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ORIGINAL PAPER

Biochemical Characterization of Sirtuin 6 in the Brain and Its Involvement in Oxidative Stress Response

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Abstract Sirtuin 6 (SIRT6) is a member of nicotinamide adenine dinucleotide-dependent deacetylase protein family and has been implicated in the control of glucose and lipid metabolism, cancer, genomic stability and DNA repair. Moreover, SIRT6 regulates the expression of a large number of genes involved in stress response and aging. The role of SIRT6 in brain function and neuronal survival is largely unknown. Here, we biochemically characterized SIRT6 in brain tissues and primary neuronal cultures and found that it is highly expressed in cortical and hippocampal regions and enriched in the synaptosomal membrane fraction. Immunoblotting analysis on cortical and hippocampal neurons showed that SIRT6 is downregulated during maturation in vitro, reaching the lowest expression at 11 days in vitro. In addition, SIRT6 overexpression in terminally differentiated cortical and hippocampal neurons, mediated by a neuron-specific recombinant adeno-associated virus, downregulated cell viability under oxidative stress condition. By contrast, under control condition, SIRT6 overexpression had no detrimental effect. Overall these results suggest that SIRT6 may play a role in synaptic function and neuronal maturation and it may be implicated in the regulation of neuronal survival.

Keywords Sirtuins · Primary neurons · Neuronal maturation · Recombinant adeno-associated virus · Neuronal survival · Oxidative stress

Introduction

Sirtuins are nicotinamide adenine dinucleotide dependent protein deacetylases and mono-ADP ribosyltransferases homologous to the yeast Sir2 protein (silent information regulator). In mammals, seven sirtuins (SIRT1-7) have been identified, which show differences in tissue specificity, subcellular localization and functions [1]. Sirtuins have been implicated in a variety of biological processes including aging, DNA repair, gene silencing, apoptosis and metabolism [2–4]. In addition, sirtuins act as stress sensors helping to organize the stress response in the cell [5].

In the brain, major roles have been reported for SIRT1 and SIRT2. In particular, SIRT1 has been shown to be involved in neuronal development by regulating axonogenesis [6], neurite outgrowth [6, 7], and dendritic arborization [8]. SIRT1 also plays important roles in adult brain modulating synaptic plasticity and regulating memory formation [9, 10]. Moreover, SIRT1 exhibits protective effects against different neurodegenerative disorders including Alzheimer's, Parkinson's, Huntington's diseases, amyotrophic lateral sclerosis and multiple sclerosis [11, 12]. By contrast, SIRT2 knock down shows beneficial effects in different models of neurodegenerative diseases [13–15]. Little is known about the function of SIRT3-7 in the brain.

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Several lines of evidence indicate that Sirtuin 6 (SIRT6) plays a major role in controlling glucose and lipid metabolism, regulating the expression of multiple glycolitic and lipid genes involved in cellular response under conditions of nutrient stress [2, 16, 17]. SIRT6 regulates the expression of a large number of genes involved in stress response and aging through Histone H3 lysine 9 (H3K9) deacetylation at NF-kB target gene promoters [18, 19], although this function has been recently questioned [20]. SIRT6 knockout mice present several metabolic defects and aging-like degenerative processes, resulting in a shortened lifespan [21]. In contrast, transgenic overexpression of SIRT6 in mice protects against high fat diet-induced metabolic damages [22, 23], suppresses inflammatory responses and joint destruction in an experimental arthritis model [24] and significantly extends lifespan in male mice [25]. SIRT6 is also implicated in cancer [26, 27] and DNA repair. Indeed, it has been shown that SIRT6 deficient mice present high levels of genomic instability, DNA-damage hypersensitivity and dysfunction of base excision repair [21, 28]. More recently, SIRT6 has been implicated both in nonhomologous end joining and homologous recombination, the two main pathways of DNA double strand breaks repair [29–31]. On the other hand, the role of SIRT6 in brain function and neuronal survival is largely unknown. Biochemical analysis of murine whole brain extracts showed that SIRT6 is expressed at high levels both in embryonic and adult brain tissues [21, 32] and SIRT6 neural deficient mice develop postnatal growth retardation and obesity [33]. In Huntington's disease and cerebral ischemia mouse models, cortical and striatal protein levels of SIRT6 are lower with respect to control mice [34, 35]. In addition, overexpression of SIRT6 in cerebellar granule cells promotes neuronal apoptosis under control and low potassium culturing conditions whereas in hippocampally derived HT22 cell line, it exhibits mild protective effects under oxidative stress conditions [36].

In this study we focused on: (1) biochemical characterization of SIRT6 in cortical and hippocampal adult tissues; (2) analysis of SIRT6 levels on cortical and hippocampal primary neurons during maturation in vitro; (3) evaluation of the effects of murine SIRT6 overexpression on survival of primary neurons, under basal and oxidative stress conditions.

Materials and Methods

Animals

Three-months old C57BL/6 mice (Harlan Laboratories) and pregnant C57BL/6 mice at gestational age 18 (E18) were used for all the experiments of this study. Experimental protocols were carried out in accordance with the European Communities Council Directive of 22 september 2010 (2010/63/UE) regarding the care and use of animals for experimental procedures and with the Italian legislation on animal experimentation (Decreto L.vo 116/92).

Primary Cell Cultures

Cortical and hippocampal neurons were prepared from the brains of E18 C57BL/6 mice as previously described [37] with some modifications. Briefly, embryos were surgically removed and the cortical and hippocampal areas were dissected from the cerebral tissue in ice-cold Hanks' balanced salt solution (HBSS, Gibco, Life Technologies), freed of meninges, digested with 0.25 % trypsin for 15 min at 37 °C, dissociated by trituration and plated (10^6 cells/dish) on 35 mm poly-L-lysine-coated wells in Earle's minimum essential medium (MEM, Gibco, Life Technologies) containing 10 % foetal bovine serum and 2 % glucose. The culture medium was replaced with neuronal-conditioned serum-free B-27/Neurobasal medium (Gibco, Life Technologies) 2 h after plating. One day after plating, cytosine arabinoside (5 μM) was added to inhibit glial proliferation. Cultures were kept at 37 °C in a humidified incubator in a 5 % CO_2 atmosphere without further medium changes until used for experiments.

Recombinant Adeno-Associated Virus Preparation and Primary Neurons Infection

The recombinant adeno-associated virus (rAAV) pAAV-CKII vector, containing mouse CaMKII promoter-driven YFP, was used to specifically express SIRT6 in neurons. The pAAV-CKII vector and AAV helper plasmids [38, 39] were kindly provided by prof. Hilmar Bading, Heidelberg, Germany [40]. The full-length cDNA coding murine SIRT6 was amplified by PCR from the SIRT6 pCMV-SPORT 6.1 vector (I.M.A.G.E. Consortium cDNA clones) and cloned into the BamHI-EcoR1 sites of a modified pCDNA3 containing an HA-tag [41] by using specific primers (MWG-Biotech, Germany). The following oligonucleotides were designed: 5′-CGGGATCCAGATGTCGGTGAATTATGC AGCAGGGTTGTCGCC-3′ and 5′-CGGAATTCTCAGCT GGGGGCAGCCTCGGTCTTCAC-3′ containing the BamH1 and EcoRI cloning sites, respectively. HA-SIRT6 cDNA was then subcloned into the pAAV-CKII vector. In this construct, the YFP of the CKII-YFP cassette was substituted with HA-SIRT6 by cutting pCDNA3-HA-SIRT6 with HindIII-EcoRV, blunting by Klenow polymerase and ligating with the sites BamH1-EcoRV blunted of the AAV backbone. The same pAAV-CKII-HA backbone carrying only HA and the pAAV-CKII-YFP vector were used as

controls. Cloning orientation was verified by diagnostic cuts followed by sequencing. Viral particles were produced and purified as described previously [38, 42]. Genomic titration was determined by quantitative Real-Time PCR (Stratagene), using primers designed to WPRE (Woodchuck Post-translational Regulatory Element) region. For viral infection, neurons were infected with 1 µl of rAAVs (5×10^{11} viral genomes/ml) on DIV 4 and processed on DIV 11.

Immunofluorescence Analysis

Cortical and hippocampal tissues preparation was performed as previously described [43] and 30 µm thick coronal sections corresponding to the cortical and hippocampal regions were analyzed for immunofluorescence. To minimize non-specific binding the sections were incubated in 10 % normal horse serum in phosphate buffer containing 0.2 % Triton X-100 for 30 min. Incubation with rabbit polyclonal anti-Sirt6 (Novus Biologicals) was carried out overnight at 4 °C and Cy2-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch) was used as secondary antibody (30 min at room temperature). For the immunofluorescence on cortical and hippocampal primary neurons, cells were grown on poly-L-lysine-coated glass coverslips, fixed with phosphate buffer containing 4 % paraformaldehyde and permeabilized with 0.2 % Triton X-100. Endogenous Sirt6 in neurons was revealed by using rabbit polyclonal anti-Sirt6 and mouse anti-Map2 (Millipore) as primary antibodies (1 h of incubation at rt) and Cy2-conjugated donkey anti-rabbit IgG and Alexa Fluor 568 donkey anti-mouse IgG (Molecular Probes, Life Technologies) as secondary antibodies (30 min at room temperature). Exogenous Sirt6 was revealed by incubation with primary antibodies mouse anti-HA (Covance) (1 h of incubation at room temperature) and rabbit anti-Map2 and by using Cy2-conjugated donkey anti-mouse IgG and DyLight 549 donkey anti-rabbit IgG (Jackson ImmunoResearch) as secondary antibodies. Nuclei visualization was performed by DAPI counterstaining and samples were mounted on glass slides, and cover slipped with antifade medium. Images were acquired with an Eclipse 80i Nikon Fluorescence Microscope (Nikon Instruments, Amsterdam, Netherlands).

Subcellular Fractionation

Biochemical fractionation of cortical and hippocampal brain regions was carried out by differential centrifugation as previously described [44]. Briefly, cortex and hyppocampus were homogenized in ice-cold TEVP buffer (10 mM Tris–HCl pH 7.5 and 320 mM sucrose) containing complete protease and phosphatase inhibitor cocktails (Sigma-Aldrich) and centrifuged at $1{,}000 \times g$ to remove nuclei and large debris (P1). The supernatant was centrifuged at $10{,}000 \times g$ to obtain crude synaptosomes, and the supernatant of the crude synaptosomes was separated in a cytosolic fraction (S3) and a microsome-enriched fraction (P3) by subsequent centrifugation at $165{,}000 \times g$. Crude synaptosomes were lysed by hypotonic shock and centrifuged at $25{,}000 \times g$ to pellet a synaptosomal membrane fraction (LP1). The resulting supernatant was centrifuged at $165{,}000 \times g$ to isolate a synaptic vesicle-enriched fraction (LP2). All collected pellet fractions (P1, P3, LP1 and LP2) were lysed for 30 min in ice-cold RIPA Buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 % Triton X-100, 0.1 % SDS and 0.5 % sodium deoxycholate) containing complete protease and phosphatase inhibitor cocktails (Sigma-Aldrich). The same buffer was used for preparation of protein extracts derived from primary neuronal cultures.

SDS-PAGE and Western Blot Analysis

Protein extracts derived from brain tissues and primary cultures were subjected to determination of protein concentration by using the bicinchoninic acid kit (Micro BCA, Pierce). Proteins (40 µg) were resolved on 10 or 12 % SDS-PAGE and electrotransferred onto nitrocellulose membrane (Hybond^{TM} C-extra, Amersham Biosciences, UK limited). After blocking, membranes were incubated overnight at 4 °C with primary antibodies and then with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Immunoreactive bands were detected by enhanced chemiluminescence detection system (EuroClone). The following primary antibodies were used: mouse anti-α-tubulin (Santa Cruz Biotechnology), rabbit anti-Sirt6 (Novus Biologicals), mouse anti-HA (Covance), rabbit anti-nucleolin (Santa Cruz Biotechnology), rabbit anti-PSD95 (Millipore) and rabbit anti-synaptophysin (Santa Cruz Biotechnology). Horseradish peroxidase-conjugated donkey anti-mouse and anti-rabbit IgG (Jackson ImmunoResearch) were used as secondary antibodies. Blots were scanned and densitometric analysis was performed by using ImageQuant software (GE Healthcare). Protein loading was monitored by normalization to α-tubulin.

Cell Treatment and Viability Assay

The effect of rAAV-HA-SIRT6 overexpression on cell viability of primary neurons, under basal and oxidative stress conditions, was assessed by MTT assay (Millipore). Details of MTT method have been previously described [45]. Briefly, neuronal cells were plated onto 96-well plates (8×10^4 cells/well) and after 11 DIV neuronal viability of SIRT6 and control-infected cells was examined by incubation with 10 µl of MTT solution (5 mg/ml) for 4 h at 37 °C, followed by solubilization with isopropyl alcohol/HCl solution and optical absorbance was measured in a

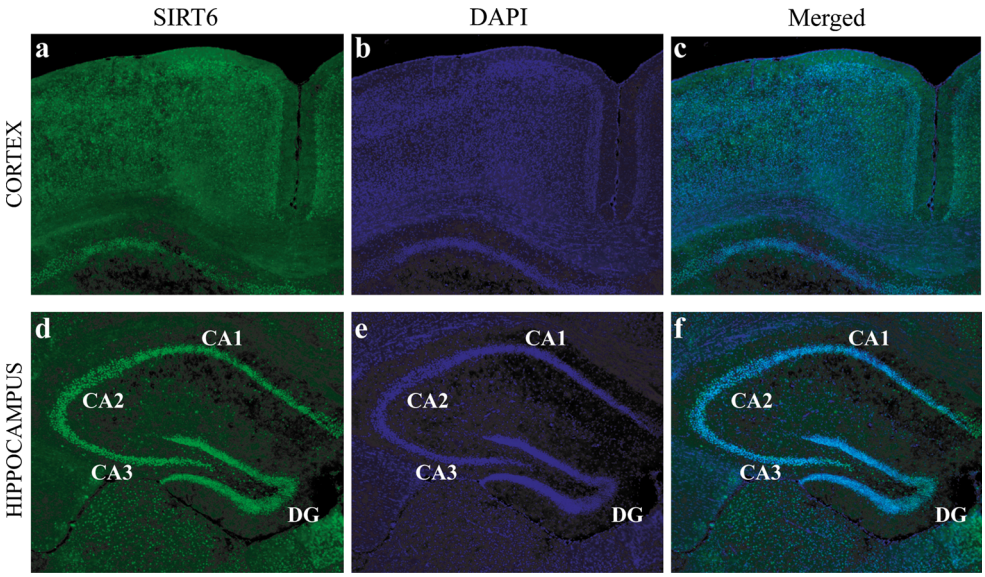

Fig. 1 Spatial distribution of SIRT6 in cortical and hippocampal regions. Coronal brain sections from 3 month-old mice were immunolabeled with anti-SIRT6 antibody (**a** and **d**) and counterstained with DAPI (**b** and **e**). Representative sections of hippocampal (**a**–**c**) and cortical regions are shown (**d**–**f**). **c** and **f** represent the merged images. CA1, CA2 and CA3, cornu ammonis subregions 1, 2 and 3; *DG* dentate gyrus

spectrophotometer microplate reader. For H_2O_2 treatment, a fresh stock of 1 mM H_2O_2 (Sigma-Aldrich) was prepared in Neurobasal medium for each experiment and added at the indicated concentrations (20 and 50 μM). In particular, following 10 DIV, neurons were exposed to H_2O_2 for 30 min and medium was replaced with conditioned medium taken from the untreated parallel cultures. MTT assay was performed 24 h after H_2O_2 treatment. MTT values of rAAV-HA and rAAV-HA-SIRT6 infected neurons treated with H_2O_2 were normalized against values for vehicle-treated controls (untreated) and expressed as a percentage of control set at 100 %.

Statistical Analysis

Data presented are representative of at least three independent experiments and were expressed as mean ± SEM. Results were analyzed using the Student's *t* test and were considered significant when $p < 0.05$. Statistical analysis was performed using GraphPad Prism (GraphPad Software).

Results

Localization of SIRT6 in Synaptosomal Membrane Fraction

Although SIRT6 is particularly abundant in the brain [21, 32], the assessment of its spatial distribution in cortical and hippocampal mouse brain regions has not been fully investigated. For this purpose, we carried out immunofluorescence analysis on cortical and hippocampal brain coronal sections of C57BL/6 mice at 3 months of age. SIRT6 immunostaining showed a wide and homogeneous distribution both in cortical and hippocampal regions (Fig. 1). In particular, in the hippocampus, a strong staining in the pyramidal cell layer of CA subregions and in the granule cell layer of the dentate gyrus (DG) was evident (Fig. 1, panels d–f). In addition, nuclear counterstaining with DAPI (Fig. 1, panels b, e), and merged (panels c and f) images indicated the prevalent nuclear localization of SIRT6 protein, as previously reported [21, 32, 46].

To investigate the subcellular localization of SIRT6 in the cortical and hippocampal regions, we carried out a biochemical fractionation [44]. Mouse cortical and hippocampal tissues were homogenized and subjected to subsequent centrifugations to isolate various subcellular compartments. As expected, SIRT6 was present in the nuclei (Fig. 2, P1 fraction) whereas it was not detected in the cytosolic or soluble subcellular fractions (S3 and LS2) and in the light membrane compartment (P3) in both cortical and hippocampal regions. Interestingly, SIRT6 was enriched in the synaptosomal membrane fraction in both brain regions (LP1). The effectiveness and the specificity of the fractionation procedure was evaluated by using specific protein markers for subcellular compartments: nucleolin for the nuclear fraction (P1), α-tubulin for the cytosolic fraction (S3), synaptophysin for the synaptic vesicle fraction (LP2) and postsynaptic density (PSD) protein PSD-95 for the synaptosomal membrane fraction (LP1).

The presence of SIRT6 in synaptosomal fraction suggests a potential role of this protein in neural plasticity.

SIRT6 Levels are Downregulated in Primary Neurons During Maturation In Vitro

Sirtuin 6 has been implicated in the regulation of neuronal survival [36] and modulation of neural chromatin structure

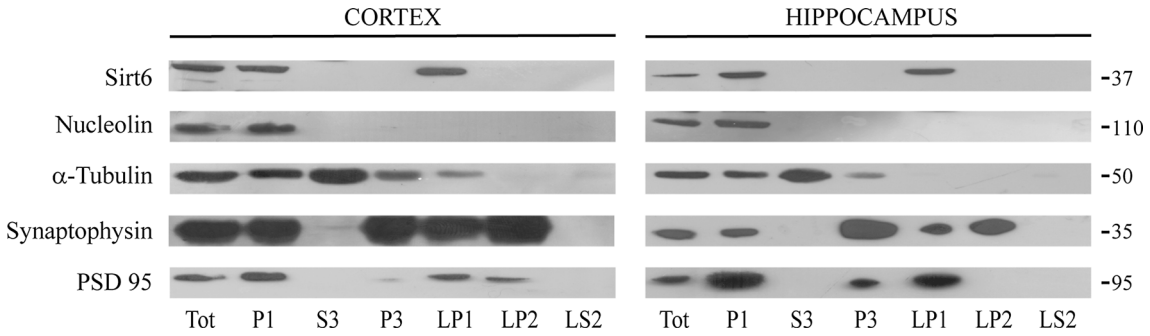

Fig. 2 Subcellular compartmentalization of SIRT6 in cortical and hippocampal regions. Tissue samples from cortex and hippocampus were subjected to biochemical fractionation and isolated fractions were analysed by Western blot. Blots were probed with anti-SIRT6, anti-nucleolin, anti-α tubulin, anti-synaptophysin and anti-PSD95 antibodies. Equal amounts of protein (40 μg) were loaded for each fraction. Molecular weights are indicated on the *right* of the blots and expressed in kDa. Tot, total homogenate; P1, nuclei and large debris; S3, cytosolic fraction; P3, light membrane fraction; LP1, synaptosomal membrane fraction; LP2, synaptic vesicle-enriched fraction; LS2, supernatant from LP2

[33], however, little is known regarding the expression pattern of SIRT6 in neuronal cells. For this purpose, we carried out immunofluorescence analysis on cortical and hippocampal mouse embryonic neurons cultured for 11 days in vitro (DIV). As shown in Fig. 3a, both types of neuronal cells displayed similar staining pattern. In particular, SIRT6 immunoreactive signal was primarily localized at nuclear level, as revealed by co-staining with DAPI and MAP2 (panels b, c, e and f). Consistently, Pfister et al. [36] reported a nuclear localization of SIRT6 also in cerebellar granule neurons and HT22 cells, a hippocampally-derived neuroblastoma cell line.

Epigenetic remodeling, such as DNA methylation and histone modifications, is a regulatory mechanism underlying maturation of post-mitotic neurons [47]. Different studies demonstrated that SIRT6 has both histone ADP-ribosylase and deacetylase activities [18, 32, 48, 49], suggesting a potential role of SIRT6 in maturation of post-mitotic neurons. Therefore, we assessed SIRT6 protein levels during maturation in vitro in cortical and hippocampal primary neurons (Fig. 3b), as they represent a useful tool to study molecular mechanisms underlying neuronal development and maturation [50]. We performed Western blot analysis on whole protein extracts derived from cortical (left panel, cortex) and hippocampal (right panel, hippocampus) neurons cultured for 1, 4, 7 and 11 DIV. In both neuronal cell types we found that SIRT6 protein levels were downregulated during maturation process, with the highest effect at 11 DIV. On the contrary, the pre-synaptic synaptophysin and post-synaptic PSD95 protein levels were increased from 1 to 11 DIV, as it would be expected for neuronal maturation markers. Densitometric analysis of immunoreactive bands showed that the down-regulation of SIRT6 at 11 DIV is more pronounced in cortical neurons compared with hippocampal cells (76 vs. 54 % of protein level reduction with respect to DIV 1) (Fig. 3c). Similar results were obtained by Real-time PCR [43] on RNA extracts derived from cortical neurons (data not shown).

Specific Overexpression of SIRT6 in Primary Neurons by Recombinant AAV-SIRT6

The finding that SIRT6 levels decreased during neuronal maturation prompted us to investigate the effect of SIRT6 overexpression in mature, terminally differentiated neurons (11 DIV in our experimental conditions). It has been reported that overexpression of SIRT6 in cerebellar granule cells promotes neuronal apoptosis under control and low potassium culturing conditions whereas in HT22 hippocampal cells, it exhibits mild protective effects under oxidative stress conditions [36]. To examine the impact of SIRT6 overexpression on survival of mouse hippocampal and cortical primary neurons, we generated rAAV-SIRT6. In particular, we used rAAV 2/1 serotype which exhibits higher transduction frequencies than other rAAV serotypes and displays specific tropism for neuronal cells with respect to glial cells [51]. We constructed rAAV 2/1 containing an expression cassette for N-terminally HA-tagged SIRT6 under the control of the neuron-specific CAMKII promoter (rAAV-HA-SIRT6). Control virus was generated using the rAAV-HA plasmid without SIRT6. Cortical and hippocampal neurons were infected after 4 DIV by rAAV-HA-SIRT6 and rAAV-HA and exogenous SIRT6 levels were examined by immunofluorescence at 11 DIV. Both for cortical and hippocampal neurons the transduction efficiency was about 60 %. The onset of transgene expression occurred at 7 DIV and the amount of expression increased gradually over the subsequent days (data not shown) resulting in robust overexpression of SIRT6 at 11 DIV (Fig. 4a, panels a and d). Anti-HA immunoreactivity was found in the nucleus, revealing that HA-SIRT6 localization was similar to endogenous SIRT6 (Fig. 4a, panels b, c and e, f). It is worth mentioning that SIRT6 overexpression by itself did not

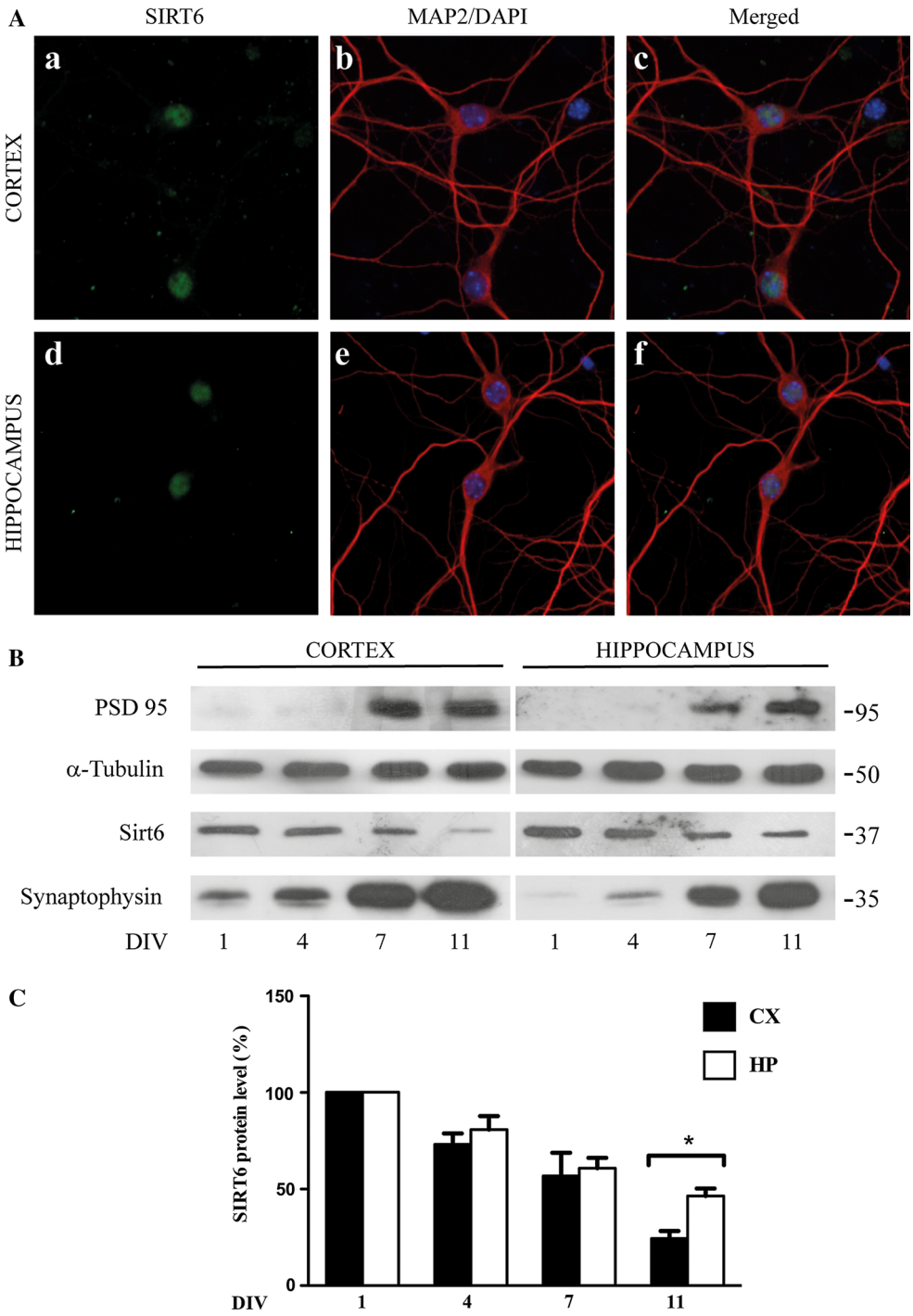

Fig. 3 SIRT6 protein levels are downregulated in primary neurons during maturation in vitro. **a** Cortical (**a**–**c**) and hippocampal (**d**–**f**) neurons from E18 mice were grown for 11 DIV on coverslips, immunostained with anti-SIRT6 and anti-MAP2 antibodies and counterstained with DAPI. **a** and **d**, Sirt6; **b** and **e**, MAP2 overlaid with DAPI; **c** and **f**, merged images. **b** Whole protein extracts derived from cortical (*left panel*) and hippocampal (*right panel*) neurons cultured for 1, 4, 7 and 11 DIV were resolved by SDS-PAGE and analysed by Western blot using antibodies against SIRT6, synaptophysin and PSD95; α-tubulin was used as loading control. Molecular weights are indicated on the *right* of the blots and expressed in kDa. **c** Densitometric quantitation of the immunoreactive bands corresponding to cortical (*black columns*) and hippocampal (*white columns*) SIRT6 levels. Values (mean ± SEM) represent the normalised percent changes in SIRT6 protein levels with respect to DIV 1 (100 %). Data are representative of 3–4 independent experiments. *Asterisk* identifies $p < 0.05$ cortical versus hippocampal Sirt6 at 11 DIV

induce alterations in neuronal morphology and dendritic branching.

The specificity of HA-SIRT6 construct was confirmed by Western analysis on protein lysates derived from rAAV-SIRT6 infected cortical neurons and immunodetection with antibodies specific for HA tag and SIRT6 (Fig. 4c). Using anti-HA antibody, expression of exogenous SIRT6 of the expected size was observed in rAAV-HA-SIRT6 neurons whereas no HA signal was detectable in uninfected and rAAV-HA lysates. (Figure 4c, left panel). Similar results were obtained using anti- SIRT6 antibody (Fig. 4b, right panel). Please, note that in protein extracts derived from uninfected and rAAV-HA infected cortical neurons, anti-SIRT6 antibodies did not reveal endogenous levels of SIRT6. This was due to the low level of SIRT6 expression at 11 DIV as showed in Fig. 3b, c.

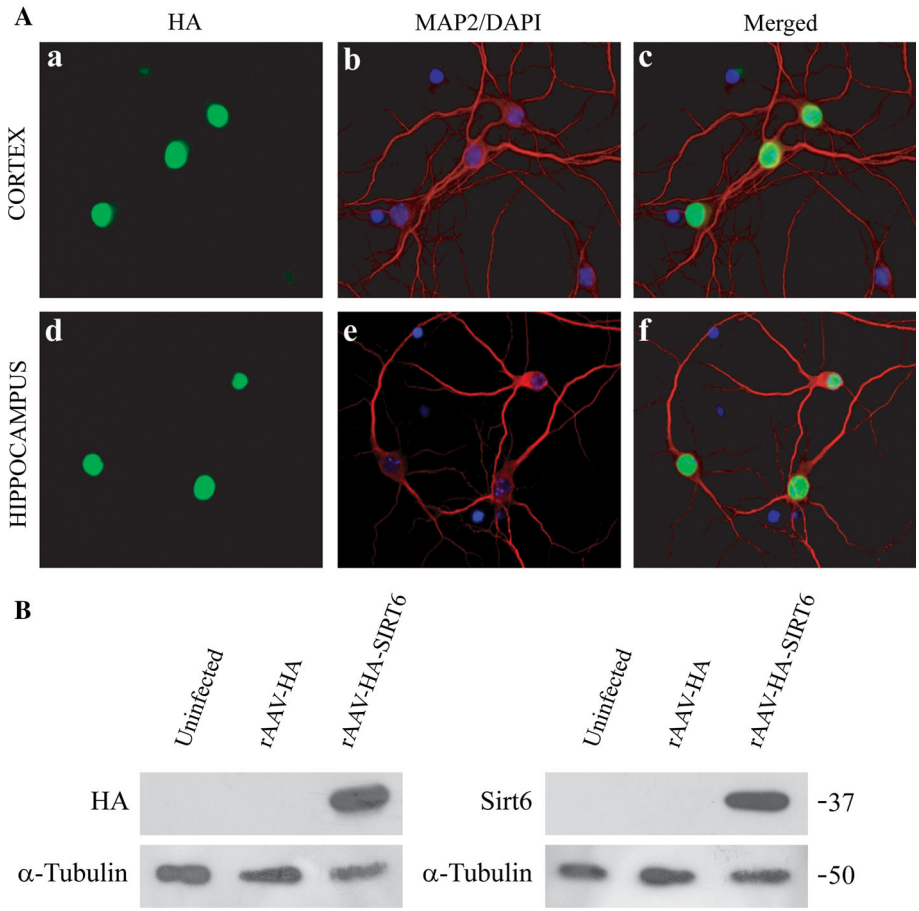

Fig. 4 rAAV-mediated SIRT6 overexpression in primary neurons. **a** Cortical and hippocampal neurons were infected after 4 DIV with rAAV-HA-SIRT6 and rAAV-HA constructs, double-immunolabeled with anti-HA and anti-MAP2 antibodies and counterstained with DAPI at 11 DIV. **a** and **d**, HA; **b** and **e**, MAP2/DAPI; **c** and **f**, merged images. **b** Whole protein extracts derived from uninfected, rAAV-HA and rAAV-HA-SIRT6 infected cortical neurons at 11 DIV were subjected to Western blot analysis by using anti-HA (*left panel*) and anti-SIRT6 (*right panel*) antibodies; α-tubulin was used as loading control. Molecular weights are indicated on the *right* of the blots and expressed in kDa

rAAV-Mediated SIRT6 Overexpression Downregulates Cell Viability in Primary Neurons Under Oxidative Stress Insult

In non neuronal cells, multiple lines of evidence [4, 18, 19, 31, 52] support the role of SIRT6 in cellular stress response. In neurons, oxidative stress has been identified as one of the major causes inducing cell death and Pfister et al. [36] found that in hippocampally-derived HT22 cell line, SIRT6 had a moderate protective effect against homocysteic acid (HCA)-induced oxidative stress.

We investigated the effect of SIRT6 overexpression on survival of primary neurons, under basal and oxidative stress conditions. To this purpose, cortical and hippocampal neurons were infected with both rAAV-HA-SIRT6 and control virus at DIV 4 and neuronal viability was assessed at DIV 11 by MTT colorimetric assay. As shown in Fig. 5a, no changes in MTT absorbance were detected in SIRT6 overexpressing cortical and hippocampal neurons with respect to controls under basal conditions.

It has been previously observed that a transient exposure (20–30 min) of cultured neurons to H_2O_2 leads to a concentration-dependent decrease of cell viability as estimated 24 h later [53–55]. To evaluate the effect of SIRT6 overexpression under oxidative stress condition, rAAV-HA and rAAV-SIRT6 infected cortical neurons were exposed to 20 and 50 μM H_2O_2 for 30 min and neuronal viability was detected 24 h later by MTT assay (Fig. 5b). After 30 min exposure to 20 μM H_2O_2, the neuronal survival of rAAV-SIRT6 and rAAV-HA infected neurons versus untreated neurons was 69 ± 11.6 % and 99 ± 12.5 % respectively. Increasing H_2O_2 concentration to 50 μM led to a significant decrease of cell viability in SIRT6 overexpressing neurons compared with rAAV-HA infected neurons (36.7 ± 5 % vs. 77 ± 12.4 % compared with untreated neurons, respectively). These results indicate that in cortical neurons, SIRT6 overexpression downregulates cell viability under H_2O_2-induced oxidative stress.

Discussion

In the present study, we characterized neural SIRT6 in murine cortical and hippocampal tissues/primary cells, and evaluated the effect of SIRT6 overexpression on neuronal survival, under control and oxidative stress conditions.

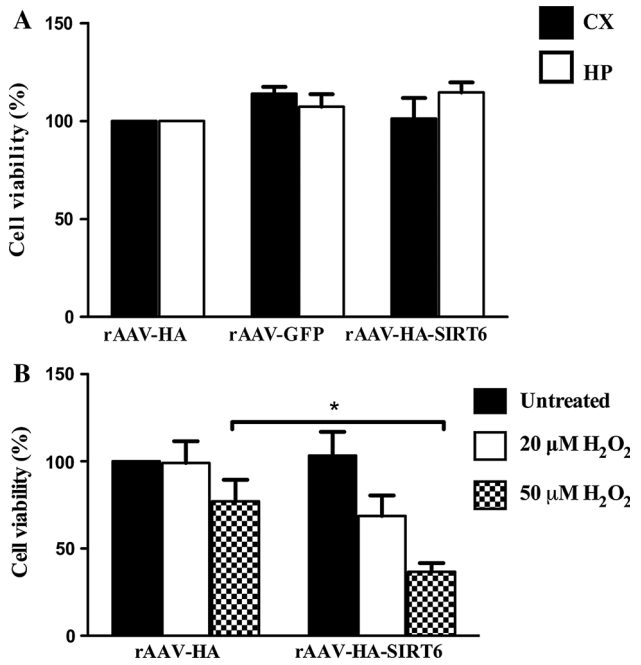

Fig. 5 rAAV-mediated SIRT6 overexpression downregulates cell viability in primary neurons under oxidative stress insult. **a** Cortical and hippocampal neurons were infected with rAAV-HA, rAAV-GFP and rAAV-HA-SIRT6 constructs and analysed by MTT assay after 11 DIV. Values are expressed as % of control (100 %, rAAV-HA). **b** MTT reduction following 30 min of H_2O_2 treatment (20 and 50 μM) on cortical neurons infected by rAAV-HA and rAAV-HA-SIRT6. SIRT6 overexpression significantly decreased MTT in neurons exposed to 50 μM H_2O_2. Results were obtained from 3 independent experiments and are mean ± SEM of MTT reduction with respect to untreated rAAV-HA infected neurons. *Asterisk* identifies $p < 0.05$ rAAV-HA-SIRT6 versus rAAV-HA group

We analyzed the spatial distribution and subcellular compartmentalization of SIRT6 in cortical and hippocampal brain tissues of 3-months old mice. Our data showed that SIRT6 is abundant in both brain regions, consistently with previous studies showing that SIRT6 is highly expressed in the brain [32, 33, 56], and it is localized in the nucleus [32, 46, 57]. Interestingly, we found that SIRT6 is also enriched in the synaptosomal membrane fraction (LP1) in both brain regions. Comparison of P1 (nuclei) and LP1 fractions revealed similar amount of SIRT6 protein levels, although immunofluorescence analysis on brain slices showed a prevalent nuclear localization or at least a major amount of SIRT6 at cellular layers level. This could be due to the masking of the epitope recognized by anti-SIRT6 antibody at synaptic membranes, where SIRT6 could be part of a multi-protein complex contained in the PSD, a complex and multi-protein organelle playing a pivotal role in synaptic plasticity [58]. Signalling from synapse to nucleus has emerged as a critical regulator for gene expression triggered by synaptic plasticity [59, 60]. By using a mass spectrometry approach, Jordan et al. [61] identified in the PSD several novel proteins that displayed both a nuclear and spiny localization. In addition, it is known that SIRT1 shuttles between the nucleus and cytoplasm [7, 62] and it is indispensable for synaptic plasticity and memory formation in mice [9, 10]. Hence, SIRT6 could be part of a nuclear-synaptic signalling which plays a role in synaptic plasticity and memory.

Neuronal maturation implies appreciable metabolism changes able to allow axonogenesis, dendritic morphogenesis and synapse formation. Primary neuronal cultures are a powerful and useful tool to study molecular mechanisms underlying neuronal development and maturation [50]. We found that SIRT6 protein levels decrease throughout maturation in vitro of cortical and hippocampal neurons, suggesting the involvement of SIRT6 in epigenetic regulation of gene expression during neuronal maturation. It is well known that during neuronal development histone acetylases promote the relaxation of chromatin to active gene expression, whereas histone deacetylases catalyze the reverse reaction, resulting in transcription repression [63]. Therefore, given that SIRT6 possesses a histone deacetylase activity, it is possible that downregulation of SIRT6 might be necessary to promote the expression of genes required during the neuronal maturation process. Consistently, pharmacological and genetic inhibition of histone deacetylases (HDACs), specifically HDAC1 and 2, in immature hippocampal neurons promotes synaptic maturation and function [64].

We showed that rAAV-mediated SIRT6 overexpression downregulates cell viability in primary neurons under oxidative stress insult. We focused on hydrogen peroxide-induced oxidative damage, because neurons generally exhibit high mitochondrial respiration and associated production of reactive oxygen species as well as have low levels of antioxidant enzymes, thus resulting particularly susceptible to oxidative damage [65]. Our results are in line with a pro-apoptotic role of Sirtuin 6 as reported in cerebellar granule cells cultured under healthy and low (apoptotic) potassium conditions and in several cancer cell lines [27, 36]. One possible mechanism explaining how SIRT6 overexpression may downregulate cell viability under oxidative stress insult is related to its ability to decrease survivin expression and thus increase cell death. In fact, it has been demonstrated that adenovirus-mediated expression of SIRT6 in a mouse liver cancer model induced by diethylnitrosamine (DEN) reduced the levels of H3K9 acetylation and p65/NF-kB binding to the survivin promoter. This results in a diminished survivin expression and increased apoptosis [66]. We found that SIRT6 overexpression in primary cortical and hippocampal neurons has no detrimental effect under control condition. One possible explanation for the lack of a toxic effect of SIRT6 is that SIRT6, acting as cellular stress sensor, would not influence neuronal survival in absence of specific stimuli.

In this regard, Mao et al. [31] reported that SIRT6 mildly promotes double-strand breaks repair in human fibroblast cell lines and that DNA repair is stimulated up to 16-fold under oxidative stress insult. Moreover, in DEN-induced-mouse liver cancer model, SIRT6 overexpression mediated the augmentation of apoptosis only in the presence of DEN, a powerful ROS inducer, strengthening the need of a specific stimuli. Another possibility is that neurotoxic effect of SIRT6 overexpression is cell type dependent. Indeed, SIRT6 induces cell death in cerebellar granule cells and cortical neurons whereas it exhibits a modest protective effect in hippocampal-derived HT22 cell line.

Overall these results suggest that SIRT6 may have an important role in synaptic function and neuronal maturation and it may be implicated in the regulation of neuronal survival.

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Conflict of interest The authors declare that they have no conflict of interests.

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