

Galanin Promotes Neuronal Differentiation in Murine Subventricular Zone Cell Cultures

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Neural stem cells of the subventricular zone (SVZ) represent a potentially important source of surrogate cells for the treatment of brain damage. Proper use of these cells for neuronal replacement depends on the ability to drive neuronal differentiation. Several neuromodulators stimulate neurogenesis. Here we examined the effects of the neuropeptide galanin, on neuronal differentiation in murine SVZ cultures. SVZ neurospheres obtained from early postnatal mice were treated with 10 nM to 2 μ M galanin. Galanin promoted neuronal differentiation, increasing numbers of NeuN-, vesicular GABA transporter- and tyrosine hydroxylase-expressing neurons. In contrast, galanin neither affected cell proliferation assessed by BrdU incorporation nor cell death evaluated by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL). Neuronal differentiation was further confirmed at the functional level by measuring $[Ca^{2+}]_i$ variations in single SVZ cells after KCl and histamine stimulations to distinguish neurons from immature cells. Galanin treatment increased the numbers of neuronal-like responding cells compared to immature cells. Using selective agonists (M617, AR-M1896) and antagonists (galantide, M871) for galanin receptors 1 and 2, we showed that both galanin receptors mediated neuronal differentiation. Early proneuronal effects of galanin included positive regulation of the transcription factor neurogenin-1 (Ngn1). In addition, galanin promoted axonogenesis and dendritogenesis, increasing both the length of phosphorylated stress-activated protein kinase- and Tau-positive axons and the numbers of microtubule associated protein-2 (MAP-2)-labelled dendrites. Moreover, galanin inhibited SVZ cell migration in the transwell assay. Our results show a proneurogenic effect of galanin and open new perspectives for future applications in stem cell-based therapies for neuronal replacement.

Introduction

IN THE ADULT MAMMALIAN brain, neural stem cells (NSCs) have been identified in the subventricular zone (SVZ). Stem cells generate transient-amplifying progenitors able to differentiate into neuroblasts that constitutively provide cells to

the olfactory bulb, improving odor discrimination accuracy in rodents [1,2]. In humans, SVZ neurogenesis stops at the 18th month of postnatal life; however, stem cells of this area retain the capacity to produce neurons in vitro [3,4], suggesting that neurogenesis may be boosted in the adult brain under proper conditions. Therefore, SVZ stem cells appear to represent a

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reservoir of cells for possibly healing the brain tissue injured by neurological diseases [5,6]. However, the use of SVZ stem cells to replace damaged neurons critically depends on the ability to induce neuronal differentiation of their progeny. Galanin is a neuropeptide consisting of 29 amino acids in mice and 30 in humans. The neuropeptide is widely expressed in the central and peripheral nervous systems. Galanin regulates numerous physiological and pathological processes ranging from food intake, nociception and nerve regeneration to memory and depression [7–14]. Galanin signals through G-protein-coupled receptors (GALR1, GALR2 and GALR3) [15]. At the cellular level, galanin has been shown to modulate noradrenergic, serotonergic and cholinergic neurotransmission in the hippocampus. Indeed, galanin inhibits the hippocampal release of acetylcholine via GALR1 and GALR1 antagonists appear to enhance cognition in patients with Alzheimer's disease [16,17]. In addition, galanin protects hippocampal neurons from glutamate and amyloid β cytotoxicity *in vitro* through activation of GALR2 and *in vivo* from seizure-induced cell death via GALR1 and GALR2 [18–22]. Regarding neurogenesis, it has been shown that GALR2, but not GALR1, mediates seizure-induced hippocampal neurogenesis [23]. Galanin was also shown to induce proliferation of hippocampal precursor cells and generation of dentate granule cells in rat cultures [24]. It is therefore, conceivable that galanin positively impacts on SVZ neurogenesis. Moreover, high galanin expression has been demonstrated in mouse and human pluripotent embryonic stem cells [25–27] and in bone marrow mesenchymal stem cells [28] suggesting that galanin may be part of the genetic signature of stem cells and has a role in maintaining their properties. The present work explores the potential effects of galanin on self-renewal, proliferation, cell survival, migration, and neuronal/glia differentiation of SVZ stem/progenitor cells. Our results show that galanin induces neuronal differentiation in SVZ cells, highlighting the possible new use of this peptide as an enhancer of neurogenesis for brain therapy purposes.

Materials and Methods

All experiments were performed in accordance with the European Community (2010/63/EU) guidelines for the care and use of laboratory animals.

SVZ cell cultures

SVZ cells were prepared from 1–3-days-old C57BL/6 mice either wild type (WT) or expressing the green fluorescent protein (GFP) under the control of the actin gene promoter, as previously described [29] (see Supplementary Data Supplementary Data are available online at www.liebertpub.com/scd). For apoptosis staining, immunocytochemistry and single cell calcium imaging assays (SCCI), 6–8-days-old primary SVZ neurospheres were collected and seeded onto glass coverslips coated with poly-D-lysine (0.1 mg/mL) placed into 24-well culture plates with 500 μ L of serum-free medium (SFM) devoid of growth factors. The SVZ neurospheres were then allowed to develop for 48 h before experimental treatments. Immunolabeling for GALRs was performed on 48 h plated neurospheres. Proliferation of neuroblasts and uncommitted progenitors was measured in neurospheres treated for 48 h in the absence or presence of

1 μ M galanin and incubated for the last 4 h of the culture session with 5-bromo-2'-deoxyuridine (10 μ M BrdU; Sigma-Aldrich). After fixation, immunostaining for BrdU, β III tubulin (marker for neuroblast) and Dlx2 (marker for uncommitted progenitor) was performed (Supplementary Fig. S1A). Neuronal commitment of proliferating progenitors was assessed in SVZ cells dissociated from neurospheres and plated at a density of 50,000 cells/cm² onto poly-D-lysine-coated glass coverslips in SFM for 48 h. Subsequently, the cultures were incubated in the absence (control) or presence of 1 μ M galanin together with BrdU (Sigma-Aldrich) for 24 h, washed, and incubated again with galanin for 6 additional days before immunodetection of NeuN- and BrdU-positive cells (Supplementary Fig. S1B). The effects of galanin on axonogenesis were determined in plated SVZ neurospheres treated for 6 h with 1 μ M galanin alone or in combination with 20 μ M SP600125 (Sigma-Aldrich), an inhibitor of the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) involved in axonogenesis [30]. After fixation, immunostaining for the phosphorylated (activated) forms of SAPK/JNK kinase (pJNK) was performed. To ascertain whether pJNK localization was associated with axons, double-labeling was performed to visualize both pJNK and Tau (marker for axonal microtubules) (Supplementary Fig. S1C). To assess the effect of galanin on dendritogenesis, primary SVZ neurospheres obtained from WT and GFP transgenic newborn mice were dissociated (Neurocult dissociation kit, Stemcell Technologies), and plated onto poly-D-lysine-coated glass coverslips at the proportion of 95% WT cells to 5% GFP+ cells, in SFM devoid of growth factors at a density of 50,000 cells/cm². The purpose is to measure microtubule associated protein-2 (MAP-2) positive dendrites from isolated GFP+ cells. The cells were allowed to grow for 7 days in the absence (control) or presence of 1 μ M galanin and were stained for MAP-2 (Supplementary Fig. S1D). Quantitative chromatin immunoprecipitation (qChIP) against the trimethylated lysine 36 of histone 3 (H3K36m3) was performed to evaluate whether galanin treatment promotes the availability of promoter regions of the proneurogenic transcription factors neurogenin-1 (Ngn1), Dlx2 and Mash1 to transcription complexes (Supplementary Fig. S1E). To confirm qChIP analysis, the expression levels of mRNA transcripts for Ngn1, Dlx2, Mash1, and of the pro-oligodendroglia transcription factor Olig2 were measured by quantitative polymerase chain reaction (qPCR). For qChIP analysis, SVZ cells were treated without (control) or with 1 μ M galanin for 6 and 24 h and for qPCR analysis for 2, 3 and 4 days (Supplementary Fig. S1E). Immunodetection of NeuN, glial fibrillary acidic protein (GFAP), Olig-2, VGAT and TH were conducted on 7-days treated neurospheres (Supplementary Fig. S1F).

^[125I]Galanin receptor autoradiography and galanin-stimulated ^[35S]GTP γ S functional binding

The procedures were previously described [31–33] and are detailed in Supplementary Data.

Self-renewal assay

SVZ cells were seeded as single cells, at 2,500 cells per well in 24-well cell culture plates in SFM containing 5 ng/mL

epidermal growth factor (EGF) and 2.5 ng/mL fibroblast growth factor 2 (FGF-2) (low EGF/FGF-2) and supplemented or not (control) with 1 μ M galanin. Four wells per condition were assayed. After 6 days, the numbers of primary neurospheres were quantified. Primary neurospheres were then collected by centrifugation, dissociated into single cell suspensions (Neurocult dissociation kit) and seeded in low EGF/FGF-2 medium as aforementioned. After 6 days, the numbers of secondary neurospheres were counted [34] (Supplementary Fig. S1G).

Cell proliferation studies

Proliferation was assessed using the ELISA BrdU colorimetric assay (Roche; #11 647 229 001). SVZ cells (40,000 per well) obtained from dissociated primary neurospheres (Neurocult dissociation kit), were plated in 96-well culture plates (4 wells per condition) and treated in the absence (control) or the presence of 1 μ M galanin in SFM devoid of growth factors or supplemented with 5 ng/mL EGF and 2.5 ng/mL FGF-2. Galanin (10 nM, 100 nM and 1 μ M, Bachem AG; #H-7450) and GALR1 and GALR2 agonists (GALR1 agonist: M617; 100 nM, #2697; GALR2 agonist: AR-M1896; 100 nM, #2699, both from Tocris) were applied for 48 h. Cell proliferation was determined by adding 10 μ M BrdU for the last 4 h of the culture session. Amount of BrdU incorporation was evaluated by densitometry (at 450 nm) after the use of a peroxidase conjugated anti-BrdU antibody and reaction with a peroxidase substrate, according to the manufacturer's instructions. The absorbance measured in control (ie, SFM-treated wells) was set at 100% and values in experimental conditions were expressed as percentages of control values.

Determination of cell apoptosis by TUNEL

Primary SVZ neurospheres were plated for 48 h on poly-D-lysine coated coverslips. Apoptosis was evaluated by the TUNEL as previously described [29] (Supplementary Fig. S1A).

Single cell calcium imaging studies

To investigate the effect of galanin on neuronal differentiation and to identify the receptor(s) mediating this effect, SVZ neurospheres were seeded on poly-D-lysine-coated coverslips. Cultures were then treated with the following drugs: galanin (10 nM, 100 nM, 1 μ M), GALR1 (M617) and GALR2 (AR-M1896) agonists both at 10 and 100 nM, 1 μ M galanin in combination with 100 nM of the GALR2 antagonist (M871; #2698; Tocris), 100 nM of the GALR1 agonist in combination with 100 nM of the GALR2 antagonist, 1 μ M galanin with 100 nM or 1 μ M of the nonselective GALR antagonist galanin (1–13)-substance P (5–11) amide (galantide, #H-1312; Bachem), and allowed to develop for 7 days at 37°C. At the end of the culture session, SCCI experiments were performed to identify functional neurons by means of their increase of free intracellular calcium levels ($[Ca^{2+}]_i$) specifically in response to 50 mM KCl but not in response to 100 μ M histamine [29,35] (see Supplementary Data and Supplementary Fig. S1F).

Immunostaining

Cells were fixed in 4% paraformaldehyde (PFA) for 15 min at room temperature (RT). BrdU staining (10 μ M; Sigma-

Aldrich) was performed as previously described [29] using the Alexa fluor 594 mouse anti-BrdU (1:100; #A21304, Invitrogen). For immunocytochemistry, SVZ cells were permeabilized and nonspecific binding sites blocked using a mixture of 1% Triton X-100 and 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 30 min at RT. SVZ cells were subsequently incubated overnight at 4°C with the appropriate primary antibodies as listed in Supplementary Table S1 and prepared in PBS containing 0.1% Triton X-100 and 0.3% BSA. Coverslips were rinsed in PBS and incubated for 1 h at RT with the appropriate Alexa Fluor 488 and 594 secondary antibodies produced in donkey (all from Invitrogen). SVZ cell nuclei were stained with Hoechst 33342 (2 μ g/mL in PBS) for 5 min at RT and mounted in Dako fluorescent medium (Dako). For immunodetection of GALR1, GALR2, and GALR3 on brain slices, adult mice were deeply anaesthetized and transcardially perfused with 0.9% NaCl followed by 4% PFA. Brains were removed and post-fixed in 4% PFA at 4°C overnight. Coronal brains sections 40 μ m-thick were cut using a Leica vibratome and then placed into anti-freeze solution (ethylene glycol 30% vol/vol, glycerol 30% vol/vol, PB 0.1 M 40% vol/vol) and conserved at -20°C. Immunohistochemistry was performed as aforementioned. Fluorescent images were recorded using a confocal microscope (LSM 510 Meta; Carl Zeiss) or a fluorescent microscope (Axioskop 2 Plus; Carl Zeiss).

Migration in Boyden chambers

Cell migration was assessed using the transwell assay in which cells migrate through a microporous membrane in response to a specific molecule. The underside of the transwell membrane was coated with 10 μ g/mL fibronectin 4 h before the addition of cells. The culture medium, supplemented with 2 μ M galanin or 2 μ M neuropeptide Y (NPY, as the positive control) or the vehicle (containing BSA, control condition), was added to each of the wells in 24-well plates. We used 2 μ M galanin because a similar NPY concentration was previously shown to induce SVZ cell migration [34]. SVZ cells (6,000 cells) were added in the upper chamber of the transwell. After 6 h incubation, the cells that were present on the underside of the transwell were fixed in 4% PFA, labeled with propidium iodide (1:100; Invitrogen), and counted on 20 microscope fields (taken with a 40 \times objective using a Zeiss microscope) by an observer blinded to the treatments. The results are expressed as percent of the control condition set to 100%. Data were obtained from three independent experiments in which each condition was assessed in three independent wells [34].

Isolation of total RNA, cDNA synthesis and qPCR analysis: galanin and GALRs

The analysis was performed as previously described [29,31] (see Supplementary Data).

Isolation of total RNA, cDNA synthesis and qPCR analysis: Mash1, Dlx2, Ngn1 transcription factors

Total RNA was isolated from SVZ neurospheres treated without (control) or with 1 μ M galanin for 2, 3, and 4 days, according to illustra RNAspin Mini RNA Isolation Kit

manufacturer's instructions (GE Healthcare Life Sciences) as detailed in the Supplementary Data section.

Quantitative chromatin immunoprecipitation

The protocol used was recently described [36] (see Supplementary Data).

Western blot

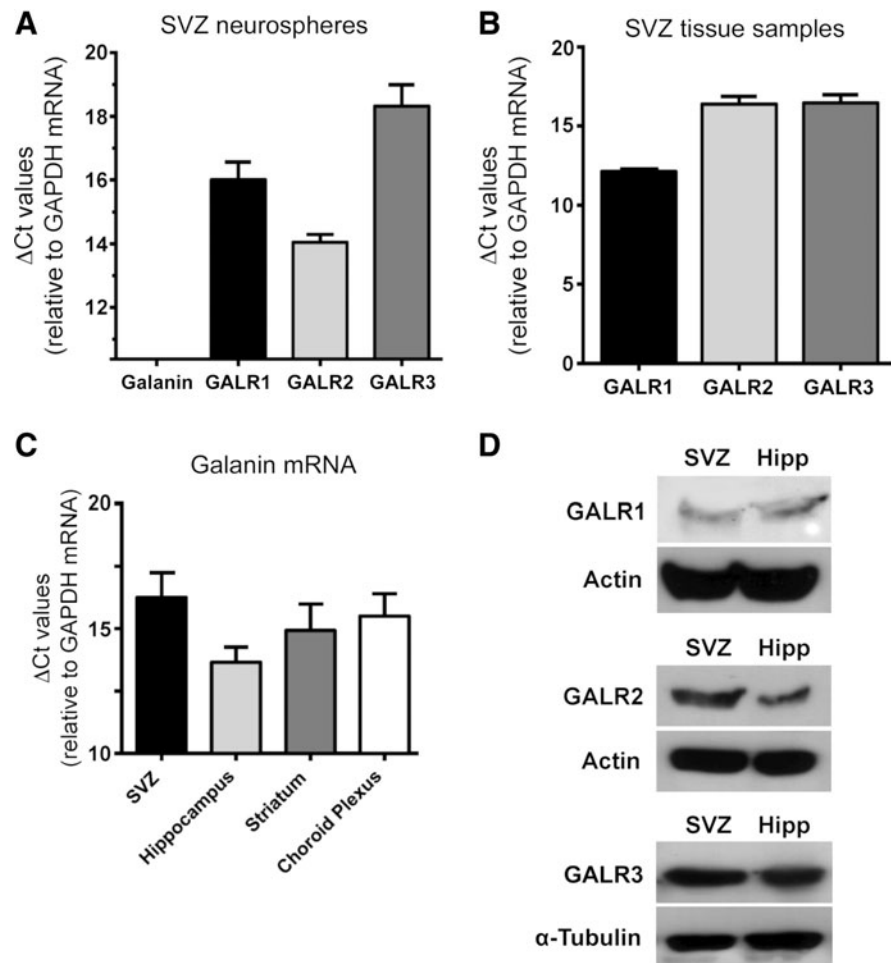
Detection of GALR1, GALR2 and GALR3 was performed by western blot in SVZ neurospheres and hippocampus tissue samples from newborn mice (see Supplementary Data).

Statistical analysis

For cell proliferation studies, each experimental condition was reproduced in four different wells of a 96-well plate and experiments were assayed four times. Background values obtained in the negative control wells were subtracted from all values. Within each experiment, the mean optical density evaluated at 450 nm in control wells were set to 100% and optical densities of experimental conditions are expressed as percent of the control condition. In all experiments involving the plating of neurospheres, measurements were performed at the border of SVZ neurospheres where migrating cells form pseudo-monolayers of cells. For SCCI experiments, the

percentage of neuronal-like responding cells (with a histamine/KCl ratio below 0.8) was calculated on the basis of one microscopic field per coverslip, containing ~100 cells (magnification, 40 \times). Except where otherwise specified, the experiments were replicated at least thrice. Three coverslips were analyzed for each condition in each experiment. The percent of NeuN, TUNEL, BrdU/NeuN, β III tubulin/BrdU, Dlx2/BrdU, β III tubulin/TH, VGAT positive cells in SVZ cell cultures was calculated from cell counts in five independent microscopic fields in each coverslip with a 40 \times objective (~200 cells per field). Quantification of the number and the total length of newly formed neurites, positive for p-JNK and emerging from the neurospheres, was performed in two independent cultures (two coverslips for each condition) in 20 nonoverlapping fields per coverslip using digital images (magnification, 20 \times). The numbers of GFP/MAP-2+ dendrites per neuron, total GFP/MAP-2+ dendrite length and mean length per GFP/MAP-2+ dendrite were evaluated in three culture preparations in ~20 nonoverlapping fields *per* coverslip (40 \times magnification). Software used was Axiovision, release 4.6 (Carl Zeiss). qChIP and qPCR experiments were performed in at least three independent cultures. Data are expressed as mean \pm standard error of the mean (SEM). Statistical significance was determined by using the unpaired two-tailed Student's *t*-test or one-way analysis of variance followed by Bonferroni or Dunnett's multiple comparison test, with *P* < 0.05 representing statistical significance.

FIG. 1. Detection of galanin and GALRs in subventricular zone (SVZ)-derived neurospheres, adult SVZ and brain tissues using quantitative polymerase chain reaction (qPCR) and western blotting. **(A)** qPCR revealed that GALR1, GALR2 and GALR3 mRNAs are expressed in both SVZ neurospheres and **(B)** adult SVZ tissue samples. **(C)** similarly, galanin mRNA is detected in tissue samples from adult SVZ, striatum and choroid plexus. Adult hippocampus tissue samples were used as the positive control for galanin expression. Total RNA was purified and relative mRNA levels were measured by qPCR. Data were normalized to GAPDH and presented as mean Δ Ct values \pm SEM. Low Δ Ct values indicate high levels of expression. **(D)** Western blot confirmed the presence of GalR1, GalR2 and GalR3 proteins in SVZ cultures from newborn mice. Dissected hippocampus tissue samples were used as positive controls. SEM, standard error of the mean.



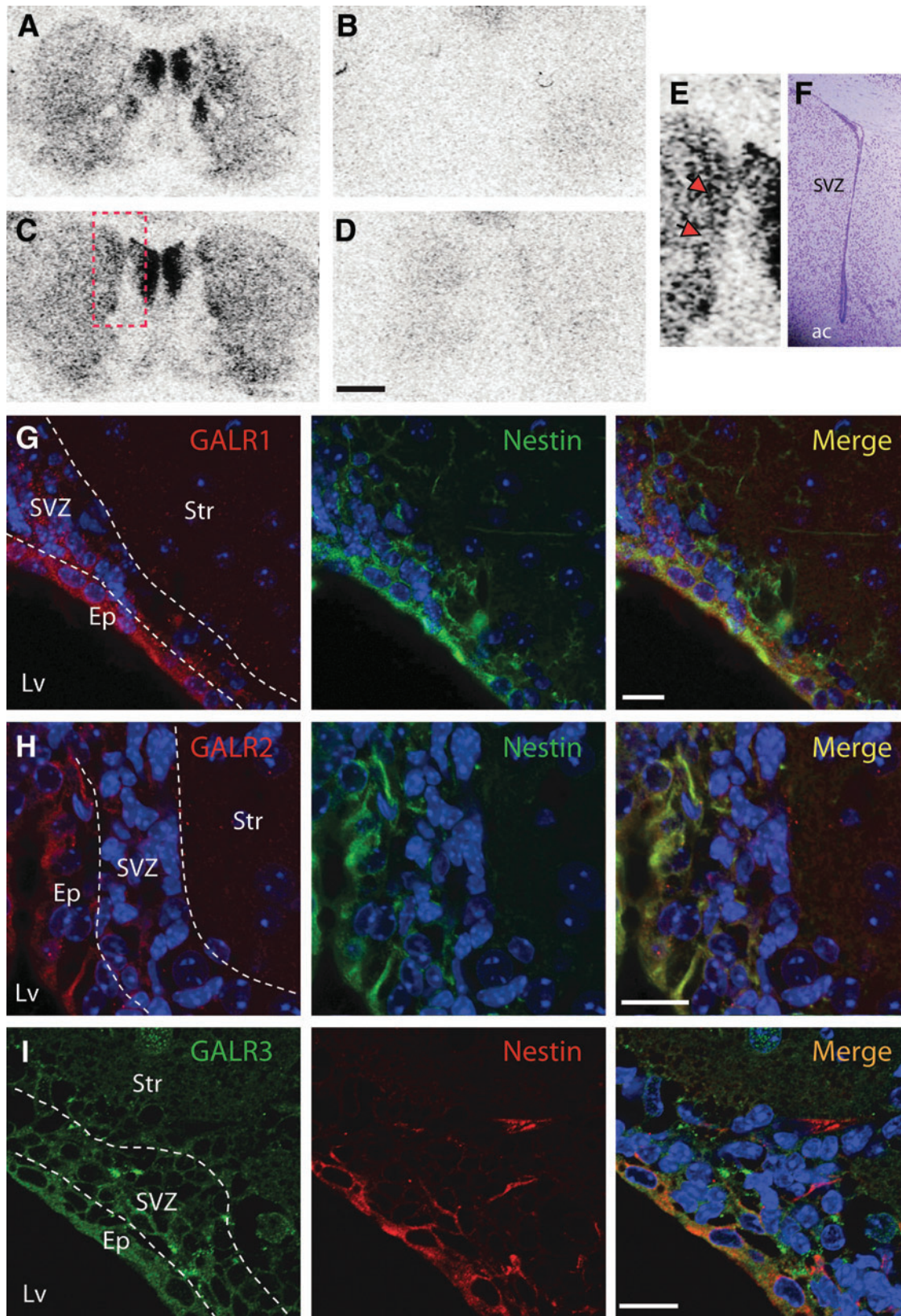


FIG. 2. $[^{125}\text{I}]$ -galanin binding and immunodetection of GALR1, GALR2 and GALR3 in the murine SVZ. Total $[^{125}\text{I}]$ -galanin binding (A, C) and corresponding nonspecific binding ($[^{125}\text{I}]$ -galanin + $1\ \mu\text{M}$ galanin; B, D) in coronal slides from adult mouse. (E) Magnification of dashed section in (C) showing $[^{125}\text{I}]$ -galanin binding corresponding to the SVZ (red arrows). (F) Slide from (E) counter-stained with Cresyl violet for identification of anatomical structures; ac, anterior commissure. Scale bar = 1 mm. Immunodetection of GALR1, GALR2 and GALR3 in the SVZ of adult mice (G–I). GALR1 (red), GALR2 (red) and GALR3 (green) are expressed in the ependymal layer (Ep) and the SVZ and faintly in the striatum (Str). Stem/progenitor cells and ependymal cells express nestin. Lv: Lateral ventricle. Scale bars = 20 μm . Color images available online at www.liebertpub.com/scd

Results

Expression of galanin and GALRs as well as galanin binding in the SVZ

Using qPCR, the expression of galanin and GALRs was investigated in the SVZ both *in vitro*, in neurosphere cultures, and *in vivo* in tissue samples relative to that of the reference gene GAPDH. In SVZ neurospheres, the galanin gene was found not to be expressed during the 40 cycles of qPCR (Fig. 1A), but GALR1, GALR2 and GALR3 were all expressed (ΔCt_{GALR1} : 16.00 ± 0.56 ; ΔCt_{GALR2} : 14.04 ± 0.25 ; ΔCt_{GALR3} : 18.32 ± 0.67 ; Fig. 1A). Similarly, in dissected SVZ tissue samples, all three GALRs were expressed (ΔCt_{GALR1} : 12.12 ± 0.22 ; ΔCt_{GALR2} : 16.39 ± 0.49 ; ΔCt_{GALR3} : 16.46 ± 0.52 ; Fig. 1B). To identify the possible source of galanin for SVZ cells *in vivo*, we assessed the expression of galanin in the SVZ, the adjacent striatum and choroid plexus that produces the cerebrospinal fluid in contact with SVZ cells. We found that galanin mRNA is expressed in all studied tissues (SVZ: $\Delta Ct_{galanin} = 16.25 \pm 1.00$; striatum: $\Delta Ct_{galanin} = 14.93 \pm 1.06$; choroid plexus: $\Delta Ct_{galanin} = 15.50 \pm 0.91$; Fig. 1C). Hippocampus tissue samples were previously found to express galanin, and therefore, used as a positive control for the specificity of the galanin primers [7] ($\Delta Ct_{galanin}$: 13.65 ± 0.61 ; Fig. 1C). Expression of GALR1, GALR2, and GALR3 receptors in SVZ cultures from newborn mice was also confirmed by western blots. Hippocampus tissue samples were used as positive controls (Fig. 1D).

Consistent with the expression of GALRs in the SVZ, specific [125 I]-galanin binding was found corresponding to the SVZ of adult mouse brain slices (Fig. 2A–F). Moreover, low levels of galanin-stimulated [35 S]GTP γ S functional binding were also detected corresponding to the SVZ in the same animals, indicating that GALRs are functional in the SVZ (basal binding: $100.00\% \pm 2.52\%$; galanin-stimulated binding: $107.30\% \pm 0.49\%$; data not shown). To further confirm that GALRs are expressed in the SVZ *in vivo*, brain slices from adult mice encompassing the ependymal layer, SVZ, and striatum were stained for nestin, a marker of ependymal and stem/progenitor cells [37], and for GALR1, GALR2 and GALR3. Colocalization of GALRs was found with nestin-positive cells both in the ependymal layer and the SVZ (Fig. 2G–I). Faint labeling for the GALRs was also found in the striatum. These data correlate with the experiment shown in Fig. 2A–F. Similarly, expression of GALRs was found in nestin-expressing cells in SVZ neurospheres (Supplementary Fig. S2).

Galanin does not affect self-renewal, proliferation or cell death

We determined whether galanin impacts on self-renewal capacity of SVZ cells by using the neurosphere assay as described previously [34,36,38]. The experiments were conducted in parallel with the hepatocyte growth factor (HGF, 50 ng/mL) known to induce self-renewal [38]. Exposure of freshly dissected SVZ cells to 1 μ M galanin for 6 days did not affect the numbers of primary neurospheres generated as compared to nontreated cultures (control) (control: $100\% \pm 2.26\%$, 1 μ M galanin: $92.03\% \pm 4.97\%$, HGF: $143.60\% \pm 13.17\%$, $P < 0.001$, data not shown). Moreover, galanin had no impact on the self-renewal capacity of stem cells, since the numbers of secondary

neurospheres were similar in control and treated conditions (control: $99.99\% \pm 2.65\%$, 1 μ M galanin: $106.00\% \pm 4.90\%$, HGF: $140.20\% \pm 11.41\%$, $P < 0.05$, data not shown). The effect of galanin on SVZ proliferation was tested in SVZ cultures using the BrdU incorporation assay. Cells were treated for 48 h with 10 nM, 100 nM, or 1 μ M galanin, and with 100 nM of either the GALR1 (M617) or GALR2 (AR-M1896) agonists. BrdU was added to the cultures for the last 4 h of exposure to neuropeptides. As depicted in Table 1, none of the treatments affected proliferation, while incubation with EGF and FGF-2, used as a positive control, increased BrdU incorporation.

To rule out the possibility that galanin may exert an effect on proliferation of stimulated cells, the assay was conducted in the presence growth factors (EGF and FGF-2). Again, no effect of galanin was obtained, while EGF and FGF-2 were highly efficacious (control: $100.00\% \pm 4.06\%$; 1 μ M galanin: $99.42\% \pm 9.01\%$; EGF + FGF-2: $163.10\% \pm 22.87\%$, $^{**}P < 0.001$, five cultures tested, data not shown). The effects of galanin and GALR agonists on cell death were evaluated by TUNEL staining to detect apoptotic nuclei. Neither galanin nor the GALR agonists affected SVZ cell death as the numbers of apoptotic nuclei did not differ from control levels (control: $19.09\% \pm 1.50\%$, galanin: $17.68\% \pm 1.98\%$, 100 nM M617: $17.89\% \pm 1.32\%$, AR-M1896: $14.31\% \pm 1.65\%$, data not shown). Taken together, these results show that galanin neither affects cell proliferation nor cell death in the SVZ.

Galanin promotes neuronal differentiation through GALR1 and GALR2 activation

The effects of galanin on neuronal differentiation were first tested in neurospheres plated on poly-D-lysine-coated coverslips and incubated for 7 days in the absence (control) or the presence of 1 μ M galanin, and then stained to reveal β III tubulin-positive neurons and GFAP-expressing astrocytes. As depicted in Fig. 3A, the neuronal network appeared denser in galanin-treated cultures as compared to the untreated cultures. To quantify neuronal differentiation, cells

TABLE 1. GALANIN EXERTS NO EFFECT ON SUBVENTRICULAR ZONE CELL PROLIFERATION

	Mean of absorbance (450 nm)	\pm SEM
Control	100.00	3.02
Galanin 10 nM	111.50	6.80
Galanin 100 nM	99.45	3.92
Galanin 1 μ M	107.10	2.60
GALR1 agonist 100 nM	95.06	6.96
GALR2 agonist 100 nM	108.06	8.27
EGF (10 ng/mL) + FGF-2 (5 ng/mL)	174.20 ^a	6.97

Table depicts the values of absorbance measured at 450 nm after revelation of the peroxidase activity associated with an anti-BrdU antibody. SVZ monolayers were treated for 48 h with galanin and GALR agonists. As a positive control, SVZ cultures were incubated with EGF and FGF-2 and, as expected, proliferation was significantly increased in these cultures. Data represent means \pm SEM of four independent cultures. ^a $P < 0.01$, using one-way analysis of variance with Dunnett's post hoc test for comparison with the control condition.

EGF, epidermal growth factor; FGF-2, fibroblast growth factor-2; SEM, standard error of the mean; SVZ, subventricular zone.

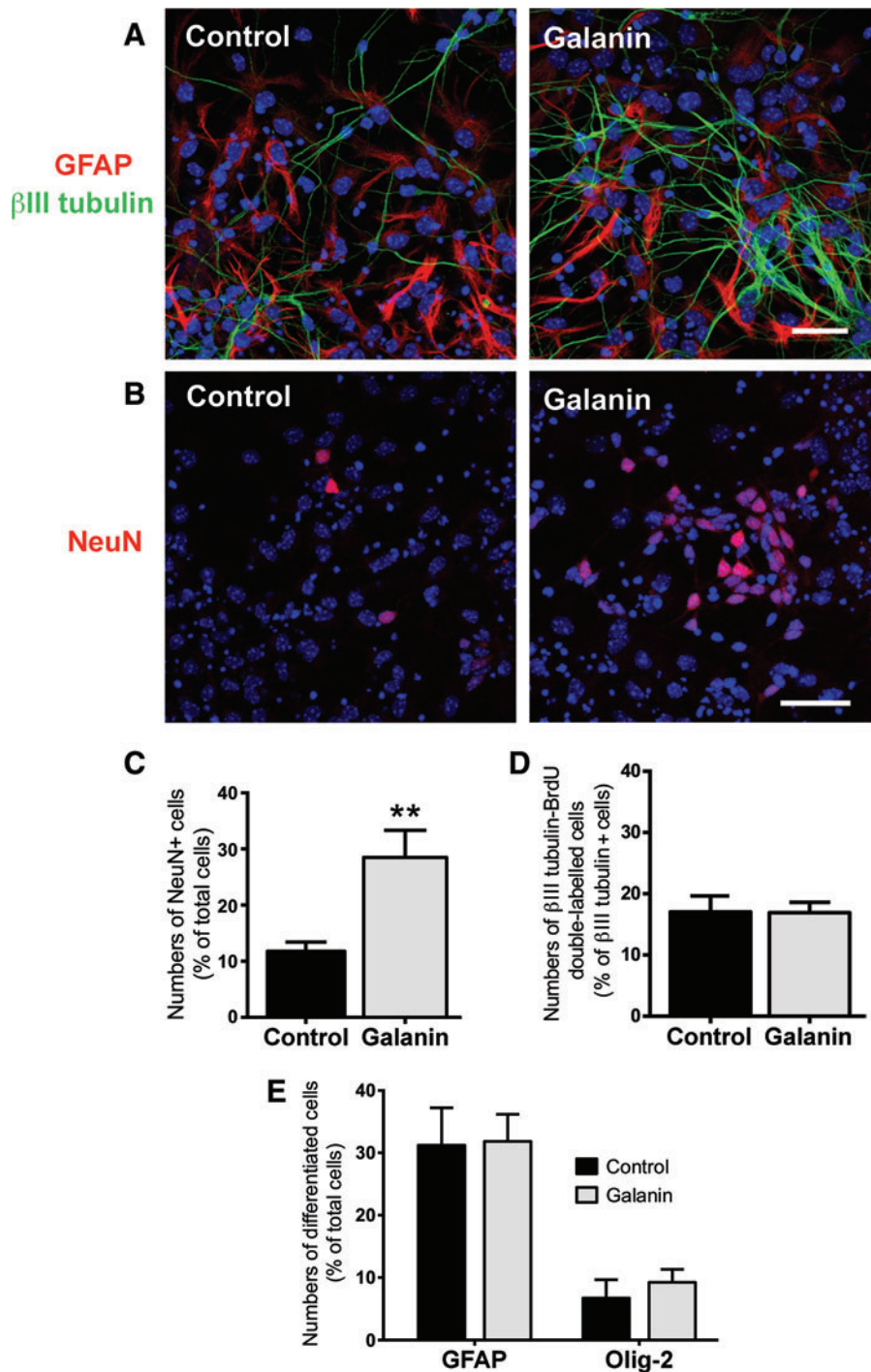


FIG. 3. Galanin stimulates neuronal differentiation. Representative digital images of SVZ cells treated or untreated (control) for 7 days with 1 μ M galanin and immunostained for glial fibrillary acidic protein (GFAP, red), β III tubulin (green) (A), and NeuN (red) (B). Nuclei were counterstained with Hoechst 33342. Scale bars = 50 μ m. (C–E) Bar graphs depict the number of NeuN+ (C), BrdU- β III tubulin double-labeled neuroblasts (D) and GFAP+ and Olig-2+ (E) cells in SVZ cultures treated or untreated (control) for 48 h (D) or 7 days (C, E) with 1 μ M galanin. Numbers are expressed as percent of total cells (C, E) or of total β III tubulin+ cells (D) and represent mean \pm SEM of two independent cultures. ** $P < 0.01$, using the unpaired Student's *t*-test. Color images available online at www.liebertpub.com/scd

were immunostained for the neuronal nuclear marker NeuN, NeuN-positive (NeuN+) nuclei were counted (Fig. 3B, C). The result shows that galanin promotes neuronal differentiation as more NeuN+ cells were counted in the presence of galanin as compared to control (control: 11.79% \pm 1.61%, galanin: 28.49% \pm 4.83%, $P < 0.01$; Fig. 3C).

As an increase in the number of neurons can result from an increase in the proliferation rate of neuronal progenitors (or neuroblasts), we incubated the cells with galanin for 48 h and marked the DNA of proliferating cells with BrdU for the last 4 h of the cultures. Proliferating neuroblasts were labeled for BrdU and β III tubulin. We counted the numbers of

double-labeled cells and found that galanin does not induce the proliferation of neuroblasts (control: 17.06% \pm 2.53%, galanin: 16.91% \pm 1.72%; Fig. 3D). To discard the possibility that galanin may enhance the proliferation of more immature progenitors, we performed the same experiments and labeled the cells for the C cell type marker Dlx2 and counted the number of BrdU-Dlx2 double-labeled cells (expressed as a percentage of proliferating cells). No significant difference was obtained between the conditions (control: 33.85% \pm 1.57%, galanin: 37.73% \pm 3.26%; three independent cultures, data not shown), indicating that galanin also does not affect proliferation of immature progenitors.

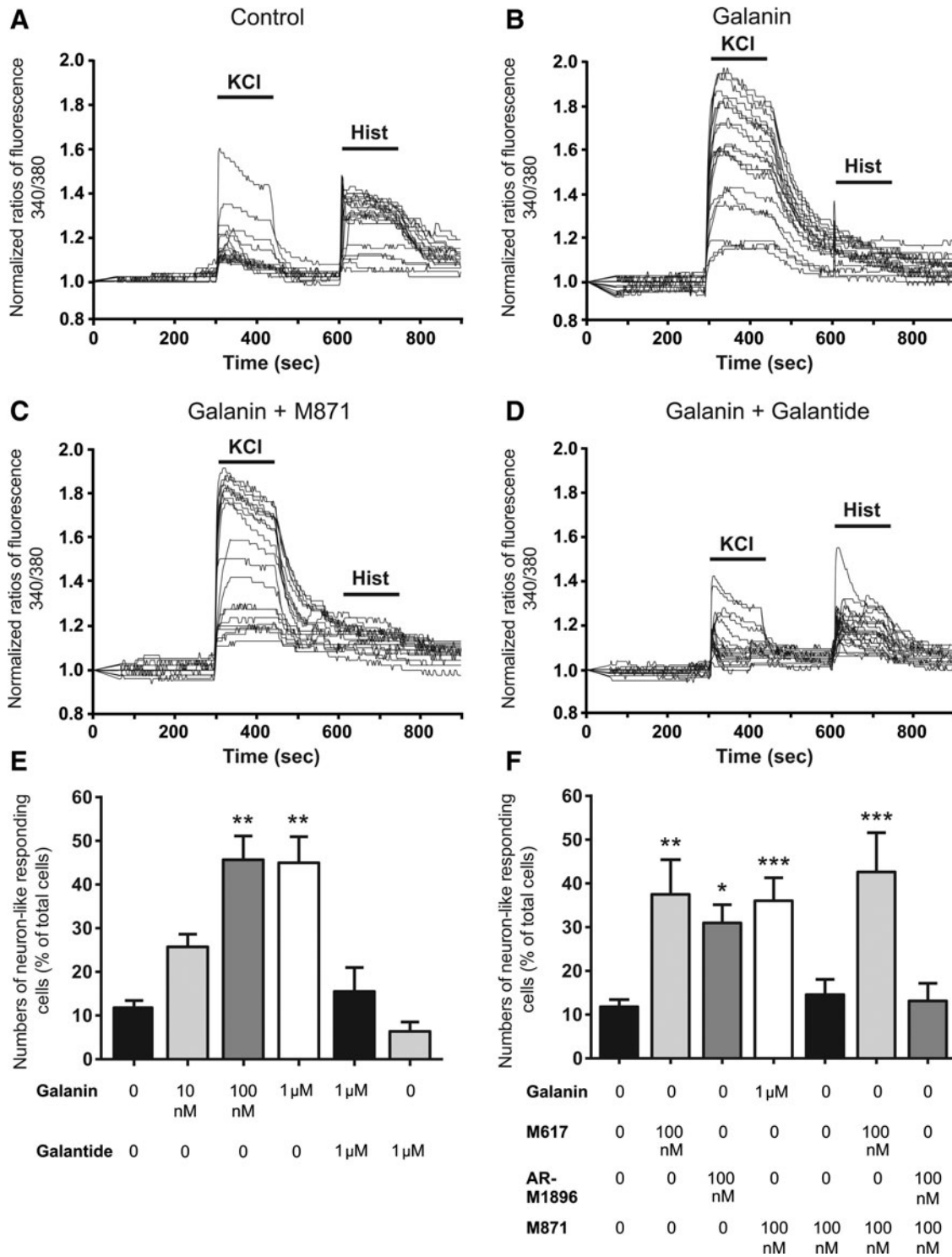


FIG. 4. Galanin induces neuronal differentiation in SVZ cultures through GALR1 and GALR2. (A–D) Representative single-cell calcium imaging profiles of the response of 20 cells in an untreated culture (A), cultures maintained 7 days with 100 nM galanin (B), 1 μM galanin + 100 nM M871 (C), or with 1 μM galanin + 1 μM galantide (D). (E, F) Numbers of neuronal-like responding cells in cultures incubated for 7 days with galanin, galantide (E), or with GALR1 (M617), GALR2 (AR-M1896) agonists and GALR2 (M871) antagonist (F). Numbers are expressed as percent of total cells. Data represent mean ± SEM of at least three independent cultures per condition. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, using one-way analysis of variance (ANOVA) with Bonferroni’s post hoc test for multiple comparisons.

The effects of galanin on glial cell differentiation were assessed by counting the numbers of GFAP+ and Olig-2+ cells in SVZ cultures incubated for 7 days in the absence (control) or the presence of 1 μ M galanin. No differences in the number of astrocytes and oligodendrocytes were observed between the conditions (GFAP, control: 31.21% \pm 6.01%, galanin: 31.84% \pm 4.34%; Olig-2, control: 6.71% \pm 2.97%, galanin: 9.24% \pm 2.08%; Fig. 3E). Moreover, quantification of GFAP expression was also performed by western blotting and we found no effect of galanin treatment (control set at 100%, 1 μ M galanin: 101.60% \pm 8.00%, three independent cultures, data not shown), indicating that galanin does not affect glial differentiation.

Functional neuronal differentiation was evaluated by measuring $[Ca^{2+}]_i$ variations in single cells after KCl and histamine stimulation. KCl depolarization leads to the massive entry of Ca^{2+} into neurons through voltage-dependent calcium channels, while stimulation with histamine increases $[Ca^{2+}]_i$ in SVZ immature cells but not in neurons or in glial cells. We previously showed that SVZ-derived neuronal-like mature cells display histamine/KCl ratios below 0.8 [29,35,39]. Figure 4A–D represents characteristic profiles of fluorescence records displayed by 20 cells of control (A), 100 nM galanin-treated cells (B), 1 μ M galanin, and 100 nM M871-treated cells (C) and 1 μ M galanin and 1 μ M galantide-treated cells (D). In control cultures, most of the cells responded to histamine but not to KCl. After 7 days of treatment with 100 nM or 1 μ M galanin, the percent of neuronal-like cells increased, suggesting a proneurogenic effect of galanin (control: 11.80% \pm 1.65%; 10 nM galanin: 25.72% \pm 2.90%; 100 nM galanin: 45.67% \pm 7.45%, $P < 0.01$; 1 μ M galanin: 44.98% \pm 5.98%, $P < 0.01$; Fig. 4E). This proneurogenic effect of galanin was abolished by cotreatment with the nonselective GALR antagonist galantide, while galantide by itself did not affect neuronal differentiation (1 μ M galanin + 100 nM galantide: 10.74% \pm 5.52%, data not shown; 1 μ M galanin + 1 μ M galantide: 15.50% \pm 5.51%; 1 μ M galantide: 6.36% \pm 2.15%; Fig. 4D, E).

To determine which receptor mediates the proneurogenic effect of galanin, SVZ cells were treated for 7 days with 10 and 100 nM of either the GALR1 agonist M617 or the GALR2 agonist AR-M1896. Single-cell calcium experiments showed that the proneurogenic effect of galanin is reproduced after incubation with 10 and 100 nM of the GALR1 agonist, suggesting that galanin effects are mediated by GALR1. However, treatment with 100 nM but not 10 nM GALR2 agonist also increased neuronal differentiation (10 nM M617: 32.47% \pm 8.39%, $P < 0.05$, data not shown; 100 nM M617: 37.49% \pm 7.95%, $P < 0.01$; 10 nM AR-M1896: 15.31% \pm 6.85%, data not shown; 100 nM AR-M1896: 30.95% \pm 4.20%, $P < 0.05$; Fig. 4F). As the IC_{50} for the GALR2 agonist AR-M1896 on GALR1s has been reported to be 879 nM [40] and as AR-M1896, in our experiments, triggered neuronal differentiation when added at 100 nM, the proneurogenic effects obtained after GALR2 stimulation seems to be specific.

To confirm that the proneurogenic effect obtained with the GALR1 agonist M617 was exclusively due to GALR1 stimulation and that AR-M1896 was selective for GALR2, SVZ cells were treated with the GALR2 antagonist M871. M871 alone at 100 nM had no effect on neuronal differentiation (14.58% \pm 3.49%). Antagonizing the GALR2 receptor with

M871 did not prevent the proneurogenic effects exerted by M617 and galanin (100 nM M871 + 100 nM M617: 42.61% \pm 8.98%, $P < 0.01$; 100 nM M871 + 1 μ M galanin: 36.04% \pm 5.26%, $P < 0.01$; Fig. 4C, F). In contrast, the antagonist M871 was able to prevent the proneurogenic effect exerted by AR-M1896 (100 nM M871 + 100 nM AR-M1896: 13.14% \pm 4.02%, Fig. 4F), indicating that GALR2 is also involved in the neurogenic effects of galanin. These results indicate that both GALR1 and GALR2 mediate the proneurogenic effect of galanin. However, GALR1 and GALR2 agonists had no synergistic effects (data not shown).

Galanin interferes with early stages of neuronal differentiation

To determine whether galanin affects early stages of neuronal differentiation, proliferating SVZ cells were labeled with BrdU for the first 24 h of treatment followed by a chase

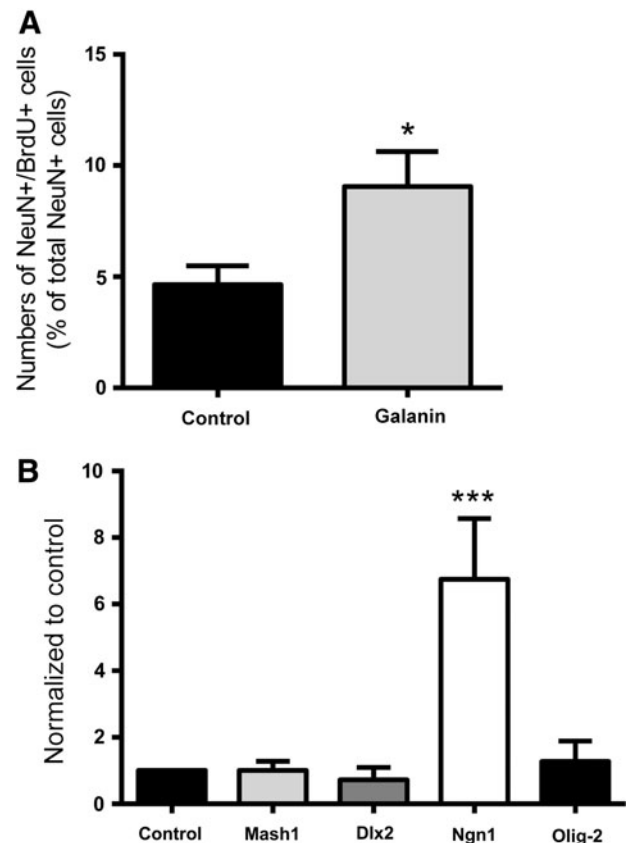


FIG. 5. Galanin induces neuronal commitment of SVZ cells. **(A)** Bar graph depicts the numbers of BrdU and NeuN+ neurons in SVZ cultures incubated for 7 days with 1 μ M galanin. Immature progenitors were labeled for the first 24 h of culture. Numbers are expressed as percent of total NeuN+ cells counted and represent mean \pm SEM of three independent cultures. * $P < 0.05$, using the unpaired Student's *t*-test. **(B)** Bar graph depicts the fold increase of mRNA expression for Mash1, Dlx2 and Ngn1 proneurogenic genes and for the prolifodendroglial gene Olig-2, measured by qPCR analysis in cultures treated for 3 days with 1 μ M galanin. Data are expressed as mean \pm SEM of at least three cultures. *** $P < 0.001$, using one-way ANOVA with Dunnett's post hoc test for comparison with the control condition (set to 1).

of 6 days in the absence (control) or the presence of galanin (Fig. 5A). Galanin treatment caused a significant increase in the proportion of NeuN- and BrdU-positive cells among the NeuN-positive cells (control: $4.64\% \pm 0.85\%$, $1\ \mu\text{M}$ galanin: $9.05\% \pm 1.57\%$, $P < 0.05$, Fig. 5A). To determine whether galanin impacts on proneuronal gene expression, levels of mRNA for the proneuronal transcription factors Mash1, Dlx2, and Ngn1 were measured in cultures treated for 2, 3, and 4 days with galanin. Galanin treatment significantly increased Ngn1 mRNA levels at 3 days (control set to 1, Mash1: 1.00 ± 0.28 , Dlx2: 0.72 ± 0.38 ; Ngn1: 6.48 ± 1.83 , $P < 0.001$, Fig. 5B) but not at 2 and 4 days (data not shown), while no effect was found on Mash1 and Dlx2 mRNA levels. Similarly, expression of the proglial transcription factor Olig-2 was not affected (3 days, Olig-2: 1.27 ± 0.61).

Moreover, galanin elicited neuronal differentiation via chromatin modification of the Ngn1 promoter region. qChIP analysis which determines the interaction between specific proteins and DNA (eg, promoter regions) was used to study if galanin treatment promoted the availability of the selected proneurogenic transcription factor Ngn1 to transcription complexes. In particular, we used qChIP against the H3K36m3, a protranscriptional histone modification known to induce gene expression. Six and 24h treatment with galanin increased H3K36m3 in the promoter region of Ngn1 as assessed (control set to 1, 6h: 3.75 ± 2.63 , 24h: 2.10 ± 0.82 , three to six independent cultures, data not shown). Together these results demonstrate that galanin triggers neuronal commitment.

Galanin promotes both GABAergic and dopaminergic differentiation

SVZ-derived neurons have been shown to develop into GABAergic interneurons with a minority of cells differenti-

ating into dopaminergic interneurons in the olfactory bulb [41]. As galanin triggered neuronal differentiation in SVZ cells, we sought to determine whether galanin would increase these former neuronal populations. SVZ cells were treated for 7 days in the absence (control) or the presence of $1\ \mu\text{M}$ galanin, and immunocytochemistry was performed to detect GABAergic neurons (expressing VGAT) and dopaminergic neurons (expressing the enzyme TH). Since glial expression of TH has been reported [42], double immunolabelling for TH and β III tubulin was performed and TH positive neurons counted. Galanin increased both the numbers of GABAergic neurons (VGAT, control: $10.55\% \pm 1.06\%$, $1\ \mu\text{M}$ galanin: $16.90\% \pm 2.38\%$, $P < 0.05$, Fig. 6A, B) and dopaminergic neurons (TH+neurons, control: $8.03\% \pm 1.13\%$, $1\ \mu\text{M}$ galanin: $13.58\% \pm 1.92\%$, $P < 0.05$; Fig. 6C, D).

Galanin promotes neuronal maturation

To examine whether galanin affects axonal formation, activation of the SAPK/JNK MAPK pathway, known to interfere with axonal formation, was investigated in branches derived from plated SVZ neurospheres incubated for 6h with $1\ \mu\text{M}$ galanin. The numbers and total length of branches which were immunoreactive for the phospho(p)JNK in SVZ cultures were measured. We ascertained that pJNK+ (green) branches colocalized with the axon-specific protein Tau (red) (Fig. 7A; see [43]). Exposure of SVZ cells to galanin significantly increased the number as well as the total length of pJNK+ branches per neurosphere as compared with control cultures (total length of ramification per neurosphere: control: $134.80 \pm 26.19\ \mu\text{m}$; $1\ \mu\text{M}$ galanin: $280.80 \pm 38.27\ \mu\text{m}$, $P < 0.01$, Fig. 7B; numbers of branches per neurosphere, control: 2.00 ± 0.38 ; $1\ \mu\text{M}$ galanin: 3.32 ± 0.37 $P < 0.05$; Fig. 7C). The same experiments were performed in the presence of

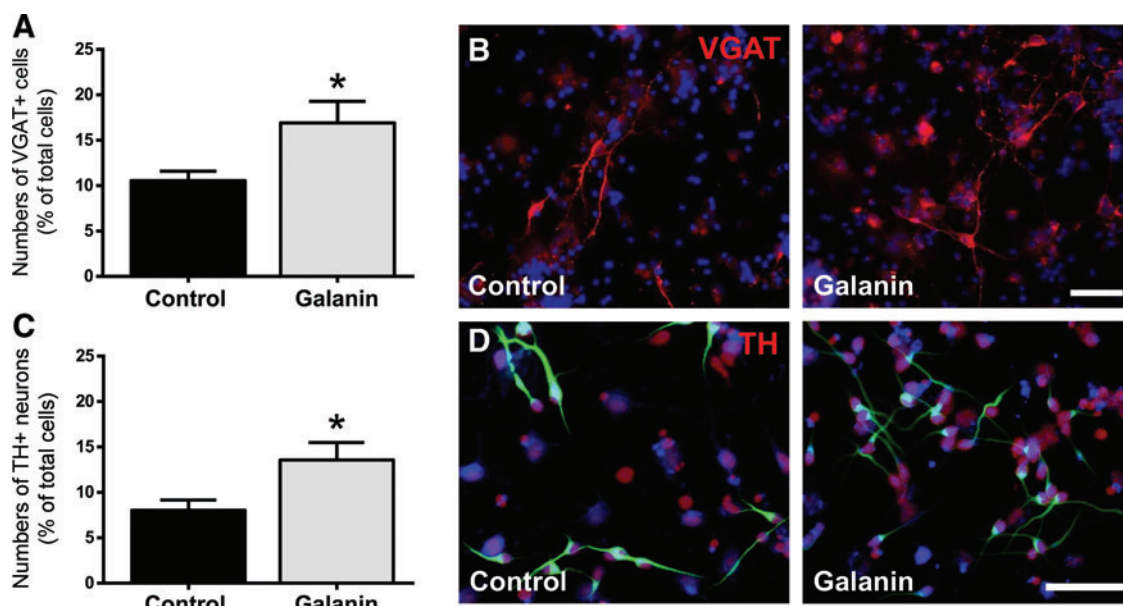


FIG. 6. Galanin promotes GABAergic and dopaminergic neuronal differentiation. Bar graph depicts the numbers of either (A) VGAT+ or (C) TH+ neurons, expressed as percent of total cells per culture. Representative fluorescent digital images of (B) VGAT+ (in red) and (D) TH+ (in red)/ β III tubulin+ (in green) neurons in control and $1\ \mu\text{M}$ galanin-treated cultures. Scale bars = $40\ \mu\text{m}$. Data represent mean \pm SEM of three independent cultures. * $P < 0.05$ using the unpaired Student's *t*-test. Color images available online at www.liebertpub.com/scd

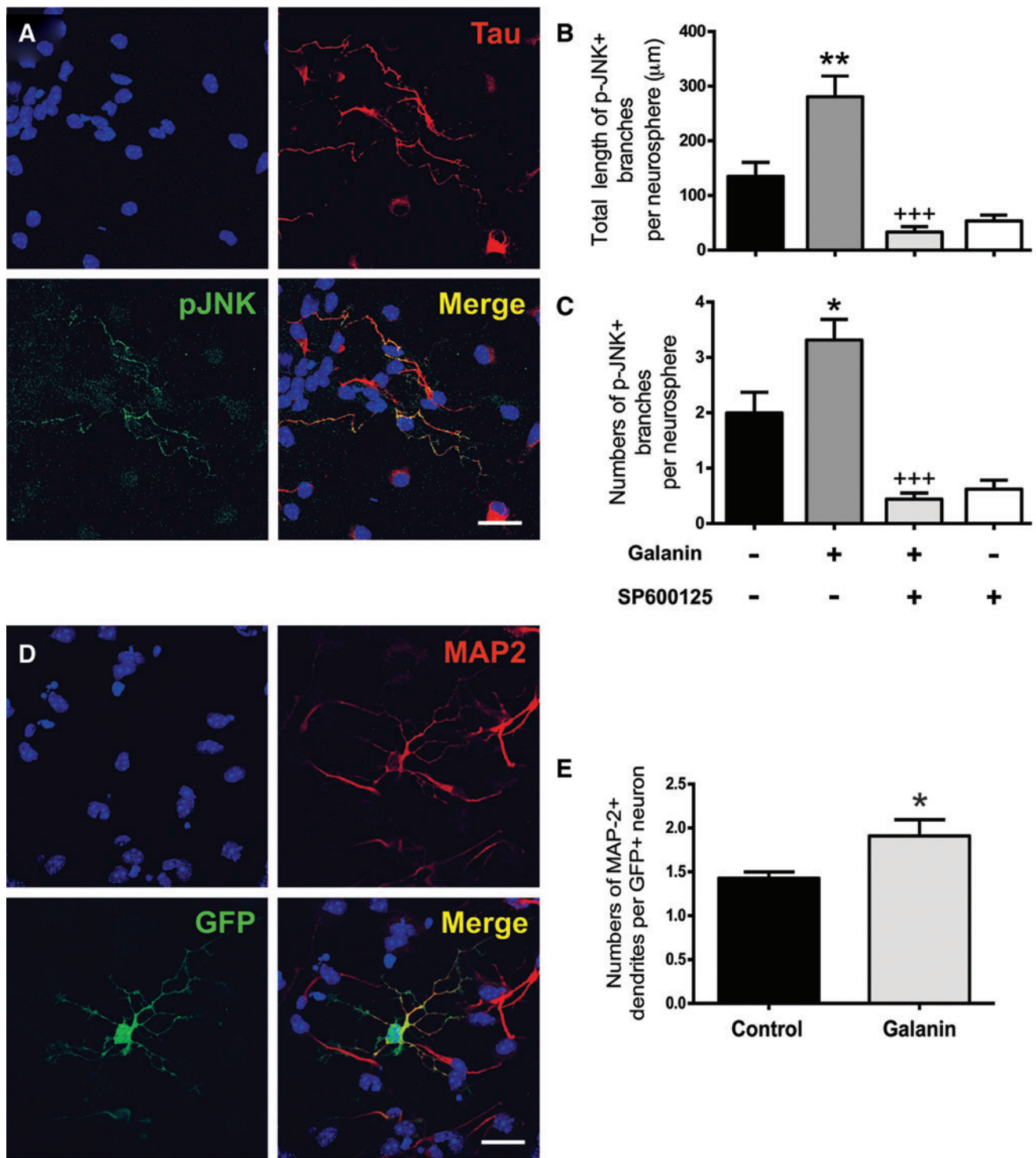


FIG. 7. Galanin promotes neuronal maturation. (A) Representative digital image of branches colabeled for pJNK+ (green) and Tau (red). Bar graphs depict total length of pJNK+ branches (B) and numbers of p-JNK+ branches (C) per SVZ neurosphere incubated for 6 h in the absence (control) or presence of 1 µM galanin, and/or with 20 µM JNK inhibitor SP600125. (D) Representative digital image of a green fluorescent protein (GFP)+ neuron with microtubule associated protein-2 (MAP-2)+ branches. (E) Bar graph depicts the numbers of MAP-2+ dendrites per GFP+ neuron in cultures treated in the absence (control) or presence of 1 µM galanin. Scale bars=20 µm. Data represent means ± SEM of three independent cultures. **P* < 0.05; ***P* < 0.01 as compared to control; +++*P* < 0.001 as compared to galanin, using one-way ANOVA with Bonferroni's post hoc test for multiple comparisons. Color images available online at www.liebertpub.com/scd

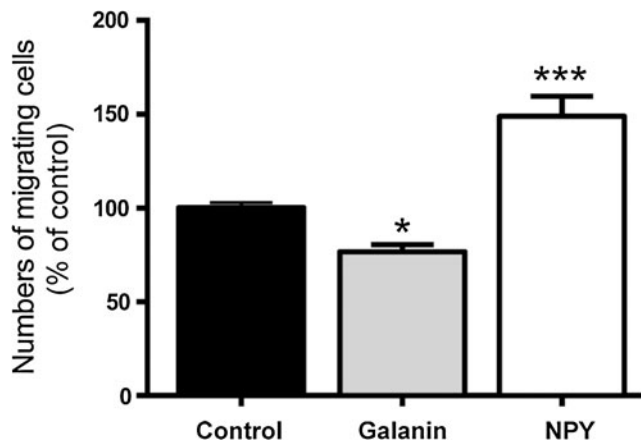


FIG. 8. Galanin reduces SVZ cell migration. Bar graph depicts numbers of migrating cells determined after 6 h incubation with 2 μ M galanin, or 2 μ M neuropeptide Y (NPY) as a positive control, and expressed as a ratio to that obtained in serum-free medium (control). Data represent means \pm SEM of three independent experiments. * $P < 0.05$, *** $P < 0.001$, using one-way ANOVA with Dunnett's post hoc test for comparison with the control condition (set to 100%).

20 μ M SP600125, an inhibitor of JNK activity. Length and number of pJNK/Tau+ branches per neurosphere decreased in cultures treated with SP600125 alone or together with 1 μ M galanin (total length of ramification per neurosphere, control + SP600125: $53.57 \pm 10.69 \mu\text{m}$; 1 μ M galanin + SP600125: $33.23 \pm 9.98 \mu\text{m}$, $P < 0.001$ as compared to galanin, Fig. 7B); numbers of branches per neurosphere, control + SP600125: 0.62 ± 0.16 ; 1 μ M galanin + SP600125, 0.44 ± 0.11 ; $P < 0.001$ as compared to galanin, Fig. 7C), attesting the crucial role of SAPK/JNK in mediating axonal elongation. Altogether, these data show that galanin promoted axonogenesis via activation of the SAPK/JNK pathway in SVZ cultures.

The effects of galanin on neurogenesis were also evaluated in WT and GFP mixed SVZ cell cultures. Neurites were immunostained for MAP-2 and numbers of GFP/MAP-2+ neurites per neuron, total GFP/MAP-2 length of neurites and mean length per GFP/MAP-2+ neurite were evaluated. Figure 7D provides a representative aspect of GFP+ neurons labeled for MAP-2. Galanin increased the numbers of dendrites per neuron (control: 1.43 ± 0.07 ; 1 μ M galanin: 1.91 ± 0.18 ; $P < 0.05$; Fig. 7E), while not affecting the other parameters significantly (total length: control: $659.00 \pm 64.75 \mu\text{m}$; 1 μ M galanin: $808.60 \pm 92.11 \mu\text{m}$; mean length per neurite: control: $32.83 \pm 2.01 \mu\text{m}$; 1 μ M galanin: $35.81 \pm 1.50 \mu\text{m}$, data not shown).

Galanin reduces SVZ cell migration

As galanin increased neuronal differentiation, we tested whether the neuropeptide would also exert an effect on the migration pattern of SVZ-derived neuroblasts using Boyden chambers. The cells were plated in the upper compartment of a transwell filter and allowed to migrate for 6 h through a microporous membrane separating the upper and lower compartments. Galanin was added in the lower compartment and found to decrease the number of migrating cells (control: $100.02\% \pm 2.68\%$; galanin: $76.75\% \pm 3.87\%$; $P < 0.05$;

Fig. 8). NPY was chosen as a positive control since its pro-migratory effects had been previously demonstrated [34].

Discussion

This study tested the effects of galanin on stem cell properties and neurogenesis in SVZ stem/progenitor cell cultures. We first showed by qPCR that galanin is not expressed in SVZ neurospheres, while the mRNA for all galanin receptors are present. However, galanin mRNA was found in situ in dissected SVZ tissue samples in accordance with a previous study [44], suggesting that SVZ cells may secrete galanin within their microenvironment. Nevertheless, we cannot conclude whether the mRNA is present in SVZ stem cells in vivo or rather in the differentiated cells of the neurogenic niche. Indeed, galanin is expressed by a variety of cell types, including non-neuronal cells, such as oligodendrocyte precursors and astrocytes [44–46] and astrocytes are cellular components of the SVZ [2,37]. Expression of galanin by mature cells of the SVZ in vivo rather than by stem/progenitor cells would explain why no galanin mRNA was found in neurospheres since the culture conditions applied sustain exclusively the expansion of stem/progenitor cells. In addition and consistent with a previous mouse study [47], galanin binding sites were found to be present in adult mice corresponding to the SVZ and, more importantly, galanin-stimulated [^{35}S]GTP γ S functional binding was also present, further establishing that galanin may exert biological effects on SVZ cells. We also identified galanin mRNA in the choroid plexus, suggesting that galanin is secreted into the cerebrospinal fluid and thereby may exert its action on SVZ cells. Another potential source of galanin could be the adjacent caudate-putamen which was shown in the present study to contain galanin mRNA and has been shown to contain galanin-immunoreactive fibres in mice [48]. Recent studies showed that galanin is expressed by pluripotent embryonic stem cells and by bone marrow mesenchymal stem cells [25–28] suggesting that galanin expression may be used as a functional marker for stemness. However, we found no expression of galanin in SVZ cells, at least in vitro, and no effect of galanin on self-renewal, indicating that galanin expression is not a hallmark for stemness. Similarly, galanin is not found in nestin positive stem cells from the adult pancreas islets [49].

The principal finding of the present work is that galanin triggers neuronal differentiation of SVZ-derived cells. The increase in neurons obtained with galanin is independent of an induced proliferation of neuroblasts and/or progenitors and does not affect glial differentiation. Our results suggest a role for galanin as an enhancer of the process of neuronal differentiation, firstly, by increasing the commitment of progenitors towards the neuronal fate and, secondly, by "accelerating" neuronal differentiation and maturation as evidenced by the increase in the proneurogenic transcription factor Ngn1 transcripts and activation of the Ngn1 gene (qChIP experiment). Moreover, we showed that galanin enhances neuronal maturation, promoting the acquisition of neurotransmitter phenotypes and extension of neurites. Therefore, galanin seems to act as an accelerator of the neuronal differentiation process. Using immunocytochemistry to detect NeuN+ neurons and functional evaluation of neuronal capacity to increase calcium after depolarization,

we showed that neuronal differentiation was achieved through the activation of GALR1 and/or GALR2 receptors. It seems that the signaling induced by each of the two GALRs is necessary and sufficient for inducing the proneuronal differentiation effect of galanin since cotreatment of the SVZ cells with both GALR agonists had no additive effects. This suggests that a common signaling pathway is activated by the receptors. GALR1 and GALR2 couple to G_i and GALR2 also signals through G_0 and $G_{q/11}$. GALR1 and GALR2 both activate the MAPK pathway via G_i and G_0 , respectively [9,50], and MAPK pathways are known to mediate neuronal differentiation [51]. Indeed, the MAPK proteins MEK1/2 and ERK1/2 mediate sensory neural development and neurite outgrowth induced by retinoic acid [52]. Moreover, galanin induces neurite outgrowth in hippocampal neural progenitor-derived neurospheres via GALR2/ G_0 and ERK activation [53], and increases neuronal differentiation in rat hippocampal precursor cell cultures [24].

In this study, galanin triggered an increase in the mRNA levels of the proneuronal gene *Ngn1*, while not affecting *Mash-1* and *Dlx2* mRNA levels. Moreover, galanin treatment increased chromatin modification of *Ngn1* promoter region consistent with active transcription of the gene. These results suggest that proneurogenic effects of galanin are mediated via *Ngn1* expression. Expression of the basic helix-loop-helix transcription factor *Ngn1* correlates with neuronal commitment and differentiation. Several studies have demonstrated that the induced expression of *Ngn1* is sufficient *per se* to commit P19 cell-line [54], embryonic stem cells [55], mesenchymal stem cells [56] into neurons, even in the absence of other neurogenic stimuli. Also, transgenic mice that lack *Ngn1/2* genes exhibit a loss of neuronal progenitor cell population [57]. Importantly, Wang and colleagues have reported that a short-interfering RNA targeting *Ngn1* abolished human recombinant erythropoietin-induced neuronal differentiation [58]. Recent studies also showed that *Ngn1* mediates the proneurogenic effects of histamine on NSCs from the SVZ and the embryonic cortex [36,59].

Numerous pathways are activated after binding of galanin to the GALR1 and GALR2 in particular the MAPK pathways. It has been demonstrated that MAPK activation can alter gene expression by directly binding to promoter regions of target genes or by inducing the recruitment of histone modification and chromatin regulation complexes (for review see [60]). For instance, novel object recognition is favored in young female mice due to the activity of the hormone 17β -estradiol in the dorsal hippocampus. Estrogen activates ERK1/2 which leads to histone 3 acetylation and DNA methylation and mediates memory consolidation [61]. Also, GALR activation regulates the cAMP Responsive Element Binding Protein (CREB) pathway: in the hippocampus, galanin impairs LTP, and this is correlated with a decrease in CREB phosphorylation [62]. The CREB pathway is involved in the regulation of proneuronal gene, such as the basic helix/loop/helix *NeuroD* [63]. It could be speculated that galanin induction of *Ngn1* expression in our cultures may be modulated by these pathways. Further studies are needed to confirm this issue.

We also showed that galanin does not specifically direct differentiation towards a specific neuronal phenotype as both populations of GABAergic and dopaminergic neurons are increased upon galanin treatment. In addition, we showed that galanin promotes neuronal maturation as it

increases axonal elongation and dendrite branching in SVZ-derived neurons. Positive effects of galanin on neurite outgrowth and branching have already been established in cultured sensory neurons of the dorsal root ganglia [64–67]. Moreover, galanin also induces neurite outgrowth in cultures of neurospheres from the hippocampus [53]. In vivo, galanin induces axonal sprouting of cranial motor neurons [67]. These effects have been attributed to activation of the GALR2 and subsequent PKC activation [64,65].

No effect on proliferation of SVZ cells was shown upon incubation with galanin, GALR1 or GALR2 agonists. Previous studies testing the effects of galanin and GALR agonists on stem/progenitor cell cultures have reported contradictory results. Indeed, the GALR2 agonist AR-M1896 has been reported to induce proliferation in rat hippocampal precursor cells and Prox1+ granule cell neurons [24], while galanin reduced proliferation in stem cells from E14 embryonic rat brain [68]. The fact that galanin did not affect SVZ cell proliferation in our study, while it promoted and inhibited proliferation in hippocampal precursor cells and in E14 brain stem cells, respectively, further demonstrates the specificity of different types of tissue-derived stem cells in terms of regulation and intrinsic properties. Interestingly, in other cell types, there is relative consensus that galanin inhibits proliferation by acting either via GALR1 or GALR2. Galanin reduced cell proliferation of glia olfactory ensheathing cells [46], head and neck cancer cells [69], and of GALR2 transfected pheochromocytoma (PC12) cells [70]. In oral squamous cell carcinoma, inhibition of proliferation of malignant cells is mediated by GALR1 activation [71,72]. Nevertheless, presence of galanin and GALRs is not always correlated to modulation of proliferation: in glioma, for instance, GALR1 and galanin are highly expressed, while no correlation between proliferation and GALR1 activation was demonstrated [73]. We found no effect of galanin or GALR1/GALR2 agonists on cell survival in contrast to other studies showing that galanin is a trophic factor, promoting the survival of basal forebrain cholinergic neurons and dorsal root ganglia cells [74,75]. Moreover, Abbosh and collaborators [24] showed that galanin exerts trophic effects on hippocampal precursor cells. Even further, galanin expression is increased upon injury both in the central and peripheral nervous systems, and galanin upregulation was correlated to neuroprotection [76]. Hippocampal neuronal cell death is exacerbated in both GALR1 and GALR2 knockout mice subjected to seizures, indicating that galanin is neuroprotective [21,22]. Galanin is upregulated in the brains of Alzheimer's disease patients and this increase has been suggested to promote the survival of cholinergic basal forebrain neurons, thereby supporting their cognitive functions [77]. Neuroprotective effects of galanin have been demonstrated in cultures of mouse septal/neuroblastoma (SN56) cells [78] and in hippocampal neuronal cells subjected to amyloid β toxicity [18].

Few studies have been conducted on the effect of galanin on cell migration. Galanin has been shown to stimulate migration of rat microglial cells [79] and bone marrow mesenchymal stem cells [28]. In contrast, our results clearly point to an inhibitory effect of galanin on SVZ-derived cell migration. The functional implications of this finding remains to be established; however, an inhibitory effect of cell migration could contribute to the processes of differentiation and

maturation. In conclusion, we here report that galanin is an efficacious inducer of neuronal differentiation and maturation in SVZ stem/progenitor cells. As SVZ is the major reservoir of stem cells in the adult brain, galanin deserves further attention as a potential inducer of neuronal differentiation in future stem cell based therapies.

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Author Disclosure Statement

No competing financial interests exist.

References

- Lledo PM, M Alonso and MS Grubb. (2006). Adult neurogenesis and functional plasticity in neuronal circuits. *Nat Rev Neurosci* 7:179–193.
- Ming GL and H Song. (2011). Adult neurogenesis in the mammalian brain: significant answers and significant questions. *Neuron* 70:687–702.
- Sanai N, T Nguyen, RA Ihrle, Z Mirzadeh, HH Tsai, M Wong, N Gupta, MS Berger, E Huang, et al. (2011). Corridors of migrating neurons in the human brain and their decline during infancy. *Nature* 478:382–386.
- Sanai N, AD Tramontin, A Quinones-Hinojosa, NM Barbaro, N Gupta, S Kunwar, MT Lawton, MW McDermott, AT Parsa, et al. (2004). Unique astrocyte ribbon in adult human brain contains neural stem cells but lacks chain migration. *Nature* 427:740–744.
- Hess DC and CV Borlongan. (2008). Stem cells and neurological diseases. *Cell Prolif* 41 Suppl 1:94–114.
- Kim SU and J de Vellis. (2009). Stem cell-based cell therapy in neurological diseases: a review. *J Neurosci Res* 87:2183–2200.
- Christiansen SH, MV Olesen, G Wortwein and DP Woldbye. (2011). Fluoxetine reverts chronic restraint stress-induced depression-like behaviour and increases neuropeptide Y and galanin expression in mice. *Behav Brain Res* 216:585–591.
- Fang PH, M Yu, YP Ma, J Li, YM Sui and MY Shi. (2011). Central nervous system regulation of food intake and energy expenditure: role of galanin-mediated feeding behavior. *Neurosci Bull* 27:407–412.
- Hobson SA, A Bacon, CR Elliott-Hunt, FE Holmes, NC Kerr, R Pope, P Vanderplank and D Wynick. (2008). Galanin acts as a trophic factor to the central and peripheral nervous systems. *Cell Mol Life Sci* 65:1806–1812.
- Hokfelt T and K Tatemoto. (2008). Galanin—25 years with a multitalented neuropeptide. *Cell Mol Life Sci* 65:1793–1795.
- Kuteeva E, T Hokfelt, T Wardi and SO Ogren. (2008). Galanin, galanin receptor subtypes and depression-like behaviour. *Cell Mol Life Sci* 65:1854–1863.
- Ogren SO, E Kuteeva, E Elvander-Tottie and T Hokfelt. (2010). Neuropeptides in learning and memory processes with focus on galanin. *Eur J Pharmacol* 626:9–17.
- Xu XJ, T Hokfelt and Z Wiesenfeld-Hallin. (2008). Galanin and spinal pain mechanisms: where do we stand in 2008? *Cell Mol Life Sci* 65:1813–1819.
- Wynick D, SW Thompson and SB McMahon. (2001). The role of galanin as a multi-functional neuropeptide in the nervous system. *Curr Opin Pharmacol* 1:73–77.
- Branchek TA, KE Smith, C Gerald and MW Walker. (2000). Galanin receptor subtypes. *Trends Pharmacol Sci* 21: 109–117.
- Mitsukawa K, X Lu and T Bartfai. (2008). Galanin, galanin receptors and drug targets. *Cell Mol Life Sci* 65:1796–1805.
- Moreno E, SH Vaz, NS Cai, C Ferrada, C Quiroz, SK Barodia, N Kabbani, EI Canela, PJ McCormick, et al. (2011). Dopamine-galanin receptor heteromers modulate cholinergic neurotransmission in the rat ventral hippocampus. *J Neurosci* 31:7412–7423.
- Elliott-Hunt CR, FE Holmes, DM Hartley, S Perez, EJ Mufson and D Wynick. (2011). Endogenous galanin protects mouse hippocampal neurons against amyloid toxicity in vitro via activation of galanin receptor-2. *J Alzheimers Dis* 25:455–462.
- Elliott-Hunt CR, B Marsh, A Bacon, R Pope, P Vanderplank and D Wynick. (2004). Galanin acts as a neuroprotective factor to the hippocampus. *Proc Natl Acad Sci U S A* 101: 5105–5110.
- Elliott-Hunt CR, RJ Pope, P Vanderplank and D Wynick. (2007). Activation of the galanin receptor 2 (GalR2) protects the hippocampus from neuronal damage. *J Neurochem* 100:780–789.
- Mazarati A, X Lu, K Kilk, U Langel, C Wasterlain and T Bartfai. (2004). Galanin type 2 receptors regulate neuronal survival, susceptibility to seizures and seizure-induced neurogenesis in the dentate gyrus. *Eur J Neurosci* 19:3235–3244.
- Schauwecker PE. (2010). Galanin receptor 1 deletion exacerbates hippocampal neuronal loss after systemic kainate administration in mice. *PLoS One* 5:e15657.
- Mazarati A, X Lu, S Shinmei, H Badie-Mahdavi and T Bartfai. (2004). Patterns of seizures, hippocampal injury and neurogenesis in three models of status epilepticus in galanin receptor type 1 (GalR1) knockout mice. *Neuroscience* 128: 431–441.
- Abbosch C, A Lawkowski, M Zaben and W Gray. (2011). GalR2/3 mediates proliferative and trophic effects of galanin on postnatal hippocampal precursors. *J Neurochem* 117: 425–436.
- Anisimov SV, KV Tarasov, D Tweedie, MD Stern, AM Wobus and KR Boheler. (2002). SAGE identification of gene transcripts with profiles unique to pluripotent mouse R1 embryonic stem cells. *Genomics* 79:169–176.
- Assou S, T Le Carrouer, S Tondeur, S Strom, A Gabelle, S Marty, L Nadal, V Pantesco, T Reme, et al. (2007). A meta-analysis of human embryonic stem cells transcriptome integrated into a web-based expression atlas. *Stem Cells* 25: 961–973.
- Tarasov KV, YS Tarasova, DG Crider, SV Anisimov, AM Wobus and KR Boheler. (2002). Galanin and galanin receptors in embryonic stem cells: accidental or essential? *Neuropeptides* 36:239–245.
- Louridas M, S Letourneau, ME Lautatzis and M Vrontakis. (2009). Galanin is highly expressed in bone marrow mesenchymal stem cells and facilitates migration of cells both

- in vitro and in vivo. *Biochem Biophys Res Commun* 390: 867–871.
29. Agasse F, L Bernardino, H Kristiansen, SH Christiansen, R Ferreira, B Silva, S Grade, DP Woldbye and JO Malva. (2008). Neuropeptide Y promotes neurogenesis in murine subventricular zone. *Stem Cells* 26:1636–1645.
 30. Oliva AA, Jr., CM Atkins, L Copenagle and GA Banker. (2006). Activated c-Jun N-terminal kinase is required for axon formation. *J Neurosci* 26:9462–9470.
 31. Christiansen SH and DP Woldbye. (2010). Regulation of the galanin system by repeated electroconvulsive seizures in mice. *J Neurosci Res* 88:3635–3643.
 32. Christensen DZ, MV Olesen, H Kristiansen, JD Mikkelsen and DP Woldbye. (2006). Unaltered neuropeptide Y (NPY)-stimulated [35S]GTPgammaS binding suggests a net increase in NPY signalling after repeated electroconvulsive seizures in mice. *J Neurosci Res* 84:1282–1291.
 33. Silva AP, J Lourenco, S Xapelli, R Ferreira, H Kristiansen, DP Woldbye, CR Oliveira and JO Malva. (2007). Protein kinase C activity blocks neuropeptide Y-mediated inhibition of glutamate release and contributes to excitability of the hippocampus in status epilepticus. *FASEB J* 21:671–681.
 34. Thiriet N, F Agasse, C Nicoleau, C Guegan, F Vallette, JL Cadet, M Jaber, JO Malva and V Coronas. (2011). NPY promotes chemokinesis and neurogenesis in the rat subventricular zone. *J Neurochem* 116:1018–1027.
 35. Agasse F, L Bernardino, B Silva, R Ferreira, S Grade and JO Malva. (2008). Response to histamine allows the functional identification of neuronal progenitors, neurons, astrocytes, and immature cells in subventricular zone cell cultures. *Rejuvenation Res* 11:187–200.
 36. Bernardino L, MF Eiriz, T Santos, S Xapelli, S Grade, AI Rosa, L Cortes, R Ferreira, J Braganca, et al. (2012). Histamine stimulates neurogenesis in the rodent subventricular zone. *Stem Cells* 30:773–784.
 37. Doetsch F, JM Garcia-Verdugo and A Alvarez-Buylla. (1997). Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. *J Neurosci* 17:5046–5061.
 38. Nicoleau C, O Benzakour, F Agasse, N Thiriet, J Petit, L Prestoz, M Roger, M Jaber and V Coronas. (2009). Endogenous hepatocyte growth factor is a niche signal for subventricular zone neural stem cell amplification and self-renewal. *Stem Cells* 27:408–419.
 39. Grade S, F Agasse, L Bernardino, CG Silva, L Cortes and JO Malva. (2010). Functional identification of neural stem cell-derived oligodendrocytes by means of calcium transients elicited by thrombin. *Rejuvenation Res* 13:27–37.
 40. Liu HX, P Brumovsky, R Schmidt, W Brown, K Payza, L Hodzic, C Pou, C Godbout and T Hökfelt. (2001). Receptor subtype-specific pronociceptive and analgesic actions of galanin in the spinal cord: selective actions via GalR1 and GalR2 receptors. *Proc Natl Acad Sci U S A* 98:9960–9964.
 41. Betarbet R, T Zigova, RA Bakay and MB Luskin. (1996). Dopaminergic and GABAergic interneurons of the olfactory bulb are derived from the neonatal subventricular zone. *Int J Dev Neurosci* 14:921–930.
 42. Kubrusly RC, R Panizzutti, PF Gardino, B Stutz, RA Reis, AL Ventura, MC de Mello and FG de Mello. (2008). Expression of functional dopaminergic phenotype in purified cultured Muller cells from vertebrate retina. *Neurochem Int* 53:63–70.
 43. Rosa AI, J Goncalves, L Cortes, L Bernardino, JO Malva and F Agasse. (2010). The angiogenic factor angiopoietin-1 is a proneurogenic peptide on subventricular zone stem/progenitor cells. *J Neurosci* 30:4573–4584.
 44. Shen PJ, JA Larm and AL Gundlach. (2003). Expression and plasticity of galanin systems in cortical neurons, oligodendrocyte progenitors and proliferative zones in normal brain and after spreading depression. *Eur J Neurosci* 18:1362–1376.
 45. Ubink R, L Calza and T Hökfelt. (2003). ‘Neuro’-peptides in glia: Focus on NPY and galanin. *Trends in Neurosciences* 26:604–609.
 46. Xia CY, CX Yuan and CG Yuan. (2005). Galanin inhibits the proliferation of glial olfactory ensheathing cells. *Neuropeptides* 39:453–459.
 47. Jungnickel SR, M Yao, PJ Shen and AL Gundlach. (2005). Induction of galanin receptor-1 (GalR1) expression in external granule cell layer of post-natal mouse cerebellum. *J Neurochem* 92:1452–1462.
 48. Kuteeva E, L Calza, K Holmberg, E Theodorsson, SO Ogren and T Hökfelt. (2004). Distribution of galanin and galanin transcript in the brain of a galanin-overexpressing transgenic mouse. *J Chem Neuroanat* 28:185–216.
 49. Zulewski H, EJ Abraham, MJ Gerlach, PB Daniel, W Moritz, B Muller, M Vallejo, MK Thomas and JF Habener. (2001). Multipotential nestin-positive stem cells isolated from adult pancreatic islets differentiate ex vivo into pancreatic endocrine, exocrine, and hepatic phenotypes. *Diabetes* 50:521–533.
 50. Wang S, T Hashemi, S Fried, AL Clemmons and BE Hawes. (1998). Differential intracellular signaling of the GalR1 and GalR2 galanin receptor subtypes. *Biochemistry* 37:6711–6717.
 51. Miller FD and AS Gauthier. (2007). Timing is everything: making neurons versus glia in the developing cortex. *Neuron* 54:357–369.
 52. Berwick DC, M Calissano, JD Corness, SJ Cook and DS Latchman. (2009). Regulation of Brn-3a N-terminal transcriptional activity by MEK1/2-ERK1/2 signalling in neural differentiation. *Brain Res* 1256:8–18.
 53. Hawes JJ, R Narasimhaiah and MR Picciotto. (2006). Galanin and galanin-like peptide modulate neurite outgrowth via protein kinase C-mediated activation of extracellular signal-related kinase. *Eur J Neurosci* 23:2937–2946.
 54. Kim S, YS Yoon, JW Kim, M Jung, SU Kim, YD Lee and H Suh-Kim. (2004). Neurogenin1 is sufficient to induce neuronal differentiation of embryonal carcinoma P19 cells in the absence of retinoic acid. *Cell Mol Neurobiol* 24:343–356.
 55. Lunn JS, C Pacut, E Stern, SA Sakowski, JM Velkey, S O’Shea and EL Feldman. (2012). Intraspinous transplantation of neurogenin-expressing stem cells generates spinal cord neural progenitors. *Neurobiol Dis* 46:59–68.
 56. Choi CI, YD Lee, H Kim, SH Kim, H Suh-Kim and SS Kim. (2012). Neural induction with neurogenin 1 enhances the therapeutic potential of mesenchymal stem cells in an ALS mouse model. *Cell Transplant* [Epub ahead of print] DOI: 10.3727/096368912x637019.
 57. Bertrand N, DS Castro and F Guillemot. (2002). Proneural genes and the specification of neural cell types. *Nat Rev Neurosci* 3:517–530.
 58. Wang L, ZG Zhang, RL Zhang, ZX Jiao, Y Wang, DS Pourabdollah-Nejad, Y LeTourneau, SR Gregg and M Chopp. (2006). Neurogenin 1 mediates erythropoietin enhanced differentiation of adult neural progenitor cells. *J Cereb Blood Flow Metab* 26:556–564.
 59. Rodriguez-Martinez G, I Velasco, G Garcia-Lopez, KH Solis, H Flores-Herrera, NF Diaz and A Molina-Hernandez. (2012).

- Histamine is required during neural stem cell proliferation to increase neuron differentiation. *Neuroscience* 216:10–17.
60. Suganuma T and JL Workman. (2012). MAP kinases and histone modification. *J Mol Cell Biol* 4:348–350.
 61. Zhao Z, L Fan and KM Frick. (2010). Epigenetic alterations regulate estradiol-induced enhancement of memory consolidation. *Proc Natl Acad Sci U S A* 107:5605–5610.
 62. Kinney JW, M Sanchez-Alavez, AM Barr, JR Criado, JN Crawley, MM Behrens, SJ Henriksen and T Bartfai. (2009). Impairment of memory consolidation by galanin correlates with in vivo inhibition of both LTP and CREB phosphorylation. *Neurobiol Learn Mem* 92:429–438.
 63. Ramirez M, J Hernandez-Montoya, SL Sanchez-Serrano, B Ordaz, S Ferraro, H Quintero, F Pena-Ortega and M Lamas. (2012). GABA-mediated induction of early neuronal markers expression in postnatal rat progenitor cells in culture. *Neuroscience* 224:210–222.
 64. Hobson SA, FE Holmes, NC Kerr, RJ Pope and D Wynick. (2006). Mice deficient for galanin receptor 2 have decreased neurite outgrowth from adult sensory neurons and impaired pain-like behaviour. *J Neurochem* 99:1000–1010.
 65. Mahoney SA, R Hosking, S Farrant, FE Holmes, AS Jacoby, J Shine, TP Iismaa, MK Scott, R Schmidt and D Wynick. (2003). The second galanin receptor GalR2 plays a key role in neurite outgrowth from adult sensory neurons. *J Neurosci* 23:416–421.
 66. Sachs HH, D Wynick and RE Zigmond. (2007). Galanin plays a role in the conditioning lesion effect in sensory neurons. *Neuroreport* 18:1729–1733.
 67. Suarez V, O Guntinas-Lichius, M Streppel, S Ingorokva, M Grosheva, WF Neiss, DN Angelov and L Klimaschewski. (2006). The axotomy-induced neuropeptides galanin and pituitary adenylate cyclase-activating peptide promote axonal sprouting of primary afferent and cranial motor neurons. *Eur J Neurosci* 24:1555–1564.
 68. Shen PJ, CG Yuan, J Ma, S Cheng, M Yao, AM Turnley and AL Gundlach. (2005). Galanin in neuro(glio)genesis: expression of galanin and receptors by progenitor cells in vivo and in vitro and effects of galanin on neurosphere proliferation. *Neuropeptides* 39:201–205.
 69. Kanazawa T, PK Kommareddi, T Iwashita, B Kumar, K Misawa, Y Misawa, I Jang, TS Nair, Y Iino and TE Carey. (2009). Galanin receptor subtype 2 suppresses cell proliferation and induces apoptosis in p53 mutant head and neck cancer cells. *Clin Cancer Res* 15:2222–2230.
 70. Tofighi R, B Joseph, S Xia, ZQ Xu, B Hamberger, T Hokfelt and S Ceccatelli. (2008). Galanin decreases proliferation of PC12 cells and induces apoptosis via its subtype 2 receptor (GalR2). *Proc Natl Acad Sci U S A* 105:2717–2722.
 71. Henson BS, RR Neubig, I Jang, T Ogawa, Z Zhang, TE Carey and NJ D'Silva. (2005). Galanin receptor 1 has anti-proliferative effects in oral squamous cell carcinoma. *J Biol Chem* 280:22564–22571.
 72. Kanazawa T, T Iwashita, P Kommareddi, T Nair, K Misawa, Y Misawa, Y Ueda, T Tono and TE Carey. (2007). Galanin and galanin receptor type 1 suppress proliferation in squamous carcinoma cells: activation of the extracellular signal regulated kinase pathway and induction of cyclin-dependent kinase inhibitors. *Oncogene* 26:5762–5771.
 73. Berger A, R Santic, D Almer, C Hauser-Kronberger, M Huemer, C Humpel, G Stockhammer, W Sperl and B Kofler. (2003). Galanin and galanin receptors in human gliomas. *Acta Neuropathol* 105:555–560.
 74. Holmes FE, S Mahoney, VR King, A Bacon, NC Kerr, V Pachnis, R Curtis, JV Priestley and D Wynick. (2000). Targeted disruption of the galanin gene reduces the number of sensory neurons and their regenerative capacity. *Proc Natl Acad Sci U S A* 97:11563–11568.
 75. O'Meara G, U Coumis, SY Ma, J Kehr, S Mahoney, A Bacon, SJ Allen, F Holmes, U Kahl, et al. (2000). Galanin regulates the postnatal survival of a subset of basal forebrain cholinergic neurons. *Proc Natl Acad Sci U S A* 97:11569–11574.
 76. Lundstrom L, A Elmquist, T Bartfai and U Langel. (2005). Galanin and its receptors in neurological disorders. *Neuro-molecular Med* 7:157–180.
 77. Counts SE, SE Perez and EJ Mufson. (2008). Galanin in Alzheimer's disease: neuroinhibitory or neuroprotective? *Cell Mol Life Sci* 65:1842–1853.
 78. Pironi S, A Giuliani, G Del Vecchio, L Giardino, T Hokfelt and L Calza. (2010). The galanin receptor 2/3 agonist Gal2-11 protects the SN56 cells against beta-amyloid 25–35 toxicity. *J Neurosci Res* 88:1064–1073.
 79. Ifuku M, Y Okuno, Y Yamakawa, K Izumi, S Seifert, H Kettenmann and M Noda. (2011). Functional importance of inositol-1,4,5-triphosphate-induced intracellular Ca²⁺ mobilization in galanin-induced microglial migration. *J Neurochem* 117:61–70.

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