs. 38% ± 25.8 in primary (n=71), P < 0.0001) (Figure 20).

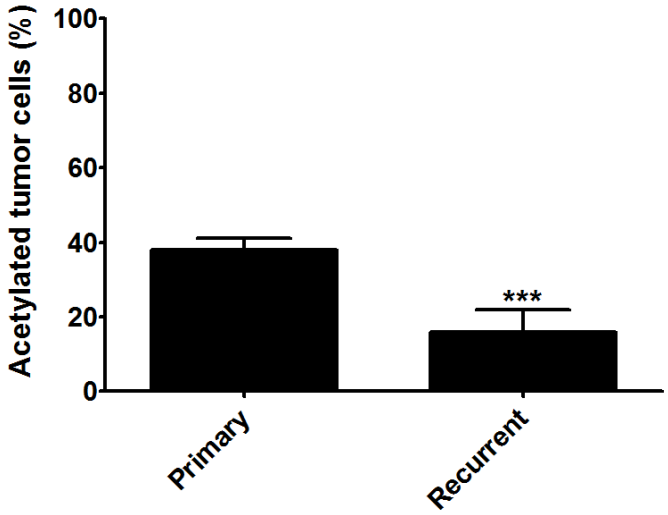

Figure 20: Global H3K9Ac levels in primary and recurrent ependymal tumors

Error bars indicate SEM, ***P < 0.0001.

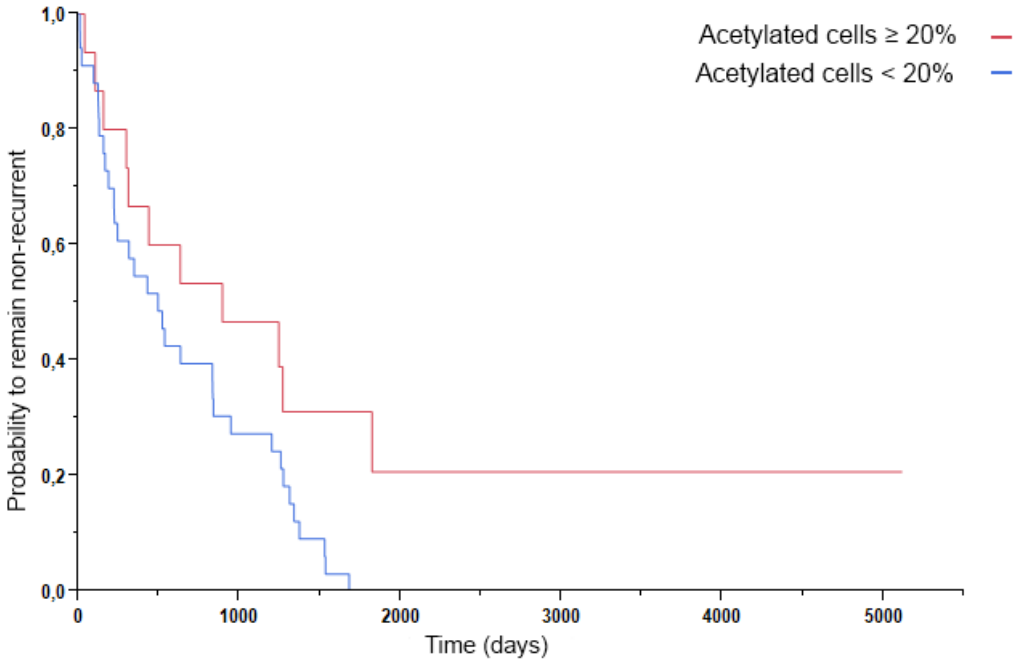

Figure 21: Survival analysis of ependymal tumors

Comparison of probability to recur in time in ependymal tumors with acetylated nuclei less than 20% compared to the tumors with 20% or more acetylated cells, *P=0.0327.

H3K9Ac pattern in ependymal tumors also revealed prognostic significance so that tumors with less than 20% acetylated cells were probable to recur faster (P=0.0327*) than tumors with 20% or more acetylated cells (Figure 21).

20. MS-275 resumes altered global H3K9 acetylation in APPPS1-21 mouse model of Alzheimer's disease

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and the most common cause of dementia among the aging population [391]. Heritable factors contribute substantially to the risk of developing AD. More than 95% onsets of AD occur later than 65 years, which are called "sporadic" AD while less than 5% of AD cases accounts for early-onset autosomal dominant forms, called familial AD (FAD) [392]. Current AD research mainly depends on the later form of the disease. Two reasons can be mentioned for this; first of all, FAD fully represents amyloid theory which is the best known and accepted theory describing molecular mechanisms of AD [393]. Secondly and most importantly, transgenic models could be designed from involved genes in familial forms which are of foremost importance for investigational purposes [394].

The amyloid hypothesis mainly emphasizes on excessive Aβ production as the core pathology of AD [393]. However, recent evidence on epigenetic mechanisms in neurodegenerative diseases has made a paradigm shift in our understanding of AD [395, 396]. Study of late o0nset AD in twins supports the involvement of epigenetic mechanisms in AD pathophysiology [397]. Latent Early-life Associated Regulation (LEARn) is a model based on environmental induction of latent epigenetic deregulation that introduce specific, testable molecular pathways, united as epigenetic mechanisms, into the etiopathology of AD and other neurodegenerative disorders [395]. Among different epigenetic modalities histone acetylation/deacetylation, dynamically regulated by histone acetyltransferases (HATs) and histone decaetylases (HDACs), has shown particular relevance in AD [398]. HDAC2 is a known regulator of memory [399]. In a recent study of cognitive function in two mouse models of neurodegeneration and in AD patients, it was shown that epigenetic blockade of transcription of the genes important for learning and memory, is mediated by HDAC2 and this blockade was reversible [400].

Moreover, non-specific HDAC inhibitors such as valproic acid, trichostatin A, and vorinostat have been shown to change the status of Aβ plaque deposition and/or tau hyperphosphorylation via different mechanisms [401]. MS-275 (entinostat) is a potent, long-lasting, selective HDAC inhibitor currently in clinical trial stage of therapeutic evaluation in various types of cancer [402, 403]. Recently the drug has gained the attention for treatment of brain disorders. Combined use of MS-275 and resveratrol has shown neuroprotective effects in a mice model of ischemic brain injury [404]. Treatment of rat astrocytes and neurons revealed an up-regulated level of the neuroprotective heat shock protein 70 (HSP70) and increased levels of H3K4Me2 at the HSP70 promoter [405]. In another study, five days treatment of a mouse model of persistent inflammatory pain with MS-275 substantially reduced the nociceptive response in the mice [406]. MS-275 has also been introduced as a potential treatment for cognitive deficits associated with schizophrenia [407]. According to a recent study, oral administration of MS-275 ameliorated neuroinflammation and cerebral amyloidosis and improved behavior in APPPS1-21 mice model of AD, carrying two important mutations of amyloid precursor protein and presenilin genes [408]. Regarding the involvement of epigenetic mechanisms in AD pathology and the relative success of HDAC inhibitors in resuming cognitive function, study of histone acetylation/deacetylation patterns in AD is warranted. Such findings not only increase our understanding of AD pathophysiology,

but also provide a platform for therapeutic development. Here we studied the patterns of global H3K9 acetylation in APPPS1-21 mice compared it to H3K9 acetylation patterns in wild-type mice. The characteristics and advantages of APPPS1-21 mice have been discussed in our review of AD murine models published in 2012 [409]. We also studied the changing patterns of H3K9Ac in response to a selective HDAC inhibitor, MS-275, in these transgenic mice.

H3K9 acetylation pattern is altered in neocortex of APPPS1-21 mice

APPPS1-21 transgenic mice of 5 months were compared to age/gender-matched wild-type littermates for global H3K9Ac status. H3K9Ac immune reactive area was localized to the cell nucleus, stained in brown, and non-reactive area was counterstained in blue. The nuclei which were stained totally brown were considered as acetylated or positive and the rest of nuclei lacking brown staining were considered as negative or non-acetylated. H3K9Ac was evaluated in striatum, hippocampal, and neocortical regions of mice brain. In hippocampus the H3K9Ac positive and negative nuclei were also counted differentially in CA1, CA2, CA3 and dental gyrus (DG). In each region the total number of nuclei, the non-acetylated and acetylated nuclei were calculated using ImageJ software. The counting was performed in 3-5 random regions in each anatomical area. A minimum of 400 cells were counted in each anatomical region. In wild-type mice, within all three regions of brain (striatum, hippocampus, neocortex), most of the nuclei were positive for H3K9Ac ($P< 0.0001$) while in APPPS1-21 mice only in hippocampus and striatum most of the nuclei were H3K9Ac positive ($P < 0.0001$) (Figure 22, table 12).

Table 12: Descriptive table of H3K9Ac status in APPPS1-21 and wild-type mice

Brain region	**H3K9 acetylation status**	**Wild-type mice**		**APPPS1-21**	
		Mean (%)	SD	Mean (%)	SD
Neocortex	Acetylated	87	6	47	24
	Non-acetylated	13	6	53	24
Hippocampus	Acetylated	98	3	89	19
	Non-acetylated	2	3	11	19
Striatum	Acetylated	94	7	99	2
	Non-acetylated	6	7	1	2

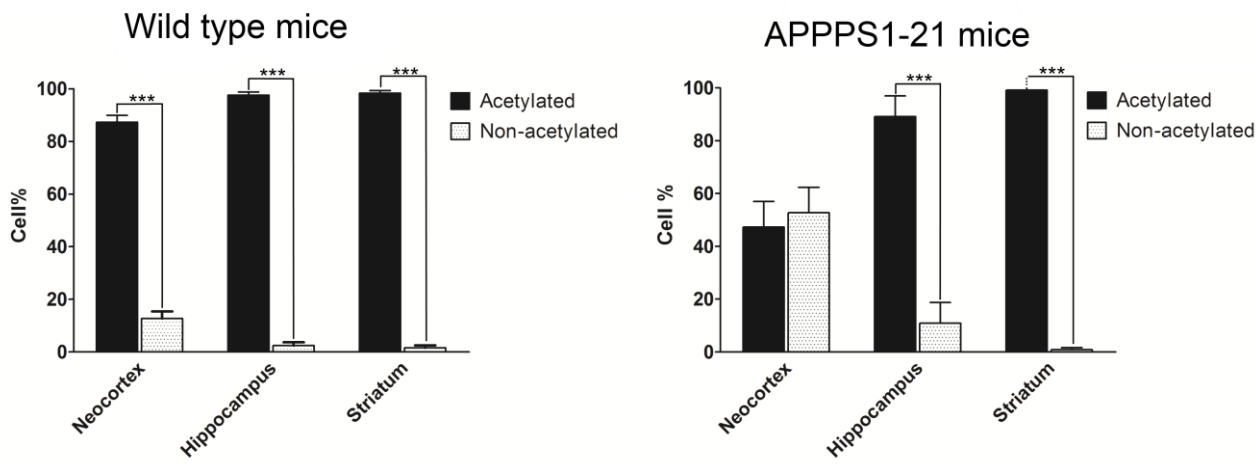

Figure 22: H3K9Ac status in brains of APPPS1-21 and wild-type mice

H3K9Ac status was studies in brains of APPPS1-21 and wild-type mice in three anatomical regions including neocortex, striatum, and hippocampus, ***P < 0.0001.

Moreover, APPPS1-21 mice had significantly lower H3K9Ac positive nuclei in their neocortex compared to wild-type littermates (P < 0.001) (Figure 23).

In order to investigate the association of H3K9Ac alteration to ageing, we also analyzed H3K9 acetylation pattern in APPPS1-21 mice of 3, 5 and 7 months old. Interestingly, the H3K9 acetylation used to decline with increased age of transgenic mice so that 3 months old APPPS1-21 mice had higher number of H3K9Ac positive nuclei in neocortex than 5 and 7 months old mice (Mean ± SD: 25% ± 14 in 7 months mice, 25% ± 18 in 5 months mice, 48% ± 12 in 3 months mice; P < 0.05) (Figure 24). There was also no difference in acetylation pattern between CA1, CA2, CA3 and DG regions of hippocampus either in APPPS1-21 or wild-type mice, and no difference in acetylation pattern of these sub-regions between the two mice groups.

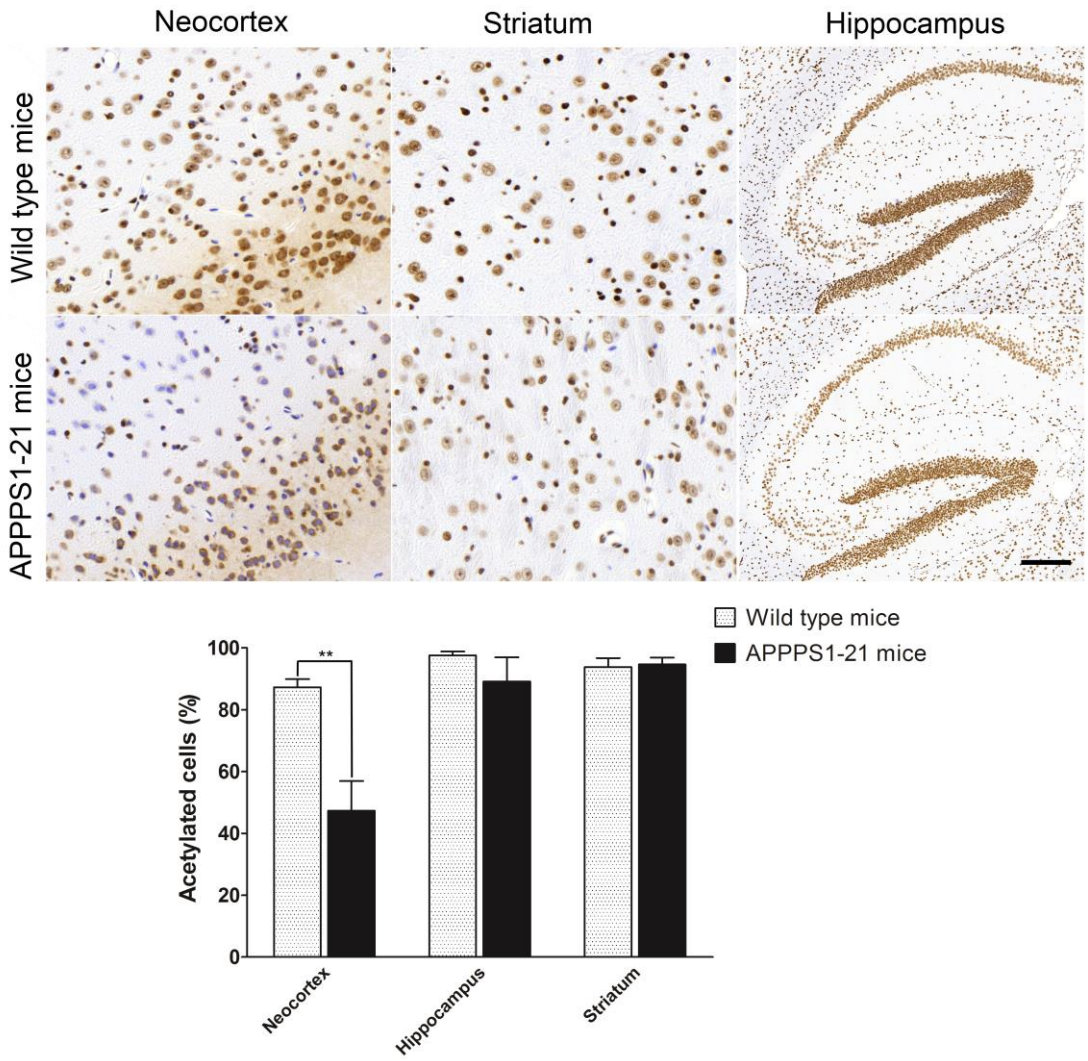

Figure 23: Brain region-specific H3K9Ac alteration in APPPS1-21 compared to wild-type mice

The acetylation pattern was altered in a region specific manner in APPPS1-21 mice so that the number of acetylated cells in neocortex was significantly lower in APPPS1-21 mice compared to the wild-type littermates. Scale bar is equal to 50 µm for neocortex and striatum and 200 µm for hippocampus frames, **P < 0.001.

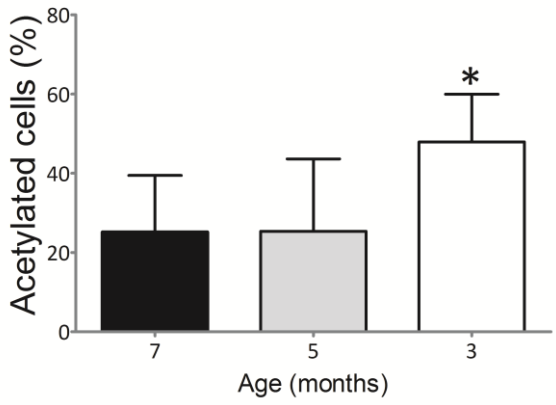

Figure 24: H3K9 acetylation of neocortex in APPPS1-21 mice of various ages

H3K9Ac status was investigated in neocortex of APPPS1-21 mice of 3, 5 and 7 months, *P < 0.05.

MS-275 resumes altered H3K9 acetylation in neocortex of APPPS1-21 mice

H3K9 acetylation pattern was studied in mice after 10 days treatment with either MS-275 or vehicle (VEH) in age/gender-matched mice. The counting process and image analysis was performed in comparable areas as described before. H3K9Ac levels were increased globally in all brain regions of APPPS1-21 mice in response to MS-275 treatment (5 mg/kg body weight). In neocortex of mice treated with MS-275, there was a significantly higher number of acetylated cells compared to VEH group (VEH group: 75.2% ± 13.1 vs. MS-275 group: 89.3% ± 5.6; $P < 0.05$) (Figure 25). In hippocampus and striatum the number of acetylated nuclei was not significantly different in MS-275 and VEH group. We also analyzed the intensity of H3K9Ac immunostaining in two groups of mice. We used the color histogram of images as described in chapter II. In neocortex and striatum the overall intensity of H3K9Ac staining was higher in MS-275 treated mice compare to VEH group ($P < 0.0001$) and there was a clear shift of the color histogram toward the higher intensity values in these two regions in MS-275 treated mice as compared to the VEH group (Figure 26). Regarding the hippocampus there was no significant difference in overall intensity of H3K9Ac staining or any significant shift of histogram in MS-275 treated mice compared to the VEH group (Figure 26).

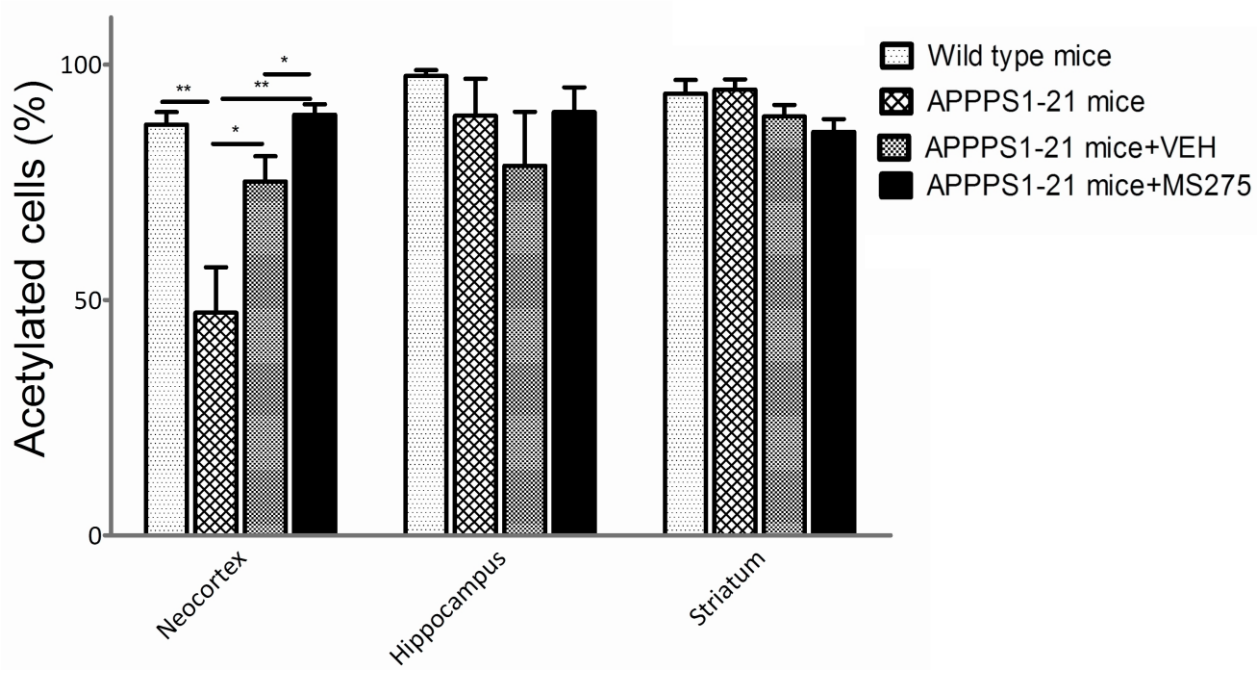

Figure 25: Effect of MS-275 treatment on H3K9Ac status of APPPS1-21 mice brain

Age/gender-matched mice were treated with either MS-275 or VEH for 10 days. MS-275 elicited an increase in the number of H3K9Ac positive nuclei in neocortex of APPPS1-21 mice which was decreased in the transgenic mice compared to the wild-type littermates, *$P < 0.05$ & **$P < 0.001$.

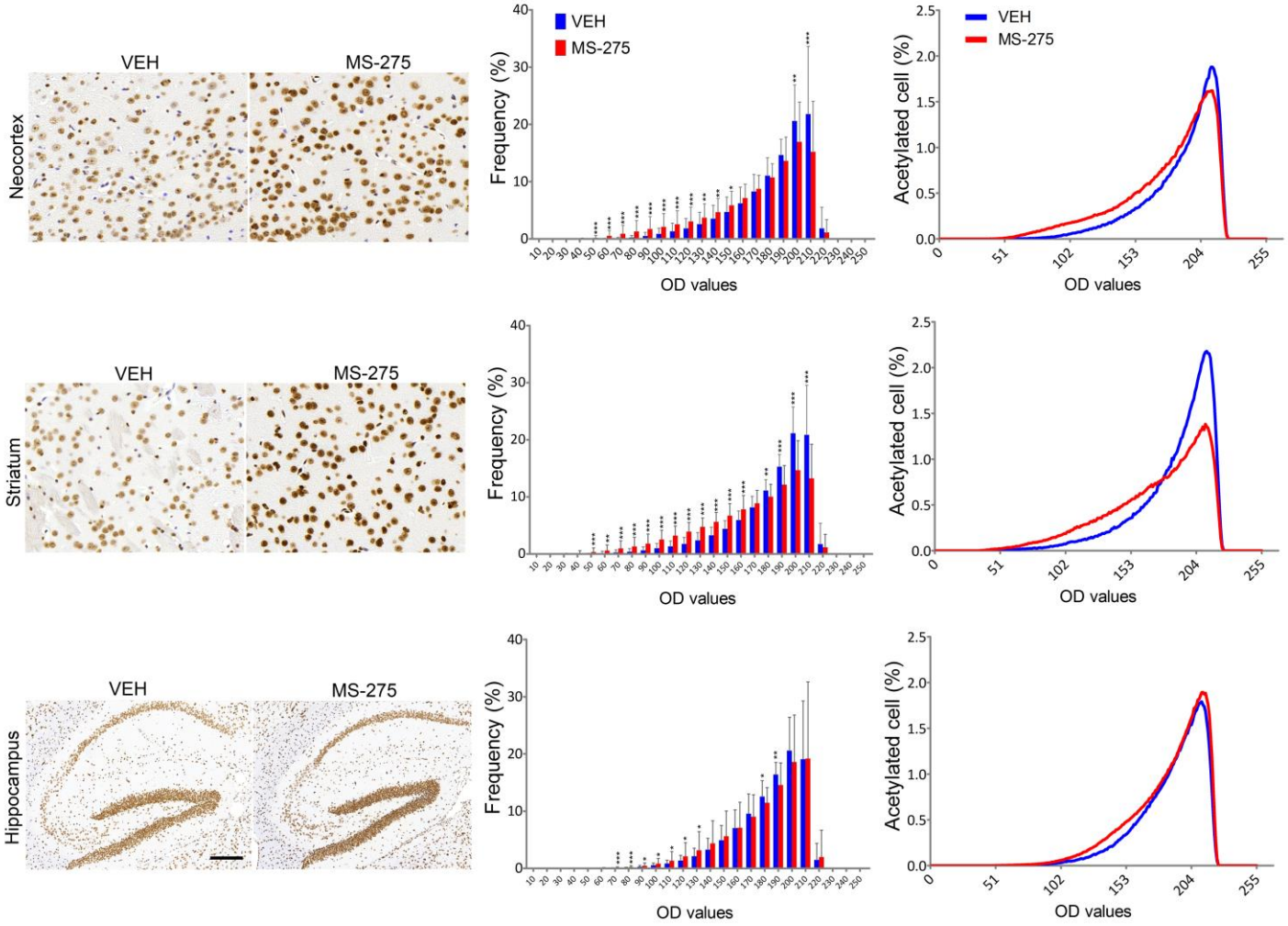

Figure 26: MS-275 elicits an increase of H3K9Ac immunoreactivity in brain of APPPS1-21 mice

In neocortex and striatum of MS-275 treated mice there is a significant shift to the left in histogram of nuclei populations with relatively higher color intensity compared to the nuclei of corresponding regions in VEH-treated mice. OD of H3K9Ac immunostaining in each nucleus with a 255 gray scale used as a reference. The left histograms represent the histogram of selected bins plotted against pixel frequency and the right histograms represent the histogram of all 255 OD levels plotted against frequency of H3K9Ac positive cells. The differences in intensity of H3K9Ac immunostaining between MS-257 and VEH treated mice were highly significant in neocortex and striatum of mice brain ($P < 0.0001$). For interpretation of OD values and histograms please refer to the chapter II. Scale bar is equal to 50 µm in neocortex and striatum, and 200 µm in hippocampus frames, *$P < 0.05$, **$P < 0.001$ & ***$P < 0.0001$.

21. Valproic acid increases the H3K9Ac levels in the brain of experimental autoimmune encephalomyelitis rats

MS is known as a chronic inflammatory disease of the CNS that leads to demyelination, axonal degeneration and consequently neurological disability mostly in young adults. It is a

prevalent neurological disease in the northern hemisphere, with 0.5-1.5 per 1000 prevalence rate [410]. The susceptibility to develop MS is mostly accredited to the human leukocyte antigen (HLA) class II region [411, 412], as well as the HLA class I region according to more recent findings [413, 414].

However, the etiology of MS is not fully known and a combination of genetic, environmental and stochastic factors is believed to drive an autoimmune response in genetically susceptible individuals through various known or yet unknown mechanisms [415, 416]. Along with new epigenetic-based definitions in various diseases, the role of epigenetic mechanisms in neurodegenerative and autoimmune diseases has started to be explored beyond targeted investigations of specific loci [417-419]. Although efforts to establish the epigenetic mechanisms in MS are just being started, studies exploring the epigenetic mechanisms of the cells implicated in MS pathology have already been done, and can be assigned to MS disease. Such findings have been a guide to the application of epigenetic modulator drugs for reprogramming of immune cells in order to reverse the pathological state in autoimmune disorders toward the healthy state. Valproic acid (VPA) is a branched fatty acid that is slightly soluble in water and highly soluble in organic solvents. VPA hyperacetylated the N-terminal tails of H3 and H4 in vitro and in vivo and was proven to directly inhibit HDAC enzymatic activity at 0.5 mM concentration in 2001 [78]. Since then, VPA has been used in different pre-clinical and clinical trials for its potential therapeutic effects against solid tumors [420]. VPA has also shown neuroprotective effects through a range of possible mechanisms, such as modulating the GABAergic and glutamatergic systems, effect on ion channels, modulating kinase pathways, anti-oxidant properties and effects on gene expression through epigenetic modulating effects [421]. In a previous study by Zhang et.al, therapeutic effect of VPA was reported in EAE model of MS in rat [384]. In present study, the changing patterns of H3K9Ac in response to VPA therapy were investigated in EAE rat brain. EAE is a murine model of MS that can be induced by active immunization of rats with auto-antigens of the CNS or adoptive transfer of myelin-reactive $CD4^+$ Th1 lymphocytes [422, 423] and H3K9Ac is a substrate for HDAC1, targeted by VPA.

VPA increases H3K9 acetylation in EAE rats

EAE rats were treated with either VPA (500 mg/kg in PBS) or vehicle (PBS) for 10 days as

described in chapter II. VPA-treated EAE rats were compared to PBS-treated ones concerning the changing patterns of H3K9Ac. H3K9Ac immune reactive area was localized to the cell nucleus, stained in brown, and non-reactive area was counterstained in blue. The nuclei which were stained totally brown were considered as acetylated or positive and the rest of nuclei lacking brown staining were considered as negative or non-acetylated. H3K9Ac was evaluated in hippocampal and neocortical regions of rat brain differentially. In each region the total number of cells, the non-acetylated nuclei and acetylated nuclei were calculated. The counting process and image analysis were performed in comparable areas using ImageJ software. The counting was performed in 3-5 randomly chosen frames in each anatomical area. A minimum of 400 cells were counted in each anatomical region. The number of non-acetylated nuclei was significantly lower in VPA-treated EAE rats compare to the PBS group. In cortical regions, there was a significantly lower number of non-acetylated cells in VPA-treated rats compare to the PBS group (Mean ± SD: 30% ± 13 in VPA group vs. 6% ± 4 in PBS group, N=6) (Figure 27).

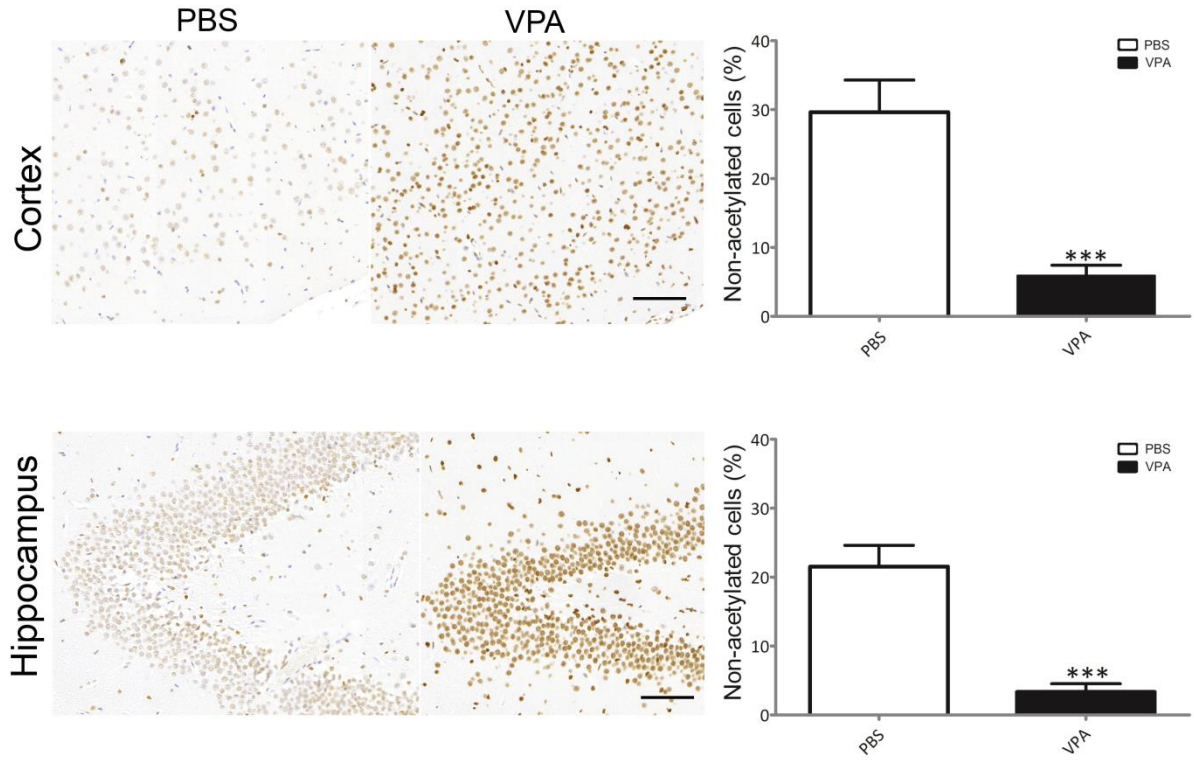

Figure 27: Alteration of H3K9Ac status in brains of EAE rats in response to VPA treatment

EAE rats were treated with either VPA (500 mg/kg in PBS) or PBS for 10 days. H3K9Ac status of nuclei was evaluated in neocortex and hippocampus in both groups after treatment. Scale bar represents 100 µm in all frames, ***P < 0.0001.

Similarly, there was a significantly lower number of non-acetylated cells in hippocampus of VPA-treated rats compare to the PBS group (Mean ± SD: 22 % ± 8 in VPA group vs. 3% ± 3 in PBS group, N=6) (Figure 27).

In this regard, not only the number of acetylated cells, but also the intensity of staining in all acetylated cells was higher in VPA-treated EAE rats compared to the PBS group both in cortical and hippocampal regions of brain (Figure 28). In order to analyze the intensity variation among treatment and control group we used the histograms of images using ImageJ software and statistically analyzed image parameters as described in chapter II [387-389].

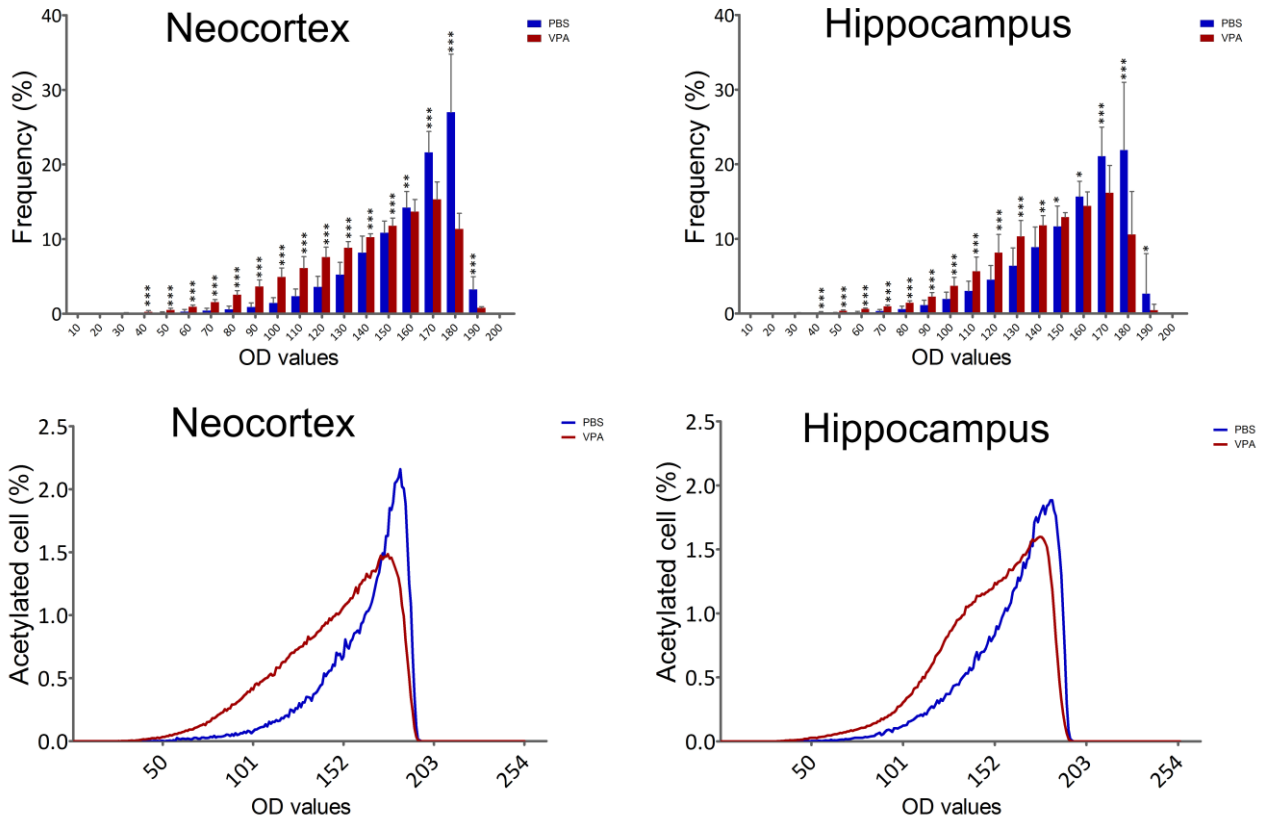

Figure 28: VPA elicits an increase of H3K9Ac immunoreactivity in brain of EAE rats

The nuclei in brain of VPA-treated rats were more strongly stained for H3K9Ac compare to PBS-treated group representing that VPA treatment is associated with increased H3K9Ac both in cortical and hippocampal region of EAE rat brain. The upper histograms represent the histogram of selected bins plotted against pixel frequency and lower histograms represent the histogram of all 255 OD levels plotted against frequency of H3K9Ac positive cells. For the details on method of analysis please refer to chapter II. X axis represent the value of various levels or bins *(h(bj)),* *P < 0.05, **P < 0.001.

VPA treatment ameliorated EAE

In previous study by Zhang Z. et.al, VPA treatment also revealed therapeutic effect in EAE rats. VPA treatment was accompanied with reduced expression of inflammatory cytokines in spinal cords and lymph nodes, decreased neurological severity and shortened duration of disease compared to PBS control [424].

22. Resveratrol effectively reduces nitric oxide production in LPS-induced N9 microglial cells without affecting their H3K9Ac profile

Microglias are immune system representative of brain and consist 10% of the total glial cell population in CNS. In a young brain, microglias function as neuroprotective cells. But in the aged brain, they have been primed to react abnormally to the stimuli, leading to neurotoxicity and neurodegeneration [425, 426]. Aging associated immune senescence is a known phenomenon which renders microglia to function abnormally, and may eventually promote neurodegeneration [427]. Immune system senescence is associated with both morphological changes and alterations in immunophenotypic expression and alteration of inflammatory profile as well as activation of inflammatory pathways that should be silent during the healthy state [428-430]. The present hypothesis of microglia senescence during brain aging and its pathological role in aging-related neurodegeneration has led to a novel perspective on potential therapeutics for neurodegenerative diseases [430-432].

Epigenetic mechanisms have been shown to contribute to this process. It has been suggested that persistent inflammation induce epigenetic changes that lead to neurodegenerative state [433]. In other words, the repressive effects of microglia and astrocyte over-activation is suggested to recapitulate permissive epigenetic conditions that induce neurodegeneration [433]. Polyphenols were already discussed as effective modulators of neuroinflammation, epigenetic modifiers and potential therapeutics for neurodegenerative diseases [432]. In the present scenery, using polyphenols that suppress neuroinflammation through epigenetic pathways would be a potential therapeutic option for neurodegenerative disorders. The best targets for such therapeutics remain to be investigated. Here, we studied the effect of three polyphenol and non-polyphenol HDAC inhibitors, resveratrol, curcumin and valproic acid (VPA), on LPS-induced murine N9 microglial cells.

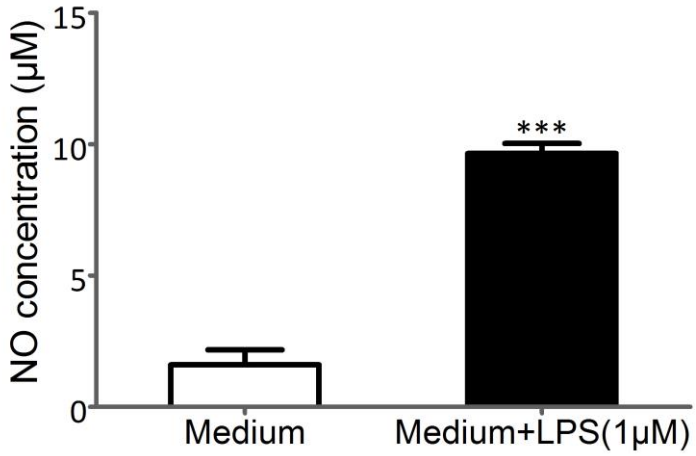

Figure 29: LPS-induced nitric oxide production in N9 cells

N9 cells were treated with LPS (1µg/ml) for 24 hours. The medium supernatants were collected and concentration of NO was measured by standard Griess assay, ***P < 0.0001.

N9 cells were seeded into 12-well cell culture plates and cultured for 48 h. After reaching the sufficient confluency, the cells were treated with LPS (1µg/ml) for 24 hours as described in chapter II. The medium supernatants were collected and concentration of nitric oxide (NO) was measured by standard Griess assay. NO concentration in medium supernatant of N9 cells was significantly increased after LPS treatment (Mean ± SD in medium: 1.6 µM ± 0.9 vs. LPS-treated group: 9.6 µM ± 0.7) (Figure 29).

After LPS induction, N9 cells were treated with a wide range of concentrations of curcumin, VPA and resveratrol (1, 10, 100, 1000, and 1500 µM) for 24 hours. MTT assay was performed on treated N9 cells and toxic concentrations were omitted for all compounds. LPS-induced N9 cells were then treated with non-toxic concentrations of curcumin (1 and 10 µM), VPA (1, 10, 100, 1000 µM) and resveratrol (1, 10, 100 µM) for 24 hours. The supernatants were collected and NO concentration was measured by standard Griess assay after treatment.

Resveratrol (100 µM) significantly reduced NO concentration in supernatant after 24 hours treatment while curcumin did not show any effect on NO production (Figure 30, table 13). Interestingly, valproic acid (1000 µM) even increased the NO production by N9 cells. DMSO was used as vehicle for curcumin and resveratrol and distilled water for VPA. As control the highest vehicle concentration in each compound was used (Figure 30, table 13). DMSO was toxic to N9 cells and curcumin and resveratrol increased the cell longevity compare to vehicle group according to MTT assay (Figure 30).

Table 13: Descriptive statistics of NO production in LPS-induced N9 cells in response to therapy

Treatment vs. control	Mean Difference	Significance	95% CI of difference
VPA (1µM)+LPS vs. Medium+LPS+H2O	0.9	ns	-4.7 to 6.4
VPA (10µM)+LPS vs. Medium+LPS+H2O	2.2	ns	-3.3 to 7.7
VPA (100µM)+LPS vs. Medium+LPS+H2O	2	ns	-3.5 to 7.5
VPA (1000µM)+LPS vs. Medium+LPS+H2O	12.6	***	7.1 to 18.1
Res(1µM)+LPS vs. Medium+LPS+DMSO	-2.6	ns	-7.5 to 2.3
Res(10µM)+LPS vs. Medium+LPS+DMSO	-2.1	ns	-6.9 to 2.8
Res(100µM)+LPS vs. Medium+LPS+DMSO	-5.7	*	-10.6 to -0.9
Curcumin(1µM)+LPS vs. Medium+LPS+DMSO	6.2	ns	-3.6 to 15.9
Curcumin(10µM)+LPS vs Medium+LPS+DMSO	5.2	ns	-4.6 to 14.9

The results were analyzed using one-way ANOVA of analysis with Bonferroni's multiple comparisons as post-hoc test. CI: confidence interval, ns: non-significant, *P < 0.05, ***P < 0.0001.

In the next step, the cells were treated with effective doses of VPA (1 mM) and resveratrol (100 µM) for 24 hours. DMSO and distilled water were respectively used as vehicle for resveratrol and VPA. After treatment, cytospin preparation of the cells was used to evaluate the effect of different therapeutics on H3K9Ac content of N9 cells, using immunohistochemistry. H3K9Ac immune reactive area was localized to the cell nucleus stained in brown. We used the same method of image analysis as in previous experiments. The brown nuclei were considered H3K9Ac positive and the nuclei lacking brown color were considered as negative. In each setting up to 200 cells were counted. Neither VPA nor resveratrol induced significant changes in global H3K9Ac levels in N9 cells (Figure 31).

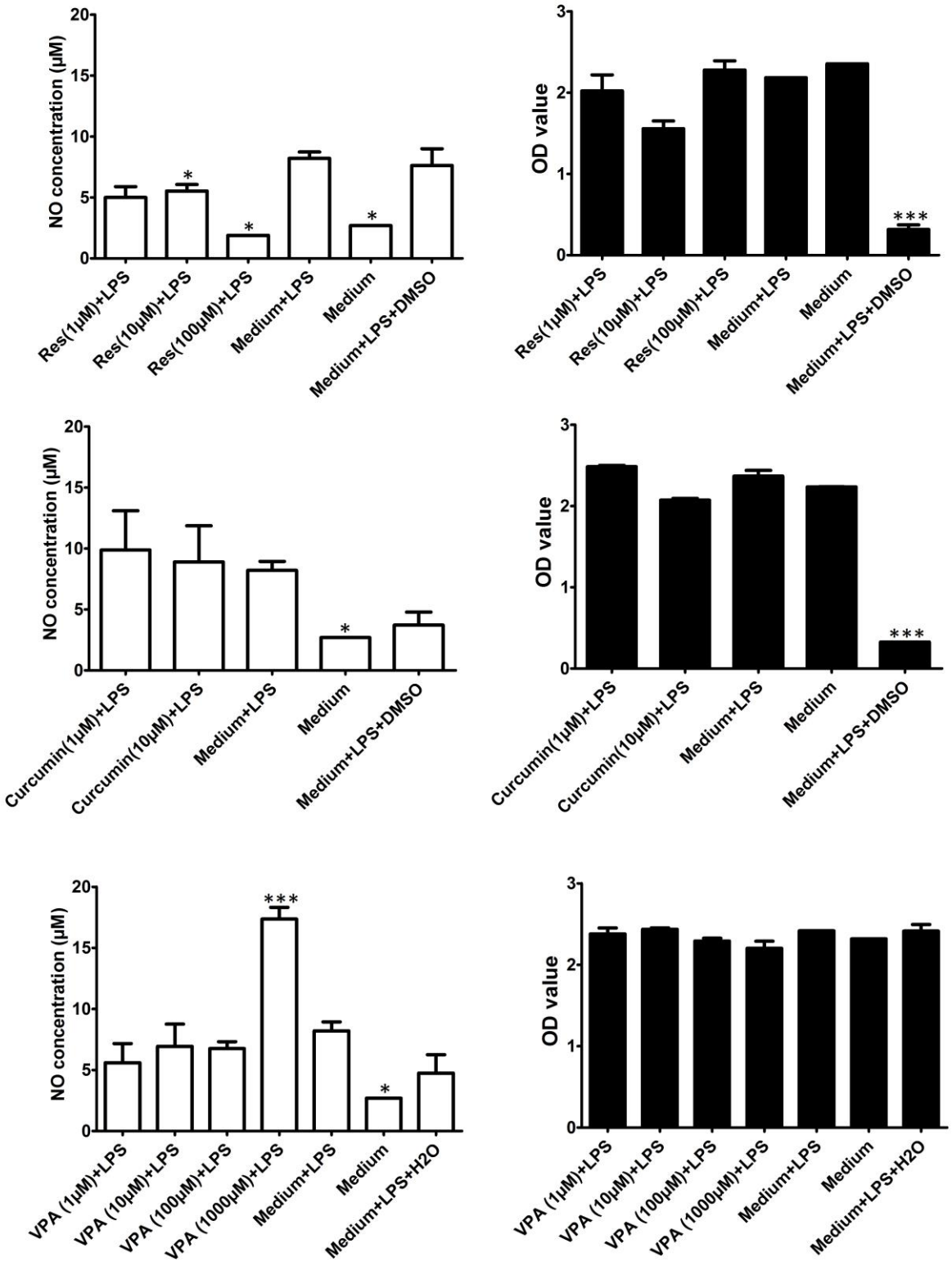

Figure 30: Effect of VPA, resveratrol and curcumin on NO production by N9 cells

LPS-induced N9 cells were treated with curcumin (1 and 10 µM), VPA (1, 10, 100, 1000 µM) and resveratrol (1, 10, 100 µM) for 24 hours. Left: Griess assay results; the values are shown as compared to Medium + LPS + Vehicle, right: MTT assay results, *P < 0.05, **P < 0.001, ***P < 0.0001.

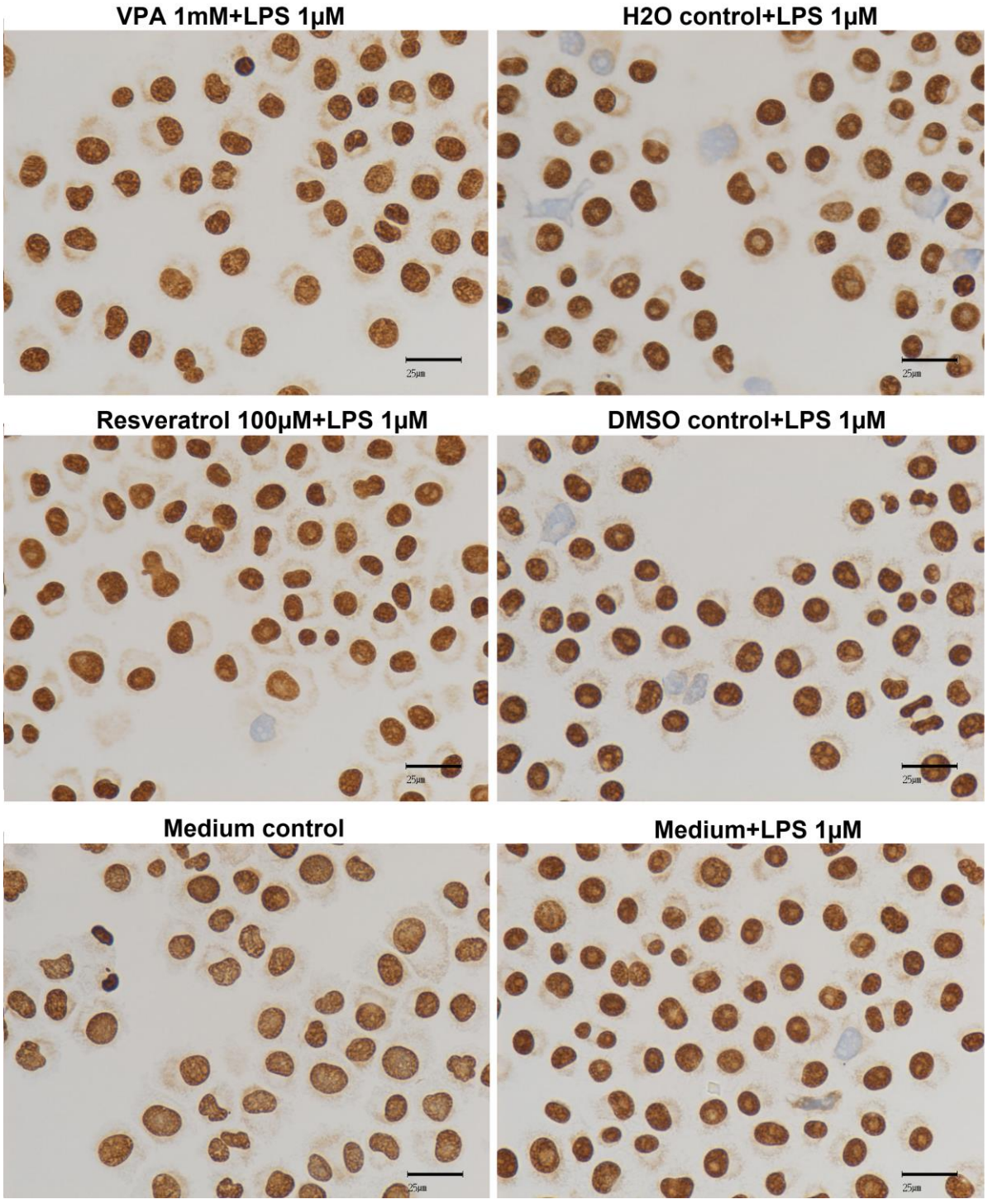

Figure 31: Immunohistochemical demonstration of global H3K9Ac in N9 cells in response to VPA and resveratrol

The cells were treated with effective doses of valproic acid and resveratrol for 24 hours. DMSO and distilled water were respectively used as vehicle for resveratrol and VPA. H3K9Ac was evaluated in cytospin preparation of N9 cells. Neither resveratrol nor VPA could significantly alter the H3K9Ac content of the N9 cells.

23. Icariin does not alter global H3K9Ac profile of APPPS1-21 mice brain

Icariin is a flavonoid and the main constituent of Epimedium brevicornum Maxim, a well-known Chinese herbal medicine. Various activities and pharmacological applications have been reported for icariin. Regulation of cardiovascular system, neurite growth stimulation, antitumor activity, anti-oxidant effect, immune regulation, improved sexual function, and an estrogen like activity are some of these properties [434-438]. Icariin has shown neuroperotective effects in several previous studies. Icariin was able to enhance neuronal viability after oxygen and glucose deprivation or hydrogen peroxidase-mediated cytotoxicity and inhibit oxidative stress by increasing SIRT1 activity and expression [364, 439]. SIRT1-dependent neuroprotective activity was also reported for icariin in an experimental model of stroke [112]. In another study icariin inhibited beta-amyloid-induced neurotoxicity through up-regulating cocaine-regulated and amphetamine-regulated transcripts [108]. Improved memory function is also one of the reported effects of icariin in several studies of icariin effects against neurodegeneration in AD mice models [440, 441].

We studied the patterns of global H3K9Ac in neocortex, hippocampus and striatum of APPPS1-21 mice, in response to icariin treatment. Age/gender-matched APPPS1-21 transgenic mice of 5 months were treated either with icariin or with 1% CMC as vehicle as described in chapter II. Immunohistochemistry was used for evaluation of brain H3K9Ac patterns. H3K9Ac immune reactive area was localized to the cell nucleus, stained in brown, and non-reactive area was stained in blue. The nuclei which were stained brown were considered as acetylated or positive and the rest of nuclei lacking brown staining were considered as negative or non-acetylated. H3K9Ac was evaluated in striatum, hippocampal, and neocortical regions of mice brain differentially. In each region the total number of cells, non-acetylated nuclei and acetylated nuclei as well as the intensity of staining in acetylated nuclei were measured using ImageJ software. The counting was performed in 3-5 random regions in each anatomical area. A minimum of 400 cells were counted in each anatomical region.

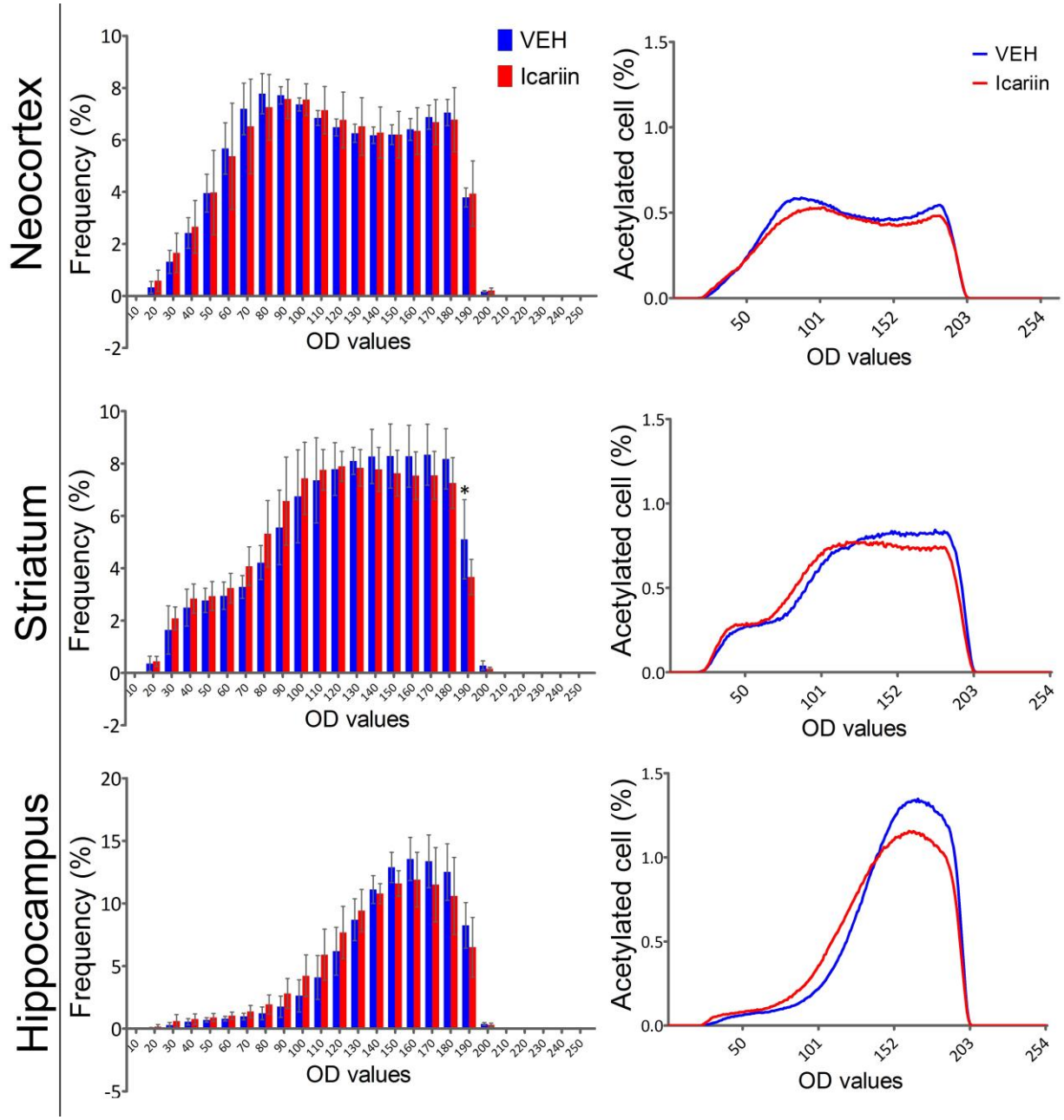

Figure 32: Histogram of H3K9Ac positive cells in APPPS1-21 mice following icariin treatment

The left graphs represent histogram of H3K9Ac positive cells in selected bins and right graphs represent the total histogram of all 255 levels in treatment group compared to the VEH group. There was no significant difference in histogram of selected bins or shift of histogram of H3K9Ac positive cells in treatment compare to VEH group. For details on method of image analysis please refer to chapter II.

There was also no difference in H3K9 acetylation pattern between icariin treated and vehicle group of transgenic mice. We did not find any significant difference of the number of acetylated nuclei or intensity of H3K9Ac staining in acetylated nuclei between vehicle and icariin treated groups in any of brain regions (Figure 32, 33).

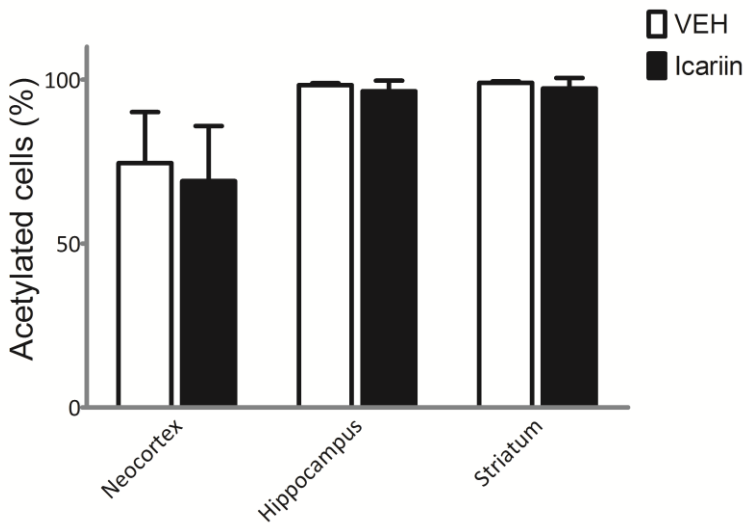

Figure 33: H3K9Ac status in APPPS1-21 mice in response to icariin treatment

APPPS1-21 mice were treated with icariin or vehicle for 10 days. Paraffin embedded sections were immunostained against H3K9Ac. Global H3K9A was evaluated in neocortex, hippocampus, and striatum of transgenic mice in response to treatment.

Chapter IV: Discussion

24. Do patterns of H3K9Ac change in various neurological diseases?

Aberrant histone modification profiles, at promoter of specific genes or at genome-wide level, have widely been studied in brain tumors and have recently begun to be described for neurodegenerative disorders such as AD, PD, HD, MS, epilepsy, and ALS [31, 51]. In fact, epigenetic mechanisms are critical to the development and function of CNS in mammals. Accordingly, modifications of histone proteins, associated with chromatin structure, play a pivotal role in transcriptional regulation of genes important for brain function. For example, neuronal differentiation is regulated in part by DNA demethylation and polycomb-mediated histone H3K27 trimethylation (H3K27me3). DNA methylation contributes to repression of pluripotency in lineage-committed neural progenitors. Also, promoters marked by H3K27me3 in neural stem cells often gain DNA methylation during differentiation [442]. In the setting of neural development several epigenetic modalities might simultaneously be involved and the interplay between the action of various epigenetic modalities (e.g. HDACs, HATs, DNAMTs, etc.) determines the pace and direction of gene transcription. In other words, context-dependent interactions between different epigenetic mechanisms guide neural differentiation, and underscore the general importance of epigenetics in normal CNS development and maintenance of cellular identity. Accordingly, epigenetic association in neurodevelopmental disorders has been described too [51]. Among various modifications that occur at the N-terminal tail of histone molecules within the nucleosome, histone acetylation has been frequently studied. Acetylation is believed to disrupt the structured arrangement of histone proteins, relax the chromatin structure via neutralizing the positive charge of histone tails, reduce their affinity for DNA and finally make DNA more accessible to transcription factors [443]. Various lysine positions over different histone types can accept this acetyl group via the action of histone acetylator/deacetylator enzymes.

Acetylation of specific lysine residues of histones plays a key role in regulating gene expression. Acetylation of H3K9 is known to be associated with active chromatin state and gene transcription [27, 444-446]. Level of H3K9 acetylation is also positively correlated with transcription rates [21]. The reduced acetylation of H3K9 is accompanied with reduced gene expression and the correlation is so significant that the genome-wide increased levels of H3K9Ac via VPA administration have shown to be contributed to the pluripotency in embryonic stem cells [447]. Alteration in acetylation of H3 either with specific notification to

the position of affected lysine or in general as global H3 acetylation has been investigated in various neurological diseases and tumors. We also studied the patterns of H3K9Ac in a selected set of brain tumors and AD as the prototype of neurodegenerative diseases.

24.1. Histone acetylation in cancer diagnostics and prognostics

Based on the current theories, aberrant increase or decrease in acetylation of H3K9 in promoter region of genes disrupts the normal gene expression pattern and chromatin architecture, and therefore could lead up to the carcinogenic state [10, 31, 32, 448, 449]. This theory applies not only to promoter-specific histone acetylation, but also to global histone acetylation levels [10, 33]. This has been supported by the evidence that changes in global levels of histone modifications correlate with their levels at individual promoters and repetitive DNA elements too [33, 34, 450].

While due to the broad variability in genetic alterations, the genetic study of brain tumors is cumbersome and frustrating, global epigenetic patterns and study of their alterations might not only provide information for developing new therapeutics, but also be an explanation why a wide range of aberration in gene expression could be found in these tumors, when no specific tumor suppressor or promoter mutation could be assigned. Defining alterations of these epigenetic components under various pathological conditions provides useful tools for the diagnosis, classification and treatment of diseases [451]. We studied three types of brain tumors, including brain gliomas, pituitary adenomas and ependymomas, for patterns of H3K9 acetyation and its probable clinico-pathological significance in these tumors.

24.2. Brain gliomas

Malignant gliomas are devastating brain tumors with no promising treatment. The prognosis of patients suffering from malignant glioma is unfavorable, despite tremendous improvement in surgery, radio- and chemotherapy. The majority of GBMs develop rapidly without clinical, radiological, or morphological evidence of a precursor lesion (primary or de novo GBMs). Secondary GBMs, in contrast, develop slowly by progression from diffuse astrocytoma grade II, or anaplastic astrocytoma grade III, and are genetically distinct from primary GBMs. Both GBM types have a rapid proliferation rate, and are capable of doubling in size within a few weeks [452]. Recent findings about epigenetic mechanisms involved in

the pathology of tumors and the simultaneous development of histone acetylation modifiers, such HDACIs, has brought new hopes for novel classification markers, better survival and therapeutic options for gliomas.

Studies on global acetylation pattern in glial tumors are scarce [63, 64, 453]. Although a few studies on global acetylation pattern of histones in brain gliomas have previously been done, the results of these studies are often variable and even contradictory [454, 455]. Global decrease in acetylation of H3 has been reported in one study [64], while in another study, an increase in global acetylation of H3 has been observed [455]. However, in none of these studies, the position of acetyl group has been specified. Alteration in global acetylation of H3K9 is associated with many cancers [21, 449, 456]. However, the changes in acetylation pattern of this specific biomarker have not been studied extensively in brain gliomas so far.

There are variable reports of altered histone acetylation patterns as well as expression or activity of histone modifying enzymes in glial brain tumors [21, 27]. Although some correlations have been described between such changes and severity or progression of these tumors, still little practical clinical conclusion has been derived. Moreover, studies on global H3K9Ac patterns of cells, which have shown practical clinical application and correlation in various tumors, are very scarce for gliomas [14, 66]. So far, only in one study global H3K9Ac levels have been shown to be important in survival prediction of low grade astrocytomas [63]; according to this study, patients whose tumors expressed H3K9Ac in less than 88% of tumor cells had a worse survival compared to patients whose tumors had at least 88% of cells expressing H3K9Ac. In another study, focusing on patterns of global H3 acetylation in different WHO grades of brain gliomas, a global increase of H3 acetylation was reported in glioblastomas compared to low-grade astrocytomas and normal brain tissue [21]. The position of acetyl group was not specified in this study [21]. In another study, investigating differential expression of 12 HDAC genes in astrocytomas and normal brain tissue revealed a negative correlation between HDAC II and IV gene expression and the glioma grade suggesting that class II and IV HDACs might play an important role in glioma malignancy [64]. Evaluation of histone 3 acetylation levels in this study showed that H3 is more acetylated in glioblastomas than normal brain tissue confirming the down-regulation of HDAC mRNA in glioblastomas. The lysine position was not specified in this study too.

Our findings regarding the decreased global acetylation of H3K9 in GBM (grade IV) compared to astrocytoma grade II and III and low H3K9Ac profile of C6 gliomas support the rationale that the levels of H3K9 acetylation correlate with the malignant progression of gliomas.

24.3. Pituitary adenomas

Pituitary tumors are tumors of the endocrine system and constitute up to 10-15% of intracranial tumors. They are classified according to histology, immunohistochemistry, ultra-structural features as well as biochemical, imaging and surgical findings [457]. These tumors mostly include benign adenomas of anterior pituitary cells. Pituitary carcinomas, which are characterized by aggressive behavior and metastasis, are much rarer [458-460]. Pituitary adenomas are classified into typical (conventional) and atypical adenomas. In typical pituitary adenomas, histologically monomorphic cells, with round uniform nuclei and finely stippled chromatin, are most prominent, although mitosis is not a significant feature and, consequently, the MIB-1 labeling index (Ki-67) is < 3% [378]. By contrast, atypical pituitary adenomas are defined as tumors with a MIB-1 labeling index > 3% and “extensive p53 immunoreactivity”, but without metastasis (according to the World Health Organization classification of Endocrine Neoplasms) [23, 461]. However, there is no general agreement about this definition and some studies prefer to consider adenomas as atypical, if the MIB-1 index is > 10%, irrespective of the p53 status [462]. A clear-cut distinction between typical and atypical pituitary adenomas is important, because both the therapeutic option to be considered and the prediction of survival depend on it. More contemporary definitions, based on epigenetic alterations, have brought not only additional therapeutic options to incurable or hardly manageable tumors, but also better criteria to classification of tumors [463]. Epigenetics, according to definition, is anything other than genes that can still affect gene expression [3]. Recently, efforts have been made to classify and describe the pathophysiology of brain tumors based on epigenetic findings and to develop drugs that can act through these epigenetic pathways [30, 31, 463]. In this respect, pituitary adenomas have shown to be candidates of epigenetic-based classifications [464-466]. HMGA2 is a low molecular weight protein, whose overexpression is involved in pituitary oncogenesis [467]. HMGA2 is activated through increased histone acetylation of a series of gene promoters that orchestrate serial tumor promoter activations and finally contribute to tumorigenesis [468]. In this scheme, not only the investigation of changes in the acetylation pattern of H3K9 in

pituitary adenomas can provide a guide to the classification of tumors based on their epigenetic changes, but also the manipulation of H3K9 modulation might be a promising target for therapeutic approaches.

We investigated the acetylation status of H3K9 as an epigenetic component in the normal and adenomatous pituitary as well as the correlation of established histopathological adenoma severity markers with acetylation status. Epigenetically mediated tumorigenesis is a common feature associated with pituitary adenomas [463]. However, the mechanisms involved in this process are still unknown. Although there are a large number of studies on methylation patterns of pituitary tumors [463-466], studies on histone acetylation patterns of these tumors are still limited [469-471].

In the study on pituitary adenomas, we observed an obvious increase in global acetylation of H3K9 in pituitary tumor cells compared to normal pituitary cells, with an increasing trend from typical to atypical pituitary adenoma. Previous studies have focused on the histone acetylation pattern of specific genes and gene promoters within pituitary tumors. For example, melanoma-associated antigen 3 (MAGEA3) is an antigen expressed in human pituitary tumors. Induced MAGEA3 expression has been shown to be associated with increased histone acetylation [472-474]. Silencing of fibroblast growth factor 4, a pituitary tumor derived antigen, has also been shown to be associated with histone deacetylation in pituitary adenomas [475]. Another study revealed that binding of HMGA2, a gene that is amplified and overexpressed in human pituitary adenomas, to retinoblastoma protein (pRB) displaces histone deacetylator enzyme 1 from the repressive pRB complex, leading to the enhanced acetylation of histones at the transcription factor E2F1 responsive sites and further gene transcription activation [468]. Studies on global acetylation pattern of human pituitary adenomas are lacking. In one study of rat pituitary adenoma cell lines GH3 and MMQ, the global acetylation status of rat pituitary adenoma cells was studied before and after treatment with suberoylanilide hydroxamic acid (SAHA; vorinostat), a prototypical histone deacetylase inhibitor [476]. A dramatic increase in histone H3 and H4 acetylation was observed by western blotting in addition to anti-proliferative and pro-apoptotic effects of SAHA. However, the position of acetyl group over the histone 3 and 4 was not specified in that study, whereas our study revealed a global increase in acetylation specifically on lysine position 9 of histone 3. It is now open to debate whether the increase in acetylation of H3K9

from normal pituitary to typical and atypical pituitary adenomas is the result of diminished HDACs or increased activities of histone acetylators.

To correlate increasing p53 expression and tumor severity with the acetylation status of H3K9, we compared two classes of atypical adenomas; one group with a MIB-1 index ≥ 3% and p53 < 10% and the other group with a MIB-1 index ≥ 3% and p53 ≥ 10%. Comparing adenomas with a MIB-1 index < 3% versus adenomas with a MIB-1 index ≥ 3%, regardless of adenoma subtype and p53 expression, revealed an increasing trend in acetylation of H3K9 with an increasing MIB-1 index. We also observed a significantly higher proportion of partially acetylated nuclei with increasing p53 expression, which indicates a shift in the global H3K9 acetylation pattern of atypical pituitary adenomas with increasing p53 expression. Epigenetic alterations have successfully been used for classification of tumors according to previous studies [477]. Demonstrating a positive correlation between established markers of tumor severity and epigenetic-based classifications will probably supplement the list of available biomarkers with newer biomarkers of higher clinical value in the classification of tumors. MIB-1, a highly repetitive epitope of Ki-67 antigen, is an important marker of tumor cell proliferation [478]. p53 is a tumor suppressor gene whose expression in human pituitary adenomas correlates with tumor invasiveness [479]. Regarding the expression status of these two antigens, different definitions have been proposed for atypical adenomas by different investigators and a clear cut-off point has not been established to date. According to the WHO classification of endocrine neoplasms, adenomas with a MIB-1 index ≥ 3.0% and so-called ‘extensive’ p53 positivity are classified as ‘atypical’ adenomas [457]. However, some studies prefer to consider adenomas as ‘atypical’, when the MIB-1 index is ≥ 10%, irrespective of the p53 expression status [462]. The reason for this disagreement in definition is a result of the variability of p53 overexpression [479-481]. In our study, we considered p53 positivity ≥ 10% as extensively positive in order to address the discrepancy in defining the extensive p53 positivity based on global H3K9 acetylation status.

Taken together, the results obtained in the present study reveal a change in acetylation pattern of high p53 expressing tumors compared to low p53 expressing tumors (with a 10% cut-off point for p53 expression) along with an increasing trend in H3K9 acetylation of tumor cells, when MIB-1 increases. This suggests a further contribution of p53 expression and MIB-1 index to the alteration of global H3K9 acetylation as well as tumor severity and malignancy

based on our epigenetic classification.

However, considering the much lower frequency of onset of atypical pituitary adenomas with p53 ≥ 10% in the population compared to atypical adenomas with p53 < 10%, these results still need to be validated by further studies conducted on a larger volume of samples. In conclusion, we found an increased acetylation of H3K9 in pituitary adenomas compared to normal pituitary. The H3K9 acetylation increases along with tumor severity, so that atypical pituitary adenomas are more acetylated than typical pituitary adenomas. In pituitary adenomas, MIB-1 (Ki-67) overexpression is highly associated with increased acetylation of H3K9 as well as tumor severity, and there is also a contribution of p53 expression to the altered global H3K9 acetylation pattern of pituitary adenomas. We suggest that H3K9 acetylation status is a relevant biomarker of tumor severity in pituitary adenomas and could be used in further tumor classification trials.

24.4. Ependymal tumors

Ependymal tumors are believed to originate from ependymal cells of the radial glial cell lineage, lining ventricular surfaces of the brain or the central canal of the spinal cord [482]. They are highly variable in location, histopathology, genetics and behavior [483]. According to WHO criteria for classification of CNS tumors, ependymal tumors are being classified as grade I including myxopapillary ependymomas and subependymomas, grade II including ependymomas, and grade III including anaplastic ependymomas [484]. However, WHO grading of these tumors neither is predictive of clinical behavior, nor very well estimates the likelihood of their recurrence [485, 486]. Regarding their heterogeneity, the outcome of ependymoma is not easily predictable. Proper cell lines, xenografts, and animal models of ependymal tumors for investigational purposes are very limited as well. Recently, a mice model of ependymal tumor was produced whose transcriptome matched only a single human cerebral ependymal tumor subgroup [487]. In this scheme, characterization and defining molecular abnormalities in ependymal tumors is a prudential way to better understanding of their initiation and progression, and might also offer improved diagnostic, prognostic and therapeutic tools. Epigenetic study of ependymal tumors is also an effort in this direction [488]. So far, epigenetic studies of ependymal tumors have mainly focused on analyzing the promoter methylation status of selected genes [73, 488, 489]. In a recent study,

vorinostat, a histone deacetylase inhibitor, induced differentiation in a novel human high-risk ependymoma stem cell model, suggesting that histone acetylation status could be a potential candidate for epigenetic study of ependymomas [490]. Among different modes of histone acetylation, global acetylation of histone 3 lysine 9 (H3K9Ac) has shown to be a relevant marker in pathological changes of a variety of tumors [5, 63, 70, 491-495].

Here, we studied 85 cases of human cerebral and spinal ependymal tumors of various clinico-pathological characteristics to explore global H3K9Ac profiling of these tumors and also to define a potential subgroup of ependymal tumors that might be a proper target for novel epigenetic-based therapeutics, HDACIs. Epigenetic studies on ependymal tumors so far have been limited to methylation status of selected genes in these tumors [488, 489]. Global histone acetylation level is shown to be a predictor of recurrence risk and malignancy grade in glioma, another type of glial tumors, as well as prostate cancer, non-small cell lung cancer, gastric adenocarcinoma and pituitary adenoma [63, 64, 70, 72, 496]. In a study on gliomas, patients with tumors of WHO grade I and II whose tumors expressed H3K9Ac in < 88% of tumor cells had worse survival compared with patients whose tumors had at least 88% of cells expressing H3K9Ac [63]. Concerning ependymal tumors, to our best of knowledge, there is no previous report on histone acetylation levels either by gene candidate approach on specific promoter regions or global histone acetylation changes in cell level and our report is the first one in this area.

Gain of chromosome 7, notably the region 7q11.23-22.1, is a commonly identified chromosomal aberration, which is associated almost exclusively with spinal ependymomas. [497, 498] HDAC9 is a candidate oncogene located at 7p21.1 [498, 499]. HDAC9 is a histone deacetylator enzyme, containing a conserved deacetylase domain and utilizes histones H3 and H4 as substrates in vitro and in vivo [500]. HDAC9 has also been shown to interact with HDAC3, another histone deacetylator [500, 501]. HDAC10 is located on chromosome 22q13 [502] while chromosome 22 loss is the most frequent overall genetic abnormality found in sporadic ependymomas [503-505]. Such findings, along with our results concerning variable patterns of H3K9Ac in different ependymal tumors reveal that histone oriented epigenetic mechanisms might be involved in pathophysiology of ependymal tumors and histone modulating enzymes could be potential targets for therapeutic approaches in these tumors. Our results, concerning lower global H3K9Ac levels in intracranial parenchymal ependymal

tumors compared to ventricular or spinal tumors, suggests that significant changes in HDAC activity or global histone acetylation might be restricted to a subgroup of ependymal tumors. In a study, evaluating the antitumor efficacy, pharmacokinetics, and pharmacodynamics of the depsipeptide, a natural HDAC inhibitor, SCID mice bearing BT41 and BT54 tumor lines of ependymoma and anaplastic ependymoma respectively did not show any significant response to therapy [506]. While in another study, using DKFZEP1NS cells, a human high-risk ependymoma stem cell model obtained from a patient with metastatic disease, the cells responded to treatment with vorinostat, a histone deacetylase inhibitor (HDACi) at therapeutically achievable concentrations [490]. This reiterates that only a subset of ependymal tumors might be responding to HDAC inhibitors and identification of this relevant target group would be of paramount importance.

Although histologically similar, ependymal tumors are believed to arise from distinct origins regarding their highly variable behavioral and molecular characteristics and heterogeneous genetic landscape [482, 487, 507]. Variable molecular and clinical patterns in ependymal tumors have previously shown to be location-specific too [508, 509]. Current observations in several tumor cohorts have shown that up to 50% of ependymal tumors of posterior fossa have balanced genomic profiles [487, 498, 510-512]. In a recent joined cohort of WHO grade II and III ependymal tumors in Heidelberg and Toronto, it was revealed that tumors of posterior fossa, which are located anatomically more laterally in the cerebellopontine angle, have a balanced genome, and are much more apt to exhibit recurrence, metastasis at recurrence, and death compared with the ones located in the midline [507]. In another smaller cohort, it was shown that ependymal tumors from different CNS locations (supratentorial, posterior fossa, and spine) had location-specific transcriptional and somatic genetic profiles, suggesting that ependymal tumors from different anatomical locations of the nervous system are separate entities [482]. To best of our knowledge, there is no previous report of location-specific epigenetic patterns in ependymal tumors. The next step would be to explore possible HDAC activity and expression changes in tumors of various CNS anatomical regions.

Taken together, ependymal tumors have a localization-specific epigenetic pattern. Global H3K9Ac has prognostic relevance in ependymal tumors so that tumors with lower H3K9Ac values have higher probability to recur in time and vice versa. Besides, subependymomas

have higher H3K9Ac profile than other ependymal tumor subclasses.

24.5. Histone acetylation in neurodegenerative disorders

As mentioned before, epigenetic mechanisms are involved in the CNS development. Accordingly, various neurodegenerative diseases have been linked to epigenetic mechanisms. The role of histone acetylator/deacetylator enzymes in between has been significant. In vitro studies of epigenetic mechanisms in HD revealed that mutant HTT fragments inhibit the HAT activity of CREB-binding protein (CBP), its close homolog p300 and CBP/p300-associated factor (P/CAF) and reduce overall H3/H4 acetylation levels in vitro and in vivo [513]. In addition, expression of a mutant expanded HTT was accompanied by reduced H3/H4 acetylation in two murine models of HD [514]. Concordant with these findings, histones associated with down-regulated genes were shown to be hypoacetylated in HD models [515]. The data also suggest HDAC inhibitors as candidate drugs for HD therapy [513, 516]. Applicability of HDAC inhibitors for HD therapy has also been confirmed in transgenic mice which mimic HD-like pathology [517, 518]. Clinical trials are now being run for evaluating the efficacy, safety and tolerability of HDACIs such as phenyl butyrate (PB) and VPA [519].

H3 was reported to be hyperacetylated in pontine tissue of spinocerebellar ataxia (SCA) type 3 patients [520]. Incorporation of polyQ-expanded ataxin-7 (92Q), involved in pathology of Autosomal dominant SCA7, into SPT3/TAF9/GCN5 acetyltransferase complex (STAGA) dramatically reduced its ability to acetylate free H3. Accordingly, a marked reduction of H3 acetylation in the promoter regions of the CRX-dependent photoreceptor genes was reported in retina of SCA7-92Q mice [521]. Due to an increasing body of evidence, suggesting transcriptional dysregulation in SCA7 as a result of reduced HAT activity, HDAC inhibition is being argued as promising pharmacologic intervention.

Disturbance of histone acetylation homeostasis has been linked to the ALS pathogenesis too. Acetylated H3 and the histone acetyltransferase CBP were severely reduced in motor neuron nuclei in the lumbar spinal cord of ALS-like mice [54]. In another study, hypoacetylation of histones H2A, H2B, H3 and H4 was observed in spinal cord of affected SOD1/G93A mice (a murine model of ALS), while PB treatment restored histone acetylation to near-normal levels [522]. ALS also features cytoplasmic aggregation of misfolded proteins, such as SOD1, TARDBP, or FUS. Overexpression of FUS was followed by hypoacetylation of H3K9 and H3K14

on the CCND1 promoter, involved in the pathology of ALS [523]. ELP3 is another gene associated with sporadic amyotrophic lateral sclerosis, which encodes the catalytic subunit of the HAT complex elongator protein [524]. 3ELP3 regulates the expression of Hsp70 through the changing histone H3K14 and H4K8 acetylation levels [525].

Histone H3/H4 hyperacetylation was observed in CK-p25 mice, a model of AD, following environmental enrichment (large cages where exploratory activity is promoted by the presence of toys, tunnels and climbing devices) [526]. Chronic levodopa therapy leads to deacetylation of histones H4K5, K8, K12, and K16. MPTP (1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine) induced PD in murine, induced H3 acetylation, which was reduced after treatment with levodopa [527]. Besides, α-synuclein, a main pathologic feature in PD, inhibits acetylation of histones which is largely linked to its effect on Sirt2, a HDAC enzyme [528].

Changes in the state of chromatin have been observed following seizure too. Such changes could affect the expression of specific genes involved in the seizure. In a study of epigenetic changes following status epilepticus, 3 hours after the induction of seizure, the histones H4 on the BDNF (brain-derived neurotrophic factor) promoter show hyperacetylation, which was correlated with an increased level of BDNF mRNA [529]. After seizures, H3 and H4 histones associated with GluR2 promoter were rapidly deacetylated, correlating with mRNA down regulation. In another study, kainic acid induction of epilepsy in vivo was followed by widespread and sustained histone H4 acetylation in hippocampal neurons [530]. H3 acetylation, in between, has been of specific relevance in human patients. Lower levels of H3 acetylation have been found in cultured lymphocytes from schizophrenic patients [531]. In a study, chronic social defeat stress in mice led to persistent increases in H3 acetylation in the nucleus accumbens (NAc), a postmortem finding in the NAc of depressed humans associated with decreased levels of HDAC2 [532].

24.6. Alzheimer's disease

AD is a progressive neurodegenerative disorder and the most common cause of dementia among the aging population [391]. The disease has been the sixth leading cause of death across all ages and the fifth leading cause of death for those aged 65 and older [533, 534].

AD is a multifactorial disease, clinically characterized by progressive cognitive loss,

neuropsychiatric and behavioral disorders. Pathological findings include extracellular Aβ plaques in brain parenchyma and arterioles, intracellular NFTs, the loss of neuronal subpopulations, mitochondrial oxidative damage, synaptic loss, proliferation and activation of astrocytes and microglia [150, 535, 536].

According to the current theories, Aβ accumulation and tau hyperphosphorylation are considered the core pathologies of AD [393, 537]. Excessive Aβ accumulation, as a result of either increased production or decreased clearance, leads to the formation of senile plaques [537]. Thereafter, a series of biological events starts which ends up with an impairment of neuronal synapses and dendrites through oxidative stress and inflammatory processes [331, 332]. NFTs, another pathological hallmark of AD, consist of abnormally hyperphosphorylated tau protein, and contribute to fulminant brain degeneration and disease progression [333]. Mitochondrial dysfunction and abnormal mitochondrial dynamics are further accompanying events that contribute to the cognitive deficits and synaptic damage [535, 538-541]. Activation and proliferation of brain glial cells, such as astrocytes and microglia will finally add up to the pathology of CNS through production of pro-inflammatory cytokines and toxins related to neurodegenerative process [542]. AD-associated vasculopathy and vascular Aβ (VAβ), is another common aspect of AD which has been focused in recent years [543]. The blood-brain barrier is thought to be disrupted as a result of amyloid deposition in vessels and lead to a passage of proteins in cerebro-spinal fluid (CSF), causing a cascade of immune responses and damage to the brain [544].

The main concept in modern translational research is to proof the drug efficacy in defined preclinical conditions after experimental manipulations and to transfer the concept to the actual clinical conditions. In this context, drug discovery efforts require valid animal models of human disease. AD transgenic models are the best tools to fulfill this concept and useful means of modern translational research in AD. Accordingly, investigation of anti-Alzheimer therapeutics in preclinical studies requires a good knowledge of AD models and potentials of each model for therapeutic development.

Heritable factors contribute substantially to the risk of developing AD. More than 95% onsets of AD occur later than 65 years and, which are called “sporadic” AD while less than 5% of AD cases accounts for early-onset autosomal dominant forms, called familial AD (FAD) [392].

Current AD research mainly depends on the later form of disease. Two reasons can be mentioned for this situation. First of all, FAD fully represents amyloid theory which is the best known and accepted theory describing molecular mechanisms of AD [393]. Secondly and most importantly, transgenic models could be designed from involved genes in familial forms which are of foremost importance for investigational purposes. So far, three different genes have been described in relation to FAD, namely the amyloid precursor protein (APP), presenilin 1 (PS1) and presenilin 2 (PS2) gene [545]. Their variations are known to be responsible for Aβ accumulation [546, 547]. These mice models are capable of recapitulating main pathological features of disease in human beings [394].

The amyloid hypothesis mainly emphasizes on excessive Aβ production as the core pathology of AD [393]. Besides, the presence of defined genes responsible for FAD has assisted making AD models overexpressing APP. These APP transgenic mice represent major pathological features of AD, including parenchymal and vascular amyloid pathology, plaque-associated dystrophic neuritis, microglial activation, synaptic impairments, and learning and memory deficits [548-551]. PS proteins are a group of multi-pass trans-membrane proteins also involved in pathology of AD and function as a part of the ɣ-secretase enzyme [552]. PS1 and PS2 are two related genes whose mutations are involved in early onset FAD [553]. PS1 mutations are the most commonly recognized causes of early-onset FAD [553]. Transgenic mice harboring both APP and PS mutations reveal extensive neuronal loss [554, 555]. These mice could produce earlier and more extensive plaque deposition as well as more profound AD morphology compared to mice models harboring only one of the two mentioned mutations [556]. APPPS1-21 is a mouse with C57BL/6J genetic background that coexpresses KM670/671NL mutated amyloid precursor protein and L166P mutated presenilin 1 under the control of a neuron-specific Thy1 promoter element [381]. The greatest advantage of the models carrying both APP and PS mutations is exhibiting a rapid neuritic-type amyloid deposition at very early age, while at the same time Aβ deposition can be detected in the cingulate and motor cortex and hippocampus. Aβ accumulation accelerates with ageing [557], and the $A\beta_{42}$/ $A\beta_{40}$ ratio increases accordingly [558, 559]. These models are associated with cognitive deficits at early age and extensive neural loss, but not with NFTs [559]. Accordingly, in APPPS1-21 mice, cerebral amyloidosis starts at an early age of 6-8 weeks and the ratio of human $A\beta_{42}$/ $A\beta_{40}$ increases form 1.5 in early pathological state to 5 in robust pathological state [381].

25. Do patterns of H3K9Ac change in response to selective or non-selective histone modulators?

We described in introduction that there are several classes of HDAC each of which have a specific group of targets. Class I HDACs consist of HDAC1, HDAC2, HDAC3, and HDAC8 that contain a nuclear localization signal (NLS) and, with the exception of HDAC3, lack a nuclear export signal. Accordingly, class I HDACs are primarily found within the nucleus, where they regulate histone acetylation. Class II HDACs includes class IIa HDACs (HDAC4, 5, 7, 9) and class IIb HDACs (HDAC6, HDAC10) having both nuclear localization and export signal. They generally shuttle between the cytoplasm and nucleus and acetylate a wide range of proteins including histones [560]. However, the precise effect of individual HATs and HDACs on acetylation of specific lysine positions or differential proteins have, to date, primarily been studied in lower organisms such as yeast [561, 562]. Based on the literature, HDAC1-5, 7-11 and SIRT1 are involved in acetylation of histones among which HDAC1 has an in vivo preference for H3K9 [401]. H3K9 is also a target for SIRT1 [563]. Other HDACs have also shown a direct or indirect effect on H3K9 acetylation. For instance, deletion of HDAC3 resulted in H3K9 acetylation in human liver cancer cells [564]. HDAC5 overexpression reduced and HDAC5 knockdown increased H3K9 acetylation in C3H10T1⁄2 cells and mouse embryonic fibroblasts [565]. HDAC9 deficiency was associated with increased site-specific H3K9 acetylation globally and localized to IL-4, roquin, and peroxisome proliferator-activated receptor-γ promoters with increased gene expression, respectively [566]. Suppressing HDAC11 expression with small interfering RNA significantly (1) increases H3K9/K14ac globally and within the MBP and PLP genes in cultures of primary oligodendrocytes [567]. HDAC6 is mainly a cytosolic deacetylase with a specified link to autophagy progression, which is of great potential for revealing disease mechanisms in neurodegeneration and therapeutic development [568], and there is no direct connection of HDAC6 and histone acetylation dynamics. However, augmented acetylation of H3K9 acetylation has been reported in absence of HDAC6 in the promoter region of PEPCK gene HDAC6-deficient (HDAC6KO) mice [569]. SIRT6 has also recently been shown to target H3K9 acetylation [570].

Based on the evidence, various HDAC inhibitors can affect the H3K9 acetylation levels either in genome wide level or in promoter specific regions; this can be accompanied with improved neurocognitive function and sometimes reduced amyloid deposition too. Targeted

inhibition of class I HDAC isoforms has shown to be a promising venue for treating the cognitive deficits associated with early stage AD. In a study of inhibitors of class 1 histone deacetylases (including sodium valproate, sodium butyrate, and vorinostat) these drugs could reverse the contextual memory deficits in APPPS1 mouse model of AD [571]. Among HDAC inhibitors, CNS-penetrant HDAC (class I) inhibitor EVP-0334, has been developed and studied in a phase I clinical trial for the treatment of AD [572] and nicotinamide is under phase I clinical trial for safety evaluation [573]. Non-specific pan-HDAC inhibitors such as valproic acid, trichostatin A, sodium phenyl butyrate, and vorinostat have been shown to affect Aβ plaque deposition and/or tau hyperphosphorylation, through diverse mechanisms [401]. However, this remains unclear whether they ameliorate AD pathology in AD mouse models through Aβ clearance, or primarily through HDAC inhibition. According to this view, exploring epigenetic mechanisms in neurodegeneration in one hand would be of critical importance. If the epigenetic blockade starts before the clinical onset of AD, reducing Aβ generation and deposition alone may not be sufficient to rescue cognitive functions, and in case of reverse condition, application of HDAC inhibitors which has no effect on amyloid deposition and pathway would be nonsense. Considering all previously discussed epigenetic mechanisms in neurological diseases, there is a growing interest to use epigenetic modulators for treatment of various brain disorders from brain tumors to psychiatric and neurodegenerative diseases. Manipulation of histone acetylation, considering the accessibility of HDAC modulators and relative success of these compounds in various neuropsychological disorders, has been of special interest [519]. Here we studied the effect of two well-known HDACIs, a non-selective (VPA) and a selective (MS-275) one, on H3K9 acetylation levels in two murine models of neurodegenerative diseases.

25.1. Effect of MS-275 on brain's H3K9Ac patterns in APPPS1-21 mice

AD is a multifactorial disease, characterized by a diverse range of pathological features as described before [150, 535, 536]. From the evidence it is apparent that AD is rather a complex syndrome than a disease, and it demands a complex and multi-dimensional definition in sense of pathology [574].

Epigenetic mechanisms in neurodegenerative disorders, especially those including histone acetylation/deacetylation, and their association to the process of learning and memory have been previously described [398, 575, 576]. HDAC2-deficient mice demonstrate improved

memories and increased synapse number, similar to mice treated with the HDAC inhibitor SAHA, while mice overexpressing HDAC2 exhibit impaired memory formation as well as reduced dendritic spine density, synapse number and synaptic plasticity [399]. Inhibition of HDAC3 increases histone acetylation, improves long term memory in the novel object recognition task, and increases expression of the genes implicated in long-term memory [577]. In addition to the previously mentioned epigenetic modalities, association of SIRT1 and HDAC6 to AD pathology has been described too [578-580].

We reported for the first time an altered global H3K9 acetylation in neocortex of APPPS1-21 mice compared to wild-type littermates. Alterations of histone modifications have been reported in AD models in previous studies with a central focus on hippocampus. Sixteen-month C57BL/6J mice revealed altered histone H4 lysine12 (H4K12) acetylation, along with decreased expression of genes involved in memory coalition in hippocampus [55]. In another study there was no dissimilarity in acetylation status of histone H4 between 4 months old wild-type and APP/PS1 mice, though contextual fear conditioning, followed by administration of HDAC inhibitor TSA, resulted in hippocampal CA3-CA1 long term potentiation and enhanced acetylated H4 levels compared to H4 acetylation level in wild-type mice [56]. In a similar study, no significant difference in acetylation levels of hippocampal H3 or H4 between 6-month APP/PS1 mice and wild-type mice was observed. However, a significant decrease in acetylation status of H4 in older APP/PS1 mice was noticeable. Besides, administration of several HDAC inhibitors such as VPA or SAHA induced expression of acetylated histone H4 and reinstated contextual memory in APP/PS1 mice [571]. In an attempt to find peripheral biomarkers for the early diagnosis of AD, peripheral blood samples of AD patients were studied for alterations of gene expression. Among 33 genes studied, HIST1H3E gene encoding histone 3 was specifically associated to the progress of the dementia stage [581]. Alterations of HIST1H3E gene have been reported in postmortem brains of Alzheimer patients too [582].

Our study in APPPS1-21 mice revealed epigenetic alterations in a relatively earlier stage of life (5 months) compared to previous studies. Besides, these changes are more remarkable in neocortex than other brain regions that signify the role of neocortex in AD pathology. We also observed an altered H3K9Ac status in 5 and 7 months old transgenic mice compared to 3 months old mice. Considering the recent evidence on involvement of epigenetic pathways in initiating late onset Alzheimer disease (LOAD), it would be interesting to find out whether

epigenetic changes are initiated, before amyloid pathology becomes detectable.

Considering the relative success of HDACIs in cancer therapy, there is a growing interest in application of this therapeutics for neurological and psychiatric disorders, such as depression, schizophrenia, AD, etc. [568, 583, 584]. MS-275 is a potent, long-lasting, selective HDAC inhibitor that has recently gained the attention for treatment of brain disorders. Combined use of MS-275 and resveratrol revealed neuroprotective effects in a mice model of ischemic brain injury [404]. Treatment of rat astrocytes and neurons up-regulated the levels of HSP70, and increased the levels of H3K4Me2 at the HSP70 promoter [405]. In another study, 5-day treatment of a mouse model of persistent inflammatory pain with MS-275 substantially reduced the nociceptive response in the mice [406]. MS-275 has been introduced as a potential treatment for cognitive deficits associated with schizophrenia too [407]. Information regarding the blood brain barrier (BBB) penetration, concentration and distribution of MS-275 in the human brain is limited and contradictive. PET study of, radioactive-labeled MS-275 revealed very poor initial BBB penetration and low brain uptake in baboon and rat [585], while several other reports have mentioned the relative permeability of BBB to MS-275 in rodents [390, 586, 587]. Interspecies differences in pharmacokinetics of MS-275 have been proposed in previous studies. For instance, MS-275 showed a significantly higher plasma concentrations in mouse, rat, rabbit, dog and pig, compared to human in a study of pharmacokinetic behavior of MS-275 [588]. The final conclusion in all these studies depends on the method of study. The direct tracking methods reveal no BBB penetration while the indirect efficacy investigations present a potential CNS effect of MS-275. According to our results, gavage of mice with MS-275 can still affect the H3K9Ac levels of the whole brain.

It has been shown that MS-275 is a brain region selective inhibitor of HDAC1 and 3 [390]. It caused an increase of H3 acetylation in the rat hippocampus (up to 3-fold) and the frontal cortex (up to 2-fold) in a dose dependent manner, but not in other brain regions such as the striatum [390]. The reason for brain region selectivity has been accredited to a number of probable factors including HDAC isoform distribution differences or MS-275 distribution and pharmacokinetics. However, this reasoning lacks a further characterization as to which the origin of this region selectivity could be attributed [390]. According to our observations, the whole brain can be affected by MS-275, but neocortex seems to be affected more than other

brain regions, that might be due to a saturation effect. This means that, due to the lower H3K9Ac levels in neocortex of transgenic compared to wild type mice, neocortex has more free positions for acetyl groups than striatum and hippocampus.

Epigenetic findings in AD have been controversial in some cases. For example, an enhancement of histone acetylation was reported in hippocampus in response to contextual learning, while at the same time reducing the levels of histone acetylation by preventing HAT activity promoted amnesia and interfered with the consolidation of hippocampus dependent memories [589]. The realization of the therapeutic development based on epigenetic mechanisms in AD, therefore, requires describing various histone modifications in AD and understanding the complex role of HDAC family members in the disease. Our finding in APPPS1 mice model of AD, concerning altered H3K9Ac levels in neocortex and resuming these changes through MS-275 administration, is an effort in this direction and hopefully provides a platform for therapeutic development against the disease progression.

25.2. Effect of valproic acid on H3K9Ac patterns of LPS-induced N9 cells

As previously mentioned, VPA is a branched fatty acid and HDAC inhibitor that hyperacetylates the N-terminal tails of histones H3 and H4 in vitro and in vivo [78]. VPA has also shown neuroprotective effects through a range of possible mechanisms, such as modulating the GABAergic and glutamatergic systems, influencing ion channels, modulating kinase pathways, anti-oxidant properties and influencing gene expression through epigenetic pathways [421]. VPA treatment of murine microglia in previous studies was associated with decreased H3 acetylation [590]. However, we did not detect any alteration of H3K9Ac immunohistochemical profiling of N9 cells after VPA treatment. Moreover, the nitric oxide (NO) production was increased in N9 cells as a result of VPA treatment. VPA is reported to induce apoptosis and dysfunction in glial cells [591-593]. In a study of NO, lipid peroxidation, and anti-oxidant enzyme levels in epileptic children using valproic acid, the NO concentration was about 10% higher in VPA group than in the control group [594]. However, there was no significant difference in serum malondialdehyde (MDA), superoxide dismutase (SOD), and catalase (CAT) levels of two groups. Increased serum NO concentration in children receiving VPA was reported in other studies too [595]. Although NO is known as an indicator of inflammation and a risk factor for neurodegeneration, there are also studies claiming the opposite role for NO, that endogenous NO may play a neuroprotective role [596]. HDAC

inhibitors, like SAHA and TSA, release NO upon oxidation, but VPA has not shown such effect [597]. Therefore, it is unlikely that the increase in NO production of N9 cells treated with VPA is resulted from drug oxidation. In a study, authors has shortly referred to an increased NO production in LPS-induced rat microglial in their discussion, but they have not reported it in the results part [593]. Although the underlying mechanisms for these conflicting results remain unclear, we speculate that HDAC inhibitors effect through a diverse range of interactions and pathways. The pathway to be modified by the drug depends on the cell type, pathophysiological condition of cell and varied interaction of affected genes, proteins and cells with other genes, proteins and cells types.

25.3. Effect of valproic acid on brain's H3K9Ac patterns in EAE rat model of MS

VPA was better known as a potential antiepileptic until HDAC1 modulating effect was reported for it [78]. VPA hyperacetylated the N-terminal tails of histones H3 and H4 in vitro and in vivo and was proven to directly inhibiting HDAC enzymatic activity at 0.5 mM concentration [78]. Since then VPA has been used in different pre-clinical and clinical trials for its potential therapeutic effects against solid tumors [420]. VPA has also shown neuroprotective effects through epigenetic pathways [421].

MS is a prevalent multifactorial neurological disease in the northern hemisphere, with 0.5-1.5 per 1000 prevalence rate [410]. The susceptibility to develop MS is mostly accredited to the HLA class I and II regions [411-414]. Along with new epigenetic-based definitions in various diseases, the role of epigenetic mechanisms in neurodegenerative and autoimmune diseases has started to be explored beyond targeted investigations of specific loci [417-419]. Accordingly, studies exploring the epigenetic mechanisms of cells implicated in MS pathology have already been done and can be assigned to the diseases involved by these cells. For example it has been shown that specialized T cell subsets are modulated by epigenetic mechanisms such as DNA methylation or histone modifications [598, 599]. Decreased DNA methylation has been reported in the IFN-γ promoter of Th1 cells [600] and DNA methylation and histone deacetylation are shown to be involved in Th2-associated IL4 silencing [601]. Moreover, dysregulation of β-arrestin-1 protein, which is thought to have a role in EAE and perhaps in MS, occurs through modulation of histone H4 acetylation at the BCL2 anti-apoptotic gene [602]. Such findings have been a guide to the application of epigenetic

modulator drugs for reprogramming of immune cells in order to reverse the pathological state toward the healthy state. For instance, application of histone modulator drugs such as sodium butyrate, an HDACI, have shown to alter the architecture of chromatin, and reduces T cell proliferation and expression of both IL12 and IFN-γ in human immune cells. These findings also emphasize the role of histone acetylation/deacetylation in immune modulation [603]. Accordingly, trichostatin A, another HDACI, reduces spinal cord inflammation, demyelination and neuronal loss in the relapsing phase of EAE [604].

About the importance of epigenetic mechanisms in MS, there is a long ray of questions to be answered. But it is probable, that the susceptibility factors and events involved in the pathology of the disease alter the chromatin state of different cell types in patients with MS [605]. There is not much evidence on alteration of histone modifications in subjects with MS. However, the evidence of epigenetic mechanisms and specially alteration of histone modifications is present in the cells implicated in MS pathology, as described in introduction. Moreover, the application of HDAC inhibitors has shown a modulating effect in the disease [606]. In the present study, we observed an increased H3K9Ac in response to VPA treatment in EAE rat brain compared to PBS-treated rats, and this increase was associated with alleviation of neurological symptoms. We did not specify the cell type, but we evaluated global or genome wide H3K9Ac status in all cell types within the brains of EAE rats. According to the literature, increasing the histone acetylation profile in EAE/MS models not only modulates the activity of immune cells involved in MS pathology, but also increases the neural survival via increasing the transcription of genes that prevent neuronal death in different models of neurodegenerative disorders [584, 606]. As a result, several cell types might be the target of HDAC inhibitor therapy in MS brain including neurons, oligodendrocytes, astrocytes, microglial cells, etc. [606]. In a study on efficacy of INF therapy in MS, INF therapy of WISH and Daudi cells, two various immune cell lines was associated with increased H3K9Ac of oligoadenylate synthetase 1 (OAS1) gene promoter, a gene involved in the innate immune response [607]. There is evidence of increased global H3K9 acetylation associated with increased neuronal survival in various neurodegenerative models [584, 608, 609]. Modulation of H3 acetylation has a specific role in oligodendrocyte progenitor differentiation, oligodendrocyte gene expression and activity [567, 610]. Reduction of microglia inflammatory signaling in vitro is associated with increased global H3K9Ac [611]. These are few examples of possible role of H3K9Ac modulation in MS

pathology.

On the other hand, any change in gene expression and protein transcription as a result of epigenetic modulating therapy can be associated with genomic reprogramming and a new chromatin status. Genomic reprogramming, including frequent transcriptional activation and gene silencing is determined by chromatin architecture and the correlation of different post-translational histone modifications with transcriptional states [612]. Histones modification is mediated by several enzymes with complex activity profiles and can occur at different sites simultaneously [613]. The combination of various histone modifications in the nucleosome exerts a functional effect on gene expression, and a single modification is not effective by itself [27]. Based on the combinations of different histone alterations, 51 different chromatin states have already been described, each of which are suggested to have different biological roles [614]. H3K9Ac is mainly located in the region surrounding the transcription start site and its elevated in promoter regions, consistent with a role in transcriptional initiation [27, 615]. Few studies have explored such chromatin states in MS patients. In one such study on discordant twins, an intriguing similarity was observed in the methylation profiles of these twins when compared to unrelated individuals [616]. The study of histone acetylation patterns in normal-appearing white matter and early MS lesions in human brains revealed increased H3 acetylation in the nuclei of oligodendrocytes in a subset of MS patients; these changes were associated with high levels of transcriptional inhibitors of oligodendrocyte differentiation (i.e., TCF7L2, ID2, and SOX2) and higher HAT transcript levels (i.e., CBP and P300) in female MS patients compared with non-neurological controls, and correlated with disease duration [617]. This study also reported a shift in histone acetylation in the white matter of the frontal lobes of aged subjects and in patients with chronic MS, suggesting that histone deacetylation is a process which occurs at the early stages of the disease, and its efficiency decreases with disease duration [617]. In another study, nuclear HDAC1 was detected in cytosol of damaged axons in brains of humans with MS and brains of mice with cuprizone-induced demyelination, in ex-vivo models of demyelination, and in cultured neurons exposed to glutamate and tumor necrosis factor. However, this was not associated with alteration of H3 acetylation which is one of the main nuclear substrates for HDAC1, suggesting a cytosolic gain of function rather than loss of its nuclear function and nuclear export of HDAC1 as a critical event for impaired mitochondrial transport in damaged neurons [618]. This shows that therapeutic effect of HDAC inhibition is not necessarily or merely due

to its effect on histone acetylation, but also due to inhibition of other functions of HDACs. However, regarding the wide range of HDACs, non-specificity/non-selectivity of most HDACIs and common histone targets for 2 to 3 different HDACs, in addition to the critical role of histone modulation in gene transcription, the alteration of histone acetylation in response to HDAC inhibitors is not far to be expected.

From all above, it is concluded that MS patient might benefit from HDAC inhibition. H3K9Ac is a target that is expected to be altered following application of non-selective HDAC inhibitors and it is relevant to the course and pathogenesis of MS too. In our study we also observed an increased H3K9Ac in brain of EAE rats treated with VPA compared to PBS treated ones, and this was associated with improved neurological scores. H3K9Ac might be a potential target for evaluation of efficacy of treatment with HDAC inhibitors in MS patients.

26. Are there natural polyphenols which target altered H3K9Ac patterns in pathological states?

Epigenetic modifications induced by natural polyphenols have been evidenced by various studies [619]. The presence of numerous evidence on epigenetic modulating effects of dietary polyphenols, has even given the title of “epigenetic diet” to these compounds [91]. Emerging evidence suggests that dietary polyphenols alter normal epigenetic states and similarly reverse abnormal gene deregulations. These compounds are able to alter the DNA methylation and histone modifications, leading to gene activation or silencing. The beneficial effects of natural polyphenols in disease treatment can be linked to their ability to modulate HDACs, DNMTs as well as many other epigenetic mechanisms involved in the diseases. Many polyphenols, like curcumin, resveratrol and catechins, are reported to modulate NF-κB expression and chromatin remodeling through modulation of HDACs and DNMTs activities [620]. The anti-inflammatory properties of many reported polyphenols are associated with their ability to induce HDAC activity, and thereby, restore the efficacy of glucocorticoids [621]. Many polyphenols have been shown to modulate epigenetic related enzymes in the cell through either activation or inhibition. Hence, the modulation of epigenetic events by natural polyphenols may be beneficial in therapeutic intervention of a variety of chronic diseases [2]. Although the epigenome is considered to represent a promising target for disease prevention and treatment, only few studies have reported the influence of natural polyphenols on prevention and therapy of diseases other than cancer. The majority of studies

available on epigenetic therapy are focused on developing DNMTs and HDACIs for treatment of cancer. However, much other pathology has been identified as a result of epigenetic defects. Although polyphenols effects certainly occur with actions on different receptors and signaling pathways, it is believed that some of these effects are mediated through epigenetic regulation. Polyphenols have shown both HDAC inhibition and activation properties.

Quercetin, a flavonoid found in foods such as citrus fruit and onions, has been shown to increased mRNA expression of PGC-1 alpha and SIRT1 in mice, activate SIRT1, increase AMPK phosphorylation in HepG2 cells and induce deacetylation of H3 in human prostate cancer cells [122, 127, 622]. Silibinin increased the expression of anti-apoptotic Bcl-2 and up regulated SIRT1 in cardiac myocytes [129]. S17834, a synthetic polyphenol, increased SIRT1 deacetylase activity in HepG2 hepatocytes [623]**.** Kaemferol activated SIRT3 in Leukemia cell line K562 and promyelocitic human leukemia cells U937 [113]. Daidzein, formononetin, DCHC, genistein, and biochainA induced the expression of SIRT1 in renal proximal tubular cells [95]. CP 205, a flavonoid fraction from the gingko biloba EGb761 extract, activated SIRT1 by promoting the deacetylation of lysine 310 of subunit p65 in neuroblasma cell line [624]. Fisetin, commonly found in strawberries and other fruits, and resveratrol activated SIRT1 in yeast, and extended the yeast's life span [104]. It is believed that resveratrol activates SIRT1 by mimicking physiological pathways that stimulate SIRT1 [625]. Icariin induced SIRT1 activity in neurons [626], whereas procyanidins stimulated SIR-2 activity in Caenorhabditiselegans worms [121]. Curcumin and catechins activate SIRT1 activity and expression directly or indirectly [96]. Phloridzin, an apple polyphenol increased SOD and SIRT1 activities in saccharomyces cerevisiae [118]. Beta-naphtoflavone increased the staining of histone deacetylase HDAC1 in rats [94], whereas resveratrol, stimulated AMPK-SIRT1 autophagy pathway in neurons [627].

The interest in HDACIs came from studies linking HDACs to a wide range of human cancers [628]. However, many polyphenols have also been reported to inhibit HDCAs, most of them contributing to cancer treatment. Quercetin has been reported to inhibit HDAC-1 in cancer cells [124]. 3, 2, 3, 4′-Tetrahydroxychalcone inhibited the deacetylase activity of SIRT1 in HEK293T cancer cells [131]. EGCG decreased the histone deacetylase activity in skin cancer cells [494]. Green tee polyphenols suppressed HDAC1 enzyme activity and its protein expression in HepG2 cells [116]. Luteolin inhibited histone deacetylase activity in human

epitheloid cancer cells [114]. Genistein reduced the endogenous SIRT1 activity in prostate cancer cells [629]. Curcumin has been reported to function as a HDAC and HAT inhibitor [630]. HDACIs have shown beneficial effects in neurodegeneration, cancer, anti-inflammatory disorders. However, most reported HDACs inhibitors appear to be non-selective.

Modulation of epigenetic effects by polyphenols has been reported to play a beneficial role in the treatment of many diseases. Neuroprotective effect of polyphenols has been more and more attributed to their anti-oxidant and anti-inflammatory properties. The acetylation balance is greatly impaired during neurodegenerative conditions [631]. It has also been reported that the epigenetic machinery is essential for cognitive function [632]. Moreover, the dysfunction of gene expression in the brain is involved in neurodegenerative diseases [633]. Epigenetic modifying effect of polyphenols has been more studied in cancer mechanisms and less in neurodegenerative conditions. HDACIs have been shown to be associated with neurodegenerative diseases [634]. It has been proven that HDACIs affect the activities of key proteins involved in AD including Aβ, GSK-3β, and therefore improve memory and learning [635]. Many other polyphenols display their neuroprotective effects through activation of several HDACs such as SIRT1. Indeed, SIRT1 has been shown to increase neuronal viability [626]. Icariin revealed protective effect against brain ischemic injury by increasing SIRT1 and PGC-1 alpha expression [112]. It has been shown that AMPK-SIRT1-autophagy pathway plays an important role in resveratrol induced neuroprotection in PD cellular models [40]. Aβ peptide toxicity is also associated with the activation of many signaling pathways such as NF-κB and MAPK. EGb71 protected neurons against Aβ-induced neurotoxicity through activation of SIRT1, reduction of NF-κB and MAPK activities [624]. NF-κB, ERK1⁄2, and JNK signaling pathways are known to be induced by Aβ [636]. EGb71 can prevent Aβ induced NF-κB signaling pathway activation through activating SIRT1 [624]. This SIRT1 activation can explain the reduction of NF-κB activity by promoting the deacetylation of lysine 310 subunit p35 [624]. It should be noticed that activation of HDACs by polyphenols may be a double sword edge. Trichostatin A, an HDACI has been shown to decrease human SH-SY5Y, mouse MN9D and rat N27 dopaminergic neuronal cell survival, and increase their apoptosis [634]. Therefore, HDACIs could influence PD pathogenesis by inhibiting neuronal survival and increasing their vulnerability to neurotoxins. Hence a lot of prudence has to be taken when using HDACIs for therapeutic purposes.

26.1. Effect of resveratrol and curcumin on H3K9Ac pattern in LPS-induced N9 microglial cells

Microglia cells are immune system representatives of brain and consist 10% of the total glial cell population in CNS. In a young brain, microglial cells function as neuroprotective cells. However, they might be primed to react to the stimuli in the aged brain abnormally, leading to neurotoxicity and neurodegeneration [425, 426]. Aging associated immune senescence is a known phenomenon which renders microglia to function abnormally and may eventually promote neurodegeneration [427]. Immune system senescence is associated with both morphological changes and alterations in immunophenotypic expression, alteration of inflammatory profile and activation of inflammatory pathways that should be silent during healthy state [428-430]. The present hypothesis of microglia senescence during brain aging and its pathological role in aging-related neurodegeneration has led to a novel perspective on potential therapeutics for neurodegenerative diseases [430-432]. The epigenetic involvement in this process has been suggested as persistent inflammation inducing epigenetic changes that lead to neurodegenerative state [433]. In other words, the repressive effects of microglia and astrocyte over-activation is suggested to recapitulate permissive epigenetic conditions that induce neurodegeneration [433]. Polyphenols were earlier in chapter I and in our review of neuroprotective properties of natural polyphenols discussed as effective modulators of neuroinflammation, epigenetic modifiers and potential therapeutics for neurodegenerative diseases [432]. In this scenery, using polyphenols that suppress neuroinflammation through epigenetic pathways would be a potential therapeutic option. The best targets for such therapeutics remain to be investigated.

Curcumin is a natural polyphenol which is known to have DNMT, HDAC and HAT inhibitory activities as mentioned in introduction [97-103]. Curcumin has shown neuroprotective activities in various studies (Table 5) [149, 155-157]. This polyphenol applies neuroprotective effect through interactions with several pathways such as NMDA pathway, PI-3K/MAPK signaling pathway, anti-inflammatory pathways and amyloid pathway (Figure 2) [233, 322, 339, 350]. Curcumin was reported to change the acetylation status of histones [103, 637]. Besides, a dose dependent inhibition of HDAC 1 and 3 following curcumin treatment of Raji cells [100], and a time and dose dependent reduction in histone acetylation of HepB3 cells was shown in previous studies [638]. In our study of curcumin effect on H3K9Ac pattern of N9 cells, it did not alter the H3K9Ac status of N9 cells. Curcumin has been reported to both

increase and decrease histone acetylation [639]. Curcumin is also able to alter the acetylation status of non-histone proteins such as GATA 4 (myocardial transcription factor) [640]. In previous studies, the acetylation alteration was reported in histone H4, but we studied acetylation pattern of H3 in response to curcumin treatment. Curcumin also inhibits HDAC1 and 3, in addition to activating HAT p300. We conclude that there might be a diverse range of mechanisms involved in neuroprotective action of curcumin. Which mechanisms to be induced by curcumin, depends largely on the methodology, context of the experiment, the cell type or the model being used, etc.

Sirtuins are highly conserved NAD+-dependent enzymes whose beneficial effects against age-related diseases have been shown frequently. They modulate main biological pathways, such as stress response, protein aggregation, and inflammatory pathways which are largely involved in neurodegenerative diseases. Resveratrol is a plant polyphenol compound, and a potent activators of SIRT1 [641]. The neuroprotective effects and mechanisms of resveratrol have been demonstrated in different models of neurodegeneration as mentioned before (Table 4-8). SIRT1 preferentially deacetylates H3K9, H3K14 and H4K16 in vitro and in vivo [642, 643]. However, it could not change the H3K9Ac status of N9 microglial cells, although it reduced LPS-induced NO production and increased cell survival according to our study. Similar to curcumin, resveratrol uses a diverse range of pathways to imply its effects, which very much depends on the conditions in which the experiment is designed and performed.

26.2. Effect of icariin on brain H3K9Ac patterns in APPPS1-21 mice model of AD

Icariin is a flavonol substance for which various activities and pharmacological applications have been mentioned according to literature. It is able to affect a wide range of signaling pathways (Table 14). Regarding the neurogenic properties, extract of Epimedium sagittatum, containing high levels of icariin and its derivatives, was shown to induce neurite outgrowth of PC12h cells [644]. In previous studies, icariin has been used in various models of neurodegeneration. Although the number of these studies is limited, icariin has shown a beneficial effect on learning, memory, neural function and survival in all these studies [108]. In a study on Aβ (25-35)-induced AD rats, icariin improved the ability of spatial learning and memory, and suppressed the beta-secretase expression [645]. In another study, icariin treatment of an in vitro AD model and AD transgenic mouse model (5xFAD) improved spatial

memory and restored axonal degeneration in rat cortical neurons [441]. Icariin also improved learning and memory abilities in senescence-accelerated mice SAMP10 [440]. Icariin has shown neuroprotective properties through epigenetic mechanisms. SIRT1 has also shown to be a target for icariin in various experiments [112, 364, 439]. According to these studies, icariin induces SIRT1 expression and activity. SIRT1 is a class III HDAC whose up-regulation has been shown to be associated with increasing longevity and neuronal survival [646].

Table 14: Signaling pathways affected by icariin treatment according to literature review

NO-cGMP signaling pathway	Up-regulation	[647-651]
mTOR signaling pathway	Down-regulation	[647]
JNK/p38/MAPK signaling pathway	Down-regulation	[55, 627, 652, 653]
	Up-regulation	[436, 439, 654-658]
Notch signaling pathway	Modification	[659]
Insulin/IGF-1 signaling pathway	Modification	[660]
PI3K-Akt signaling pathway	Up-regulation	[436, 661-663]
NF-κB signaling pathway	Down- regulation	[55]
Toll-like receptor signaling pathway	Down- regulation	[664]
TGF-beta signaling pathway	Up-regulation	[665, 666]
	Down- regulation	[667, 668]

SIRT1 catalyzes the removal of acetyl groups from a number of histone and non-histone proteins (Figure 34) [578, 642, 643, 669-680]. All histone proteins affected by SIRT1 are involved in maintenance of active chromatin state and gene transcription [29, 681-683]. SIRT1 deacetylates H3 at Lys9 and Lys14, and it also deacetylates H4 at Lys16, leading to genomic silencing [642]. Genomic silencing could further reduce protein synthesis and energy consumption, a common strategy for cells and organisms to survive through unfavorable conditions, such as calorie restriction, hypothermia, or hibernation. This mechanism also implies to SIRT1-mediated neuroprotection [684]. In addition, acetylation of H3K14 has been linked to depression, schizophrenia and bipolar disorders through controlling the expression of BDNF gene [685-688]. Reduced acetylation of H3K9 was associated with memory impairment induced by brain iron overload [609] and hypoacetylation of H3K9, H3K14 and H4K16 was reported in association with Friedreich's ataxia [689]. H3K56 acetylation has been implicated in the regulation of gene activity and

chromatin structure, in various brain regions including those involved in neuroendocrine regulation [690]. SIRT1 also deacetylates a number of non-histone proteins (Figure 34). These proteins are linked to various important cell signaling pathways whose aberrations are shown to be involved in neurodegeneration too [578, 673, 675, 691-694].

SIRT1 overexpression prevents oxidative stress-induced apoptosis and increases resistance to oxidative stress through regulation of the FOXO family of fork head transcription factors [646]. SIRT1 has been shown to affect amyloid production through the ADAM10 gene modification [578]. Up-regulation of SIRT1 can also induce the Notch pathway and inhibit mTOR signaling [227, 646]. SIRT1 is highly expressed in the brain with high levels in the cortex, hippocampus, cerebellum, hypothalamus, spinal cord and dorsal root ganglion [695].

SIRT1 is predominantly in neurons and also in microglia when co-cultured with neurons [246, 696-698]. In the case of icariin, SIRT1 is necessary for the neuroprotective effect of the substance, since the knockdown of SIRT1 diminishes the flavonol's protective effect [112]. In our study, we did not find any changes in H3K9Ac profile of APPPS1-21 mice. Considering, our previous results on reduced H3K9Ac status of APPPS1 mice compared to wild type mice, we would have expected that icariin treatment further reduces H3K9Ac in brain of mice. But it did not change the H3K9Ac profile showing that icariin probably induces neuroprotective effects through pathways rather than targeting H3K9Ac by SIRT1. In most of previous studies on icariin neuroprotective effects, the genes whose expression profile in response to icariin therapy has been studied are not almost exclusively specific for SIRT1 induced pathways. In order to reach a higher specificity in definition of icariin neuroprotection mechanisms it might be helpful to study more specific genes and protein targets within epigenetic pathways. Our study on H3K9Ac profile of APPPS1-21 mice brain in response to icariin treatment was an effort in this respect.

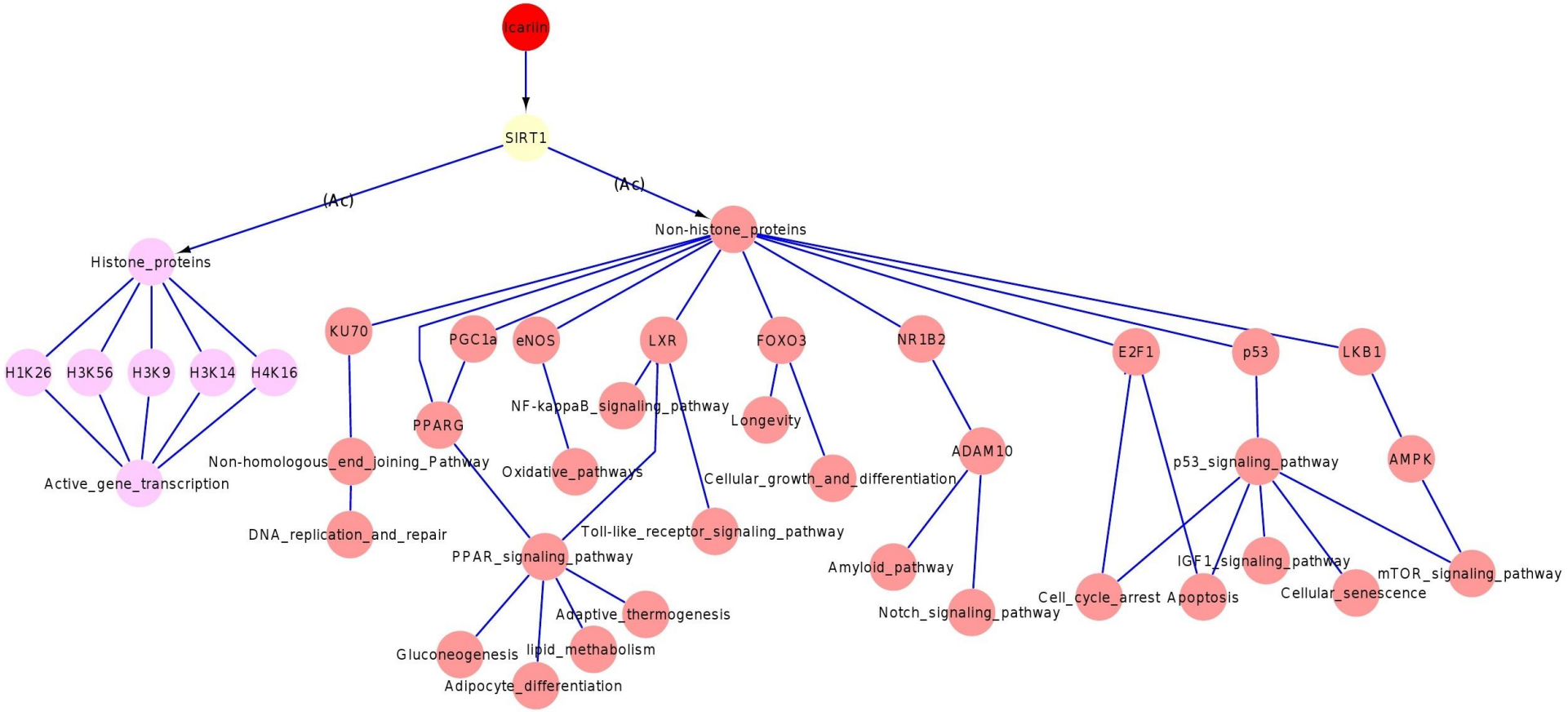

Figure 34: Network of icariin targeted proteins and signaling pathways via SIRT1 activation

Icariin induces SIRT1 expression and activity according to several studies. SIRT1 is a class III HDAC that catalyzes the removal of acetyl groups from a number of histone and non-histone proteins. Histone proteins affected by SIRT1 are involved in maintenance of active chromatin state and gene transcription in addition to their link to some brain psychosomatic pathological conditions. Besides, non-histone proteins affected by icariin are involved in neural survival and correspondently in neurodegenerative conditions directly or indirectly through mediating several cell signaling pathways. The network was produced using Cytoscape v.2.8.3. Cytoscape is a free software package for visualizing, modeling and analyzing molecular and genetic interaction networks [699].

Chapter V: Conclusion

Neurological diseases are being increasingly characterized with contribution to variable forms of epigenetic disruption. The rapidly evolving field of neuroepigenetics has provided a platform for development of epigenetic-based therapeutics against neuropathological conditions. However, in many of these pathological conditions, the epigenetic machinery seemingly operates in a highly complex and multifunctional manner at a large number of genomic loci. For the numerous emerging chromatin-modifying drugs that show promising effects in preclinical studies, it will be important to identify their key mechanisms of action and their main targets.

Among various epigenetic modalities, we selected histone acetylation profile and specifically acetylation of H3K9 as a highly relevant candidate whose alteration has been reported in many neuropathological conditions and a target, based on which, numerous therapeutics have been developed. In the present study, we tried to pinpoint the role of global H3K9 acetylation, as a target of many HDACs and HATs, in brain pathological conditions, from benign and malignant brain tumors to neurodegenerative diseases to find out whether it acts as a relevant target for development of epigenetic-based therapeutics. We also tried to drive appropriate natural products with known neuroprotective properties to find out whether they influence or alter acetylation status of H3K9.

Based on our findings, global H3K9Ac status alters in a variety of pathological condition including malignant brain tumors such as gliomas, benign brain tumors such as ependymal tumors as well as neuroendocrine tumors such as pituitary tumors. In cancers, global H3K9Ac status has an association to the degree of malignancy and the disease aggressiveness. The more malignant, aggressive or atypical a pathological condition, the higher amount of alteration we see in global H3K9Ac profile of the disease. In non-cancerous conditions such as AD, we also noticed an aberration of H3K9 acetylation profile in brains of APPPS1-21 mice, carrying both APP and PS mutations with high association to brain amyloid pathology. In our study on H3K9Ac profile of LPS-induces N9 cells, we also noticed that prototypical HDAC inhibitors either selective or non-selective, such as resveratrol or valproic acid, do not necessarily change global H3K9Ac profile, suspecting us to two possible reasons: (1) The insufficient sensitivity of immunohistochemistry to detect very subtle changes of global H3K9Ac status or (2) alternative mechanisms of action for HDACIs other than changing acetylation profile of histones.

An emerging question in the field of epigenetic-based neurotherapeutics is whether the beneficial effects of HDACIs in various acute and chronic neurodegenerative disorders and cancerous conditions are due to their broad effects on histone modifications, modifications of non-histone proteins acetylation or a combination of both. According to our in vitro experiment, treatment of LPS-induced N9 microglial cells with resveratrol, an HDACI, reduced NO production without affecting the global H3K9Ac profile of the cells, while in our in vivo experiment, treatment of APPPS1-21 mice and EAE rats with MS-275 and VPA, increased H3K9Ac status of brain. Moreover, treatment of APPPS1 mice with icariin, a SIRT1 activator flavonol, did not change H3K9Ac profile of mice brain significantly. From our results, we conclude that a combination of both mechanisms might be involved in neuroprotective actions of both polyphenol and non-polyphenol HDAC modifiers. The field of neuroepigenomeics is growing in a rapid pace and neuroprotective effects of epigenetic-based therapeutics are highly promising in preclinical studies. However, there is still a challenging way to the proof of clinical efficacy of these drugs against neurological diseases.

Chapter VI: References

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