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α_1-ADRENERGIC RECEPTORS REGULATE NEUROGENESIS AND GLIOGENESIS

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Abbreviations: AR, adrenergic receptor; bHLH, basic helix-loop-helix; BrdU, 5-bromo-2'-deoxyuridine; BSA, bovine serum albumin; CAM, constitutively active mutant; CNS, central nervous system; DAPI, 4', 6'-diamidino-2-phenylindole; EGF, epidermal growth factor; EGFP, enhanced green fluorescence protein; FBS, fetal bovine serum; FGF, fibroblast growth factor; LV, lateral ventricle; NSC, neural stem cells; PBS, phosphate-buffered saline; PFA, paraformaldehyde; Phe, phenylephrine; RMS, rostral migratory stream; RT, room temperature; SGZ, subgranular zone; SVZ, subventricular zone; TAP, transient amplifying progenitor; TUNEL, terminal deoxynucleotidyl transferase; VEGF, vascular endothelial growth factor.

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ABSTRACT

The understanding of the function of α_1-adrenergic receptors in the brain has been limited due to a lack of specific ligands and antibodies. We circumvented this problem by using transgenic mice engineered to over-express either wild-type receptor tagged with enhanced green fluorescent protein or constitutively active mutant α_1-adrenergic receptor subtypes in tissues in which they are normally expressed. We identified intriguing α_{1A}-adrenergic receptor subtype expressing cells with a migratory morphology in the adult subventricular zone that co-expressed markers of neural stem cell and/or progenitors. Incorporation of 5-bromo-2-deoxyuridine *in vivo* increased in neurogenic areas in adult α_{1A}-adrenergic receptor transgenic mice or normal mice given the α_{1A}-adrenergic receptor selective agonist, cirazoline. Neonatal neurospheres isolated from normal mice expressed a mixture of α_1-adrenergic receptor subtypes, and stimulation of these receptors resulted in increased expression of the α_{1B}-adrenergic receptor subtype, proneural basic helix-loop-helix transcription factors, and the differentiation and migration of neuronal progenitors for catecholaminergic neurons and interneurons. α_1-adrenergic receptor stimulation increased the apoptosis of astrocytes and regulated survival of neonatal neurons through PI3K signaling. However, in adult normal neurospheres, α_1-adrenergic receptor stimulation increased the expression of glial markers at the expense of neuronal differentiation. *In vivo,* S100 positive glial and βIII tubulin neuronal progenitors co-localized with either α_1-adrenergic receptor subtype in the olfactory bulb. Our results indicate that α_1-adrenergic receptors can regulate both neurogenesis and gliogenesis that may be developmentally dependent. Our findings could lead to new therapies to treat neurodegenerative diseases.

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INTRODUCTION

It is now recognized that neurogenesis continues in the mammalian brain after birth. The areas of the most active neurogenesis are the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the hippocampus (Lie et al., 2004). The SVZ contains neural stem cells (NSCs) whose progenitors migrate through defined pathways, such as the rostral migratory stream (RMS) that directs neuroblasts to the olfactory bulb where they mature into interneurons. In the hippocampus, new neurons are born in the SGZ and become functioning granule cells (Santarelli et al., 2003). The prevailing view is that NSCs are glial fibrillary acidic protein (GFAP)-positive cells of a radial glial lineage (Morshead and van der Kooy D, 2004). NSCs are self-renewing and multipotent cells that generate neurons, astrocytes and oligodendrocytes (Lie et al., 2004). Under normal conditions, neurogenesis in other central nervous system (CNS) regions is minimal, suggesting that specific mechanisms regulate where new neurons are produced.

The α_1-adrenergic receptor (AR) subtypes (α_{1A}, α_{1B}, α_{1D})[1] are G-protein coupled receptors that regulate the sympathetic nervous system by binding and transducing the signals of norepinephrine and epinephrine. Within the peripheral nervous system, α_1-AR activation is known to regulate the cardiovascular and other organ systems. Within the CNS, it has proven more difficult to ascribe α_1-AR functions, let alone the subtype to a particular function, due to poorly selective ligands and weak antibodies (Jensen et al., 2009). However, evidence links central α_1-ARs to regulation of plasticity (Sirvio and MacDonald, 1999) and stimulation of GABAergic interneurons (Papay et al., 2006). Studies have also indirectly suggested a potential role of α_1-ARs in neurogenesis. α_1-AR activation increases vascular endothelial growth factor (VEGF) mRNA (Gonzalez-Cabrera et al., 2003), and VEGF has been suggested to increase proliferation of neuronal precursors (Jin et al., 2002). VEGF localizes to the choroid plexus (Maharaj et al., 2006), which

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receives strong adrenergic innervation to regulate its secretory functions (Lindvall and Owman, 1981). The α_1-ARs stimulate the shedding of epidermal growth factor (EGF) and fibroblast growth factor (FGF) (Chen et al., 2006), factors needed to maintain NSC niches. [^{3}H]-Prazosin binding sites are found in SVZ neuroepithelia in rat E13 embryos (Pabbathi et al., 1997) and in adult mice engineered to over-express α_{1A}-ARs tagged with enhanced green fluorescent protein (EGFP) to localize the receptor (Papay et al., 2006). Using α_1-AR promoters expressing EGFP tags with or without the receptor (Papay et al., 2004; Papay et al., 2006), we have identified a cell type in the SVZ *in vivo* that co-expressed markers of NSCs and/or their progenitors that can be labeled by 5-bromo-2-deoxyuridine **(**BrdU). Subsequent studies on isolated neonatal neurospheres derived from normal mice and mice engineered to over-express constitutively activate mutant (CAM) receptors or with their α_1-ARs knocked-out (KO) revealed that α_1-ARs play an important role in the regulation of NSC/progenitors and their differentiation into neurons. In contrast, α_1-ARs expressed on adult neurospheres and isolated from normal mice regulated gliogenesis. However, α_1-ARs co-localized with both glial and neuronal progenitors in the adult mouse olfactory bulb.

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MATERIALS AND METHODS

Animal use. Mice were housed and provided veterinary care in an AAALAC-accredited animal care facility. The experimental protocols employed in this study conform to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health and was approved by the Animal Care and Use Committee at our institutions.

Immunohistochemistry. For *in vivo* analysis, mice were cardiac perfused and brain sections made using a vibrotome as previously detailed (Papay et al., 2004; Papay et al., 2006). Primary antibodies used were: rabbit notch1 at 1:50 (Santa Cruz), mouse nestin at 1:100 (Chemicon), rabbit Dlx2 at 1:200 (Chemicon), rabbit vimentin at 1:5000 (Abcam). For *in vitro* analysis, cells were fixed in 4% PFA for 30 min. Cover slips were blocked in 6% BSA and 0.3% Trition-X100 in PBS for 1hr at room temperature (RT), then incubated with the primary antibody chicken microtubule-associated protein-2 (MAP2) at 1:5000 (Novus Biolog); rabbit NG2 at 1:7000 (a gift from Bill Stallcup), or mouse GFAP at 1:3000 (Chemicon) in blocking buffer for 24 h at 4°C. The cover slips were washed 3 times in PBS, then incubated with the corresponding secondary antibodies (donkey anti-chicken cy5 at 1:500 (Jackson immunoresearch), goat anti-rabbit 488 at 1:4000 (Molecular probes), goat anti-mouse 568 at 1:4000 (Molecular probes) for 1 h at RT. Cover slips were washed 3 times in PBS, transferred to a microscope slide with Vectashield containing 4', 6'-diamidino-2-phenylindole (DAPI) (Vector labs). Sections were analyzed using a confocal laser-scanning microscope (Model Aristoplan; Leica, Inc., Deerfield, IL). Confocal images represent optical sections of 2-3 μm axial resolution and an average of 3-6 line scans. Fluorescence in red, green, blue and purple channels were collected simultaneously. Confocal images were reassembled and the

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images presented as a projection. Co-localization was confirmed in single confocal sections in which EGFP fluorescence is present in the same cell with each cell-type marker.

Neonatal Neurosphere isolation and culture. Neurospheres were isolated from the periventricular regions of postnatal day 3 pups of neonatal normal, CAM α_{1A}, CAM α_{1B}, and their corresponding KO mice. Periventricular regions were digested for 20 min at 37°C in 3 ml of 0.05% trypsin, 0.53mM EDTA, 0.001% DNAase in D-PBS (10 mM HEPES, pH 7.6 and 0.5g glucose/500 ml). Six ml of B27 complete media (DMEM/F12, 1x B27 (Gibco), 20 ng/ml rhEGF (Stem Cell Technologies), 10 ng/ml rhFGF (Abcam), 0.0002% heparin (Sigma), and 100u/ml Pen/Strep) was added and the sample centrifuged at 100 g for 7min. The supernatant was removed and the pellet titurated in 5 ml D-PBS with a 5ml pipet for 5min. Cells were passed through a large 70 µm cell strainer into a 50 ml conical tube and centrifuged at 100 g. Cells were resuspended in D-PBS, centrifuged and the pellet resuspended in D-PBS. Cells were passed through a 30 µm cell strainer (CellTrics), centrifuged, and resuspended into 10 ml of B-27 complete media and counted. Cells were split and fed every 2-3 d by centrifugation and re-plating. After 1-2 wk, we picked 20 neurospheres from the culture of dissociated tissue. Of these isolated neurospheres, about 60% maintained good neurosphere growth when dissociated. We finally isolated 6 neurospheres for cell lines, which were all positive for nestin/notch and α_1-AR expression, except for the KO cell lines. After reaching sufficient density, neurospheres were passaged by mechanical dissociation or with accutase (Sigma). Neurospheres used for experimental purposes were all taken from third or later passages.

Radioligand binding. Membranes were prepared from neurospheres as previously described (Zuscik et al., 1998). Saturation or competition binding was performed using the α_1-AR antagonist [^{125}I]-HEAT or the β-AR antagonist [^{125}I]-CYP as the radioligand as previously described (Zuscik

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et al., 1998). Data were analyzed using Prism software (GraphPad, San Diego, CA). Various AR antagonists used in radioligand binding were purchased from Sigma-Aldrich.

Neurosphere assay. Neurospheres were dissociated with accutase and mechanical tituration, and diluted to single cell level for re-plating in individual uncoated 24 well plates. The percentage of single cells (% cloning efficiency) that regenerate neurospheres was determined.

Differentiation assay. Neurospheres were centrifuged at 100 g for 7 min to remove growth factors and resuspended in B-27 Media without EGF/FGF but supplemented with 2% FBS. Cells were then transferred to a 24-well plate containing a sterile precoated poly-D-lysine/laminin coverslip (Biocoat, BD Biosciences). For phenylephrine (Phe)-induced differentiation, 10 μM Phe was added daily to B27 complete media in the presence of 1 μM propranolol and 0.1μM rauwolscine to block β-and α_2-ARs respectively. Cover slips were fixed with 4% PFA in PBS for 30 min at RT, then washed twice with PBS for 5 min and used for immunohistochemistry or stored in PBS at 4°C. At least 3 different cover slips (each containing 40-300 cells) were analyzed from three separate experiments.

In vivo BrdU incorporation. Mice were injected i.p. with BrdU at 150 mg/kg body weight. Two hours after the injection, the mice were anesthetized, cardiac perfused, and brains sectioned as previously described (Papay et al., 2006). In chase experiments, normal mice or mice that received bottle water containing cirazoline at 10mg/L for 12 wk were injected twice daily i.p. with BrdU at 50 mg/kg body weight for 2 wk, then sacrificed at 2 d, 7 d, and 14 d after the last BrdU injection. Sections were first rinsed in 0.9% NaCl, then incubated in 1N HCl in 0.9% NaCl at 37°C for 30 min. Sections were rinsed with 0.1 M borate buffer, pH 9.0, then rinsed with TBS. Sections were then incubated with mouse anti-BrdU (Chemicon MAB3510) at 1:5000 in blocking buffer for 2 d at 4°C, then incubated with Alexa Fluor 568 goat anti-mouse IgG (Molecular Probes A11004) at

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1:4000 in blocking buffer for 1 h at RT. Specimens were rinsed twice with distilled water prior to incubation with 2 ml of 10 mM copper sulfate in 50 mM ammonium acetate for 1 h at RT to remove autofluorescence. Samples were then rinsed twice with distilled water before being returned to PBS, transferred to a microscope slide containing Vectashield with DAPI, and BrdU nuclei counted using confocal microscopy followed by stereology.

Stereology. The optical dissector technique was used to estimate the density of BrdU$^+$ and/or Nestin$^+$ cells in the SGZ of the dentate gyrus of the hippocampus and the SVZ of the lateral ventricles. Random slices were selected and the number of BrdU$^+$ cells and volume of each structure was computed with Stereo Investigator software (MBF Bioscience, MicroBrightField Inc., Williston, VT). Cell density was expressed in cells per mm^3 and the number of BrdU- and Nestin-labeled cells per section was then averaged.

Real time PCR. Neurospheres were treated with Phe (10 μM) or 1% FBS for 1, 3, or 7 days in the presence of rauwolscine (0.1 μM) and propranolol (1 μM). Total RNA was isolated using the RNAesy kit (Quiagen) and 2 μg RNA was reverse transcribed using oligodT primer with Superscript II Reverse Transcriptase (Invitrogen). Quantitative real time PCR was performed with an iCycler (Biorad) using iQ SYBR Green Supermix (Bio-Rad). The cDNA was amplified with primers for various neural transcriptional factors. Primers used included: Dlx2: 5'-aagggtgtctgtgcagatttc-3' and 5'-cgtcgcagctttcacaact-3'; Mash-1: 5'-catctcccccaactactcca-3' and 5'-ccagcagctcttgttcctct-3'; Math-1: 5'-acatctcccagatcccacag-3' and 5'-gggcatttggttgtctcagt-3'; Ngn-1: 5'-gagccggctgacaatacaat-3' and 5'-ctcaggttcttcctggagca-3'; Nestin: 5'-gaggggacctggaacatgaa-3' and 5'-gtccattctccatcctccca-3'; NeuroD: 5'-gtgatgctggtactactggaattg-3' and 5'-gcaactgcatgggagttttc-3'; α_{1A}-AR: 5'-ggttcccaaaggaaacctgt-3' and 5'-ggtttcataccagggtggtg-3'; α_{1B}-AR: 5'-tctcagccaagtcctggttt-3' and 5'-gcgaacacctttacctgctc-3'; α-tubulin: 5'-ggctgccctagagaaggatt-3' and

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5'-aaacatccctgtggaagcag=3'. Samples were analyzed for relative gene expression using the $2^{-\delta\delta CT}$ method (Livak and Schmittgen, 2001), and normalized to α-tubulin gene expression as an internal control. Data were obtained from three independent experiments, performed in duplicate.

Pathway analysis. Normal neonatal neurospheres were pretreated 1 h with individual inhibitors (either 20 μM PD98059 (Calbiochem), 10 μM SB203580 (Calbiochem), 10 μM SP600125 (Calbiochem), 20 μM LY294002 (Cell Signaling Technology), or 0.5 μM Go6983 (Calbiochem), then stimulated with Phe (10 μM) for 3 d. Neurospheres were then lysed and subjected to SDS PAGE, transferred to PVDF membrane and probed with antibodies against mouse GFAP at 1:1000 or chicken MAP-2 at 1:3000. Membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies. Chemiluminescence detection was performed by incubating membranes with SuperSignal West Pico Chemiluminescent Substrate (Pierce) followed by exposure onto X-ray film.

Neuronal cell types. Normal neurospheres were stimulated with 10 uM Phe for 0, 3, 7, or 14 d and lysates subjected to western blot analysis. Nonspecific binding was blocked by incubation with 5% milk and 0.1% Tween 20 in TBS (TBST) for 2 h at RT. The membranes were then incubated with primary antibody in blocking buffer against goat anti GAD 65/67 (1:100), rabbit anti-β_1-integrin (1:500, Santa Cruz), sheep anti-tyrosine hydoxylase (1:200), rabbit anti-NMDAR1 (1:100, Chemicon), sheep anti-dopamine β-hydroxylase (1:3000, Novus Biologicals) or monoclonal anti-β-actin (Sigma) overnight at 4ºC with gentle agitation. Membranes were washed 3 times with TBST and incubated with the appropriate horseradish peroxidase coupled secondary antibodies for 1 h at RT, followed by chemiluminescence detection.

Apoptosis assay. Neurospheres were grown and analyzed for apoptosis by using the FlowTACS Apoptosis Detection Kit (Trevigen, Gaithersburg, MD). Terminal deoxynucleotidyl

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tranferase (TdT) end labeling of the free 3' hydroxyl residues in the fragmented DNA was performed according to manufacturer's instructions. In brief, normal neonatal neurospheres were treated with 10 μM Phe for 0 d (control), 2 d, or 3 d, fixed in 3.7% formaldehyde, permeabilized, labeled with primary antibodies against MAP2 and GFAP, and the TdT enzyme, followed by Strep-Fluoresein treatment and appropriate secondary antibodies. Cells were also treated with propidium iodide to label necrotic cells, which were not counted. Cells were analyzed under a fluorescence microscope. Total cells counted ranged from 40-240 per cover slip using at least three different cell preparations.

Migration assay. The effect of α_1-AR stimulation on progenitor cell migration was determined by boyden chamber assay as described previously (Sun et al., 2001). In brief, neurospheres derived from normal, CAM and KO mice were dissociated in DMEM/F-12 medium without growth factors at a density of $1x10^6$ cells per transwell (Costar) using a 0.4 μM membrane. 10 μM Phe was added to the lower well and after 48 h, the media from each of the upper and lower wells was aspirated, cells scraped, and assayed for DNA content using the CyQuant Cell Proliferation assay kit (Invitrogen) and a CytoFlour II fluorescent plate reader.

Isolation of adult normal neurospheres. Adult normal neurospheres were isolated from the periventricular regions of 2-3 mo normal mice. Periventricular regions were dissected in a Petri dish containing 10ml of NeuroCult Tissue Collection Solution (05715A; StemCell Technologies, Vancouver, BC, Canada). After dissection, the collection solution was discarded and the tissue divided into two piles, minced for one minute in 1 ml of NeuroCult Dissociation Solution, and transferred to a 15 ml sterile conical tube being careful not to introduce air bubbles. The mincing was repeated twice. The pooled minced tissue was incubated for 7 min at 37ºC in a beaker of pre-warmed water. The suspension was gently mixed with 3 ml of NeuroCult Inhibition Solution

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(05715C), avoiding air bubbles. The mixture was centrifuged at 100 g for 7 min and the supernatant removed. The pellet was resuspended in 1 ml of NeuroCult Resuspension Solution (05715D) and triturated using a sterile P1000 pipette tip 15 times until a smooth and creamy suspension was achieved. NeuroCult Resuspension Solution was added to a final volume of 10 ml and the sample gently mixed. The cells were washed three times by centrifugation at 100 g for 7 min and the pellet resuspended in 1 ml of NeuroCult Resuspension Solution. The pellet was first resuspended in 1 ml of B27 Adult Proliferation Media (StemCell Technologies), followed by Resuspension Solution to a total volume of 10 ml. Cells were passed through a large 70 µm cell strainer into a 50 ml conical tube, passed through two more 70 µM cell strainers, into a sterile 15 ml polypropylene tube. A cell sample was assessed for viability with trypan blue and the remaining cells pipetted into two T-162 flasks each containing 40 ml of B27 complete media. The next day, the media was collected and the cells centrifuged and re-plated into six T-162 flasks. After 1-2 wk, several large neurospheres were isolated and expanded for experimental studies.

FACS analysis. The SVZ regions were dissected from normal and α_{1A}-promoter-EGFP mouse brains and dissociated in 0.25% trypsin solution in DMEM. The number of $EGFP^+$ cells was determined by fluorescence-activated cell sorting (FACS).

Statistical analysis. One-way ANOVA followed by the Bonferroni post-hoc test was used for multiple data sets. For comparisons between two data sets, a student's paired t-test was used. The data are presented as mean ± SEM. *P* values less than 0.05 were considered to be significant.

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RESULTS

Transgenic Mice. We previously created several mouse models that systemically express either the α_{1A}- or α_{1B}-AR subtypes using large fragments of the isogenic promoters isolated from mouse genomic DNA (**Fig.1**). Two of our models overexpress the α_1-ARs with CAMs that cause the receptor to be activated without the need for agonists and to increase subtype-specific signaling (**Fig. 1A, B**). We have previously characterized these mutations both *in vitro* and *in vivo* to verify their constitutive activity (Zuscik et al., 2000; Hwa et al., 1997). Since antibodies against α_1-ARs have poor avidity in endogenous tissues (Jensen et al., 2009), we also created α_1-AR transgenic mice with and without EGFP tags (**Fig. 1C, D**). All of these mice have been previously characterized (Papay et al., 2004; Papay et al., 2006).

To verify promoter fidelity, the mouse α_{1A}- and α_{1B}-AR promoters used in generating the transgenic mice were expressed in α_1-AR expressing and null cell lines (Zuscik et al., 1999; O'Connell et al., 2001) and demonstrated predicted fidelity and regulation by transcription factors and known stimuli. Protein comparison studies were also performed using α_{1A}-promoter EGFP, α_{1A}-AR-EGFP fusion, and α_{1A}-AR KO mice in which the α_{1A}-AR locus was knocked-in with the β-galactosidase gene. EGFP and β-galactosidase expression patterns throughout the brain appeared to be exact (Papay et al., 2006). Since the α_{1B}-AR KO mice were not made with the β-galactosidase knock-in, we performed additional studies to verify this subtype‘s promoter fidelity. We confirmed α_{1B}-AR expression in transgenic mice expressing WT and CAM receptors under the direction of the α_{1B}-AR promoter by measuring receptor levels in both positive and negative tissues, and by *in situ* mRNA hybridization studies in the mouse brain (Zuscik et al., 2000). The promoters appear to

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direct similar if not identical expression to the endogenous gene, although temporal studies were not investigated.

α_1-AR SVZ Localization In Vivo. In performing localization studies in the brain using adult EGFP-expressing mice, we noticed an intriguing cell type in the SVZ that expressed EGFP using the α_{1A}-AR promoter (**Fig. 2A**). We showed that these assumed α_{1A}-AR expressing cells are located near the ependymal layer, the SVZ, and RMS with a migratory morphology. These SVZ cells do not express markers for any glia or neuronal cell type (S100, CC1, NG2, NeuN, βIII-tubulin). To determine if these cells could be stem/progenitor cells, we performed immunohistochemistry in the EGFP-expressing mice using a series of NSC/progenitor markers. EGFP co-localized in the SVZ with cells that express nestin (**Fig. 2B, 2C**), notch-1 (**Fig. 2D, 2E**), and vimentin (**Fig. 2F**). The transcription factor Dlx2 is expressed in transient amplifying progenitors (TAP) and neuroblasts. Some of the EGFP cells in the SVZ expressed Dlx2 but others did not (**Fig. 2G**). We notice that EGFP cells in the SVZ were of a mixed population; cells near the ependymal layer rarely expressed Dlx2 but did express other NSC markers, suggesting they were pre-TAP cells (**Fig. 2G**; yellow arrows). In contrast, EGFP cells further inside the SVZ expressed Dlx2 (**Fig. 2G**, white arrows), but not other NSC markers, suggesting they were TAPs or neuroblasts. These unique EGFP cells were also located in all of the ventricles, such as the 4th ventricle (**Fig. 2H**), but did not co-localize with stem/progenitor markers. In contrast, α_{1B}-AR-EGFP cells did not localize within the SVZ (**Fig. 2I**) but were observed further downstream in the migratory path. FACS analysis of EGFP cells present in the SVZ of adult α_{1A}-promoter-EGFP mice suggests that approximately 44% of cells contain EGFP (**Fig. 2J**). However, not all of these cells may be progenitors since the α_{1A}-AR-EGFP is also expressed on mature cell types (Papay et al., 2006). These results are consistent with the α_{1A}-AR being expressed in NSCs and/or TAP cells *in vivo* and regulating the neurogenic process.

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Neurosphere Characterization. One way to functionally define NSC/progenitors is to isolate and culture cells so that they form neurospheres, which express mostly progenitor cells. Since we are not sure of all the types of progenitors that express the α_{1A}-AR, neurosphere analysis is likely addressing the bulk of cell responsiveness. We initially picked 20 neurospheres from each culture of dissociated tissue. Of these isolated neurospheres, about 60% maintained good neurosphere growth when dissociated. We finally isolated 6 neurospheres to establish cell lines, which were all positive for nestin/notch, To first determine if α_1-ARs were expressed in neonatal neurospheres, we performed direct radioligand binding experiments. Saturation binding indicated that neonatal neurospheres expressed 174 fmoles/mg protein of α_1-ARs with a K_d of 176 pM, results similar to brain tissue known to express α_1-ARs (**Fig. 3A**). EGFP-derived neonatal neurospheres also express α_1-ARs as assessed by detection of EGFP (data not shown).

To reveal the α_1-AR subtype composition, we performed competition ligand binding using 5-methylurapidil, which has 100-fold higher affinity for the α_{1A}-AR than the α_{1B}-AR subtype. We know from real-time PCR that mouse neonatal neurospheres do not express the α_{1D}-AR subtype (data not shown). We found that the composition of α_1-ARs expressed in neonatal neurospheres was 33% α_{1A}-AR and 67% α_{1B}-AR (red line, **Fig. 3B**). Upon incubation with 10 μM Phe for 24 h, which differentiates the cells, the subtype composition switched to 14% α_{1A}-AR and 86% α_{1B}-AR (blue line, **Fig. 3B**). Competition binding with the β-AR radiolabel [I^{125}]-ICYP and the β_2-AR selective antagonist ICI-118, 551 indicate that normal neonatal neurospheres also express 86% of the β_1-AR subtype (**Fig. 3C**).

Neonatal Neurosphere Assay. The ability to self-renew is a key trait of neurospheres that contain NSCs. Neurospheres were dissociated and diluted to a single cell density to determine what proportion of the single cells reformed a neurosphere (*i.e.* % of NSCs contained within the

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neurosphere) (**Fig. 3D**). CAM α_{1A}-AR derived neurospheres had a significantly lower efficiency to regenerate neurospheres than normal or α_{1A}-AR KO, consistent with the CAM α_{1A}-AR neurospheres containing more differentiated progenitors.

Serum-induced Pluripotency. If neurospheres contain NSCs, they should be pluripotent and differentiate into all three of the major cell types (neurons, oligodendrocytes, astrocytes) in the brain. To determine what type of progenitors were present in each neonatal neurosphere culture, we differentiated normal neurospheres with serum (2%FBS) and found all three cell-types present (**Fig. 3E**). Neurospheres isolated from CAM α_{1A}-AR mice (**Fig. 3F**) differentiated into all three cell-types but were mostly neurons. Neurospheres isolated from the α_{1A}-AR KO mice (**Fig. 3G**) had greater numbers of astrocytes but reduced levels of neurons. Quantization of serum-differentiated cell types for neurospheres isolated from all mouse models is shown in **Fig 3H**. CAM α_{1B}-AR neurospheres also differentiated into significantly more neurons and less astrocytes than normal but were not as robust as the CAM α_{1A}-AR, as evident in the lack of response in the neurospheres from the α_{1B}-AR KO.

Progenitor Proliferation. Effects of α_1-AR stimulation on proliferation rates were then confirmed using *in vivo* BrdU incorporation. NSCs are considered a relatively quiescent cell while TAP and progenitor cells are actively dividing (Morshead et al., 1994) which are more likely to incorporate BrdU within a 2 h period. We found that the number of cells that incorporated BrdU in the adult CAM α_{1A}-AR mice increased in the SVZ of the lateral ventricle and SGZ of the hippocampus (**Fig. 4A, B**), compared to normal, CAM α_{1B}-AR, or either KO. We confirmed that BrdU positive cells were co-expressed with cells containing the α_{1A}-AR by EGFP expression, *in vivo* (**Fig 4C**).

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α_1-AR Induced Progenitor Differentiation. To determine direct effects of α_1-AR stimulation on the ability to induce differentiation, normal neonatal neurospheres (basal, **Fig. 4D, 4I**) were incubated with Phe (10 μM). Phe induced differentiation of all three cell-types (**Fig. 4E, 4I**) but under prolonged stimulation (20 d) only neurons and NG2 oligodendrocytes survived (**Fig. 4F, 4I**). Under basal conditions, CAM α_{1A}-AR derived neurospheres were composed mostly of MAP2-positive neuroblasts with few GFAP-positive astrocytes (**Fig. 4G, 4I**). KO of the α_{1A}-AR increased GFAP expression but reduced MAP2 expression (**Fig. 4H, 4I**).

α_{1A}-AR Induced Stem Cell Proliferation. Since CAM α_{1A} mice have increased BrdU incorporation in fast dividing progenitor cells, we determined if stimulation of α_{1A}-ARs increases proliferation of NSCs in normal mice. We found increased BrdU incorporation in normal mice treated with the α_{1A}-AR selective agonist, cirazoline. Furthermore, approximately 25-50% of the $BrdU^+$ cells were $nestin^+$ in the normal and α_{1A}-AR stimulated mice. Although both BrdU and nestin staining was slightly diminished after 7 and 14 d of chase, BrdU incorporation remained significantly higher at all days of chase in the cirazoline-treated mice (**Fig. 5**).

Regulation of Proneural Transcription Factor RNA. To determine how α_1-ARs regulate neonatal neurogenesis, we performed real-time PCR from each neonatal neurosphere line and targeted key bHLH transcription factor genes involved in regulating neuronal differentiation (Ross et al., 2003). Relative fold values over one indicate an increased in mRNA expression while fold values under one indicate decreased mRNA expression. We differentiated using 1% instead of 2% serum to lengthen the time to complete differentiation. When differentiated by 1% serum as a control, normal neonatal neurospheres significantly increased the RNA of the α_{1B}-AR subtype, Dlx2, Mash1, Math1, Ngn1, and NeuroD, consistent with neuronal differentiation (**Fig. 6**, grey triangles). When stimulated by Phe, CAM α_{1A}-AR neurospheres increased its own α_{1A}-AR RNA as

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well as that of the Dlx2, Mash1, and NeuroD (**Fig. 6**, green circles), while CAM α_{1B}-AR neurospheres increased the RNA of Math1 and Ngn1 (**Fig. 6**, red circles). While all other genes decreased its mRNA levels in the Phe-stimulated α_{1A}-AR KO, nestin RNA was the only gene to increase its mRNA levels (**Fig. 6**, magenta triangles), suggesting neurosphere progenitors were more undifferentiated. The RNA of notch-1, zic-1 and sox-2, were not changed in neurospheres derived from the CAMs or KOs, suggesting these factors are not regulated by α_1-ARs.

α_1-ARs Regulate Specific Neural Cell Types. To determine which neuronal cell types are being differentiated, we found that 10 μM Phe stimulated normal neonatal neurospheres to have increased protein expression of tyrosine hydroxylase, dopamine β-hydroxylase, and GAD65/67, markers for catecholaminergic neurons and interneurons. α_{1A}-AR KO neonatal neurospheres displayed decreased levels of each of the same cell type markers (**Fig 7A**). α_1-AR stimulation also decreased the expression of β_1-integrin. However, the presence of the R1 subunit of the NMDA receptor was not expressed even though the antibody detected its presence in a whole normal brain extract.

α_1-AR Regulation of Differentiation Through PI3K. To determine which signaling pathways were regulating α_1-AR mediated neuronal differentiation, we used a battery of PKC, PI3K, and MAPK inhibitors and assessed the appearance of the post-mitotic neuronal marker MAP2 in normal neonatal neurospheres. After 2 d of stimulation, 10 μM Phe increased the expression of MAP2 (**Fig. 7B**), which decreased upon co-incubation with the PI3K inhibitor, LY294002 (20 μM). The PKC inhibitor, Go 6983, or the MEK, P38, or JNK inhibitors, PD98059, SB203580, SP600125, respectively, did not decrease Phe-induced MAP2 expression. Furthermore, the reciprocal loss of GFAP protein expression induced by Phe was blocked using LY 294002 (**Fig. 7C**).

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α1-ARs Induce Apoptosis of Astrocytes. Since MAP2 expression increases but GFAP expression decreases upon α_1-AR stimulation, we assessed the ability of α_1-ARs to induce apoptosis of astrocytes. Phe can increase the number of TUNEL-positive nuclei in normal neonatal neurospheres after 2-3 d of stimulation (**Fig. 8A**). However, the percentage of GFAP expressing astrocytes with TUNEL-positive nuclei was significantly greater than TUNEL-positive cells expressing MAP2 (**Fig. 8B**).

α1-ARs Regulate Neonatal Neuronal Progenitor Migration. Since α_1-ARs were expressed *in vivo* in cells with a migratory morphology (**Fig. 2A**), we explored whether α_1-ARs can affect migration of neural progenitors using *in vitro* transwell assays. We found that normal neurospheres stimulated with Phe, or unstimulated CAM α_{1A}-AR derived neurospheres increased migration of progenitors similar to 2% serum controls (**Fig. 8C**), while α_{1A}-AR KO derived progenitors displayed decreased migration.

α1-ARs Regulate Adult Gliogenesis. To determine if effects of α_1-AR regulation of neonatal neurogenesis extended to adult mice, we performed western analysis on adult (2-3 mo) neurospheres isolated from the periventricular region of normal mice. We confirmed that two different lines of adult normal mouse neurospheres contained the stem cell markers nestin, notch 1, and vimentin, but decreased their expression with α_1-AR stimulation, consistent with the role of α_1-ARs in regulating differentiation. Interestingly, adult neurospheres did not contain or alter the neuronal markers that were present in neonatal neurospheres (tyrosine hydroxylase, dopamine β-hydroxylase, and GAD 65/67), but expressed and increased the glial markers, CC1 and GFAP, upon α_1-AR stimulation (**Fig. 9A**). However, adult neurospheres did contain the NR1 neuronal marker, but its expression decreased with α_1-AR stimulation. We confirmed in adult EGFP mice *in vivo* that

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either α_1-AR subtype was expressed in glial progenitors (S100 positive cells) and neuronal progenitors (βIII tubulin) in the olfactory bulb (**Fig. 9B-E**).

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DISCUSSION

While α_1-ARs are abundant in the CNS, there is limited information about neural function and subtype signaling because of the lack of highly selective ligands and avid antibodies. Using unique and systemically expressing mouse models and KOs that circumvent these limitations, we report that α_1-AR subtypes are involved in neurogenesis and gliogenesis that may be developmentally dependent. Immunohistochemistry using stem cell markers (**Fig. 2**) suggest the presence of α_{1A}-ARs on stem cells and/or early progenitors.

The use of neurospheres as an *in vitro* model of NSCs has limitations. While a single NSC *in vitro* is able to generate a neurosphere, in addition to NSCs, neurospheres contain neuronal and glial progenitors in different states of differentiation. Therefore, neurospheres become very heterogeneous and exhibit intra-clonal neural cell-lineage diversity (Suslov et al., 2002).

Regulation of the ability to regenerate neonatal neurospheres from single cells *in vitro* (**Fig. 3D**) provides evidence for the presence of α_{1A}-ARs on stem cell/early progenitors in neonatal mice. However, α_1-ARs regulate differentiation of neurons in the neonate while switching to astrocyte and oligodendrocyte differentiation in adult mice. Further studies may indicate exactly the time in development the switching of cell type differentiation occurs.

In the neonate, α_1-ARs stimulated the migration and differentiation of neurosphere progenitors into catecholaminergic neurons and interneurons **(Fig. 7).** Neuronal differentiation was dependent upon a PI3K-sensitive pathway that protects neurons from apoptosis (**Fig. 7, 8**). α_1-ARs regulated both early (Dlx2, Mash1) and late stage (NeuroD) homeobox and bHLH mRNA in neonatal neurospheres, suggesting their regulation of neuronal maturation (**Fig. 6**).

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However, in adult mice, α_1-ARs appeared to regulate gliogenesis. Isolated adult neurospheres increased markers for CC1 and GFAP-positive glia upon α_1-AR stimulation while down regulating NMDA receptor levels which are located predominately on neurons (Köhr, 2006) (**Fig. 9**). *In vivo*, S100-positive glial and βIII tubulin-positive progenitors co-localized with α_1-ARs in the olfactory bulb while α_1-AR cells in the SVZ were negative for either marker. In previous reports, we found that α_1-AR subtypes were present in NG2-positive oligodendrocytes and weakly expressed in CC1-positive mature oligodendrocytes in adult mice (Papay et al., 2004; Papay et al., 2006).

α_{1A}-ARs are likely to also regulate the proliferation of adult neural stem and/or TAP cells. Upon long-term exposure to BrdU and several days of chase, α_{1A}-AR stimulation increased the number of $BrdU^+$ cells in adult SVZ, which was not diluted upon increasing chase (**Fig. 5**). Since NSCs slowly divide (Morshead et al., 1994), incorporated BrdU would not be lost with increased chase in this cell type. Upon short-term BrdU incorporation, which would label rapidly dividing progenitors (Morshead et al., 1994), α_1-AR stimulation increased BrdU incorporation in the SVZ and SGZ (**Fig. 4A, B**). The unexpected high incorporation of BrdU in the SGZ in the α_{1A}-AR KO could be due to spontaneous seizures reported in these mice (Knudson et al., 2007). Seizure activity has been reported to increase neurogenesis in the SGZ (Parent et al., 1997). These data suggests that α_{1A}-ARs are expressed in and regulate the proliferation of both NSCs and progenitors. Further studies are needed to determine if α_1-ARs also affect the maturation rate of progenitors.

In contrast, α_{1B}-ARs were expressed in the adult RMS *in vivo* but not in the SVZ (**Fig. 2I**), and regulated late neuronal maturation factors (Ngn1, Math1) in neonatal neurospheres (**Fig. 6**). Furthermore, α_{1B}-AR expression was increased in normal neonatal neurospheres undergoing differentiation by phenylephrine (**Fig. 3B**) or by 1% serum (**Fig. 6**). Since we do not see a decrease

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in α_{1A}-AR RNA upon serum-induced differentiation (**Fig. 6**), our results suggest that the α_{1B}-AR is being induced to increase expression instead of the α_{1A}-AR becoming down regulated. Overall, this data suggests that the role of the α_{1B}-AR is more important in the later maturation stages of neurogenesis.

Our work suggests that the mechanism of α_{1A}-AR induced neonatal neurogenesis is due to an initial expansion of NSC/TAP and progenitor cells (**Fig. 4A**), followed by increased transcription of proneural factors (**Fig. 6**), and ultimately to the PI3K-dependent survival and maturation of catecholaminergic neurons and GABAergic interneurons (**Fig. 7, 8**). The role of PI3K in neonatal neuronal differentiation suggests that α_1-ARs increase pro-survival signals. PI3K has been shown to increase proliferation of adult hippocampal progenitors (Peltier et al., 2007), and induce neuronal differentiation in cell lines (Sánchez et al., 2004). In addition to pro-survival signals, the PI3K/Akt pathway enhances the assembly and activity of bHLH complexes that promote neuronal differentiation (Vojtek et al., 2003). The coordinated increases in the activity of Mash1 and Ngns that are regulated by α_1-ARs (**Fig. 6**) have been shown to transition neurogenesis from proliferation to neuronal differentiation (Ross et al., 2003). Interestingly, each α_1-AR subtype regulates its own distinct set of proneural transcription factors. Mash1 regulates Dlx2's temporal expression (Poitras et al., 2007) and both genes are regulated by the α_{1A}-AR.

The α_{1A}-AR has been previously confirmed to functionally regulate interneurons (Hillman et al., 2007) and now confirmed in our work to be involved in their differentiation in the neonate. The decreased expression of β1 integrin by α_1-AR stimulation is also consistent with differentiation since β1-integrins have been shown to maintain NSC function (Campos et al., 2004).

The mechanism of α_1-AR regulation of adult gliogenesis remains to be determined. While α_1-ARs are localized to neurons, progenitors, and glia, we cannot rule out that α_1-AR regulation of

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the cerebral microvasculature system does not contribute to neurogenesis or gliogenesis. α_1-ARs regulate the secretion of growth factors and extracellular matrix from endothelium that may affect the NSC niche (Ramírez-Castillejo et al., 2006).

A neuron to glia switch occurs naturally in neuroepithelial precursor (NEP) cells. Early in development, NEPs generate all types of neurons and glia. At later stages, NEP potential becomes restricted to glia (Kessaris et al., 2008). All of the factors that drive this neuron-glial switch are not known. However, down regulation of bHLH transcription factors is an important step, driving stem cell transition from a neurogenic towards a gliogenic potential. A microarray study of gene expression changes in the brain of the CAM α_{1B}-AR mice revealed several cell fate genes being regulated that directs both glial (BMP2 receptor, insulin-like growth factor receptor, thyroid hormone and Eph receptors) and neuronal development (brain-derived neurotrophic factor, prolactin) (Yun et al., 2003). It is possible that differential regulation of bHLH transcription factors from neonatal to adult by α_1-ARs accounts for the neuron-glial transition.

Our studies highlight the ability of α_1-ARs to regulate neurogenesis and gliogenesis and offers new treatment options for cell loss due to neurodegeneration. Since proliferation in adult rodents decreases with age (Kuhn et al., 1996), any agent that can increase proliferation of NSCs and direct differentiation in the adult may be a valuable strategy.

However, chronic activation of the α_{1B}-AR would likely be detrimental as a therapy since CAM α_{1B}-AR mice have an age-progressive neurodegeneration that manifests at 7-12 mo of age (Zuscik et al., 2000), confirmed in KO mice (Pizzanelli et al., 2009), which may be due to chronic glutamate (Marek and Aghajanian, 1999) or dopamine excitation (Villégier et al., 2003). α_{1A}-AR activation co-joined with specific α_{1B}-AR antagonism may be a preferred treatment option since α_{1B}-AR blockage protects against α-synuclein aggregates and neurotoxicity (Papay et al., 2002). In

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addition to regulating neurogenesis, the α_{1A}-AR is neuroprotective against seizures and protects interneurons against age-related death (Knudson et al., 2007). Chronic α_{1A}-AR stimulation is also cardioprotective (Huang et al., 2007), which would be an additional benefit of such a class of drugs.

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Footnotes: [1]After the α_{1C}-AR was reclassified as the α_{1A}-AR, the α_{1C}-AR designation is no longer used.

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Figure Legends

FIGURE1. Constructs used for systemic over expression in transgenic mice. A, CAM α_{1A}-AR; B, CAM α_{1B}-AR; C, α_{1A}-AR promoter driving EGFP only; D, α_{1B}-AR EGFP-tagged receptor. CAM, constitutively active mutant; EGFP, enhanced green fluorescent protein.

FIGURE 2. **α_{1A}-AR promoter-EGFP cells localize in the SVZ *in vivo.*** A, α_{1A}-AR promoter-EGFP expressing cells (green) are found in the SVZ and RMS of adult mice. B, α_{1A}-AR promoter-EGFP cells are abundant in the SVZ, where Nestin (in red) is located. C, Magnification of the boxed area in B. α_{1A}-AR promoter-EGFP cells co-localize with nestin. D, α_{1A}-AR promoter-EGFP cells are abundant in the SVZ, where Notch-1 (in red) is located. Some α_{1A}-AR promoter-EGFP cells co-localize with Notch-1 (E) or vimentin (F) near the LV border. G, Some α_{1A}-AR promoter-EGFP cells express Dlx2 in the nucleus (white arrows) in the SVZ and RMS, while other α_{1A}-AR promoter-EGFP cells do not (yellow arrows). H, α_{1A}-AR promoter-EGFP cells line the 4th ventricle. I, α_{1B}-AR-EGFP tagged cells are not localized near the ependymal border. J, FACS of dissociated periventricular cells from normal mice (control) or three different α_{1A}-promoter EGFP mice (FITC 1-3). Approximately 44.4% of periventricular cells express EGFP. Mice were 2-3 mo. White bar=10 μm.

FIGURE 3. AR expression in neonatal neurospheres and pluripotency. A, [I^{125}]-HEAT saturation binding of normal neonatal neurospheres. B, Competition binding with 5-methylurapidil indicates that normal neonatal neurospheres expressed 33% of the α_{1A}-AR subtype and 67% of the α_{1B}-AR (red circles). Upon incubation with 10μM phenylephrine (Phe)

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for 24 h, the subtype composition became 14% of the α_{1A}-AR and 86% of the α_{1B}-AR subtype (blue circles). C, Competition binding with [I^{125}]-CYP and ICI-118,551, a β_2-AR selective antagonist indicates that normal neonatal neurospheres also express a majority (86%) of the low affinity β_1-AR subtype. D, Neurosphere self-renewal assay. CAM α_{1A}-AR has a lower efficiency to regenerate neurospheres than normal or KOs. E, Normal neonatal neurospheres differentiate into all three cell-types (red, GFAP; magenta, MAP2; green, NG2 upon incubation with serum (2%FBS). F, Neurospheres isolated from CAM α_{1A}-AR mice differentiated into all three cell-types but were mostly neurons. G, Neurospheres isolated from the α_{1A}–AR KO mice were mostly astrocytes with reduced number of neurons. H, Quantitation of cell-type differentiation by 2% FBS for neurospheres isolated from all mouse models. At least 3 different cover slips (each containing 40-300 cells) were analyzed from three separate experiments. White bar=10 μm. # $p<0.05$, * $p<0.01$.

FIGURE 4. α_{1A}-AR induces progenitor proliferation and differentiation. A, *In vivo* BrdU incorporation for 2 h indicates that 2-3 mo CAM α_{1A}-AR mice have increased the number of proliferating progenitor cells in the SVZ (A) and SGZ (B) compared to CAM α_{1B}-AR or either KO mice. C, α_{1A}-promoter EGFP cells in the SVZ express BrdU (arrows). Normal neonatal neurospheres and Phe-stimulation for 0 (D, control), 3 d (E), or 20 d (F) differentiates into neurons (Map2 in magenta), astrocytes (GFAP in red) and oligodendrocyte progenitors (NG2 in green). G, CAM α_{1A}-AR neurospheres at 0 d, basally expressed more neurons than normal controls. KO of the α_{1A} -AR increased expression of astrocytes (H, red). I, Quantified cell types for figures (D-H). At least 3 different cover slips (each containing 40-300 cells) were analyzed from three separate experiments. White bar=10 μm. *$p<0.05$.

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FIGURE 5. α_{1A}-AR agonist increases BrdU incorporation *in vivo*. *In vivo* BrdU incorporation for 2 wk was followed by 2 d (A), 7 d (B), or 14 d (C) of chase in normal mice (control) or mice that were treated with the α_{1A}-AR selective agonist, cirazoline (Ciraz), for 3 mo. A, α_{1A}-AR stimulation increases the number of Nestin and/or $BrdU^+$ cells at 2 d of chase. At 7 d (B) and 14 d (C), α_{1A}-AR stimulation increased BrdU+ cells compared to control. *$p<0.05$ compared to matched control. B + N stands for $BrdU^+$/$Nestin^+$ co-labeled cells.

FIGURE 6. α_1-ARs regulate key transcription factor mRNA involved in neonatal neuronal differentiation. RNA was isolated from neurospheres and subjected to real time PCR. When differentiated by 1% serum, normal neonatal neurospheres significantly increased the RNA of the α_{1B}-AR subtype, Dlx2, Mash1, Math1, Ngn1, and NeuroD, consistent with neuronal differentiation (grey triangles). When Phe-stimulated, CAM α_{1A}-AR neurospheres increased the RNA of Dlx2, Mash1, and NeuroD (green circles), while CAM α_{1B}-AR neurospheres increased the RNA of Math-1 and Ngn-1 (red circles). KO of the α_{1A}-AR subtype increased the RNA of nestin (magenta triangles), suggesting a reversion to a more undifferentiated state. Other factors tested (Notch-1, Zic-1, Sox-2) were not regulated by α_1-ARs. α_{1B}-AR KO (blue triangles). Samples are obtained from three independent experiments, performed in duplicate, and were normalized to α-tubulin gene expression as internal controls. *$p<0.05$, **$p<0.001$.

FIGURE 7. α_1-ARs differentiate interneurons, and catecholaminergic neurons through PI3K in neonatal neurospheres. A, Western blot analysis of normal neonatal neurospheres stimulated with phenylephrine (Phe 10μM) increased while α_{1A}-AR KO decreased the

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expression of tyrosine hydroxylase, GAD 65/67, and dopamine β-hydroxylase. β_1-integrin decreases expression with Phe-stimulation while no R1 subunit of the NMDA receptor could be detected. BR, brain extract control. B, Neuronal differentiation as assessed by MAP2 expression levels via western analysis is stimulated by Phe, but decreased when co-incubated with the PI3K inhibitor LY294002 (20μM). C, Decreased astrocyte expression as assessed by GFAP western analysis was reduced with Phe, but increased when co-incubated with LY294002. Other inhibitors used were: 10 μM PD98059 (MEK inhibitor, PD), 10 μM SB203580 (p38 inhibitor, SB), 10 μM SP600125 (JNK inhibitor, SP), 0.5 μM Go 6983 (PKC inhibitor, Go). *$p<0.05$; **$p<0.01$.

FIGURE 8. α_1-ARs regulate apoptosis and migration of neonatal progenitors. A, Normal neonatal neurospheres treated with 10 μM Phe for 0 d (control), 2 d, or 3 d increase the number of TUNEL-positive cells. B, TUNEL-positive cells expressed GFAP, but not MAP2. C, Dissociated neonatal normal neurospheres treated with 10 μM Phe or CAM α_{1A}-AR derived neurospheres increased migration comparable to 2% serum. α_{1A}-AR KO derived neurospheres displayed decreased migration. Total cells counted ranged from 40-240 per cover slip using at least three different cell preparations tested. *$p<0.05$; **$p<0.01$.

FIGURE 9. α_1-ARs differentiate normal adult neurospheres into glial cells. A, Western blot analysis of normal adult neurospheres stimulated with phenylephrine (Phe 10μM) decreased the expression of markers associated with undifferentiated stem cells, such as Notch-1, Nestin, and Vimentin, but increased the expression of GFAP and CC1, markers for astrocytes and oligodendrocytes, respectively. The NMDA receptor neuronal marker NR1, decreased in

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expression upon α_1-AR stimulation. Similar results repeated in another normal adult neurosphere cell line. Both adult neurosphere cell lines were negative for the neuronal markers, tyrosine hydroxylase, GAD 65/67, and dopamine β-hydroxylase. α_{1A}-AR promoter-EGFP (B, D) or α_{1B}-AR-EGFP tagged (C, E) cells (green) co-localized (arrows) with S100 positive or βIII tubulin positive cells in the adult olfactory. Mice were 2-3 mo. White bar=10 μm.

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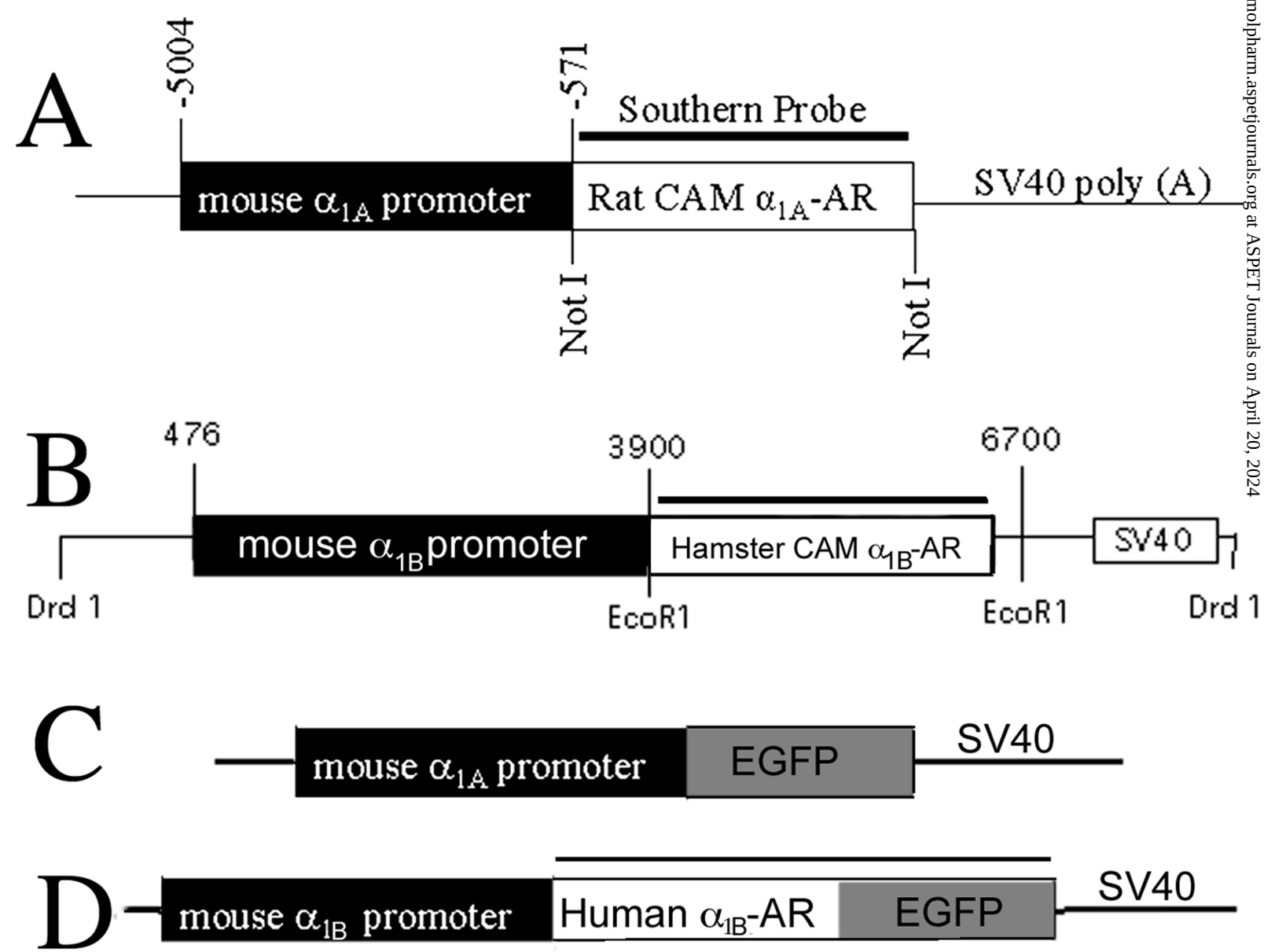
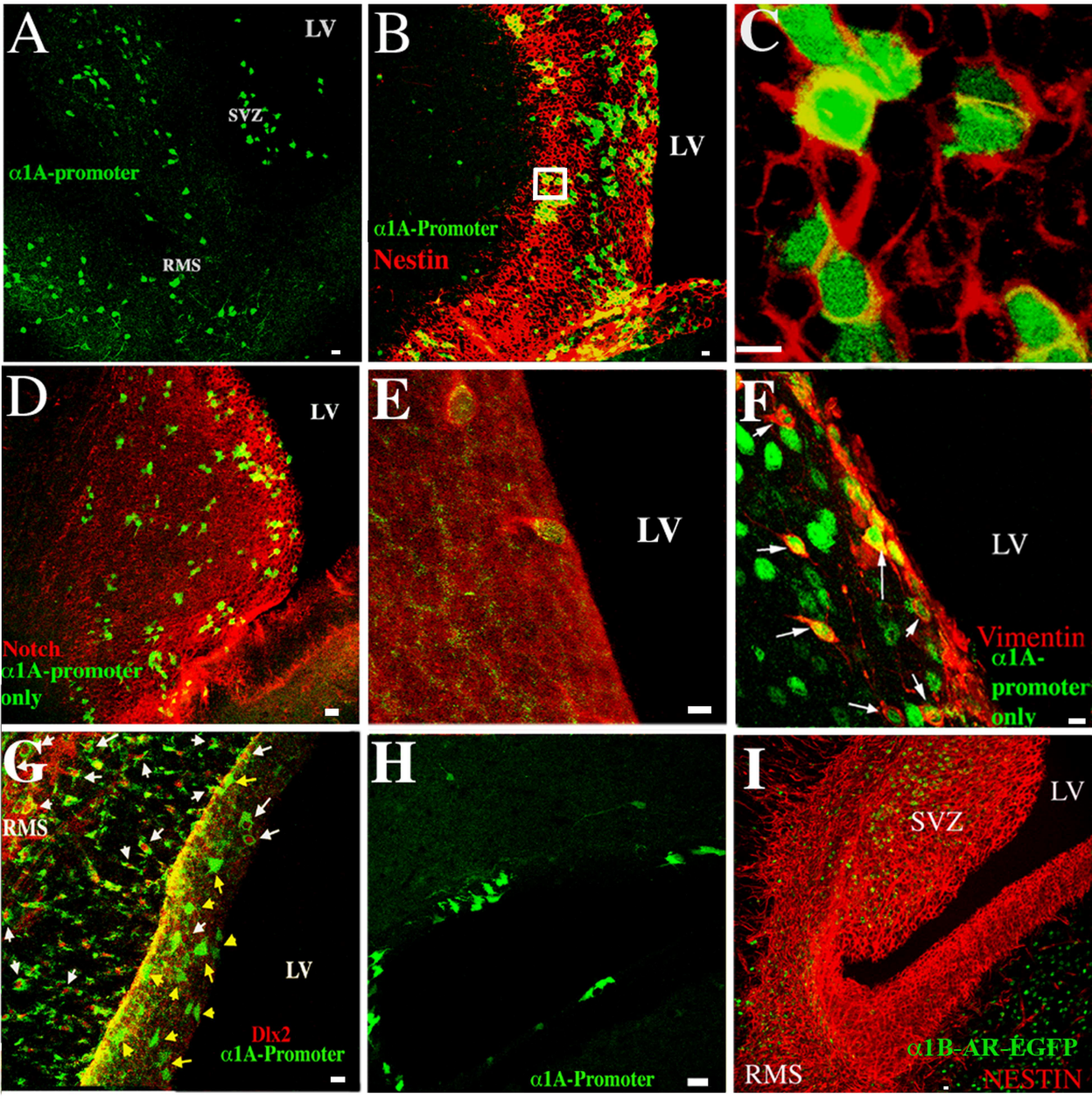


Figure 1


A
LV
SVZ
α1A-promoter
RMS
B
LV
α1A-Promoter
Nestin
C
D
LV
Notch
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only
E
LV
F
LV
Vimentin
α1A-
promoter
only
G
RMS
LV
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α1B-AR-EGFP
RMS
NESTIN

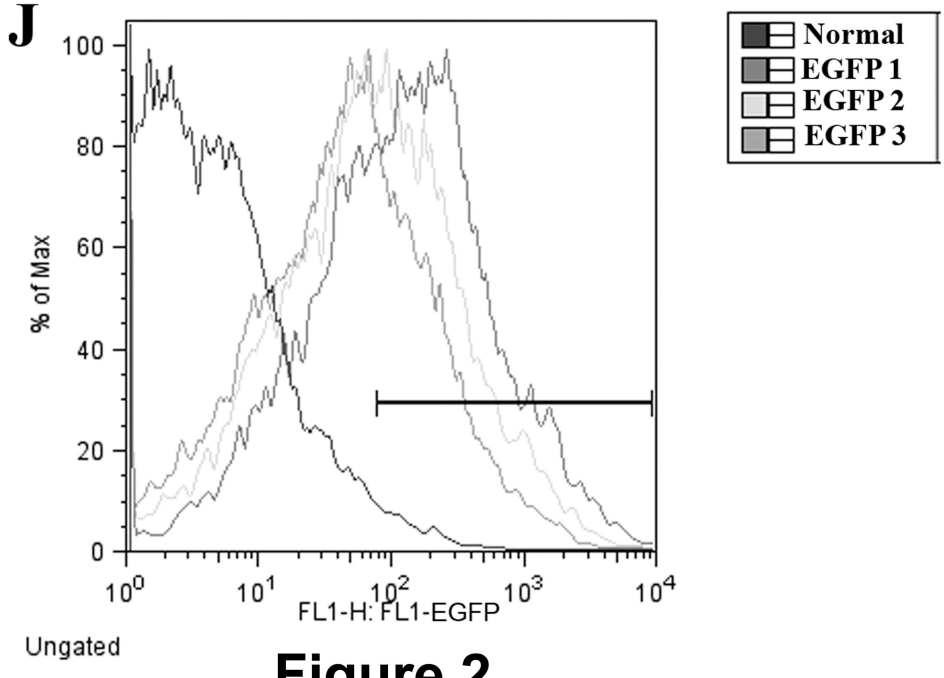
J
Normal
EGFP 1
EGFP 2
EGFP 3
% of Max
100
80
60
40
20
0
10^0
10^1
10^2
10^3
10^4
FL1-H: FL1-EGFP
Ungated

Figure 2

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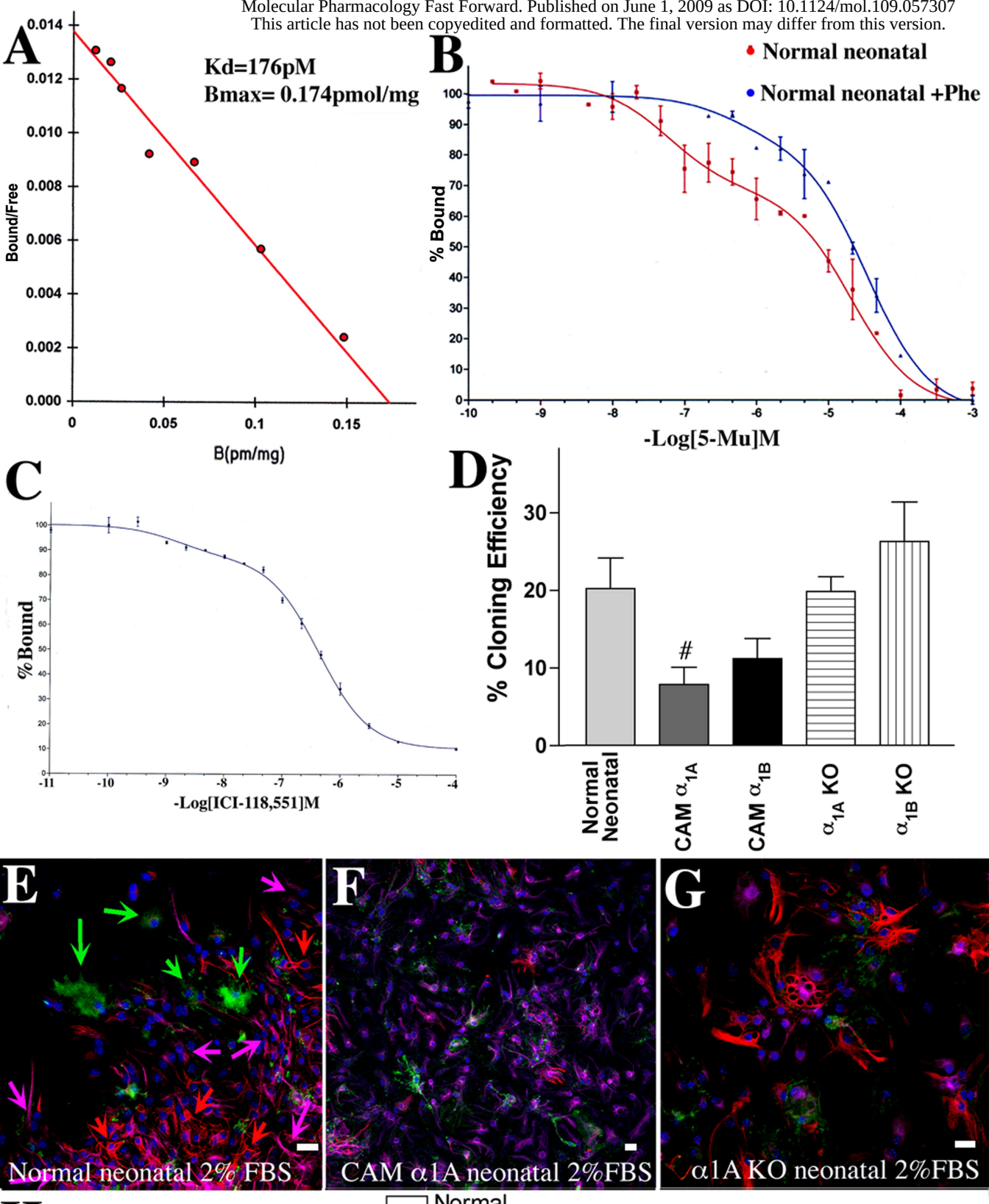

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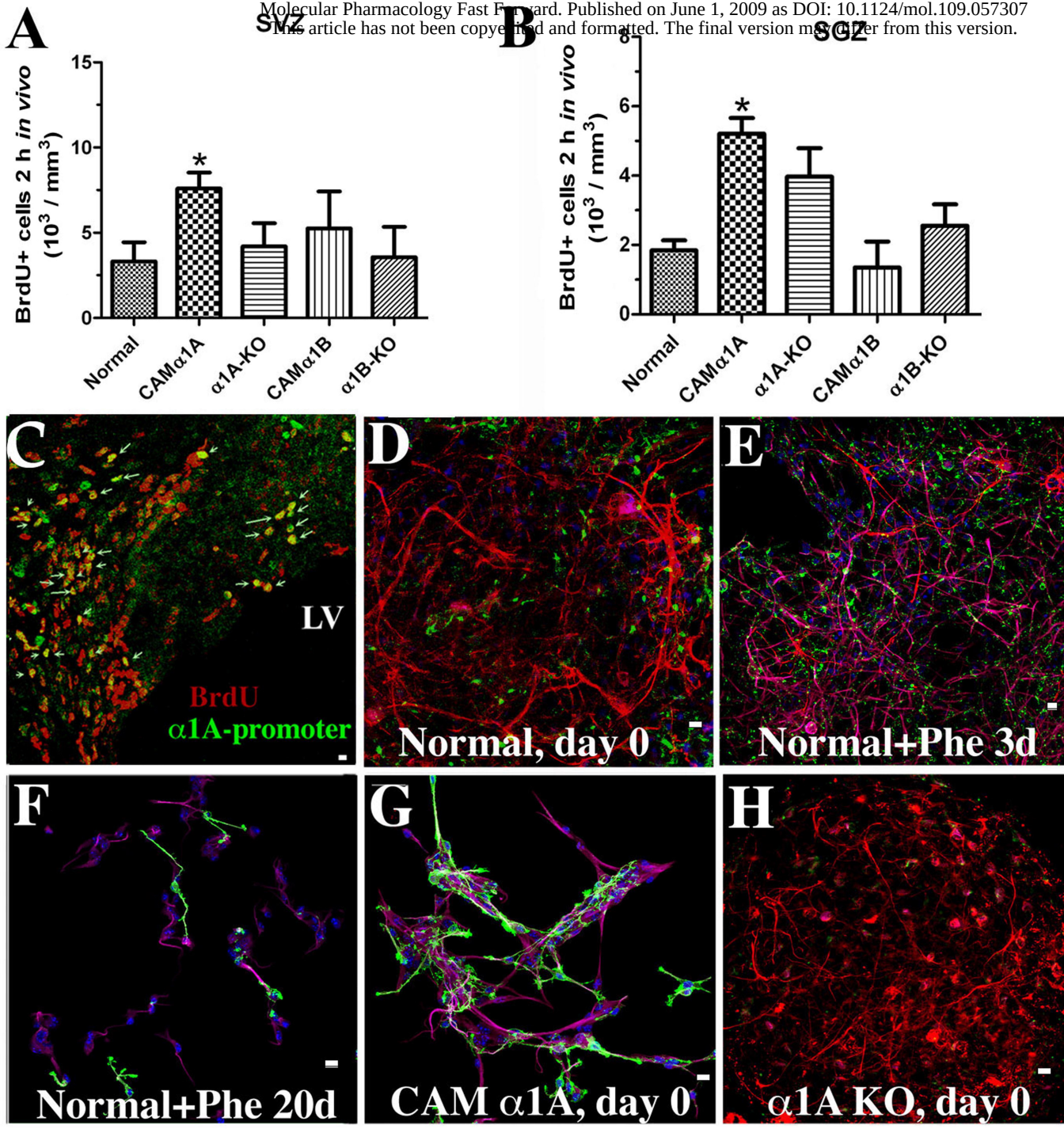

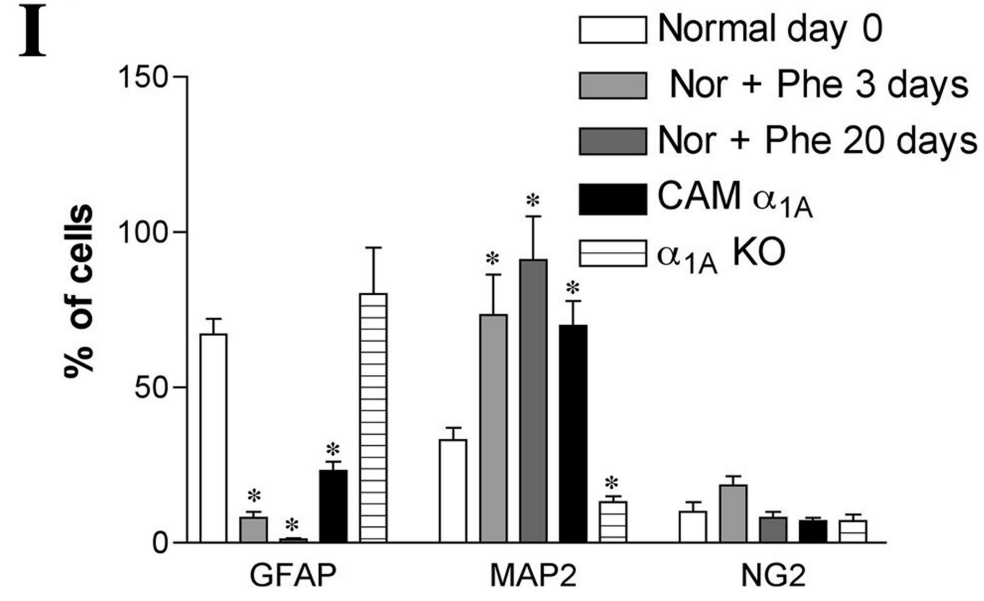

Figure 4

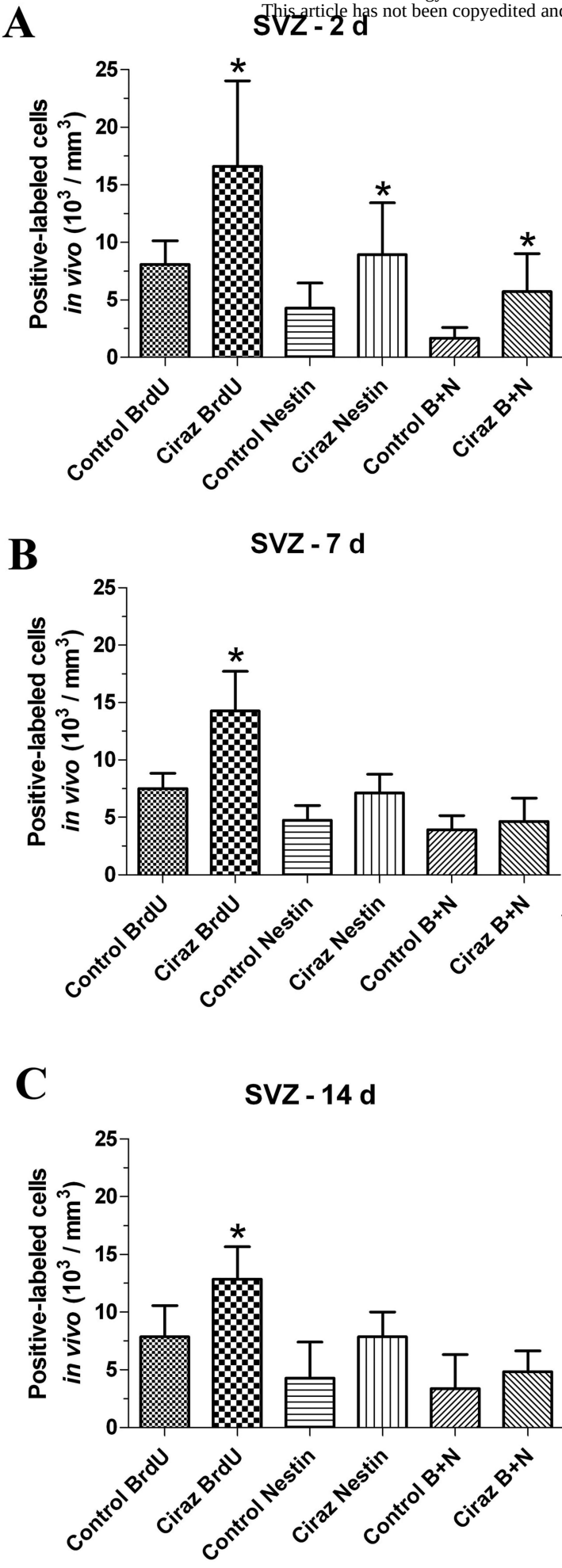

Figure 5

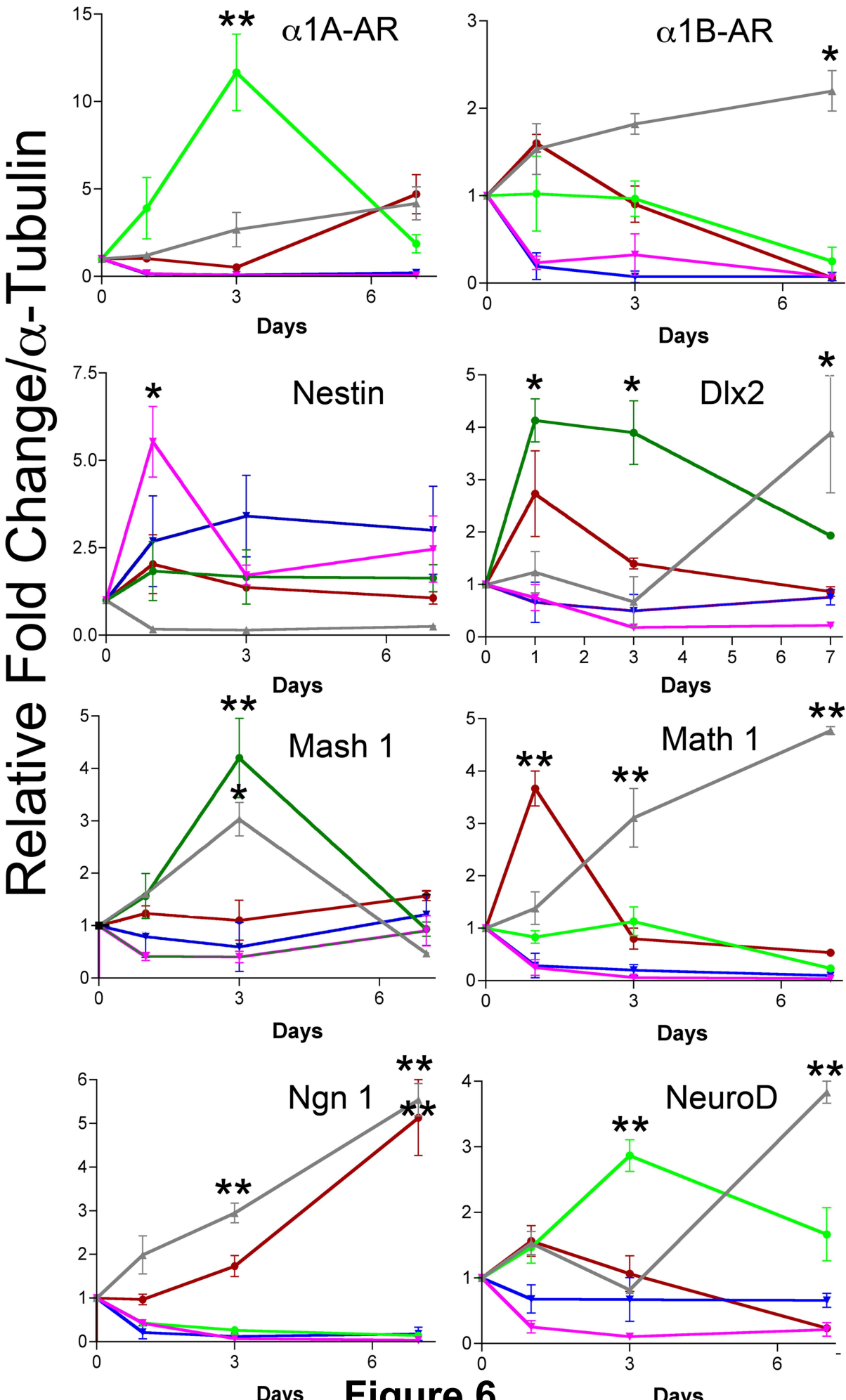

Figure 6

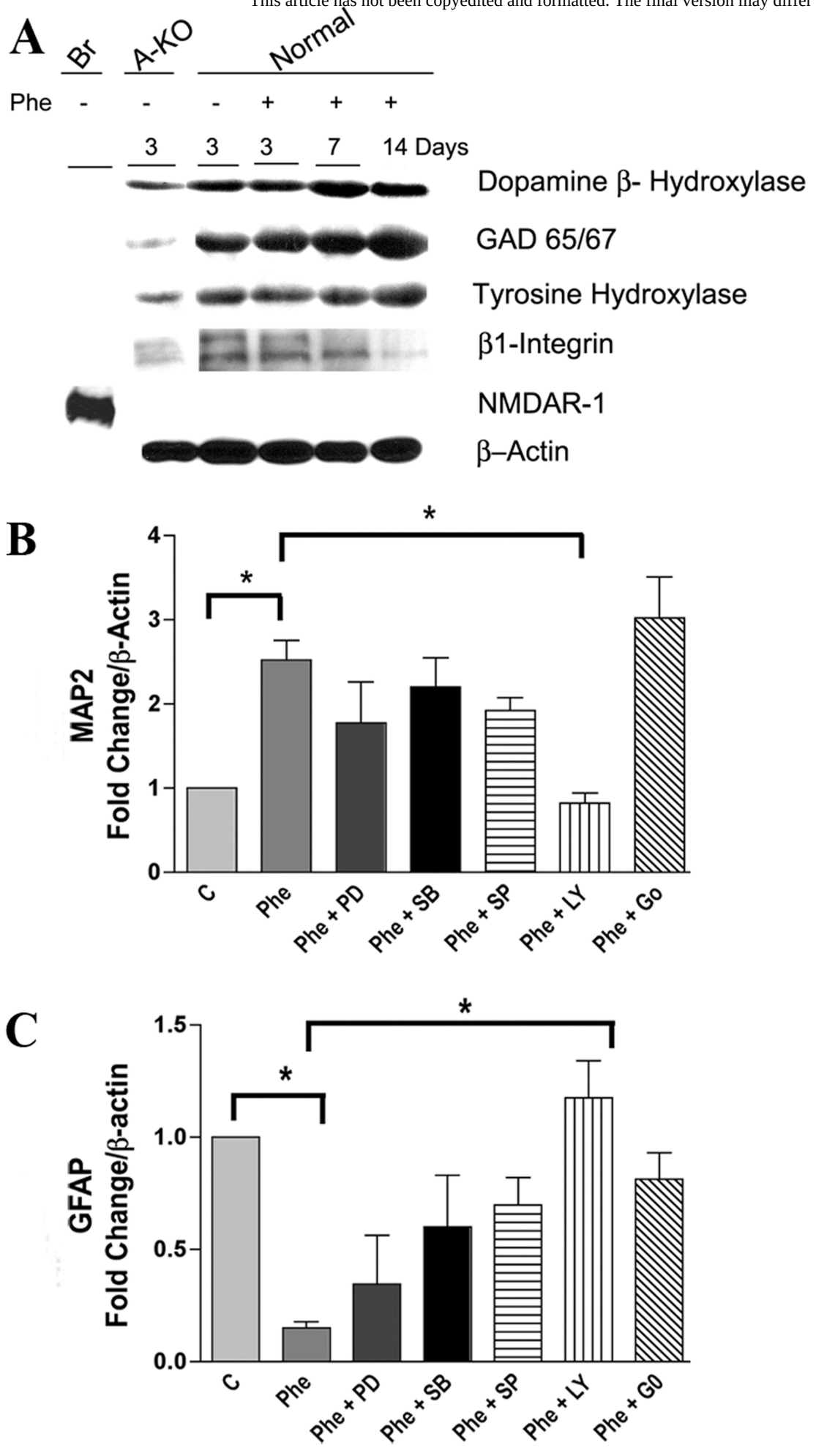

Figure 7

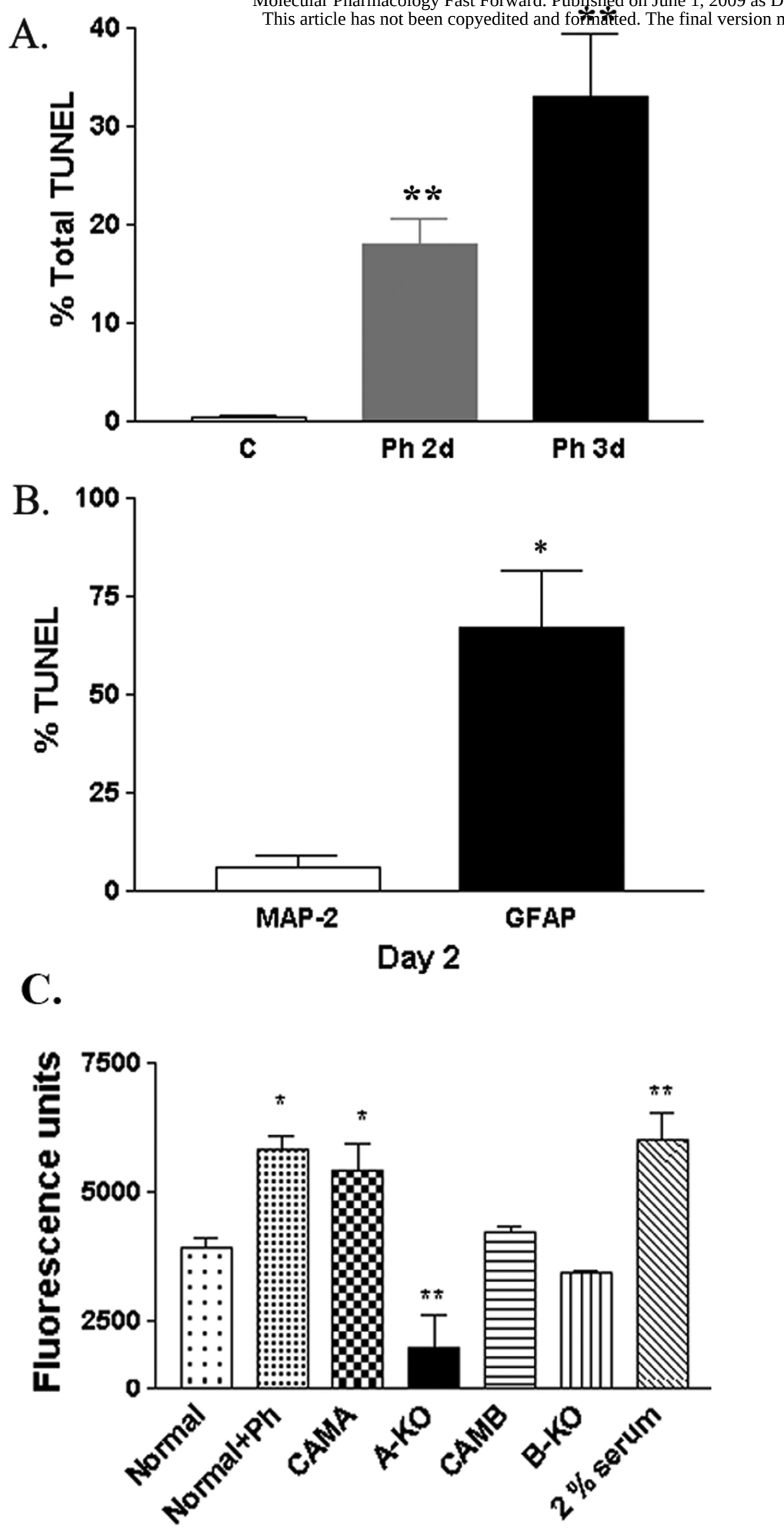

Figure 8

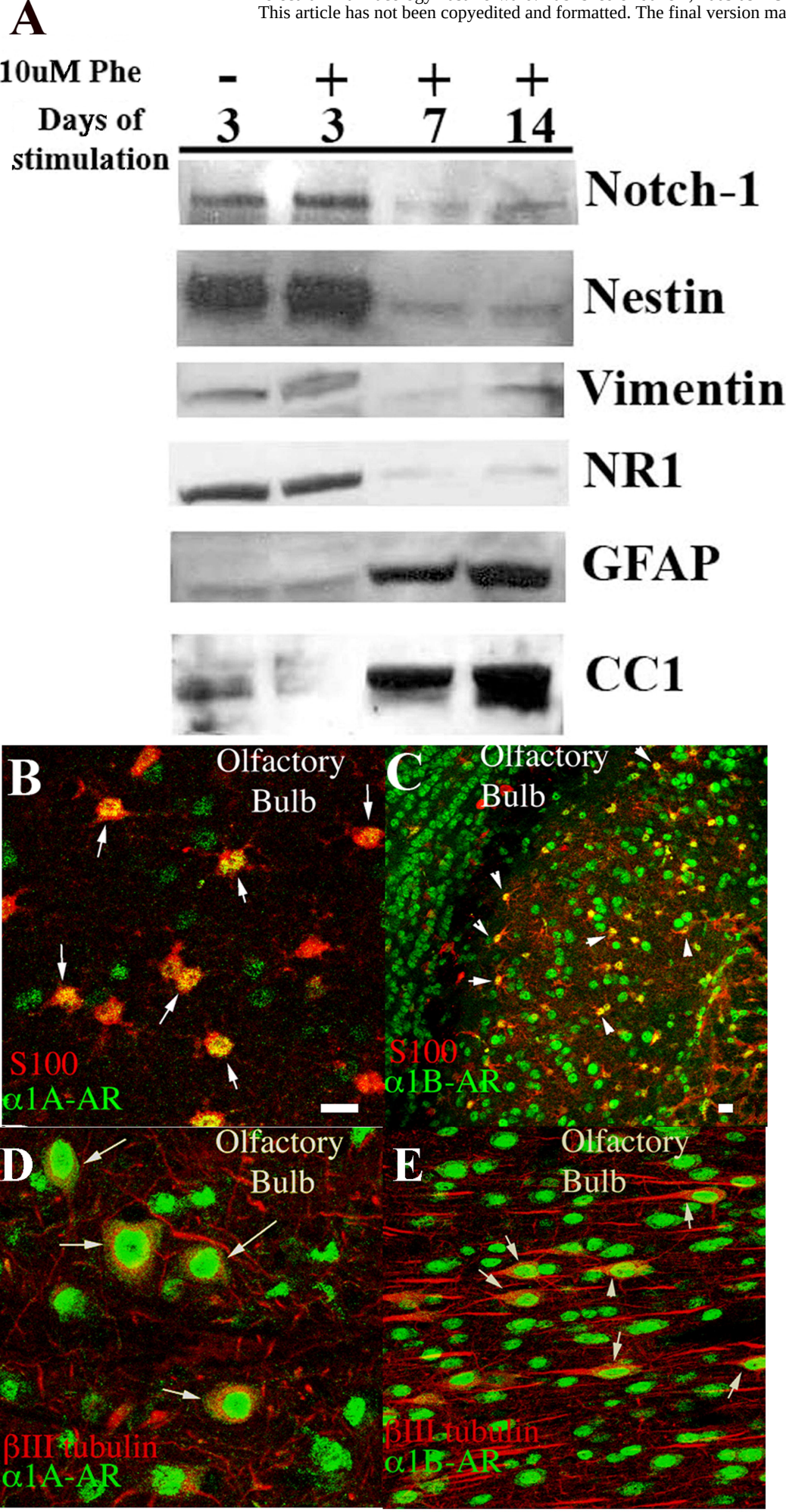

Figure 9