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Auzmendi-Iriarte, J, Moreno-Cugnon, L, Saenz-Antoñanzas, A et al. (5 more authors) (2022) High levels of HDAC expression correlate with microglial aging. Expert Opinion on Therapeutic Targets, 26 (10). pp. 911-922. ISSN 1472-8222

https://doi.org/10.1080/14728222.2022.2158081

High levels of HDAC expression correlate with microglial aging

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Running title : HDAC, microglia and aging

The authors declare no potential conflicts of interest

ABSTRACT

Background: With aging, cellular damage gets gradually accumulated, promoting a time-dependent functional decline of the brain. Microglial cells play an important regulatory role in the maintenance of cognitive activity, phagocytosing cell debris and apoptotic cells during neurogenesis. The activity of different Histone Deacetylases (HDACs) regulates microglial function during development and neurodegeneration. However, no evidence has been published describing the role of HDACs in microglia during physiological aging.

Research design and methods: HDAC and microglial marker levels were examined in microglia cells after induction of senescence *in vitro*, and mouse and human hippocampal biopsies *in vivo* by quantitative real-time PCR. Public available datasets were used to determine the expression and function of HDACs in different brain areas during physiological aging.

Results: We show that HDAC expression is upregulated upon induction of senescence with bleomycin or with serial passage in microglia cultures. High levels of HDACs are also detected in both mice and human aged brain samples *in vivo*. In particular, human hippocampal samples show a positive correlation of expression of HDAC1, 3 and 7 with CD68 and IBA1 microglial and $p21^{CIP}$ and $p16^{INK4A}$ senescence markers. Finally, HDAC1 and 3 levels are enriched in purified microglia population.

Conclusions: These results reveal that several HDACs are elevated with senescence induction in microglia *in vitro* and with aging in the brain *in vivo* where they correlate with microglial and senescence biomarkers.

Keywords : HDAC, microglia, senescence, hippocampus, aging

INTRODUCTION

Aging is a time-dependent functional decline of organs and tissues of most living organisms that occurs due to the gradual accumulation of cellular damage [1,2]. Although human aging affects the entire organism, brain aging is especially distinctive. The brain is considered the most structurally and functionally complex organs in mammals [3]. Human brain contains billions of neurons and hundreds of trillions of nerve connections [4,5]. Moreover, neurons are surrounded by a vast number of glial cells, in numbers that are equivalent or even greater than neuronal numbers [5]. Aging affects both neurons and glial cells and their alterations, mainly in neurogenic niches, contribute to a decline in cognitive abilities, sensory perception, and motor function and coordination. In detail, glial cells are quite susceptible to brain aging. In fact, a gene expression study has demonstrated that glial-specific genes predict age with greater precision than neuron-specific genes, as their regional specific pattern is shifted upon human brain aging [6].

Microglia are part of the innate immune system, playing a key role in the detection of signals of external danger such as invading pathogens and internal damaged or dying cells, and orchestrating subsequent tissue repair. Moreover, microglial function is also crucial for brain homeostasis through the control of neuronal proliferation and differentiation, as well as influencing formation of synaptic connections and removing excess synapses [7].

During aging, cellular composition of neurogenic niches and their response to local or systemic signals changes significantly [8]. Interestingly, studies performed in rats and monkeys reported that microglial cells present more dramatic alterations than other glial cell types upon aging, [9,10]. Those alterations, as well as morphological changes in glia, such as increased soma volume and shortening of processes, were also observed in old mice compared to young ones [11]. Human brains show a distinctive microglial morphology upon aging, with beaded and fragmented processes and a disrupted cytoplasmic structure [12]. As this phenotype reflects the degeneration of microglia, the difference observed in mice and human aged brains could be partly explained by the different lifespan of each species [13]. Regarding microglial function, both murine and human aged microglia adopt an aberrant activation (primed) and pro-inflammatory status, accompanied by a dysfunction in phagocytosis and impaired *in vivo* motility, among others [14,15]. Overall, these alterations in microglial cells contribute to the aged phenotype of other cell types, altogether limiting brain function [14]. In this direction, microglial impaired phagocytosis in brain aging may contribute to the accumulation of debris and aggregates in the neural stem cell (NSC) niches [8]. Besides, the aberrant pro-inflammatory factor secretion by aged microglia reduces NSC proliferation in both the sub ventricular zone (SVZ) and hippocampus in mice [16-18]. In this sense, the rapid depletion of aged microglia by the administration of an inhibitor of the colony-stimulating factor 1 receptor (CSF1R) in mice, and subsequent natural cell repopulation showed an improved spatial learning and increased hippocampal dendritic spine density and neurogenesis [19].

Histone modification through acetylation of lysine residues is a main epigenetic modification which promotes the conversion into a more relaxed chromatin state and transcriptional activation [20]. Acetylation is controlled by two antagonistic enzyme families: the ‘writers’ histone

acetyltransferases (HAT) and the 'erasers' histone deacetylases (HDAC). HDACs catalyze the removal of acetyl groups from histone tails and thus provoke a 'compacted' chromatin status, while HATs have the opposite effect. To date, 18 human HDACs have been identified, which are classified in four classes based on sequence similarity with yeast HDACs. Among them, class I, II, and IV HDACs, also known as classical HDACs, depend on Zn^{2+} ion located in their catalytic pocket, whereas class III HDACs, known as Sirtuins (SIRTs), are nicotinamide adenine dinucleotide (NAD^+)-dependent enzymes [21].

Among HDACs, class I include HDAC1, 2, 3 and 8 and their catalytic activity depend on their respective co-repressor complexes. HDAC1 and HDAC2 have similar structures and they are often recruited to the same co-repressor complexes, such as NuRD, RCOR1/CoREST, Sin3A and MiDAC [20]. On the contrary, HDAC3 associates with NCoR and SMRT complexes [20]. Class II HDACs are further divided in two subgroups: Class IIa includes HDAC4, 5, 7, and 9, while class IIb includes HDAC6 and 10. Members of class IIa include an extended N-terminal domain that contains conserved serine residues, which can be phosphorylated by several kinases which facilitates nuclear export of HDACs [20]. For the opposite effect, class IIa HDACs also contain nuclear localization sequences (NLS), indicating their ability to shuttle between the nucleus and the cytoplasm.

HDACs are implicated in a wide range of processes, as a result of the direct and indirect modulation of multiple histone and non-histone protein acetylation [20,22]. In fact, knock out mice of almost all HDACs present embryonic and perinatal aberrations and lethality [23], indicating the pivotal role of HDACs in cellular homeostasis. In particular, knock out mice for *HDAC1, 3* and *7* present embryonic lethality. Other HDACs dysregulation presents a milder but still significant effect, as it is the case of *HDAC2, 5* and *9*, whose knock out mice present cardiac defects, or *HDAC4* knock out, which show premature and ectopic ossification [23]. On the contrary, knock out mice for *HDAC6*, which show a global tubulin acetylation, are viable and do not present significant defects [24]. The epigenetic activity of HDACs mainly results in the negative regulation of gene transcription, forming a complex with transcription factors or by indirectly regulating their transcription or acetylation [25]. In addition to transcription factors, HDACs also modulate the activity of super enhancers [26], and the maintenance of DNA methylation [26].

Epigenetic mechanisms are increasingly appreciated to be crucial for a variety of processes related to aging, such as cellular and organismal senescence, genomic instability, and carcinogenesis [27]. Several evidence have suggested the impact of classical HDACs on brain function and aging. In this sense, recent studies performed in rodent models have revealed that both classical HDAC expression [28,29] and activity [30] are increased with age in the hippocampus. In this line, HDAC1, 3, 5, and 7 are highly expressed in NSCs, whereas HDAC2 is more widespread in the brain [31,32]. Importantly, treatment of adult NSCs with HDACis induce differentiation and upregulates neuronal-specific genes, such as NeuroD, Neurogenin 1, and Math1 [33], altogether indicating the relevance of classical HDAC activity in NSC maintenance. Consistent with this, HDAC1 [34], HDAC2 [35], HDAC3 [36], HDAC4 [37] and HDAC6 [38] play a key role in associative and spatial memory, learning, and synaptic plasticity. The impact of HDAC

enzymes is also extended to the maintenance of other cell-types, such as microglia, Particularly, HDAC1 and HDAC2 activity regulate microglial function during development and neurodegeneration [39]. However, the implication of HDACs in microglial aging has not been explored so far. In this study, we characterized the expression of several HDACs in 2 different models of senescence induction *in vitro* and in different mouse and human brain samples of individuals of different ages.

MATERIAL AND METHODS

Human and mice brain samples of young and aged individuals

Hippocampal samples from 33 individuals from the Basque Country were analyzed. In particular, this cohort comprises young individuals ranged from 27-45 years old (female n=8, male n=8) and elderly people from 76 to 96 (female n=8, male n=9). Samples were stored in the Basque Institute of Legal Medicine and the post-mortem interval was 24 hours.

RNAseq data from white matter of forebrain, hippocampus, parietal neocortex and temporal neocortex from aged human individuals were obtained from 'Aging, Dementia and TBI Study' (https://aging.brain-map.org/rnaseq/search) [40]. This cohort encompasses 30 participants from 78 to 100+ years old (female n=14, male n=16), which did not present any neurological disorder. For the study of the expression of *HDAC*s directly in aged human microglia, RNAseq data originated from the bulk human dorsolateral prefrontal cortex (n=540) and from purified human microglia (n=10) from the same brain region were studied (http://shiny.maths.usyd.edu.au/Ellis/MicrogliaPlots/) [41].

Transcriptomic data for *HDACs* and *REST* in different human tissues (consensus dataset consisting on the normalized expression (nTPM) levels, combining the HPA and GTEx transcriptomic datasets using the internal normalization pipeline), and single cell RNA sequencing data from the brain were extracted from human protein atlas (https://www.proteinatlas.org/). For protein interactions between HDACs and REST, STRING platform was used (https://string-db.org/).

Mice of different ages from C57BL/6J background ((young of 1-2 months-old, (female n=7, male n=6); and aged of 13-32 months-old, (female n=7, male n=16)) were housed in specific pathogen-free barrier areas of the Biodonostia Health Research Institute, maintained in ventilated racks, under controlled humidity, light cycle, temperature, food, and water. Animal research regulations specified in the European Union Directive [2010/63/EU] and guidelines from the Animal Care and Use Committee from Biodonostia Health Research Institute have been followed and fulfilled in this work.

In situ hybridization data were obtained from sagittal sections of P56 male adult C57BL/6J mice from Allen Brain Atlas data portal (https://mouse.brain-map.org/).

Microglial cultures

Human microglial cells (HMC3) were purchased from ATCC and cultured as a monolayer in treated culture plates, in StableCell™ DMEM – high glucose medium (D0819, Sigma-Aldrich), supplemented by 10 % FBS, 2 mM L-Glutamine and 100 U/mL penicillin and 100 µM/mL streptomycin. Cells were passaged by detaching from the plates using, 0.05 % trypsin-EDTA for 2 min at 37 °C, then centrifuged at 1,000rpm for 5 min before resuspending in fresh growth media and distributing to new culture plates. Cells were maintained in standard culture conditions, at 37 °C, 95 % humidity, 21 % O_2 and under 5 % CO_2. All cell culture procedures were performed in class II security laminar flow hoods (Class II Biohazard Safety Cabinets, ESCO). Cell lines were tested regularly for Mycoplasma (90021, Biotools). Bleomycin (19692, Cayman Chemicals) and LPS (L2630, Escherichia coli serotype O111:B4, Sigma) were dissolved in DPBS.

Microglia cultures from newborn rat brain. Primary cultures of microglial cells were obtained from new-born (P0) to 2-day-old (P2) Wistar rat forebrains. Pups were sexed via measurement of anogenital distance and a separate cohort of animals was used for each experiment. Homogenized forebrains from male or female pups were grown separately in DMEM medium supplemented with 10% FBS, 10% HS and P/S (DMEM 10:10:1) in 75-cm^2 flasks, coated with poly-L-lysine (10 µg/mL) After reaching confluence, cells were shaken at 230 rpm for 3 h at 37°C. Detached cells were centrifuged at 168g for 10 min. To avoid the estrogenic effects of phenol red, purified microglia were plated in warm antibiotic- and phenol-red free RPMI 1640 supplemented with 0.5% FBS.

***In vitro* transfections**

For *HDAC1* and *HDAC2* overexpression in HMC3 microglia, transient transfections were performed, using empty *pGal4*, *pGal4-HDAC1 OE* and *pGal4-HDAC2 OE* plasmids, kindly provided by Dr. Ian C. Wood. HMC3 cells ($7 \cdot 10^5$) were seeded in each 100 mm plate and transfected after 24 h. Ten µg of plasmid and 1.5 mL DMEM without FBS were incubated for 5 min at room temperature. Meanwhile, 25 µL lipofectamine™ 2000 (11668019, Invitrogen™) and 1.5 mL DMEM without FBS were incubated for 5 min at room temperature. Both cocktails were mixed and incubated for 20 min at room temperature. Next, HMC3 cells were washed with DPBS and 5 mL DMEM without FBS were added. Finally, transfection cocktail was added dropwise to the cells, which were then incubated for 4 h at 37 °C. After this time, cell medium was replaced by 8 mL DMEM 10 % FBS and cells were incubated overnight at 37 °C and 5 % CO_2. Finally, transfected cells were treated with bleomycin and/or LPS.

Senescence induction

HMC3 cells were firstly treated with 50 µg/mL bleomycin for 24 h for senescence induction (adapted from [42]), and after that period of time, 1 µg/mL LPS [43] was applied into the culture media for additional 12 h. In detail, HMC3 cells were cultured in 6-well-treated plates in a density of $3 \cdot 10^4$ cells per well, in appropriate culture medium with vehicle or 50 µg/mL bleomycin. This culture was maintained for 48 h and subsequently, cells were counted. Next, cells were seeded again in the same density, and the procedure was repeated for another 48 h. With these data, cumulation of population doubling level (ΔPDL) was calculated, which represents the number of times in which cells were doubled. For that, the following formula was used: PDL = Log_{10} (number of HMC3 at day 2/number of HMC3 at day 0)/Log_{10} (2).

Senescence was induced in rat microglia cultures as previously described [44,45], with minor modifications. After microglia purification from new-born brains, the cells were seeded on 6 well plates coated with poly-L-lysine (10 µg/mL) at a density of 100,000 cells/cm^2 for PCR analysis. Cells were maintained for 1 or 18 days at 37º C and 5% CO2 in RPMI medium containing 0.5% FBS. The cells were incubated for at least 12 h in antibiotic-free serum-free RPMI prior to be lysed to gene expression study.

Senescence-associated-β-Galactosidase assay

For the analysis of the activity of β-galactosidase in senescent cells, a commercial kit was used (9860S, Cell Signaling). For the performance of the assay, manufacturer's instructions were

followed. Briefly, $2.5 \cdot 10^4$ HMC3 cells were seeded in 6-well-treated plates in 2 mL of appropriate culture medium with vehicle or 50 µg/mL Bleomycin. After 48 h of treatment, cells were washed with PBS fixed and stained. In particular, staining was done keeping cell plates overnight with the pH 6-adjusted-staining-reagent in a non-CO_2 incubator. Cell senescence was quantified using an optical microscope, and.

mRNA expression analysis

Total RNA was extracted with trizol (Life Technologies). Reverse transcription was performed using random priming and Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Quantitative real-time PCR was performed by Absolute SYBR Green mix (Thermo Fisher Scientific) in a CFX384 real-time thermal cycler (Bio-Rad). Variations in input RNA were corrected by substracting PCR threshold cycle values obtained for *GAPDH* in human samples and *β-actin* in murine samples. The ΔΔCT method was used for relative quantification. List of primers is included as Table Suppl. 1.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM) with the number of experiments (n) carried out for each assay. Unless otherwise indicated, statistical significance (p-values) was calculated using a two-tailed Student´s t-test. Asterisks (≠,p≤0.1; *,p≤0.05; **,p≤0.01; ***,p≤0.001; ****,p≤0.0001). Correlation analysis was performed using the spearman correlation coefficient, as samples followed a non-normal distribution. All the statistical analyses and graphics were performed using Microsoft Office Excel, IBM SPSS Statistics 20 and GraphPad Prism 8 software.

Data Availability Statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials. List of abbreviations is included as Table Suppl. 2.

Study approval

This study was approved by the Clinical Research Ethics Committee of the Donostia University Hospital (AMM-MHP-2019-1) and adhered to the tenets of the Declaration of Helsinki by the World Medical Association regarding human experimentation. All study participants signed informed consent form approved by the Institutional Ethical Committee. All processes involving animals were subjected to approval by the Research Animal Care of Biodonostia Institute.

RESULTS

High levels of HDAC expression are associated with microglial senescence *in vitro*

As a first approach for characterizing the impact of HDACs on microglial aging, we aimed to recapitulate it by two independent *in vitro* models in which we induced senescence. First, we cultured HMC3 human microglial cells with the senescence inductor bleomycin [42]. Herein, we confirmed that 48 h of bleomycin treatment increased the number of HMC3 cells positive for the senescence-associated β-galactosidase activity staining (***Fig. 1A***), and stopped proliferation of HMC3 cells (***Fig. 1B***). Regarding molecular changes, we found that bleomycin treated microglia cells presented significant upregulation of *p21^{Cip1}* (***Fig. 1C***) and also elevated mRNA levels of members of Caspase family (***Fig. 1D***). However, cells did not display clear induction of proapoptotic PARP protein (***Fig. Suppl. 1***). In this context, we checked the expression of *HDACs*. We selected *HDAC1, 2, 3, 6* and *7*, based on their link with age-associated brain disorders [46-51]. Interestingly, bleomycin treated microglia presented an increase of *HDAC1* and *HDAC3* mRNA levels (***Fig. 1E***). Of note, when activating microglia with lipopolysaccharide (LPS) for 12 h, microglial activation markers *CD68*, *ITGAM*, *AIF1* and *IL6* expression were increased in both control and bleomycin induced senescent cells (***Fig. 1F***). However, no clear changes were observed in any of the studied HDACs (***Fig. 1E***).

Next, we performed an experiment that triggers senescence in microglia cells in rats *in vitro*, which has shown to be sex dependent [44,45]. In particular, primary microglia cells from rats were maintained in culture for 18 days. In this context, we compared the expression of different HDACs at 1 and 18 days in vitro (DIV) in male and female microglial cells. *HDAC6* and *HDAC7* were increased at 18 DIV in cells from both sexes, whereas *HDAC1* and *HDAC3* were higher in microglia derived females at DIV18 compared to DIV1 (***Fig. 1G***).

HDAC1 levels are associated with *p21^{CIP}* and *REST* in microglial senescence *in vitro*

We next wondered if the upregulation of HDACs could have an impact on microglial senescence. Among HDACs studied, *HDAC1* was the most significantly dysregulated upon bleomycin treatment, whereas *HDAC2*, although not altered in our study, share some redundant functions with HDAC1 [52]. Thus, we performed their individual ectopic overexpression in HMC3 cells. After validating the upregulation of both transcripts (***Fig. 2A)***, we studied the expression of *p21^{Cip1}* in these cells. No changes were observed at basal level, but bleomycin treated cells presented a higher induction of *p21^{Cip1}* in HMC3 cells overexpressing *HDAC1* or *HDAC2* compared to control cells (***Fig. 2B***). Besides, HDAC1, but no HDAC2, overexpressing cells showed an upregulation of *CD68* expression, after bleomycin treatment, compared to control cells (***Fig. 2C***).

Repressor Element 1 Silencing Transcription Factor (REST) is an important player of aging and cognitive activity that directly interacts with HDACs in a repressor complex as shown by us (***Fig. 2D***) and others [53,54]. In this line, we observed that *REST* tended to be also increased upon bleomycin treatment in HMC3 (***Fig. 2E***) and that this upregulation was greater in HDAC1 overexpressing senescent HMC3 (***Fig. 2F***).

Hdac and microglial marker expression is increased in aged dentate gyrus in mice

Next, we studied the link of HDACs and microglia *in vivo*. First, we analyzed the expression of *HDACs* in adult mice brain sections taking advantage of Allen Brain Atlas data portal. *Hdac1, 2, 3, 6* and *7* were highly expressed in the dentate gyrus **(**DG) from adult C57BL6 mice (***Fig. 3A***). Indeed, *Hdac2* and *Hdac3* were also expressed in other regions of the brain, but in a lower intensity. When studying microglial marker expression in the same samples, *Cd68* was as well highly enriched in the DG, whereas no strong signal was detected for *Iba1* (***Fig. 3B***).

Thus, we subsequently moved to study Hdac expression in DG samples from a set of young and aged mice. Although variability between samples was high, aged mice DG samples presented higher levels of *Hdac3* and *7* among the studied Hdacs (***Fig. 3C***). Moreover, we observed that *Iba1* was upregulated in aged mice compared to young ones (***Fig. 3D***), We also detected significantly increased expression of the senescence marker $p16^{Ink4a}$ *and a tendency in the case of* $p21^{Cip1}$ *(**Fig. 3D**).*

HDAC and microglial marker expression is elevated in aged human hippocampus

With the aim of translating the obtained results in mice into human samples, we analyzed the same set of *HDACs* in hippocampal samples from young and aged human individuals. Herein, we found that aged individuals presented general increase on the expression of *HDACs*, with significant results in the case of *HDAC1, 3* and 7 (***Fig. 4A***). For the characterization of microglial content, we measured *AIF1 (IBA1) as well* as *CD68* and *ITGAM*. Notably, the 3 microglial markers were significantly elevated in hippocampus samples from aged individuals compared to young ones (***Fig. 4B***). Moreover, aged hippocampus showed significant upregulation of the senescence markers $p16^{INK4A}$ and $p21^{CIP1}$ (***Fig. 4C***). To test the link between *Hdac* expression and microglial aging in this brain region, we performed correlative analysis. Intriguingly, *HDAC1, 3* and *7* showed positive and significant correlation with *CD68*, *ITGAM* and *AIF1* (***Fig. 4D***), being *HDAC1* and *HDAC7* the ones with strongest Spearman correlation coefficients. Moreover, there was also a robust correlation between *HDAC2* and *HDAC6* (***Fig. Suppl 2A***). These positive correlations were extended into $p21^{CIP1}$ (***Fig. 4E, Fig. Suppl. 2B***) and $p16^{INK4A}$ senescence markers (***Fig. Suppl. 2C***) and *REST* (***Fig. 4F***). These data indicate a significant association between the levels of HDACs and microglia in human hippocampus with aging. Consistent with this, there was also a positive correlation between microglial and senescence markers (***Fig. Suppl. 2D,E***).

Taking advantage of transcriptomic studies in publicly available datasets, we further characterized the expression of HDACs in human aged individuals. Herein, we firstly observed that HDAC expression pattern was variable among different organs and tissues (***Fig. Suppl. 3)***. Moreover, we found that the expression of *HDAC1* and *HDAC2* was enriched in the hippocampus (***Fig. 4G***). Similarly, *CD68*, *ITGAM* and *AIF1* microglial markers were enriched in this brain region (***Fig. 4G***), as well as REST levels (***Fig. 4G***). Additionally, *HDAC1, 2, 6* and *7,* as well as *REST,* expression was also high in white matter of forebrain, where glial cells are predominantly found (***Fig. 4G***). Accordingly, high expression of microglial markers was also observed in this brain region (***Fig. 4G),*** reinforcing the positive correlation between HDAC expression, REST and microglia.

HDAC and microglial marker expression is upregulated in human microglia *in vivo*

Next, we checked *HDAC* expression directly in aged microglia. For that, we first analyzed their expression in a publicly available single cell RNA sequencing study performed in the brain. Herein, we observed that glial cells presented a greater expression of *HDAC1, 7* and *REST* than neurons (***Fig. 5A***), whereas *HDAC2, 3* and *6* showed a more homogeneous expression pattern (***Fig. 5A, Fig. Suppl. 4A***). As expected, microglial cells expressed high levels of *CD68* and *ITGAM* (***Fig. Suppl. 4B***). Next, we took advantage of RNAseq data from aged human bulk dorsolateral prefrontal cortex samples and purified microglia from the same region. Herein, as expected, we observed that microglial markers *CD68, ITGAM* and *AIF1* were clearly elevated in microglia compared to bulk cortex (***Fig. 5B***). Interestingly, we found that the expression of *HDAC1, 2, 3* and *REST* was also enriched in purified microglia (***Fig. 5C***).

DISCUSSION

Previous studies in rodent models showed that *Hdac2* expression [28,29] and overall HDAC enzymatic activity [30] increased with age in the hippocampus. In this study, taking advantage of Allen Brain Atlas, we observed that the expression of *Hdac1, 2, 3, 6* and *7* was specifically intense in DG from adult mice. Moreover, we detected that aged murine dentate gyrus (DG) presented higher levels of *Hdacs* compared to young samples, although differences were only significant for the case of *Hdac3* and *7*. This could be attributed, at least in part, to the low number of samples and the heterogeneity between them. Importantly, our work further extends these results into human samples, wherein we observed significant elevation of *HDAC1, 3* and *7* in hippocampus from aged individuals. A similar but not significant tendency was observed in *HDAC2*, suggesting a generalized upregulation of *HDAC* expression. Accordingly, a study based on *in vivo* neuroimaging of aged human individuals reported not significant, but higher expression of *HDAC1, 2* and *3* in human hippocampus [55]. We did not perform cognitive behavioural experiments neither in the young and old control animals or with genetic deletion or overexpression of Hdacs to extrapolate results directly to aging and cognitive decline. However, the dysregulation of HDAC activity has been associated with memory and learning cognitive impairments [35,56,57], Besides, HDAC2-overexpressing mice presented reduced synapse number and learning impairment, which were ameliorated by chronic HDAC inhibitor (HDACi) treatment [35]. In the same line, *HDAC* knock down or the use of HDACis at organismal level have extended lifespan and healthspan in yeast, worm, fly and rodent models [58,59].

Microglia is the most abundant immune cell type within the brain and HDACs seem to be involved in its regulation [39]. Thus, single cell RNA sequencing study of human brain showed that *HDAC1, 7* and *REST* levels were enriched in glial cells, with special emphasis in microglia. At functional level, the deletion of *Hdac1* and *Hdac2* in mice led to enhanced apoptosis and reduced survival of microglia in developmental stages [39]. The same study found that the deletion of *Hdac1* and *Hdac2* did not affect survival of adult microglia during homeostasis, but it enhanced microglial amyloid phagocytosis in a mouse model of Alzheimer's disease [39]. However, no studies have characterized the expression and role of HDACs in microglia aging.

We found that the expression of different HDAC members is increased in microglial aging using different *in vitro* and *in vivo* models. *In vitro*, we found that bleomycin, an inducer of senescence but also apoptosis, promoted a strong senescence induction with over 20-fold elevation in $p21^{CIP1}$ levels and in Senescence-associated-β-Galactosidase positive cells. In this context, we detected an increase of ~20% in the levels of *HDAC1* and *HDAC3* in human microglia cells. Similarly, microglia cells derived from rats maintained in culture for 20 days also displayed significantly higher levels of Hdacs compared to cells cultured for 1-2 days. Moreover, the ectopic overexpression of *HDAC1* or its homologous *HDAC2* [52] in human microglia cells promoted the upregulation of $p21^{CIP1}$. When analyzing *in vivo* samples, we unraveled that the expression of *HDAC1*, *HDAC3* and *HDAC7* was increased in aged hippocampal samples. Intriguingly, expression of HDACs was positively associated with *CD68, ITGAM* and *AIF1* microglial markers in human hippocampal samples. Although the detected rise was slight, it is noteworthy to say

that small changes in HDACs can have a large impact at functional level due to pleiotropic effects [55]. In this line, genetic knock down of *Hdac2* expression by 25-30%, increased transcription of memory-related genes and improved spatial memory in an Alzheimer's disease rodent model [60]. Similarly, aged samples from the WM of forebrain showed an enriched expression of *HDAC1, 2, 6, 7*, REST and microglial markers together supporting that elevation of HDACs activity promotes microglial senescence and aging. In the previously mentioned *in vivo* neuroimaging study, authors found a significant increase in the protein expression of HDAC1 and 2 with age in cerebral WM [55], but no mention was made regarding any specific cell-type. Indeed, reduced expression of HDAC2 in microglia has been shown to promote neurological functional recovery and to reduce WM injury after intracerebral hemorrhage [61]. Moreover, WM hyperintensities, common age-associated findings that impact cognition [62], have been associated with decreased prefrontal cortex (PFC) activity in elderly individuals [63]. Intriguingly, our data show that microglia from dorsolateral PFC of aged individuals presented enriched expression of *HDAC1, 2* and *3* compared to bulk tissue. In summary, our results confirm increased expression of HDACs in microglial aging in different brain regions where they correlate with senescence markers and REST.

CONCLUSION

Our work reveals that expression of different HDACs is increased in two independent *in vitro* models upon senescence induction and in murine and human brain samples *in vivo* with age, where their expression positively correlates with microglia markers and REST. Among all studied HDACs, HDAC1 and 3 seem to be the strongest member associated with microglial aging, as they followed the positive tendency in almost all experiments and cohorts analyzed. These results link HDAC expression and microglia aging and suggest that alterations in the expression of HDACs, in particular rise, could play a role in brain aging, particularly in the hippocampus neurogenic niche microenvironment. This information is novel and relevant since it could serve to propose new approaches of HDAC inhibitors to treat brain aging and cognitive decline.

ACKNOWLEDGMENTS: J.A-I (PRE_2016_1_0375), L.M-C (PRE_2014_1_92) and A.S-A (FI17/00250) received predoctoral fellowships from Department of Education, University and Research of the Basque Government and Carlos III Institute, respectively. J.A-I received an EMBO Scientific Exchange Grant (number 8775) for developing experiments in the group of IC. W in the University of Leeds. This work is supported by grants from Instituto de Salud Carlos III cofinanced by European Union (PI19/01355, DTS20/00179) and Health Department of the Basque Country.

AUTHOR CONTRIBUTIONS: J.A-I performed experiments and analyzed the results with the help of L.M-C and A.S-A. J.A-I also wrote the draft of the manuscript. DG and MA.A performed and analyzed the experiments of microglia cultures in rats. M.M-P provided biological samples. I.W offered reagents and experience and helped with supervision of experiments performed in Leeds. All revised the manuscript. A.M directed the project, contributed to data analysis and wrote the manuscript.

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FIGURE LEGENDS

Figure 1. HDAC expression is increased with microglial senescence. (A) Representative image (left) and quantification (right) of microglial cells positive for senescence-associated β-galactosidase (S-A-β-Gal) activity staining upon 48 h treatment of 50 µg/mL bleomycin (n=4). **(B)** Cumulative population doubling level (PDL) at day 2 and day 4 treatment of 50 µg/mL bleomycin (n=4). **(C,D)** Relative mRNA expression of *p21^{CIP}*, *Caspase3, Caspase7 and Caspase9* with control, 1 µg/mL LPS, 50 µg/mL bleomycin or 50 µg/mL bleomycin plus 1 µg/mL LPS (n=3) for 24h + 12h. **(E,F)** Relative mRNA expression of the indicated transcripts in above described conditions (n=3). **(G)** Relative mRNA expression of the indicated HDAC transcripts in male and female rat microglia cultured for 1 or 18 days.

Figure 2. HDAC1 expression is associated with *p21^{CIP}* and *REST*. (A) Relative mRNA expression of *HDAC1*, *HDAC2* and **(B,C)** *p21^{CIP1}* and *CD68* in HMC3 transfected with control and HDAC1 or HDAC2 overexpression (OE) plasmids, and treated with control, 50 µg/mL bleomycin or bleomycin plus 1 µg/mL LPS for 24 h + 12 h (n=4). **(D)** Map of interactions between REST, HDAC1, 2, 3, 6 and 7 at protein level. **(E)** Relative mRNA expression of *REST* with control, 1 µg/mL LPS, 50 µg/mL bleomycin or 50 µg/mL bleomycin plus 1 µg/mL LPS (n=3). **(F)** Relative mRNA expression of *REST* in HMC3 transfected with control and HDAC1 or HDAC2 overexpression (OE) plasmids, and treated with control or 50 µg/mL bleomycin for 24 h (n=4).

Figure 3. HDAC expression is high in dentate gyrus of adult C57BL6. (A) Images of *in situ* hybridization (up) and expression (down) of *Hdac1, 2, 3, 6* and *7* and **(B)** microglial markers *Iba1* (*Aif1)* and *Cd68* in brain sections from P56 *C57BL6* mice. **(C)** Relative mRNA expression of the indicated *Hdac* transcripts in dentate gyrus from young (1-2 months, n=13) and aged (13-32 months, n=23) C57BL6 mice. **(D)** Relative mRNA expression of microglial marker Iba1 (*Aif1),* *p16^{nk44a}* and *p21^{Cip1}* in dentate gyrus from the same young and aged C57BL6 mice.

Figure 4. HDAC expression is high in hippocampus of aged human individuals. (A) Relative mRNA expression of the indicated *Hdac* transcripts in hippocampus from young (27-45 years old) and aged (76-96 years old) human individuals (n=16 vs n=17, respectively). **(B)** Expression of *CD68*, *ITGAM* and *Aif1* microglial markers and **(C)** Expression of *p16^{INK4A}* and *p21^{CIP1}* senescence markers were also analyzed in same human samples. **(D)** Correlation analysis of *HDAC1, 2, 3, 6 and 7* with *CD68*, *ITGAM* and *AIF1* microglial markers in young and aged human hippocampus (n=33). Linear regression is shown. **(E,F)** Correlation of *HDACs* with *p21^{CIP1}* and *REST* expression. For *p21^{CIP1}* gene, due to the variability, Kolmogorov–Smirnov (KS) test was used to confirm the association with the rest of the genes: *HDAC1* (KS p= 0.0103), *HDAC3* (KS p= 0.0005), *HDAC7* (KS p< 0.0001). **(G)** RNAseq results for *HDACs,* microglial markers and REST expression represented by z-score in white matter of forebrain (FWM), hippocampus (HIP), parietal neocortex (PCx) and temporal neocortex (TCx) of aged human individuals ranged between 78-100+ years old (n=30). Data obtained from https://aging.brain-map.org/rnaseq/search.

Figure 5. HDACs are enriched in isolated microglia from aged individuals. **A)** Single cell RNAseq data from whole brain for the expression of *HDAC1, 3, 7* and *REST*, obtained from human protein atlas (https://www.proteinatlas.org/). RNAseq data for the expression of **(B)** microglial markers *CD68, ITGAM* and *AIF1*, and **(C)** *HDAC1, 2, 3, 6, 7* and *REST* in bulk dorsolateral prefrontal cortex (n=540) *vs.* purified microglia from the same region (n=10). Data obtained from http://shiny.maths.usyd.edu.au/Ellis/MicrogliaPlots/.

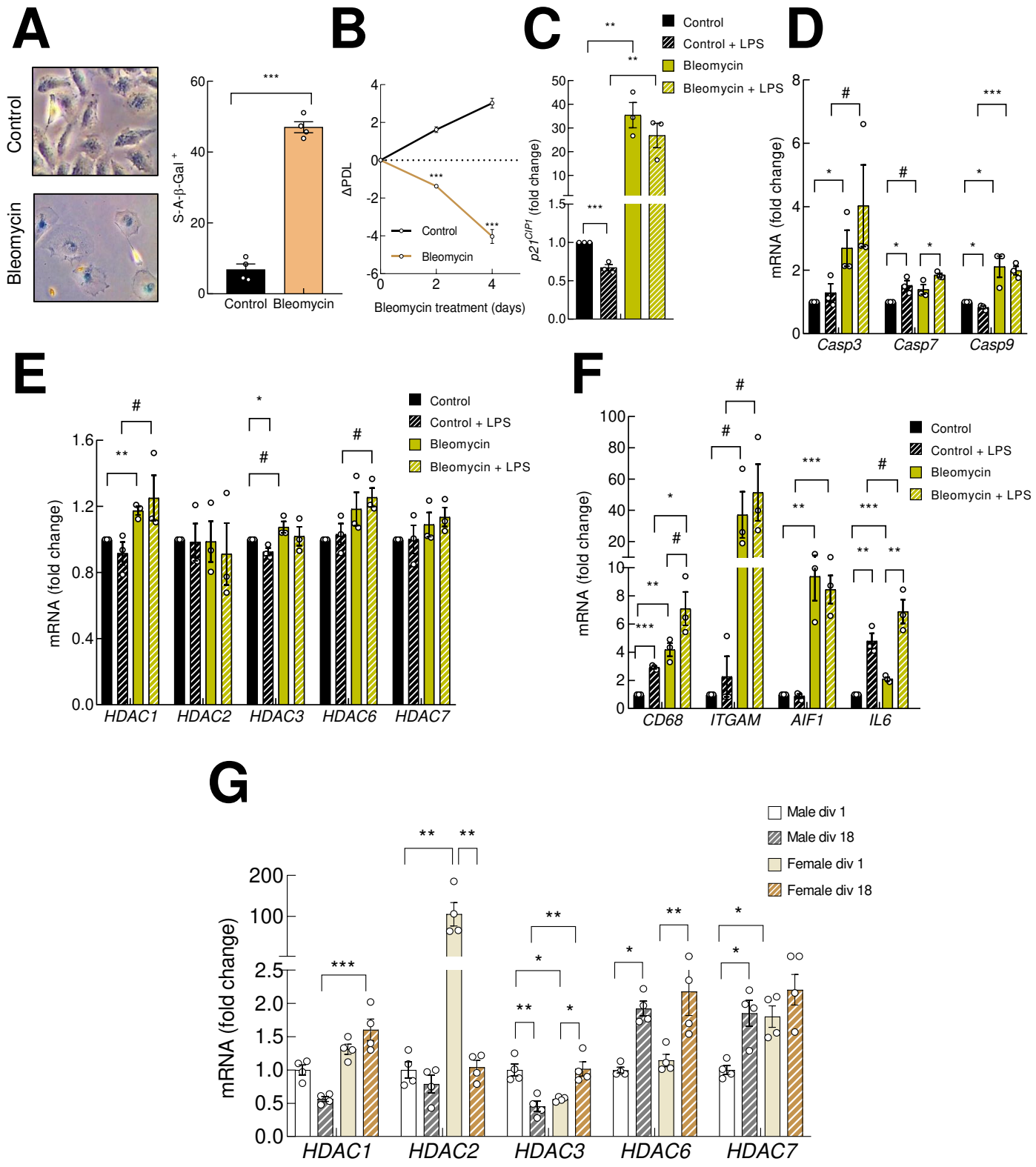


Fig. 1. Auzmendi-Iriarte et al.

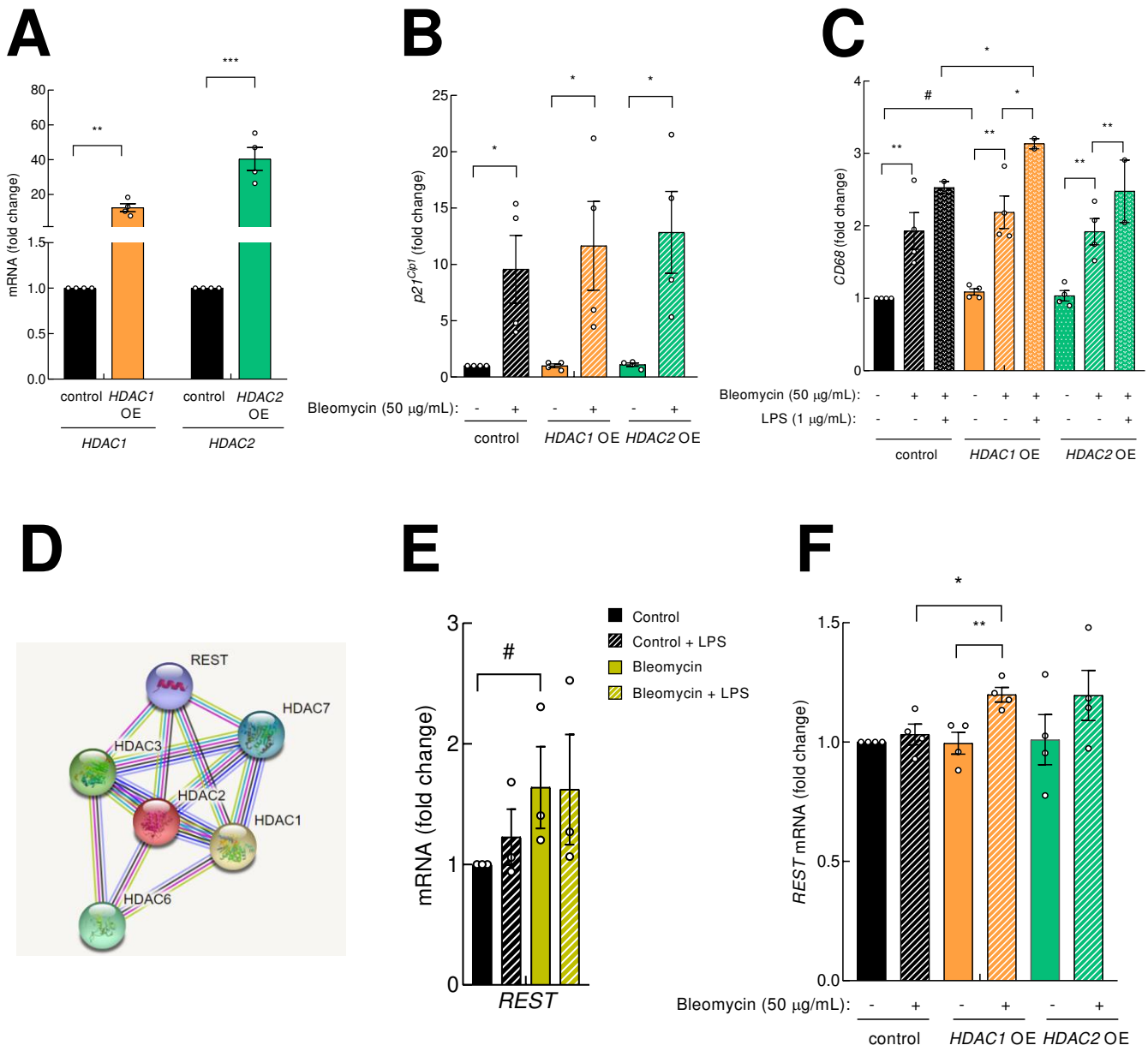


Fig. 2. Auzmendi-Iriarte et al.

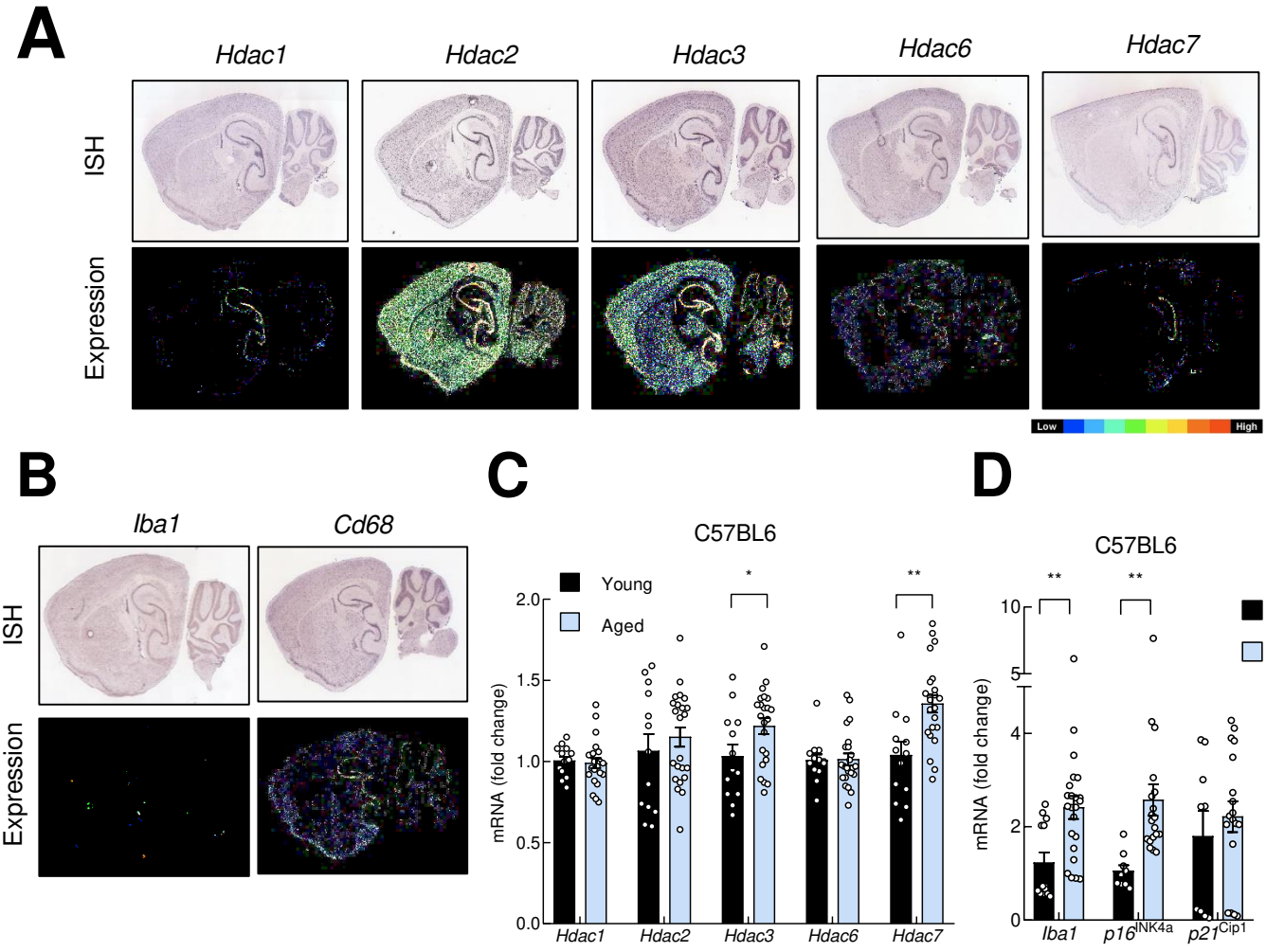


Fig. 3. Auzmendi-Iriarte et al.

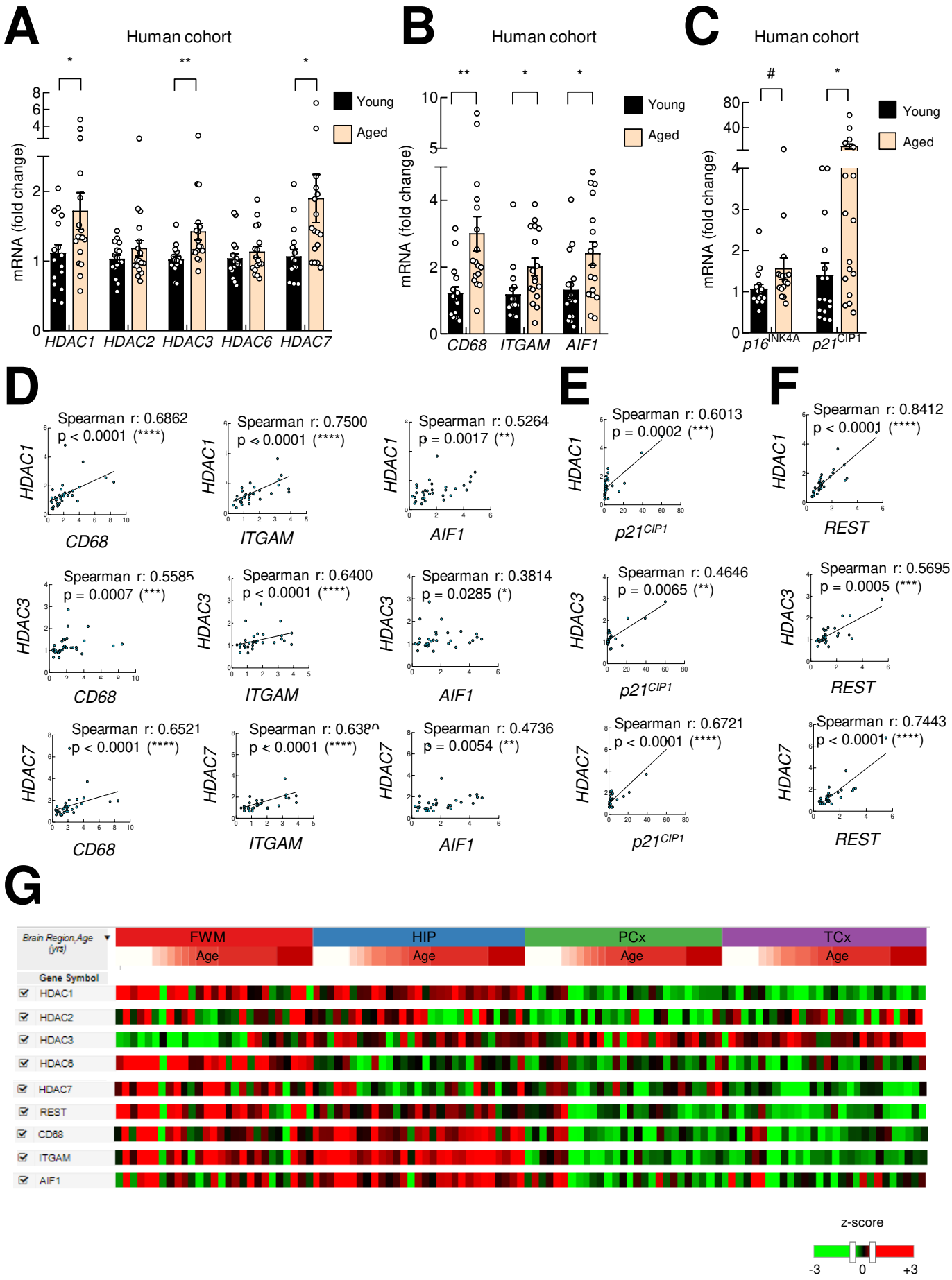


Fig. 4. Auzmendi-Iriarte et al.

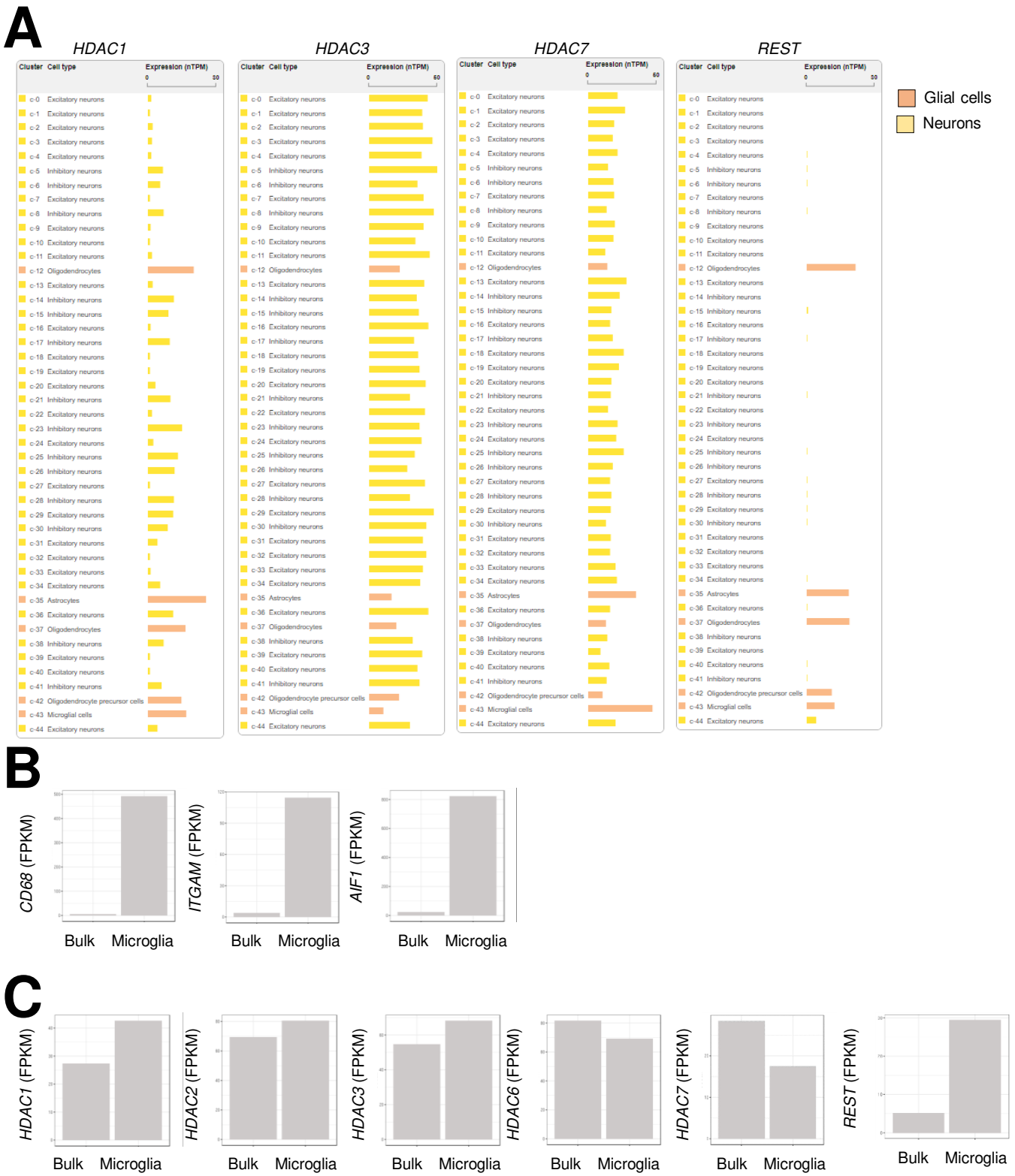
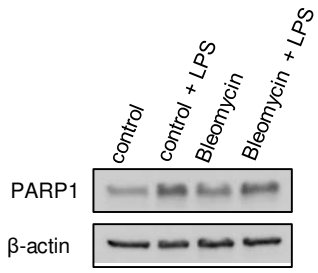
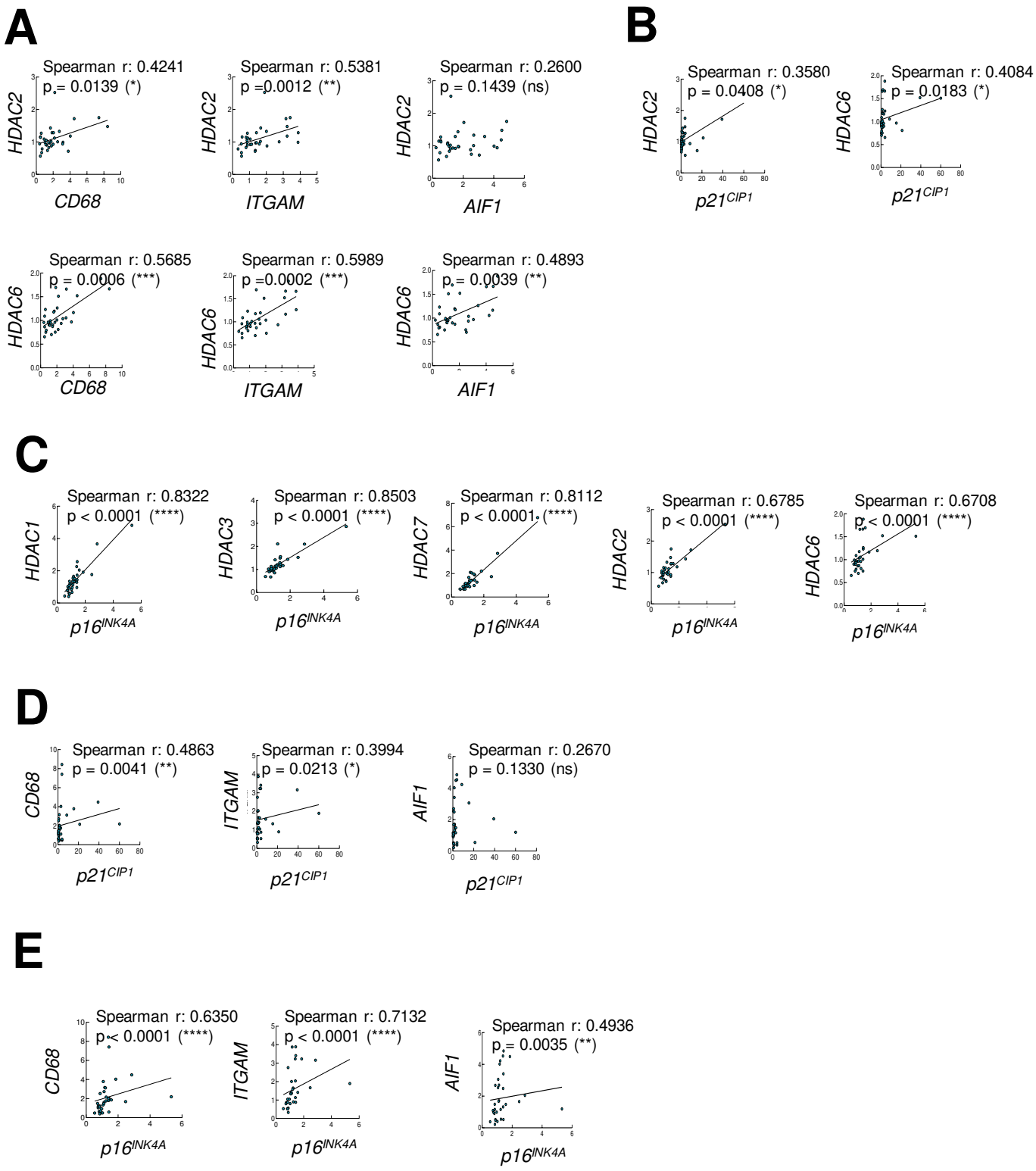


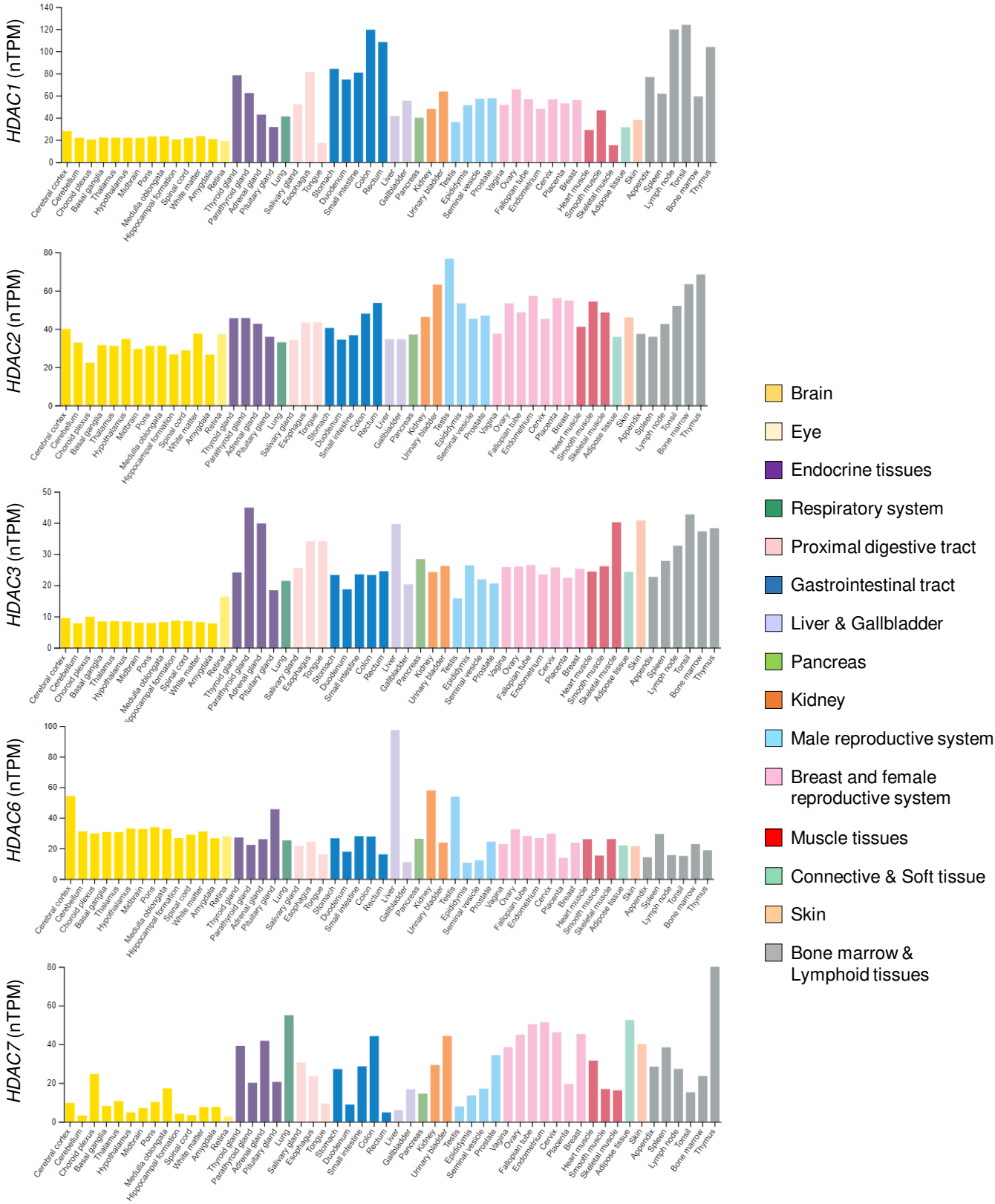
Fig. 5 Auzmendi-Iriarte et al.



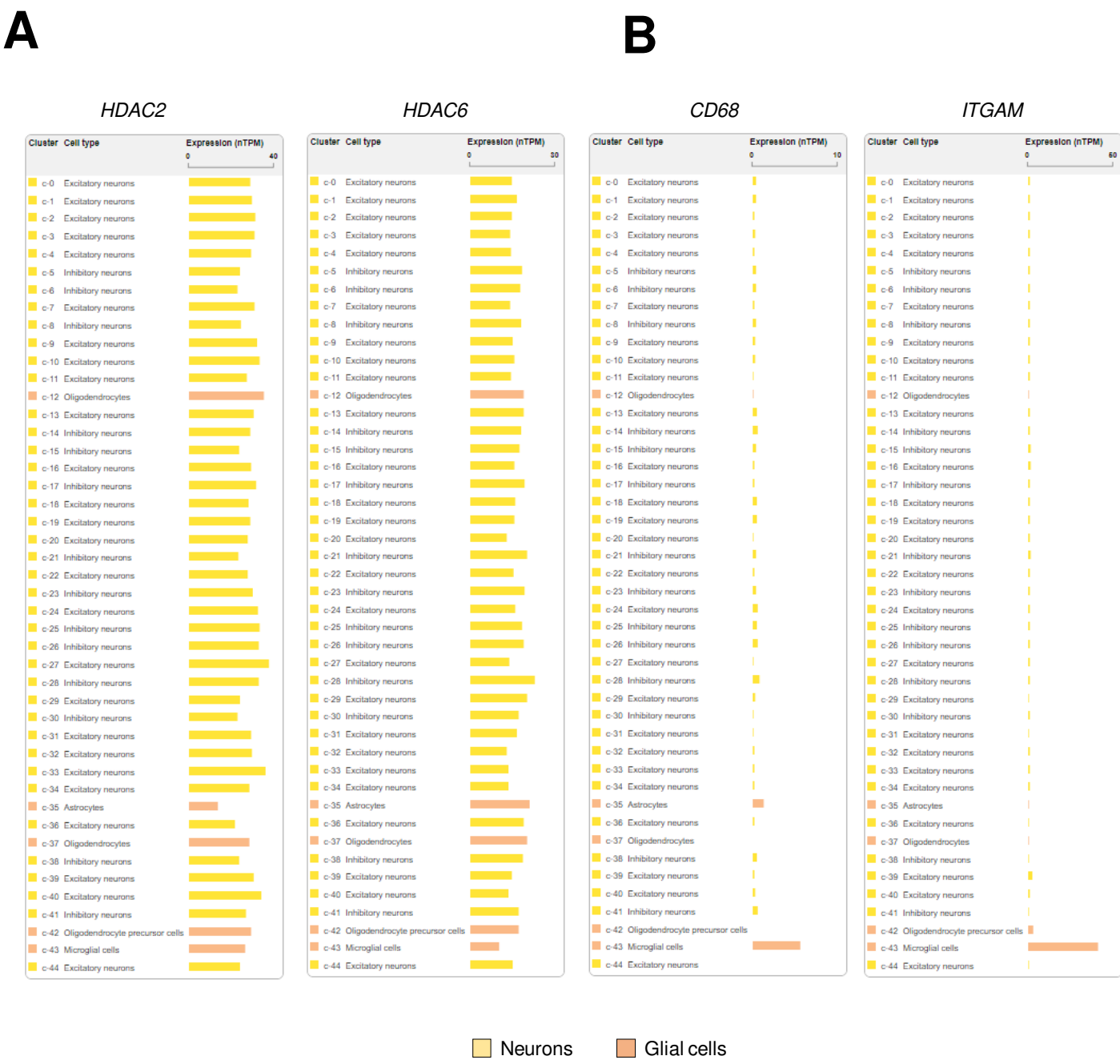
Suppl. Fig. 1. PARP1 is not induced upon bleomycin and LPS treatments. Immunoblot of apoptosis marker PARP1 and β –actin in HMC3 treated with control, 1 µg/mL LPS, 50 µg/mL bleomycin or 50 µg/mL bleomycin plus 1 µg/mL LPS for 24h + 12h conditions.



Suppl. Fig. 2. *HDAC* expression correlate with microglia, senescence and REST markers in human hippocampus. (A-E) Correlation analysis of *HDACs* with the indicated transcripts. Linear regression is shown when correlation is significant. For *p21CIP1* gene, due to the variability, Kolmogorov–Smirnov (KS) test was used to confirm the association with the rest of the genes: *HDAC2* (KS p= 0.0616), *HDAC6* (KS p= 0.0121), *CD68* (KS p =0.0005), *ITGAM* (KS p= 0.0095), *AIF1* (KS p= 0.0052).



Suppl. Fig. 3. HDAC expression is variable among human tissues. Normalized expression (nTPM) levels for the indicated tissue types, created combining the HPA and GTEx transcriptomic datasets using the internal normalization pipeline.



Suppl. Fig. 4. ***HDAC2*** **and** ***HDAC6*** **expression pattern is similar in neurons and glial cells.** Single cell RNAseq data from whole brain for the expression of **(A)** *HDAC2* and *6*; **(B)** and *CD68* and *ITGAM*, obtained from human protein atlas (https://www.proteinatlas.org/).

Suppl. Fig. 4 Auzmendi Iriarte et al.