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Histamine Stimulates Neurogenesis in the Rodent Subventricular Zone

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ABSTRACT

Neural stem/progenitor cells present in the subventricular zone (SVZ) are a potential source of repairing cells after injury. Therefore, the identification of novel players that modulate neural stem cells differentiation can have a huge impact in stem cell-based therapies. Herein, we describe a unique role of histamine in inducing functional neuronal differentiation from cultured mouse SVZ stem/progenitor cells. This proneurogenic effect depends on histamine 1 receptor activation and involves epigenetic modifications and increased expression of *Mash1*, *Dlx2*, and *Ngn1* genes. Biocompatible poly (lactic-*co*-glycolic acid) microparticles, engineered to release histamine in a controlled and prolonged manner, also triggered robust neuronal differentiation *in vitro*. Preconditioning with histamine-loaded microparticles facilitated neuronal differentiation of SVZ-GFP cells grafted in hippocampal slices and in *in vivo* rodent brain. We propose that neuronal commitment triggered by histamine per se or released from biomaterial-derived vehicles may represent a new tool for brain repair strategies. STEM CELLS *2012;30:773–784*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Neural stem cells reside in two niches of the adult brain: the subventricular zone (SVZ) lining the lateral ventricles and the subgranular zone (SGZ) in the dentate gyrus (DG) of the hippocampus [1]. SVZ-derived neuroblasts migrate through the rostral migratory stream (RMS) toward the olfactory bulb where they differentiate into functional interneurons [2]. Interestingly, upon brain injury, some neuroblasts can leave the RMS to migrate toward damaged areas where they differentiate into the dead cells' neuronal-specific phenotype [3]. Therefore, taking advantage of these unique stem cell's properties, much attention has been given to the design of experimental strategies for promoting brain repair [4, 5]. For that purpose, it is crucial to identify new proneurogenic factors that can enhance neural stem cells capabilities.

Histamine, an amine acting as a neurotransmitter and neuromodulator at central nervous system (CNS) and peripheral nervous system, is present in the brain in three main cellular stores: neuronal, mast, and microglial cells [6]. Its actions can be mediated through several receptors: two postsynaptic (H1R and H2R), one presynaptic (H3R), and one more receptor mainly present in the immune system (H4R). All receptors belong to the family of rhodopsin-like class A receptors coupled to guanine nucleotide-binding proteins (G proteins) [6, 7]. Histaminergic neurons, present in the tuberomammillary nucleus, project numerous ramifications throughout the entire adult brain. This makes histamine involved in a broad range of physiological functions, such as sleep-wake control, emotions, learning, and memory [6–8]. Histamine has also been reported to be involved in several pathologic conditions, such as neuroinflammation, epilepsy, and brain infarction [9–11]. Regarding adult neurogenesis, it has been shown

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that histamine receptors are expressed in undifferentiated neural progenitors. We and others have shown that histamine transiently increases intracellular free calcium levels ($[Ca^{2+}]_i$) in SVZ immature/stem cells, embryonic stem cells, and carcinoma cells [12, 13]. Moreover, Molina-Hernandez and Velasco [14] showed that histamine induces embryonic neural stem proliferation and neuronal differentiation. Despite these reports, histamine effects on the SVZ neurogenic niche have not yet been addressed. Herein, we unravel the effects of histamine in SVZ cell proliferation, survival, and neuronal differentiation as well as the underlying molecular mechanisms involved. Furthermore, we also developed histamine-loaded poly (lactic-*co*-glycolic acid) (PLGA) microparticles in order to promote a more efficient and controlled delivery of histamine. Overall, our results identify histamine as a pro-neurogenic factor for SVZ cells, whose effects are potentiated through delivery by microparticles, highlighting its possible use in future brain repair strategies.

Materials and Methods

All experiments were performed in accordance with European Union (86/609/EEC) guidelines for the care and use of laboratory animals. Both wild-type (WT) and green fluorescent protein (GFP) transgenic C57BL6 mice were used.

SVZ Cell Cultures

SVZ cell cultures were prepared from 1 to 3-day-old mice as described in [15] (Supporting Information). SVZ cells were grown in serum-free media (SFM) supplemented with 10 ng/ml epidermal growth factor (EGF) and 5 ng/ml fibroblast growth factor 2 (FGF-2) (Invitrogen, Carlsbad, CA, http://www.invitrogen.com). Six days afterplating, the resulting SVZ neurospheres were seeded onto glass coverslips coated with 0.1 mg/ml poly-D-lysine in SFM medium devoid of growth factors. Two days after plating, the medium was renewed with or without a range of concentrations for histamine or PLGA histamine-releasing microparticles.

Pharmacological Treatments

To investigate the influence of histamine on neuronal differentiation, SVZ cells were allowed to develop for 7 days with histamine or histamine-releasing microparticles. Free histamine (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com) was added to the cell cultures at the following concentrations: 10, 100, and 500 μM. A dilution series of microparticles were also tested as follows: 10 ng/ml, 100 ng/ml, and 1 μg/ml (weight of particles per microliter of culture medium).

To determine which histamine receptor was involved in the proneurogenic effect, SVZ cells were treated with 100 μM histamine together with antagonists for each receptor: mepyramine (H1R antagonist, 1 μM; Tocris, Bristol, U.K., www.tocris.com), cimetidine (H2R antagonist, 5 μM; Tocris), or thioperamide (H3R and H4R antagonist, 1 μM; Sigma-Aldrich). On the second day, the treatments were renewed. To investigate the involvement of the c-Jun N-terminal kinase mitogen-activated protein kinase (JNK MAPK) signaling pathway in response to histamine stimulation, SVZ neurospheres were exposed to 100 μM histamine for only 6 hours.

To access whether histamine promotes trimethylated lysine 4 form of histone H3 (H3K4m3) on the promoter region of *Mash1*, *Dlx2*, and *Neurogenin1* (*Ngn1*), cells were incubated with 100 μM histamine for 24 or 48 hours and then processed for quantitative chromatin immunoprecipitation (qChIP). To confirm qChIP analysis, the mRNA expression levels of the aforementioned genes were determined by qRT-PCR in SVZ cells treated or not (control) with 100 μM histamine for 48 and 72 hours.

Cell-Fate Studies: *Sox2* Cell Pair Assay

Dissociated SVZ cell suspensions obtained during the cell culture procedure were plated onto poly-D-lysine-coated glass coverslips at a density of 6,400 cells per square centimeter. After seeding, SVZ cells were grown in SFM containing 5 ng/ml EGF and 2.5 ng/ml FGF-2 (low EGF/FGF-2) supplemented or not (control) with 100 μM histamine for 24 hours. Cells were then fixed in methanol for 15 minutes at -20°C and processed for immunocytochemistry against Sox2 and Dlx2.

Preparation and Characterization of PLGA Microparticles

Single emulsion technique was used to prepare microparticles of approximately 2 μm in diameter. PLGA (resomer 752H) (100 mg; Boehringer Mannheim, Mannheim, www.roche.com) was dissolved in 2.5 ml of a solvent mixture (1:4, methylene chloride/trifluoroethanol) and 5 mg histamine was added. This solution was added to a stirred chilled polyvinyl alcohol solution (100 ml, 5% (w/v)). The resulting suspension was stirred for 3 hours, washed with distilled water, and finally freeze-dried. The morphology and diameter of PLGA particles were evaluated by scanning electron microscopy according to our previous reports [16]. Release experiments in phosphate-buffered saline (PBS) at 37°C were performed in order to evaluate the release profile of histamine for more than 30 days and to assess the loading efficiency of the microparticles (details in Supporting Information). Blank microparticles, that is, without histamine, were also prepared to test the effect of the microparticle formulation per se in SVZ cells.

Single-Cell Calcium Imaging

To determine the functional differentiation pattern of SVZ cells, the variations of intracellular calcium concentrations in single cells following stimulation with 50 mM KCl (Merck, Darmstadt, Germany, www.merck.com) or 100 μM histamine (Sigma-Aldrich) were analyzed [13]. Histamine/KCl peak values for Fura-2 ratio were calculated to determine the extent of neuronal differentiation in SVZ cultures (Supporting Information).

qChIP

Chromatin was crosslinked to proteins with 1% formaldehyde and then fragmented by sonication. Chromatin was incubated overnight with the mouse monoclonal antibody against histone H3 (trimethyl K4) (H3K4me3; Abcam, ab1012, Cambridge, U.K., www.abcam.com) or the unrelated antibody as a negative control (rabbit polyclonal anti-tumor necrosis factor receptor 1 (TNFR1); Santa Cruz Biotechnology, Santa Cruz, CA, www.scbt.com) and then collected by incubation with protein G-Sepharose beads (Sigma-Aldrich). DNA eluted from the washed immune complexes was extracted, precipitated, and then subjected to quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis. Recovery of genomic DNA as a percentage input was calculated as the copy number ratio in the precipitated immune complexes to the input control (Supporting Information).

Isolation of Total RNA and Real-Time RT-PCR Analysis (*Mash1*, *Dlx2*, and *Ngn1*)

Total RNA was isolated from SVZ cells according to illustra RNAspin mini RNA Isolation Kit manufacturer's instructions (GE Healthcare Life Sciences, Hillerød, Denmark, http://www.gehealthcare.com). The full content of a six-well culture plate was pooled for each experimental condition. Precipitated

RNA was resuspended in 40 μl of diethylpyrocarbonate-treated water, and samples were stored at −80°C until further use. cDNA synthesis was made according to Roche Molecular Biochemicals transcriptor first strand cDNA synthesis kit instructions (Roche, Basel, Switzerland, http://www.roche-appliedscience.com). PCR cycles and primers design are detailed in Supporting Information.

Organotypic Hippocampal Slice Cultures

Hippocampal slice cultures were prepared from 7-day-old WT mice as described in [17]. Culture trays were placed in an incubator with 5% CO_2 and 95% atmospheric air at 37°C, and the medium was changed twice a week for 2 weeks. Before grafting, the medium was replaced with 1 ml of serum-free neurobasal medium (Invitrogen) containing 1 mM L-glutamine (Sigma-Aldrich), 2% B27 (Gibco, Invitrogen), 50 U/ml penicillin, and 50 μg/ml streptomycin (Supporting Information).

SVZ-GFP Neurosphere Grafting on Hippocampal Slices

Histamine-loaded or blank (unloaded) microparticles (both at 1 μg/ml) were added to SVZ neurospheres obtained from GFP mice 48 hours before grafting. Grafting experiments were done under sterile conditions with manual delivery of SVZ neurospheres on the surface of hippocampal slices facilitated by a P10 pipette and a blunt Pasteur pipette. In each experimental condition, one SVZ-GFP neurosphere was grafted on the DG of a hippocampal slice. These cocultures were kept in an incubator for 1 week before fixing with 4% paraformaldehyde (PFA). The whole hippocampal slice was processed for immunohistochemistry.

In Vivo Transplantation of SVZ-GFP Cells

Histamine-loaded or blank microparticles (1 μg/ml) were added to GFP-SVZ neurospheres 48 hours before grafting. SVZ neurospheres were dissociated, and the cells were counted and resuspended in SFM to a concentration of 50,000 viable cells per microliter. Weight-matched (generally 25–30 g; 8–9 weeks old) male WT mice were anesthetized with 2,2,2-tribromoethanol (Avertin; Sigma-Aldrich) (240 mg/kg, i.p.) and received stereotaxic unilateral injections of 2 μl of cell suspension in the right DG (anteroposterior (AP): −1.9 mm, mediolateral (ML): −1.2 mm, and dorsoventral (DV): −1.8 mm from bregma) or striatum (AP: 0.6 mm, ML: 1.8 mm, and DV: −3.0 mm). After 3 weeks, mice were anesthetized with an overdose of ketamine (Merial, U.K., http://uk.merial.com) and perfused transcardially with 4% PFA. Brains were stored overnight at 4°C in PFA and were then transferred to 30% sucrose in PBS at 4°C for cryoprotection. Twenty micrometer coronal sections were cut on a microtome (Leica Microsystems GmbH, Wetzlar, Germany, http://www.leica.com) and collected for immunohistochemistry.

Statistical Analysis

Analysis of experiments involving immunocytochemistry and single-cell calcium imaging (SCCI) was performed in the border of SVZ neurospheres where migrating cells form a cell monolayer. Percentages of neuronal nuclear protein (NeuN), terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), or bromodeoxyuridine (BrdU) immunoreactive cells were calculated from cell counts in five independent microscopic fields (approximately 200 cells per field) in each coverslip with a ×40 objective. These experiments were at least triplicated, and, within each experiment, experimental conditions were assayed in two different wells of cultures. For SCCI experiments, the percentage of neuronal-like responding cells (histamine/KCl below 0.8) was calculated on the basis of one microscopic field per coverslip, containing approximately 100 cells (×40 magnification), in a total of at least three independent cultures where each conditions are triplicated. Percentages of Sox2 cell pair populations were obtained from counting approximately 60 cell pairs in triplicate coverslips obtained from five independent cultures. Quantification of the number of neuritic ramifications as well as the total neuritic length positive for phospho-JNK (P-JNK) per neurosphere was performed in six culture preparations in approximately 20 nonoverlapping fields per coverslip (×20 magnification). Software used was Axiovision, release 4.6 (Carl Zeiss, Göttingen, Germany, www.zeiss.com). For the grafting experiments, the percentage of doublecortin (DCX)-/GFP-positive cells per total GFP cells was calculated on the basis of five confocal nonoverlapping fields per hippocampal slice, where SVZ-GFP migrating cells emerge from the core of neurosphere. Each experiment included a series of control cultures not subjected to any drugs or to blank microparticles whenever histamine-loaded microparticles were used. Data are expressed as mean ± standard error of the mean (SEM). Statistical significance was determined by using analysis of variance (ANOVA) followed by Dunnett's test or unpaired two-tailed Student's t test, with $p < .05$ considered to represent statistical significant.

Results

Histamine Promotes Neuronal Differentiation in SVZ Cell Cultures Via H1R Activation

We have shown that histamine receptors are functional in SVZ immature cells by means of $[Ca^{2+}]_i$ oscillations driven by H1R activation [15]. Accordingly, in this work, we show that SVZ cells express not only histamine receptor 1 (H1R) but also receptors 2 and 3 (H2R and H3R) mRNA (Supporting Information Fig. S1A). To study the effect of histamine on neuronal differentiation, SVZ cells were treated with histamine for 7 days. Then, to phenotypically evaluate neuronal differentiation, immunocytochemistry against NeuN, a marker for mature neurons, was performed. As shown in Figure 1A, histamine increased significantly the percentage of NeuN-positive neurons when compared with control cultures (control: 6.7% ± 0.8%; histamine 100 μM: 17.9% ± 2.4%; histamine 500 μM: 21.1% ± 0.6%; $p < .001$ vs. control). Histamine treatment, however, had no effect on glial differentiation, as determined by immunostaining and Western blotting against the astrocytic marker glial fibrillary acidic protein-GFAP (data not shown). We then analyzed the variations of $[Ca^{2+}]_i$ at single cell level upon KCl and histamine stimulations, as previously reported by us [15]. The ratio of cellular responses due to histamine or KCl exposure (Hist/KCl) differs significantly between cell types, with a low Hist/KCl ratio (below 0.8) being characteristic of SVZ-derived neurons. Therefore, we quantified the percentage of cells with a low Hist/KCl ratio as a functional measure of neuronal differentiation. As expected, upon histamine treatment, cells with a neuronal-like response significantly increased when compared with the control (control: 13.8% ± 3.1%; histamine 100 μM: 35.2% ± 3.9%; histamine 500 μM: 33.4% ± 3.3%; $p < .001$ vs. control) (Fig. 1B). A significant shift from a typical immature profile found in control cultures toward a neuronal-like profile in 100 μM histamine-treated cultures is shown in the right panel of Figure 1B.

At all concentrations tested, histamine induced no significant changes in cell proliferation and cell death (as assessed using the BrdU incorporation assay and by TUNEL staining, respectively; Supporting Information Fig. S1B, S1C). Moreover, we also observed that 100 μM histamine neither alter the percentage of Brdu+Ki67+ cells (as a % of total number of

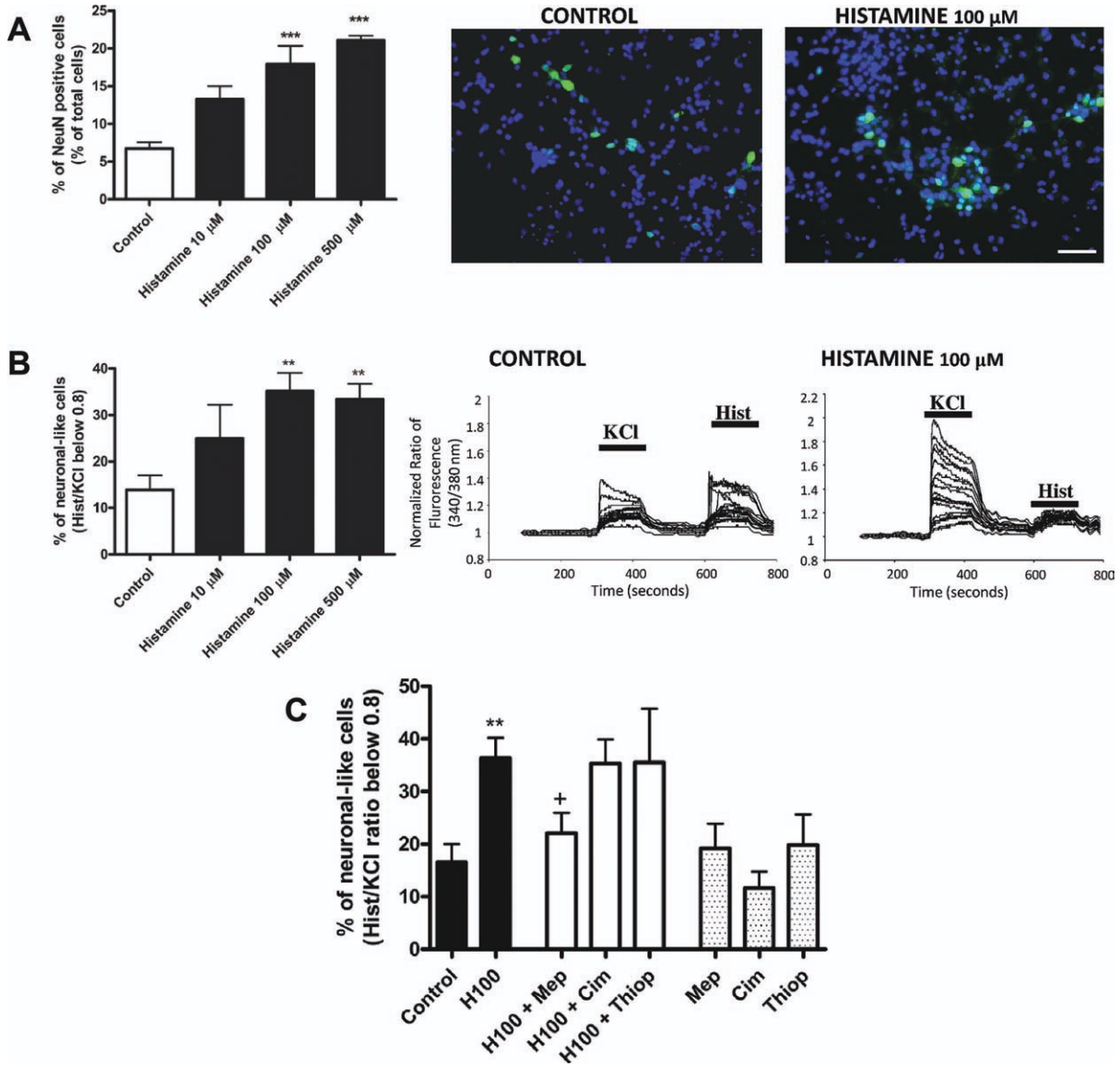

Figure 1. Histamine induces neuronal differentiation via H1R activation. **(A):** Bargram depicts the number of NeuN-positive cells expressed as percentage of total number of nuclei per culture. Data are expressed as mean percentage of total cells ± SEM. $n = 3$–5 coverslips. ***, $p < .001$ using Dunnett's test for comparison with the control. Representative fluorescent photographs of NeuN-positive neurons (green) and Hoechst 33342 staining (blue). Scale bar = 50 μm. **(B, C):** Bargrams depict the percentages of neuronal-like responding cells as accessed by single cell calcium imaging (SCCI) analysis; $n = 4$–12 coverslips. **, $p < .01$ using Dunnett's test for comparison with the control. Representative SCCI response profiles of 20 cells in a control culture and in a culture treated with 100 μM histamine. (C): Bargram depicts the percentages of neuronal-like responding cells. $n = 3$–11 coverslips. Mep, 1 μM; Cim, 5 μM; Thiop, 1 μM. **, $p < .01$ and $^{+}$, $p < .05$ using unpaired Student's t test for comparison with the control and with 100 μM histamine-treated (H100) cultures, respectively. In all experiments, data are expressed as a mean ± SEM. Abbreviations: Cim, cimetidine; Mep, mepyramine; NeuN, neuronal nuclear protein; Thiop, thioperamide.

Ki67+ cells) in differentiation conditions (control: 77.2% ± 2.0%; histamine: 82.9% ± 2.9%; $n = 3$; data not shown) nor affected the percentage of cells in G1 (control: 48.1 ± 5.9; histamine: 47.1 ± 5.1; $n = 5$ and 4, respectively), G2 (control: 13.2 ± 1.5; histamine: 15.5 ± 1.1; $n = 5$ and 4, respectively), or S phase (control: 38.8 ± 6.5; histamine: 37.4 ± 5.1; $n = 5$ and 4, respectively), as accessed by using a DNA-selective stain (Vybrant Dye Cycle Orange) in flow cytometry experiments (proliferative conditions, data not shown). These experiments reinforce our previous conclusion that histamine does not affect proliferation of SVZ cells.

Based on this data, we then used 100 μM histamine concentration in subsequent experiments. To disclose which receptor mediates the proneurogenic effect, SVZ cells were coincubated for 7 days with histamine and the antagonists for each histamine receptor. Mepyramine (1 μM), an H1R antagonist completely abolished the increase of the percentage of neuronal-like cells induced by histamine (histamine 100 μM [H100]: 36.4% ± 3.8%; histamine plus mepyramine [H100 + Mep]: 22.1% ± 3.9%; $p < .01$). Neither cimetidine (5 μM), an H2R antagonist, nor thioperamide (1 μM), a broad antagonist of H3R and H4R, was able to block the proneurogenic effect mediated by histamine (Fig. 1C). These results suggest that histamine induces functional neuronal differentiation in SVZ cells via activation of H1R receptor, without affecting cell proliferation or survival. We also characterized the population of GABAergic (vesicular GABA transporter (VGAT)+ cells) and glutamatergic (vesicular glutamate transporter

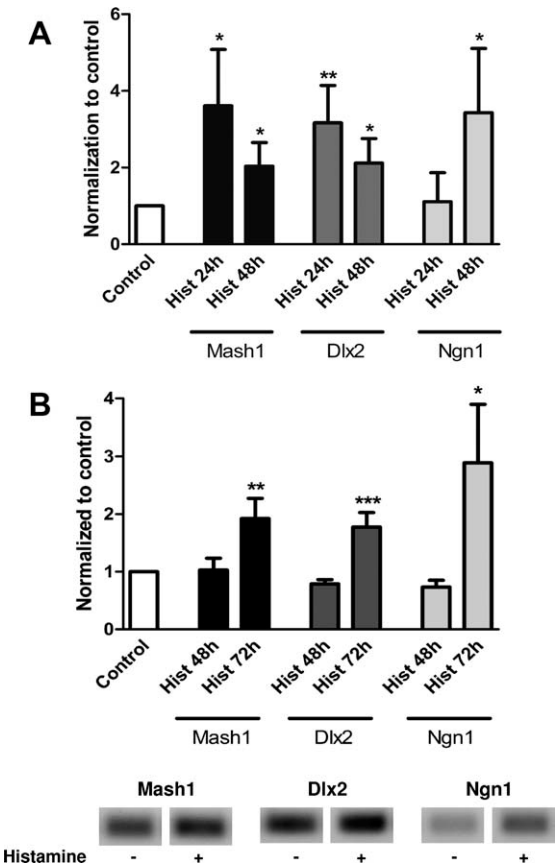

Figure 2. Histamine promotes the expression of the proneurogenic genes *Mash1*, *Dlx2*, and *Ngn1*. **(A):** Bargram depicts the fold increase of trimethylated lysine 4 form of histone H3 modifications in the promoter regions of *Mash1*, *Dlx2*, and *Ngn1* genes measured by quantitative chromatin immunoprecipitation analysis. **(B):** Bargram depicts the fold increase of mRNA expression for *Mash1*, *Dlx2*, and *Ngn1* genes measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis. Below the graph, representative agarose gels illustrate the mRNA expression levels of each gene in cultures under the absence (control, −) or presence of 100 μM histamine for 72 hours (+). Data are expressed as mean ± SEM. $n = 4$–12 subventricular zone cell cultures. *, $p < .05$; **, $p < .01$; ***, $p < .001$ using Dunnett's test for comparison with control (set to 1). Abbreviation: *Ngn1*, *Neurogenin1*.

(VGLUT)+ cells) cells in the presence of histamine (Supporting Information Fig. S2A, S2B). Indeed, 100 μM histamine induced an increase in the percentage of VGAT+ cells when compared with untreated controls (control: 16.9% ± 1.6%; histamine: 29.8% ± 1.2%; $p < .001$; $n = 6$), suggesting that histamine triggers the GABAergic phenotype. In addition, we observed no effect of histamine treatment on the subpopulation of glutamatergic neurons labeled by VGLUT (control: 19.1% ± 2.6%; histamine: 17.6% ± 1.9%; $n = 6$).

Histamine Facilitates Epigenetic Modifications and Subsequent Expression of Proneurogenic Genes

To disclose whether histamine has proneurogenic effects through histone modifications associated with the transcription of proneurogenic genes (*Mash1*, *Dlx2*, and *Ngn1*), we then performed qChIP targeting the H3K4m3 followed by a qPCR analysis. This specific histone methylation is associated with the facilitation of proneurogenic genes transcription and therefore reflects cell state and lineage potential [18, 19]. As shown in Figure 2A, SVZ cells exposed to 100 μM histamine, for 24 hours, showed an approximately 3.5-fold increase in the association of *Mash1* (3.6 ± 1.5; $n = 5$ SVZ cell cultures; $p < .05$) and an approximately threefold increase in the association of *Dlx2* (3.2 ± 0.9; $n = 7$ SVZ cell cultures; $p < .05$) promoters with the trimethylated form of H3K4, when compared with the control cultures. Interestingly, no statistical increase was seen regarding the association of H3K4Me3 with the *Ngn1* promoter at 24 hours (1.1 ± 0.8; $n = 5$ SVZ cell cultures). After 48 hours of incubation, the association of H3K4Me3 to *Mash1* (2.0 ± 0.6; $n = 5$ SVZ cell cultures; $p < .05$), *Dlx2* (2.1 ± 0.6; $n = 6$ SVZ cell cultures; $p < .05$), and *Ngn1* (3.4 ± 1.7; $n = 4$ SVZ cell cultures; $p < .05$) promoters was increased, when compared with control (set to 1). These data suggest that histamine promotes trimethylation of H3K4 on the promoter regions of the studied proneurogenic genes with different kinetics and intensities. To confirm that these epigenetic modifications indeed make DNA more prone for transcription, we then performed qRT-PCR analysis. Accordingly, we show that incubating SVZ cells with 100 μM histamine, for 72 hours, triggered a significant increase in mRNA levels for *Mash1* (1.9 ± 0.3; $p < .01$), *Dlx2* (1.8 ± 0.3; $p < .001$), and *Ngn1* (2.9 ± 1.0; $p < .05$), when compared with controls (set to 1) (Fig. 2B). At 48 hours, no changes were observed between mRNA levels in controls and 100 μM histamine-treated cultures, confirming that recruitment of H3K4m3 is a previous event that prones the DNA for the subsequent mRNA transcription of these proneurogenic genes.

Histamine Induces a Shift from Early Immature Cells Toward Committed Progenitor Cells

Having demonstrated that histamine triggered neuronal differentiation, we further assessed whether histamine induces the commitment of stem/progenitor cells. Uncommitted stem/progenitor cells divide symmetrically into two stem/progenitor cells or asymmetrically into a stem/progenitor and a committed cell, whereas cells committed to differentiate will terminally divide into two progenitor cells. We examined the capacity of histamine to modify the type of cell division in freshly isolated single SVZ cells plated for 24 hours with or without histamine. Sox2 labeling was performed to identify multipotent neural stem cells with the ability to self-renewal, whereas committed progenitors were labeled with an anti-Dlx2 antibody. Cell pairs resulting from the division of single SVZ stem/progenitor cells were counted. These pairs were categorized in three groups according to Sox2 expression: Sox2+/+, Sox2+/−, and Sox2−/− (Fig. 3A, 3B, 3C, respectively). Histamine at 100 μM induced a significant decrease in the percentage of Sox2+/+ cell pairs (control: 45.2% ± 5.1%; histamine: 31.5% ± 2.3%; $p < .01$) with a concomitant increase of Sox2−/− cell pairs when compared with controls (control: 30.2% ± 3.1%; histamine: 39.6% ± 3.3%; $p < .05$) (Fig. 3D). These data suggest that histamine induced the terminal division of stem/progenitor cells into committed progenitors. Moreover, the reduction of Sox2+/+ cell pairs upon treatment suggests that histamine could reduce self-renewal capacity. Indeed, SVZ neurospheres treated with 100 μM histamine generated lower numbers of secondary neurospheres (histamine: 86.3% ± 4.1%; control set to 100%; $n = 20$ SVZ cell cultures; $p < .05$; data not shown). These results show that histamine is actively involved in the reduction of the immature cells' pool toward a neural commitment.

Histamine Induces Activation of the JNK MAPK Pathway on Growing Axons

To evaluate whether histamine promotes neuronal maturation and axonogenesis, the phosphorylation and subcellular

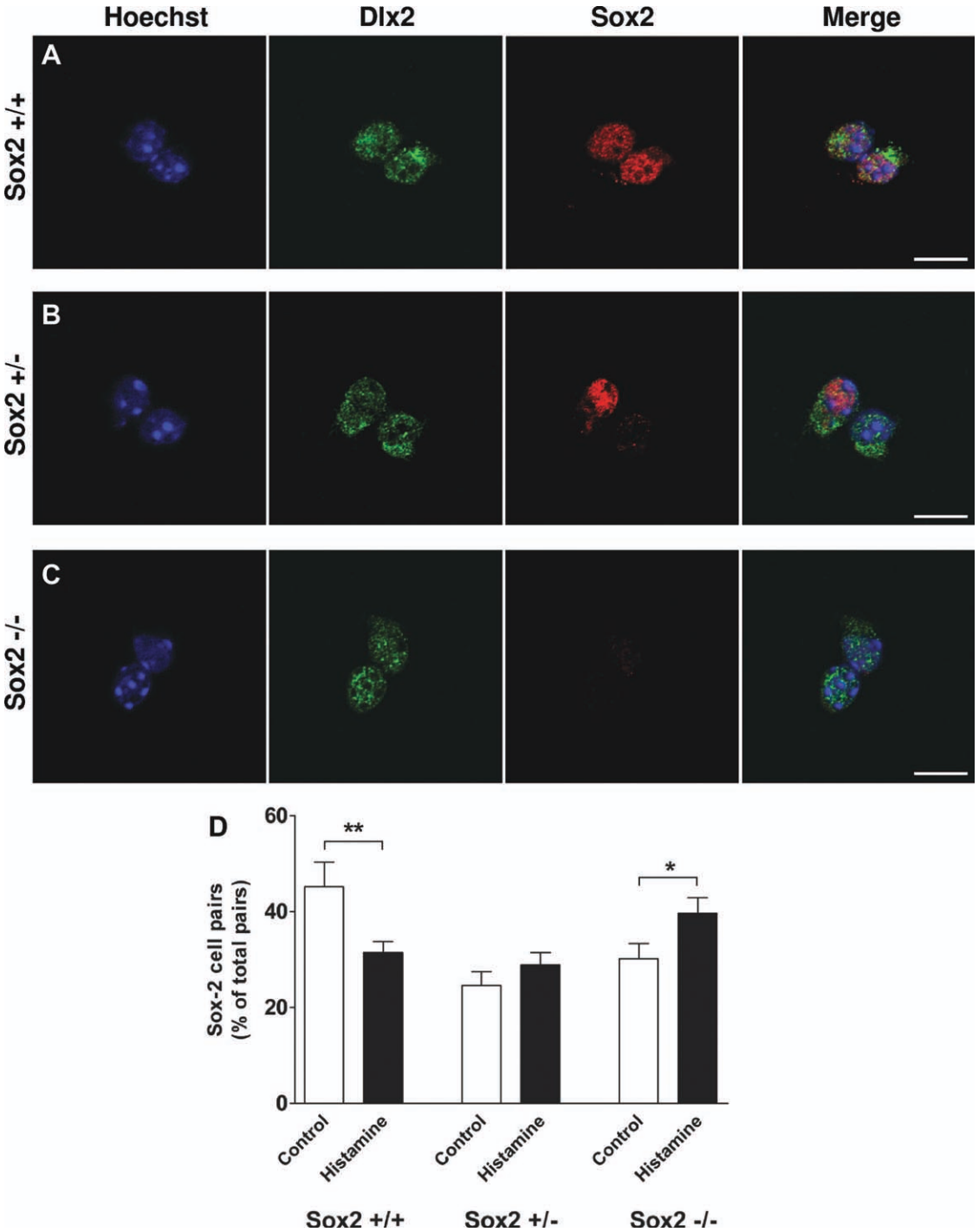

Figure 3. Histamine induces a reduction of the *Sox2*-positive stem-like population and an increase of *Sox2*-negative progenitor cells. Types of cell pairs characterized by the expression of *Sox2* (red) and *Dlx2* (green), namely: **(A)** two daughter cells (pairs) that coexpress *Sox2* and *Dlx2* (Sox2+/+); **(B)** two daughter cells that coexpress *Dlx2* but just one express *Sox2* (Sox2+/−); **(C)** two daughter cells that only express *Dlx2* (Sox2−/−). Scale bar = 10 μm. **(D):** Bargram illustrates the effects driven by 100 μM histamine (more than 24 hours) on the fate of newly born daughter cells by their expression of *Sox2* and *Dlx2* (Sox2+/+, Sox2+/−, and Sox2−/−). Data are expressed as the percentages of types of cell pairs per total cell pairs and are represented as mean ± SEM. $n = 5$ subventricular zone cell cultures. *, $p < .05$ and **, $p < .01$ using Bonferroni's multiple comparison test for comparison with the respective controls.

localization of the JNK MAPK pathway were studied. SVZ cultures exposed to 100 μM histamine, for 6 hours, showed a robust increase in P-JNK immunoreactivity located in neuritis. In contrast, control cultures showed a faint staining, with few P-JNK-positive neurites and growth cone structures. Quantification of the number of ramifications per neurosphere (control: 1.3 ± 0.3; 6 coverslips; histamine: 3.5 ± 0.5; 6 coverslips; $p < .01$) as well as the total length of ramifications and growth cone-like projections positive for P-JNK per neurosphere (control: 155.5 ± 28.3 μm;

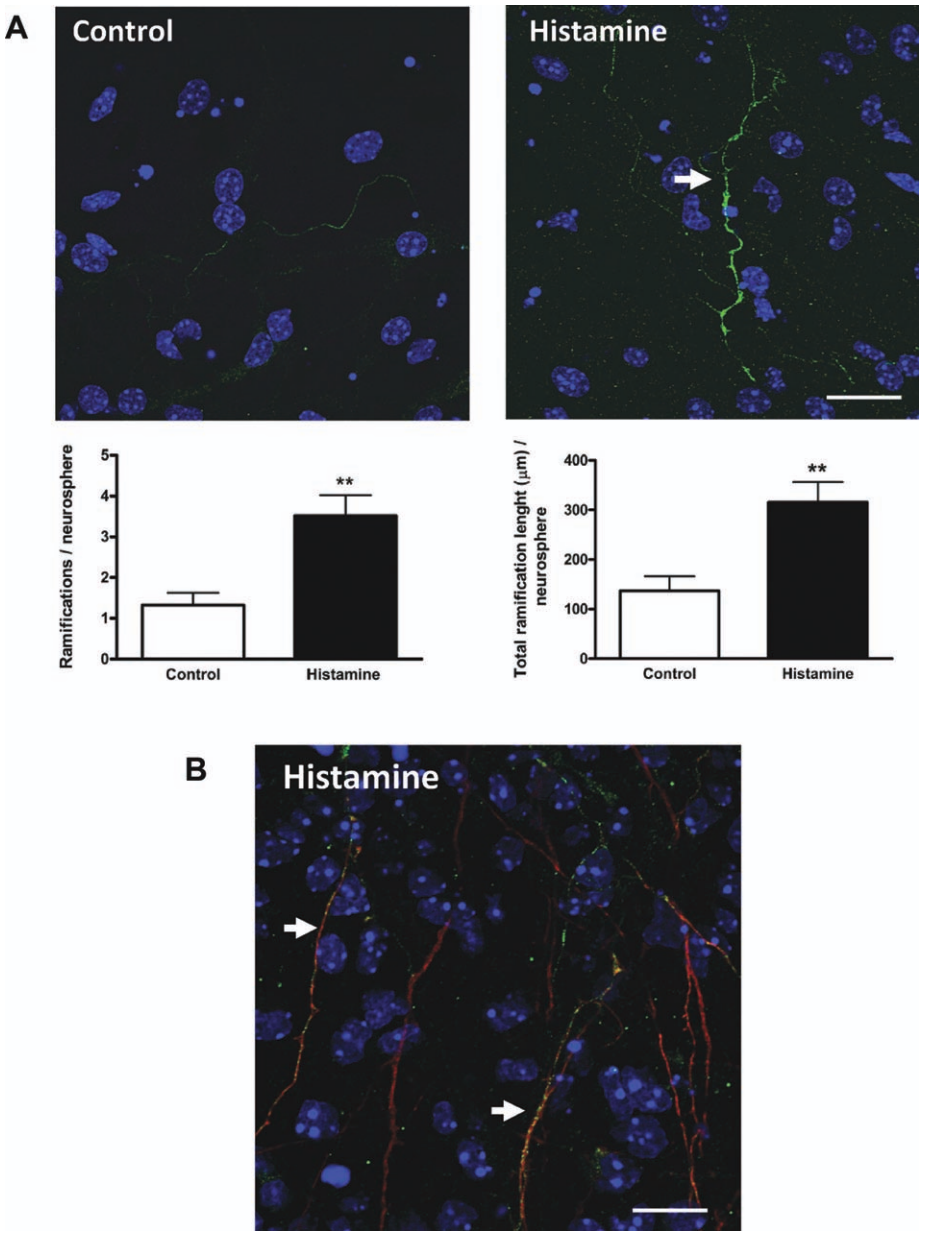

Figure 4. Histamine induces activation of the c-Jun N-terminal kinase, mitogen-activated protein kinase (JNK MAPK) pathway on growing axons. **(A):** Representative fluorescent confocal digital images of the phospho-JNK (P-JNK) (green) and Hoechst staining (blue), in control cultures and in cultures exposed for 6 hours to 100 μM histamine. Arrow depicts P-JNK immunoreactivity in neurites. Scale bar = 20 μm. Bargrams depict numbers of ramifications and total length (μm) of the P-JNK-positive ramifications per neurosphere. $n = 6$ culture preparations. **, $p < .01$ using unpaired Student's t test for comparison with the control. **(B):** Representative fluorescent confocal digital images of P-JNK (green), Tau (red), and Hoechst 33342 (blue) in cultures exposed for 6 hours to 100 μM histamine. Scale bar = 20 μm. Arrows depict axons double labeled for P-JNK and Tau.

histamine: 315.5 ± 40.8 μm; 6 coverslips; $p < .05$) showed that 100 μM histamine significantly increased both parameters, compared with control cultures (Fig. 4A). Interestingly, the P-JNK immunoreactivity induced by histamine was associated with Tau, a microtubule-associated protein that promotes bundling and stabilization of axonal microtubules of both immature and mature neurons (Fig. 4B). All together, these data suggest that histamine favors axonogenesis and neuronal maturation.

We may also argue that the JNK MAPK may be involved in previous events of neurogenesis, such as the initial phases of cell commitment. Therefore, we performed cell pair experiments, as previously explained, in the presence of 20 μM SP600125, a specific inhibitor of JNK activity. In fact, we observed that SP600125 prevented the effects mediated by histamine on cell commitment, namely, the decrease of the percentage of Sox2+/+ (histamine: 27.0% ± 3.9%; histamine+SP600125: 45.1% ± 3.9%; $p < .05$; control: 50.9% ± 2.6%) and the concomitant increase of Sox2−/− cell pairs (histamine: 51.3% ± 2.4%; histamine+SP600125: 25.6% ± 1.7%; $p < .001$; control: 24.9% ± 1.9%), to levels similar to untreated cultures (data not shown). Moreover, SP600125 per se did not alter the levels of any of the categorized groups of Sox2 cell pairs. Therefore, these results suggest that P-JNK can be also involved in the initial phases of cell commitment induced by histamine.

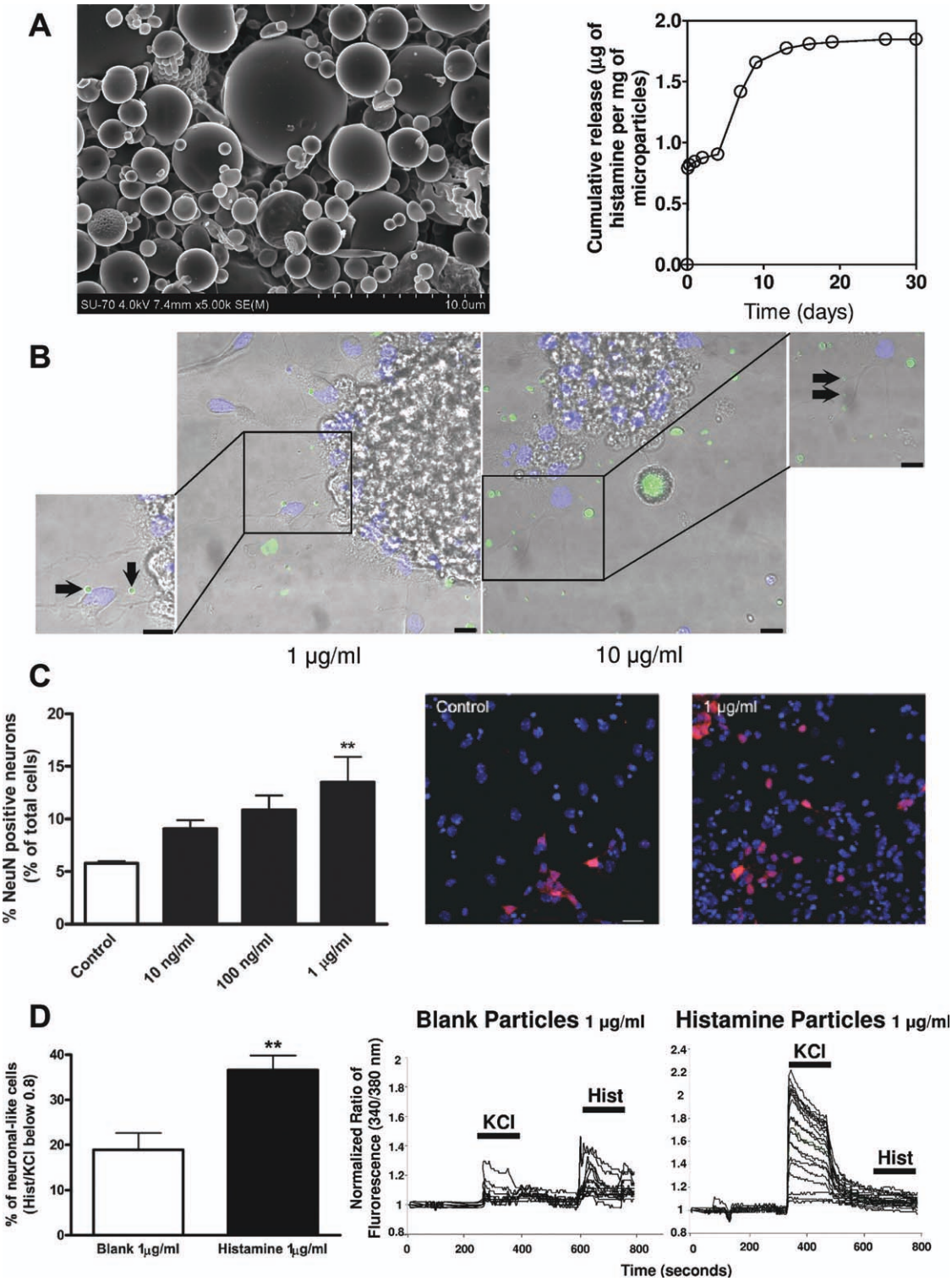

Figure 5. Histamine released from microparticles induces neuronal differentiation *in vitro*. **(A):** Characterization of poly (lactic-*co*-glycolic acid) microparticles. Left: Scanning electron micrographs of microparticles obtained by single emulsion. Right: Histamine-release profile from microparticles in phosphate-buffered saline (PBS) aqueous solution at 37°C. **(B):** Distribution of histamine-releasing microparticles in subventricular zone (SVZ) cell cultures. Representative confocal photos of SVZ cells incubated for 1 hour in the presence of 1 μg/ml (right) and 10 μg/ml (left) histamine-releasing microparticles (fluorescein isothiocyanate (FICT) labeling-green). Cell nuclei were stained with Hoechst 33342 (blue). Arrows point to microparticles. Scale bars = 20 μm. **(C):** Left: Bar graph represents number of NeuN-positive cells expressed as percentages of total number of cells per culture. n = 5–6 coverslips. **, $p < .01$ using Dunnett's test for comparison with the control. Right: Representative confocal digital images of NeuN-positive neurons (red) and Hoechst staining (blue). Scale bar = 20 μm. **(D):** Left: Bargram depicts the percentages of neuronal-like responding cells analyzed by single cell calcium imaging (SSCI). n = 5–6 coverslips. **, $p < .01$ using unpaired Student's t test for comparison with the control. Right: Representative SCCI profiles of response of 20 cells in a blank or histamine microparticle-treated cultures. In all experiments, data are expressed as mean ± SEM. Abbreviation: NeuN, neuronal nuclear protein.

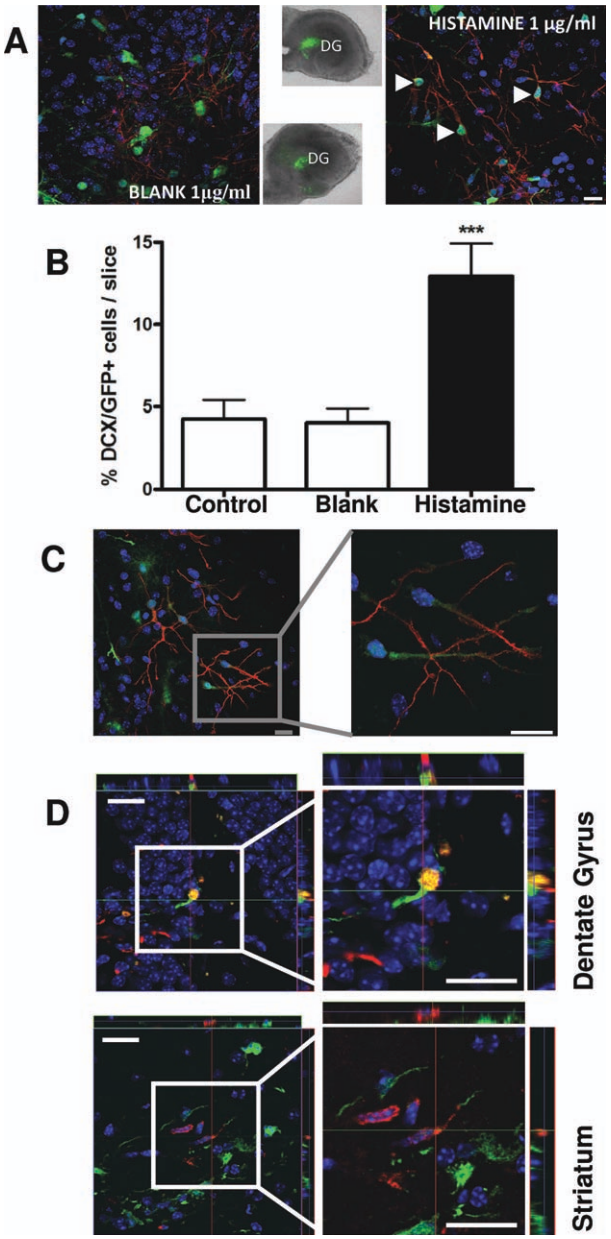

Figure 6. Histamine microparticles induce neuronal differentiation of subventricular zone (SVZ)-GFP cells grafted on the DG of hippocampal slices and *in vivo*. **(A):** Representative confocal digital images of DCX (red), SVZ-GFP cells (green), and Hoechst (blue) in organotypic hippocampal slices transplanted with SVZ-GFP cells previously treated for 48 hours with 1 μg/ml histamine-releasing or blank microparticles. Arrows depict DCX and GFP double-labeled cells. **(B):** Bar graph represents the percentage of DCX+/GFP+ cells per SVZ GFP total cells counted in hippocampal slices. Data are expressed as mean ± SEM. $n = 6$–15 hippocampal preparations. ***, $p < .001$ using Dunnett's test for comparison with blank-treated cultures. **(C):** Representative confocal digital images of DCX-positive neurons (red), SVZ-GFP-transplanted cells (green), and Hoechst staining (blue) in hippocampal slices transplanted with SVZ-GFP cells pretreated with 1 μg/ml histamine-releasing microparticles. Fluorescent picture at the right side consists in a cropped image from the limited field in the left figure. Scale bar = 20 μm. **(D):** Representative fluorescent confocal digital images of DCX-positive neurons (red), GFP (green), and Hoechst (blue) observed in the grafting site of the DG and striatum of the WT mouse in vivo. SVZ-GFP cells were pretreated with 1 μg/ml histamine-releasing microparticles for 48 hours and then intracerebrally injected in different regions of the mouse brain for 3 weeks. Scale bar = 20 μm. Abbreviations: DCX, doublecortin; DG, dentate gyrus; GFP, green fluorescent protein.

Histamine Released from PLGA Microparticles Induces Neuronal Differentiation in SVZ Cell Cultures

To provide a more efficient and controlled delivery of histamine in the SVZ neurogenic niche, PLGA microparticles were prepared. These microparticles have an average diameter of 2 μm (Fig. 5A) and a loading capacity of approximately 5.3 μg of histamine per mg of microparticles. When suspended in PBS aqueous solution (pH 7.4), they release histamine over a period of 30 days (Supporting Information Methods; Fig. 5A). Furthermore, blank (unloaded) or histamine-loaded microparticles (up to 1 μg/ml of microparticles) incubated with SVZ cells have no effect on cell viability or proliferation (Supporting Information Fig. S1D, S1E), and they distribute homogeneously at the cells' membrane (Fig. 5B).

A significant increase in the percentage of NeuN-positive cells was observed in SVZ cells incubated with histamine-loaded microparticles (1 μg/ml) for 7 days (control: 5.73% ± 0.34%; histamine-loaded microparticles [1 μg/ml]: 13.51% ± 2.38%; $p < .01$) (Fig. 5C), as evaluated by immunofluorescence. In addition, the treated cells show a significant increase in the percentage of neuronal-like cells (Hist/KCl ratio below 0.8) when compared with blank (unloaded) microparticle-treated cultures (histamine-loaded microparticles [1 μg/ml]: 36.6% ± 3.2%; blank microparticles [1 μg/ml]: 15.6% ± 2.1%; $p < .01$). In fact, a neuronal-like profile of $[Ca^{2+}]_i$ was also observed in histamine microparticle-treated cultures (Fig. 5D). It should be noted that histamine-releasing microparticles did not affect astrocytic differentiation, as detected by Western blotting against GFAP (data not shown). Overall, our data suggest that histamine released from PLGA microparticles induces a robust neuronal differentiation without affecting cell viability or proliferation.

Histamine Microparticle-Induced Neuronal Differentiation of Grafted SVZ-GFP Cells

To further dissect the role of histamine on neurogenesis, an *ex vivo* model was developed by grafting SVZ neurospheres (obtained from GFP transgenic mice, previously treated with histamine-loaded microparticles for 48 hours) on the DG of WT organotypic hippocampal slice cultures. After 1 week, cultures were fixed and immunostained for DCX, a marker for neuroblasts. As shown in Figure 6A, 6B, 1 μg/ml of histamine-loaded microparticles promoted a robust increase in DCX-/GFP-positive cells per total GFP cells from 4% to 13% (blank microparticles: 4.0% ± 0.8%, 2,077 cells counted; histamine-loaded microparticles: 12.9% ± 1.9%, 1,754 cells counted; $p < .001$). New neurons (DCX+/GFP+) derived from the SVZ-GFP neurospheres pretreated with histamine-releasing microparticles showed a complex morphology with spines, similar to the structure of endogenous granule neurons present in the host DG (Fig. 6C). Therefore, the SVZ-GFP-transplanted cells in the hippocampal slice survive and differentiate into new neurons. A similar approach was used *in vivo*, grafting SVZ-GFP cells into the hippocampal DG or striatum of WT mice. Three weeks after transplantation, some SVZ-GFP cells pre-exposed to histamine-loaded microparticles integrated the DG and striatum and differentiated into DCX-positive neuroblasts (Fig. 6D).

DISCUSSION

Since the discovery that classic antihistamines have a sedative action, it has become clear that histamine affects the CNS.

Surprisingly, the putative effects of histamine on postnatal neurogenesis are still unknown.

We previously showed that immature nestin-positive cells, coexpressing or not GFAP, can be distinguished on the basis of the selective increase of $[Ca^{2+}]_i$ in response to histamine via H1R activation [13]. Indeed, it was also shown by others that embryonic stem and carcinoma cells express functional H1R, suggesting a role for this receptor as a marker for undifferentiated neural progenitors [12]. Herein, we found that histamine induces functional neuronal differentiation in SVZ cell cultures via H1R activation. Moreover, we showed that in vitro, postnatal SVZ cells retain the potential to differentiate in GABAergic and glutamatergic neurons, and that GABAergic differentiation is favored by histamine treatment. In fact, Molina-Hernandez and Velasco [14] showed that histamine induces an increase in neuronal differentiation and a decrease in astrocytic cell proportion in neuroepithelial stem cells derived from the embryonic rat cerebral cortex in vitro. However, in our case, neuronal differentiation was not occurring at the expense of glial differentiation since GFAP protein levels were not altered. Moreover, this proneurogenic effect occurred without affecting proliferation or cell viability. It has been reported by others that histamine may promote cell proliferation of embryonic stem cells as well as cancer stem cells [14, 20]. In opposite, Medina et al. [21] showed that histamine impairs proliferation on human malignant melanoma cells, probably by increasing hydrogen peroxide levels. These contradictory effects may rely on the specificities displayed by each cell types (embryonic stem/progenitor cells vs. neonatal SVZ stem/progenitor cells vs. cancer stem cells).

Emerging information indicates that epigenetic modifications comprising histone modifications and chromatin remodeling may be inherent to the differentiation of neural stem cells. Recent studies indicate that lineage control genes such as *Mash1*, *Dlx2*, and *Ngn1* are epigenetically modified with a unique combination of activating histone modifications that prime them for potential activation upon cell lineage induction and differentiation [22]. Thus, we then investigated whether histamine may promote H3K4m3 recruitment to the promoter of genes involved in neuronal cell fate lineage and consequently prompt their transcription. Our results suggest that histamine induced the trimethylation of H3K4 on *Mash1*, *Dlx2*, and *Ngn1* promoters that ultimately leads to increased mRNA expression of the former proneurogenic genes. In fact, Lim et al. [23] showed that mixed-lineage leukemia 1 is required for neurogenesis in the mouse postnatal brain by epigenetic modulation of the expression of the proneurogenic gene *Dlx2*. They showed that in differentiating WT SVZ cells, *Mash1*, *Olig2*, and *Dlx2* loci have high levels of H3K4me3, consistent with their transcription. *Mash1* (*Ascl1*) is a key transcription factor essential during embryogenesis, being involved in the production and commitment of neural precursor cells while inhibiting their astrocyte potential [24, 25]. *Ngn1* was described to be induced in neuronal precursor cells and therefore essential for neurogenesis [26]. In accordance, Wu et al. [27] showed that the expression of *Ngn1* increases drastically in retinoic acid-induced neuronal differentiation of P19 cell lines. All these reports strength the conclusion that histamine signaling triggers favorable histone modifications for the transcription of proneurogenic genes in SVZ cells. Moreover, we found that histamine induces terminal division of stem/progenitor cells into committed *Sox2−/−* progenitors and decreases self-renewal capacity of SVZ cells without affecting cell proliferation. In line with our results, it has been shown by others that the neuropeptide Y (NPY) promotes proliferation in SVZ cell cultures; however, NPY is not inducing an increase in the number of secondary neurospheres [28]. Moreover, the hepatocyte growth factor has a positive impact on self-renewal, increasing the number of secondary neurospheres, but does not have any effect on cell proliferation [29]. Accordingly, these data show that histamine reduces stemness in SVZ cells, promoting late precursors pool expansion and neuronal differentiation.

Another key process during neuronal differentiation and maturation is neurite outgrowth and axon formation. Previously, we showed that tumor necrosis factor (TNF)-α and NPY induce axonal formation and maturation in SVZ cells by activating the JNK MAPK pathway [15, 30]. This pathway is involved in axonal sprouting and neurite outgrowth but not in dendritic growth [31]. Therefore, JNK may mediate axonal outgrowth, microtubule dynamics, and neuronal polarization by DCX or MAP-2 phosphorylation [32, 33]. Our P-JNK immunolabelings pointed to an increase in the active form of this kinase upon histamine treatment. Not only just the number of ramifications per neurophere but also the total length of these ramifications was significantly higher in histamine-treated cultures. The association of this increased labeling with Tau suggests that histamine might be involved in the axonal formation of SVZ cells.

Moreover, we showed that histamine-stimulated JNK activation can be also involved in the initial phases of cell commitment. This is in line with previous reports showing that this pathway is involved in neural differentiation from embryonic stem cells [34].

For a potential delivery of proneurogenic drugs into neurogenic niches, we developed biocompatible PLGA microparticles that release histamine in a controlled way. PLGA is a Food and Drug Administration approved copolymer for therapeutic devices and extensively investigated for the preparation of drug delivery formulations [35–37]. It is degradable in aqueous solutions into lactic and glycolic acid, by-products of metabolic pathways. Microparticles with an average diameter of 2 μm were selected to prevent their cellular uptake and consequently to release histamine at the extracellular millieu. The histamine released by the microparticles had no effect on cell proliferation or death, neither the microparticle formulation was toxic per se. Thus, PLGA microparticles are suitable biocompatible carriers for histamine release on SVZ cell cultures. Not only histamine-releasing microparticles induced a robust neuronal differentiation from SVZ cell cultures but also facilitated neuronal differentiation of SVZ-GFP neurospheres grafted in ex vivo or in vivo. Importantly, the real histamine cumulative released from microparticles was far smaller ($\times$1,000 less) than the initial histamine concentrations tested for the single-pulse experiments (100 μM). Several studies reported that in physiologic conditions histamine is present at nanomolar concentrations in the cerebrospinal fluid and brain parenchyma of humans and rodents [38–44]. Importantly, circulating levels of histamine and histaminergic innervations are increased following brain injury and degeneration or infection [39, 45-49]. Histamine may therefore have an important role modulating injury-induced neurogenesis since it is part of the repertoire of soluble factors released in the lesioned microenvironment. This suggests that the concentration of 100 μM used in our work is of robust pathophysiological relevance.

In this context, biocompatible histamine-PLGA microparticles represent an advantage in histamine delivery since this amine is released in a continuous manner and in concentrations that are closer to physiological levels at the SVZ neurogenic niche. In addition, the histamine released by these microparticles had comparable effects to the ones observed with single-pulse histamine treatments. This is likely due to the spatio-temporal controlled delivery of histamine to the

cells. Microparticles tend to locate at the proximity of cell membrane, creating a concentration gradient of histamine. Therefore, low amounts of histamine are required to exert the same biological effect. Similar results have been obtained previously by one of us in the vascular differentiation of human embryoid bodies [50]. The incorporation of polymeric biodegradable particles loaded with specific growth factors into human embryoid bodies had a minimal effect on cell viability and proliferation but a great impact on vascular differentiation. Therefore, microparticles constitute a novel approach to promote brain repair by local and continuous delivery of histamine into neurogenic niches.

Our ex vivo data showed that the pre-exposure of SVZ-GFP cells with histamine microparticles increased neuronal differentiation of grafted cells in the DG of hippocampal slices. These data support the concept that pretreatment of neural stem cells with known proneurogenic factors favors neuronal differentiation of transplanted cells. In vivo, a comparable approach showed a more discrete but still positive effect on survival, integration, and differentiation of the pretreated transplanted cells into the host hippocampus and striatum. Previous studies have shown that in the intact adult rodent brain, expanded SVZ cells in vitro preferentially produce neurons upon transplantations in homotypic (SVZ) and heterotypic (SGZ) neurogenic niches, whereas upon transplantation in non-neurogenic regions such as the cortex or the striatum, SVZ cells generate mainly glial cells [51, 52]. These transplantation approaches indicate that the neuronal phenotype determination is tightly regulated by local signals derived from both the SVZ and SGZ neurogenic niches.

Conclusion

As a proof of principle, we showed that SVZ-GFP cells pretreated with histamine microparticles differentiate into new neurons at both neurogenic (DG) and non-neurogenic (striatum) niches. Taken together, these results suggest that histamine either added in a one-pulse way or released by a PLGA microparticle drug delivery systems induces neuronal differentiation in SVZ stem/progenitor cells highlighting its use in brain repair strategies.

Acknowledgments

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