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Nascent histamine induces α-synuclein and caspase-3 on human cells

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ABSTRACT

Histamine (Hia) is the most multifunctional biogenic amine. It is synthetized by histidine decarboxylase (HDC) in a reduced set of mammalian cell types. Mast cells and histaminergic neurons store Hia in specialized organelles until the amine is extruded by exocytosis; however, other immune and cancer cells are able to produce but not store Hia. The intracellular effects of Hia are still not well characterized, in spite of its physiopathological relevance. Multiple functional relationships exist among Hia metabolism/signaling elements and those of other biogenic amines, including growth-related polyamines. Previously, we obtained the first insights for an inhibitory effect of newly synthetized Hia on both growth-related polyamine biosynthesis and cell cycle progression of non-fully differentiated mammalian cells. In this work, we describe progress in this line. HEK293 cells were transfected to express active and inactive versions of GFP-human HDC fusion proteins and, after cell sorting by flow cytometry, the relative expression of a large number of proteins associated with cell signaling were measured using an antibody microarray. Experimental results were analyzed in terms of protein–protein and functional interaction networks. Expression of active HDC induced a cell cycle arrest through the alteration of the levels of several proteins such as cyclin D1, cdk6, cdk7 and cyclin A. Regulation of α-synuclein and caspase-3 was also observed. The analyses provide new clues on the molecular mechanisms underlying the regulatory effects of intracellular newly synthetized Hia on cell proliferation/survival, cell trafficking and protein turnover. This information is especially interesting for emergent and orphan immune and neuroinflammatory diseases.

1. Introduction

Histamine (Hia) is a biogenic amine involved in a broad spectrum of both physiological and pathological processes that includes allergy, gastric-acid secretion, neurotransmission and immunomodulation [1]. The biosynthesis of Hia is catalyzed by histidine decarboxylase (HDC), a short-lived enzyme showing complex mechanisms of regulation [2]. Despite the pleiotropic character of Hia, only a limited number of cell types produce it. Among these cells, some of them store the amine in secretory granules until an exogenous signal elicits its secretion (e.g. mast cells, gastric enterochromaffin-like cells and histaminergic neurons), but others release it following its synthesis (e.g. macrophages). In most cases, Hia is synthesized in differentiated and low proliferative stages. Interestingly, Hia-producing neoplasias such as mastocytosis and rare gastric cancers, although displaying several hallmarks of cancer, usually present lower proliferation rates than other cancer types [3,4].

Other biogenic amines produced by the decarboxylation of cationic aminoacids are the arginine/ornithine-derived polyamines: putrescine, spermidine and spermine. Polyamines are small polycations present in the majority of living organisms, where they are absolutely necessary for cell growth and survival. In contrast to Hia, polyamines are present in almost every cell type, where the most important enzymes participating in their metabolism (ornithine decarboxylase, ODC; S-adenosyl methionine decarboxylase, SAMDC; and spermidine/spermine acetyl transferase, SSAT) are usually expressed, at least during active proliferation and/or differentiation [5].

Since both Hia and polyamines share chemical properties and metabolic reactions [6], we previously investigated the possibility of a metabolic interplay between both sorts of amines in mast cells, one of the cell types in which both metabolisms coexist. Indeed, by using established mouse and rat mast cell and basophilic cell lines, and also mouse bone marrow derived mast cells, we observed multiple evidence suggesting an antagonistic metabolic relationship between growth-related polyamine and Hia in these cell types

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[7–10]. From this previous experience, evidence accumulated supporting a negative effect of Hia production on cell growth. Consequently, we pursued to characterize the effects of intracellular "nascent" Hia on polyamine metabolism in a cellular model unable to store Hia and insensitive to external administration of this amine. For this purpose, we chose human embryonic kidney 293 (HEK-293) cells, a cell line widely used for transfection-based experiments that demonstrated no natural expression of HDC [11]. Transfection of these cells with several constructs allowed us to achieve biosynthesis of Hia without the putative side effects of pharmacological stimuli. In this model, we observed that the ability to synthesize Hia provoked a dramatic reduction in ODC activity, and a decrease both in spermidine levels and protein biosynthesis accompanied by a partial blockade of the cell-cycle progression.

To get further insight into the mechanisms underlying these observations, i.e. to advance in the knowledge on the molecular bases of the anti-proliferative effect of "nascent" Hia, in this work we have used an antibody microarray to investigate the relative expression levels of a large number of proteins related to cell signaling. To avoid the interference of non-transfected cells usually present after transient transfection experiments, cells expressing GFP fusion proteins were sorted by flow cytometry and subsequently used for the experiments. The results were analyzed in terms of protein–protein and functional interaction network, and show that expression of an active form of HDC induces a cell cycle arrest through alteration of the levels of key cell cycle proteins such as cyclin D1, cdk6, cdk7 and cyclin A. Our results also indicate an undescribed regulatory role of nascent histamine on expression of both caspase-3 (a proapoptotic effector) and α-synuclein, a protein involved in vesicle trafficking, which takes part of protein aggregates observed in several neurodegenerative disorders [12].

2. Materials and methods

2.1. Cell culture and treatments

HEK-293 cells were cultured in DMEM and Kelly cells were cultured in RPMI 1640 both supplemented as described previously [11]. Transfections were performed with FuGENE HD Transfection Reagent (Promega), using 3 μL of reagent per μg of DNA and 2.8×10^6 cells on P100 dishes. Twenty-four hours after transfection, cells were sorted by flow cytometry with a MoFlo cell sorter (DakoCytomation) before analysis.

2.2. Recombinant plasmids and proteins

hHDC proteins tagged at the C-terminus with the Green Fluorescence Protein (GFP) were generated by cloning the encoding sequences in frame into the *Hind*III/*Bam*HI sites of the pEGFP-N1 vector (Clontech). hHDC DNA inserts were previously obtained in our lab by RT/PCR from human blood, and their corresponding proteins were previously characterized as catalytically active (hHDC1/512-GFP) and inactive (hHDC1/512Δ262-277-GFP), respectively, [11]. Plasmids were purified using the HISpeed Plasmid Maxi Kit (Quiagen) following the manufacturer's recommendations.

2.3. Histidine decarboxylase activity

Histidine decarboxylase (HDC) enzymatic activity of transfected cells (sorted or not) was determined by measuring the release of $^{14}CO_2$ from [U-^{14}C]-histidine, as reported previously [8].

2.4. Antibody microarrays

The Panorama™ Antibody Microarray-Cell Signaling Kit was purchased from Sigma–Aldrich. 24 h after transfection, 5×10^6 cells expressing GFP fusion proteins were sorted by flow cytometry and proteins were extracted following the antibody microarray manufacturer's instructions. Protein concentrations were adjusted to 1 mg/mL, before labeling with Cy3 (hHDC1/512Δ262-277-GFP) or Cy5 (hHDC1/512-GFP) Monofunctional Reactive dye (GE Healthcare). Afterwards, the non-conjugated free dyes were removed from the labeled samples by using SigmaSpin columns and the dye to protein molar ratios (D/P ratio) were determined. Only samples with D/P ratios >2 were used. Next, equal amounts (50 μg) of labeled protein of both extracts were incubated on the same slide. Finally, slides were scanned with an Aquirescanner (Genetix). Two independent experiments were performed.

2.5. Bioinformatics analysis

The scanned data were analyzed by GenePix Pro 6.0 (Axon Instrument). Fluorescence in each channel was normalized to signal of actin and tubulin antibodies and fluorescence ratio (Cy5/Cy3, corresponding to HDC active/inactive) was calculated. Proteins whose expression levels changed more than 2.2-fold were processed through the ClueGo [13] plug-in of Cytoscape [14] in order to identify enrichment in Gene Ontology (GO) biological processes, Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome pathways. Comparison cluster analysis was performed using downregulated (cluster 1) versus upregulated genes (cluster 2).

2.6. Western blot analysis

Protein expression levels for HDC, α-synuclein and caspase-3 were assayed by Western blot as described previously [9]. Primary antibodies were anti-hHDC [15], anti-α-synuclein (Sigma–Aldrich, S3062) and anti-caspase-3 (Biorbyt, Orb10237). Normalization for sample loads was performed by re-probing membranes with the anti-actin (Millipore, mab1501), anti-tubulin (Abcam, Ab11323) or anti-GAPDH antibody (Cell Signaling, 2118).

2.7. Caspase-3/7 activity

After transfection, cells expressing either hHDC1/512-GFP or hHDC1/512Δ262-277-GFP were sorted by flow cytometry, resuspended in PBS and transferred to 96-well plates (15,000 cells/well). Then, Caspase-Glo® 3/7 reagent (Promega Biotech) was added to the wells according to the manufacturer's instructions and the luminescence was recorded after 30 min with a GLOMAX 96 microplate luminometer. Measurements were relativized to those obtained after 24 h treatment with 10 μM 2-methoxyestradiol.

3. Results

In a previous study [11], we described an inhibitory effect of newly synthetized Hia on the proliferation of HEK-293 cells. Treatments with exogenous Hia up to 0.5 mM suggested that this effect is not attributable to an interaction of the amine with Hia receptors or to an intracellular accumulation of Hia under our experimental conditions [11]. To address the signaling pathways involved in the antiproliferative effect of "nascent" Hia, HEK-293 cells were transiently transfected with either an active or an inactive version of hHDC and were analyzed with an antibody array that allows the simultaneous study of the relative expression levels of a large number of cell signaling-related proteins. However, although slight differences in the expression of certain proteins could be observed, statistical significance was not achieved (data not shown), most possibly due to the use of heterogeneous cell populations, i.e. cell

populations including both transfected and non-transfected cells. Therefore, in order to enrich the final cell suspensions in those cells expressing the fusion proteins, HEK-293 cells were transfected to express either inactive or active hHDC-GFP fusion proteins and were subsequently sorted by flow cytometry. Before FACS enrichment, only cells expressing the active version of HDC presented HDC enzymatic activity (163.8 pmol of $CO_2/h \times 10^6$ cells). After sorting, HDC activity in hHDC1/512-GFP-enriched cells was increased up to 291.4 pmol of $CO_2/h \times 10^6$ cells. Then, protein extracts were labeled with Cy3 (hHDC1/512Δ262-277-GFP, inactive form) and Cy5 (hHDC1/512-GFP, active form) and analyzed in parallel. After normalization, the mean ratio was of 1.07. A ratio equal or greater than 2.5 was observed for 6.7% of the spots, and 14.3% of the spots presented a fluorescence ratio equal or lower than 0.4 (Supplementary Fig. 1 and Table 1).

Fig. 1 shows the fluorescence ratio of functionally grouped proteins whose expression changed by more than 2-fold. We used the Cytoscape plug-in ClueGO [13] to achieve a functional enrichment analysis of the events induced by "nascent" Hia. Fig. 2 shows functional relationships established by ClueGO among the proteins mentioned in Fig. 1, on the basis of the functional terms associated with them by GO (Fig. 2A), KEGG (Fig. 2B) and Reactome (Fig. 2C). Between the major groups found we can observe processes related with the G1 phase of cell cycle and to apoptosis.

The analysis performed using the GO biological processes database showed a total of 53 enriched GO terms (with adjusted *p*-value <0.05). Out of these terms, 29 contained elements corresponding to downregulated genes, 22 to upregulated genes, and 2 contained elements of both groups (Supplementary Table 2). Among the best represented processes, an important percentage of them (83.01%) are related to activation of cysteine-type endopeptidase activity involved in apoptotic processes, with 21 genes associated (12 downregulated and 9 upregulated) (Fig. 2A and Supplementary Table 2). By using the KEGG database, a total of 14 pathways were significantly enriched. Eleven of them are related to cell cycle (9 downregulated and 6 upregulated genes) (Fig. 2B and Supplementary Table 3). Finally, enrichment analysis in the Reactome database showed a total of 34 represented pathways. Three pathways are related to G1 phase of cell cycle (1 upregulated and 3 downregulated genes); 7 pathways are related to caspase-mediated cleavage of cytoskeleton proteins (3 upregulated and 2 downregulated genes) and 22 pathways are related to signaling to RAS (2 downregulated and 7 upregulated genes) (Fig. 2C and Supplementary Table 4).

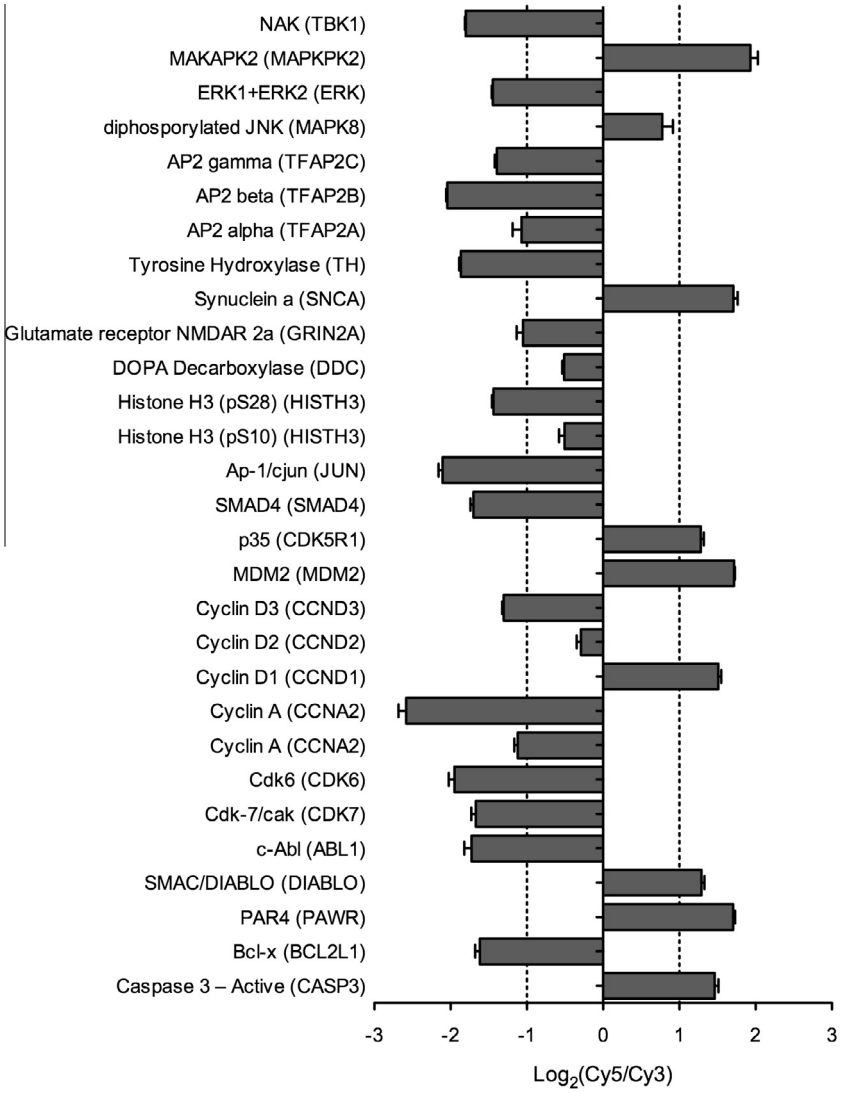

Fig. 1. Proteins considered for biocomputational analysis and their relative expression level after active hHDC-GFP overexpression. For ratios lower than 1, fold change was calculated as −1/ratio. Antibody names are expressed as in the commercial microarray brochure. Entrez Gene Symbols corresponding to the protein recognized by each antibody are provided in brackets. Data represent mean ± SEM of fluorescent ratio (duplicates of two independent experiments).

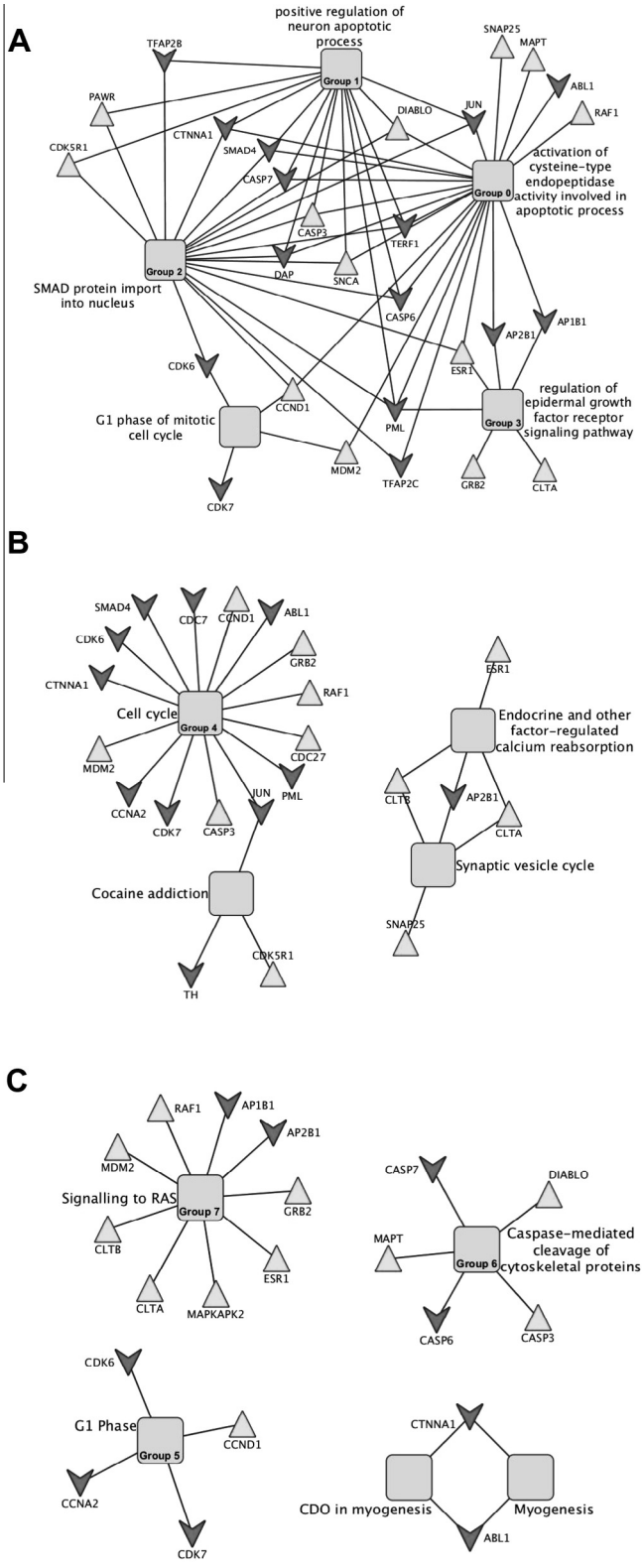

Fig. 2. Functional relationships obtained using ClueGO among proteins affected by active hHDC-GFP overexpression, as retrieved from GO biological process (A), KEGG (B) and Reactome (C) databases. Upregulated genes: light triangles; downregulated genes: dark grey arrows. Squares represent functional modules as considered in the respective database. Abbreviations are specified in Fig. 1.

As shown in Fig. 1, α-synuclein (SNCA) is one of the polypeptides that increased its signal to a major extent in the HEK-293 cells transfected to express the active form of hHDC (ratio 3.30 versus control). This increase was also observed using unsorted samples (results not shown). Besides, the functional network provided by ClueGO showed SNCA as a clear connector of those GO biological processes related to cell death (Fig. 2A). Therefore, we chose SNCA for validation by Western blotting, and Fig. 3A shows a representative result. Indeed, a specific band, with the expected Mr for SNCA, clearly increased in samples from cells transfected with active version of hHDC-GFP. Since the expression level of SNCA was low in HEK-293 cells, the transfection experiments were repeated in neuroblastoma Kelly cells. Expression of the active version of HDC in these cells led also to an increase in SNCA levels (Fig. 3B).

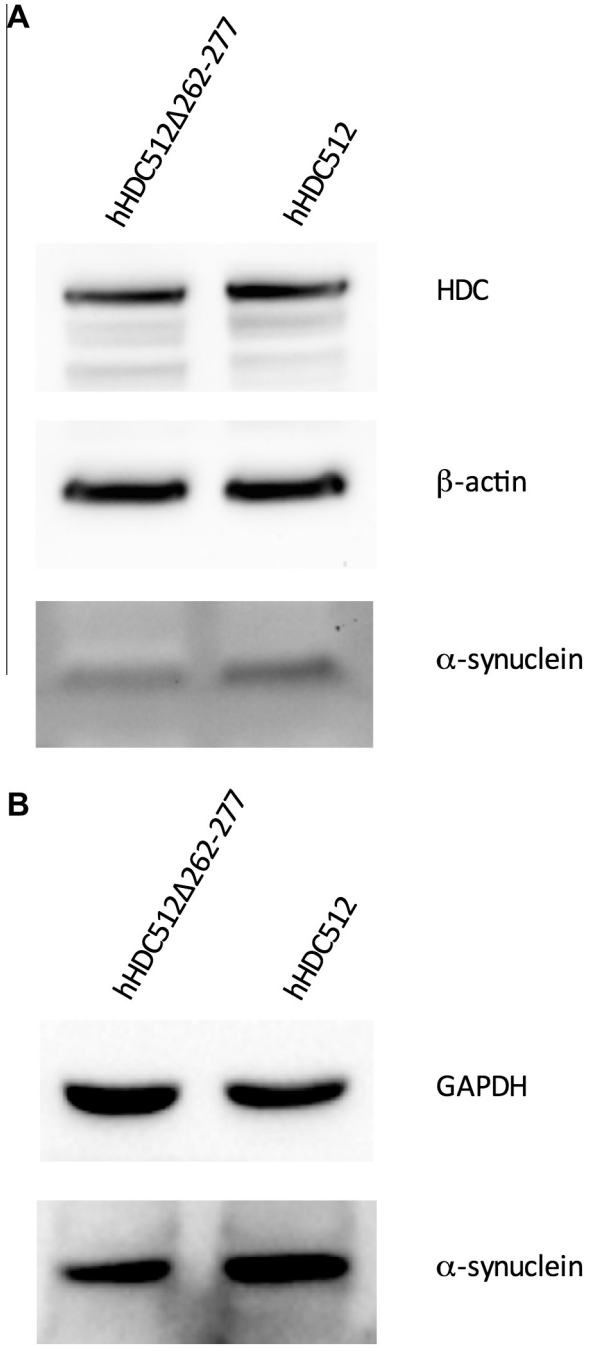

Fig. 3. α-Synuclein expression after transfection with different versions of hHDC-GFP. α-Synuclein protein levels were assayed in HEK-293 sorted cells (A) and in Kelly non-sorted cells (B) by Western blot. HDC was used as a control of transfection (A) and ß-actin and GAPDH as a control of sample loads (A, B). Results shown are representative of three independent experiments.

In addition to SNCA, this work provides an enriched profile of other cell death key elements being altered as a consequence of newly synthetized Hia. In this sense, it is remarkable the increase in protein levels of both DIABLO (ratio 2.455 ± 0.128) and active caspase-3 (ratio 2.778 ± 0.188) (Fig. 1), although signals for other caspases (6 and 7), located upstream in the canonical apoptotic cascade, were depressed in our model (Fig. 1). To validate the caspase-3 results obtained, we measured protein levels by Western blot and enzyme activity. Fig. 4A shows active caspase-3 levels after transfection with the hHDC versions used. Normalized signal of active caspase-3 was increased by 2.77-fold after transfection of active form of hHDC. On the other hand, a clear increase in caspase enzymatic activity was also observed (Fig. 4B), though the method used cannot distinguish between caspase-3 and 7 activities.

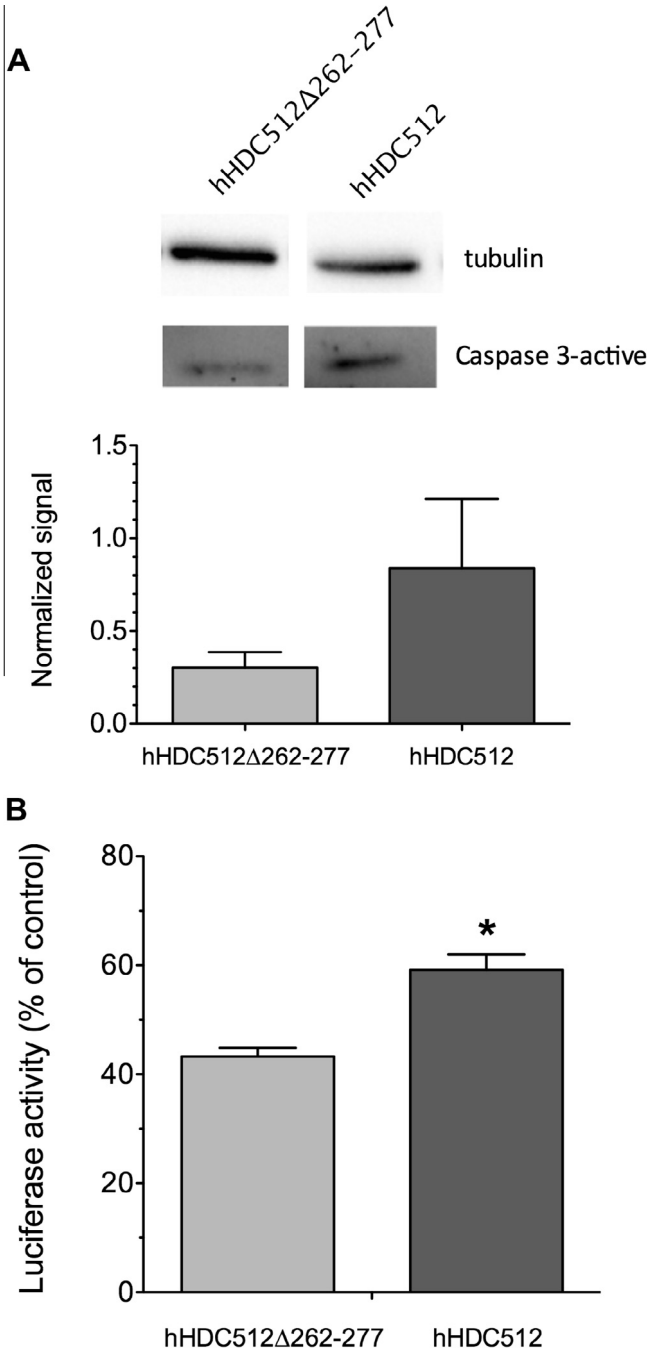

Fig. 4. Active caspase-3 protein level and caspase 3/7 activity in HEK-293 cells transfected with different versions of hHDC-GFP. (A) Active caspase-3 levels were analyzed by Western blot, and signals were normalized to that of tubulin (mean ± SEM, $n = 3$). (B) Caspase-3/7 activity was determined as described in Section 2. Measurements of luciferase activity were normalized to those obtained after treatment with 10 μM 2-methoxyestradiol for 4 h (mean ± SEM, $n = 3$; $^{*}p < 0.01$ Student's unpaired sample t-test).

4. Discussion

In a previous study, we described an inhibitory effect of newly synthetized Hia on the proliferation of HEK-293 cells. The major target of this inhibitory effect seems to be protein synthesis. "Nascent" Hia produced in HEK-293 cells provokes a reduction of ODC levels, which leads to a decrease in spermidine in these cells [11]. Spermidine is the polyamine precursor of the hypusine biosynthesis, an amino acid essential for activation of eIF5A translation factor, and consequently for cell-cycle progression and proliferation [16].

In order to advance in the knowledge on the molecular bases of the anti-proliferative effect of "nascent" Hia, we have used an antibody microarray to investigate the relative expression levels of a large number of proteins related to cell signaling in cells expressing active and inactive versions of hHDC-GFP fusion proteins sorted by flow cytometry. Results were analyzed using ClueGO, which is able to perform functional grouping of GO terms and pathways in a systematic way generating dynamical network structures. It is remarkable that, in all three used databases, processes related to cell cycle, and specifically G1 phase of the cell cycle, appear to be enriched. This observation is in full agreement with our previous results showing that the expression of an active form of HDC induces an accumulation of cells in G1 phase of cell cycle [11]. Among the genes associated with this process/pathway, we observed an upregulation of cyclin D1 (CCND1) and a downregulation of cdk6, cdk7 and cyclin A (CCNA2). An active cyclin D-cdk4/6 complex is necessary for G1-S transition, and one of its targets is retinoblastoma protein (Rb). Phosphorilated Rb (pRb) releases from the E2F transcription factor, which in turn activates transcription of several genes required for the transition from the G1 to S-phase and for DNA replication [17]. Our data show an increase in cyclin D1 (fluorescent ratio 2.86 ± 0.14) and a decrease in cdk6 (0.26 ± 0.03). In addition, pRb (although it was not considered in our list of altered genes, Fig. 1) is reduced by 0.68 ± 0.02. Several authors have reported a decreased level of cyclin D1 associated with a G1 phase block of the cell cycle [18,19], but in PC12 cells, NGF induces a G1 phase block with accumulation of cyclin D1 and p21CIP/WAF1 and a decrease in other cell cycle regulatory proteins [20]. All these facts could contribute to the accumulation of cells in G1 phase observed in response to newly synthesized Hia.

In previous works with different amine handling cells, we got evidence of an interplay between intracellular content of Hia and other biogenic amines such as serotonin and polyamines [6]. In rat basophilic leukemia models RBL-1 and RBL-2H3, induction of HDC activity with phorbol-12-myristate-13-acetate and dexamethasone reduced cell proliferation but increased intracellular levels of both Hia and serotonin. However, HDC induction had the opposite effect on intracellular serotonin in mouse C57.1 mast cells [8]. During the differentiation of mouse mast cells from bone-marrow precursor cells in culture, serotonin also accumulated at the end of the differentiation process, when intracellular Hia levels were decreased (unpublished results). Even though HEK-293 cells are not typical amine handling cells, the antibody array revealed differential signals for several key enzymes of both serotonin and dopamine synthesis, i.e. tryptophan hydroxylase (TPH), tyrosine hydroxylase (TH) and dopa decarboxylase (DDC), in line with our previous observations of an interplay between Hia and other bio-

genic amines. In HEK-293 cells transfected to express active hHDC-GFP, the TPH signal increased by more than twofold (ratio 2.095 ± 0.219), the TH signal was reduced by more than three times (ratio 0.274 ± 0.008) and a slight reduction of the DDC signal was also observed (ratio 0.704 ± 0.027).

HEK-293 cells are derived from human embryonic kidney cells. In kidney, it has been reported that SNCA is involved in ER-Golgi trafficking; SNCA aggregates can also be formed in this tissue [12]. As far as we know, this is the first time that Hia is related to SNCA-related processes in humans. This finding can be very interesting for inflammatory, neuroinflammatory and neurodegenerative diseases, as Hia and the DDC products (dopamine and serotonin) can be produced simultaneously by different nervous and immune cell types. It has been reported that these biogenic amines have multiple metabolic/signaling elements in common (oxidases, transporters, receptors, among others) [21]. Furthermore, the present work adds a new element that could be responsible for pathophysiological interferences among these biogenic amines in humans. In addition to diseases like Parkinson or Alzheimer, this fact can be involved in other less frequent (i.e. multiple sclerosis) and even rare diseases. Indeed, in a recent text mining-based work, Hia has been related to more than 20 orphan diseases, in some cases associated with DDC product-related elements [22]. Moreover, recent publications have shown the role of the homeostasis of the biogenic amines (i.e. dopamine) in addictions and/or in Parkinson disease [23,24].

In this paper, in addition to the increase in SNCA expression, the caspase-3 expression pattern seems to be altered by "nascent" Hia. Also an increase in caspase 3/7 activity has been observed. Curiously, both caspase-3 and SNCA have been related to apoptosis regulation in HEK models by the amines staurosporine [25] and 6-hydroxidopamine [26], as well as in telencephalon-specific murine neurons and in SH-SY5Y neuroblastoma cells [27]. This is explained by a non-direct interaction that involves the C-terminal portion of the polypeptide synphilin-1, an *in vivo* interactor of SNCA and an apoptosis regulator activated by caspase-3 action [27]. This work adds Hia to the list of amines that are able to modify this apoptosis feedback system in mammalian cells.

Summarizing, the present systemic approach to the study of the effects of "nascent" Hia on mammalian cells has provided new molecular data to explain the relatively reduced proliferation rates of mammalian Hia-producing cells. As far as we know, this is the first time that Hia is related to regulation of SNCA and caspase-3 expression, both proteins related to cell death. In addition to these data, the work can also provide multiple insights for other groups working on the molecular and cell biology of Hia producing cells and Hia-related pathologies. Our results also draw attention to the convenience of considering cross-regulation events among amine metabolic pathways, as the resulting interferences can have important physiopathological consequences for emergent and rare neurological and immune diseases.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.08.022.

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