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SIRT1 deacetylase protects against neurodegeneration in models for Alzheimer's disease and amyotrophic lateral sclerosis

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A progressive loss of neurons with age underlies a variety of debilitating neurological disorders, including Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS), yet few effective treatments are currently available. The SIR2 gene promotes longevity in a variety of organisms and may underlie the health benefits of caloric restriction, a diet that delays aging and neurodegeneration in mammals. Here, we report that a human homologue of SIR2, SIRT1, is upregulated in mouse models for AD, ALS and in primary neurons challenged with neurotoxic insults. In cell-based models for AD/tauopathies and ALS, SIRT1 and resveratrol, a SIRT1-activating molecule, both promote neuronal survival. In the inducible p25 transgenic mouse, a model of AD and tauopathies, resveratrol reduced neurodegeneration in the hippocampus, prevented learning impairment, and decreased the acetylation of the known SIRT1 substrates PGC-1alpha and p53. Furthermore, injection of SIRT1 lentivirus in the hippocampus of p25 transgenic mice conferred significant protection against neurodegeneration. Thus, SIRT1 constitutes a unique molecular link between aging and human neurodegenerative disorders and provides a promising avenue for therapeutic intervention.

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Introduction

Although neurodegenerative disorders are relatively cell type specific, many of the underlying pathogenic processes are similar, including protein misfolding, oxidative stress, cytoskeletal abnormalities, disruption of calcium homeostasis, and inflammation, all of which increase during aging (Bossy-Wetzel *et al*, 2004; Forman *et al*, 2004; Selkoe, 2004). The existence of related mechanisms underlying neurodegeneration raises the possibility of developing a class of therapeutic interventions that treat a variety of neurological disorders by activating the body's own defenses against age-related deterioration and cell death (Bossy-Wetzel *et al*, 2004; Forman *et al*, 2004; Selkoe, 2004). Studies from yeast identified the evolutionarily conserved NAD^+-dependent deacetylase Sir2 as a critical regulator of the aging process (Kaeberlein *et al*, 1999; Imai *et al*, 2000; Anderson *et al*, 2003a, b; Howitz *et al*, 2003; Cohen *et al*, 2004b). An additional copy of the *SIR2* gene extends lifespan in yeast and metazoans by a process seemingly analogous to caloric restriction (Lin *et al*, 2000; Anderson *et al*, 2003a, b), a diet that delays diseases of aging in mammals including neurodegeneration (Luo *et al*, 2001; Vaziri *et al*, 2001; Langley *et al*, 2002; Howitz *et al*, 2003; Brunet *et al*, 2004; Cohen *et al*, 2004a, b; Motta *et al*, 2004; Qin *et al*, 2006). Mammals possess seven Sir2 homologues (SIRT1-7) whose biological functions remain poorly defined. The SIRT1 gene is believed to provide cell protection during times of cell stress (Brunet *et al*, 2004; Cohen *et al*, 2004a, b; Chen *et al*, 2005; Tang, 2006). Consistent with this, knockdown of the *SIRT1* gene in cultured mouse dorsal roots ganglion sensory neurons abrogates the protective effects of increased NAD^+ synthesis on axonal degeneration following acute axotomy (Araki *et al*, 2004). On the other hand, a recent study suggests that SIRT1 is not required for NAD-dependent protection, rendering the role of SIRT1 in peripheral axotomy unclear (Wang *et al*, 2005). Furthermore, Sir2 seems to block extreme lifespan in post-mitotic cells in yeast, raising the possibility that SIRT1 may play a dual role in the CNS (Fabrizio *et al*, 2005). Most importantly, the role of SIRT1 *in vivo* in age-dependent chronic neurodegenerative disorders remains undefined.

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Results

Levels of SIRT1 in models of neurodegeneration

We hypothesized that SIRT1 levels may increase as a protective response to neurodegenerative conditions and examined levels of SIRT1 in various mouse models for human age-dependent neurodegeneration. Mice inducibly overexpressing a toxic coactivator of cyclin-dependent kinase 5 (CDK5), p25, display massive degeneration of forebrain with features of AD (Cruz *et al*, 2003, 2006), whereas transgenic mice expressing a mutant form of superoxide dismutase 1 (SOD1G37R), which has been linked to human amyotrophic lateral sclerosis (ALS), exhibit severe motor neuron and axon degeneration in spinal cord (Gurney *et al*, 1994; Wong *et al*, 1995). Interestingly, in the forebrains of p25 transgenic mice ($n=9$), SIRT1 protein levels increased as early as 2 weeks after p25 induction and persisted throughout the progression of the pathology to 12 weeks (Figure 1A and B). In accordance with increased protein levels of SIRT1, there was a decrease in the acetylation state of PGC-1alpha, a target for SIRT1 deacetylase activity (Nemoto *et al*, 2005; Rodgers *et al*, 2005; St-Pierre *et al*, 2006) (Figure 1C). In the spinal cords of mutant SOD1G37R mice, SIRT1 was only slightly upregulated at 4 months ($n=4$), a stage with limited degeneration; however, levels of SIRT1 were significantly upregulated when severe neurodegeneration was evident at 10–12 months ($n=8$) (Nguyen *et al*, 2001) (Figure 1D and E). Mice expressing a mutant form of amyloid precursor protein (APP) linked to Familial AD (PDAPP-V717F, $n=7$; 2–12 months) (Games *et al*, 1995) do not exhibit significant neuronal loss, although they display, in an age-dependent manner, substantial β-amyloid plaques, a hallmark of AD (Games *et al*, 1995). These mice showed no significant increase in SIRT1 in the forebrain (Supplementary Figure 1). Together, these results indicate that SIRT1 levels correlate with neurodegeneration accompanied by progressive and severe loss of neurons, but not with β-amyloid plaque pathology in the absence of neuronal loss. In all the mouse models analyzed, as well as in human brains, SIRT1 is not only enriched in the nucleus but also localized in the cytoplasm (Supplementary Figure 2; unpublished data).

Since p25 and mutant SOD1 trigger disruption of calcium homeostasis and generate oxidative stress (Bruijn *et al*, 2004; Cruz and Tsai, 2004), we tested whether SIRT1 is induced in neurons in response to ionomycin (1 μM), a calcium ionophore, or hydrogen peroxide (H_2O_2) (25 μM), a free radical generator. These specific stresses have previously been shown to trigger the deterioration of neuronal morphology and the formation of p25 in cultured neurons (Kusakawa *et al*, 2000; Lee *et al*, 2000; Nath *et al*, 2000). Treatment of primary cortical neurons with either ionomycin or H_2O_2 rapidly induced SIRT1 protein expression, and did so in a dose-dependent manner (Figure 1F and G). Thus, SIRT1 is not only induced in mouse models of neurodegeneration but also in primary cultured neurons under neurotoxic stresses.

Resveratrol-mediated SIRT1 activation protects against p25 and mutant SOD1

To understand the physiological significance of SIRT1 activation in context of p25 and mutant SOD1 toxicity, we first tested the effects of resveratrol, a polyphenolic SIRT1-activating compound (STAC) (Howitz *et al*, 2003), on the viability of primary mouse neurons overexpressing p25 or a mutant form of SOD1 linked to ALS (SOD1G93A). The SOD1G93A mutation, which has been linked to ALS, has been widely used in primary neurons to examine neurotoxicity related to ALS. Doses of up to 500 nM resveratrol showed no evidence of toxicity to primary neurons transfected with GFP (Figure 2A). As previously reported (Patrick *et al*, 1999; Lee *et al*, 2000; Zhang *et al*, 2002; Hamdane *et al*, 2003), transfection with p25-GFP resulted in a high degrees of cell death (54% after 24 h), which were scored on the basis of neuritic integrity and nuclear morphology, as described in Materials and methods (Figure 2B and C). Remarkably, resveratrol treatment significantly reduced the extent of cell death caused by p25 (Figure 2B and C) (54 versus 27%; $P(T\leqslant t)$ two tails: 0.003). The protective effect of resveratrol against p25-mediated toxicity was further confirmed by examination of propidium iodide uptake as a marker for loss of membrane integrity and viability (Supplementary Figure 3A and B). In this experiment, resveratrol treatment reduced p25-induced propidium uptake (37 versus 18.2%; $P(T\leqslant t)$ two tails: 0.007). We also examined whether resveratrol protected against neurotoxicity elicited by expression of SOD1G93A. Approximately 52% of primary neurons transfected with mutant SOD1G93A for 48 h exhibited cytoskeletal disruption and SOD1 aggregates, two hallmarks of ALS-associated SOD1 toxicity (Figure 2D and E). Resveratrol treatment significantly attenuated SOD1G93A-mediated neurotoxicity (52 versus 28%; $P(T\leqslant t)$ two tails <0.001) (Figure 2E). These results are in line with a recent report showing that in cultured neurons derived from transgenic mice overexpressing a mutant (109Q) huntingtin, resveratrol suppressed the neurotoxic effects of the mutant protein (Parker *et al*, 2005). In addition, we examined whether the acetylation of PGC-1alpha was decreased following resveratrol treatment. Indeed, we observed a decrease in PGC-1alpha (Supplementary Figure 4), suggesting that SIRT1 activity is increased following resveratrol treatment of primary neurons.

Deacetylase activity of SIRT1 confers neuroprotection against p25 and mutant SOD1

To directly verify the protective role of SIRT1 in neurodegeneration, we transfected primary neurons with p25-GFP or SOD1G93A, together with either SIRT1 or SIRT1 lacking catalytic activity (H363Y). Overexpression of SIRT1, but not H363Y, significantly rescued the rate of p25-GFP-mediated cell death (54 versus 28.7%; $P<0.01$; 54 versus 56.5%; $P>0.05$; one-way ANOVA with *post hoc* Newman–Keuls Multiple Comparison Test) (Figure 3A and B). The morphology of the p25-GFP/SIRT1-overexpressing neurons appeared normal and indistinguishable from control GFP-transfected neurons. This protective effect was unlikely to be an effect of SIRT1 on the stability of p25-GFP, because similar levels of p25-GFP were detected in the presence or absence of SIRT1 overexpression in CAD cells (Figure 3C). Similarly, a neuroprotective effect of SIRT1, but not the H363Y mutant, was observed when examining p25-induced propidium iodide uptake (37 versus 17.7%; $P<0.05$; 37 versus 41.6%; $P>0.05$; one-way ANOVA with *post hoc* Newman–Keuls Multiple Comparison Test) (Supplementary Figure 3A and C).

We also sought to determine whether SIRT1 overexpression protected against mutant SOD1-induced neurotoxicty. The overexpression of SIRT1, but not H363Y, protected

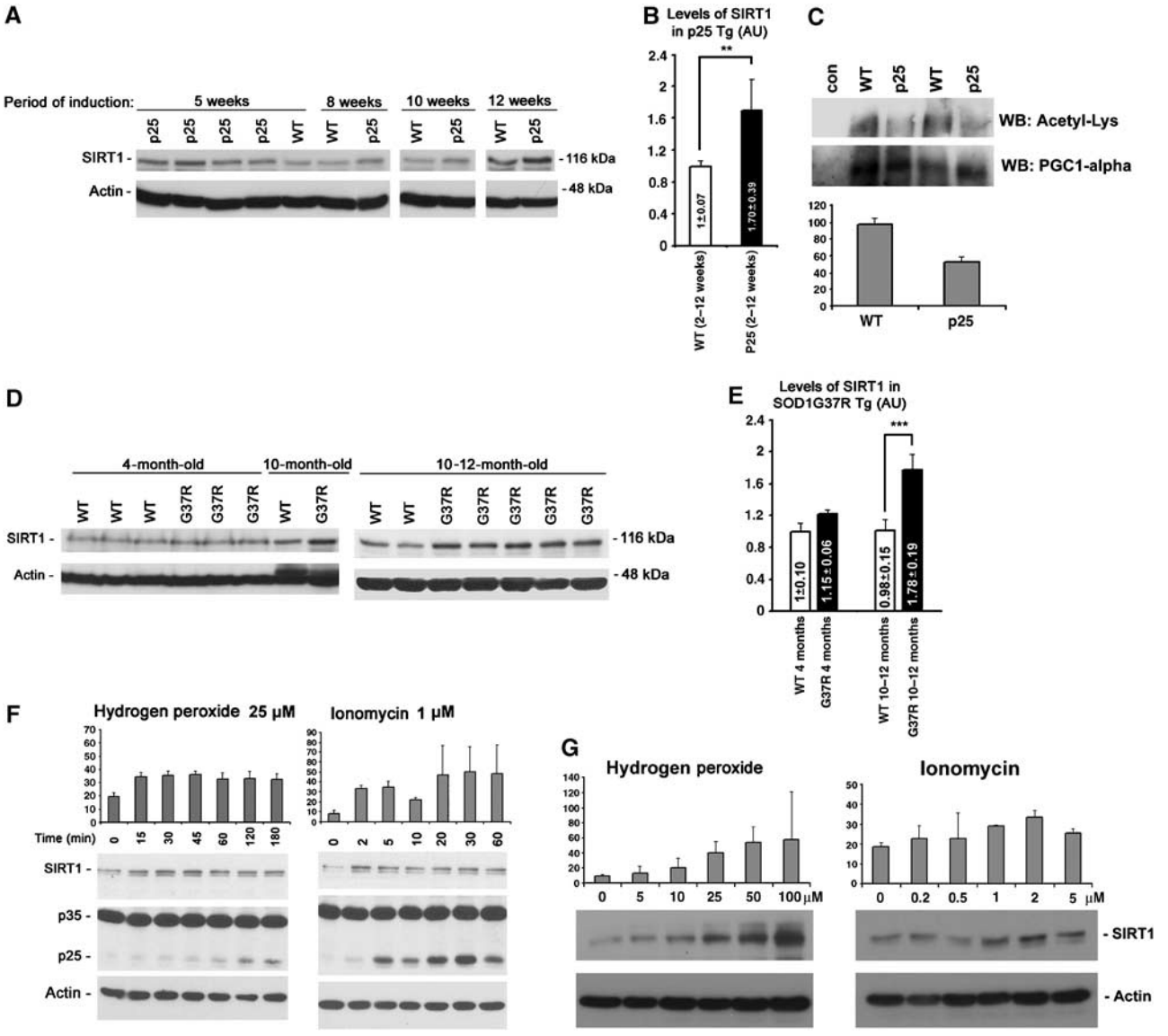


Figure 1 Upregulation of SIRT1 in mouse models displaying progressive and severe neurodegeneration. (**A**) Upregulation of SIRT1 in p25 transgenic mice during progressive neurodegeneration (after 2–12 weeks of induction). (**B**) Quantifications of levels of SIRT1 in p25 transgenic mice. $^{**}P(T \leqslant t)$ two tails: 0.007. (**C**) Acetylation levels of PCG-1alpha is decreased in forebrains of p25 transgenic mice (10–12 weeks of induction). Densitometry-based analyses of acetylated PCG-1alpha levels is shown in the lower panel. (**D**) Progressive increase of SIRT1 in mutant SOD1G37R (line 29) peaking at stage of massive neurodegeneration (10–12 months). (**E**) Quantifications of levels of SIRT1 in SOD1G37R mice. $^{***}P(T \leqslant t)$ two tails: 0.0004. (**F**) Treatment of primary cortical neurons with low concentrations of ionomycin (1 μM) and H_2O_2 (25 μM) induces rapid upregulation of SIRT1 associated with generation of p25. Time expressed in minutes. Top panels show densitometry-based analyses of SIRT1 levels. (**G**) Treatment of primary cortical neurons with increasing doses of ionomycin or H_2O_2 for 20 min demonstrates a dose-dependent induction of SIRT1 by these neurotoxic stimuli. Top panels show densitometry-based analyses of SIRT1 levels.

against SOD1G93A toxicity (52 versus 32.3%; $P<0.001$; 52 versus 48%; $P>0.05$; one-way ANOVA with *post hoc* Newman–Keuls Multiple Comparison Test) (Figure 3D and E). Together, these results indicate that increased levels of SIRT1 in primary neurons confer protection against neurotoxicity induced by p25 or mutant SOD1. The observation that the H363Y mutant did not confer protection demonstrates that the deacetylase activity of SIRT1 is required for neuroprotection. Interestingly, siRNA-mediated knockdown of SIRT1 in primary neurons was not neurotoxic *per se* and did not result in increased sensitivity of neurons to neurotoxic stimuli (Supplementary Figure 5). This suggests that in contrast to the survival benefit of increased SIRT1 activation, SIRT1 knockdown *per se* does not sensitize neurons to acute neurotoxic stimuli; alternatively, other SIRT family members may compensate following loss of SIRT1 function.

Resveratrol prevents neurodegeneration and cognitive decline in p25 transgenic mice

To test the neuroprotective effects of resveratrol *in vivo*, resveratrol (Resv) or vehicle (Veh) was introduced by intracerebroventricular (ICV) injection in 2 week induced p25 mice for 3 weeks at a dose of 5 μg/μl, 0.5 μl bilateral injections injected every 2–3 days (Veh-treated animals, $n=5$; Resv-treated animals, $n=9$) (Figure 4A). We determined that levels of acetylation of the SIRT1 substrate PGC-1alpha were

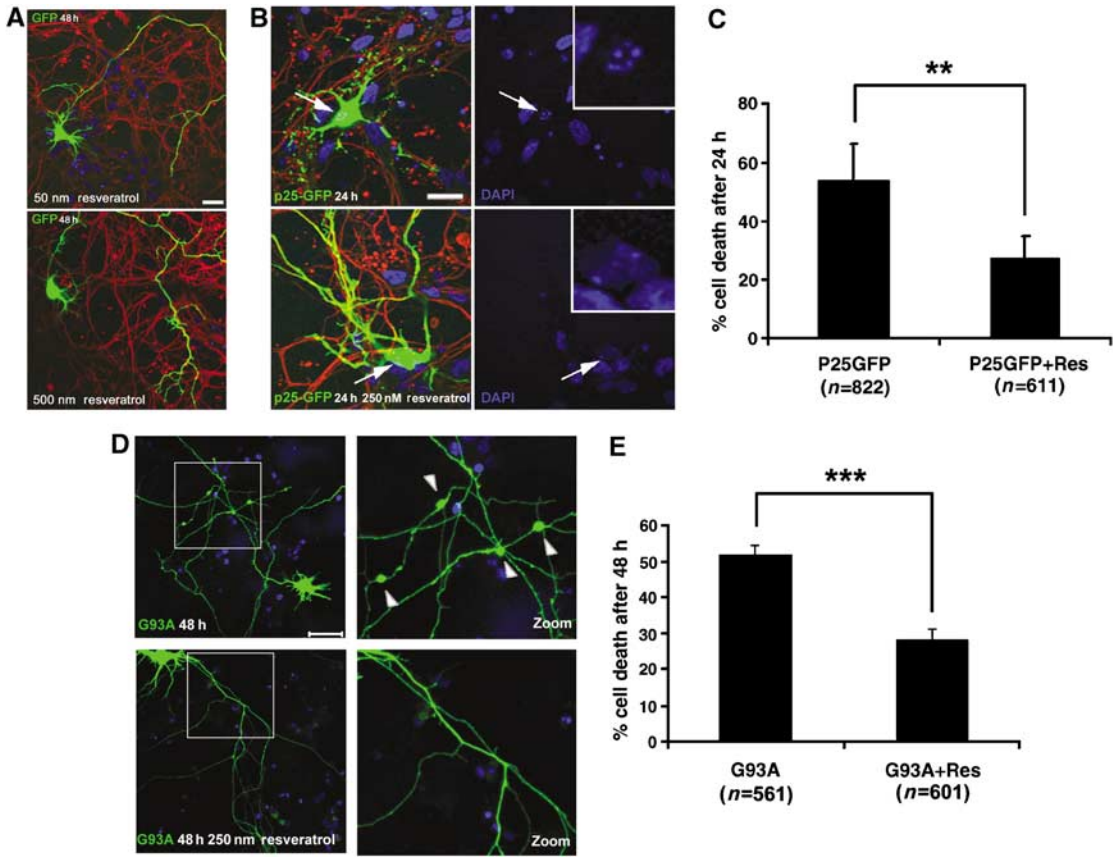


Figure 2 Resveratrol protects against p25 and mutant SOD1 toxicity. (**A**) No toxicity observed in GFP-transfected neurons treated with resveratrol for 48 h (50–500 nM). For all experiments (A–E), primary rat neurons were transfected at DIV 5–7 with plasmids, and resveratrol was added to the medium at 3 h after transfection. Characterization of neuronal integrity was performed 24–48 h after transfection. Scale bar, 40 μm. (**B**) Representative confocal images of dying and healthy neurons transfected with p25-GFP, and untreated or treated with resveratrol (250 nM) for 24 h, respectively. Inset in DAPI-only panel is a magnification of the nucleus of the transfected neuron, as indicated by arrow. Scale bar, 20 μm. (**C**) Quantifications of p25-GFP-induced cell death in neurons untreated or treated with 250 nM resveratrol for 24 h expressed in percent (%) (54 ± 12.2 versus $27 \pm 8\%$; $^{**}P(T \leqslant t)$ two tails: 0.003). (**D**) Representative confocal images of neurons transfected with SOD1G93A-FLAG, and untreated or treated with 250 nM resveratrol for 48 h. Right panels are magnifications of the boxed area shown in the left panel. Arrowheads indicate regions of SOD1 aggregation. Scale bar, 50 μm. (**E**) Quantifications of SOD1G93A-FLAG-induced cell death in neurons untreated or treated with 250 nM resveratrol for 48 h expressed in percent (%) (52 ± 3 versus $28 \pm 3\%$; $^{***}P(T \leqslant t)$ two tails: <0.001).

decreased in these resveratrol-treated animals compared to the vehicle-treated controls, suggesting that ICV injection of resveratrol resulted in the activation of SIRT1 (Figure 4B). After 5 weeks of p25 induction, cell death and neurodegeneration were evident in the hippocampus of the vehicle-treated animals, consistent with previous observations (Cruz *et al*, 2003). In contrast, administration of resveratrol reduced neurodegeneration in CA1 and CA3 regions of the hippocampus, as revealed by lower levels of the apoptotic marker activated caspase 3, and a marker of astrogliosis, GFAP (Figure 4C–E). Of note, GFP immunostaining, which labels p25-GFP expressing neurons, was more robust in the hippocampus of resveratrol-treated animals, suggesting the neurons were better able to tolerate and survive p25 expression (Figure 4E). We previously reported that by 4–6 weeks of induction, p25 mice have dramatically decreased associative learning capability, as revealed by contextual fear conditioning paradigm (Fischer *et al*, 2005). Associative learning was significantly rescued in p25 mice treated with resveratrol for 3 weeks, in comparison to the vehicle group or untreated p25 mice (Figure 4F). Together, these results show that resveratrol provides neuroprotection and prevents cognitive decline in an animal model of CNS degeneration that features massive neuronal loss and tau pathologies.

Deacetylation of p53, a SIRT1 target, by resveratrol protects against p25 toxicity

Next, we sought to gain insights into the mechanism by which resveratrol confers neuroprotection *in vivo*. We hypothesized that the tumor suppressor p53, an important mediator of cell death, may, play an important role in mediating neuroprotection for the following reasons. First, p25/Cdk5 is known to phosphorylate p53 and upregulate its transcriptional activity (Zhang *et al*, 2002). Second, we found that p53 protein levels are significantly increased in p25 transgenic mice (Figure 5A). Third, this increase is accompanied by an increase in the acetylation status of lysine 382 of p53 (Figure 5B), a modification known to stabilize p53 and potentiate its apoptotic function (Luo *et al*, 2001; Vaziri *et al*, 2001; Langley *et al*, 2002). Fourth, lysine 382 of p53 (K382-p53) is a well-characterized target for SIRT1 deacetylase activity, and activation of SIRT1 by resveratrol would be consistent with the neuroprotective effects we observed *in vivo*.

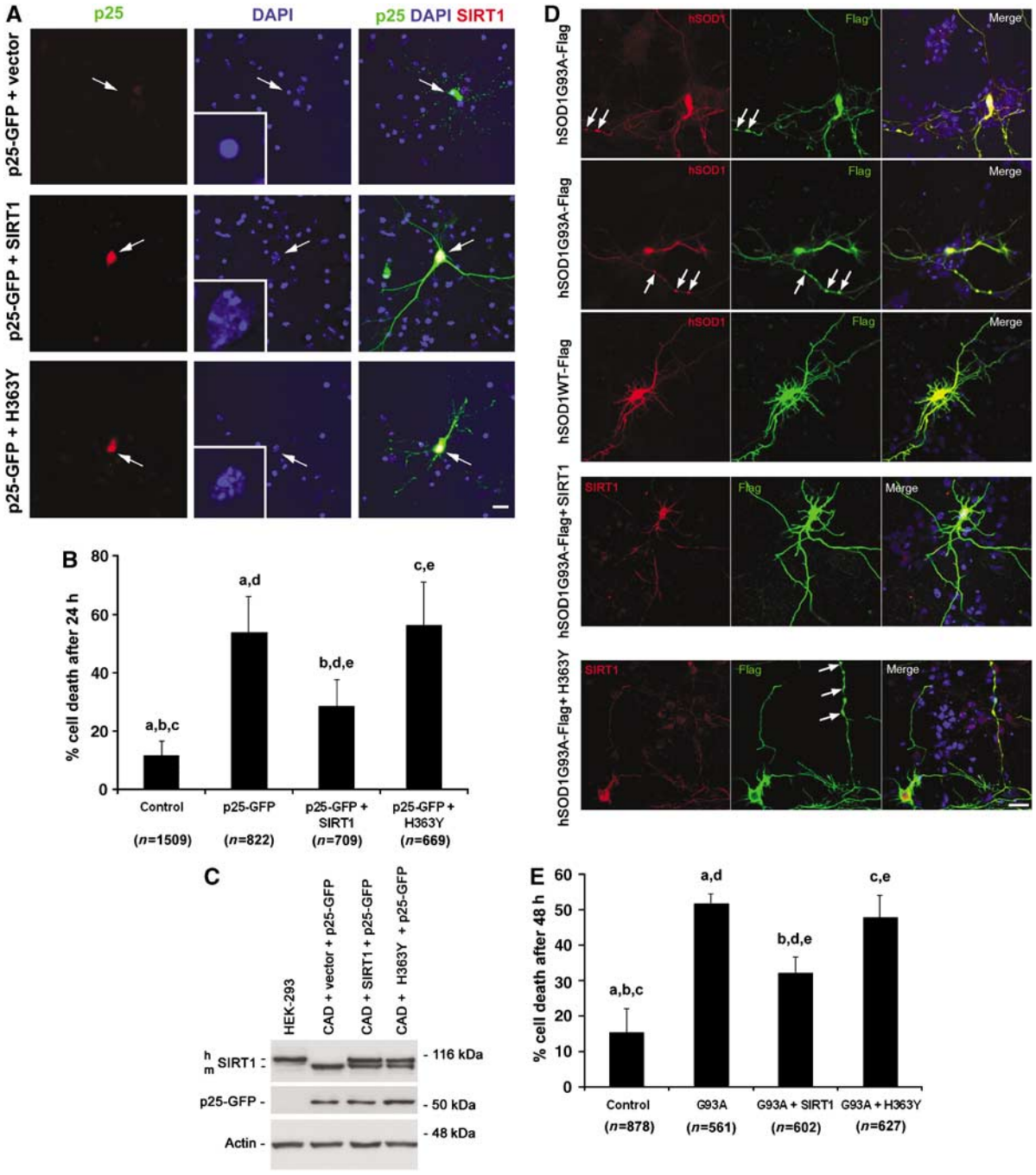


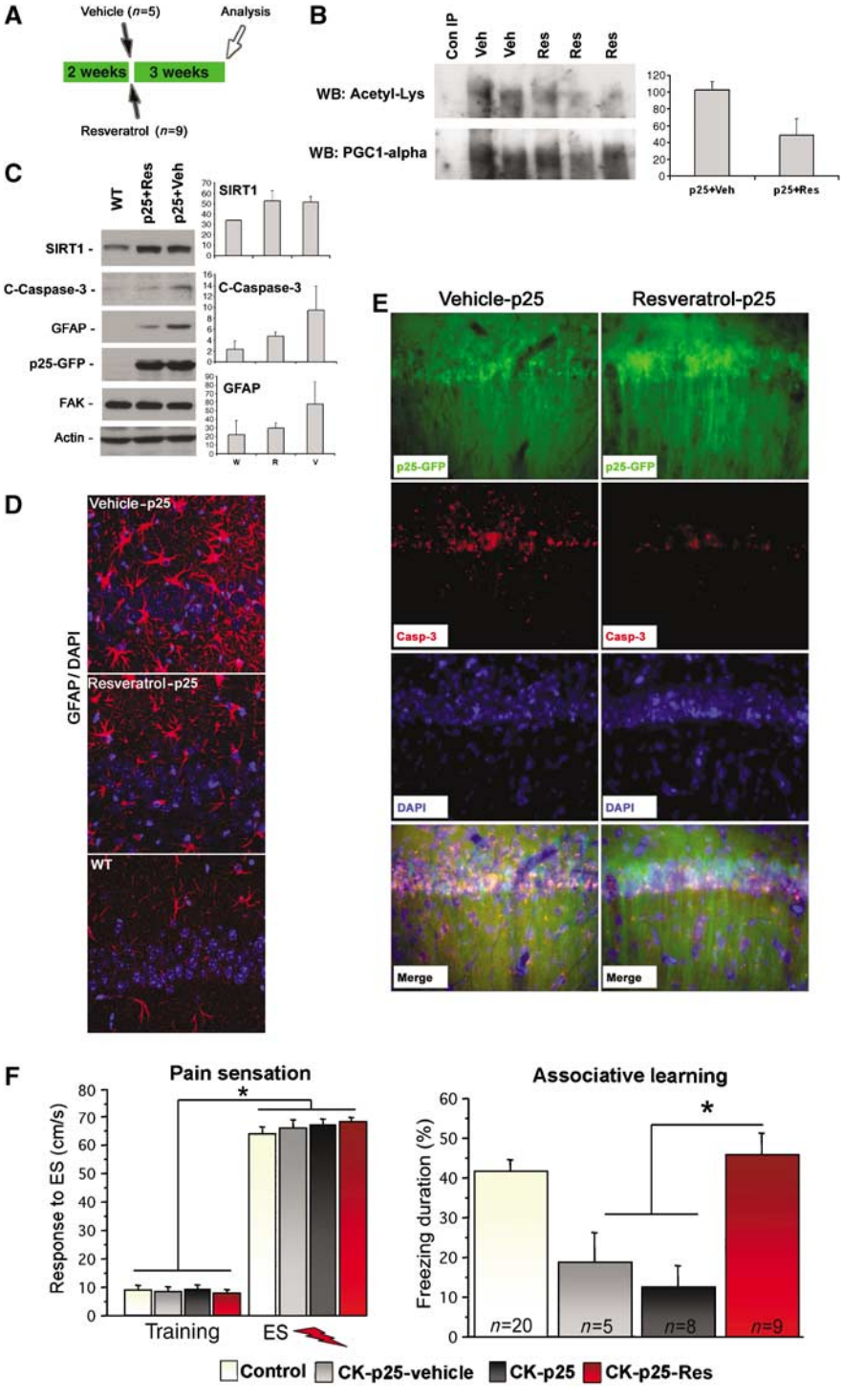
Figure 3 Overexpression of SIRT1 protects against p25 and mutant SOD1 toxicity. (**A**) Effects of overexpression of SIRT1 or SIRT1 lacking catalytic deacetylase activity (H363Y) on p25 GFP toxicity. Arrows indicate neurons transfected with p25-GFP with or without SIRT1 (red). Inset in DAPI-only panel is a magnification of the nucleus of the transfected neuron, as indicated by arrow. Scale bar, 20 μm. (**B**) Quantifications of cell death in p25-GFP expressing neurons with or without ectopic expression of SIRT1 or H363Y. a, control versus p25-GFP, $P<0.001$; b, control versus p25-GFP + SIRT1, $P<0.05$; c, control versus p25-GFP + H363Y, $P<0.001$; d, p25-GFP versus p25-GFP + SIRT1, $P<0.01$; e, p25-GFP + SIRT1 versus p25-GFP + H363Y, $P<0.01$. p25-GFP versus P25GFP + H363Y is non-significant ($P>0.05$). One-way ANOVA with Neuman–Keuls Multiple Comparison Test. (**C**) Unchanged levels of p25-GFP following expression of SIRT1 or H363Y. h, human; m, mouse. Comparison of HEK and CAD cells for SIRT1 expression. (**D**) Effects of overexpression of SIRT1 or H363Y on SOD1G93A toxicity. Arrows indicate to SOD1 aggregates as detected with FLAG Ab. WT SOD1 is not toxic. Scale bar, 25 μm. (**E**) Quantifications of cell death in SOD193A and WT SOD1-expressing neurons with or without ectopic expression of SIRT1 or H363Y. a, control versus G93A, $P<0.001$; b, control versus G93A + SIRT1, $P<0.001$; c, control versus G93A + H363Y, $P<0.001$; d, G93A versus G93A + SIRT1, $P<0.001$; e, G93A + SIRT1 versus G93A + H363Y, $P<0.001$. G93A versus G93A + H363Y is non-significant ($P>0.05$). One-way ANOVA with Neuman–Keuls Multiple Comparison Test.

We first tested whether p53 contributes to p25-induced cell death by cotransfecting cortical neurons with p25-GFP, together with either a control siRNA vector or p53 siRNA vector. Knockdown of p53 provided a 25% increase in cell survival (Figure 5C), which is similar to what we observed for resveratrol-treated neurons (Figure 2). Next, we asked whether resveratrol had a similar effect *in vivo*. As shown in Figure 5E, the acetylation status of K382-p53 was lower in

resveratrol-treated hippocampal tissue of p25 transgenic mice, relative to vehicle. Overall p53 levels were also decreased by resveratrol treatment, which is consistent with the deacetylated form being less stable (Luo *et al*, 2001).

Injection of SIRT1 lentivirus into the CA1 of p25 transgenic mice protects against neurodegeneration

Finally, to directly ascertain a role for SIRT1 in neuroprotection *in vivo*, we introduced lentivirus carrying HA-tagged SIRT1, or control virus, into the hippocampus of p25 mice ($n=4$; SIRT1-injected and control-injected hemispheres compared) by stereotaxic injection, as described in Materials and methods. Briefly, 2 week induced p25 transgenic mice were subjected to a single injection of control or SIRT1 expressing virus in each side of the brain. Mice were killed 3 weeks after the viral injections. GFP immunofluorescence staining showed that GFP-positive p25-expressing neurons are more prominent in the CA1 regions receiving the SIRT1 virus, compared to those receiving the control virus (Figure 6A–E), indicating that they can tolerate higher levels of p25, as seen in resveratrol-treated animals (Figure 4). In general, the SIRT1 virus-treated side exhibited 38±13%



higher number of neurons than the control virus-treated side (Figure 6A; Supplementary Table 1), and the neurons were morphologically healthier. At higher magnification, it was clear that the surviving neurons expressed SIRT1, as detected by co-immunostaining with HA and GFP antibodies (Abs) (Figure 6F–H). These results provide further evidence that that resveratrol's protective effects are due to SIRT1 activation and demonstrate a neuroprotective role of SIRT1 *in vivo*.

Discussion

Collectively, our results demonstrate that it is possible to slow *in vitro* cell death as well as *in vivo* neurodegeneration and cognitive decline with resveratrol, a SIRT1-activating molecule, and by expression of SIRT1. We also provide evidence that the neuroprotective effect is due, at least in part, to deacetylation of K382-p53 (Figure 5). We do not rule out the possibility that other known substrates of SIRT1 are involved, such as Ku70, a protein that sequesters the apoptotic protein Bax from mitochondria (Brunet *et al*, 2004; Cohen *et al*, 2004a). Resveratrol may also stimulate the deacetylation of FOXO3/4 transcription factors, thereby enhancing gene expression of antioxidative molecules and upregulating DNA repair (Nguyen *et al*, 2002; Brunet *et al*, 2004; Smith *et al*, 2004). Another interesting candidate target for SIRT1 deacetylation activity is PGC-1alpha, which we have shown for the first time to be deacetylated in a model for neurodegeneration (p25 Tg) and in the brain, in response to resveratrol treatment. PGC-alpha activity was recently shown to play an important role in neuronal metabolism and detoxification of reactive oxygen species (St-Pierre *et al*, 2006), and our finding suggests that deacetylation and activation PGC-1-alpha by enhanced SIRT1 activity may also be involved in

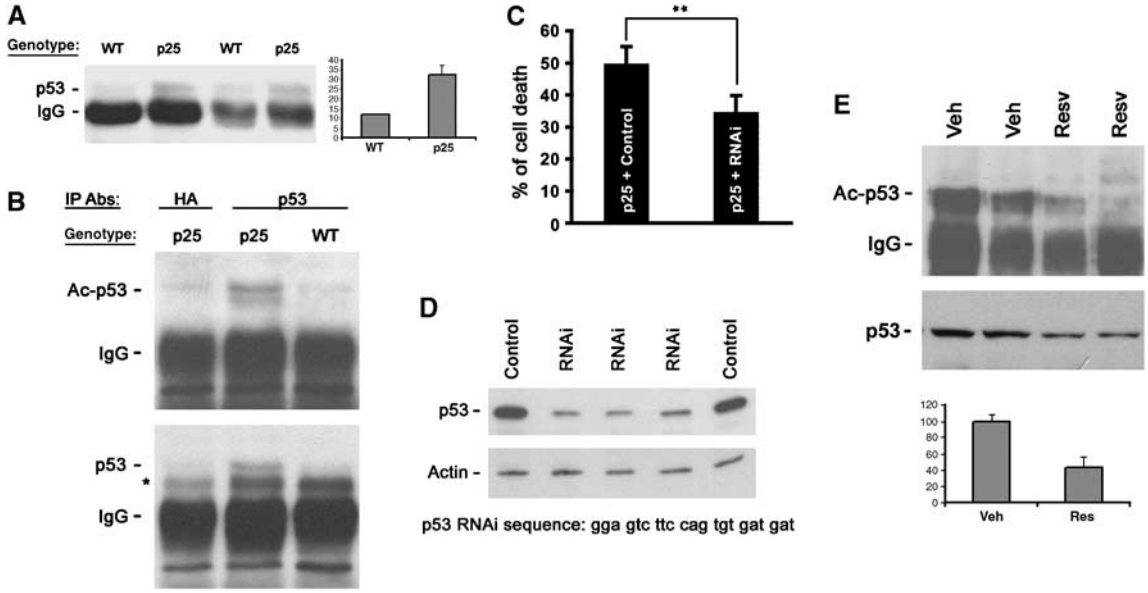


Figure 5 Acetylation of p53, a SIRT1 substrate, in p25 transgenic mice reversed by resveratrol. (**A**) Upregulation of p53 in p25 transgenic mice ($n=4$) detected by immunoprecipitation followed by Western blot. Densitometry analyses of p53 levels are shown on right. (**B**) Acetylation of p53 at lysine 382 in p25 transgenic mice ($n=3$) detected by immunoprecipitation, followed by Western blot. * Indicates nonspecific band. (**C**) P53 knockdown in p25-expressing primary hippocampal neurons rescues p25 neurotoxicity by 25%. $^{**}P(T\leqslant t)$ two tails: 0.001. (**D**) Efficient knockdown of p53 by RNAi in cell line transfected with p53. (**E**) Reduced acetylation of p53 at lysine 382 and downregulation of p53 in p25 transgenic mice ($n=3$) treated with resveratrol. Densitometry analyses of acetylated p53 levels is shown in the bottom panel.

Figure 4 Resveratrol prevents neurodegeneration in p25 transgenic mice. (**A**) Experimental design for ICV injection of resveratrol (Resv) or vehicle (Veh) in p25 transgenic mice ($n=5$ for vehicle (Veh); $n=9$ for resveratrol (Resv)). (**B**) Acetylation levels of PGC-1alpha is decreased in p25 animals treated with resveratrol, compared to p25 animals injected with vehicle. Shown also is densitometry-based analysis of acetylated PCG-1alpha levels. (**C**) Downregulation of activated caspase 3 and GFAP, markers of cell death and astrogliosis, in the hippocampus of p25 transgenic mice treated with resveratrol ($n=3$), compared to p25 animals injected with vehicle ($n=2$), as revealed by Western blots. Uninjected age-matched WT mice ($n=2$) were also compared as a control. SIRT1 levels are increased in p25 transgenic mice compared to WT mice, but are similar between resveratrol and vehicle-treated mice. Densitometry values for activated caspase 3, GFAP and SIRT1 are also shown. Actin and FAK are used for loading controls. (**D**) Reduction of GFAP-expressing cells in CA1 of p25 transgenic mice treated with resveratrol ($n=3$), compared to p25 animals injected with vehicle ($n=2$) and uninjected WT controls ($n=2$) as revealed by immunofluorescence staining. Scale bar, 15 μm. (**E**) Immunofluorescence staining revealed reduced caspase 3 activation and higher number of p25-GFP expressing cells in CA1 of p25 transgenic mice ($n=2$) injected with resveratrol versus vehicle ($n=2$). Scale bar, 50 μm. (**F**) Two weeks induced p25 transgenic mice were injected ICV with either resveratrol ($n=9$) or vehicle ($n=5$) 2–3 × /week for 3 weeks. An additional control group of p25 transgenic mice was not injected with vehicle or resveratrol ($n=8$). Subsequently all groups and WT mice ($n=20$) were subjected to contextual fear conditioning. Left: resveratrol had no effect on the total activity and escape response to the electric foot shock during the training procedure. ES, electric foot shock. Right: vehicle treated and non-treated p25 transgenic mice displayed reduced freezing behavior during the memory test when compared to WT littermates ($P=0.0032$, $t_{(1,23)}=3.295$; $P<0.0001$, $t_{(1,26)}=5.048$). However, when compared to the vehicle group ($P=0.0109$, $t_{(1,12)}=3.009$) or non-treated p25 transgenic mice ($P=0.0005$ $t_{(1,15)}=4.407$) resveratrol-treated p25 transgenic mice showed significantly improved freezing behavior during the memory test. * Denotes significant difference; ES, electric foot shock.

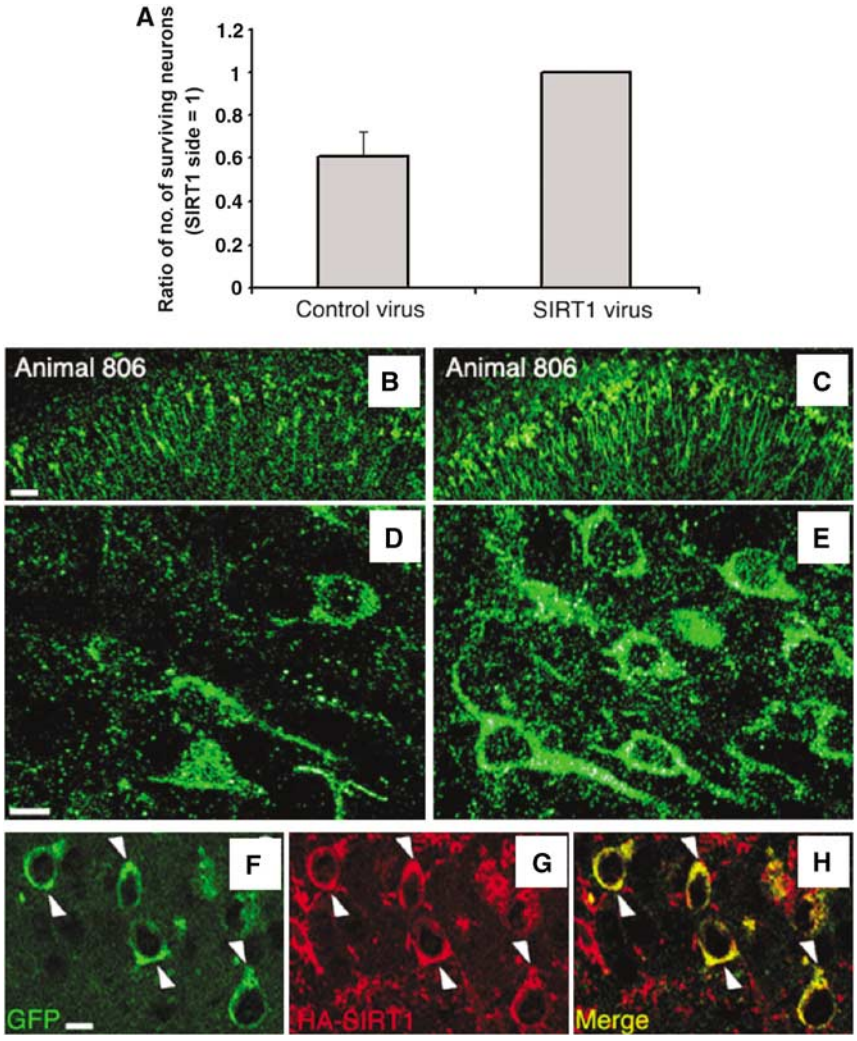


Figure 6 SIRT1 expression prevents neurodegeneration in p25 transgenic mice. (**A**) Less p25-GFP-positive neurons are present in the CA1 of control virus-injected hemispheres compared to the CA1 of the SIRT1 lentivirus-injected hemispheres. For each animal, the ratio number of neurons in control side: SIRT1 side was calculated, where the SIRT1 side equals 1. Count data are shown in Supplementary Table 1. (**B, C**) Representative confocal images of CA1 hippocampal GFP-positive neurons in control (left) and SIRT1-injected (right) hemispheres of p25 mouse 806, showing decreased number of GFP-positive neurons in the control hemisphere. Scale bar, 100 μm. (**D, E**) High-magnification confocal images of CA1 hippocampal GFP-positive neurons in control (left) and SIRT1-injected (right) p25 mouse 807. Neuronal integrity in SIRT1-injected p25 transgenic mice is better preserved when compared to the contralateral control-injected side. Scale bar, 15 μm. (**F–H**) GFP-positive neurons express SIRT1, as revealed by co-staining with HA antibody. Scale bar, 15 μm.

the neuroprotection observed in our experiments. On the other hand, while our study and previous reports suggest that the activation of SIRT1 constitutes an important aspect of resveratrol action, we cannot rule out that resveratrol may interact with other biomolecules, besides SIRT1, to exert its neuroprotective effects.

SIRT1 is thought to be a key regulator of an evolutionarily conserved pathway that allows organisms to cope with adversity. Consistent with this notion, yeast Sir2 and mammalian SIRT1 are upregulated by various biological stresses, including caloric restriction, which has been shown to prevent numerous diseases of aging in mammals such as Alzheimer's disease (AD) (Lamming *et al*, 2004; Bordone and Guarente, 2005; Lombard *et al*, 2005). Of note, a reduction of β-amyloid peptide, a hallmark of AD, occurs in brain of calorie-restricted animals and can be reproduced in mouse neurons *in vitro* by manipulating cellular SIRT1 expression/activity (Marambaud *et al*, 2005; Tang, 2005; Qin *et al*, 2006). In this study, we show for the first time the ability of SIRT1 and SIRT1-activating molecules to prevent an age-dependent neurodegenerative disease caused by the toxic Cdk5 coactivator, p25, which has been implicated in various neurotoxic conditions such as AD, ALS and stroke. The induction of SIRT1 expression levels in various neurotoxic conditions may be interpreted as a neuroprotective adaptation response, implying role for SIRT1 as an important stress sensor molecule that links aging to neurodegeneration. Future research efforts will investigate the underlying mechanism for SIRT1 induction by neurotoxic stresses. Microarray analyses of p25 transgenic mice indicated elevated mRNA levels of SIRT1 (not shown), suggesting a transcriptional component for SIRT1 induction. On the other hand, rapid induction of SIRT1 in cultured neurons (Figure 1F) implies that post-translational mechanisms may also be involved.

Our results predict that positive intervention into SIRT1 activity, such as through intake of SIRT1-activating molecules, may have profound therapeutic benefits against various age-dependent neurodegenerative diseases. Conversely, it may be worthwhile to explore whether mechanisms that decrease SIRT1 activity or levels result in enhanced susceptibility to age-dependent neurodegeneration. While knockdown of SIRT1 did not appear to result in increased susceptibility to acute neurotoxic stimuli in cultured neurons (Supplementary Figure 5), the long-term effects of decreased SIRT1 levels *per se* or in chronic neurodegenerative conditions is an important question for future studies. Interestingly, the SIRT1 gene resides in a locus on chromosome 10 that is associated with familial AD (WIPO, international publication WO 2005/004815 A2) and future studies are planned to determine whether mutations or polymorphisms in SIRT1 affect the susceptibility of individuals to AD pathology.

Materials and methods

Protein preparation and Western blots

Total protein extracts of mouse spinal cord, mouse forebrain, mouse hippocampus or human prefrontal cortex were obtained by homogenization in SDS–urea β-mercaptoethanol (0.5% SDS, 8 M urea in 7.4 phosphate buffer) or Triton X-100 (10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA (pH 8.0) and 1% Triton). The protein concentration was estimated by the Bradford procedure (Bio-Rad Laboratories, Hercules, CA). Proteins were fractionated on 7.5% SDS–PAGE and blotted on a nitrocellulose or PVDF membrane for Western blot analysis. Membranes were incubated with Abs against SIRT1 (07–131, Upstate), α-tubulin (B512, Sigma), actin (MAB 1501, Chemicon), FAK (C-20, Santa Cruz Biotechnology), Bax (N-20, Santa Cruz) and GFP (B-2, Santa Cruz). The Western blots were examined using RENAISSANCE, a Western blot chemiluminescence kit from NEN Life Science (Boston, MA). Quantitations were corrected with levels of actin, α-tubulin and FAK and performed with the Labscan program (Image Master, 2D software v 3.10, Amersham Pharmacia Biotech).

Culture, transfection and treatment of primary neurons

Rat cortical primary neurons were isolated, cultured and transfected at DIV 5–7 with Lipofectamine 2000, according to Nguyen *et al* (2004), in a ratio of 3:1 ((SIRT1 or SIRT1 H363Y or p53 RNAi): (p25-GFP, WT SOD1 or SOD1G93A or GFP)). Treatment of primary cortical neurons with ionomycin (1 μM), H_2O_2 (25 μM) or resveratrol (50–500 nM) were performed according to Lee *et al* (2000).

Determination of cell death

Primary neurons transfected with various constructs were scored as healthy or dying, on the basis of neuritic integrity and neuronal morphology, and in the case of mutant SOD1 overexpression, neuritic aggregation of SOD1. Specifically, fragmented neurites, nuclei with pyknosis or karyorrhexis, and SOD1 beading along neurites were considered as signs of degeneration. A neuron with one or more of these features was scored as dying, and only neurons with none of these features were scored as healthy. Over 100 transfected neurons were scored per condition per experiment, and experiments were carried out at least five times. Results are shown as percentage of transfected dying neurons out of the total transfected neurons. Counts were performed in a blind manner by multiple researchers.

In addition, p25-GFP-mediated neurotoxicity was also scored by propidium iodide uptake. Briefly, neurons transfected with the various constructs were stained in a 1:500 dilution of propidium iodide staining stock solution (500 μg/μl of propidium iodide in 0.038 M sodium citrate, pH 7.0) for 1 h before fixation and immunocytochemistry. Transfected neurons were scored for presence of propidium iodide signal in the nuclei, which indicates permeabilization of the membrane and subsequent binding of propidium iodide with DNA. Thus, neurons that are positive for propidium iodide are considered to be in an advanced state of degeneration in which membranes have been permeabilized. Over 100 transfected neurons were scored per condition per experiment in a blind manner, and experiments were carried out at least three times. Results are displayed as a percentage of transfected propidium iodide-positive neurons out of the total transfected neurons.

Immunofluorescence of primary neurons and human prefrontal cortex tissues

Cells were stained according to Nguyen *et al* (2004) with Abs against tubulin (α-tubulin, Sigma Aldrich), GFP (Molecular Probes), SOD1 (Biodesign), FLAG (M2, Sigma), SIRT1 (Upstate). Staining of spinal cord tissues was performed according to Cruz *et al* (2003) with Abs against SIRT1 (Upstate).

Generation of SOD1^{G37R} transgenic mice and p25 inducible transgenic mice

Transgenic mice overexpressing SOD1^{G37R} (line 29) (G37R) and p25-CK transgenic have been generated as described previously, and have been maintained on a pure C57BL6 background (Nguyen *et al*, 2001; Cruz *et al*, 2003).

Cannulation and injections

Double cannulae (Plastic1) were implanted 7 days before the experiments, under 1.2% avertin anesthesia (0.4 ml/mouse), as described previously (Fischer *et al*, 2004). For resveratrol injection, the cannulae were placed in both lateral brain ventricles, AP—0.5 mm, lateral 1 mm, depth 2 mm. Resveratrol (5 μg/μl) or vehicle was injected bilaterally 2–3 ×/week using a microinjector (CMA/microdialysis) over a 60 s period, so that a volume of 0.5 μl was injected into each side. Resveratrol (25% DMSO/artificial cerebrospinal fluid) was prepared fresh immediately before each injection. For SIRT1 lentivirus injection, cannulae were placed in the dorsal hippocampus, AP—1.5 mm, lateral 1 mm, depth 2 mm. SIRT1-HA lentivirus (1.5 μl) was injected as described above into the left hippocampus, whereas SIRT1-HA lentivirus (1.5 μl) was into the right hippocampus of 1 week induced CK-p25 mice. Number of GFP neurons was counted 1–2 mm caudal to the injection site. A ratio neurons control side/neurons SIRT1 side was calculated to quantify variations in percentage of neurons between both sides.

Fear conditioning

The fear conditioning apparatus (TSE Systems) consisted of two test boxes with defined light and background noise that were connected to a control unit and a PC computer. The experimental protocols were designed and performed using TSE fear conditioning software. Box 1 contained a grid to apply the electric foot-shock and was cleaned with 70% ethanol before each training or test session. The second test box had no grid and was cleaned with 1% acetic acid before each test. This box was used to analyze tone-dependent fear memories. Fear conditioning consisted of a single exposure to context (box 1; 3 min) followed by a foot shock (2 s, 0.7 mA, constant current). Context-dependent freezing was measured 24 h later The experimental boxes were equipped with light beams that allowed movement detection along the *x*-, *y* and *z*-axes. The system was configured so that freezing was automatically counted if no movement was observed for more than 3 s. The freezing duration is expressed as the percentage of time spent freezing, during the 180 s memory test. In addition, to verify these data, two observers scored freezing behavior every tenth second over 180 s in a blind manner.

Generation of RNAi

P53 RNAi sequence were selected based on the criteria proposed by Sui *et al* (2002). Complementary hairpin sequences were commercially synthesized and cloned into pSilencer 2.0 under promoter U6 (Ambion). Sequence for p53 are basepairs: gga gtc ttc cag tgt gat gat. A random sequence without homology to any known mRNA was used for control RNAi. All RNAi constructs were tested in cell lines and primary neuronal cultures.

Immunoprecipitation

Immunoprecipitations were performed according to Nguyen *et al* (2004) on eight forebrains from p25 transgenic mice and wild-type

(WT) mice, with a monoclonal Ab against p53 (Ab-3, Calbiochem/Oncogene). Membranes were probed with a homemade Ac-p53 Ab and a mouse monoclonal p53 Ab (pAb-240, Abcam).

For examination of PGC-1alpha acetylation, brain samples or cultured primary neurons were lysed in RIPA buffer then diluted three-fold with PBS and protease inhibitors. Samples were immunoprecipitated using an anti-PGC-1alpha Ab (H-300, Santa Cruz), washed extensively with a 1:2 solution (RIPA:PBS + protease inhibitors, nicotinamide, and TSA) and membranes were probed using acetylated lysine Abs (Cell Signaling) and PGC-1alpha Abs (H-300, Santa Cruz).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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