

# Retinoic acid regulates postnatal neurogenesis in the murine subventricular zone-olfactory bulb pathway

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## Summary

Neurogenesis persists throughout life in the rodent subventricular zone (SVZ)-olfactory bulb pathway. The molecular regulation of this neurogenic circuit is poorly understood. Because the components for retinoid signaling are present in this pathway, we examined the influence of retinoic acid (RA) on postnatal SVZ-olfactory bulb neurogenesis. Using both SVZ neurosphere stem cell and parasagittal brain slice cultures derived from postnatal mouse, we found that RA exposure increased neurogenesis by enhancing the proliferation and neuronal differentiation of forebrain SVZ neuroblasts. The RA precursor retinol had a similar effect, which was reversed by treating cultures with the RA synthesis inhibitor disulfiram. Electroporation of dominant-negative retinoid receptors

into the SVZ of slice cultures also blocked neuroblast migration to the olfactory bulb and altered the morphology of the progenitors. Moreover, the administration of disulfiram to neonatal mice decreased *in vivo* cell proliferation in the striatal SVZ. These results indicate that RA is a potent mitogen for SVZ neuroblasts and is required for their migration to the olfactory bulb. The regulation of multiple steps in the SVZ-olfactory bulb neurogenic pathway by RA suggests that manipulation of retinoid signaling is a potential therapeutic strategy to augment neurogenesis after brain injury.

Key words: Neural stem cell, Neuronal migration, Retinoid signaling

## Introduction

Retinoic acid (RA) plays an important role in the developing mammalian nervous system. RA is essential for initial anteroposterior neural patterning and the subsequent development of spinal cord and hindbrain structures (reviewed in Maden, 2002). Recent evidence indicates that RA also influences the embryonic development of forebrain structures such as the striatum and olfactory bulb (Anchan et al., 1997; Valdenaire et al., 1998; Toresson et al., 1999). In terms of the postnatal rodent forebrain, retinoid binding proteins are expressed in olfactory bulb and lateral ventricle ependyma (Zetterstrom et al., 1999), and RA receptors persist into adulthood in the olfactory bulb (Krezel et al., 1999). The RA synthesizing enzyme retinaldehyde dehydrogenase-3 (RALDH3) is expressed in the SVZ, RMS and olfactory glomerular layer (Wagner et al., 2002). Importantly, adult transgenic mice expressing a RA response element (RARE)-reporter construct show reporter expression in the SVZ and olfactory granular and glomerular layers, indicating RA-induced transcriptional activation in this pathway (Thompson Haskell et al., 2002).

Although retinoid signaling components persist in the mammalian forebrain (Zetterstrom et al., 1999), its role in postnatal brain development is poorly understood. Accumulating data suggest that retinoid signaling influences neurogenesis in persistent germinative zones of the neonatal and adult rodent forebrain. Neurogenesis continues throughout life in the mammalian subventricular zone (SVZ)-olfactory

bulb pathway and hippocampal dentate gyrus (Altman and Das, 1965; Altman, 1969; Luskin, 1993; Lois and Alvarez-Buylla, 1994; Eriksson et al., 1998). In neonatal and adult rodents, the forebrain SVZ generates neuroblasts that migrate along the rostral migratory stream (RMS) to the olfactory bulb and differentiate into granule and periglomerular neurons (Luskin, 1993; Luskin, 1998; Lois et al., 1996). Recent work suggests that RA modulates cell proliferation or neurogenesis in regions where neural progenitors persist postnatally. RA exposure stimulates neurogenesis in neural stem cell cultures isolated from the embryonic striatal SVZ or adult hippocampus (Wohl and Weiss, 1998; Takahashi et al., 1999). Prolonged oral RA administration also increases adult rat SVZ cell proliferation (Giardino et al., 2000). Moreover, the lateral ganglionic eminence (LGE), which gives rise to the striatum under the influence of RA, evolves into the postnatal forebrain SVZ and is a source of olfactory bulb neurons (Anderson et al., 1997; Wichterle et al., 1999; Toresson and Campbell, 2001; Stenman et al., 2003).

The presence of retinoid signaling components in the SVZ-olfactory bulb pathway and evidence that RA influences neural development in persistent germinative zones led us to explore the role of retinoid signaling in postnatal SVZ-olfactory bulb neurogenesis. We found that RA and its precursor retinol increased SVZ neurogenesis in dissociated and explant cultures. Moreover, blockade of retinoid signaling decreased SVZ cell proliferation *in vitro* and *in vivo*, and inhibited SVZ neuroblast migration to the olfactory bulb in explants. These

findings suggest that RA signaling regulates multiple aspects of postnatal forebrain SVZ neurogenesis.

## Materials and methods

### Postnatal mouse neurosphere cultures

Neurosphere (NS) cultures were prepared as previously described (Gritti et al., 1996) with modifications. Postnatal day 15 (P15) CD-1 mice (Charles River) were anesthetized with CO<sub>2</sub>, the brains removed and cut into 2 mm-thick coronal blocks. The lateral SVZ was dissected, minced and dissociated with trypsin. Approximately 3–8 × 10<sup>4</sup> SVZ cells (four brains) per 60 mm dish were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1, Gibco) containing 20 ng ml<sup>-1</sup> epidermal growth factor (EGF; Sigma), 10 ng ml<sup>-1</sup> basic fibroblast growth factor (bFGF; Sigma), 0.6% glucose, 5 mM HEPES buffer, 3 mM sodium bicarbonate, 2 mM glutamine, and a defined hormone and salt mixture (100 μg ml<sup>-1</sup> transferrin, 25 μg ml<sup>-1</sup> insulin, 60 μM putrescine, 30 nM sodium selenite and 20 nM progesterone). Primary NS were cultured for 6–7 days *in vitro* (DIV), mechanically dissociated, and passaged to form secondary NS or plated for differentiation at 1 × 10<sup>5</sup> cells per well in 24-well plates (Corning-Costar) coated with poly-ornithine (Sigma). To examine RA effects on progenitor differentiation, dissociated primary NS were differentiated for 7 days in DMEM/F12 containing 1 μM bromodeoxyuridine (BrdU; Roche) and 1% fetal bovine serum (FBS; Gibco). All-trans RA (0.2, 1.0 or 5.0 μM; Sigma), retinol (1 μM; Sigma) ± disulfiram (100 nM; Sigma), or vehicle was added for the entire culture duration. In other experiments, dissociated NS were cultured for 4 days in differentiation medium with 1 μM RA or vehicle. BrdU (1 μM) was added for 2 hours prior to washout and fixation on day 4. In a third set of experiments to examine NS expansion and self-renewal, secondary NS were cultured with mitogens for 6 days in the presence of 1 μM RA or vehicle, and then passaged to form tertiary NS without RA exposure. For all experiments, half of the medium was replaced every 3 days.

### Reverse transcriptase polymerase chain reaction (RT-PCR) for retinoid receptors

mRNA was isolated from secondary NS after 6 DIV using Trizol reagent (Invitrogen). RT-PCR was performed with the Titan one-step kit (Roche Molecular) using a PCR cycler (RoboCycler Gradient 40; Stratagene). RT-PCR was carried out at 55°C (50 minutes) for cDNA synthesis followed by 36 cycles at 94°C (30 seconds), 58°C (45 seconds), 72°C (90 seconds) and a final extension step at 72°C (7 minutes). Specific primer pairs were (1) RA receptor α (RARα) (forward 5'-agagcagttccgaagatag-3', reverse 5'-cgactgtccgcttagagt-gtccaa-3'); (2) RARβ (5'-gcaggaatgcacagagagctatgagat-3' and 5'-ggt-gactgactgactcactgttctccact-3'); (3) RARγ (5'-tgtatgcaatgacaagtcttctgg-3' and 5'-atgaggcagatagcactgagtagc-3'); (4) retinoid X receptor α (RXRα) (5'-cactgaggatatacaagccgacct-3' and 5'-ggtgtca-caccagctctgctatgc-3'); and (5) RXRγ (5'-tgtgtacagctgtaaggttgc-3' and 5'-tctgagaagtgggggatgc-3'). PCR products were analyzed using 1% agarose gel electrophoresis. RT-PCR for actin served as an internal control; no PCR product was detected without RT in the RNA mixture.

### Explant culture and electroporation

Neonatal CD-1 mouse brains were hemisectioned, embedded in 4% LMP agarose (Gibco), vibratome-sectioned into 250 μm-thick parasagittal slices, and cultured on polycarbonate membrane filters (Whatman nucleopore 25 mm) floating on 1 ml of serum-free medium (Neurobasal A/pen-strep/glutamax/B27 supplement, all from Gibco). Only sections containing SVZ-olfactory bulb pathway were used; typical yields were two explants per hemisphere. After 1 hour, RA [10 μM in dimethyl sulfoxide (DMSO)] or vehicle was added. BrdU (5 μM) was added on day 2 for 24 hours and explants fixed 48 hours

later (4 DIV), or slices were incubated with BrdU for 2 hours prior to washout and fixation at 2 or 4 DIV. Pilot studies were performed using explants from P2-P10 mice (Fig. 3). P2 mice were used for subsequent experiments.

Electroporation was performed on hemispheres prior to explant preparation (Fig. 3). DNA (1 μg μl<sup>-1</sup>) was electroporated as follows: (1) co-electroporation of enhanced green fluorescent protein (EGFP) or dsRed plasmids under the control of an ubiquitin promoter (US2-EGFP, US2-dsRed; gifts from D. Turner); or (2) electroporation of an EGFP reporter under the control of a simian CMV promoter (pCS2+EGFP, gift of D. Turner) (Farah et al., 2000) alone or combined with dominant-negative (dn) RARα or dnRXRα constructs (gifts from P. Chambon) (Feng et al., 1997; Xiao et al., 1999). DNA was injected into the anterior portion of the open lateral ventricle, hemispheres incubated in D-PBS on ice (10 minutes), and electroporation (85 V, 10 pulses, 50 milliseconds per pulse, 1 second inter-pulse interval) performed in a platinum petridish plate electrode (Protech International) using a square-wave electroporator (BTX). For additional experiments, dnRARα was subcloned into a CMV-IRES-EGFP vector (BD Biosciences). CMV-dnRARα-IRES-EGFP or CMV-IRES-EGFP control construct was electroporated into slices as above. Hemispheres were embedded, sectioned sagittally at 250 μm and cultured for 4 DIV.

### Immunofluorescence histochemistry and TUNEL stain

Cultured cells were fixed at 4 or 7 DIV for 30 minutes in 4% paraformaldehyde (PFA). After washes and blocking, cells were incubated overnight at 4°C with the following primary antibodies: mouse anti-β-tubulin (TuJ1, diluted 1:1000; Covance); rabbit anti-calretinin (1:2000; Chemicon); rabbit anti-glial fibrillary acidic protein (GFAP; 1:500; Sigma); rat anti-myelin basic protein (MBP; 1:1000; Chemicon) or mouse anti-activated caspase-3 (1:1000; BD PharMingen). Secondary antibodies (all from Molecular Probes) included: anti-rat Alexa Fluor 594; anti-rabbit Alexa Fluor 594 or 488; or anti-mouse Alexa Fluor 488 at 1:400 dilution. Bis-benzimide (Molecular Probes) was used for counterstaining. To detect double labeling for BrdU and β-tubulin, GFAP, or MBP, cells were incubated with the first antibody overnight at 4°C, washed and post-fixed in 4% PFA for 30 minutes, and denatured as above. After blocking, cells were incubated with rat anti-BrdU antibody (1:200; Serotec) overnight at 4°C, and then with secondary antibodies as described above. Immunofluorescence was analyzed using a DMIRB inverted epifluorescence microscope (Leica).

Explant cultures were fixed with 4% PFA for 3 hours and vibratome-resectioned at 40–50 μm thickness. For immunofluorescence, sections were rinsed, blocked and incubated with the following primary antibodies (48 hours, 4°C): rabbit anti-calretinin (1:2000); mouse anti-nestin (1:10; rat-401 clone, University of Iowa); or mouse IgM anti-PSA-NCAM (1:500; 5A5 clone, University of Iowa). Rabbit anti-GFP antibody (1:2000; Molecular Probes) was used for double-labeling with nestin or PSA-NCAM. After washes, sections were incubated with Alexa Fluor 594-conjugated goat anti-rabbit IgG (1:200; for single labeling), or for double-labeling with Texas Red-conjugated anti-mouse IgG or IgM and FITC-conjugated anti-rabbit IgG (all 1:200; Jackson Laboratories) for 1 hour, washed, mounted on slides and coverslipped with anti-fade medium (ProLong, Molecular Probes). For BrdU immunohistochemistry, sections were denatured, incubated with rat anti-BrdU antibody (1:200; 4°C, 48 hours), and then processed as above. For BrdU/calretinin or PSA-NCAM double-labeling, tissue was incubated with non-BrdU primary antibody, washed, post-fixed with 4% PFA for 20 minutes, denatured and processed for BrdU labeling as above.

For Tdt-mediated dUTP nick end labeling (TUNEL), sections were treated with 0.3% Triton X-100 and incubated with 0.3 mg ml<sup>-1</sup> proteinase K (37°C, 20 minutes). Sections were then rinsed, incubated in 0.25% acetic anhydride and treated with 0.2% H<sub>2</sub>O<sub>2</sub> in methanol.

The manufacturer's protocol for ApopTag Fluorescein Kit S7110 (Intergen) was then used.

### In vivo disulfiram injections and BrdU labeling

P7 mice were injected intraperitoneally (i.p.) with the RALDH inhibitor disulfiram (5 mg kg<sup>-1</sup> in DMSO/olive oil) daily for 4 days. BrdU (100 mg kg<sup>-1</sup>) was injected i.p. once on P7 (6 hours after disulfiram). Animals gained weight normally and appeared healthy. On P10, mice were perfused with 4% PFA. Brains were post-fixed, cryoprotected with 20% sucrose and sectioned coronally at 40  $\mu$ m. All animal experimentation was approved by the University of Michigan Committee on Use and Care of Animals.

### Image analysis and quantification

Microscopic images were acquired with a SPOT-RT digital camera (SPOT Diagnostic Instruments). For differentiated NS cell counts, labeled and total cells were counted in five random fields (20 $\times$  objective). Explants were randomly assigned to treatment condition by a blinded observer. BrdU-positive cells within a defined SVZ area (0.0676 mm<sup>2</sup>; Fig. 4) were counted in three resectioned slices/hemisphere (two explants/condition; four replicates) with a grid-lined reticle (20 $\times$  objective) without knowledge of treatment condition. TUNEL-positive cells in two resectioned explants/treatment (three slices/explant; three replicates) were counted from the SVZ to a point in the RMS bisected by a line drawn from the rostral edge of frontal cortex, perpendicular to the RMS (see inset, Fig. 7H). Maximum migration distance of GFP-labeled cells was calculated as the linear distance from the anterior border of the ventricle to the most rostral GFP-positive cell (see inset, Fig. 7G) using NIH Image v.1.63 software. GFP-positive cells in the RMS and olfactory bulb were counted rostral to a point in the line bisecting the RMS when drawn perpendicularly from the rostral edge of the frontal

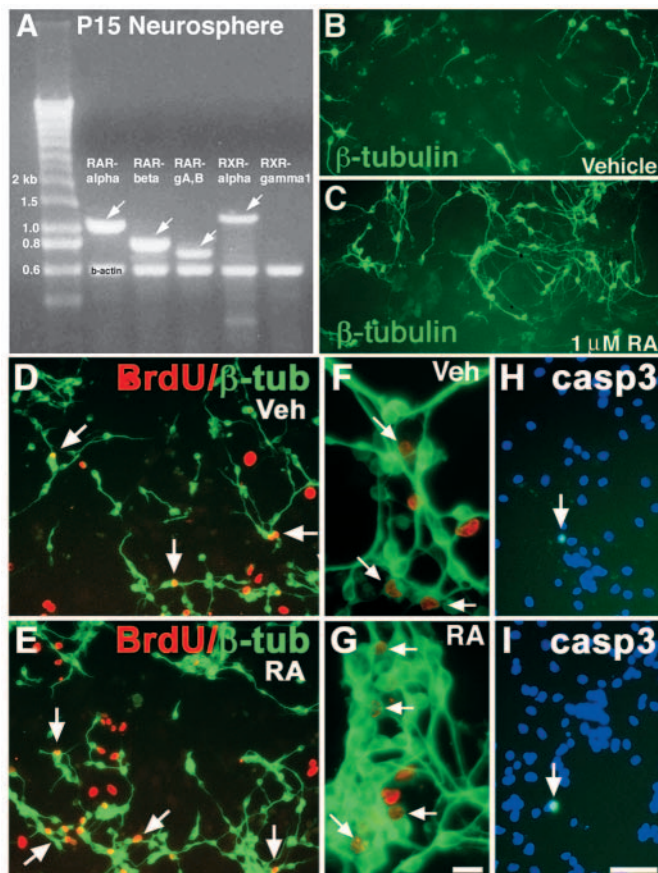
cortex (Fig. 7H). Data were obtained from two explants/condition in 3-4 separate experiments. For in vivo BrdU labeling, percentage area of BrdU-immunoreactivity in a 0.05 mm<sup>2</sup> region of dorsolateral striatal SVZ (10 $\times$  objective) was measured using NIH Image (Parent et al., 2002). Statistical analyses were performed using StatView 4.1 (Abacus Concepts). Multiple comparisons were analyzed by one-way ANOVA (analysis of variance) with post hoc test (Fisher's PLSD); two-tailed Student's *t*-test was used for two-group comparisons. Results are shown as means $\pm$ s.e.m.; significance level was *P*<0.05.

## Results

### RA stimulates neuroblast proliferation in postnatal SVZ neural stem cell cultures

Neural stem cells from the postnatal rodent forebrain SVZ expand in vitro when cultured with mitogens as floating neurospheres (NS) (Reynolds and Weiss, 1992; Gritti et al., 1996; Tropepe et al., 1999; Seaberg and van der Kooy, 2002). Upon mitogen removal, the cells differentiate into neurons, astrocytes and oligodendrocytes. RA exposure during differentiation increases neurogenesis in EGF-expanded, embryonic mouse striatal NS cultures (Wohl and Weiss, 1998). We used the NS culture method to examine the effects of RA on neural stem cells from postnatal (P15) mouse striatal SVZ expanded with EGF and bFGF. RA binds to its nuclear receptor, typically a RAR/RXR heterodimer, and activates the transcription of genes containing RAREs (reviewed in Chambon, 1996). To first determine whether postnatal neural stem cells are capable of responding to RA, primary NS were passaged after 6 DIV, and total RNA harvested from secondary NS expanded for 6 DIV. RT-PCR using primers for different retinoid receptors confirmed the presence of multiple RAR and RXR subtypes in the NS cultures (Fig. 1A), including RAR $\alpha$ ,  $\beta$ , and  $\gamma$  and RXR $\alpha$ , but not RXR $\gamma$ . These findings indicate that, similar to NS cultured from embryonic mouse striatum (Wohl and Weiss, 1998), progenitors in single passage (secondary) NS derived from postnatal mouse striatal SVZ express retinoid receptors. This result is consistent also with findings of RA-responsive cells in the striatal SVZ of adult RARE- $\beta$ -galactosidase transgenic mice (Thompson Haskell et al., 2002).

To directly test whether RA influences neurogenesis in forebrain SVZ NS cultures, we differentiated primary NS for 7 days in the presence of different concentrations of RA or vehicle. RA exposure significantly increased the mean percentage and numbers of  $\beta$ -tubulin-immunoreactive neurons in a concentration-dependent manner (Fig. 1B,C; Fig. 2A,B). Similar results were obtained when a subset of calretinin-



**Fig. 1.** Retinoid receptors and RA effects in P15 mouse SVZ neurosphere (NS) cultures. (A) RT-PCR reveals that secondary NS expanded for 6 days express mRNA for RAR $\alpha$ ,  $\beta$ ,  $\gamma$  and RXR $\alpha$ , but not RXR $\gamma$  retinoid receptor subtypes. (B,C) Differentiation of NS for 7 DIV with RA increases  $\beta$ -tubulin-immunoreactive neurons (C) compared with vehicle (B). (D-G) BrdU (red)/ $\beta$ -tubulin ( $\beta$ -tub; green) double labeling (arrows) demonstrates that RA treatment of differentiating NS for 4 DIV increases neuroblast proliferation (E,G) versus vehicle (D,F). (H,I) Activated caspase-3 (casp 3; green) immunolabeling (arrows) shows no influence of RA treatment (I) on cell death versus control (H). Bis-benzimide counterstain in blue. Scale bars: in I, 100  $\mu$ m for A,B and 50  $\mu$ m for D,E,H,I; in G, 20  $\mu$ m for F,G.

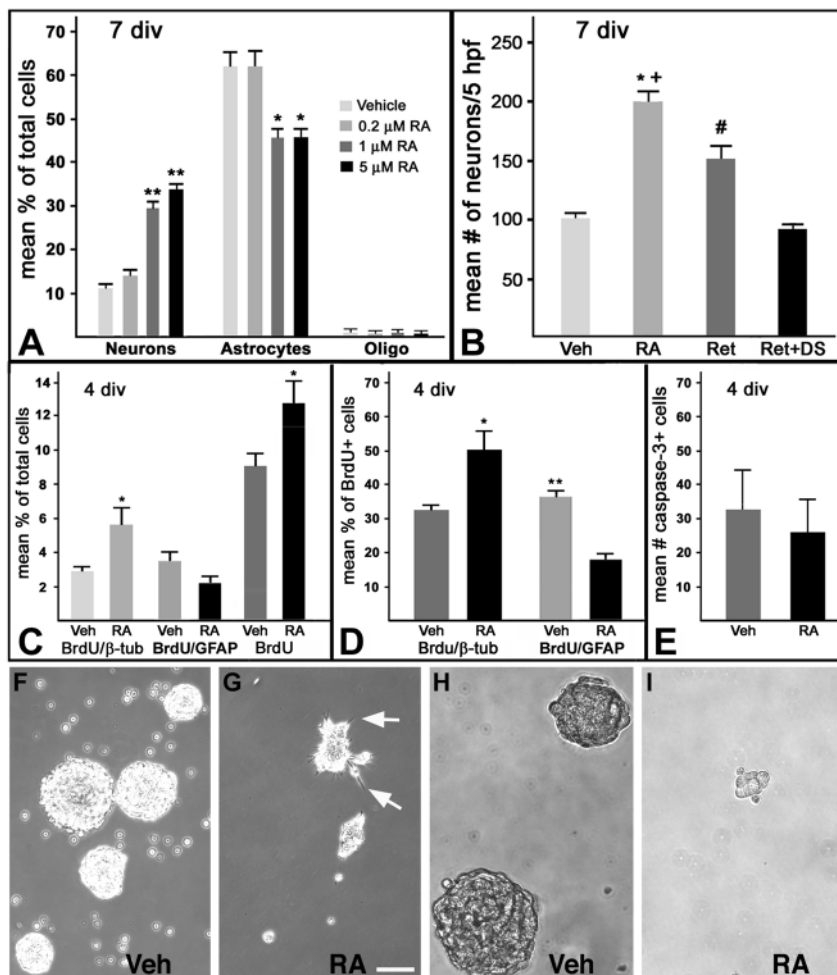


expressing neurons was analyzed (data not shown). The enhancement of neurogenesis by RA treatment occurred at the expense of astrocyte differentiation, as the percentage of GFAP-immunoreactive astrocytes decreased after RA exposure (Fig. 2A). The percentage of oligodendrocytes was small (<2% on average) and did not vary with RA treatment (Fig. 2A). We next examined whether the RA precursor retinol would increase neurogenesis in the cultures. P15 mouse SVZ-derived NS were exposed to RA, retinol or vehicle for 7 days. Retinol significantly increased neuronal differentiation in the cultures, although to a lesser extent than RA (Fig. 2B). This effect was blocked by the RALDH inhibitor disulfiram (Fig. 2B). These findings suggest that retinoid signaling increases postnatal forebrain SVZ neurogenesis. Moreover, SVZ NS appear capable of synthesizing RA from its precursor retinol, consistent with the reported expression of the RA-synthesizing enzyme RALDH3 in the postnatal murine striatal SVZ (Wagner et al., 2002).

RA may stimulate neurogenesis through a number of mechanisms. To examine whether RA increases the proliferation or survival of neuronal progenitors, NS cultures were treated with RA or vehicle for 4 days. BrdU incorporation and activated caspase-3 expression were assessed to examine proliferating and dying cells, respectively. Cultures were exposed to BrdU 2 hours prior to washout and fixation on day 4, and were double-immunostained for BrdU and neuronal or

glial antigens. Compared with control cultures, RA exposure significantly increased BrdU-positive neurons, both as a percentage of total or BrdU-labeled cells (Fig. 1D-G and Fig. 2C,D). Conversely, RA treatment significantly decreased the proportion of BrdU-labeled cells that expressed GFAP; a trend towards decreased BrdU/GFAP double-labeled cells as a percentage of total cells was observed. The mean percentage of total cells incorporating BrdU (Fig. 2C) also significantly increased after RA treatment ( $12.8 \pm 1.4\%$  RA vs.  $9.0 \pm 0.7\%$  vehicle). The absolute number of BrdU-immunoreactive cells, however, was slightly but non-significantly increased (mean cells/5 hpf: RA-treated  $161 \pm 5$ ; control:  $118 \pm 28$ ;  $P=0.20$ ). RA did not influence cell death, as measured by activated caspase-3-immunoreactivity at 4 DIV (Fig. 2E). Moreover, total cell number (per 5 hpf) was not influenced by RA treatment at either 4 (RA  $1 \mu\text{M}$ :  $1471 \pm 152$  cells vs. Control:  $1328 \pm 224$  cells;  $P=0.63$ ) or 7 DIV (vehicle:  $711 \pm 49$ ; RA  $0.2 \mu\text{M}$ :  $698 \pm 48$ ; RA  $1 \mu\text{M}$ :  $701 \pm 26$ ; RA  $5 \mu\text{M}$ :  $772 \pm 32$ ;  $P=0.53$ ). The lack of altered cell numbers after RA exposure despite increased BrdU labeling likely indicates that RA produced only a small percentage increase in overall cell proliferation; i.e. stimulation of neuroblast proliferation largely was offset by reduced astrocytic proliferation.

To examine whether RA treatment influences NS expansion, secondary NS were cultured in the presence of growth factors and either vehicle or RA. In RA-treated cultures, NS were



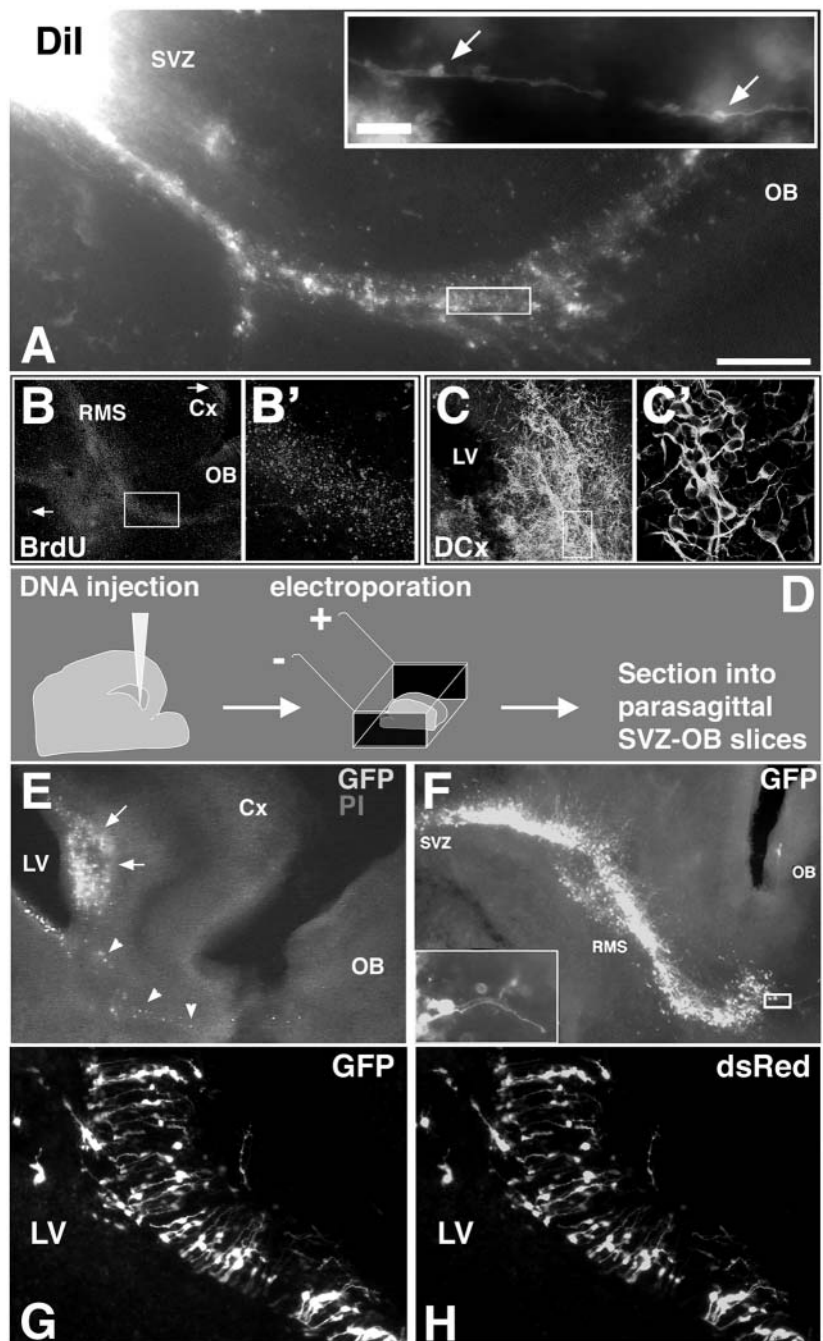
**Fig. 2.** Retinoid signaling increases neurogenesis in P15 mouse SVZ NS cultures. (A) RA treatment of differentiating NS for 7 DIV increases the percentage of neurons and decreases that of astrocytes in a concentration-dependent manner;  $**P < 0.0001$  versus vehicle or  $0.2 \mu\text{M}$  RA groups;  $*P < 0.001$  versus vehicle or  $0.2 \mu\text{M}$  RA groups. Oligodendrocyte differentiation is unchanged by RA exposure. (B) RA ( $1 \mu\text{M}$ ) or retinol (Ret;  $1 \mu\text{M}$ ) treatment increases differentiating neurons after 7 DIV versus vehicle (Veh); the addition of disulfiram (DS) inhibits the retinol effect.  $*P < 0.0001$  versus Veh or Ret/DS; +,  $P < 0.001$  versus Ret; # $P < 0.0005$  versus Veh or Ret/DS. (C,D) At 4 DIV,  $1 \mu\text{M}$  RA increases the proportion of proliferating neuroblasts (BrdU/ $\beta$ -tubulin double-labeled) compared with total or BrdU-positive cells, and decreases the proportion of BrdU/GFAP double-labeled astrocytes. The proportion (%) of cells incorporating BrdU also significantly increases after RA exposure (C). For C and D, \*denotes  $P < 0.05$  and \*\*denotes  $P < 0.005$ . (E) RA does not influence numbers of activated caspase-3-immunoreactive (dying) cells. (F,G) Secondary NS cultures were treated with vehicle (A) or  $1 \mu\text{M}$  RA (B) for 6 days in expansion conditions. RA-treated NS are smaller and appear more differentiated with irregular morphology and process outgrowth (arrows). (H,I) When RA- or vehicle-treated secondary NS were passaged and expanded without RA, the tertiary NS not previously exposed to RA were larger and more numerous (H) compared with those treated with RA before passaging (I). Scale bar:  $50 \mu\text{m}$ .

smaller and appeared to prematurely differentiate compared with controls (Fig. 2F,G). These findings suggest that RA can override the mitogenic effects of EGF and FGF and induce neural progenitor differentiation. When secondary NS exposed to RA or vehicle were passaged to form tertiary NS, the previously RA-exposed progenitors formed fewer and smaller NS, with some also showing premature differentiation (Fig. 2H,I and data not shown). Along with the above results, these data suggest that RA exerts a mitogenic effect on committed neuronal progenitors, and induces premature differentiation of NS-forming cells. Moreover, RA treatment stimulates SVZ-derived neuroblast proliferation at the expense of astrocyte progenitor proliferation, but does not affect cell survival over 4-7 DIV.

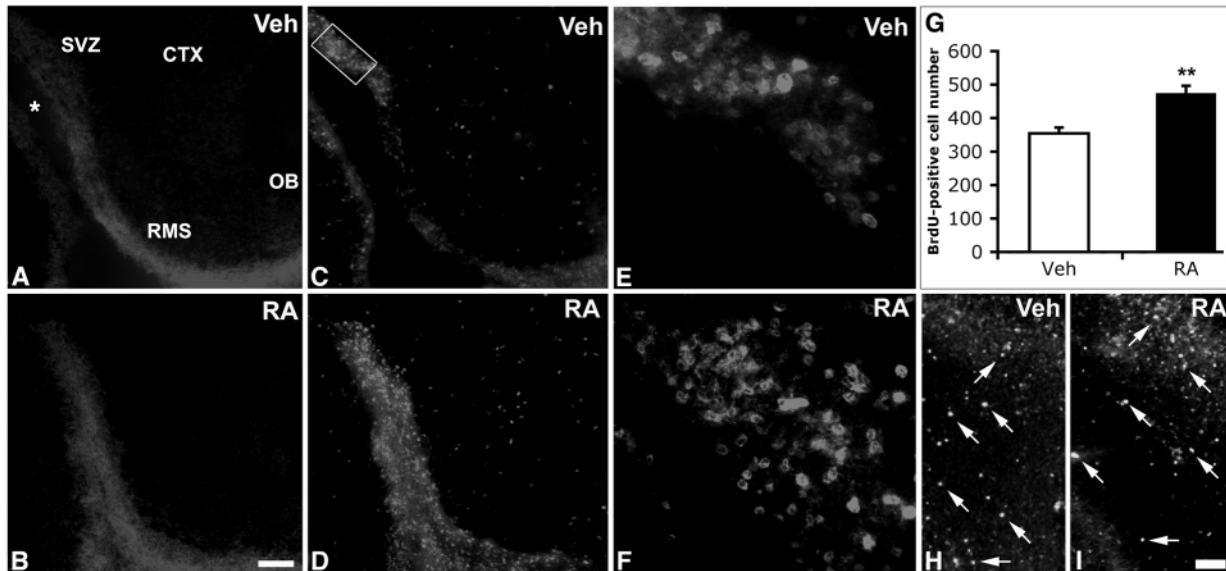
### SVZ-olfactory bulb neurogenesis persists in slice cultures

To study the effects of RA on postnatal neurogenesis in an *in vitro* system that more closely resembles *in vivo* conditions, we developed a SVZ-olfactory bulb slice culture preparation. Parasagittal, 250  $\mu\text{m}$ -thick slices derived from neonatal (P2-10) mice were cultured for up to 4 days in serum-free medium. SVZ-olfactory bulb neurogenesis has been observed in similar cultures (De Marchis et al., 2001; Alifragis et al., 2002). We first confirmed SVZ neural

precursor proliferation and migration by BrdU and DiI labeling. DiI-labeled SVZ cells migrated to the olfactory bulb over several days (Fig. 3A). BrdU administered *in vivo* prior to slice preparation or in the culture medium mainly labeled proliferating cells in the SVZ-olfactory bulb pathway (Fig. 3B,B' and data not shown). Less concentrated BrdU labeling also appeared in the corpus callosum, cortex and striatum (Fig. 3B and data not shown), probably reflecting proliferating glial precursors (Levison and Goldman, 1993; Zerlin et al., 2004). Immunohistochemistry for neuroblast markers, such as doublecortin, showed expression patterns in the SVZ, RMS and olfactory bulb similar to that seen *in vivo* (Fig. 3C,C'). Neuroblasts in this pathway also expressed calretinin, PSA-



**Fig. 3.** Slice culture model of SVZ-olfactory bulb neurogenesis. (A) DiI labeling of cells migrating from SVZ to olfactory bulb (OB) in a P10 explant 3 days after DiI crystal placement in SVZ. The inset shows the bipolar appearance of migrating cells (arrows) at higher magnification. (B) BrdU labeling of cells in the rostral migratory stream (RMS) of a P10 slice culture. Arrows in B show scattered, putative glia that incorporated BrdU. (B') Higher magnification of the boxed area in B. BrdU was given *in vivo* 6 hours before slice preparation and culture for 3 days. (C) Doublecortin-expressing neuroblasts in the SVZ and RMS of a P10 explant after 3 DIV. (C') Higher magnification of the boxed area in C. (D) Schematic of the electroporation technique. Plasmid DNA was placed in the open lateral ventricle of a hemisphere, electroporated, and parasagittal slices prepared. (E) GFP labeling of cells in the SVZ (arrows) and RMS (arrowheads) of a P2 slice 3 days after electroporation of a GFP reporter. Propidium iodide counterstain (PI). (F) Four days after electroporation of a GFP reporter, labeled cells appear in the SVZ-olfactory bulb pathway. The inset is a higher magnification view of the boxed area showing a cell with migratory morphology. LV, lateral ventricle; Cx, frontal cortex. (G,H) To examine dual plasmid electroporation efficiency, P2 slices were co-electroporated with GFP and dsRed reporters, cultured for 4 DIV, fixed and resectioned at 50  $\mu\text{m}$ . Nearly 100% co-electroporation efficiency was found in the SVZ. All explant images in this and subsequent figures display parasagittal sections with anterior to the right and dorsal at top. Scale bars: in A, 150  $\mu\text{m}$  for A,B',C, 250  $\mu\text{m}$  for B,F and 500  $\mu\text{m}$  for E; in inset in A, 75  $\mu\text{m}$  for C' and insets in A and F; and 100  $\mu\text{m}$  for G,H.



**Fig. 4.** RA increases SVZ cell proliferation in explants. (A,B) Bis-benzimide staining of P2 slices cultured for 2 DIV with vehicle (Veh, A) or 10  $\mu$ M RA (B) and re-sectioned at 40  $\mu$ m. The SVZ and RMS stain as a dense band that expands after RA treatment. (C-F) BrdU immunostaining of the sections in A and B; slices were exposed to BrdU for 2 hours prior to fixation. More SVZ cells incorporated BrdU with RA treatment (D,F) compared with control (C,E). (E,F) Show regions of SVZ approximated by the boxed area in C. (G) Quantification of BrdU-positive cell numbers per fixed SVZ area (boxed region in C). \*\*Denotes  $P < 0.01$ . (H,I) TUNEL staining of control (H) and RA-treated (I) explants. No difference in TUNEL-stained cells (arrows) was seen (see text for quantitation). CTX: cortex. OB: olfactory bulb. Asterisk marks lateral ventricle in A. Scale bar: in B, 100  $\mu$ m for A-D and 25  $\mu$ m for E,F; in I, 50  $\mu$ m for H,I.

NCAM and neuron-specific  $\beta$ -tubulin (Figs 5, 8 and data not shown).

We also developed an electroporation technique to express genes in SVZ cells of the explants. P2 mouse brains were hemisectioned and a CMV-GFP reporter construct was injected into the anterior aspect of the open lateral ventricle (Fig. 3D). After electroporating plasmid DNA across the ventricle, sagittal slices were cultured for 3-4 days. GFP-positive cells were found mainly in the SVZ-olfactory bulb pathway (Fig. 3E,F). Many GFP-labeled cells also incorporated BrdU and expressed PSA-NCAM (Fig. 7 and data not shown). To determine SVZ cell co-transfection efficiency, we simultaneously electroporated two reporter constructs (US2-EGFP and US2-dsRed), made slices and cultured the explants for 4 DIV. Nearly 100% of SVZ cells expressed both reporters (Fig. 3F), and approximately 96% co-localization appeared in the RMS (not shown). Along with previous reports (De Marchis et al., 2001; Alifragis et al., 2002), these data indicate that SVZ-olfactory bulb explants are useful for studying postnatal forebrain neurogenesis.

### RA increases neurogenesis in SVZ-olfactory bulb explants

We next examined whether RA influences SVZ cell proliferation or survival in slices. P2 mouse explants containing the SVZ-olfactory bulb pathway were cultured with 10  $\mu$ M RA for 2 days, and BrdU (5  $\mu$ M) was added for 2 hours prior to fixation at 2 DIV. RA treatment increased SVZ BrdU-labeled cell numbers by about 33% above control values (mean cells/0.0676  $m^2$ : RA-treated: 473 $\pm$ 23; control: 354.5 $\pm$ 17;  $P = 0.004$ ; Fig. 4A-G). Similar results were found after 4 DIV (data not shown). A portion of the neural progenitors in the

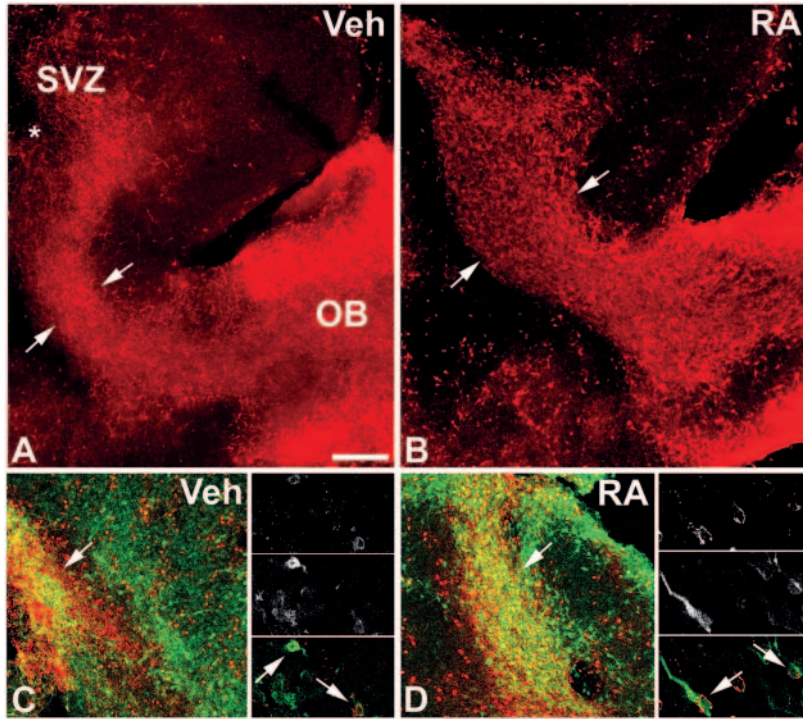
neonatal and adult rodent SVZ-olfactory bulb pathway normally undergoes cell death (Biebl et al., 2000). To examine potential survival effects of RA, P2 slices were cultured with 10  $\mu$ M RA for 4 DIV and apoptotic cells were identified by TUNEL staining. No difference in apoptotic cell numbers were found in the SVZ or RMS between RA- and vehicle-treated cultures (mean cell numbers: 112 $\pm$ 13 for RA; 101 $\pm$ 6 for controls;  $P = 0.42$ ; Fig. 4H,I). These results suggest that RA increases SVZ cell proliferation but not survival.

To explore whether RA specifically stimulates SVZ neuroblast proliferation, we cultured slices in the presence of 10  $\mu$ M RA or vehicle for 4 days and examined the expression of calretinin, which labels SVZ-olfactory bulb neuroblasts (Kato et al., 1999). RA treatment markedly increased calretinin immunoreactivity in the SVZ and RMS of explants (Fig. 5A,B). To confirm that the calretinin-positive cells were proliferative, BrdU was added to the medium for 24 hours during the second DIV and slices were cultured for 2 additional days (4 DIV total). Most calretinin-positive cells in the SVZ and RMS incorporated BrdU (Fig. 5C,D). Similar to the NS culture data, these results indicate that RA treatment augments neurogenesis in the neonatal SVZ.

### The RA precursor retinol stimulates SVZ neurogenesis in explants

RA is synthesized from retinol by the successive actions of alcohol dehydrogenase and RALDH enzymes (Duester et al., 2003). The RALDH3 isoform persists in the postnatal SVZ-olfactory bulb pathway (Wagner et al., 2002); therefore, RA is likely synthesized locally in these regions. To test whether retinol has effects similar to RA, we exposed P2 explants to retinol for 2 days and labeled proliferating cells with BrdU. We





**Fig. 5.** Exposure of explants to RA increases neurogenesis in the SVZ-olfactory bulb pathway. (A,B) Explants were treated with vehicle (Veh; A) or RA (10  $\mu$ M; B) for 4 DIV and then immunostained for calretinin. Note increased calretinin immunoreactivity and thicker RMS (arrows) in the RA-treated explant (B) compared with Veh (A). (C,D) Confocal images of re-sectioned explants (50  $\mu$ m thick slices) double immunostained for calretinin (green) and BrdU (red) after a 4-day exposure to Veh (C) or 10  $\mu$ M RA (D). Explants were incubated with BrdU from 24–48 hours in vitro. Most BrdU-labeled cells in the RMS (arrows) express calretinin; the RMS is expanded in the RA treated slice. Insets in panels C and D show double labeled cells (arrows; BrdU at top; calretinin middle; merged at bottom). OB: olfactory bulb. \*Denotes the lateral ventricle. Scale bar: 200  $\mu$ m for A,B; 100  $\mu$ m for C,D; 40  $\mu$ m for insets.

found that 10  $\mu$ M retinol increased cell proliferation and neuroblast numbers similar to RA treatment (Fig. 6A,B,I). To test whether this effect was mediated via RA synthesis, some retinol-treated cultures were simultaneously exposed to the RALDH inhibitor disulfiram. Disulfiram treatment blocked the proliferative effects of retinol (Fig. 6A–D,I). Retinol also mimicked the effect of RA on SVZ neurogenesis, as calretinin expression in the SVZ-olfactory bulb pathway of the vehicle, disulfiram, or disulfiram plus retinol groups was less than in retinol-treated slices (Fig. 6E–H). Compared with control, disulfiram treatment alone did not significantly decrease BrdU incorporation in SVZ cells. This lack of effect may be due to a relative paucity of ‘endogenous’ retinol remaining in the cultures for conversion to RA, and also suggests that the disulfiram was not toxic to SVZ cells. Alternatively, RA may be unnecessary for basal SVZ cell proliferation, although this idea is not supported by our *in vivo* studies (below). Taken together, these results suggest that RA is produced locally in the SVZ from retinol and augments postnatal neuroblast proliferation.

### Expression of dominant-negative retinoid receptors inhibits SVZ cell migration

RAR- and RXR-type retinoid receptors are ligand-activated transcription factors. In the presence of RA, they bind to DNA sequences containing RAREs and transactivate gene expression. Retinoid signaling probably involves heteromeric receptors containing both RAR and RXR (reviewed in Chambon, 1996). Dominant-negative (dn) receptors have been designed that lack transactivation domains and block signaling by competing with endogenous RA receptors (Feng et al., 1997; Xiao et al., 1999).

To inhibit retinoid signaling in the SVZ, we electroporated dnRAR $\alpha$  or dnRXR $\alpha$  into P2 mouse SVZ and prepared

explants (see Fig. 3D). A GFP reporter was co-electroporated to identify transfected cells, or electroporated alone as a control. Expression of dominant-negative RA receptors, alone or in combination, into the SVZ of P2 slices markedly affected SVZ cell migration. After 4 DIV, most SVZ cells expressing dnRAR $\alpha$  or both dnRAR $\alpha$  and dnRXR $\alpha$  failed to migrate toward the olfactory bulb (Fig. 7A–D). Expression of

dnRAR $\alpha$  or both dominant-negative receptors significantly shortened migration distances (Fig. 7G), and fewer GFP-labeled cells expressing dominant-negative receptors appeared in the distal RMS or olfactory bulb (Fig. 7H). Indeed, many cells expressing dominant-negative RA receptors appeared to migrate into the septum (Fig. 7B,D), and displayed a more undifferentiated or disorganized morphology than SVZ cells in control explants (Fig. 7E,F; Fig. 8).

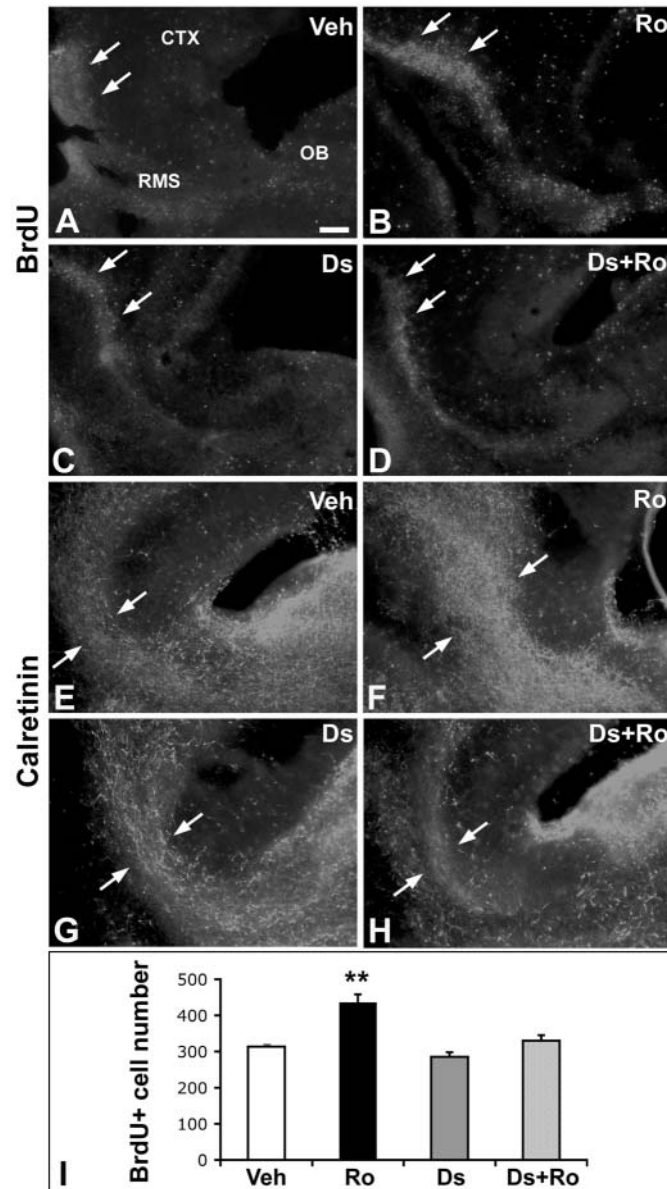
The vast majority of GFP-labeled cells likely expressed dominant-negative receptors given that we found an extremely high degree of dual expression when two reporter plasmids were co-electroporated (Fig. 3F). To ensure that GFP-positive cells also expressed a dominant-negative receptor construct, however, we electroporated a CMV-dnRAR-IRES-EGFP construct or CMV-IRES-GFP alone into P2 slices and cultured them for 4 days. After electroporation of the CMV-IRES-GFP control, GFP-positive cells migrated along the SVZ-olfactory bulb pathway (Fig. 7I). In contrast, GFP-labeled cells failed to migrate into the RMS after electroporation of the dnRAR-IRES-EGFP construct (Fig. 7J). Most GFP-positive cells appeared caudal and ventral to the SVZ/RMS in a pattern similar to that seen in the co-electroporation experiments (Fig. 7B).

In addition to the altered morphology and impaired migration of dominant-negative retinoid receptor-expressing cells, double labeling for GFP and the neural precursor marker nestin revealed that more GFP-positive cells in dnRAR $\alpha$ -, dnRXR $\alpha$ - and dnRAR $\alpha$ /dnRXR $\alpha$ -transfected cultures co-expressed nestin than in control cultures (Fig. 8A–F and data not shown). Also, fewer GFP-positive cells in the RMS expressing dominant-negative constructs co-expressed calretinin or PSA-NCAM (Fig. 7A–D; Fig. 8G–L). These results suggest that, in addition to stimulating SVZ neurogenesis, retinoid signaling is required for the normal differentiation and olfactory bulb migration of SVZ neuroblasts.

### Inhibition of RA synthesis decreases SVZ cell proliferation in vivo

The results of our in vitro experiments indicate that RA has multiple effects on postnatal SVZ progenitors. To examine the influence of RA on postnatal neurogenesis in vivo, mice

received disulfiram (5 mg kg<sup>-1</sup>, i.p.) or vehicle daily from P7-10 to inhibit endogenous RA synthesis. Proliferating SVZ cells were labeled with BrdU on P7, 6 hours after the first disulfiram injection. Four days of disulfiram treatment significantly decreased BrdU labeling in the dorsolateral SVZ (Fig. 9). Double-label immunofluorescence for BrdU and PSA-NCAM showed that, as expected, most BrdU-labeled cells in the SVZ also expressed PSA-NCAM (insets in Fig. 9A,B). These results therefore suggest that RA regulates the proliferation of forebrain SVZ neuroblasts in vivo.



**Fig. 6.** Retinol stimulates SVZ neurogenesis in explants. (A–D) BrdU immunolabeling of SVZ cells (arrows) in slices incubated with vehicle (Veh; A), retinol (Ro; B), disulfiram (Ds; C), or Ds and Ro (D). Explants were treated for 2 days, exposed to BrdU for 2 hours before fixation, and then resectioned at 40  $\mu$ m. Note the increased BrdU labeling in the Ro-treated slice (B) compared with vehicle (A) or Ds (C), and the lack of increased BrdU-positive cells in the explant cultured with both Ro and Ds (D). E–H: Confocal images of explants treated as in A–D and immunostained with an anti-calretinin antibody. Ro treatment (F) expanded the RMS (arrows) compared with the other groups, and the effect was blocked by co-treatment with Ds (H). I: Quantification of BrdU-positive cell numbers per fixed SVZ area (see Fig. 4C). \*\*Denotes  $P < 0.01$ . CTX: cortex. RMS: rostral migratory stream. OB: olfactory bulb. Scale bar: 100  $\mu$ m.

### Discussion

RA regulates multiple aspects of embryonic neural development, but little is known about its role in the postnatal forebrain. Here we show that RA-responsive cells persist in the postnatal mouse SVZ-olfactory bulb pathway. Both RA and retinol increase SVZ neurogenesis in NS and explant cultures, and blockade of retinoid signaling in SVZ cells inhibits their migration to the olfactory bulb and alters their morphology in slice cultures. Moreover, inhibition of RA synthesis in vivo decreases SVZ cell proliferation. These data indicate that RA plays an important role in regulating persistent neurogenesis in the postnatal forebrain SVZ-olfactory bulb pathway.

#### The role of RA in postnatal neurogenesis

Recent work suggests that the striatum and olfactory bulb share similar developmental mechanisms. The LGE contains two distinct progenitor populations that give rise to striatal projection neurons and olfactory bulb interneurons (Stenman et al., 2003). LGE cells transplanted into the adult SVZ migrate to the olfactory bulb (Wichterle et al., 1999), and mutations of DLX1/2 or GSH1/2 homeobox genes cause abnormal development of both the striatum and olfactory bulb (Anderson et al., 1997; Toresson and Campbell, 2001). Because embryonic striatal development is regulated in part by RA (Wohl and Weiss, 1998; Toresson et al., 1999), the common LGE origin of striatal and postnatally-derived olfactory bulb interneurons suggests that RA may influence persistent SVZ-olfactory bulb neurogenesis. Our data indicate that RA does indeed modulate postnatal forebrain development, and that it regulates multiple steps in SVZ-olfactory bulb neurogenesis. Taken together with in vivo evidence that RA-responsive cells persist in the adult rodent SVZ-olfactory bulb pathway (Thompson Haskell et al., 2002) and findings that RA stimulates neurogenesis in adult hippocampal neural stem cell cultures (Takahashi et al., 1999), these results suggest that RA promotes neurogenesis throughout life in the rodent forebrain.

We examined the influence of RA on SVZ neurogenesis in NS cultures as well as an explant culture system that better reflects the in vivo environment. Our data show that RA treatment of SVZ-olfactory bulb slices stimulates SVZ neuroblast proliferation and expands the SVZ-olfactory bulb pathway without influencing cell survival. Moreover, we found that retinol stimulated neurogenesis in vitro while the RA synthesis inhibitor disulfiram decreased SVZ cell proliferation in vivo, suggesting that RALDH3 in the postnatal SVZ synthesizes RA to regulate SVZ neural precursors. In addition to demonstrating a mitogenic effect of RA, the results of the dominant-negative retinoid receptor experiments indicate that inhibition of RA signaling alters the morphology and



migratory behavior of SVZ cells. Because the morphology and antigen expression patterns suggest that dominant-negative retinoid receptor expression inhibits SVZ progenitor differentiation, the altered migration may be a secondary consequence of impaired differentiation. Indeed, a differentiation effect of RA on postnatal SVZ progenitors is supported by our finding of premature differentiation of NS expanded in the presence of RA (Fig. 2). RA also may directly influence both differentiation and migration of SVZ neuroblasts. These data indicate that RA regulates multiple steps in postnatal SVZ-olfactory bulb neurogenesis, and underscore the utility of combining NS, slice culture and *in vivo* approaches.

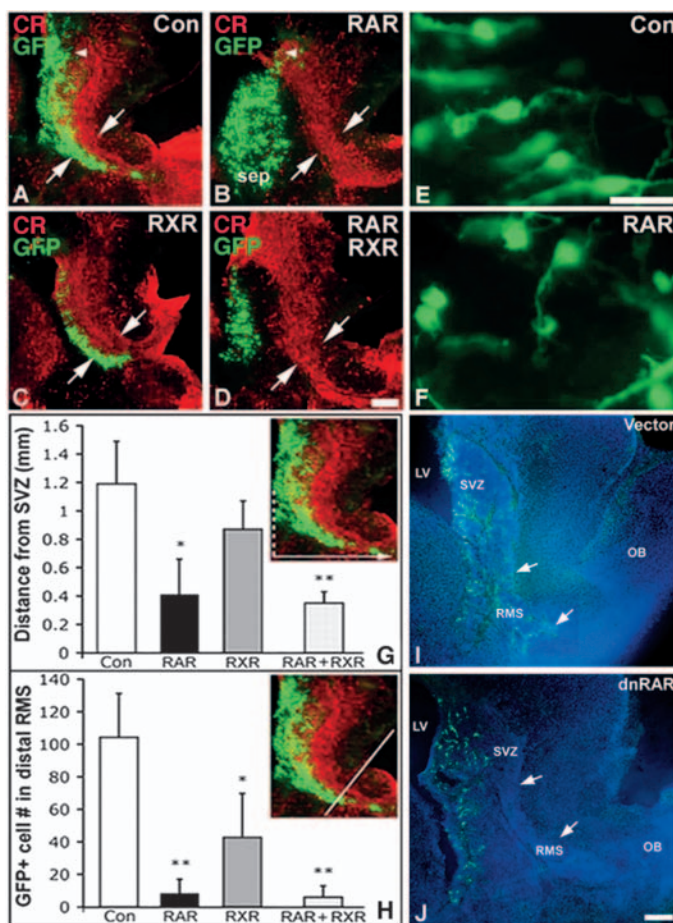
### Potential mechanisms underlying RA-induced SVZ neurogenesis

The precise SVZ cell type(s) and stage(s) of neurogenesis influenced by RA remain unclear. Postnatal SVZ-olfactory bulb neurogenesis involves multiple steps and progenitor cell states: the generation of transit-amplifying progenitors from neural stem cells (Doetsch et al., 1997, Doetsch et al., 1999; Johansson et al., 1999); the differentiation of transit-amplifying cells into neuroblasts (Doetsch et al., 2002b); the migration of neuroblasts to the olfactory bulb (Luskin, 1993; Lois and Alvarez-Buylla, 1994; Doetsch and Alvarez-Buylla, 1996; Lois et al., 1996; Hack et al., 2002); and the differentiation, survival and integration of adult-generated olfactory interneurons (Bayer, 1983; Betarbet et al., 1996; Brunjes and Armstrong,

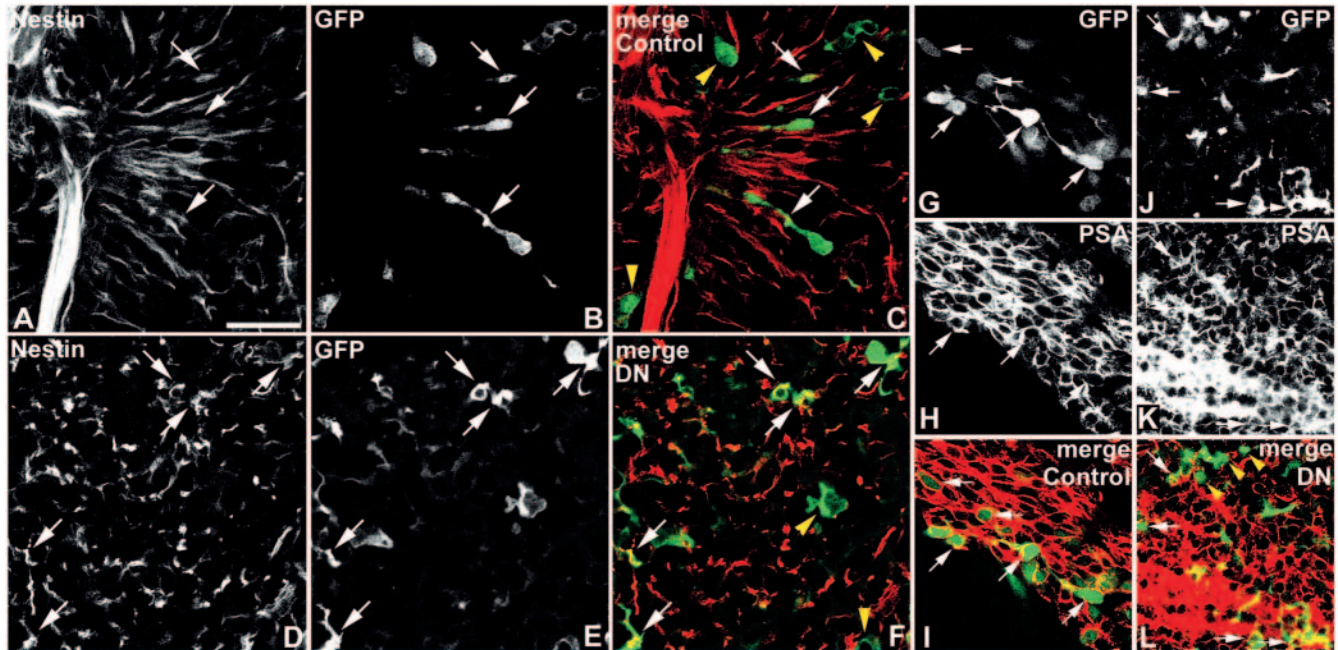
1996; Biebl et al., 2000; Petreanu and Alvarez-Buylla, 2002; Belluzzi et al., 2003). Prior work using RARE- $\beta$ -gal transgenic reporter mice showed that RA-responsive cells persist in the adult mouse SVZ-olfactory bulb pathway, but the cell types expressing the reporter were not defined (Thompson Haskell et al., 2002).

We found that RA exerted proliferative effects on differentiating NS and slice cultures within two-four days. In NS cultures, RA stimulated neuroblast proliferation and decreased that of astrocytes, leading to increased numbers of neurons and fewer astrocytes after 7 DIV. These results are consistent with effects of RA on committed progenitors, although modulation of multipotent progenitors in the NS cultures by RA cannot entirely be excluded (Doetsch et al., 2002a). The multiple passage NS and slice culture experiments suggest that RA also directly influences SVZ multipotent progenitors. RA treatment of expanding NS appeared to promote premature differentiation and impair NS self-renewal. Inhibition of retinoid signaling with dominant-negative receptors also altered the morphology of nestin-expressing, putative neural stem cells with radial glial-like morphology in the SVZ (Fig. 7E,F, Fig. 8A-F).

Our slice culture results suggest that altered RA signaling directly affects neuronal- or glial-restricted progenitors to modify SVZ cell proliferation and migration. RA synthesized in the SVZ-olfactory bulb pathway may act on neuroblasts to keep them from migrating out of the pathway, perhaps by



**Fig. 7.** Inhibition of RA signaling alters SVZ cell morphology and impairs migration to the olfactory bulb. (A-D) Images of GFP (green) and calretinin (red) immunofluorescence double labeling after electroporating SVZ cells with EGFP (A), or EGFP plus dominant-negative (dn) RAR $\alpha$  (B), dnRXR $\alpha$  (C), or both dominant-negative constructs (D) as depicted in Fig. 3D. Explants were cultured for 4 DIV after electroporation and resectioned at 50  $\mu$ m. After electroporation of EGFP (Con; A) or EGFP plus dnRXR $\alpha$  (C), GFP-labeled cells appear in the SVZ and RMS and overlap with calretinin-expressing cells. Expression of EGFP with dnRAR $\alpha$  (B) or dnRAR $\alpha$  plus dnRXR $\alpha$  (D) decreases the migration of GFP-positive cells; many appear in the septum and are calretinin-negative. Arrows in A-D outline the RMS. (E,F) Higher magnification images of GFP+ SVZ cells near the regions marked with arrowheads in A and B, respectively. GFP-labeled cells in the Con explant show a radial cell-like morphology (E), while those co-expressing dnRAR $\alpha$  appear disorganized (F). (G) Quantification of migration distance from the SVZ to the most rostral GFP-positive cell in the distal RMS/olfactory bulb. Maximum migration distance was calculated by measuring the linear distance from the anterior border of the lateral ventricle to the most rostral GFP-positive cell (see inset and methods). GFP+ cells that express dnRAR $\alpha$  or dnRAR $\alpha$ /dnRXR $\alpha$  migrate significantly shorter distances. (H) Quantification of GFP-positive cell number in the distal RMS and olfactory bulb (rostral to the line shown in the inset) reveals significantly fewer GFP+ cells migrating to the bulb in explants expressing dominant-negative retinoid receptors. For G and H, single asterisk denotes  $P < 0.05$ , and double asterisk  $P < 0.01$ . (I,J) P2 slices were electroporated with a dnRAR-IRES-GFP plasmid (J) or IRES-GFP control (I) and cultured for 4 DIV, fixed and resectioned at 50  $\mu$ m. After electroporation of IRES-GFP vector alone (Vector), GFP-labeled cells migrated in the RMS toward the olfactory bulb (OB). SVZ cell expression of dnRAR-IRES-GFP (dnRAR) inhibits the rostral migration of GFP-positive cells. Arrows in I and J point to the RMS. Scale bar: in D, 200  $\mu$ m for A-D; in E, 25  $\mu$ m for E,F; in J, 200  $\mu$ m for I,J.

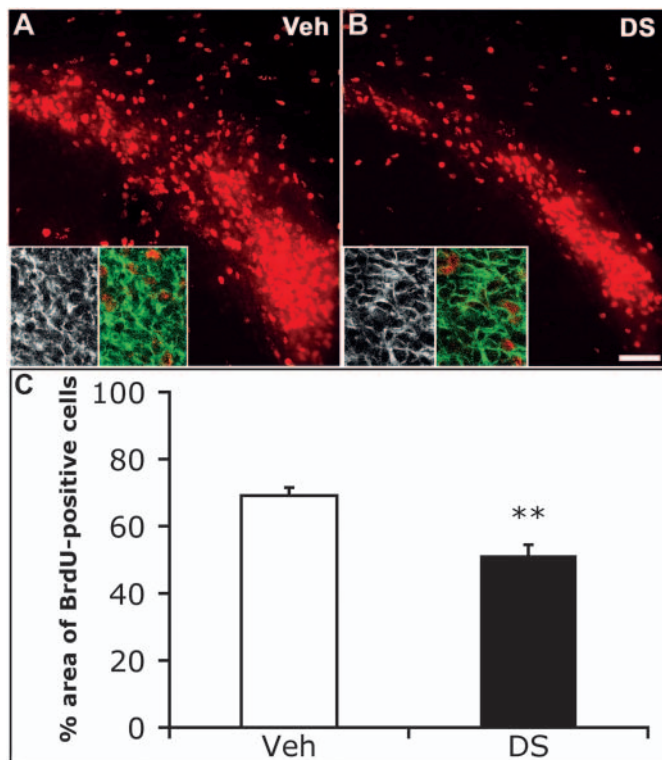


**Fig. 8.** Inhibition of RA signaling alters SVZ cell morphology and differentiation. Confocal images of 50  $\mu\text{m}$ -thick resected explants immunostained for GFP (green) and nestin (red in C, F) or PSA-NCAM (red in I, L). In the EGFP-electroporated control (A-C), some GFP-labeled SVZ cells express nestin (arrows) and have typical radial cell morphology. Others express GFP only (arrowheads). After GFP reporter co-electroporation with dnRAR $\alpha$  (D-F), GFP-labeled cells lack radial morphology, have shorter processes, and more co-express nestin (arrows). Arrowheads show GFP single-labeled cells. G-L: In a typical control explant (G-I), most GFP+RMS cells express PSA-NCAM (arrows) and have a migratory morphology (fusiform shape and leading/lagging processes); in contrast, RMS cells co-expressing GFP and dnRAR $\alpha$  (J-L) have more irregular cell bodies (arrows); many appear outside of the RMS and lack PSA-NCAM expression (arrowheads). Scale bar: 25  $\mu\text{m}$ .

regulating the expression of migration factor receptors. Alternatively, blockade of RA signaling could act on the

transit-amplifying cells to inhibit their differentiation or change the identity of their progeny, perhaps from neurons to astrocytes, and thereby alter their migratory behavior. This latter mechanism is suggested by the altered morphology of SVZ cells expressing dominant-negative receptors that fail to migrate into the RMS in explants (Fig. 7). The finding that RA treatment inhibits astrocyte generation while increasing neurogenesis in differentiating NS cultures also is consistent with this idea. Further study is needed to determine which specific cell types synthesize RA, which are transcriptionally activated by retinoid signaling, and what downstream genes are influenced to regulate neurogenesis.

In terms of potential downstream molecules influenced by retinoid signaling in SVZ progenitors, the transcription factors



**Fig. 9.** Blockade of endogenous RA synthesis inhibits forebrain SVZ cell proliferation. (A,B) Coronal sections through the anterior SVZ show confocal images of BrdU immunostaining after treatment of mice with vehicle (Veh, A) or disulfiram (DS; B) daily from P7-10. A single BrdU injection was administered 6 hours after the first DS/Veh injection. DS treatment decreases SVZ BrdU labeling compared with Veh. Insets in A and B are high magnification images of PSA-NCAM (left panels) or merged PSA-NCAM/BrdU double immunolabeling (right panels) showing that most BrdU-positive cells (red) in the SVZ are PSA-NCAM-positive (green) neuroblasts. (C) Quantification of BrdU immunoreactivity in a fixed dorsolateral SVZ area shows decreased BrdU labeling in the SVZ of DS-treated mice compared with controls. \*\*Denotes  $P < 0.01$ . Scale bars: 50  $\mu\text{m}$  for A,B; 20  $\mu\text{m}$  for insets.



PBX1 and MASH1 (ASCL1 – Mouse Genome Informatics) are attractive candidates. PBX1 and MASH1 are expressed during RA-induced neurogenesis in P19 cells (Johnson et al., 1992; Knoepfler and Kamps, 1997). They also persist in the adult rodent forebrain SVZ (Redmond et al., 1996; Murray et al., 2003). Upstream of RA signaling, the transcription factor PAX6 may be important for SVZ neurogenesis. PAX6 acts via retinoid signaling to regulate eye development (Enwright and Grainger, 2000). Interestingly, the embryonic olfactory bulb does not form in PAX6 mutant mice, possible due to a lack of RA production in the telencephalon (Anchan et al., 1997). PAX6 also promotes neurogenesis in postnatal SVZ NS cultures (Heins et al., 2002). Recent work, however, suggests a more complicated relationship between PAX6 expression and retinoid signaling during forebrain development (Marklund et al., 2004; Waclaw et al., 2004). Future studies directed at identifying the mediators of RA signaling in the postnatal SVZ-olfactory bulb pathway will be necessary to better understand the complex regulation of persistent forebrain neurogenesis.

### RA and injury-induced neurogenesis in the SVZ-olfactory bulb pathway

Prior studies of SVZ neurogenesis in adult rat stroke models suggest that new striatal medium spiny neurons are generated from SVZ neural precursors after focal ischemia (Arvidsson et al., 2002; Parent et al., 2002). Our finding that RA regulates postnatal neurogenesis in the SVZ-olfactory bulb pathway, together with previous data showing that RA regulates the differentiation of embryonic striatal neurons (Valdenaire et al., 1998; Wohl and Weiss, 1998; Toresson et al., 1999), raise the possibility that RA may be useful for augmenting injury-induced striatal neurogenesis. Retinoid signaling is also a potential mediator of ischemia-induced striatal neurogenesis. RA increases neurogenesis from adult hippocampal-derived neural stem cells, in part by upregulating their expression of neurotrophin receptors (Takahashi et al., 1999). Several groups have shown that increasing forebrain levels of brain derived neurotrophic factor (BDNF) in adult rats induces striatal neurogenesis (Benraiss et al., 2001; Pencea et al., 2001). Therefore, increased retinoid signaling after stroke may induce the expression of trkB, the BDNF receptor, on SVZ progenitors to stimulate striatal neurogenesis. Such a role fits with the regenerative effects of RA in other systems (reviewed in Maden and Hind, 2003). A better understanding of how RA regulates postnatal neurogenesis may therefore offer regenerative strategies to treat brain injury or degeneration.

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