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Distinct Patterns of Sirtuin Expression During Progression of Alzheimer's Disease

Mirjam I. Lutz · Ivan Milenkovic · Günther Regelsberger · Gabor G. Kovacs

Abstract Aging is one of the major risk factors for Alzheimer's disease (AD). Sirtuins are associated with prolonged life span. To examine whether the expression levels of sirtuins associate with the progression of AD or not, we performed a comparative immunoblotting and immunohistochemical study of SIRT1, 3, and 5 in the entorhinal cortex and hippocampal subregions and white matter in 45 cases grouped according to Braak and Braak stages of neurofibrillary degeneration. In addition, we compared the expression levels with the local load of tau and amyloid-beta deposits, evaluated using morphometry. Our study revealed that (1) the neuronal subcellular redistribution of SIRT1 parallels the decrease in its expression, suggesting stepwise loss of neuroprotection dependent on the neuronal population; (2) in contrast to SIRT1 and 3, expression of SIRT5 increases during the progression of AD; (3) which might be related to its appearance in activated microglial cells. The complex patterns of the expression of sirtuins in relation to tissue damage should be taken into account when searching for therapies interacting with sirtuins.

Keywords Aging · Alzheimer's disease · Hippocampus · Sirtuin · SIRT1 · SIRT3 · SIRT5

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Introduction

Aging is one of the major risk factors for Alzheimer's disease (AD) and therefore an increasing challenge for the society (Goedert and Spillantini 2006). AD is a neurodegenerative disease characterized clinically by slowly progressive cognitive decline and neuropathologically by intracellular deposition of protein tau in the form of neurofibrillary tangles and extracellular deposition of amyloid-β (Aβ) (Duyckaerts et al. 2009). These alterations are termed as AD-related neuropathologic changes (Montine et al. 2012) that overlap with normal aging. Neurofibrillary degeneration is classified into six stages according to BB stages (Braak and Braak 1991), while Aβ deposition follows five phases according to Thal et al. (2002). Although AD is known since more than a century (Alzheimer et al. 1995), there is still a lack of curative treatment.

Due to the overlap of AD- and age-related neuropathologic changes, recent studies focused on the identification of genetic factors for healthy aging with the hope of finding a rationale for anti-AD therapy. Indeed, a recent high-density genome-wide linkage analysis of exceptional human longevity identified a locus on chromosomes 3, 9, 12, and 4 (Boyden and Kunkel 2010). The most suggestive LOD scores were found for 3p24-22 and 9pq31-34. From the candidate genes, *DBC1* (chromosome 9) warrants mention. This directly interacts with *SIRT1*, which is a member of the sirtuin family. Sirtuins are NAD$^+$-dependent histone deacetylases (group III HDACs) with additional non-histone substrates (Herskovits and Guarente 2013). The sirtuin family members play a key role in the life span of *S. cerevisiae*, *C. elegans*, and *D. melanogaster* by mediating the effects of caloric restriction, which is until now the only effective life-prolonging behavior (Polito et al. 2010; Hall et al. 2013; Herskovits and Guarente 2013). Sirtuins have been recently suggested to regulate the aggregation and

removal of misfolded proteins, stress responses, cell survival, mitochondrial functions and act as energy sensors and anti-inflammatory mediators (Gan and Mucke 2008; Polito et al. 2010; Houtkooper et al. 2012; Hall et al. 2013). In spite of the extensive studies on experimental models, there is a relative paucity of data on sirtuins in human neurodegenerative diseases, in particular in aging and AD. A single study on human AD (Julien et al. 2009) reported that the loss of SIRT1 expression evaluated by immunoblotting and in situ hybridization inversely correlates with the accumulation of tau evaluated by immunoblotting. Another study, using qRT-PCR, in situ hybridization, and immunohistochemistry, focused on amyotrophic lateral sclerosis (ALS), a disorder affecting predominantly motor neurons (Körner et al. 2013). They reported various patterns of SIRT1, 2, and 5 expression (both protein and mRNA), depending on which method was used or which anatomical region (motor cortex or spinal cord) was analyzed.

Due to the paucity of data on the cellular/subcellular distribution of sirtuins in the human brain, in particular in anatomical regions vulnerable to AD and brain aging, we conducted a detailed comparative study of major members of the sirtuin family (SIRT1, 3, and 5). By the evaluation of subregions of the hippocampus and entorhinal cortex, and white matter, we show subcellular and subregional differences between these sirtuins. In addition, we found opposing effects during the progression of AD-related neuropathologic change, suggesting a complex scenario of the role of sirtuins in relation to tissue damage.

Materials and Methods

Human Brain Tissue

Human brain samples were collected at the Institute of Neurology, Vienna. This study was approved by the ethics committee of the Medical University of Vienna (number 1295/2012). Comparative immunohistochemical and immunoblotting investigations were performed in a cohort of 16 cases (age range 39–91 years, average 77.5 years; 11 women; 5 men), including 4 cases, BB stage 0–II, 7 cases BB stage III–IV, 5 cases BB stage V–VI. We evaluated SIRT1 immunostaining in a group of further 29 cases (age range 78–88 years, average 83.6 years; 17 women; 12 men) including 11 cases, BB stage 0–II, 10 cases BB stage III–IV, 8 cases BB stage V–VI. For the immunoblotting study of subcellular fractions, we used one case with BB stage II and one with BB stage VI.

Immunohistochemistry

In addition to formalin-fixed, paraffin-embedded 4-μm-thick sections, we used 8-μm-thick frozen tissue sections post-fixed 10 min in 4.5 % formalin. This post-fixation was chosen after a pilot-fashion testing of various methods, including no fixation, 10 min in 4 % PFA, 10 min in 4.5 % formalin, and 10 min in acetone. Sections were stained with the following polyclonal antibodies: anti-SIRT1 (1:500, EMD Millipore Corporation, Billerica, MA, USA), anti-SIRT3 (clone C73E3, 2627, 1:100, Cell Signaling Technology, Inc. Danvers, MA, USA), anti-SIRT5 (1:2,000, EMD Millipore Corporation, Billerica, MA, USA), and the following monoclonal antibodies: anti-tau (clone AT8, pS202, 1:200, Pierce Biotechnology, Rockford, IL, USA) and anti-amyloid-β (clone 6F/3D, M0872, 1:50, Dako, Glostrup, DK). After a pilot-fashion testing of antigen retrieval methods for paraffin sections, including 10-min citrate buffer (pH 6), 10-min Tris/EDTA buffer (pH 9), proteinase K, and citrate buffer (pH 6) followed by 1 min 80 % formic acid, we used the 10-min citrate buffer (pH 6) pretreatment for SIRT1, SIRT3, and SIRT5; 1 h 80 % formic acid for Aβ while no pretreatment was applied for antibody AT8. For frozen section, we did not use any antigen retrieval. For nuclear staining, we used Mayer's hemalum solution (Merck KGaA, Darmstadt, Germany).

Specificity of the Sirtuin Antibodies

To test whether sirtuin antibodies recognize the protein specifically, antibody-competition experiments were performed as described elsewhere (Vasiljevic et al. 2012). Briefly, sirtuin antibodies (SIRT1 and SIRT5) were incubated either with appropriate blocking peptide (SIRT1: R&D Systems, Inc., Minneapolis, MN; SIRT5: EMD Millipore Corporation, Billerica, MA, USA) or with pure diluent (Dako, Glostrup, DK; as intrinsic positive control) for 4 h at 4 °C. Subsequently, both mixtures were added to the sections (mixture of antibodies and the blocking peptide was diluted additionally in Dako diluent to reach appropriate antibody concentration). Blocking peptides abolished immunoreaction for both SIRT1 and SIRT5 completely (Suppl.Fig. 1).

Double Immunolabeling and Laser Confocal Microscopy

Monoclonal anti-TPPP/p25 (1:1,000, Höftberger et al. 2010) and monoclonal anti-GFAP (1:25, Dako Cytomation, Glostrup, DK) antibodies were used with Alexa Fluor 488 (1:200, Jackson Immuno Research Laboratories, Inc. PA, USA). Antibodies anti-SIRT1 and anti-SIRT5 were used with Cy3 (1:100, Jackson Immuno Research Laboratories, Inc. PA, USA). Nuclei were stained with Topro 3 (1:8,000, Molecular Probes Europe BV, Leiden, NL). Immunofluorescent signal was elicited with argon 488 nm and helium/

neon 550 nm lasers. Immunofluorescence labeling was evaluated using Leica TCS SP5 II confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) using HCX PL APO 40x/1.2-0.75 oil CS objective.

Morphometric Analysis of tau and Aβ Load

For the densitometric analysis, we used the software ImageJ (nih.gov). For each case, at least 8 serial images in $400\times$ magnification of the most densely stained area in the entorhinal cortex were evaluated. Densitometry plugins were applied to exclude background noise as well as threshold was set to a defined level. Threshold level was defined after comparison of low- and high-density areas. All images were run with a fixed macro (Densitometry 3 channel, 8-bit, set threshold) to exclude subjective variations.

Immunoblotting for Sirtuins

Small punches of either gray matter (GM) or white matter (WM) of the parahippocampal gyrus were taken from fresh frozen tissues. Brain tissues were homogenized at the end 7 volumes of $2\times$ Laemmli buffer. $2\times$ Laemmli buffer was prepared stepwise as described subsequently. Tissue was homogenized in a mixture of 0.5 M Tris–HCl, pH 6.8, $25\times$ protease inhibitor, and ddH$_2$O. Protein concentration was determined by Bradford protein assay. Afterward, 100 % glycerol, 10 % SDS, ddH$_2$O, $10\times$ reducing agent, and bromphenol blue were added to make the $2\times$ Laemmli buffer. Tissue homogenate was heated to 42 °C for 30 min. These tissue homogenates contained different cell types as well as all subcellular compartments. Thus, to confirm our immunohistochemical observations, we performed subcellular fractionation of two selected cases (BB stage II and BB stage VI).

For this subcellular fractionation, 50 mg of gray matter was processed as described elsewhere (Dimauro et al. 2012) with small modifications to meet the requirements of brain tissue. Briefly, tissue was pottered for 1 min at 800 rpm (Potter S., B. Braun Biotech International, Germany) on ice using glass-Teflon douncer in 500 µl STM buffer (250 mM sucrose, 50 mM Tris–HCl, pH 7.4, 5 mM MgCl$_2$ supplemented with protease and phosphatase inhibitor cocktails). The homogenate was kept on ice for 30 min before centrifugation at $800\times g$ for 15 min. The pellet (P0) was resuspended in STM buffer and centrifuged at $500\times g$ for 15 min. The subsequent pellet (P1) was resuspended in STM buffer and centrifuged ($1,000\times g$ for 15 min) twice. The final pellet (P5), containing the nuclear fraction, was resuspended in NET buffer (20 mM HEPES, pH 7.9, 1.5 mM MgCl$_2$, 0.5 M NaCl, 0.2 mM EDTA, 20 % glycerol, 1 % Triton X-100 supplemented with protease and phosphatase inhibitor cocktails) and frozen until

further use. The supernatant after the first centrifugation (S0) was centrifuged at $800\times g$ for 10 min. The subsequent supernatant (S2) was again centrifuged at $11,000\times g$ for 10 min. The supernatant (S3) was precipitated in cold acetone at -20 °C for at least 1 h and then centrifuged at $12,000\times g$ for 5 min. The pellet (P7), containing the cytosolic fraction, was resuspended in STM buffer and frozen until use. Corresponding pellet (P3) was resuspended in STM buffer and centrifuged at $11,000\times g$ for 10 min. The subsequent pellet (P4), containing the mitochondrial fraction, was resuspended in SOL buffer (50 mM Tris–HCl, pH 6.8, 1 mM EDTA, 0.5 % Triton X-100 supplemented with protease and phosphatase inhibitor cocktails) and frozen until further use.

The efficacy of the subcellular fractionation was tested by immunoblotting and labeling with histone H3 antibody (96C10, 1:1,000, Cell Signaling Technology, Inc. Danvers, MA, USA) for the nuclear fraction, TOM22 antibody (clone 1C9-2, 1:10,000, Sigma-Aldrich Co. LLC., St. Louis, MO, USA) for the mitochondrial fraction, and GAPDH antibody (ab8245, 1:10,000, Abcam, Cambridge, UK) for the cytosolic fraction.

Total protein of brain tissues was extracted as described above and separated on 10 % SDS-PAGE. Proteins were transferred onto PVDF membranes at RT. Membranes were blocked in 5 % non-fat dry milk in $1\times$ TBST for 1 h at RT. Afterward, they were probed overnight at 4 °C with primary antibodies against SIRT1 (1:1,000), SIRT3 (1:1,000), and SIRT5 (1:2,000) followed by incubation with the appropriate secondary antibody (goat anti-rabbit 1:5,000, Dako, Glostrup, DK) for 1 h at RT and chemiluminescence reagents. Membranes were exposed to film (Amersham Hyperfilm ECL, GE Healthcare, Ltd., Buckinghamshire, UK) for 30 s to 15 min and developed with Agfa Curix 60 (Agfa HealthCare Corporation, Greenville, SC, US). Membranes were stripped and reused for β-actin (1:10,000, clone AC-15, Sigma-Aldrich Co. LLC., St. Louis, MO, USA). SIRT1 shows its main band at 75 kDa, and additional isoforms are known (Lynch et al. 2010; Oppenheimer et al. 2012; uniprot.com 2013). SIRT3 shows a single band at 28 kDa, which is in accordance with several publications (Schwer et al. 2002; Onyango et al. 2002). SIRT5 shows its main band at 35 kDa; according to the datasheet and earlier reports, additional isoforms are known (Mahlknecht et al. 2006; Matsushita et al. 2011). The relative expression was quantified with densitometry using the software ImageJ (nih.gov). Measurements were processed with Microsoft Excel.

Statistical Analysis

Spearman's correlation test was performed to compare the variables examined using SPSS software (version 20). $p < 0.05$ was considered as significant.
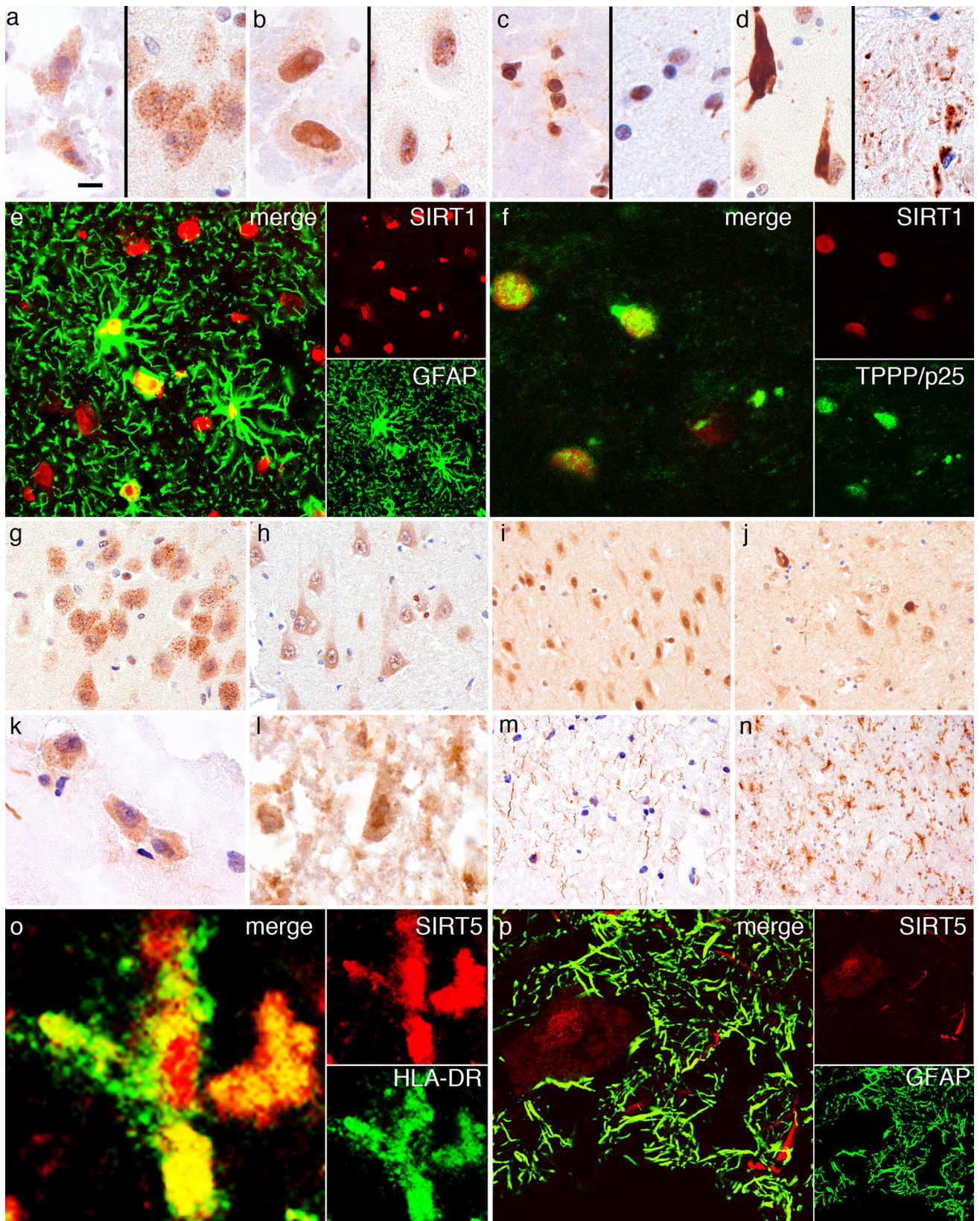
◀ **Fig. 1** Immunostaining for SIRT1, 3, and 5 in human hippocampus and entorhinal cortex samples. Immunostaining for SIRT1 revealed immunoreactivity of the neuronal nuclei (**a**), neuronal cytoplasm (**b**), glial nuclei (**c**; for **a–c** left represents frozen and right paraffin-embedded sections), neurofibrillary tangles (**d**, *left*) and dystrophic neurites (**d**, *right*). Double immunolabeling revealed that both GFAP (astroglial marker; **e**) and TPPP/p25 (oligodendroglial marker; **f**) co-localize with SIRT1. Prominent cytoplasmic staining of neurons in the CA2 is lost during the progression of AD neuropathologic change (**g**: Braak and Braak stage II; and **h**: Braak and Braak stage VI). In contrast, the entorhinal cortex showed only few cells with cytoplasmic staining and more with nuclear staining in early stages (**i**: Braak and Braak stage II), which was decreased in later stages (**j**: Braak and Braak stage VI). Frozen sections showed SIRT5 immunoreactivity of the neuronal cytoplasm (**k**), neuronal nuclei (**l**), neuronal processes (**m**), and glial cells reminiscent of microglia (**n**). Double immunolabeling for HLA-DR and SIRT5 (**o**). SIRT5 immunoreactive processes in the WM did not co-localize with GFAP (**p**)

Results

Subcellular Redistribution and Expression of SIRT1 Inversely Correlates with the Progression of AD Neuropathologic Change

First, we tested the SIRT1 antibody using frozen and paraffin sections from non-diseased controls and cases with AD neuropathologic change. In both types of sections, we observed immunoreactivity of the neuronal nuclei (Fig. 1a), neuronal cytoplasm (Fig. 1b), and glial nuclei (Fig. 1c). In addition, we observed immunoreactivity of neurofibrillary tangles (Fig. 1d) and a few dystrophic neurites and neuropil threads in the brains showing AD neuropathologic change. Double immunolabeling for GFAP (astroglial marker) and TPPP/p25 (oligodendroglial marker) revealed that both astro- and oligodendroglial nuclei show SIRT1 immunoreactivity (Fig. 1e, f).

Next, we compared the subcellular distribution and load of immunoreactivity, evaluated semiquantitatively, in two subregions: entorhinal cortex, representing a highly vulnerable region in AD, and CA2 subregion, representing a less vulnerable region in AD. Our semiquantitative evaluation revealed that the prominent cytoplasmic staining of neurons in the CA2 is lost during the progression of AD neuropathologic change (Spearman's correlation, $R = -0.44$, $p = 0.002$; Fig. 1g, h), while the nuclear staining in this subregion did not show significant variability ($p = 0.13$). In contrast, the entorhinal cortex showed only few cells with cytoplasmic staining, mainly in the early stages of AD neuropathologic change ($p = 0.26$). Furthermore, nuclear staining was prominent in the entorhinal cortex already in early stages and showed inverse correlation with the progression of AD neuropathologic change ($R = -0.35$, $p = 0.018$; Fig. 1i, j). Glial immunoreactivity was seen in all stages and did not show significant variability.

Our immunohistochemical observations were strongly supported by the immunoblotting for SIRT1. First, we showed that corresponding to our immunohistochemical observations, SIRT1 reacts in the nuclear, mitochondrial, and cytosolic fraction of both early- and late-stage AD tissue (Fig. 2a). Then, the densitometric analysis of total homogenate (Fig. 2b) showed strong negative correlation with the groups of BB stages (i.e., 0–II, III–IV, and V–VI): pooled WM and GM samples, $R = -0.47$, $p = 0.006$. This remained significant for the GM ($R = -0.58$, $p = 0.016$), and a tendency was seen for the WM ($R = -0.33$, $p = 0.22$). Similar tendencies were noted for the tau load in the GM ($R = -0.33$, $p = 0.24$) and WM ($R = -0.41$, $p = 0.16$) samples. There was a lack of correlation between Aβ and SIRT1 for the GM ($R = -0.29$, $p = 0.28$); WM did not show Aβ deposits.

Expression of SIRT3 Inversely Correlates with the Progression of AD Neuropathologic Change

We tested SIRT3 immunohistochemically on formalin-fixed, paraffin-embedded brain tissue as well as on frozen sections. We did not detect unequivocal cell-specific staining in any of the sections (data not shown), so we based our investigations on immunoblotting. Using immunoblotting on subcellular fractions (Fig. 2a), we show that SIRT3 reacts with the mitochondrial and cytosolic fractions. The expression of SIRT3 in total homogenates (Fig. 2b) showed strong negative correlation with groups of BB stages (pooled WM and GM samples, $R = -0.48$, $p = 0.006$), which remained significant for the GM-only samples ($R = -0.52$, $p = 0.03$), while for WM samples a similar tendency was noted ($R = -0.42$, $p = 0.13$). For the total tau load, similar tendencies were observed (GM samples: $R = -0.42$, $p = 0.13$; WM samples: $R = -0.83$, $p < 0.001$). There was a lack of correlation between Aβ and SIRT3 for the GM ($p > 0.1$); WM did not show Aβ deposits.

Expression of SIRT5 Correlates with the Progression of AD Neuropathologic Change

First, we tested the SIRT5 antibody using frozen and paraffin sections from non-diseased controls and cases with AD neuropathologic change. Only in the frozen sections, we observed immunoreactivity of the neuronal cytoplasm (Fig. 1k), which is less in the neuronal nuclei (Fig. 1l), neuronal processes (Fig. 1m), and glial cells reminiscent of microglia (Fig. 1n). Indeed, double immunolabeling for HLA-DR (microglial marker) revealed that microglial cells show SIRT5 immunoreactivity (Fig. 1o). The SIRT5 immunoreactive processes did not co-localize with GFAP (Fig. 1p).
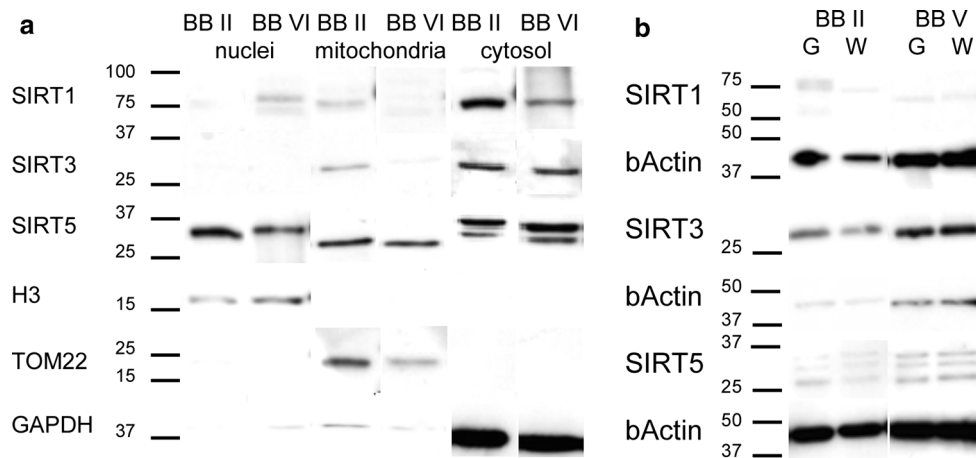
Fig. 2 Expression of SIRT1, 3, and 5 in human hippocampus samples. Immunoblots of subcellular fractions of an early-stage (BB II) and a late-stage (BB VI) AD patient sample. SIRT1 and 5 react with all three fractions, while SIRT3 is only present in mitochondrial and cytosolic fractions. Efficacy of the fractionation was tested by immunolabeling with histone H3 (nuclear marker), TOM22 (mitochondrial marker), and GAPDH (cytosolic marker) (**a**). Representative immunoblots of human *gray matter* (G) or *white matter* (W) homogenates for SIRT1, 3, and 5. β-Actin represents the loading control (**b**)

Immunoblotting of the subcellular fractions showed that SIRT5 reacts with nuclear, mitochondrial, and cytosolic fractions (2a). Immunolabeling of the total homogenates revealed a positive correlation with the groups of BB stages (pooled WM and GM samples, $R = 0.44$, $p = 0.011$; only GM $R = 0.68$, $p = 0.004$; only WM $R = 0.42$, $p = 0.11$). The total tau load did not correlate with the SIRT5 expression (for GM or WM: $p > 0.1$). There was a lack of correlation between Aβ and SIRT5 for the GM ($p > 0.1$); WM did not show Aβ deposits. The opposite correlation of SIRT1 and SIRT5 expression with BB stages was supported by a negative correlation of SIRT1 and SIRT5 expression (pooled samples: $R = -0.24$, $p = 0.17$; GM: $R = -0.59$, $p = 0.015$; WM: $R = -0.23$, $p = 0.4$).

Discussion

The major observations of our study can be summarized as follows: (1) Expression of SIRT1 and SIRT3 inversely correlates with the progression of AD accompanied by subcellular redistribution of SIRT1 immunoreactivity; and (2) SIRT5 expression shows an opposite effect. The strength of our study is that we were able to compare highly vulnerable subregions with less affected areas in the human brain using comparative immunohistochemical, morphometric, and immunoblotting examinations.

Our results indicate that during the progression of AD neuropathologic change, represented by BB stages, a loss of SIRT1 expression is paralleled by a subcellular redistribution. With the local load of tau immunoreactivity, we found a strong tendency but no significant inverse correlation. We interpret this finding that later BB stages (V–VI)

of AD neuropathologic change reflect an advanced state of a neurodegenerative process, while the local tau load in the entorhinal cortex does not increase significantly further after the limbic stages (III–IV). A previous study on brain tissue derived from individuals with AD, defined as "definite" or "probable" according to CERAD (Mirra et al. 1991), found decreased protein and mRNA expression of SIRT1 in the parietal cortex but not in the hippocampus or cerebellum samples (Julien et al. 2009). Similar to our observations, they found inverse correlation with insoluble hyperphosphorylated paired helical filament tau and total insoluble tau, and a weaker correlation with Aβ (Julien et al. 2009). In summary, apart from slight differences in the level of significance, most likely due to distinct sampling, both studies showed a decrease in SIRT1 associated with AD. Since homogenates include different cell types and compartments, we expanded our observations using immunohistochemistry. Indeed, we observed differences in subcellular distribution of SIRT1, which was confirmed by the subcellular fractionation study. This difference should be discussed in two contexts. First, the higher cytoplasmic expression of SIRT1 in the relatively unaffected CA2 subregion as compared to the early vulnerable entorhinal cortex suggests that cytoplasmic localization of SIRT1 might be cytoprotective. This supports the concept that some neuronal groups (i.e., entorhinal cortex) act as "weakest links" in the brain that are prone to degeneration due to various pathogenetic impulses (Adori et al. 2005), while others that are capable to express cytoprotective proteins are less vulnerable (i.e., CA2). In experimental models, SIRT1 is predominantly localized in nuclei (Luo et al. 2001; Vaziri et al. 2001; Sakamoto et al. 2004; Michishita et al. 2005) but has nuclear import and export

sequences (Tanno et al. 2007). There are controversial observations regarding the physiological role of cytoplasmic SIRT1 expression; interestingly, this also depends on which cell type is investigated. One study showed that cytoplasmic SIRT1 enhances apoptosis in different cultured cells, such as HeLa, HEK, and C2C12 (Jin et al. 2007), while another one suggested that it promotes neurite outgrowth in PC12 cells (Sugino et al. 2010). In cultured NPCs, the translocation of SIRT1 from cytoplasm to the nucleus occurred after transferring into differentiation medium and led to 84.7 % differentiated cells (Hisahara et al. 2008). However, the opposite was also reported, where the nucleocytoplasmic shuttling occurs in response to differentiation stimulus (Tanno et al. 2007). In summary, there is a general loss of SIRT1 expression during the progression of AD associated with distinct subcellular predominance of SIRT1 immunoreactivity. Since our observation shows that the latter may be different in distinct subregions, the pathogenic role should be evaluated in the context of the subregion. This might help to understand the various results obtained in cell culture models. The SIRT1 immunoreactivity seen in neurofibrillary tangles might represent a subcellular redistribution associated with pathological protein aggregates; thus, most likely has a less efficient cytoprotective role.

The decreased expression of SIRT1 suggests that a neuroprotective role of SIRT1 is lost during progression of AD. SIRT1 is the most studied member of the sirtuin family, as reviewed recently (Herskovits and Guarente 2013); it is involved in several crucial pathways linked to the pathogenesis of AD. This includes deacetylation of tau marking it for proteosomal degradation (Min et al. 2010); promotion of the non-amyloidogenic APP processing pathway (Qin et al. 2006); protection against Aβ toxicity through inhibiting NFkB signaling (Chen et al. 2005); and suppression of Aβ production by activating the α-secretase gene ADAM10 (Donmez et al. 2010). Kim et al. (2007) suggested that SIRT1 levels correlate with neuronal loss but not with Aβ plaque burden, which was supported by our observation also, since BB stages reflect rather a progression in the neurodegeneration process. Our study, together with the one by Julien et al. 2009), provides morphological evidence in the human brain that loss of SIRT1 can mirror the progression of AD, supported by a recent in vivo finding of a decline in SIRT1 concentration in the serum in patients with AD (Kumar et al. 2013). This renders SIRT1 also as a potential biomarker (Kumar et al. 2013). In addition, SIRT1 deacetylates LXR, which regulates whole-body cholesterol levels and lipid homeostasis (Li et al. 2007), negatively regulates PPARγ and UCP2 (Ramadori et al. 2008), which influence adipogenesis and insulin secretion, and together with SIRT3, it interacts, via PGC-1α activity, with the mitochondrial biogenesis (Brenmoehl and Hoeflich 2013).

Our study is the first to report that in the human brain, the decrease in SIRT1 is paralleled by SIRT3 during the progression of AD. We were unable to map the cellular distribution of SIRT3 immunohistochemically in the human brain; however, SIRT3 is thought to be a mitochondrial member of the sirtuin family (Michishita et al. 2005; Imai and Guarente 2010; Herskovits and Guarente 2013). Indeed, we did not observe reactivity in the nuclear fraction in our immunoblotting study. Therefore, SIRT3 is suggested to have an important role in energy production or maintenance, cell signaling, and apoptosis (Rice et al. 2012; Han and Someya 2013). SIRT3 deacetylates substrates, including LCAD, GDH, IDH2, complexes I/II/III of electron transport chain, SOD2, and cyclophilin D (Houtkooper et al. 2012; Rice et al. 2012). In addition, a genetic variability at position 477 of SIRT3 seems to play a role in longevity of men (Rose et al. 2003). Interestingly, at the same locus on chromosome 11p15.5, polymorphisms associated with longevity have already been reported for tyrosine hydroxylase, proinsulin, and insulin-like growth factor 2 (De Luca et al. 2001). Furthermore, SIRT3 is suggested to retard the progression of age-related hearing loss in mice (Han and Someya 2013).

Interestingly, our study revealed opposing effects of SIRT5, since it increased during the progression of AD. In ALS, a progressive neurodegenerative disease affecting different neuronal populations as in AD, a similar increase was noted in an immunohistochemical level in ALS when compared to healthy controls (Körner et al. 2013). Another study (Michishita et al. 2005) demonstrated that, contrasting SIRT1, 3, 4, 6, and 7, an increase in SIRT5 mRNA expression can be seen in adult compared to fetal brain. SIRT5 is a mitochondrial member of the sirtuin family (Michishita et al. 2005; Glorioso et al. 2011). It not only acts as deacetylase but also removes malonyl and succinyl moieties from its substrates (Du et al. 2011; Park 2013), which makes it outstanding within the sirtuin family. However, the effects of demalonylation and desuccinylation are not very well elicited to date as they are new discoveries (Zhang et al. 2011; Peng et al. 2011). Thus, only two substrates are known; cytochrome c, which plays a key role in energy metabolism and apoptosis of cells (Geng et al. 2011), and CPS1, which is involved in the nitrogen disposal in the urea cycle and therefore necessary to remove ammonium from the cells as well as the synthesis of pyrimidines (Du et al. 2011). We confirm that SIRT5 is reactive with the mitochondrial fraction; however, we observed a nuclear signal as well. Although in the present study we could not clarify the functional meaning of this, an N-terminal nuclear export sequence was recently shown for SIRT5 (Costantini et al. 2013). Moreover, our observation that SIRT5 appears in activated microglia cells provides an explanation for its increase during progression

of disease. In later stages of neurodegenerative diseases, the amount of microglia increases in the brain, indicating an attempt of the immune system to break down protein aggregates, which accumulate during neurodegenerative processes (Mrak and Griffin 2005). Thus, microglia cells act as markers of disease progression (Perry et al. 2010). Our observation is supported by a recent study in rats where "additional cell types besides neurons" were immunoreactive for SIRT5 (Geng et al. 2011).

In summary, our study revealed novel insights into the involvement of sirtuins during progression of AD. In particular, we showed that (1) the subcellular redistribution of SIRT1 parallels the decrease in its expression, suggesting a stepwise loss of neuroprotection dependent on the neuronal population. (2) In contrast to SIRT1 and 3, SIRT5 behaves differently, which might be related to its appearance in activated microglial cells. This provides a rationale for the application of therapies aiming to target the sirtuin system. So far, resveratrol was identified as a small-compound activator, which uses SIRT1 as effector (Howitz et al. 2003). Various studies in model organisms showed a prolongation of life after over-expression of SIRT1, or application of natural or chemical resveratrol (Howitz et al. 2003; Wood et al. 2004; Baur et al. 2006; Cantó and Auwerx 2009; Polito et al. 2010). An additional way to prolong life was shown to be calorie restriction (Koubova and Guarente 2003). Accordingly, the sirtuin family was proposed to work as effectors of reduced caloric intake (Kim et al. 2007; Boily et al. 2008; Polito et al. 2010). Hence, the first studies on sirtuins connected it with metabolic diseases, such as obesity, lipid and glucose metabolism, and type 2 diabetes (Ramadori et al. 2008; Houtkooper et al. 2012). Consequently, the first clinical trials investigate the effect of small-compound activators such as resveratrol or STAC1720 on metabolic diseases (Howitz et al. 2003; Wood et al. 2004; Baur et al. 2006; Boily et al. 2008; Dai et al. 2010). All of the mentioned diseases not only involve problems in metabolism but bring an increased risk of neurodegenerative processes with them (Panza et al. 2010; Misiak et al. 2012; Cai 2013). Accordingly, treatment for these diseases could also positively influence the risk for neurodegeneration. However, therapeutic interventions aiming to intervene with the sirtuin system must take the complex scenario, presented in our study, into account. Therapies that increase the expression of SIRT1 may have different effects in distinct neuronal cell populations, which may lead to unexpected therapeutic effects.

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