JPET Fast Forward. Published on December 2, 2014 as DOI: 10.1124/jpet.114.219345
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MS-275 inhibits A1254-induced SH-SY5Y neuronal cell toxicity by preventing the formation of the HDAC3/REST complex on the Synapsin-1 promoter

Luigi Formisano, Natascia Guida, Giusy Laudati, Luigi Mascolo, Gianfranco Di Renzo and Lorella M. T. Canzoniero

LF, LMTC Division of Pharmacology, Department of Science and Technology, University of Sannio, via Port'Arsa 11, 82100 Benevento, Italy.

LF, NG, GL, LM, GDR, LMTC Division of Pharmacology, Department of Neuroscience, Reproductive and Odontostomatologic Sciences, School of Medicine, "Federico II" University of Naples, Via Pansini, 5, 80131 Naples, Italy.

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Running title: *Role of HDAC3/REST complex in PCB-induced neurotoxicity*

Corresponding author contact information:

Prof Luigi Formisano, Division of Pharmacology Department of Science and Technology, University of Sannio, Via Port'Arsa 11, 82100 Benevento, Italy. E-mail: cformisa@unisannio.it

Abbreviations: PCBs, Polychlorinated biphenyls, A1254, Aroclor 1254, HDAC, Histone deacetylase, siREST, small interfering RNA against REST; siHDAC3 small interfering RNA against HDAC3; siCTL, scrambled control; MS-275, N-(2-aminophenyl)-4-[N-(pyridin-3-yl-methoxycarbonyl)aminomethyl]-benzamide; MC-1568, 3-[5-(3-(3-Fluorophenyl)-3-oxopropen-1-yl)-1-methyl-1H-pyrrol-2-yl]-N-hydroxy-2-propenamide; DMSO, dimethyl sulfoxide; MTT, 3[4,5-dimethylthiazol-2-y1]-2,5-diphenyltetrazolium bromide; ChIP, chromatin immunoprecipitation; IP, Immunoprecipitation, PI, preimmune; PCR, polymerase chain reaction; Lactate Dehydrogenase (LDH) ; PI, propidium iodide.

No. pages: 25

No. Tables: 0

No. Figures: 4

No. Supplemental Figures :0

No. References: 33

No. Abstract words: 236

No. Introduction words: 476

No. Discussion words: 517

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Abstract

PCB exposure has been associated with neurodegenerative diseases like Parkinson's disease, amyotrophic lateral sclerosis, and dementia. Neuronal death elicited by the PCB mixture Aroclor 1254 (A1254) has been attributed to an increase in RE-1-silencing transcription factor (REST), which, in turn, correlates with a decrease in Synapsin -1 promoter gene. Although histone deacetylase (HDAC) inhibitors are known to be neuroprotective in several neurological disorders, the core mechanisms governing this effect are not yet understood. Here, to examine how HDAC class I (MS-275) and HDAC class II (MC-1568) inhibitors prevent A1254-induced neuronal cell death, we exposed SH-SY5Y neuroblastoma cells to A1254. Exposure to A1254 (30.6 μM) for 24 and 48 h resulted in a time-dependent cell death. Indeed, after 48 h, MS-275, but not MC-1568, reverted A1254-induced cell death in a dose-dependent manner. Furthermore, A1254 significantly increased HDAC3, but not HDAC1 and HDAC2. Interestingly, REST physically interacted with HDAC3 after A1254 exposure. Chromatin immunoprecipitation assays revealed that MS-275 reverted the increased levels of HDAC3 binding and decreased acetylation of histone H3 within the Synapsin-1 promoter region, thus reverting Synapsin-1 mRNA reduction. Moreover, REST knockdown by small interfering RNA (siRNA) prevented HDAC3 from binding to the Synapsin-1 promoter. Likewise, HDAC3 siRNA significantly reduced A1254-induced cell toxicity in SH-SY5Y cells and cortical neurons. Hence, this study demonstrates that inhibition of HDAC class I attenuates A1254-induced neuronal cell death by preventing HDAC3 binding and histone deacetylation within the Synapsin-1 promoter region.

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Introduction

PCBs are developmental neurotoxins (Chen et al., 2011) that may damage a variety of neuropsychological functions in children, including general cognition, memory, attention, visual-spatial, executive, and motor functions (Boucher et al., 2012),(Humphrey et al., 2000). Aroclor 1254 (A1254), a commercial mixture of PCBs (Webb and McCall, 1972), frequently found in various foods and in human specimens at contaminated sites (Tilson and Kodavanti, 1997), is widely used for studying PCB toxicity (Canzoniero et al., 2006; Adornetto et al., 2013). At the neuronal level, A1254 can induce mitochondrial dysfunction in dopaminergic neurons (Lee and Opanashuk, 2004; Lee et al., 2012), and neuronal death of cerebellar granule cells (Mariussen et al., 2002) and cortical neurons (Inglefield et al., 2001; Formisano et al., 2014). Neuronal death induced by A1254 is likely due to the activation of the transcription factor REST via the ERK2\Sp1\Sp3 pathway (Formisano et al., 2014), a phenomenon that, in turn, down-regulates Synapsin-1 expression (Formisano et al., 2011). Since REST requires histone deacetylase (HDAC) activity to repress neuronal gene transcription in both non-neuronal (Iannotti et al., 2013) and neuronal cell lines (Formisano et al., 2013), it is likely that HDAC is also involved in the mechanisms leading to PCB-induced neuronal toxicity. Indeed, several lines of evidence indicate that alterations in the expression and function of HDAC are involved in neurodegeneration. Conversely, inhibition of HDAC can ameliorate stress-related behavior in a wide range of neurological disorders like Huntington's disease and Parkinson's disease, as well as psychiatric disorders like anxiety, mood disorders, Rubinstein-Taybi syndrome, and Rett's syndrome (Abel and Zukin, 2008).

The hypothesis that HDACs might play a role in the mechanisms leading to neuronal toxicity after PCB exposure is substantiated by several neurological studies. One

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study shows that MS-275, a class I histone deacetylase inhibitor (Beharry et al., 2014), reduces cell death following traumatic brain injury (Cao et al., 2013). Likewise, another study indicates that inhibition of class II histone deacetylases in the spinal cord attenuates inflammatory hyperalgesia (Bai et al., 2010). Still other studies demonstrate that MC-1568, a class II HDAC inhibitor (Beharry et al., 2014), can prevent di(2-ethylhexyl)phthalate-induced neurotoxicity (Guida et al., 2014) and promotes neurite growth and arbourisation protecting neurite arbours against neurotoxic insult (Collins et al., 2014). Furthermore, in animal models of brain ischemia, injection of the pan-HDAC inhibitor trichostatin A (TSA), an inhibitor of the key components of the REST–corepressor complex of HDAC1 and HDAC2, ameliorates neuronal injury (Noh et al., 2012). Similarly, TSA also prevents A1254-induced neurotoxic effects in a dose-dependent manner (Formisano et al., 2011).

Here, we investigated the effect of the class I HDAC inhibitor MS-275 and the class II HDAC inhibitor MC-1568 on A1254-induced cell death, in order to verify which of these two classes is involved in the toxic effect of PCBs, and the involvement of the HDAC isoform with REST in A1254-induced Synapsin-1 reduction and cell death.

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Material and Methods

Drug and Chemicals

Aroclor 1254 (A1254) (lot: LB794306V cod:488586) (stock solution 153 mM) was purchased from Supelco (Bellefonte, PA, USA). Culture media and sera were obtained from Invitrogen (Milan, Italy). The HDAC inhibitors N-(2-aminophenyl)-4-[N-(pyridin-3-yl-methoxycarbonyl)aminomethyl]-benzamide (MS-275; cod. EPS002; stock solution 100 μM), and 3-[5-(3-(3-Fluorophenyl)-3-oxopropen-1-yl)-1-methyl-1H-pyrrol-2-yl]-N-hydroxy-2-propenamide (MC-1568; cod. M1824;stock solution 1 M), were obtained from Sigma-Aldrich (St. Louis, MO). The calpain inhibitor, calpeptin (cod. sc-202516) was from Santa Cruz Biotechnology (Santa Cruz, CA) (stock solution 1 mM). All chemicals were diluted in cell culture medium as previously reported (Formisano et al., 2011; Guida et al., 2014)

Cell lines and culture conditions

Human neuroblastoma SH-SY5Y cells (IRCCS Azienda Ospedaliera Universitaria San Martino-IST-Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy) were grown as previously reported (Formisano et al., 2011; Guida et al., 2014). All experiments were conducted in cultures containing cells between the 10th and 25th passage. After 24 h of cell seeding, A1254 (30.6 μM) was added to DMEM medium containing 5% FBS .Thus, cells were exposed to A1254 for 24 and 48 h. The experiments on cortical neurons were approved by the Animal Care Committee of “Federico II”, University of Naples, Italy. Cortical neurons were prepared and cultured from mixed sex embryonic day 17 (E17) Sprague-Dawley rats (Charles River, Calco (Milan, Italy) as previously described

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(Formisano et al., 2014; Guida et al., 2014; Vinciguerra et al., 2014) To study the effects of HDAC and calpain inhibitors, cells were seeded and treated with 0.1% DMSO (vehicle), or pre-treated with MS-275 (0.05, 0.5 and 5 μM in SH-SY5Y cells and 1 μM in cortical neurons) and MC-1568, (0.05, 0.5 and 5 μM in SH-SY5Y cells) for 2 h, and with calpeptin (30 μM) for 6 h. After pretreatments, cells were exposed to A1254 (30.6 μM) for 48 h in SH-SY5Y cells and for 24 h in cortical neurons. SH-SY5Y cells were plated at a density of $2x10^5$ in 24-multiwell plates for MTT assay, LDH assay, and propidium iodide staining, and in 100-mm well plates at a density of $10x10^5$ for qRT-PCR, Western Blot, ChIP, and immunoprecipitation analyses. Cortical neurons were plated at a density of $1x10^6$ in 24-well plates for LDH and MTT assays and in 100-mm well plates at a density of $15x10^5$ for Western Blot.

Cell transfection

Gene silencing by small interfering RNA (siRNA) was carried out by transfecting cells with scrambled control (siCTL; sc-37007), with siRNAs against human HDAC3 (siHDAC3; sc-35538), or against rat HDAC3 (siHDAC3; sc-270161), at 400 nM (Santa Cruz Biotechnology,Santa Cruz, CA). They were also transfected with siRNAs against human REST (siREST; SI04333588), or against rat REST (siREST; SI01969905) at 20 nM (Qiagen, Milan, IT). siRNAs for human and rat, transfected into SH-SY5Y cells and cortical neurons, respectively, were performed as previously reported (Formisano et al., 2011; Guida et al., 2014). Transfection efficiency was ≅70% for SH-SY5Y cells and ≅60% for cortical neurons (data not shown). Finally, cells were exposed to A1254 (30.6 μM) in neurons for 24 h and in SH-SY5Y cells for 48 h.

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Determination of Cell Viability

Cell viability was determined as previously described (Guida et al., 2014) using the dimethylthiazolyl-2-5-diphenyltetrazolium-bromide (MTT, Sigma-Aldrich [St.Louis, MO]) staining. In particular, cells were incubated in 0.5 mg/ml MTT solution for 2 h at 37°C, after treatment with A1254 in cortical neurons and in SH-SY5Y cells for 24 and 48 h, respectively.

Lactate Dehydrogenase (LDH) assay

Cell injury was assessed by measuring LDH efflux into the medium after cortical neurons and SH-SY5Y cells had been treated with A1254 for 24 and 48 h, respectively. LDH activity is correlated with the number of necrotic cells in the medium. Cytosolic levels of LDH in the extracellular medium were measured with the LDH Cytotoxicity Kit (1000882) from (Cayman, DBA, Milan, IT). Briefly, after A1254 exposure, the medium was removed and sampled for LDH content by measuring absorbance at 490 nm on a spectrophotometer (BioPhotometer Eppendorf, Hamburg, Germany). Cell lysate, prepared with 1% Triton X100 (St. Louis, MO), was used as a positive control and its value was considered 100%.

Propidium iodide (PI) uptake

After 24 h of seeding, SH-SY5Y cells were pre-treated for 2 h with MS-275 (5 μM) and with MC-1568 (5 μM), and for 6 h with calpeptin (30 μM); then, they were treated with A1254 for 48 h. After A1254 exposure, they were washed with ice-cold PBS and collected

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on ice. To evaluate necrosis, cells were stained with propidium iodide (PI) for 30 min in ice-cold PBS containing PI (1.5 μM), and finally deposited in suspension on slides. A negative sample was acquired for control staining. The analysis was performed, on 10 fields for each experimental condition (Guida et al., 2014). In addition, the cells were at a concentration of 300,000/ml and analyses were performed on 75,000 cells for each experimental condition.

Western Blot Analysis and Immunoprecipitation

Western blot analysis was performed as described elsewhere (Formisano et al., 2011; Sirabella et al., 2012; Formisano et al., 2014; Guida et al., 2014). Cells were lysed in lysis buffer. Samples (80 μg for REST, HDAC1, HDAC2 and HDAC3, and 60 μg for Calpain) were subjected to SDS-PAGE. After electrophoresis, samples were transferred onto a polyvinylidene difluoride membrane (Amersham Biosciences) and were immunoblotted with antibodies. Immunoprecipitation was performed as previously described (Formisano et al., 2011; Guida et al., 2014). In brief, cell lysates (1500 μg) were immunoprecipitated overnight at 4 °C using antibodies with Protein A/G Plus agarose beads (Santa Cruz, CA). The precipitated samples were then subjected to Western Blot analysis. Specific antibodies were used against calpain (1:500 cat: sc-48453; polyclonal goat antibody; Santa Cruz Biotechnology, Santa Cruz, CA, USA), HDAC1 (cat: 5356), HDAC2 (cat: 5113) (1:1000, monoclonal antibodies Cell-Signaling, EuroClone, Milan, IT), HDAC3 (1:1000, cat: sc-11417, polyclonal antibody, Santa Cruz Biotechnology, Santa Cruz, CA, USA), REST (1:1000) (Formisano et al., 2011), and β-Actin (1:1000) (Formisano et al., 2011).

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Chromatin Immunoprecipitation (ChIP) assay

ChIP was performed as previously described (Formisano et al., 2011; Formisano et al., 2014; Guida et al., 2014). In brief, after 24 h of seeding, cells were exposed to A1254 (30.6 μM) alone, after pretreatment with MS-275 and calpeptin, or after transfection with siCTL and siHDAC3. SH-SY5Y were cross-linked with 1% formaldehyde (10 min), collected, and then lysed in a buffer containing 50 mM Tris, pH 8.1, 1% SDS, 10 mM EDTA, and antiprotease. DNA was sheared by sonication. Cell lysates were prepared and immunoprecipitated with 5 μl anti-HDAC3 (cat: sc-11417, polyclonal antibody, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and 5 μl anti-H3 acetyl (06-599; polyclonal rabbit antibody; Millipore, Milan, Italy). IgG rabbit antibody was used as a negative control. Data of real time PCR were normalized for the DNA input. IgG controls were performed (not shown), and the values were subtracted from results for ChIP samples. The oligonucleotides used for amplification of the immunoprecipitated DNA of Synapsin-1 promoter were the same as those reported elsewhere (Ekici et al., 2008)

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Reverse Transcription-Real Time PCR

Total RNA isolation and quantitative real-time RT-PCR (qRT-PCR) were performed as previously reported (Formisano et al., 2011). The primer pairs used for Synapsin-1 and β-Actin were the same as those reported elsewhere (Formisano et al., 2011). Samples were amplified simultaneously in triplicate in one assay run, and the threshold cycle (Ct) value for each experimental group was determined. Normalization of data was performed using β-actin. To evaluate the differences in mRNA content between the groups, normalized values were entered into the formula $2^{-\Delta\Delta ct}$ (Sirabella et al., 2012).

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Statistical analysis

Data are expressed as mean ± SEM. Statistical comparisons between the experimental groups were performed using one way ANOVA followed by Newman Keuls test. P value <0.05 was considered statistically significant.

Results

A1254-induced necrotic cell death is inhibited by the class I HDAC inhibitor MS-275 in SH-SY5Y cells

Exposure of SH-SY5Y neuroblastoma cells to A1254 (30.6 µM) for 24 and 48 h resulted in a time-specific reduction in mitochondrial activity, as revealed by MTT analysis (Figure 1A). Because 48 h of exposure damaged approximately 50% of cells, this time point was chosen for all experiments unless stated otherwise. To investigate the role of a specific class of HDACs involved in A1254-induced toxicity, cells were treated with the class I HDAC inhibitor MS-275 (Lanzillotta et al., 2012; Guida et al., 2014) and the class II HDAC inhibitor MC-1568 (Nebbioso et al., 2010; Spallotta et al., 2013; Guida et al., 2014) at 0.05, 0.5, and 5 µM. MTT assays showed that cell viability significantly improved in a dose dependent manner when cells were pre-treated with MS-275, as opposed to cells exposed to A1254 alone (Figures 1B,C). By contrast, no improvements were observed after MC-1568 pre-treatment. HDAC specificity for MS-275 and MC-1568 in SH-SY5Y cells has previously been demonstrated by our group (Guida et al., 2014).

At 48 h, A1254-induced calpain expression, an index of necrosis (Liu et al., 2004), was reverted by MS-275 treatment, but not by MC-1568 treatment (Figure 1D). Necrosis,

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quantified with PI staining and analyzed with a Tali™ Image Cytometer, was determined by LDH efflux of damaged cells into the medium. After 48 h of exposure to A1254 (30.6 μM), 45% and 55% of SH-SY5Y cells were positive for PI staining and LDH release, respectively, compared to control. By contrast, treatment with HDAC inhibitors alone had no effect on either PI staining or LDH efflux (Figures 1E,F). Noticeably, MS-275, but not MC-1568, reversed A1254-induced necrosis (Figures 1E,F). Further evidence for A1254-induced necrosis is that cell death was also prevented by the calpain inhibitor calpeptin (30 μM) (Figures 1E,F).

A1254-induced REST and HDAC3 complex in SH-SY5Y cells

MS-275 is a class I HDAC inhibitor of HDAC1, HDAC2, and HDAC3, but not HDAC8 (Beharry et al., 2014). Thus, to detect which of these isoforms was possibly involved in PCB-induced toxicity, we evaluated the expression of HDAC1, HDAC2, and HDAC3 protein levels using Western Blot after treating SH-SY5Y cells with A1254 for 48 h. A1254 (30.6 μM) significantly increased HDAC3, but not HDAC1 and HDAC2, compared to vehicle (Figures 2A-C). In addition, co-immunoprecipitation analysis revealed that A1254 induced REST binding to HDAC3 (Figure 2F), but not to HDAC1 and HDAC2 (Figures. 2D,E).

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A1254 (via REST) induced HDAC3 binding and deacetylation of histone H3 within the Synapsin-1 promoter sequence, determining Synapsin mRNA reduction and cell death

Since A1254-induced cell death is due to the binding of REST to the Synapsin-1 promoter sequence, thus decreasing its expression (Formisano et al., 2011), we investigated whether HDAC3 might be involved in REST-induced Synapsin-1 reduction in cells treated with A1254. The cells were treated with REST siRNA (siREST) and with HDAC3 siRNA (siHDAC3) for 48 h. siREST decreased REST protein expression by 50% (Figure 3A, upper panel). Similarly, siHDAC3 decreased HDAC3 by 60% (Figure 3A, lower panel). As shown by ChIP analysis, HDAC3 binding to the Synapsin-1 promoter increased after 48 h of A1254 treatment; however, this binding was resolved following incubation with the class I HDAC inhibitor MS-275 and siHDAC3 (Figure 3B). Cells exposed to A1254, MS-275, and siHDAC3 were able not only to revert H3 deacetylation within the Synapsin-1 promoter (Figure 3C) but also to block its mRNA reduction (Figure 3D). Similarly, REST knockdown in A1254-treated cells was able to revert HDAC3 binding to Synapsin-1 (Figure 3B) and to block H3 deacetylation (Figure 3C). These findings thus suggest that REST mediates the specific interaction between HDAC3 and the Synapsin-1 promoter. Consistently, whereas siREST blocked the decrease in A1254-induced Synapsin-1 mRNA (Figure 3D), siHDAC3 counteracted A1254 neurotoxicity at 48 h (Figure 3E).

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The HDAC class I inhibitor MS-275, REST and HDAC3 knockdown reverted A1254-induced cell death in cortical neurons

Cortical neurons were treated with A1254 (30.6 μM) for 24 h (Formisano et al., 2014). This time point was chosen because it reduced cell survival only by 50%, as opposed to longer periods which completely killed the neurons (data not shown). The PCB mixture increased both REST and HDAC3 protein expression (Figures 4 A,B). By contrast, A1254-induced calpain expression was reverted at 24 h by MS-275 and siHDAC3 treatments (Figures 4 C,D). Notably, siHDAC3 significantly reduced HDAC3 expression by 70% (Figure 4 E). Then, to evaluate the roles of HDAC3 and REST in A1254-induced neurotoxicity, HDAC3 and REST were knocked down in cortical neurons (Figure 4 E, upper and lower panel, respectively).

Interestingly, both siREST and siHDAC3 transfection reverted A1254-induced neurotoxicity (Figures. 4 F, G). Moreover, cell death, induced after 24 h of A1254 exposure, (30.6 μM) was inhibited when cells were pre-treated with MS-275 at 1 μM (Figures. 4F,G), a concentration that is known to be neuroprotective in cortical neurons (Lanzillotta et al., 2012)

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Discussion

The present study demonstrates that exposure to A1254 induces a time-dependent reduction in cell viability in SH-SY5Y neuroblastoma cells. Our finding that PCB neurotoxicity is prevented by the class I HDAC inhibitor MS-275, suggests the involvement of class I HDACs in A1254-induced cell death. Indeed, of the three class I HDAC isoforms studied (HDAC1, HDAC2, and HDAC3) only HDAC3 was increased by A1254, whereas HDAC1 and HDAC2 remained unaffected. We thus hypothesized that HDAC3 expression was responsible for triggering a cell death mechanism. Indeed, we found that HDAC3 siRNA prevented A1254-induced cell death both in SH-SY5Y cells and in cortical neurons. Additional evidence for HDAC3-mediated cell death is that the HDAC3 inhibitor MS-275 blocked A1254-induced cell viability reduction in SH-SY5Y cells and neurons while decreasing calpain expression and LDH release. A remarkable finding of the present paper is that A1254 exposure caused an interaction between HDAC3 and REST. To our knowledge, this is the first evidence showing that (1) the transcription factor REST binds to HDAC3 and (2) that this specific HDAC isoform is involved in A1254 neurotoxicity. These results fully echo the well-documented role of HDAC in promoting cell death. In fact, recent evidence shows that shRNA-mediated suppression of HDAC3 expression protects against low-potassium-induced neuronal death (Bardai and D'Mello, 2011) and that expression of mutant huntingtin (Htt) liberates HDAC3 from wild type Htt, thus de-repressing its neurotoxic activity (Bardai et al., 2013).

Another important aspect of this study is the characterization of a possible mechanism through which the REST-HDAC3 protein complex induces cell death after A1254 treatment. In particular, we found that exposure to A1254 caused a decrease in Synapsin-1 gene expression that was though inhibited by REST or HDAC3 knockdown,

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and MS-275 treatment. These findings suggest the involvement of class I HDACs, specifically HDAC3, in A1254-induced Synapsin-1 mRNA reduction.

Evidence for the role of HDA3 in reducing Synapsin-gene expression after A1254 exposure is further supported by the fact that MS-275 antagonized the suppressive effects of A1254 on Synapsin-1 gene expression by reducing HDAC3 binding to, and H3 deacetylation on the Synapsin-1 promoter. Our results are in accordance with previous studies demonstrating that MS-275 reduces cell death in brain ischemia (Baltan et al., 2011; Lanzillotta et al., 2012; Murphy et al., 2014) and after traumatic brain injury (Cao et al., 2013). Recruitment of HDACs to a gene locus is achieved through the binding of specific transcription factors. Interestingly, after A1254 treatment, the transcription factor REST was identified as an important repressor for the expression of Synapsin-1 gene. Indeed, REST knockdown experiments in SH-SY5Y cells confirmed that after A1254 treatment HDAC3 requires REST in order to bind to the Synapsin-1 promoter. Thus, REST or HDAC3 knockdown, and MS-275 treatment were all able to counteract the neurodetrimental effect of A1254 on cortical neurons.

Collectively, the present study suggests that A1254-induced necrotic cell death reflects accumulation of HDAC3 and formation of REST-HDAC3 complex. Binding of the latter to the Synapsin-1 promoter suppresses transcription due to deacetylation of promoter chromatin. Hence, these findings provide a novel mechanism for the neuroprotective effect of HDAC class I inhibitors on PCB-induced cell death.

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Acknowledgments

We thank Dr Mario Galgani from (Laboratorio di Immunologia, Istituto di Endocrinologia e Oncologia Sperimentale, Consiglio Nazionale delle Ricerche (IEOS-CNR) , Napoli, Italy) for providing reagents for Tali Image based Cytometer. We also thank Mr. Carmine Capitale for technical support and Dr Paola Merolla for stylistic editing .

Author contributions

Participated in research design: Formisano, Guida and Di Renzo.

Conducted experiments: Formisano, Guida, Laudati, and Mascolo.

Contributed new reagents or analytic tools: Formisano

Performed data analysis: Formisano, Guida, Laudati, and Mascolo

Wrote or contributed to the writing of the manuscript: Formisano, Guida, Di Renzo, and Canzoniero.

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Footnotes

This work was supported by grants: from [COFIN2008], Ricerca finalizzata [2009] and Ricerca-Sanitaria progetto Ordinario [2008] by Ministero della Salute.

Figure Legends

Figure 1. ***Effect of HDAC inhibitors on SH-SY5Y cell survival.***

A) Effect of 24 and 48 h A1254 (30.6 μM) exposure on mitochondrial activity. Bars represent mean ± S.E.M. obtained in three independent experiments. *P<0.05 vs vehicle, **P<0.05 vs vehicle and A1254 at 24 h groups. **B,C)** Effect of 30.6 μM A1254 at 48 h, alone or with 0.05, 0.5, and 5 μM of MS-275 or MC-1568 on mitochondrial activity. Bars represent mean ± S.E.M. obtained in four independent experiments. Cells were pre-treated with MS-275, and MC-1568 for 2 h before A1254 addition. *P<0.05 vs vehicle; **P<0.05 vs A1254 alone and A1254+MS-275 0.05 μM, ***P<0.05 vs A1254 alone, A1254+MS-275 0.05 μM and A1254+MS-275 0.5 μM**. D)** Western blots of calpain protein levels in vehicle, after 48 h of A1254 (30.6 μM), alone or after pre-treatment with MS-275 (5 μM) or MC-1568 (5 μM). Graphs show quantification of the ratio of calpain to β-actin. Bars represent mean ± S.E.M. obtained from three independent experiments. *P<0.05 vs vehicle; **P<0.05 vs A1254. **E,F)** Effect of 30.6 μM A1254 at 48 h, alone or after pre-treatment with 5 μM MS-275, 5 μM MC-1568 and 30 μM calpeptin on necrosis, determined using PI staining and by following LDH release. Bars represent mean ± S.E.M. obtained in three independent experiments. Cells were pre-treated with MS-275 and MC-1568 for 2 h and with calpeptin for 6 h before A1254 addition. For LDH experiments, 1% Triton X100 was used as a positive control and its value was considered as 100% *P<0.05 vs CTL (0.1%DMSO); MS-275 and MC-1568 alone, **P<0.05 vs A1254 alone.

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Figure 2. ***A1254-induced HDAC3 protein increase and its binding to REST in SH-SY5Y cells.***

A-C) Western blots of HDAC1, HDAC2, and HDAC3 protein levels in vehicle and after 48 h of A1254 (30.6 μM), Graphs show quantification of the ratio of HDAC1, HDAC2, and HDAC3 to β-Actin. Bars represent mean ± S.E.M. obtained from three independent experiments. *P<0.05 vs vehicle **D-F)** Representative Western blot showing immunoprecipitation between REST and HDAC1, REST and HDAC2, and REST and HDAC3 after 24 h of A1254 treatment. Input are aliquots of the extracts before immunoprecipitation. PI (Pre-Immune IgG) was used as negative control.

Figure 3. ***A1254-induced HDAC3 binding on Synapsin-1 promoter sequence causing cell death.***

A) Representative Western blot of the effect of siCTL, siHDAC3 and siREST on HDAC3 and REST protein expression in SH-SY5Y cells. Bars represent mean ± S.E.M. obtained from three independent experiments. *, P <0.05 versus siCTL **B-D)** Quantification by ChIP assay and by real time RT-PCR of the effects of A1254 (30.6 μM) on HDAC3 binding and histone H3 acetylation to the Synapsin-1 gene promoter and on Synapsin-1 mRNA, in SH-SY5Y after (1) transfection with siCTL, (2) A1254 exposure alone or after 2 h pre-treatment with MS-275, or (3) transfection with siHDAC3 and siREST. Bars represent mean ± S.E.M. obtained from three independent experiments for ChIP and real time RT-PCR experiments. **E)** Quantification of the effects of A1254 (30.6 μM) on cell survival after transfection with siCTL and when the toxicant was administered alone or after transfection with siHDAC3. Each bar represents the mean (± S.E.M.) obtained from four independent experiments. For all the experiments *P<0.05 vs siCTL , **P<0.05 vs A1254.

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Figure 4. ***Effect of MS-275, siREST and siHDAC3 on A1254-induced cell death in cortical neurons.***

A,B) Representative Western blot of REST and HDAC3 under control conditions or after 24 h of exposure to vehicle or A1254 (30.6 μM). Values are expressed as percentage mean ± SEM of three independent experiments. * P <0.05 versus vehicle. **C)** Representative Western blot of calpain in cells treated with vehicle and after 24 h of exposure to A1254 (30.6 μM) with or without MS-275 * P< 0.05 vs vehicle and ** P < 0.05 vs A 1254 alone **D)** Representative Western blot of calpain in cells treated with siCTL and after 24 h of exposure to A1254 (30.6 μM) with or without siHDAC3. Values are expressed as percentage mean ± SEM of three independent experiments. *, P <0.05 versus siCTL and **, P <0.05 versus A1254 alone: **E)** Effect of siCTL, siHDAC3 and siREST on HDAC3 and REST protein expression in cortical neurons. Bars represent mean ± S.E.M. obtained from three independent experiments. *, P <0.05 versus siCTL. **F,G)** Effects of 24 h exposure to A1254 (30.6 μM) on cortical neurons survival and death, measured as MTT and LDH assays, respectively, in (1) vehicle, (2) siCTL**,** and (3) when the toxicant was administered alone or after transfection with siREST and siHDAC3 or pre-treatment with MS-275. For LDH experiments, 1% Triton X100 was used as positive control and its value was considered as 100%. Each bar represents the mean ± S.E.M. obtained from 4 independent experiments. *, P < 0.05 versus vehicle or siCTL, **, P <0.05 versus A1254 alone.

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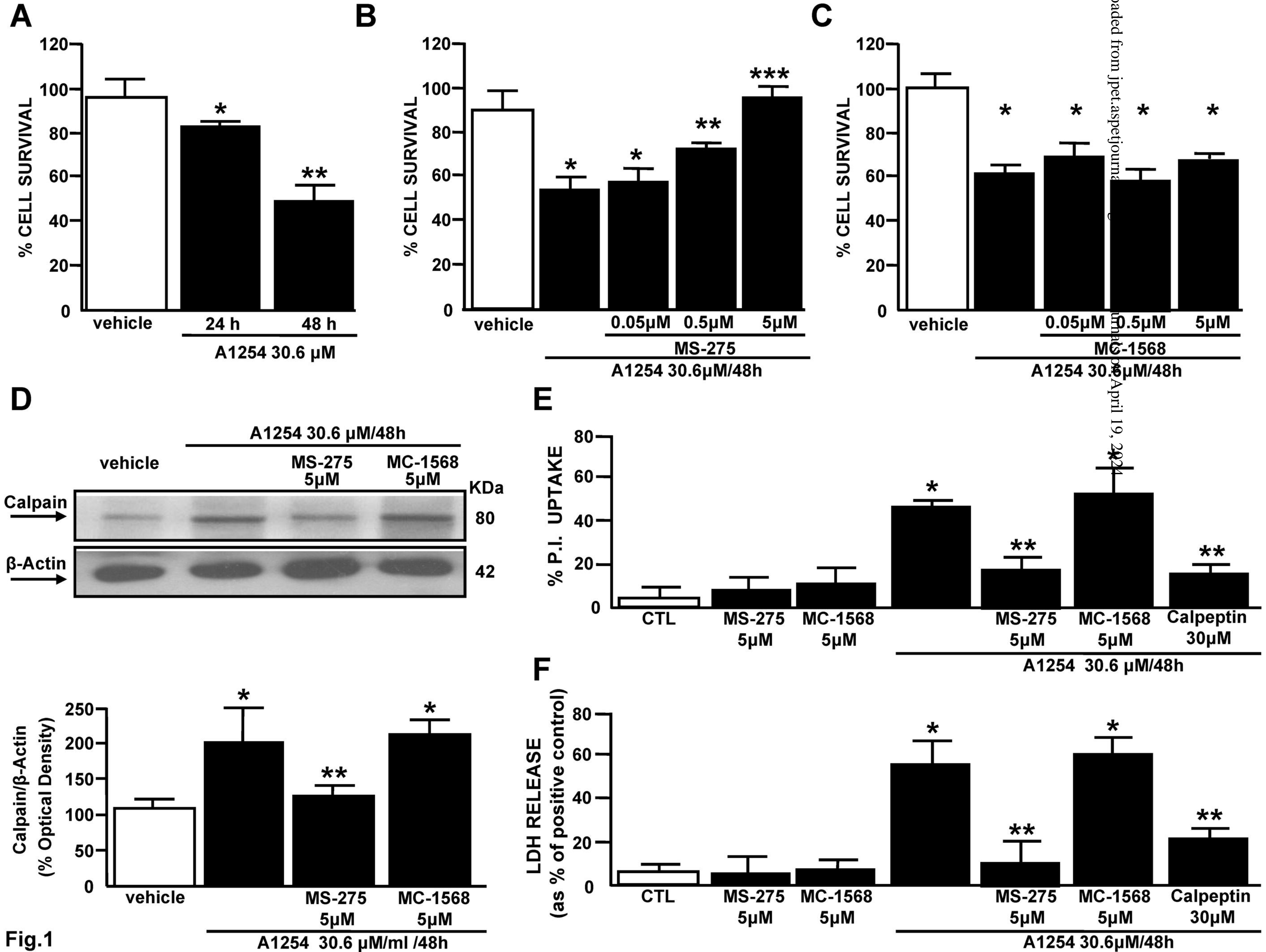

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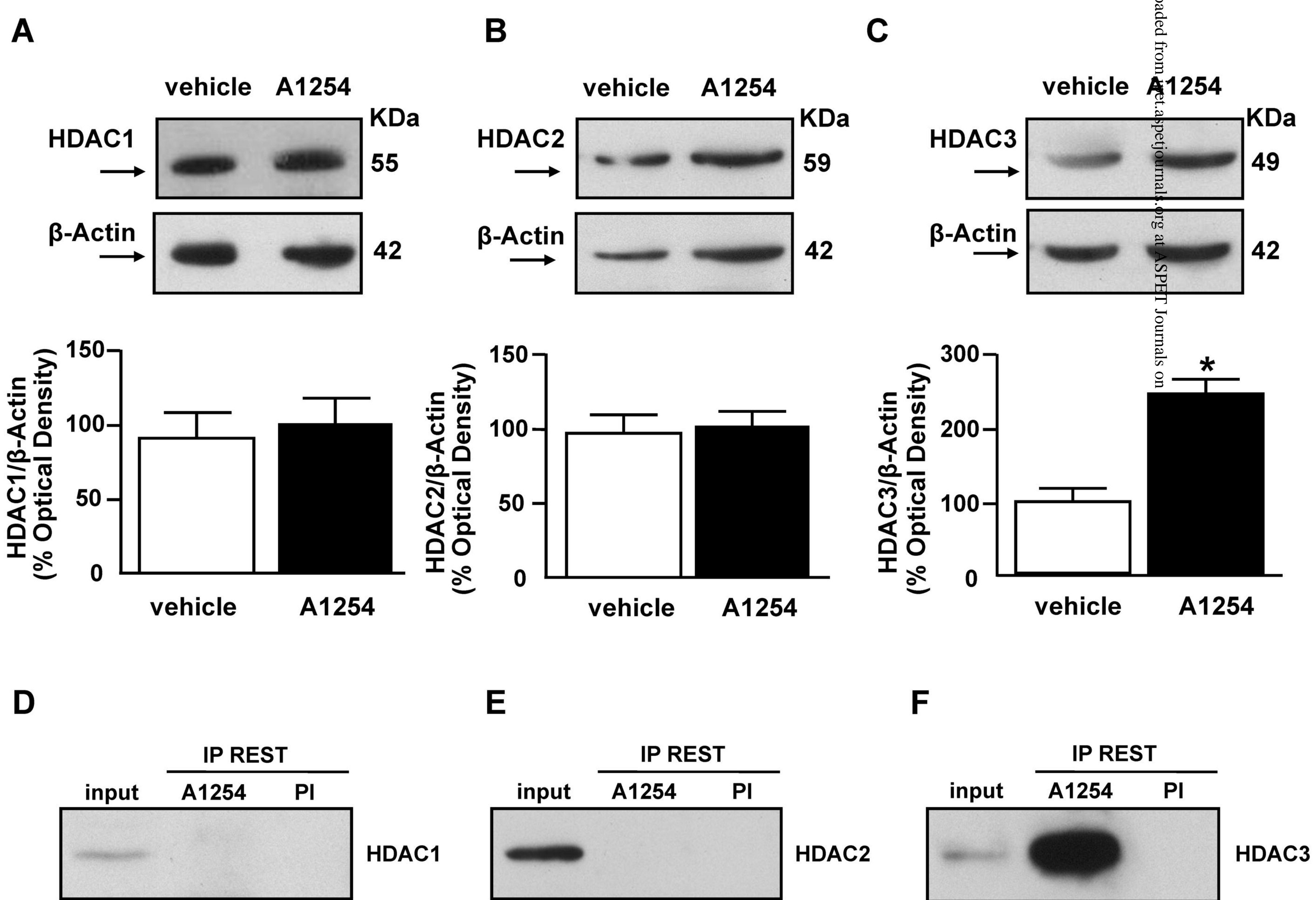

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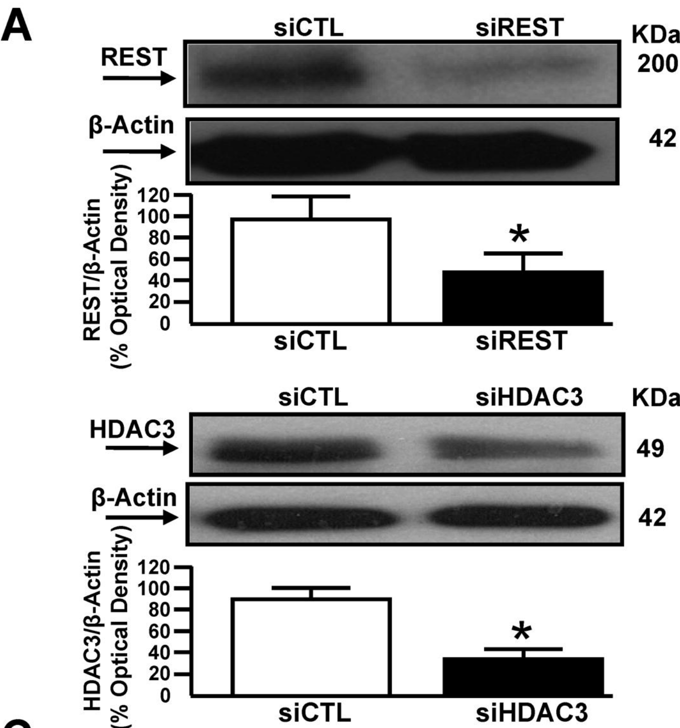

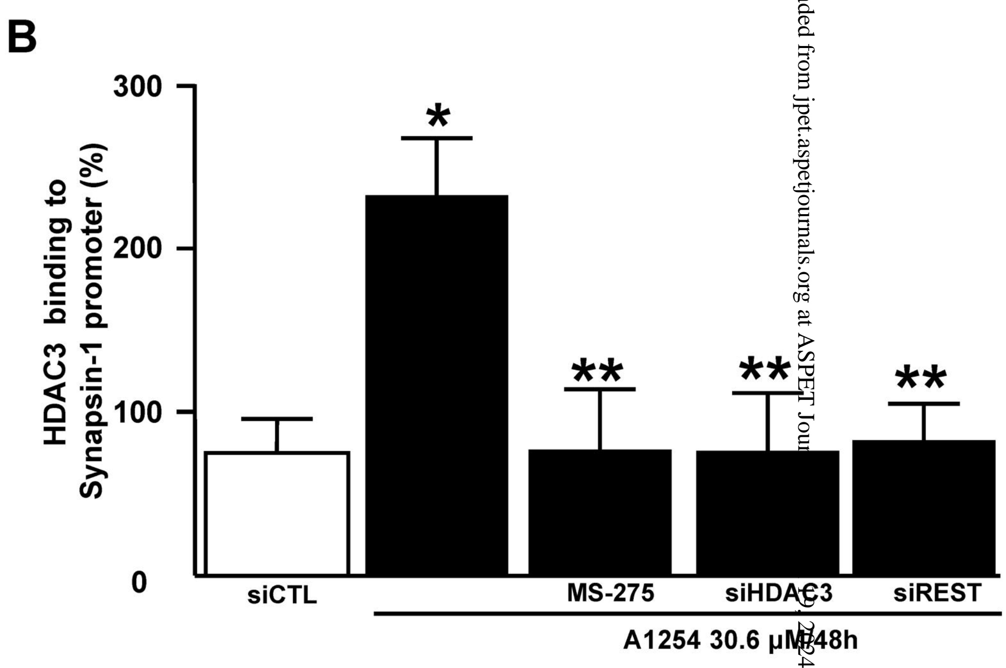

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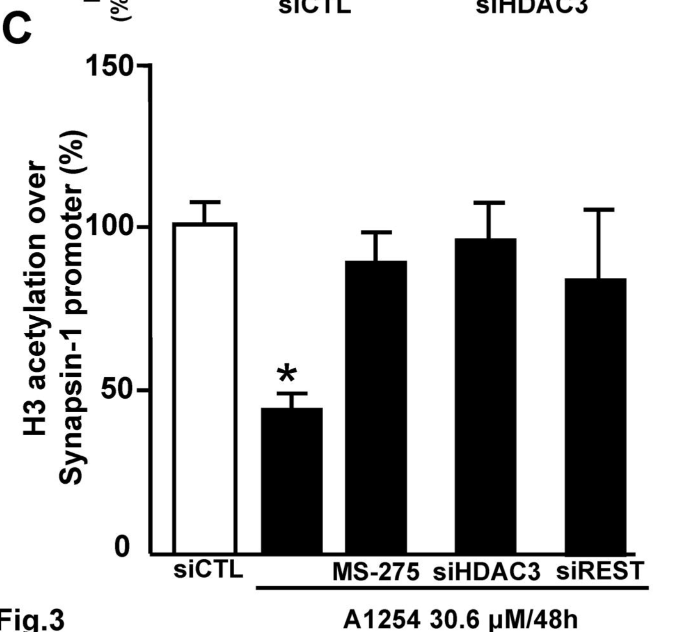

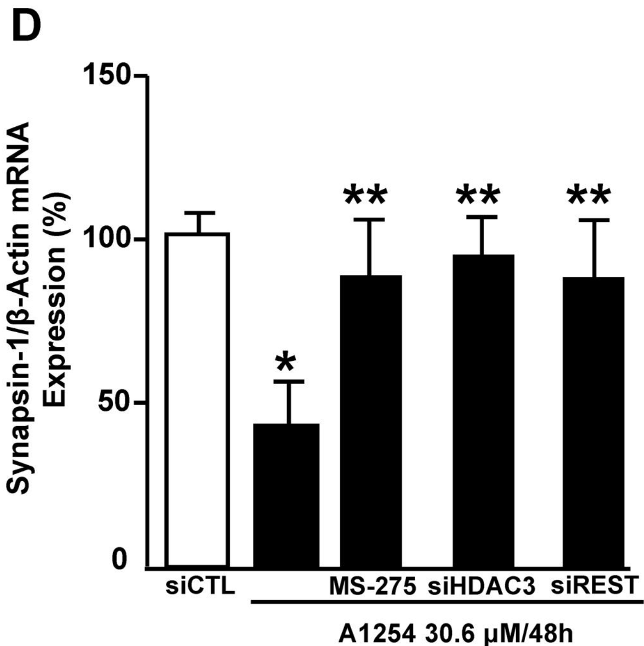

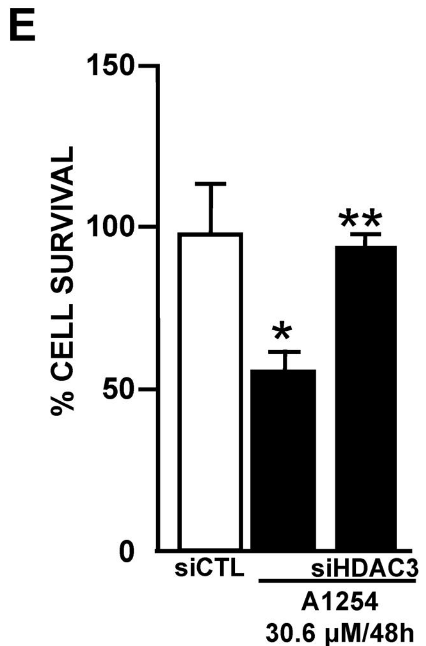

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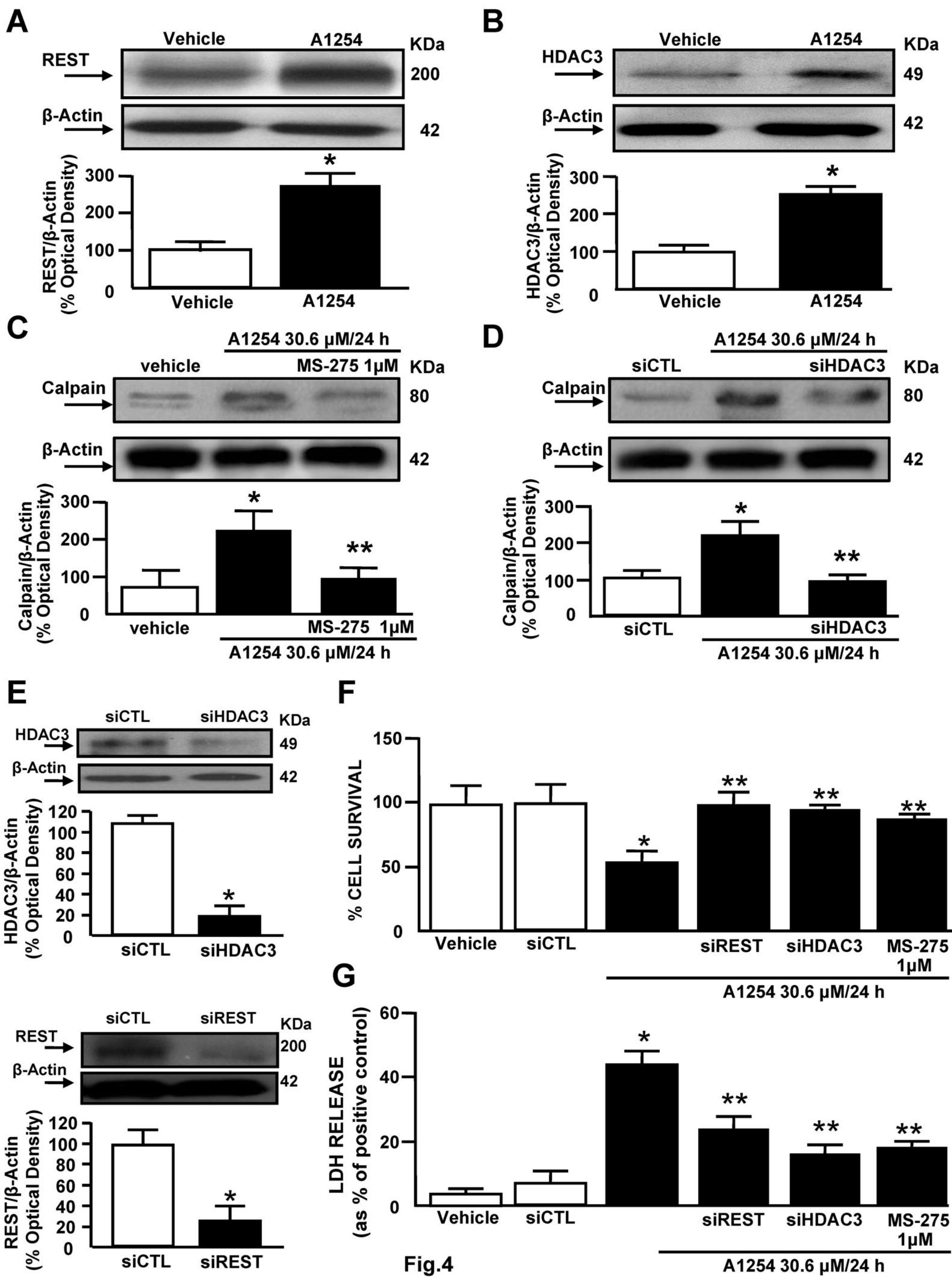

Fig.4