BRAIN
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Loss of REST associated with Alzheimer's disease pathology is ameliorated by NAD+

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Downregulation and inactivation of the Repressor Element 1-Silencing Transcription factor (REST) is shown in Alzheimer's disease (AD) and likely contributes to its progression, but the exact molecular mechanism linking REST reduction to AD remains unclear.

We examined changes in REST expression in the entorhinal cortex and hippocampus across different Braak stages of tauopathy. We show that alterations in REST expression and sub-cellular localization are partially responsible for AD pathology, as REST overexpression improves cognition, reduces amyloid-β and phosphorylated Tau deposition, and restores mitochondrial and synaptic homeostasis. Mechanistically, the NAD+/SIRT1 axis modulates REST expression through chromatin remodelling in the promoter region of REST, leading to changes in the expression of REST target genes involved in mitophagy and synaptic function.

These findings reveal a new mechanism of action for NAD+ and highlight REST as a promising therapeutic target for AD therapy.

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Introduction

Alzheimer's disease (AD) is an irreversible multifactorial neurodegenerative disorder primarily characterized by progressive memory impairment and cognitive decline.[1-5] At the neuropathological level, AD is defined by the accumulation of extracellular amyloid-β (Aβ) plaques, concomitant with hyperphosphorylated Tau (p-Tau) protein-formulated intracellular neurofibrillary tangles (NFTs).[6-8] The neuropathological progression of AD begins years before clinical onset with the accumulation of Aβ in the neocortex, and in the early stages in the medial temporal lobe.[9] This accumulation arises from an imbalance between production and clearance of Aβ in the brain. In parallel or shortly after, microtubule-associated protein Tau (MAPT) phosphorylates and aggregates inside neurons, starting from the neurons in layer II of entorhinal cortex (EC), spreading to hippocampus (HIP).[9] The convergence of Aβ and p-Tau in the temporal lobe further accelerates the progression of AD, ultimately compromising axonal transport, leading to neural damage and eventual cell death.[10] Nevertheless, the exact molecular mechanisms driving these processes remain to be fully elucidated.

Ageing is one of the primary risk factors for most neurodegenerative diseases, including AD.[11,12] A specific transcription factor, known as RE1-Silencing Transcription factor (REST), has been implicated in the process of healthy ageing.[11,13] REST is a key negative regulator of neuronal transcription, playing a crucial role in neural activity and neurodifferentiation.[14] *REST* might generate up to 45 mRNA variants through alternative splicing, leading to several isoforms with distinct functions and structural differences.[15,16] While the primary *REST* isoform contains functional domains necessary for DNA binding, transcriptional repression, and interactions with co-repressors, the biological functions of the various isoforms remain unclear. Plasma levels of total REST have been directly correlated with age, as well as increased nuclear levels during healthy ageing and notably lower in AD.[13,17,18] Therefore, upregulation of REST in humans may be associated with prolonged longevity, heightened cognitive performance, and reduced risk of AD.[13,17] Recently, it has been demonstrated that overexpression of REST may confer neuroprotection in AD by mitigating Aβ deposition and accumulation of misfolded p-Tau proteins.[19] Furthermore, REST regulates mitochondrial biogenesis and mitochondrial autophagy (mitophagy) via activation of the transcription factor fork head box protein O1 (FOXO1)[13] and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α).[20] FOXO1 upregulates genes involved in autophagosome formation including *Atg14b, Atg12* and *Pi3k3*,[21] while PGC-1α regulates transcription of mitochondrial genes and the expression of genes encoding proteins involved in mitophagy/autophagy, such as *p62, LC3*[22] and *FUNDC1*.[23]

Accumulation of damaged mitochondria due to impaired mitophagy has been proposed as a key mechanism contributing to the age-related risk of AD.[2,9,24,25] Mitophagy is a sub-type of macroautophagy through which damaged or superfluous mitochondria are specifically recognized for degradation and then recycling.[26,27] Mitochondria are responsible for producing cellular energy in the form of adenosine triphosphate (ATP), while also serving as a centre for metabolism and as a node in the broad spectrum of cell signalling pathways that participate in neuronal development, Ca^{2+} firing, and even in dictating cellular survival or death.[28,29] Impairment of mitochondrial metabolism can increase reactive oxygen species (ROS) and potentially lead to significant mitochondrial and cellular injury.[28,30] Maintaining a healthy mitochondrial pool is crucial for the optimal functioning of cells and, consequently, the entire organism. Moreover, accumulation of damaged mitochondria is detrimental to the process of healthy ageing and can initiate neurodegenerative processes.[31] For example, compromised clearance of damaged mitochondria leads to impaired metabolic profiles, imbalances in redox status and nicotinamide adenine dinucleotide (NAD^+) metabolism, and damage to mitochondrial dependent signalling pathways. Additionally, it induces innate immunity, such as cGAS–type I interferon (IFN-I) and NLRP3-Caspase1 signalling pathways. These changes are associated with an increased risk of AD, and normalizing these alterations to wild-type (WT) levels showed cognitive benefits in AD-like animals.[24,32]

Among the mitochondria-related changes, we focus on NAD^+, a small metabolite essential for life and health, which has been shown to be reduced during ageing and AD.[12] NAD^+ is a rate-limiting substrate for sirtuin 1 (SIRT1), a class III histone deacetylase and master regulator of metabolism, neuronal resilience and survival, and brain health and healthspan.[24,33-35] In AD, SIRT1 has been shown to be reduced.[36-38] Recent studies point to the NAD^+/SIRT1-mitophagy axis playing an important role in neuroprotection and memory preservation against both Aβ and Tau pathologies.[24,25] Specifically, supplementation with NAD^+ precursors has been shown to elevate SIRT1 levels, which in turn contributes to neuroprotective effects. Furthermore, genetic and pharmacological augmentation of NAD^+ restores mitophagy, leading to reduced Aβ and Tau pathologies, as well as memory retention.[24]

Although there is some evidence suggesting that SIRT1 may regulate REST, the mechanisms underlying their interconnection, regulation and/or interaction remain largely unknown.[39] In this

study, we investigate the regulation of the NAD^+/REST axis and provide new insights into its role in mitochondrial quality control in AD. Using a cross-species approach, we demonstrate that downregulation of REST negatively affects lifespan by disrupting mitochondrial homeostasis and worsening AD pathology. Conversely, upregulation of REST enhances mitochondrial health and organismal lifespan, and delays the pathology of AD. Furthermore, NAD^+ augmentation through treatment with NAD^+ inducers increases nuclear translocation and activity of REST, as well as the levels of SIRT1 and its binding to the promoter region of *REST*. As a result, SIRT1 regulates the transcription of different REST isoforms and modulates REST target genes, revealing a novel mechanism with potential clinical applications.

Materials and methods

This study employed an integrative approach using post-mortem human brain tissue, cellular models, *C. elegans* and transgenic mouse models (5xFAD and hTau[P301S]). A range of methodologies was applied, including behavioural testing, immunohistochemistry, immunofluorescence, western blotting, electrophysiology and RNA sequencing. Mechanistic investigations involved REST and SIRT1 knockdown, mitophagy quantification and reporter assays across systems. Induced pluripotent stem cell (iPSC)-derived cortical neurons and multiple stress paradigms in *C. elegans* further supported functional validation. Statistical analyses were rigorously performed using appropriate parametric and non-parametric tests. This multimodal design enabled a comprehensive interrogation of REST-associated pathways in AD, spanning molecular, cellular and organismal levels. Detailed descriptions of all materials, procedures and statistical approaches are provided in the Supplementary material, 'Materials and methods' section.

Results

Altered REST levels and localization in the entorhinal cortex and hippocampus across AD stages

The expression and subcellular localization of REST across different Braak stages in different brain regions had not been systematically evaluated, so we aimed to address this question first. Two of the brain areas most seriously affected by Tau pathology from the very early stages of AD are the EC and the HIP.[11,36] Since REST has been shown to correlate with Aβ levels in the blood, and to decrease in the prefrontal cortex and HIP of AD patients,[17] we decided to examine REST protein levels in greater detail in EC and HIP regions across different Braak stages using immunofluorescence and western blotting (Fig. 1).

In EC, REST protein levels increased 208.4% in Braak III/IV compared to cognitive unaffected control samples and subsequently decreased in Braak V/VI compared to Braak III/IV (Fig. 1B). Immunofluorescence quantification showed a significant decrease (approximately 40%) in the percentage of nuclear REST-positive ($REST^{nucleus}$) cells in all Braak stages compared to the control group (Fig. 1C). Conversely, the percentage of cytoplasmic REST-positive ($REST^{cytoplasm}$) cells (Fig. 1D) and the number of cells with REST expression, regardless of subcellular localization ($REST^{total}$) (Fig. 1E) increased significantly from the very early stages of AD (Braak I/II) compared to control group. Nevertheless, the density of $REST^{total}$ cells significantly decreased between Braak III/IV and V/VI, accompanied by a shrinkage in their size (Fig. 1A and E). In post-mortem HIP tissue, REST levels also increased 102.0% in Braak III/IV, followed by a tendency to decrease by 33% in Braak V/VI compared to Braak III/IV (Fig. 1G), showing a similar pattern to that observed in the EC. For the quantification of the subcellular localization of REST, the percentage of $REST^{nucleus}$ cells significantly decreased (−35.5% reduction) in Braak I/II and showed a significant gradual increase (~50%) during disease progression (Fig. 1H). This was the opposite of $REST^{cytoplasm}$, which significantly increased (+319.2%) in Braak I/II and gradually decreased throughout the later Braak stages (Fig. 1I). The density of $REST^{total}$ also showed a significant decrease (−30%) in Braak III/IV and Braak V/VI (Fig. 1J). Collectively, we detailed changes of total REST protein, and subcellular localization of REST in EC and HIP post-mortem tissue from different Braak stages, with a Braak staging-dependent reduction of these parameters in some of the cases.

Recent progress in clinical studies supports the possibility and reliability of developing blood-based diagnosis and staging biomarkers for AD.[40,41] While an exceptional example is pTau217,[42] other candidate proteins which can be considered as biomarkers of AD include autophagy proteins such as PINK1, BNIP3L, TFEB[43] and REST.[18] Through the application of the European Medical Information Framework (EMIF)-AD cohort, we analysed a set of proteins in plasma samples from controls (Ctrl) ($n = 220$), individuals with mild cognitive impairment (MCI) ($n = 382$) and AD ($n = 183$) patients,[44,45] obtained via aptamer capture array (SomaScan; detailed in the 'Materials and methods' section). We found that some proteins involved in synaptic plasticity and mitochondrial homeostasis changed in concentration in at least one of the comparison groups. Intriguingly, among the changed protein list, up to eight of these proteins were encoded by REST-target genes—GRIA4, DLG4, SNAP25, GFAP, BDNF, APP, PLG and IL-2.[17] For example, REST-regulated proteins GRIA4 and SNAP25 were reduced in the AD versus Ctrl and AD versus MCI groups. While others, like GFAP, BDNF, APP and IL-2 showed changes in very early stages of cognitive impairments (MCI versus Ctrl) (Fig. 1K). Further protein-protein interaction networks (from https://string-db.org/) pointed to broad linkages among these REST-regulated proteins and classical autophagy proteins, which were in our list of changed proteins, such as LRPPRC, NDP52, PARKIN-1, BECLIN-1, NBR1, ATG-5, AMPK, LC-3, PSD95, DNM2, TBK-1 and IL-1BR (no change in plasma levels in the last two) (Fig. 1K and L). Collectively, all these data suggest a likelihood that the protein level and transcriptional activity of REST are changed in AD, reflected by changes of REST protein level and subcellular localization in post-mortem brain samples (EC and HIP) in different Braak stages, and changes of REST-regulated proteins in plasma samples from Ctrl, MCI and AD.

REST overexpression extends lifespan and improves health span

Although we did not observe clear changes in REST protein levels through western blot analysis, histological analysis revealed a decrease in nuclear REST and $REST^{total}$-positive cells during tauopathy progression. Decreased nuclear REST is in line with previously reported findings from the prefrontal cortex[17,19] and supports the hypothesis that REST regulation may influence the progression of AD pathology. To test this hypothesis, we first aimed to down- and up-regulate REST expression in healthy conditions to assess the implications in healthy ageing using *C. elegans* as a model organism (easy for genetic editing). The *C. elegans* orthologues of mammalian REST are *spr-4* and *spr-3,* which retain the main biological functions of REST, such as suppression neuronal excitability

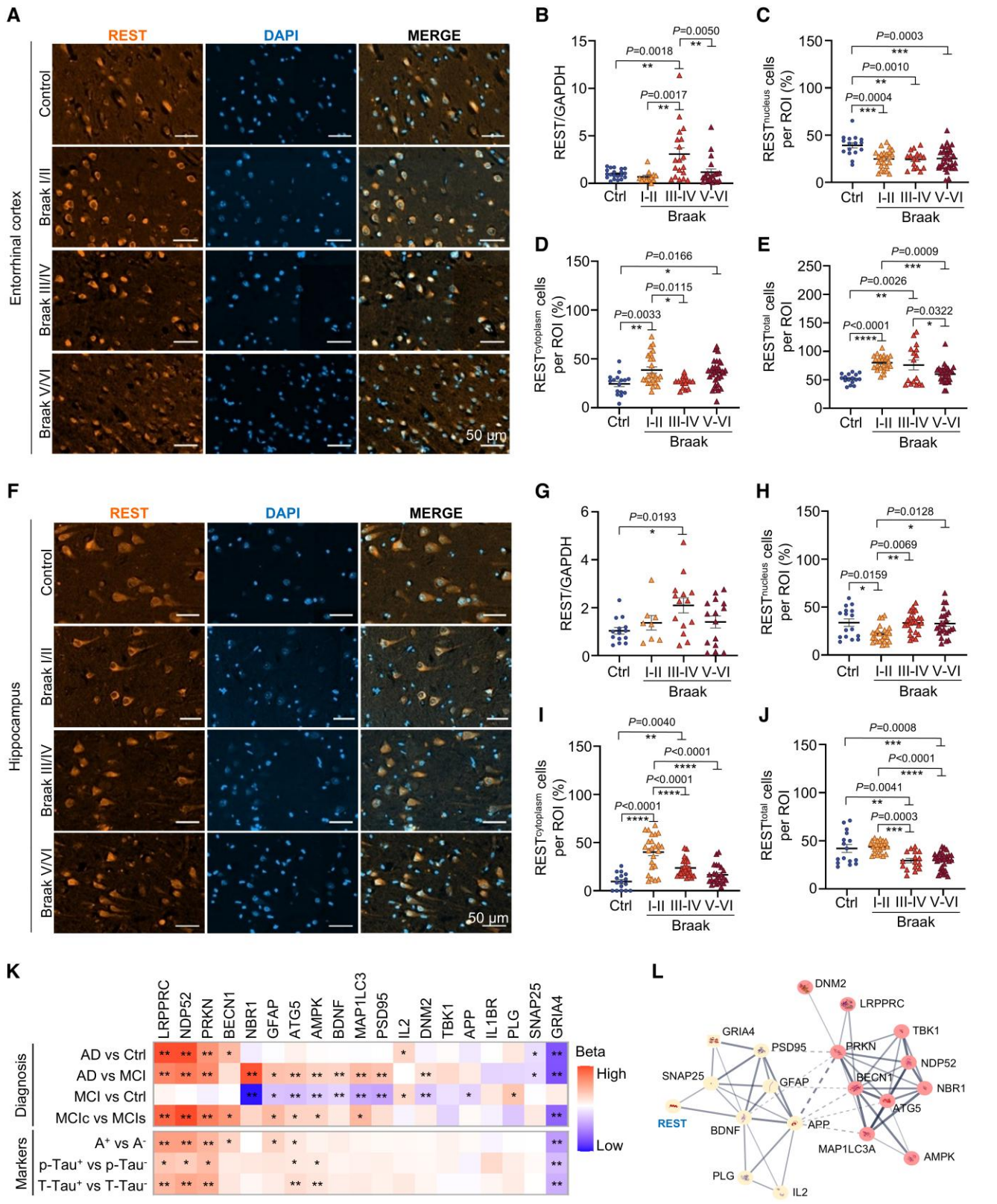

Figure 1 Changes in REST at different Braak stages. (**A** and **F**) Representative immunofluorescence images for REST and DAPI staining in the entorhinal cortex (EC) (**A**) and CA1-hippocampus (HIP) (**F**) of control (Ctrl), Braak stage I/II, Braak stage III/IV, and Braak stage V/VI. Scale bars, 50 = µm. (**B** and **G**) Quantification of the level of REST protein in homogenized tissue of EC (12–20 samples per group) (**B**) and HIP (9–15 samples per group) (**G**) from non-Alzheimer's disease (AD) and AD at different Braak stages. (**C** and **H**) Percentage of the total number of REST-positive cells with signal in the nucleus in the EC (**C**) and HIP (**H**). (**D** and **I**) Percentage of the total number of REST-positive cells with signal in the cytoplasm in the EC (**D**) and HIP (**I**). (**E** and **J**) Total number of REST-positive cells increases in the EC (**E**) and HIP (**J**). (**K**) Heat map of genes related to mitophagy/autophagy and synaptic markers and their potential use for diagnosis or markers of AD. (**L**) STRING analysis of the genes in (**K**) and their relationship with REST (https://string-db.org/). Quantitative data are presented as mean ± standard error of the mean. Statistical analysis performed by one-way ANOVA with Tukey's multiple comparison test. *ns* = not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. A = amyloid, Ctrl = Control; MCI = mild cognitive impairment; MCIc = MCI converting; MCIs = MCI stable; p-Tau = phosphorylated tau; T-Tau = total tau; REST = Repressor Element 1-Silencing Transcription factor.

and lifespan extension.[13] Here we used four well-characterized worm strains (gifts from the Yankner laboratory),[13] including three mutants *spr-3(ok2525)*, *spr-4(tm465)* and *spr-3(ok2525);spr-4(tm465)*, as well as one overexpressed strain *spr-4*^{ov}, all with WT N2 as the background. Similar to a previous report,[13] *spr-3(ok2525)* and *spr-4(tm465)* had shorter lifespans (approximately 22.2%) than the WT, and the lifespan of the double mutant *spr-3(ok2525); spr-4(tm465)* was shortened by 33.3% (Fig. 2A). In line with the role of *spr-3/spr-4/REST* in healthy longevity, *spr-4* overexpression extended the lifespan of WT worms by approximately 37.5% (Fig. 2B).

Our next step was to assess whether upregulation of REST could slow AD progression in both *C. elegans* and a mouse model (see later section). We tested this in two well-characterized AD-mimic strains, including a Tau-like strain with pan-neuronal overexpression of the human hTau[P301L] (CK12) and an Aβ-like strain with pan-neuronal overexpression of $A\beta_{1-42}$ (CL2355). Both hTau[P301L] and $A\beta_{1-42}$ animals showed shorter lifespans (40% and 10% shorter, respectively) compared to the WT (Supplementary Fig. 1A), which is compatible with a previous report.[42] Similar to its benefit to the WT lifespan, *spr-4* overexpression extended the lifespan of both hTau[P301L] (16.7%) and $A\beta_{1-42}$ (11.1%) animals (Fig. 2C and Supplementary Fig. 1B). As lifespan extension does not always correlate with improved health span,[46] we then asked whether genetic manipulation of REST has similar effects on health span using classical parameters, including pharyngeal pumping rate and development. While there were trends of reduced pumping rate in the mutant models with WT background (Supplementary Fig. 1C) (data from several points showed statistical differences), *spr-4* overexpression significantly improved pumping rates in WT, hTau[P301L] and $A\beta_{1-42}$ at a late life stage (adult Day 10) (Supplementary Fig. 1D). Furthermore, *spr-4* overexpression displayed a tendency to increase reproduction, L4 development and development of adulthood in WT and the two AD-like strains. It statistically increased larval and adulthood development in hTau[P301L] animals (Supplementary Fig. 1E–G). Collectively, our data indicated that *spr-3/spr-4/REST* increases lifespan and extends health span in both WT and two AD-like (Tau and Aβ) nematode animal models.

REST increases neuronal mitophagy and resistance to multiple mitochondrial stresses in *C. elegans*

Mitophagy-dependent maintenance of a healthy mitochondrial pool plays an important role in metabolic homeostasis, signalling transduction, cell survival and healthy longevity.[24,27] A recent study reported that REST regulates both mitophagy and mitochondrial biogenesis,[20] but whether REST plays a role in neuronal mitophagy in live animals is not known. To determine this, a neuronal mitochondria-targeted Rosella mitophagy reporter strain (mtRosella) was used.[24] This is a transgenic strain expressing a pan-neuronal mtRosella biosensor with a GFP variant sensitive to the acidic environment of the lysosomal lumen fused to the pH-insensitive DsRed protein. In the results, lower ratios indicate increased mitophagy. First, we evaluated whether REST overexpression might increase mitophagy in WT and AD strains. As we reported before,[24] the basal level of neuronal mitophagy in the hTau[P301L] worms was 36.6% lower than the WT. Importantly, *spr-4* overexpression increased neuronal mitophagy by 44% in WT, and a trend was observed ($P = 0.0542$, 23% increase) in hTau[P301L] worms (Fig. 2D). Surprisingly, neuronal *spr-4* knockdown did not change the mitophagy signals (Fig. 2E), possibly due to the compensation by *spr-3*. As we had challenges in evaluating the role of *spr-3/spr-4/REST* on mitophagy in *C. elegans* neurons, we continued addressing this question using a human HeLa cell line which expresses mt-Keima (a mitophagy reporter). mt-Keima is a fluorescent protein in the mitochondria, which shows excitation in green (440 nm) in an alkaline environment, but shifts towards red (586 nm) within the acid pH of the lysosomes. Therefore, higher values of red signal mean higher mitophagy levels.[47] In this setting, the mitochondrial uncoupler CCCP was used as a positive control (Fig. 2F). Here, siRNA knockdown of REST reached 40% reduction of mitophagy. Compared with the Scrambled + CCCP group, the REST (siRNA) + CCCP group showed a 22% reduction in mitophagy, suggesting CCCP-induced cellular mitophagy was likely REST-independent (Fig. 2F). These data indicated that REST regulates neuronal mitophagy, but not CCCP-dependent mitophagy.

We then asked whether *spr-3/spr-4/REST* affects mitochondria in the neurons, since impaired mitophagy could lead to mitochondrial dysfunction. We crossed a GABAergic neuron-expressing TOMM20::GFP (EG6531) strain with the *spr-3(ok2525); spr-4(tm465)* strain. Neuronal mitochondrial mass was reduced by 70% in the *spr-3(ok2525); spr-4(tm465)* strain, with more fragmented mitochondria identified within both axons and soma of this strain (Fig. 2G).

It has been reported that mitophagy also plays an essential role in resilience against oxidative stress and other exogenous stresses. Therefore, we asked whether REST also affects resilience to exogenous stressors. After exposure to 15 μM CCCP and starvation (from L1), *spr-4(tm465)* and hTau[P301L] worms exhibited lower mitochondrial stress resistance than WT and *spr-4*^{OV} worms (Fig. 2H and I). However, the benefit of SPR-4 overexpression to heat shock (37°C for 4 h) and UV resistance was not clear. Under heat shock conditions, *spr-4*^{OV} animals showed 148.2% greater survival compared to *spr-4(tm465)* animals, while there were no significant differences among other groups. In the UV exposure group, hTau[P301L]; *spr-4*^{OV} animals were substantially (+15.4%) more sensitive to UV than hTau[P301L]; *spr-4(tm465)* animals (Fig. 2J and K). Thus, REST protects the animals against multiple exogenous stressors.

REST overexpression restores memory in AD models

As REST overexpression improved lifespan, health span and resilience in the *C. elegans* strains, we wondered whether it also improved memory using the same model. A well-established chemotaxis-based short-term associative memory assay was applied for the experiments.[25,48] The hTau[P301L] animals show a memory-loss-like attribute that is similar to that seen in AD patients. Intriguingly, SPR-4 overexpression improved associative memory in hTau[P301L] (Fig. 3A). Thus, SPR-4/REST overexpression prevented short-term memory loss in AD-mimic hTau[P301L] worms.

Encouraged by the potential improvements in memory retention capacity imparted by REST in worms, we asked whether this benefit was also conserved in a murine model of AD. The AD Tau pathology-like mouse model, P301S,[45] and its WT counterpart were used. We overexpressed REST in the HIP of 9–10-month-old P301S$^{+/-}$ mice (both hemispheres) via AAV-REST^{OV} vector injection and using AAV-Empty vector as control. We waited 2 months for protein expression to occur and then performed behavioural and pathological studies. After this period, both WT mice injected with the AAV-Empty vector and those injected with the AAV-REST^{OV} vector exhibited similar performance in novel object location (NOL), novel object recognition (NOR), and spontaneous

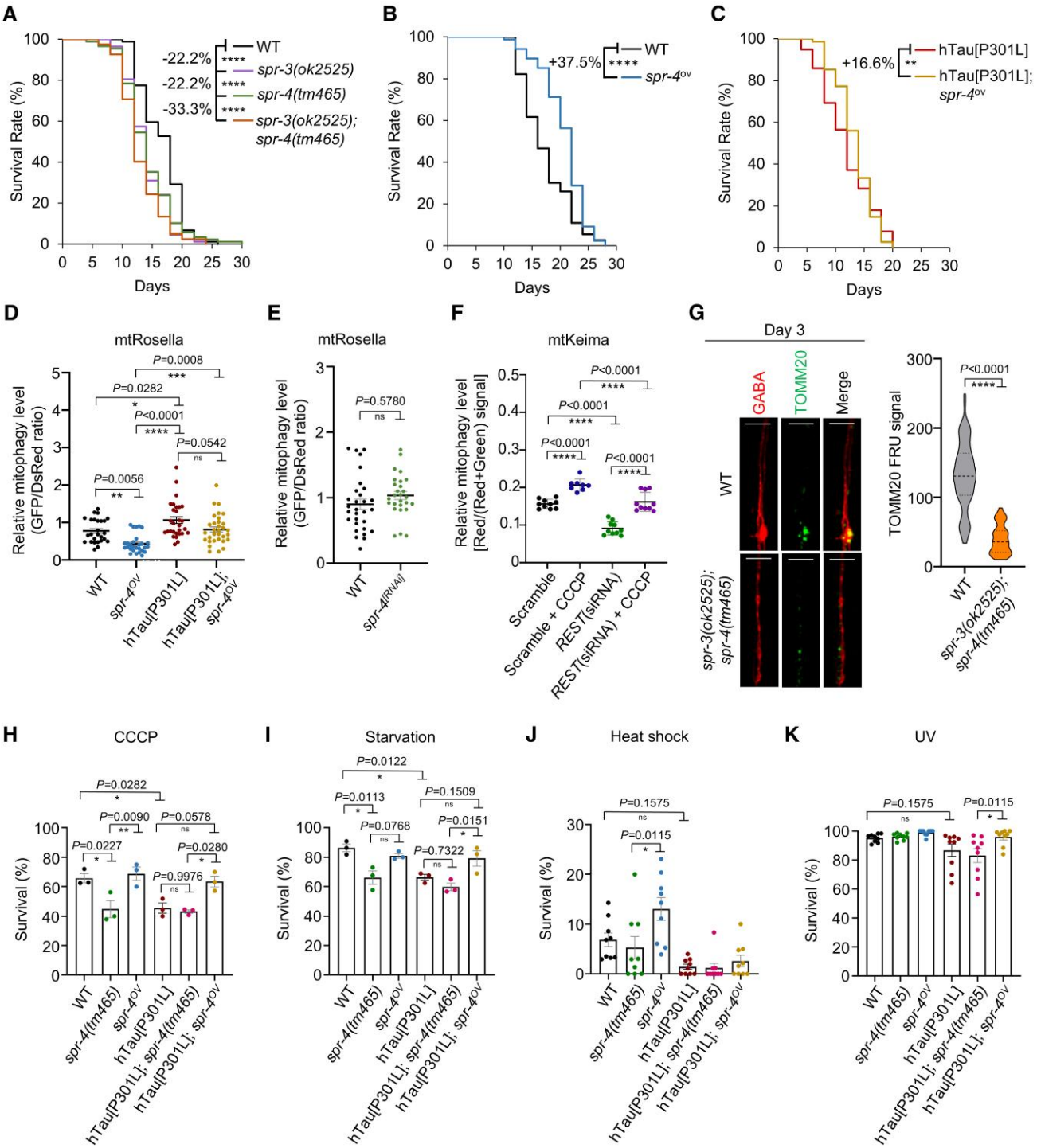

Figure 2 Genetic manipulation of REST regulates lifespan and mitochondrial health. (**A** and **B**) Effect on the lifespan of REST orthologue mutants [*spr-3*(*ok2525*), *spr-4*(*tm4655*) and *spr-3*(*ok2525*);*spr-4*(*tm465*)] and ubiquitous overexpression (*spr-4*^{OV}). Data shown are from one biological repeat with ~100 worms per condition. (**C**) Effect in lifespan of overexpression of REST (*spr-4*^{OV}) in an Alzheimer's disease (AD) model of *C. elegans* (hTau[P301]). Only one biological repeat (n = 90–100 worms/condition) is shown. (**D**) Quantification of mitophagy levels when *spr-4/REST* is overexpressed in normal conditions and tau pathology (hTau[P301L]). Data shown are pooled from three biological repeats (n = 5–10 worms/strain). (**E**) Quantification of mitophagy levels using an mtRosella construct in WT background with a knockdown of REST. Data shown are pooled from three biological repeats (n = 5–10 worms/strain). (**F**) Quantification of mitophagy activity using mt-Keima in HeLa cells upon REST knockdown and treatment with 5 μM of mitophagy inducer CCCP (carbonyl cyanide chlorophenylhydrazone) for 24 h. One biological repeat with 10 technical repeats per condition is shown. (**G**) Representative confocal images and quantification of TOMM20 fluorescent intensity in the GABA neurons of *C. elegans* WT and *spr-3*(*ok2525*); *spr-4*(*tm465*) strains on Day 3 after 2 days of treatment with 10 nM paraquat (from Day 1). Experiment was repeated 6–9 times with n = 5–20 worms/condition. Scale bars = 10 μm. (**H**–**K**) Evaluation of stress resistance in normal *C. elegans* and AD model when *spr-4/REST* is genetically manipulated (mutation and overexpression). Worms were subjected to different types of stress: (**H**) 2 h of 15 μM CCCP, (**I**) 5-day starvation from larva stage 1, (**J**) 7 h of 37°C heat shock and (**K**) 500 J/m^{-2} UV stress. Experiments were repeated three times with 100–150 worms per strain. Quantitative data are presented as mean ± standard error of the mean. Statistical analysis performed by log-rank test for lifespan data (**A**–**C**), unpaired t-test (**D** and **E**) and one-way ANOVA with Tukey's multiple comparison test (**F**–**K**). ns = not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. REST = Repressor Element 1-Silencing Transcription factor; WT = wild-type strain of *C. elegans*.

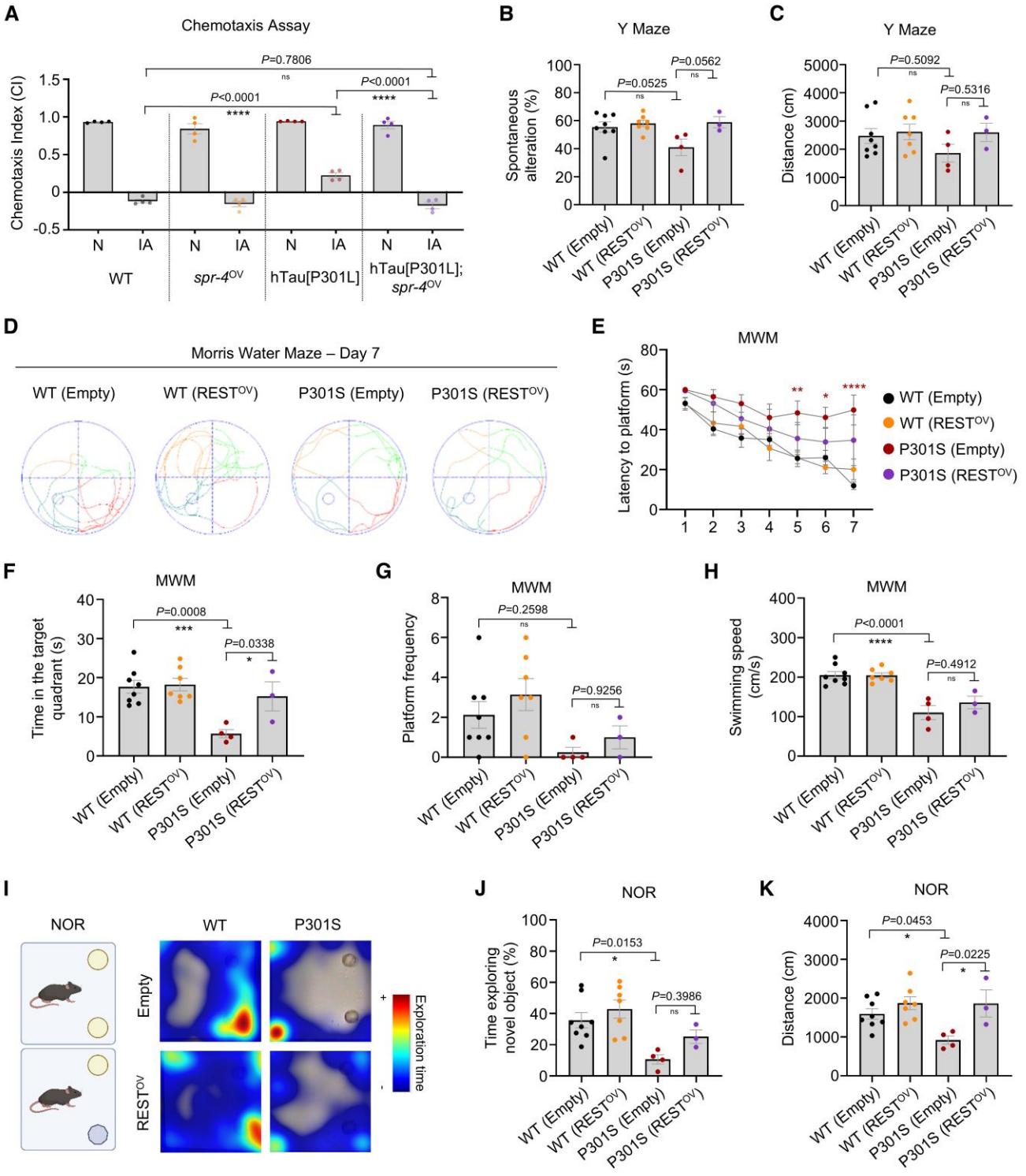

Figure 3 Overexpression of REST improves cognition in a Tau model of Alzheimer's disease. (**A**) Memory-like behaviour in adult Day 1 *C. elegans* wild-type (WT) and Alzheimer's disease (AD) models with REST overexpression (*spr-4*^{OV}). Four biological repeats (n = 150–300 worms/strain) are shown. (**B** and **C**) Y maze results of 12.5-month-old WT and P301S mice 3 months post-virus injection with AAV-empty vector (Empty) or AAV injection with over-expression of REST (REST^{OV}). (**D**) Representative images from swimming trajectories of Morris Water Maze (MWM) on Day 7 of WT (Empty), WT (REST^{OV}), P301S (Empty) and P301S (REST^{OV}) mice. (**E**) Latency to platform during MWM test in the training days performed with WT (Empty), WT (REST^{OV}), P301S (Empty) and P301S (REST^{OV}) mice. The *P*-values shown at the top of each panel indicate significant differences in the comparison between WT and P301S (in red), otherwise there is no significance. (**F**–**H**) MWM probe trial results: (**F**) time in the target quadrant, (**G**) number of times the mice passed the platform location, and (**H**) swimming speed, performed in 12.5-month-old WT (Empty), WT (REST^{OV}), P301S (Empty) and P301S (REST^{OV}) mice. (**I**) Representative heat maps of movement in novel object recognition (NOR) assay for WT (Empty), WT (REST^{OV}), P301S (Empty) and P301S (REST^{OV}) mice. Panel created in BioRender. Escobar Doncel, B. (2025) https://BioRender.com/n07wq25. (**J** and **K**) NOR assay results showing time spent exploring novel object (**J**) and distance travelled (**K**) of WT (Empty), WT (REST^{OV}), P301S (Empty) and P301S (REST^{OV}) mice at 12.5-month-old. In all mouse experiments, one dot represents one mouse. Quantitative data are presented as mean ± standard error of the mean. Statistical analysis performed by one-way ANOVA with Šídák's multiple comparisons test. ns = not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. REST = Repressor Element 1-Silencing Transcription factor.

alternation-based Y-maze tests. For P301S mice, while they displayed an inferior performance in comparison to WT, 2 months of REST overexpression did not significantly improve results in these memory tests (though a tendency) (Supplementary Fig. 2).

We wondered if the negative data from 2 months REST overexpression could be due to short REST exposure and therefore performed a new set of experiments at 3 months post-injection. At 3 months, we performed the Y-maze, NOR, and additionally added the Morris water maze (MWM) test. Like the observations at 2 months post-AAV injection, no differences were observed between WT mice injected with the AAV-Empty vector and those injected with the AAV-REST^{OV} vector. For spontaneous alternation (Y-maze), REST overexpression showed a trend toward improved scores for the P301S mice (Fig. 3B and C). The MWM test showed that REST overexpression likely improved spatial learning and memory in P301S mice (Fig. 3D–H). Finally, we performed the NOR test, where REST overexpression significantly enhanced the exploration distance, and a trend towards increases in the percentage of time exploring a novel object compared to the P301S control mice was seen (Fig. 3I–K). In summary, long-term (3 month) REST overexpression improved some of the memory tests in the P301S mice.

REST reduces Tau pathology and Aβ plaques

As *REST* overexpression improved memory in AD-mimic Tau mice, we further explored the underlying molecular mechanisms. As hyper-phosphorylation of Tau is considered to be the driver of Tau pathology, and especially Tau aggregation, we checked hippocampal p-Tau using a monoclonal AT8 antibody that recognizes p-Tau (Ser202, Thr205). Compared to P301S with an AAV-Empty vector group, P301S with an AAV-REST^{OV} vector had 36% less AT8 + signal (Fig. 4A and B). Encouraged by the anti-Tau pathology capacity of REST, we further challenged ourselves to investigate whether REST also inhibits Aβ pathology. The 5xFAD mouse, which shows significant Aβ plaque accumulation and memory loss, was used.[44] For this experiment, 7-month-old 5xFAD mice were bilaterally injected with AAV-REST^{OV} in one side and AAV-Empty vectors in the other side of the CA1 region of the HIP, followed by a 3-month waiting period to allow protein expression to develop (Fig. 4C and Supplementary Fig. 3A and C). When the two sides of the brain were compared for each mouse, the size (Supplementary Fig. 3B) and the total number (Fig. 4D) of Aβ plaques were dramatically reduced (around 29% and 71%, respectively) in the REST-overexpressed region. Collectively, REST reduced both Tau and Aβ pathologies in AD-like mouse models.

Next, we asked whether functional connectivity could be restored by overexpressing REST in the 5xFAD mice. For this purpose, a set of long-term potentiation (LTP) experiments (Supplementary Fig. 3D) was conducted on the HIP of 6- to 7-month-old 5xFAD and WT control mice to evaluate changes of synaptic strength. However, while there was a trend towards reduced LTP in 5xFAD in comparison to WT mice, no significant differences were noted (Supplementary Fig. 3E). This could be due to variations within each group. Thus, we did not continue the LTP perspective but focused on other cellular/molecular mechanisms as detailed below.

Hippocampal REST overexpression changed hippocampal neural activity and homeostasis

To better understand how hippocampal REST overexpression affects molecular pathways, we performed RNA sequencing in the hippocampal tissue of WT and P301S mice. The data were generated using tissue from WT, WT (REST^{OV}), P301S and P301S (REST^{OV}) mice and subjected to two-dimensional (2D) principal component analysis (PCA). Transcriptomic profiles revealed that REST overexpression altered gene expression compared to vehicle in both WT and P301S mice (Supplementary Fig. 3F), particularly affecting genes in clusters 4, 6 and 7 (Fig. 4E and F and Supplementary Table 3). These clusters were mainly related to gene regulation (Supplementary Fig. 4C), developmental processes (Supplementary Fig. 4E) and synaptic plasticity (Supplementary Fig. 4F). More interestingly, we identified another set of genes which were changed in P301S (compared with WT) but were normalized to that of WT after REST overexpression. These genes were enriched in clusters 1, 3 and 8 (Fig. 4E and F and Supplementary Table 3) and were involved in Aβ clearance (Supplementary Fig. 4A) and chromatin remodelling (Supplementary Fig. 4H). Overall, pathway analysis shows that most of the pathways that exhibited changes in expression in the AD model and subsequently recovered after REST overexpression were mainly involved in cognition, memory and synaptic homeostasis (Fig. 4G and Supplementary Figs 3G and 4A–H).

Since REST is a transcription factor, we further evaluated transcriptional profiles of REST-regulated genes, directly and indirectly (Supplementary Table 4).[13] Compared with WT, changes in expression of the REST-regulated genes in the P301S group were involved in immune response, neural differentiation and synaptic homeostasis (Supplementary Fig. 3H). Overexpression of full-length REST changed the expression of several genes that are targets of REST. This overexpression was able to restore the expression levels of some of these genes in the P301S mice to that of WT mice, including *Cirbp*, *Egr1*, *Egr4*, *Marchf7* and *Npas4* (Fig. 4H, Supplementary Fig. 3H and Supplementary Table 4). *Cirbp* is associated with cold-induced stress,[49] while *Egr1* and *Egr4* are early growth response genes[50]; *Marchf7* plays a role in protein ubiquitination,[51] and *Npas4* regulates gene expression critical for synaptic plasticity and neuron survival.[52] Additionally, upregulation of REST in the HIP led to changes in mRNA expression of genes involved in pathways important for neuronal survival and function, such as endo-lysosomal pathway, oxidative stress and chromatin remodelling (Fig. 4H and Supplementary Fig. 3H). Thus, our transcriptional data unveiled potential REST-targeted pathways related to neuronal function and brain health that are affected in AD and can be restored by overexpression of REST.

NAD+ modulates REST expression and activity to promote lifespan, mitophagy and synaptic function

Given that *spr-3/spr-4/REST* regulates lifespan, health span, mitophagy and mitochondrial homeostasis, as well as being reduced during ageing, we wondered whether any pharmacological intervention could compensate for the loss of cellular REST. We here applied an NAD+ precursor, nicotinamide riboside (NR). NR induces mitophagy and further supports mitochondrial homeostasis, leading to healthy longevity in cross-species model systems, overlapping functions with REST.[33,53,54] In WT worms, 2 mM NR achieved a 25% lifespan extension, similar to that of *spr-4*^{OV} group. Moreover, NR treatment further resulted in a mild lifespan extension of 9.1% in the *spr-4*^{OV} group (Fig. 5A). In the case of the hTau[P301L] group, NR significantly extended the lifespan in hTau[P301L] (+16.7%); tau worms overexpressing *spr-4* also experienced this, but to a lesser extent (+9%) (Fig. 5B). However, this was not exactly the case in hAβ$_{1-42}$ worms since NR alone did not extend lifespan. On the contrary, when *spr-4* was overexpressed, the lifespan-extending benefits of NR became apparent in the hAβ$_{1–42}$

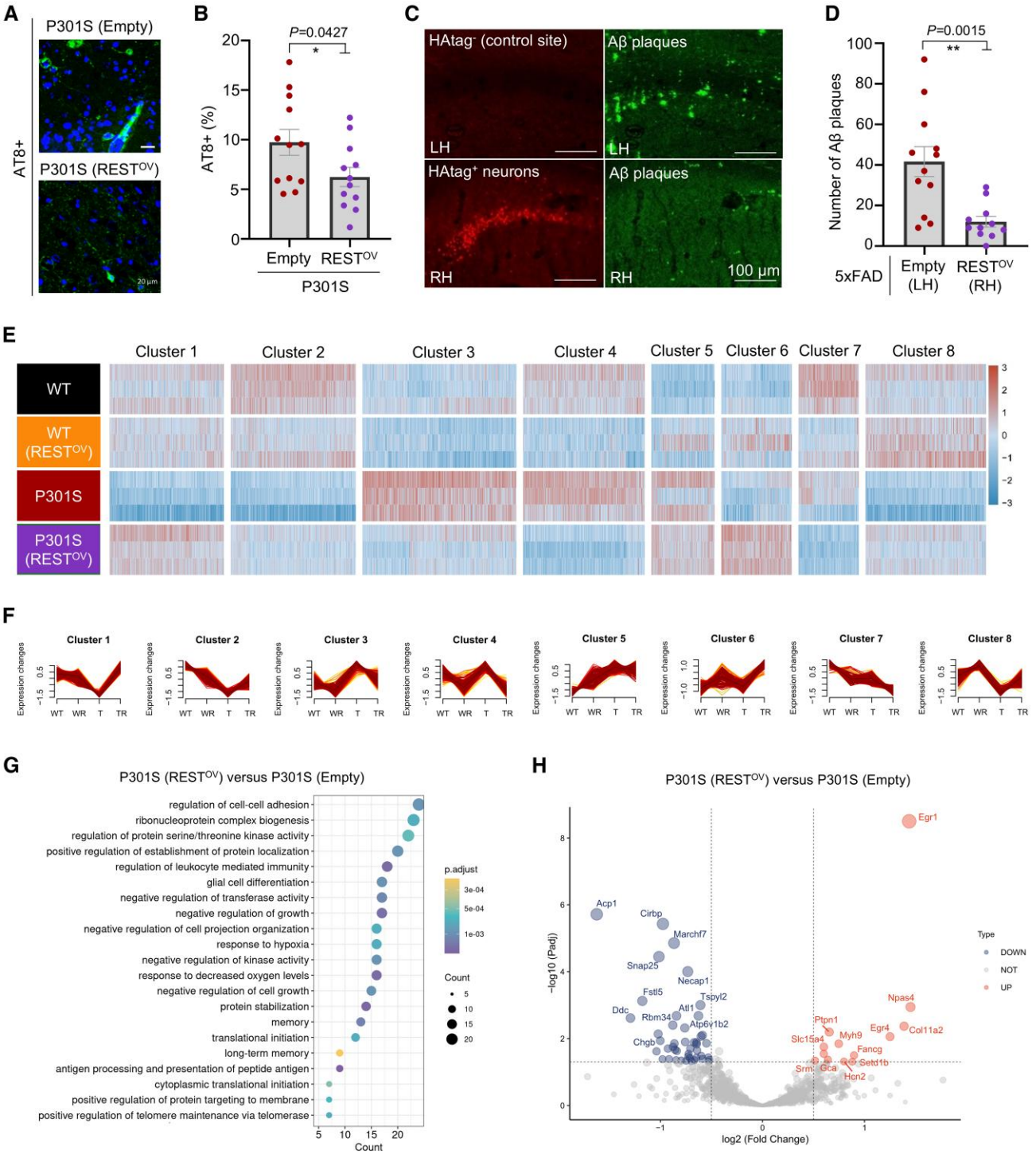

Figure 4 Overexpression of REST ameliorates Alzheimer's disease pathology and restores transcriptomic changes. (**A**) Representative images of hippocampal brain region stained for AT8+ (Tau) and DAPI. Scale bars, 20 µm. (**B**) Percentage of AT8+ cells in P301S (Empty) and P301S (REST^{OV}) mice at 13-months-of-age. Quantitative data are presented as mean ± standard error of the mean. Data shown are from three mice, with four images each. (**C**) Representative images of hippocampal brain, with the left hemisphere (LH) not injected and the right hemisphere (RH) expressing REST^{OV}. Scale bars, 100 µm. (**D**) Quantification of amyloid-β (Aβ) plaques per area quantified in hippocampus of LH and RH of 5xFAD mice at 7-months-of-age. Quantitative data are presented as mean ± standard error of the mean. Data shown are from three mice, with 3–4 hippocampal images per mouse. (**E** and **F**) Hippocampal gene expression analysis of (**E**) differentially expressed genes (DEGs) and (**F**) hierarchical clustering of DEGs differentially up- or downregulated performed in wild-type (WT), WT (REST^{OV}), P301S and P301S (REST^{OV}) mice at 13-months-of-age. Data collected from three mice per condition. (**G**) Gene ontology (GO) terms enriched in DEGs in the hippocampus of 13-month-old P301S (REST^{OV}) mice when compared to P301S (Empty). (**H**) Volcano plot of REST-targeted genes (negative numbers: downregulated, positive numbers: upregulated) in the hippocampus when REST is overexpressed in 13-month-old P301S mice compared to controls (Empty vector). Statistical analysis performed using unpaired *t*-test. ns = not significant, $^{*}P < 0.05$, $^{**}P < 0.01$. REST = Repressor Element 1-Silencing Transcription factor.

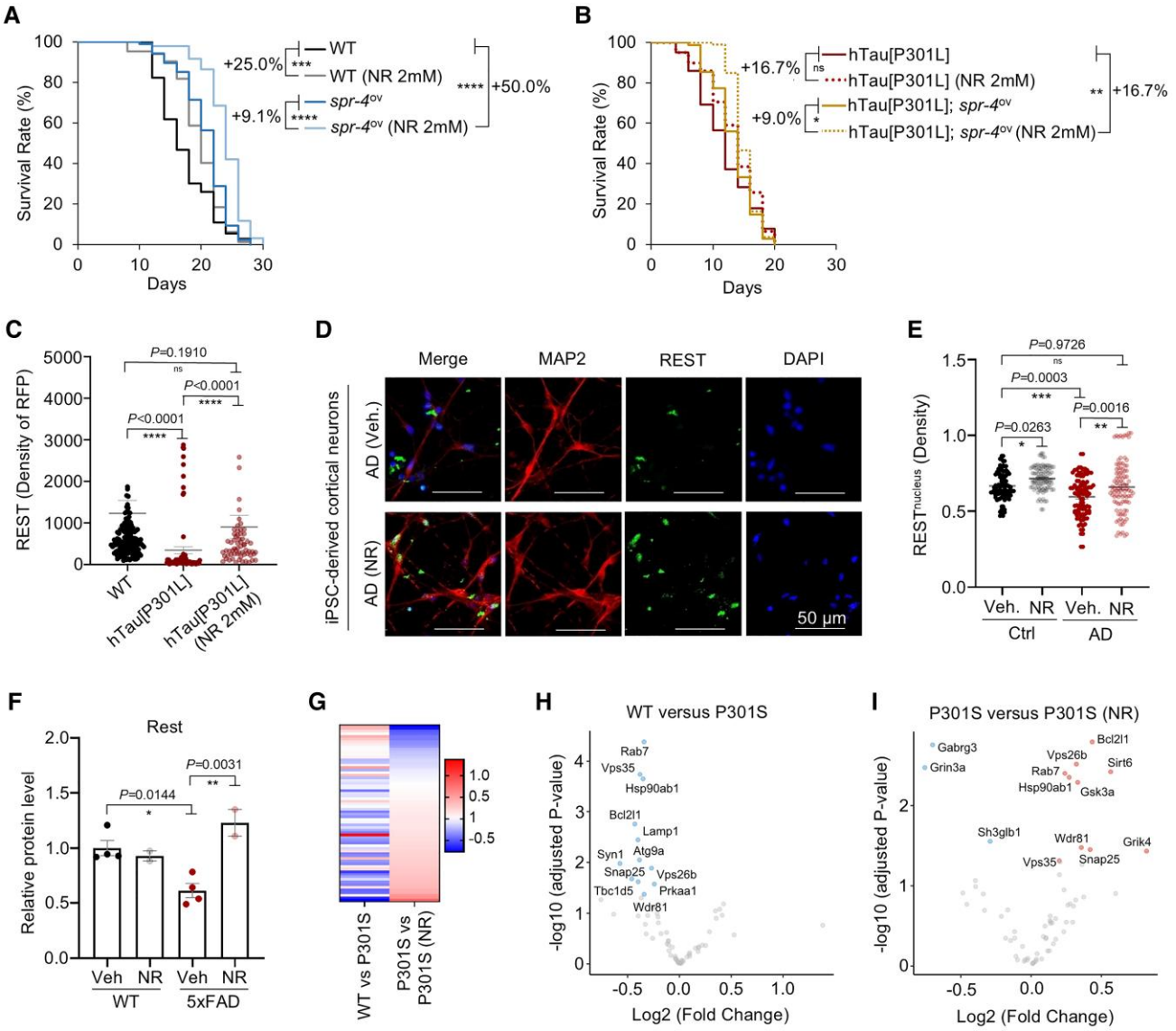

Figure 5 Nicotinamide riboside regulates nuclear translocation of REST and REST-related molecular pathways. (**A** and **B**) Combinatorial effect on lifespan of REST overexpression and 2 mM nicotinamide riboside (NR) treatment in (**A**) normal and (**B**) Alzheimer's disease (AD) model using *C. elegans*. All quantitative data are presented as medians. For the comparison between treated and untreated hTau[301L]; *spr-4*^{OV} strains, the data are means. Data shown from only one biological repeat with approximately 100 worms per condition. (**C**) Quantification of REST levels (RFP integrated density) in 3-day-old *C. elegans* AD model upon treatment for 3 days with vehicle or 2 mM NR from egg. Data are from three biological repeats ($n = 5–10$ worms/condition). Quantitative data are presented as mean ± standard error of the mean. (**D**) Representative images of REST localization upon NR treatment in AD iPSC-derived neurons after 1 mM NR treatment for 24 h. In total, 30–40 images were analysed from three biological repeats. (**E**) Quantification of REST signal in the nucleus in control (Ctrl) and AD iPSC-derived neurons upon treatment with vehicle or 1 mM NR. Quantitative data are mean ± standard error of the mean. (**F**) Protein levels of REST in the whole brain of wild-type (WT) and 5xFAD upon treatment with vehicle or 12 mM NR for 8 weeks when they were 7 months old. Data collected from 3–4 mice per group. Data are mean ± standard error of the mean. (**G**) Volcano plot of $\log_2$(fold-change) of 69 well-characterized genes related to mitophagy/autophagy and synaptic activity in the hippocampus of 14-month-old WT and P301S, and P301S treated with vehicle or 6 mM NR for 3 months. Data collected from three mice per group. (**H** and **I**) Volcano plot of genes shown in (**G**). Statistical analysis performed by log-rank test for lifespan data (**A** and **B**), Kruskal–Wallis test with Dunn's multiple comparisons test (**C**) and one-way ANOVA with Tukey's multiple comparison test (**E** and **F**). ns = not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. iPSC = induced pluripotent stem cell; REST = Repressor Element 1-Silencing Transcription factor.

group. (Supplementary Fig. 5A). Similarly, the *spr-4* overexpression combined with NR treatment did not have a clear effect on the health span (Supplementary Fig. 5E–G). Nevertheless, REST mutants treated with NR showed an extension in lifespan but not in health span (Supplementary Fig. 5H). Overall, these findings suggests that NAD$^+$ inducers and the regulation of REST-mediated pathways work synergically, but that NAD$^+$ benefits are not fully dependent on REST.

To address how NAD$^+$ might be influencing REST and REST-mediated molecular pathways, we used *C. elegans*, iPSC-derived cortical neurons, and mouse models. We observed that *spr-4* expression was significantly lower in hTau[P301L] *C. elegans* than in WT (~72% versus WT), which was increased almost 3-fold with NR treatment (Fig. 5C). To evaluate the cellular translocation of REST and the potential beneficial effects of NR treatment through REST in AD and healthy ageing, we used an iPSC-derived cortical

neuronal system from cognitively normal humans and AD patients. The nuclear localization of REST was significantly lower (10.8%) in AD iPSC-derived neurons compared to the iPSC-derived cortical neurons of healthy controls, consistent with previously reported data.[55] Interestingly, after NR exposure (1 mM for 24 h), both groups of cortical neurons showed a significant increase in the translocation of REST to the nucleus (Fig. 5D and E). Additionally, the results in the 5xFAD mouse model were consistent with these findings. The brain of 12-month-old 5xFAD mice showed a decrease in REST protein levels compared to WT, and NR treatment (12 mM NR for 8 weeks) dramatically increased whole-brain REST expression by approximately 2-fold (Fig. 5F). Our cross-species data suggested that NR increases protein expression and nuclear translocation of REST.

Up to this point, we have demonstrated that NR treatment increases nuclear REST, raising the question of its impact on downstream regulation. For this purpose, we selected 69 well-characterized genes involved in REST-regulated molecular pathways related to autophagy/mitophagy and synaptic activity (Supplementary Table 5). In the HIP of P301S mice, most of these genes exhibited altered expression in AD, but NR treatment restored their levels to values closer to WT (Fig. 5G). Among these, 43 of 69 genes were targets of REST, and 13 of these showed significant changes following NR treatment. Some of those targets of REST included *Rab7*, *Vps35*, *Hsp90ab1*, *Bcl2l1* and *Snap25* genes, which were downregulated in AD mice (Fig. 5H) and restored upon treatment with NR (Fig. 5I). In addition, in the whole brain of 5xFAD mice, western blotting data showed the expression levels of selected proteins related to mitophagy/autophagy and synaptic activity. Some of these proteins were significantly increased after NR treatment in the 5xFAD mice, such as the autophagy proteins PKRN/Parkin and LC3B-II, as well as a trend of increase in the neurotrophin brain-derived neurotrophic factor (BDNF) (Supplementary Fig. 6).

Collectively, all these data point to the hypothesis that NAD^+ regulates REST expression and its nuclear localization in *C. elegans*, human iPSC-derived neurons and mice.

SIRT1 regulates the expression of REST by binding to its promotor

NAD^+ is a cofactor for a group of NAD^+-dependent consuming enzymes such as Sirtuins (especially SIRT1, SIRT3, SIRT6 and SIRT7), PARPs [Poly (ADP-ribose) Polymerase], CD38/157 (Cluster of Differentiation 38/157) and SARM1 (Sterile Alpha and TIR Motif containing 1).[34,56,57] Among them, we observed a reduction of almost a half in hippocampal Sirt1 in 5xFAD compared with WT, while NR increased the level of REST expression 2-fold (Fig. 6A). Indeed, SIRT1 safeguards neuronal metabolism, function and brain health, and is reduced during ageing and in AD.[35-37,53,58,59] Therefore, we asked whether NAD^+/SIRT1 might have a potential role in the regulation of REST.

We hypothesized the existence of an NAD^+/SIRT1-REST axis. First, we asked whether SIRT1 and REST interact. Immunofluorescent images of 5xFAD mouse hippocampal brain tissues showed co-localization of Rest and Sirt1 (Fig. 6B). In addition, downregulation of SIRT1 in neuronal cells (SH-SY5Y cells) resulted in reduced protein levels of REST (Fig. 6C and Supplementary Fig. 7A). In contrast, downregulation of REST did not affect the protein concentration of SIRT1 at the lowest concentration (Supplementary Fig. 7B and C). Together, these data suggested that SIRT1 may regulate REST at transcriptional level.

We further investigated whether SIRT1 regulates *REST* expression. We observed that NR regulated specific isoforms of *REST*, such as pre-mRNA, Exon 1 and Exons 1–3, but not Exon 4 (Fig. 6D and E and Supplementary Fig. 7D and E). Specifically, this regulation was dependent on SIRT1. Downregulation of SIRT1 decreased the transcriptional expression of the studied isoforms of *REST* (Fig. 6F–H and Supplementary Fig. 7F and G). To explore this in more detail, we used ChIP-qPCR experiments. Our data showed that SIRT1 binds to the promoter region of *REST*, and NR treatment enhanced this binding (Fig. 6I). Because of an enhanced binding of SIRT1 after NR treatment, acetylation of H3K9 at the *REST* promoter region decreased, leading to changes in chromatin organization (Fig. 6J and K). As a consequence, different isoforms (Exon 1, Exons 1–3 and pre-mRNA) of *REST* are regulated. Moreover, NR increased mRNA levels of specific REST target genes, such as *GABR*, *GAD2*, *SNAP25* and *ULK2* (Supplementary Fig. 7H–K). Collectively, all the data suggested that NAD^+-dependent SIRT1 binding on the promoter region of *REST*, as shown in the schematic working model (Fig. 6L), leads to increased REST at transcriptional, protein and activity levels.

Discussion

In this study, first we carefully characterized the changes in REST throughout the progression of AD according to its level of tau pathology (Braak stages),[60] not only in the HIP but also in the EC, another region severely affected by this neurodegenerative disease but far less studied.[9,61] We observed that the total amount of REST protein significantly increases in both EC and HIP at Braak stages III/IV, with a reduction in REST levels occurring at the later stages (Braak V/VI), likely due to cell death, which occurs in more advanced stages of AD.[62]

At the cellular level, a decrease in the percentage and density of cells with positive $REST^{nucleus}$ was observed, accompanied by an increase in the percentage of cells with positive $REST^{cytoplasm}$ in both EC and HIP. However, between Braak I/II and Braak V/VI, a gradual decrease in the density of positive $REST^{total}$ cells was observed only in the HIP, suggesting that REST regulation was more severely affected in the HIP than in the EC. Nevertheless, clear shrinkage of the cells was observed in the EC of Braak V/VI stages, indicating that some of the quantified cells were likely undergoing necroptosis or other forms of cell death stress,[63] processes associated with cell death and neurodegeneration in AD.[64] These findings are consistent with previous publications showing alterations in the REST protein in the HIP and prefrontal cortex of AD patients, in addition to a brain-protective role in long-lived elderly individuals.[17] This is further supported by the fact that loss of REST expression is associated with a loss of mitochondrial integrity,[20] which is affected in the later stages of AD.[62]

Our experiments demonstrated, on the one hand, that the downregulation of REST altered mitochondrial integrity, decreased mitophagy and shortened lifespan in healthy ageing, which is in line with previous studies.[13,20] On the other hand, REST overexpression increased mitophagy and extended lifespan in both normal ageing and in AD. REST plays a beneficial role not only under pathological conditions, but also in normal ageing; however, at the cognitive level, we did not observe any improvement when REST was overexpressed in the WT of both our *C. elegans* and murine models. Under healthy conditions, cognitive function is not significantly impaired at this age, and we likely need to study older WT animals to observe a change. In contrast, REST overexpression restored memory in both *C. elegans* and mouse models of AD. It is worth noting that in the murine model this effect was more moderate. This could be because in mice, REST was overexpressed locally only in the dorsal HIP and at an age when Tau pathology had already

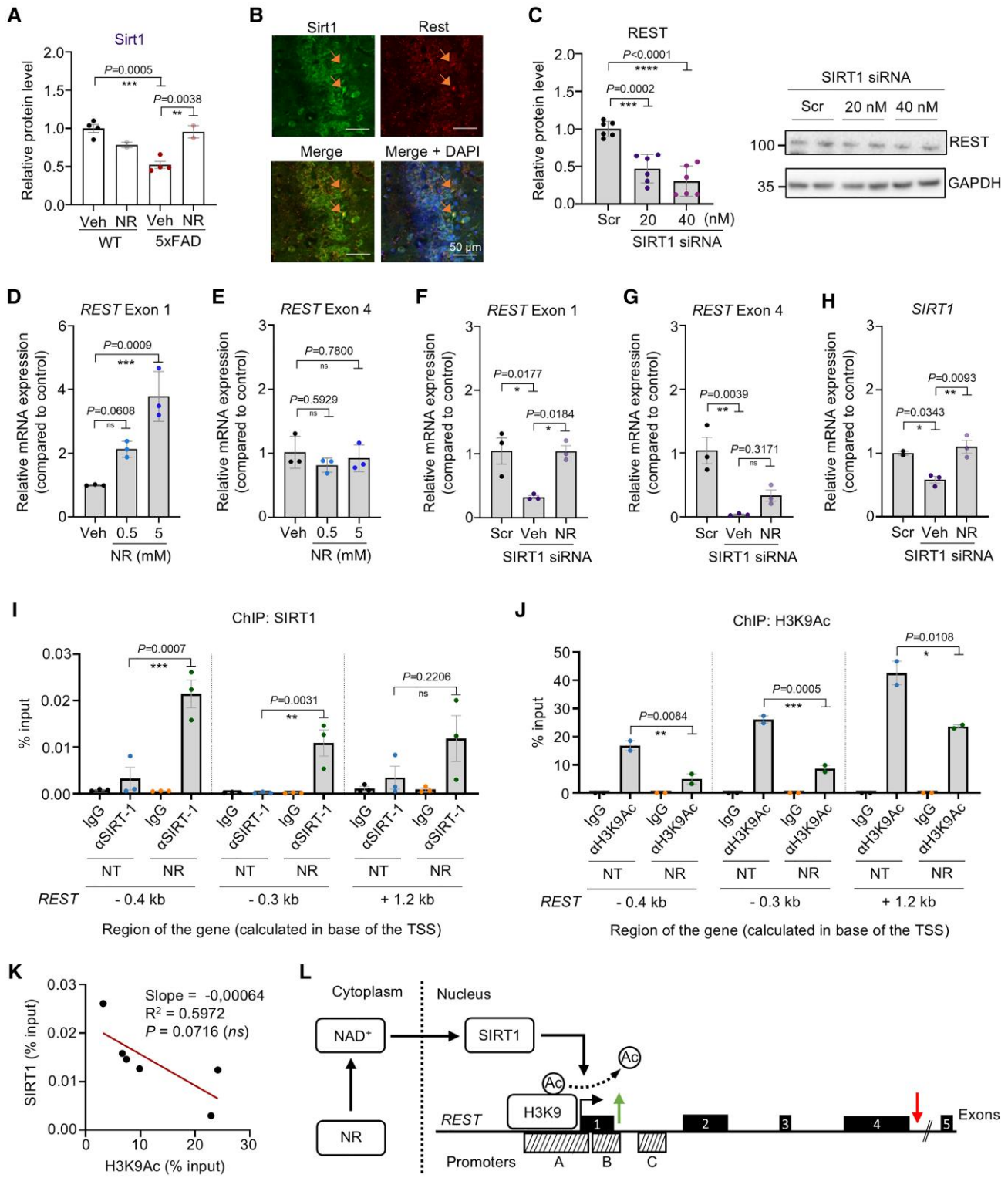

Figure 6 Nicotinamide riboside regulates transcription of REST through SIRT1 binding to the promoter region. (**A**) Protein levels of Sirt1 in the brain of wild-type (WT) and 5xFAD mice upon treatment with vehicle or 12 mM nicotinamide riboside (NR) in drinking water for 8 weeks. Data presented are from 2–4 mice per condition. (**B**) Representative images of Sirt1 (green) and Rest (red) co-localization in the pyramidal cell in the hippocampus of mouse brains. Scale bars = 50µm. (**C**) Quantification and western blot analysis of REST protein levels upon knockdown of SIRT1 in undifferentiated SH-SY5Y cells. Analysis was performed with five biological repeats; representation of only two biological repeats is shown here. (**D** and **E**) mRNA expression of *REST* isoforms (**D**) Exon 1 and (**E**) Exon 4 upon treatment with different concentrations of NR for 24 h in undifferentiated SH-SY5Y cells. Data shown from three biological repeats. (**F**–**H**) mRNA expression of *REST* isoforms Exon 1 (**G**) and Exon 4 (**H**), and *SIRT1* (**F**), when SIRT1 is knocked-down and upon treatment with vehicle or 2 mM NR for 24 h in undifferentiated SH-SY5Y cells. Representation of three biological repeats. (**I** and **J**) ChIP-qPCR assays within the promotor region of *REST* in differentiated SH-SY5Y cells, treated with 1 mM NR 24 h, with (**I**) SIRT1 and (**J**) H3K9Ac. Data shown are from two biological repeats. (**K**) Correlation between the levels of SIRT-1 and H3K9Ac in the different promoter regions of *REST* upon NR treatment. (**L**) Schematic diagram showing the NAD+/SIRT1-REST axis proposed in this study. Quantitative data are presented as mean ± standard error of the mean. Statistical analysis performed by one-way ANOVA with Tukey's multiple comparison test (**A** and **C**–**J**), and simple linear regression (**K**). ns = not significant, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. REST = Repressor Element 1-Silencing Transcription factor.

developed. Nonetheless, the cognitive results were also reflected at the tissue level, as the increase in REST in the HIP led to decreased levels in both p-Tau and Aβ plaques in this area. These results are similar to those recently published by the Yankner laboratory[19] where, in other murine AD strains, REST overexpression reduced amyloidosis and tau pathology.

Based on our RNA-sequencing results, the restoration of cognitive function in AD mice overexpressing REST was primarily due to the restoration of expression levels of genes involved in synaptic plasticity, vesicular trafficking and stress response. Additionally, we observed an upregulation of genes related to telomerase maintenance, which might explain why *C. elegans* with REST overexpression live longer, since telomere length is positively associated with healthy ageing.[65] Although REST appears to have this positive effect in AD through the modulation of synapses and stress response, the molecular mechanisms underlying its effects remain poorly understood, and these seem to have rather complex regulation.[66,67] While REST is mainly known as a transcriptional repressor, its overexpression may indirectly upregulate certain genes, such as those repressing negative regulators (i.e. microRNA), modulating transcription factor networks, or enhancing transcriptional activators like PGC-1α. However, these pathways require further experimental validation.[20,68-72]

In this study, we provided new insights into the molecular regulation of REST, demonstrating that SIRT1 regulates REST at transcriptional level. SIRT1 requires NAD^+ to function, a key metabolite that is decreased in AD and other neurodegenerative diseases.[12] We observed that NR significantly increased nuclear REST levels in AD, which in turn enhanced the expression of REST target genes associated with synaptic plasticity, reaching values close to those of WT mice. Thus, NAD^+ supplementation increased the nuclear localization of REST, suggesting that it was activated and performing its function, similar to other transcription factors that translocate to the nucleus when active.[73-76]

While our study highlighted a previously unexplored potential NAD^+/SIRT1-REST-mitophagy axis in AD aetiology, there are further questions to be explored. First, the mechanisms by which SIRT1 upregulates *REST* transcription are still largely unknown. Thus, while our data suggest that enhancing NAD^+-SIRT1 signal upregulates *REST* Exon 1 expression, it remains unclear whether this occurs through a direct mechanism (deacetylating H3K9 at one of REST promoter regions), an indirect pathway (e.g. by repressing another repressor, such as a microRNA), or the existence of both effects of SIRT1 on *REST* Exon 1 expression. These questions remain unanswered and need to be studied further. Second, while SIRT1 deacetylates histones normally, leading to increased expression of the targeted gene(s), how could it be upregulating REST? In the *REST* promoter region selected for this study, NR decreased H3K9 acetylation, implying chromatin remodelling. Typically, chromatin deacetylation is associated with decreased gene expression[77]; however, in genes with complex promoter architecture (e.g. canonical and alternative promoters), deacetylation can be linked to isoform-specific regulation rather than transcriptional repression.[16] This has been demonstrated for genes like *BDNF* and *PGC-1α*, where different promoter regions are used depending on tissue type and metabolic state to express specific isoforms.[78,79] In the case of REST, transcription is regulated by at least three promoter regions, including one located within an intron, and possibly by microRNAs.[80] Therefore, the observed decrease in acetylation at a specific promoter by SIRT1 after NR treatment does not contradict the fact that SIRT1 could also increase REST expression. Instead, H3K9 deacetylation may reflect a shift in promoter activation or isoform preference. This is further supported by evidence showing that SIRT1 overexpression can differentially modulate histone acetylation at various promoter regions across the genome. In at least two cases, increased gene-specific transcriptional outcomes occur in parallel with the deacetylation of its specific promoter regions.[81] Third, how are different REST isoforms regulated by NAD^+ in neurons and what are their differential roles in AD? The fact that NAD^+ increases the transcription of exons 1, 2 and 3 but not exon 4 of *REST* supports the idea that changes in chromatin conformation at the studied promoter region are related to changes in the expression of specific *REST* isoforms. Although different *REST* isoforms have been described, the exact roles of most of them are not clearly understood.[16] Of note, there is a REST isoform, REST4, which lacks exon 4 and appears to play a crucial role in the activation of neuron-specific genes, potentially having a neuroprotective function.[82] Importantly, it is likely that REST4 could complement and sometimes also compete with full-length REST in modulating the expression of a broad spectrum of genes and protein activities.[82-84] Future studies on how cells regulate all the REST proteins, and their independent roles in neuronal function and brain diseases are needed. Fourth, it is important to check the pathological effects of overexpression of *REST* exon 1 (and exon 2, exon 3, and with different combinations) in AD models. Additionally, could other NAD^+-dependent SIRTs, such as SIRT3 (important for mitophagy and mitochondrial homeostasis),[85] SIRT6 (DNA repair and healthy longevity)[86,87] and SIRT7 (DNA repair and epigenetic regulation)[88] be involved in the NAD^+-REST-mitophagy axis? Finally, how a defective NAD^+/SIRT1-REST-mitophagy axis exists in AD iPSC-derived cortical neurons (as well as in glial cells and organoids, compared with isogenic controls), and its relation to AD genetic mutation risks factors (e.g. *APP* and *PS1/2* mutations, as well as *APOE4* genotype) and gender (male and female) still needs to be explored.

Here we have presented an NAD^+/SIRT1-REST axis influencing mitophagy and neuronal survival, as well as health span and lifespan. Better understanding of the NAD^+/SIRT1-REST axis could be highly relevant, as treatments with NAD^+ precursors for neurodegenerative diseases have already passed the initial phases of clinical trials.[89-91] A deeper knowledge on the functions of this NAD^+/SIRT1-REST axis in health and brain function is necessary.

Data availability

All data are available in the Supplementary material and GEO database. Data that support the findings of this study are available from the corresponding author upon request.

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Competing interests

E.F.F. is a co-owner of Fang-S Consultation AS (Organization number 931 410 717) and NO-Age AS (Organization number 933 219 127). He has an MTA with LMITO Therapeutics Inc (South Korea), a CRADA arrangement with ChromaDex (USA), a commercialization agreement with Molecule AG/VITADAO, and MTAs with GeneHarbor (Hong Kong) Biotechnologies Limited and Hong Kong Longevity Science Laboratory (Hong Kong). He is a consultant to MindRank AI (China), NYO3 (Norway), AgeLab (Vitality Nordic AS, Norway), and Hong Kong Longevity Science Laboratory (Hong Kong). The other co-authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

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