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Opposing effects of retinoid signaling on astrogliogenesis in embryonic day 13 and 17 cortical progenitor cells

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Abstract

All-trans retinoic acid (RA) is a differentiation factor in many tissues. However, its role in astrogliogenesis has not been extensively studied. Here, we investigated the effect of RA on the regulation of astrogliogenesis at different cortical developmental stages. We prepared rat cortical progenitor cells from embryonic day (E) 13 and E17, which correspond to the beginning of neurogenic and astrogliogenic periods, respectively. Surprisingly, RA promoted astrogliogenesis at E17 but inhibited astrogliogenesis induced by ciliary neurotrophic factor (CNTF) at E13. The inhibitory effect of RA on astrogliogenesis at E13 was not due to premature commitment of progenitors to a neuronal or oligodendroglial lineage. Rather, RA retained more progenitors in a proliferative state. Furthermore, RA inhibition of astrogliogenesis at E13 was independent of STAT3 signaling and required the function of the α and β isoforms of the RA receptors (RAR). Moreover, the differential response of E13 and E17 progenitors to RA was due to differences in the intrinsic properties of these cells that are preserved *in vitro*. The inhibitory effect of RA on cytokine-induced astrogliogenesis at E13 may contribute to silencing of any potential precocious astrogliogenesis during the neurogenic period.

The mammalian cortex is derived from neural stem/progenitor cells that line the ventricular cavities and give rise to all three major cell types found in the central nervous system (CNS), neurons, astrocytes and oligodendrocytes. The generation of these three cell-types occurs sequentially: neurons are made first, followed by astrocytes, and then oligodendrocytes (Sauvageot & Stiles 2002). This temporally sequential pattern of differentiation observed *in vivo* is preserved even in dissociated cultures *in vitro*, suggesting that an intrinsic developmental program governs lineage specification of neural stem/progenitor cells (Davis & Temple 1994, Luskin *et al.* 1988). However, extrinsic cues, such as growth factors, cytokines, and hormones, can alter the sequence of differentiation. For example, astrogliogenesis can be induced during the neurogenic period by Notch (Tanigaki *et al.* 2001), bone-morphogenic proteins (BMPs) (Gross *et al.* 1996), and the CNTF family cytokines (Bonni *et al.* 1997, Molne *et al.* 2000, Nakashima *et al.* 1999, Yanagisawa *et al.* 1999, Ochiai *et al.* 2001).

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Several key questions remain to be addressed regarding the orderly production of neurons and astroglia. Although pro-astrogliogenic cytokines are expressed during the neurogenic period in the developing cortex (Derouet *et al.* 2004, Uemura *et al.* 2002), astrocytes are not produced and the mechanism responsible for silencing astroglialogenesis during the neurogenic period is unclear. Furthermore, mechanisms governing the exact timing and the developmental switch from neurogenesis to astroglialogenesis remain controversial. There is evidence suggesting that astroglialogenesis is initiated by induction of pro-glial factors at distinct developmental stages (Lillien *et al.* 1988, Stockli *et al.* 1991). However, other reports indicate that changes in the competence of progenitors to respond to respective ligands may play a pivotal role in regulating specific responses and differentiation patterns throughout development (Mehler *et al.* 2000, Molne *et al.* 2000).

One of the extensively studied signalling pathways underlying astroglialogenesis is the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) 1 and 3 (Bonni *et al.* 1997, Nakashima *et al.* 1999). Upon receptor binding and activation, CNTF and related ligands activate the receptor-associated tyrosine kinase JAK, which phosphorylates STAT1 and STAT3. Once phosphorylated, STAT1 and STAT3 dimerize and translocate to the nucleus where they induce STAT-dependent expression of astrocyte-specific genes including glial fibrillary acidic protein (GFAP) (Bonni *et al.* 1993).

All-trans retinoic acid (RA), a natural derivative of vitamin A (retinol), was first recognized to be critical for body axis formation as well as for the development of heart, lung, and retina (Wilson *et al.* 1953). RA, acting as a caudalizing factor, is crucial for the proper development of the spinal cord and hindbrain (White *et al.* 1998, Niederreither *et al.* 2000, Wendling *et al.* 2001). Furthermore, *in vitro* studies suggest a major role for RA in regulating the differentiation of neurons (Henion & Weston 1994), astrocytes (Wuarin *et al.* 1990), and oligodendrocytes (Noll & Miller 1994, Staines *et al.* 1996). Therefore, RA is believed to act as a general differentiation factor for many different tissues rather than favoring differentiation towards any specific lineage.

Little is known about the specific actions of RA on astroglialogenesis at different developmental stages. Here, we show that RA has opposing effects on astroglialogenesis in cortical progenitors derived from early vs. late developmental stages. While RA specifically inhibits CNTF-induced astroglialogenesis at E13 by retaining the progenitors in a proliferative state, it promotes astroglialogenesis in progenitors derived from E17 cortex. Furthermore, we demonstrate that the differential response of cortical progenitors to RA is due to changes in their intrinsic cellular properties.

Experimental Methods

Materials

RA, the pan-RAR-agonist TTNPB, the RAR α -specific agonist Am580 and the RAR γ -specific agonist CD437 were purchased from Sigma-Aldrich, Inc. (Saint Louis, MO). LE135, a RAR β -specific antagonist, was purchased from Tocris Bioscience (Ellisville, MO). BMS 213309, a RAR β -specific agonist, and BMS 189453, a RAR-pan-antagonist, were kindly provided by Bristol-Myers Squibb (Princeton, NJ). The selectivity and biological effects of these retinoids have been previously characterized in several reports (Li *et al.* 1999, Gehin *et al.* 1999, Matt *et al.* 2003, Schoenemark *et al.* 1999, Vivat-Hannah *et al.* 2001, Chapellier *et al.* 2002). RA and related retinoids were dissolved in DMSO. Stock solutions (10 mM) were aliquoted and stored at -80°C . EGF (epidermal growth factor), bFGF (basic fibroblast growth factor) and CNTF were purchased from Chemicon- International (Temecula, CA).

The following plasmids have been described: UB6-LacZ (Poser *et al.* 2003), human GFAP-luc (positions -1873 to +130) (Tanigaki *et al.* 2001), the STAT-3 responsive luciferase reporter (4×APRE-luc) (Nakajima *et al.* 1996), and the expression vector for dominant negative RAR (LRAR α 403SN) (Tsai *et al.* 1992).

E13 and E17 cortical progenitor cell culture

E13 and E17 dorsal telencephalon were dissected out from Sprague-Dawley rats, enzymatically digested with cysteine-activated papain (Worthington Biochemical Corporation, Lakewood, NJ), and mechanically dissociated into single cells with a 5-ml pipette. Dissociated cells were plated as a monolayer on poly-D-lysine/laminin (BD Bioscience, Bedford, MA) coated plates in culture medium containing Neurobasal Medium and 2% B27 (Invitrogen, Inc., Carlsbad, CA), 35 mM glucose, 1 mM L-glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 20 ng/ml bFGF $\pm$ 20 ng/ml EGF as indicated in figure legends. The cultures were maintained in a humidified incubator with 5% CO₂ at 37 °C. On the day after plating of E13 cultures, approximately 90% of the cells were nestin⁺ and 21% of the cells were β III tubulin⁺. On the day after plating of E17 cultures, 25% and 59% of the cells were nestin⁺ and β III tubulin⁺, respectively. However, after culture for 4–5 days in the presence of bFGF and EGF, neural progenitor cells greatly outnumbered the neurons in E17 cultures due to the proliferative effects of bFGF and EGF.

For neurosphere-forming assays, freshly dissociated E13 cortical progenitor cells were plated at clonal density on uncoated plates in the regular culture medium plus 1% N2 supplement (Invitrogen, Inc) as described (Liu *et al.* 2006). After treatment with RA and CNTF (50 ng/ml), spheres were transferred to poly-D-lysine/laminin coated plates on the 5th day *in vitro* (DIV). Three hours after the transfer, the medium was replaced with fresh media lacking bFGF and EGF to allow differentiation. Spheres were fixed and processed for immunocytochemistry 3–4 days later.

[³H] Thymidine incorporation assay

E13 cortical progenitors were cultured on 24-well plates as described above and then processed for [³H] thymidine incorporation as described (Faigle *et al.* 2004). Briefly, cells were treated on DIV1 with RA for 24 hr. During the final 4 hr of RA incubation, cells were pulsed with [*methyl*-³H] thymidine (0.5 μ Ci/ml). Cells were then washed with phosphate-buffered saline (PBS) and precipitated with 5% trichloroacetate for 30 min on ice. The precipitate was subsequently dissolved in 1 M NaOH for 20 min and neutralized with 1M HCl. After transferring the solution into vials containing scintillation fluid, radioactivity was measured using a β -counter.

Transient transfection of E13 cortical progenitors and reporter gene assay

Freshly dissociated E13 cortical progenitors were spun down at 735 $\times$ g for 5 min and resuspended in Rat Neural Stem Cell Nucleofector Solution (Amaxa Bioscience, Inc., Gaithersburg, MD) to $2\text{--}4 \times 10^6$ cells/100 μ l. After addition of 5–10 μ g of total plasmid DNA, the DNA/cell-mixture was transferred to an Amaxa electroporation cuvette and electroporated using the Amaxa Nucleofector device. Immediately after electroporation, pre-warmed culture medium was added to the cells and the cell suspension was incubated at 37°C for another 15 min. The cells were then plated into 24-well plates at a density of 1.5×10^5 cells/well. Twenty-four hours after plating, cells were treated with RA for 24 hr, followed by CNTF treatment for another 24 hrs. The human GFAP promoter-driven luciferase reporter activity was normalized to the co-transfected β -galactosidase (UB6-LacZ) activity. Each experiment was performed in triplicate or quadruplicate.

Western analysis

The following primary antibodies were used: mouse monoclonal anti-GFAP (1:800; Sigma-Aldrich, Inc.), mouse monoclonal anti- β III tubulin (1:1000; Promega, Madison, WI), mouse monoclonal anti- β -actin (1:10,000; Sigma-Aldrich, Inc.), mouse monoclonal anti-Nestin (1:1000; BD Bioscience, Bedford, MA), mouse monoclonal anti-PLP (1:250; Chemicon International, Temecula, CA), rabbit polyclonal anti-gp130 (1:250; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-phospho-STAT3 (1:1000; Cell Signaling Technology, Beverly, MA), and rabbit polyclonal anti-STAT3 (1:1000; Cell Signaling). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin G was used as secondary antibodies at concentrations of 1:15,000. Signals were detected by enhanced chemiluminescence.

Immunocytochemistry

Cultured cells were fixed in 4 % paraformaldehyde for 30 min at room temperature, then incubated with PBS blocking buffer containing 0.2 % Triton-X, 5 % BSA and 3 % goat serum for one hr and incubated overnight at 4 °C with primary antibodies diluted in the blocking buffer. After several washes, the cells were incubated with anti-rabbit Alexa Fluor 488 or 594, anti-mouse Alexa Fluor 488 or 594 (1:1000, Molecular Probes, Eugene, OR) for two hr. For nuclear counterstaining, the cells were incubated in Hoechst 33258 for 20 min in the last wash prior to mounting. The following primary antibodies were used: rabbit polyclonal anti-GFAP (1:500; Dako, Carpinteria, CA), mouse monoclonal anti- β III tubulin (1:1000; Promega, Madison, WI), mouse monoclonal anti-MAP2 (1:1000; Sigma-Aldrich, Inc), mouse monoclonal anti-BrdU (1:1000; Oncogene, Boston, MA), mouse monoclonal anti-S100 β (1:500, Sigma-Aldrich, Inc), rabbit anti-active caspase 3 (1:1000, R&D systems, Minneapolis, MN), rabbit polyclonal anti-Olig2 (1:40,000, a kind gift from Dr. John Alberta, Dana-Farber Cancer Institute, Boston, MA), and rabbit polyclonal anti-NG2 (1:300, a kind gift from Dr. William Stallcup, Burnham Institute for Medical Research, San Diego, CA).

Quantification of total apoptosis—Apoptosis was determined by nuclear condensation and/or fragmentation after Hoechst staining; healthy cells have evenly and uniformly stained nuclei (Liu *et al.* 2003). Apoptosis was also quantified by active-caspase 3 or TUNEL staining.

TUNEL labeling

TUNEL assay for DNA fragmentation was performed using DeadEnd™ Fluorometric TUNEL system (Promega, Madison, WI) per manufactory's instruction. Briefly, cells were fixed with 4% paraformaldehyde and permeablized in PBS containing 0.2% Triton X-100. Cells were then incubated at 37°C for 1 hr with TUNEL mixture containing Nucleotide Mix and rTdT enzyme. The fluorescein-12-dUTP-labeled DNA was visualized by fluorescence microscopy. The image was pseudo-colored to red for better co-visualization with Hoechst staining (blue).

BrdU incorporation

Cells were treated with RA on DIV1 for 20 hrs, then pulsed with 10 μ M BrdU for another 4 hr. Cells were then fixed and processed for anti-BrdU immunostaining.

RT-PCR

Total cellular RNA was extracted using TRIZOL Reagent and the synthesis of cDNA and PCR was carried out using one-step RT-PCR kit (Invitrogen) according to the manufacturer's protocol. The cycling parameters were as follows: reverse transcription at 55°C for 30 min, first denaturation at 94°C for 3 min followed by 25–35 cycles of denaturation at 94°C for 1 min, annealing step at 51°C for 1 min, and extension step at 72°C for 1.5 min. The last cycle consisted of one last extension step at 72°C for 10 min. The following oligonucleotide primers

were used: RAR α sense 5'-CAG ATG CAC AAC GCT GGC and antisense 5'-CCG ACT GTC CGC TTA GAG; RAR β sense 5'-GCG GAG AGA TCA TGT TTG AC and antisense 5'-TGG CAT CGA TTC CTA GTG AC; RAR γ sense 5'-GTG GAG ACC GAA TGG ACC and antisense 5'-GAC AGG GAT GAA CAC AGG; CNTF-R- α sense 5'-CTC CAG AAA ATG TGG TG and antisense 5'-TCA CAG ATC TTC GTG GTG; β -actin sense 5'-AAG ATG ACC CAG ATC ATG TTT GAG and antisense 5'-AGG AGG AGC AAT GAT CTT GAT CTT. The samples were analyzed on a 1% agarose gel containing ethidium bromide.

Statistical analysis

All experiments were replicated at least three times. Overall significance was determined by submitting the data to one-way analysis of variance (ANOVA). The significance of between-group differences was determined by the Scheffé post-hoc test or the student t-test, two-tailed analysis. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not statistically significant. Error bars are standard error of the mean (SEM).

Results

RA inhibits CNTF-induced astrogliogenesis in E13 cortical progenitor cultures

Very few GFAP⁺ astroglial cells were detected when cortical progenitors prepared from E13 were cultured for 4 days (Fig. 1a), consistent with the notion that cortical progenitors primarily undergo neurogenesis at this time point (Bonni et al. 1997, Liu et al. 2006, Sun *et al.* 2001). As anticipated, treatment with CNTF on DIV2 for 2 days increased the number of GFAP⁺ cells from almost undetectable to about 37%. Although treatment with RA alone had no effect on the number of GFAP⁺ cells, a 24 hr pre-treatment with RA significantly reduced the number of CNTF-induced GFAP⁺ astrocytes in a dose-dependent manner. The inhibitory effect of RA on CNTF-induction of astrogliogenesis was confirmed when GFAP expression was analyzed by Western blotting (Fig. 1b). Furthermore, RA pre-treatment suppressed CNTF stimulation of GFAP-promoter driven luciferase activity (Fig. 1c). The production of astrocytes was monitored by another astroglial marker S100 β (Fig. 1d), a protein expressed in newly generated, immature astrocytes. CNTF induction of S100 β ⁺ cells was also greatly inhibited by 24 hr pre-treatment of RA.

The results described above were obtained using progenitors grown as a monolayer in adherent cultures. We also analyzed the effect of RA on astrogliogenesis using a neurosphere-forming assay. It is generally believed that only multipotent, neural stem/progenitor cells, but not fate-committed precursor cells, have the capacity to form neurospheres in suspension culture (Reynolds & Weiss 1992). Spheres were comparable in terms of the number and size among the different treatment groups. In cultures treated with vehicle control or RA alone, virtually all spheres contained less than 10% GFAP⁺ astrocytes (Fig. 1, e and f). However, almost 80% of the spheres contained >40% GFAP⁺ astrocytes after CNTF treatment. Pre-treatment with RA before addition of CNTF greatly inhibited CNTF-induced astrogliogenesis in the neurosphere assay.

The above experiments suggested that RA antagonizes CNTF-induced astrogliogenesis in E13 cortical progenitors after 2 days in culture. One might argue that 2 days in culture was not long enough for differentiation to occur and that RA merely delayed terminal differentiation of astrocytes. To address this issue, CNTF-induced astrogliogenesis after 6 days in culture was investigated. RA-treatment significantly reduced the number of GFAP⁺ and S100 β ⁺ cells even after 6 days in culture (Fig. 2). These data support the conclusion that RA inhibits CNTF-induced astrogliogenesis in cortical progenitors during the neurogenic period.

Pre- but not co-incubation of RA is sufficient to inhibit CNTF-induced astroglialogenesis of E13 cortical progenitors

In the experiments described in Figures 1 and 2, RA or vehicle control was added to the cell culture 24 hr before and was continuously present during CNTF treatment (Pre/Co). To define the time frame in which RA treatment is effective at suppressing astroglialogenesis, RA was added to the culture simultaneously with CNTF (Co), or only for the 24 hr pre-treatment before CNTF was added (Pre). Co-treatment of RA did not affect CNTF induction of GFAP⁺ cells (Fig. 3a) or the level of GFAP protein expression (Fig. 3b). However, pre-treatment with RA for 24 hr was equally effective at inhibiting CNTF-induced astroglialogenesis regardless of whether RA was continuously present during the subsequent CNTF treatment. Therefore, a 24 hr pre-treatment of RA but not its continued presence is necessary for RA to exert an inhibitory effect on CNTF-induced astroglialogenesis.

Lack of competence of E13 cortical progenitors to respond to CNTF after RA pre-treatment is not due to premature commitment of progenitors to a non-astroglial lineage

Since a 24 hr pre-treatment is necessary for RA to inhibit CNTF-induced astroglialogenesis, we considered the possibility that progenitors prematurely commit to a non-astroglial lineage within 24 hr of RA treatment, and thus lose competence to differentiate into astroglia in response to CNTF. We performed [³H] thymidine incorporation and BrdU-labelling experiments to determine whether the cells exit the cell cycle within 24 hr of RA treatment. Neither the amount of [³H] thymidine incorporation nor the percentage of BrdU⁺ cells was significantly changed when E13 cortical progenitor cultures were exposed to RA for 24 hr (Fig. 4, a and b). This suggests that a 24 hr RA pre-treatment does not significantly reduce the progenitor pool by inducing cell cycle exit.

Since RA has been implicated in neuronal differentiation, we tested the hypothesis that a 24 hr pre-treatment with RA irreversibly induces the E13 cortical progenitors to a neuronal fate. After 24 hr treatment of RA, E13 cortical progenitor cultures were thoroughly washed to remove RA, and the cells were placed in fresh medium and cultured for an additional 7 days. Neuronal differentiation was measured by immunostaining for MAP2 (Fig. 4, c and d), a marker for mature neurons. Treatment of RA for 24 hr did not change the percentage of MAP2⁺ cells. A 24 hr RA pre-treatment also did not affect the number of cells expressing β III tubulin (Fig. 4e), a marker for newly generated, immature neurons, or the expression of β III tubulin protein levels (Fig. 4, f and g). Similarly, the protein expression levels for nestin, a marker for neural stem/progenitor cells, and phospholipoprotein (PLP), a marker for oligodendrocytes, were unaffected by 24 hr RA treatment (Fig. 4g). These data suggest that a 24 hr RA exposure reduces the competence of E13 cortical progenitors to differentiate into astroglial cells upon CNTF-stimulation, without affecting cell fate-specification towards neuronal or oligodendrocyte lineage.

RA retains E13 cortical progenitors in a proliferative state

What is the fate of those E13 progenitors that do not become astroglia in the presence of both CNTF and RA? They did not differentiate into oligodendrocytes since there was no increase in the number of cells staining positive for Olig2 or NG2, markers for oligodendrocytes (Fig. 5, a and b). They also did not become neurons because the number of cells staining positive for MAP2 or β III tubulin was not significantly different when progenitors were treated with CNTF either in the presence or absence of RA (Fig. 5c and data not shown). Furthermore, RA did not affect apoptosis, assessed by nuclear fragmentation/condensation (Fig. 5d), positive labelling for active caspase 3 (Fig. 5e) or TUNEL (Fig. 5f). However, there were more BrdU⁺ cells in cultures treated with CNTF together with RA than with CNTF alone (Fig. 5g).

bFGF and EGF were included in the medium for the entire duration of the experiments described so far. To rule out the possibility that bFGF and EGF interfere with astrocyte differentiation or CNTF induction of astrogliogenesis in E13 cortical progenitors, cultures were treated and analyzed as in Fig. 5 except that bFGF and EGF were removed from the medium during CNTF treatment. The results were essentially the same as before; RA inhibited CNTF induction of astrogliogenesis (Fig. 6a) with no statistically significant effect on the number of neurons (Fig. 6b) or oligodendrocytes (Fig. 6c). However, more cells were nestin⁺ and BrdU⁺ when cultures were treated with CNTF+RA than with CNTF alone (Fig. 6, d and e). Together, these data suggest that RA retains more E13 cortical progenitors in a proliferative state in the presence of CNTF, thereby preventing CNTF from inducing astrogliogenesis.

RAR activation is necessary and sufficient to inhibit CNTF-induced astrogliogenesis at E13

The diverse and complex biological functions of all-trans retinoic acid (RA) are mainly mediated through the RAR class of nuclear receptors, RAR α , RAR β , and RAR γ which are encoded by distinct genes. RA binds to and activates RARs which induce the expression of target genes (Mark *et al.* 2006, Lane & Bailey 2005). However, recent studies suggested that RA could function independently of RAR (Radomska-Pandya *et al.* 2000, Aggarwal *et al.* 2006). To determine whether RA inhibits CNTF-induced astrogliogenesis via a RAR-dependent mechanism, we transfected E13 cortical progenitors with a truncated RAR, which lacks the transcriptional activation domain but retains DNA binding and ligand-binding domains, thus functioning as a dominant negative RAR to block RA-induced transcription afforded by endogenous RAR (Tsai *et al.* 1992). The effect of expressing this mutant RAR on RA inhibition of astrogliogenesis was examined in the total cell population after transient transfection, since the transfection efficiency by the Nucleofection method was more than 80% (Supplemental Fig. 1). Transient transfection of the dominant negative RAR almost completely attenuated the inhibitory effect of RA on CNTF-induced astrogliogenesis (Fig. 7a). These data suggest that RAR-dependent signaling and transcription are required for RA inhibition of astrogliogenesis in E13 cortical progenitors.

To determine if RAR activation is sufficient to inhibit CNTF-induced astrogliogenesis, we utilized TTNPB, a pan-RAR-agonist that specifically binds to RAR (Sheikh *et al.* 1994). Pre-treatment with as little as 10 nM TTNPB for 24 hr inhibited CNTF-induction of GFAP protein expression as effectively as pre-treatment with 1 μ M RA (Fig. 7b). To identify which subtypes of the RAR mediate the inhibitory effect of RA, we utilized Am580, a RAR α specific agonist (Brooks *et al.* 1996), BMS 213309, a RAR β specific agonist (Koubova *et al.* 2006), and CD437, a RAR γ specific agonist (Martin *et al.* 1992). Pre-incubation of E13 cortical progenitors with as little as 1 or 10 nM of the RAR α specific agonist blocked CNTF-induction of GFAP⁺ cells (Fig. 7c) or GFAP protein expression (Fig. 7d) to the same extent as 1 μ M RA. Similarly, pre-treatment with 10 nM of the RAR β specific agonist was as effective as 1 μ M RA at blocking CNTF-induction of GFAP protein expression (Fig. 7e). In contrast, treatment with up to 100 nM of the RAR γ -specific agonist failed to alter GFAP protein expression (Fig. 7f). These data suggest that activation of RAR α and RAR β , but not RAR γ , inhibits CNTF stimulation of astrogliogenesis in E13 cortical progenitors.

RA inhibits CNTF-induced astrogliogenesis at E13 independently of JAK-STAT3-signaling

CNTF-induced astrogliogenesis requires JAK-STAT3 signaling (Bonni *et al.* 1997, Nakashima *et al.* 1999). Since a 24 hr pre-incubation, but not co-application of RA is required to inhibit CNTF-induced astrogliogenesis, and over-expression of a dominant negative RAR abolishes the inhibitory effect of RA, we investigated whether RA inhibition of CNTF-induced astrogliogenesis at E13 is mediated by perturbation in the expression or activities of components in the JAK-STAT3 signaling pathway. Treatment with 0–1 μ M RA for 24 hr did not affect the mRNA levels for CNTFR- α (Fig. 8a) or the protein levels of the gp130 CNTF

co-receptor (Fig. 8b). Furthermore, RA did not interfere with CNTF-induced activating tyrosine phosphorylation of STAT3 (Fig. 8c), nor did RA affect CNTF stimulation of STAT-3-luciferase activity (Fig. 8d). These data suggest that RA inhibition of CNTF-induced astroglial differentiation is independent of the JAK-STAT3 signaling cascade.

RA promotes astroglial differentiation in E17 cortical progenitor cultures

To determine whether RA inhibition of astroglial differentiation is developmentally restricted, we examined the effect of RA on astroglial differentiation in cortical progenitor cultures prepared from E17 cortex, a time when endogenous astroglial differentiation begins. In contrast to the observations made in E13 cultures, pre-treatment with 0–1 μ M RA synergized with CNTF in a dose-dependent manner to increase the levels of GFAP protein expression (Fig. 9a) and the number of GFAP⁺ cells in E17 cultures (Fig. 9b). Similarly, RA synergized with CNTF to induce astroglial differentiation even when bFGF/EGF were removed from the culture medium during CNTF treatment (Fig. 9c). Furthermore, treatment of E17 cultures with RA alone for 6 days is sufficient to induce astroglial differentiation by itself (Fig. 9, d and e). These data suggest a pro-astroglial function of RA in cortical progenitors derived from E17 cortex.

We also pre-treated E17 cortical progenitors with BMS 189453, a pan-RAR-antagonist (Gehin et al. 1999, Schulze *et al.* 2001) for 24 hr followed by incubation with CNTF or vehicle control for 6 d. Significantly, treatment with the pan-RAR-antagonist reduced both endogenous and CNTF-induced GFAP expression (Fig. 9f). Since the culture media and supplements do not contain retinoids but have Vitamin A, a precursor of RA, our data suggest that RAR-mediated signaling by endogenously produced retinoids may be involved in astroglial differentiation in E17 cortical progenitor cultures.

Activation of RAR α and RAR γ , but not RAR β , is sufficient to promote astroglial differentiation in E17 cortical progenitors

To determine if RAR stimulation is sufficient to induce astroglial differentiation at E17, cortical progenitor cultures were treated with subtype specific agonists of RAR as in Fig. 7. Treatment with the RAR α agonist alone for 6 d induced the expression of GFAP protein; 100 nM RAR α agonist was as effective as 1 μ M RA (Fig. 10a). Furthermore, treatment of cultures with the RAR α agonist on DIV1 for 24 hr synergistically induced CNTF-induced GFAP protein expression (Fig. 10b). Similar observations were made with the RAR γ -specific agonist (Fig. 10, c and d). By contrast, treatment with the RAR β specific agonist did not induce GFAP expression (Fig. 10, e and f). These data suggest that activation of RAR α or RAR γ but not RAR β , is sufficient to promote astroglial differentiation in E17 cortical progenitors.

Differential expression of RAR β may underlie the opposing effect of RA on astroglial differentiation in E13 and E17 cortical progenitors

Because studies using subtype specific agonists implicated different RAR subtypes in glial differentiation at E13 and E17, we determined whether the three genes encoding the RAR subtypes are differentially expressed at these two developmental stages. Semi-quantitative RT-PCR analysis revealed that the mRNAs encoding RAR α and RAR γ were expressed at comparable levels in E13 and E17 cortical progenitor cultures (Fig. 11a). However, RAR β mRNA was decreased at E17, which could explain why the RAR β agonist does not promote astroglial differentiation at E17. Furthermore, pre-incubation of E13 cortical progenitor cultures with LE135, a RAR β specific antagonist (Li et al. 1999), attenuated RA inhibition of CNTF-induced GFAP-expression (Fig. 11b). These data suggest that RAR β activation is not only sufficient by itself to inhibit glial differentiation, but also necessary to mediate RA inhibition of glial differentiation in E13 cortical progenitors. Furthermore, differential expression of RAR β may be responsible for the distinct effect of RA on astroglial differentiation in early (E13) and late (E17) cortical progenitors.

The differential response of E13 vs. E17 cortical progenitors to RA is due to dynamic changes in their intrinsic cellular properties during development

In the experiments described above, cortical progenitor cells were initially cultured in the presence of both EGF and bFGF. It is known that while E13 cortical progenitors do not express EGF receptors, cortical progenitors isolated from E17 contain both bFGF- and EGF-responsive populations (Eagleson *et al.* 1996, Burrows *et al.* 1997). Furthermore, EGF facilitates CNTF-mediated astrogliogenesis (Viti *et al.* 2003). To determine if the observed differences between the E13 and E17 progenitors simply reflect the inclusion of EGF in the culture medium, we repeated the experiments using bFGF only. Exclusion of EGF from the culture medium did not change the differential responsiveness of progenitors to RA: while RA inhibited CNTF-induced astrogliogenesis in E13 cortical progenitors (Fig. 12a), it promoted astrogliogenesis at E17 (Fig. 12c). RA also did not affect apoptosis in cultures maintained in bFGF alone (Fig. 12, b and d). Thus, the contrasting RA responsiveness in E13 vs. E17 cortical progenitors was not due to the absence or presence of the EGF- responsive progenitor cell population.

Our data indicate that cortical progenitors isolated from E13 and E17 differ in their response to RA. This could either be due to changes in cellular properties of the same progenitor cell population over time or due to different origins of the progenitor cell population isolated from E13 vs. E17 cortex. To determine whether progenitor cells display changes in intrinsic properties over time, we cultured progenitors from E13 cortex for 4 days in bFGF-containing medium to artificially mimic E17 *in vitro*. Cells were then transferred to new plates and treated the next day with CNTF $\pm$ RA using the same conditions as the regular E17 progenitor cultures. Interestingly, RA increased CNTF induction of GFAP⁺ and S100 β ⁺ cells in these E13-derived “pseudo E17” progenitor cultures (Fig. 13). These data suggest that cortical progenitors change their intrinsic properties from E13 to E17; this change is preserved *in vitro*, and that the differential effects of RA on astrogliogenesis reflect intrinsic differences between E13 and E17 progenitors.

Discussion

The objective of this study was to elucidate the role of retinoid signaling in astrogliogenesis during cortical development. We discovered an opposing effect of RA on astrogliogenesis in cortical progenitors derived from neurogenic (E13) vs. astrogliogenic (E17) periods. While RA inhibited CNTF-induced astrogliogenesis in cultured E13 cortical progenitors, it promoted astrogliogenesis by itself or in synergy with CNTF in cultured progenitor cells derived from E17 cortex. The effects of RA were mediated by RAR signaling. RA is commonly believed to be a general pro-differentiation factor in neural and non-neural tissues (Mey & McCaffery 2004, Maden 2001). To our knowledge, this is the first report that RA inhibits differentiation towards a specific lineage, and particularly, towards astrogliogenesis during development.

Only a few classes of molecules are known to promote differentiation of progenitors to opposing fates with a dependency on the developmental stage. For example, BMP signaling induces neuronal differentiation in E12–E14 rodent cortical progenitors at the expense of astrogliogenesis (Li *et al.* 1998). However, BMP promotes astrogliogenesis in cortical progenitors prepared from the gliogenic period at the expense of neuronal differentiation (Gross *et al.* 1996, Mabie *et al.* 1997). The effect of BMP on astrogliogenesis was dependent on JAK-STAT3 signaling (Sun *et al.* 2001). However, the inhibitory effect of RA on CNTF-induced astrogliogenesis at E13 reported here is independent of JAK-STAT3 signaling. This observation is quite unique and novel since the JAK-STAT3 pathway is obligatory for CNTF-induced astrocyte differentiation. Nevertheless, RA inhibition of CNTF-induced astrogliogenesis is still likely mediated by RAR induction of gene expression since expression of a dominant negative RAR, which lacks a transactivation domain, reversed the inhibitory effect of RA.

Although RAR mediates most of the functions of RA, only a few reports have begun to address functional differences among various subtypes of RARs in the central nervous system (Goncalves *et al.* 2005, Jang *et al.* 2004). Here we report that selective activation of RAR α or RAR β , but not RAR γ at E13 is sufficient to inhibit CNTF-induced astrogliogenesis, while selective activation of RAR α or RAR γ promotes astrogliogenesis at E17. Our data also show that the switch of RA's function from anti-astrogliogenic at E13 to pro-astrogliogenic at E17 correlates with down regulation of RAR β , whose function is required for RA to suppress astrogliogenesis at E13. These data suggest distinct function of the subtypes of RAR in astrogliogenesis.

It is interesting that RA inhibition of CNTF-induced astrogliogenesis requires only a 24 hr pre-treatment of RA but not its continued presence during CNTF treatment. This finding is in contrast to the prolonged exposure that is needed for RA to promote neuronal differentiation (Takahashi *et al.* 1999). Moreover, RA inhibits CNTF-induced astrogliogenesis in E13 progenitors without affecting neuronal or oligodendrocyte differentiation under our conditions; rather, it retains progenitors in a proliferative state even in the presence of an instructive pro-astrogliogenic factor such as CNTF. Thus, RA renders early cortical progenitor cells unresponsive to extracellular astrogliogenic signals.

During embryonic development of the rat cortex, neurons and glial cells are generated in distinct phases: an early phase with mainly neuronal differentiation peaking around E12–E15, and a late phase with predominantly astrogliogenesis beginning at approximately E16 and ending postnatally. A number of mechanisms, both intrinsic and extrinsic, have been proposed to contribute to this orderly differentiation program which ensures no premature astrogliogenesis during the neurogenic period while maintaining a timely switch to astrogliogenesis after neurogenesis has completed (Ross *et al.* 2003, Miller & Gauthier 2007). For example, gliogenesis is repressed during neurogenic period by intrinsic mechanisms. Neurogenins, a family of basic helix-loop-helix (bHLH) transcription factors, are highly expressed during the neurogenic period; they promote neurogenesis at the expense of gliogenesis by binding to and sequestering the transcription co-activator CBP/p300-Smad1 complex away from activated STAT3 (Sun *et al.* 2001). Furthermore, epigenetic silencing of astrocyte specific genes as well as the gp300-JAK-STAT signalling pathway by DNA methylation and/or chromatin modifications also contributes to gliogenic repression during the neurogenic period (Fan *et al.* 2005, Takizawa *et al.* 2001). The intrinsic properties of multipotent neural progenitors also change over time such that they become more biased towards making astrocytes during the gliogenic period. There is less DNA methylation of the genes in the JAK/STAT signaling pathway which suppresses neurogenesis and promotes astrogliogenesis in the developing cortex. Studies in developing spinal cord implicated the induction of a family of transcription factors, nuclear factor (NF) I, and combinatorial actions of patterning (Pax6, Olig2, Nkx2.2) and bHLH transcription factors (neurogenins, hes1 and Id1) in controlling the onset of astrogliogenesis (Deneen *et al.* 2006, Sugimori *et al.* 2007). These or similar mechanisms discovered in spinal cord may also regulate the transition from neurogenesis to astrogliogenesis in the developing cortex.

In addition to the intrinsic mechanisms, extrinsic environmental signals also play a key role in the fate of neural progenitors. For example, embryonic cortical precursors generate neurons when co-cultured with embryonic cortical slices but differentiate into astrocytes when cultured on postnatal cortical slices (Morrow *et al.* 2001). A number of cytokines and growth factors that are expressed in the embryonic cortex, including cardiotrophin-like cytokines, neuropoietin, Notch, BMP, bFGF and EGF (Uemura *et al.* 2002, Derouet *et al.* 2004, Miller & Gauthier 2007) have been implicated in the regulation of the neurogenic to gliogenic switch. Many of these extrinsic signals induce astrogliogenesis through, at least in part, modulation of the JAK/STAT signaling pathway. The change in the responsiveness of the progenitor cells to

RA signaling described in this study may also underlie the orderly differentiation program. Our data support the hypothesis that progenitors change their intrinsic cellular features during development; one such change is manifested as their responsiveness to RA signaling. Since mRNAs for both the enzymes responsible for RA synthesis (supplemental Fig. 2) and the receptors for RA (Fig. 11) are expressed in E13 cortical progenitors, endogenous RA signaling may be part of the orchestration to ensure that untimely astrogliogenesis does not occur during the neurogenic period (e.g. at E13). However, RA signaling during astrogliogenic phase may cooperate with other cues such as CNTF to warrant proper astrogliogenesis.

It will be interesting to determine if the pro-astrogliogenic effect of RA in E17 cultures is mediated by directly or indirectly modulating JAK/STAT signaling, inducing expression of NFI, or changing the relative expression of pro-neural vs. pro-astrogliogenic transcription factors. However, the anti-astrogliogenic activity of RA at E13 is independent of STAT signaling and requires RAR β function. Perhaps RA antagonizes the astrogliogenic activity of CNTF at E13 by promoting DNA methylation and silencing of astrocyte specific genes downstream from the JAK/STAT signaling pathway. Alternatively, RA may antagonize a novel, but yet unidentified signaling pathway that is necessary for astrogliogenesis.

The fact that RA has contrasting effects on astrogliogenesis in E13 vs. E17 cortical progenitors is quite novel and interesting. In some of our studies, bFGF and EGF were included during the entire culture period for both E13 and E17 cultures although similar results were obtained when bFGF and EGF were only included in the first 48 hr of culture but were removed during CNTF treatment. It is known that bFGF influences positional identity and therefore cellular potential in cultured cells (Hack *et al.* 2004, Santa-Olalla *et al.* 2003, Gabay *et al.* 2003). For example, a 48 hr bFGF treatment of cortical progenitors isolated from mouse E14.5, which is equivalent to the rat E17 culture used in our study, up-regulates Olig2 (Hack *et al.* 2004). Another study showed that bFGF treatment of E14 mouse cortical progenitors caused a down regulation of Ngn1 and Ngn2 concurrent with the Olig2 induction (Abematsu *et al.* 2006). Olig2 inhibits LIF-induced astrocyte differentiation in E14.5 mouse telencephalic neural progenitors (Fukuda *et al.* 2004) and its nuclear export is required for CNTF to induce astrocyte differentiation (Setoguchi & Kondo 2004). It is possible that bFGF and EGF, even if only present for 48 hr, exert distinct effects on E13 vs. E17 progenitors including differential regulation of transcription factors critical for positional specification and astrogliogenesis, leading to the differential response to RA.

There were also differences in the cell types present in the initial preparations of E13 vs. E17 cultures. While the majority of cells dissociated from E13 rat cortex were nestin⁺ progenitors, most of the cells dissociated from E17 cortex were β III tubulin⁺ newborn neurons. The proportion of neurons in the E17 cultures decreases as a result of progenitor cell proliferation in the continued presence of bFGF and EGF. It has been reported that newborn neurons secrete cardiotrophin-1 to instruct multipotent cortical precursors to produce astrocytes (Barnabe-Heider *et al.* 2005). It is conceivable that the difference in the presence or absence of newborn neurons in the starting point of the culture may be responsible for the differential effect of RA on CNTF-induced astrogliogenesis in the E13 vs. E17 cultures. However, our data showed that E13 progenitors cultured for 4 days *in vitro* displayed the same response towards RA-signaling as progenitors isolated from E17 cortex. Thus, the switch of RA's function from anti-astrogliogenic at E13 to pro-astrogliogenic at E17 reflects, at least in part, a change in the intrinsic astrogliogenic competence of progenitors from E13 to E17.

The percentage of GFAP⁺ cells in E17 cultures treated with CNTF + RA were much greater than the sum of each individual treatment alone (Fig. 9, b and c). Therefore, the synergistic effect of RA and CNTF on astrogliogenesis at E17 is unlikely due to the existence of different

subpopulations of progenitors that respond to RA and CNTF. Rather, the synergism is most likely at the molecular level such as synergistic activation of the JAK/STAT signaling pathway.

Neural stem/progenitor cells are thought to be a promising source of cells for cell replacement therapy in treating neurodegenerative diseases. However, differentiation of the grafted cells into astrocytes rather than neurons is one of the major obstacles in transplantation attempts to date. The gliogenic environment in the adult brain is believed to be due to extrinsic environmental factors, such as CNTF. Therefore, it is of great interest to elucidate positive and negative regulators of astroglialogenesis. We have found that developmentally restricted response patterns of neural progenitors towards RA signaling render distinct cell phenotypes in the presence of pro-astroglialogenic factors. Data reported here provide important insights into astroglialogenesis during cortical development and suggest clinical strategies to reduce astroglialogenesis during transplantation of neural progenitor/stem cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. RA inhibits CNTF-induced astroglialogenesis in E13 cortical progenitors

E13 cortical progenitor cells were cultured in bFGF- and EGF- containing medium throughout the experiment. (A) A representative fluorescence photomicrograph and quantification of RA inhibition of CNTF-induced astroglialogenesis of E13 cortical progenitors. Cells were treated with vehicle control DMSO or 0–1 μ M RA on DIV1 for 24 hr, followed by vehicle control or 50 ng/ml CNTF treatment for 2 d in the continued presence of RA. Cells were then immune stained for astrocyte marker protein GFAP (green) and counterstained with the nuclei dye Hoechst (blue) to identify all cells. (B) Dose-dependent inhibition of CNTF-induced GFAP-protein expression by RA treatment. GFAP expression was analyzed by Western blotting and normalized to the loading control β -actin. Cells were treated as in panel A. (C) RA inhibits

CNTF-stimulated GFAP-transcription. Freshly dissociated E13 cortical progenitors were transiently transfected with a GFAP promoter driven-luciferase reporter and treated with 1 μ M RA or DMSO on DIV1 for 24 hr. Cells were then stimulated with CNTF in the continued presence of RA for another 24 hr before harvesting for luciferase assay. **(D)** A representative fluorescence photomicrograph and quantification of RA inhibition of cells expressing S100 β , a marker for immature astrocytes. Cells were treated as in panel A. **(E)** Representative fluorescence photomicrographs of E13 cortical progenitors in a neurosphere assay. E13 cortical progenitors were seeded at clonal density to allow neurosphere formation. Cells were treated with 1 μ M RA or DMSO on DIV1 for 24 hr, followed by 50 ng/ml CNTF or vehicle control treatment in the continued presence of RA for another 3 d. The neurospheres were then transferred to coated plates to allow differentiation for 3–4 d. Cells were stained as in panel A. **(F)**, Quantification of the data in panel E. Data shown are the percentage of the total neural spheres expressing <10%, 10–40%, or >40% GFAP⁺ cells per sphere.

Figure 2. RA inhibits CNTF-induced astroglialogenesis in E13 cortical progenitors even after prolonged treatment in culture

E13 cortical progenitor cells were cultured in bFGF- and EGF- containing medium throughout the experiment. Cells were treated with vehicle control DMSO or 1 μ M RA on DIV1 for 24 hr, followed by vehicle control or 50 ng/ml CNTF treatment for 6 days in the continued presence of RA. **(A)** RA inhibits CNTF induction of cells expressing GFAP. **(B)** RA inhibits CNTF induction of cells expressing S100 β .

Figure 3. Pre- but not co-incubation of RA is sufficient to inhibit CNTF-induced astrogliogenesis in E13 cortical progenitors

E13 cortical progenitor cells were cultured in bFGF- and EGF- containing medium throughout the experiment. Cells were treated on DIV1 with 1 μ M RA (+) or vehicle control (-) for 24 hr, followed by 50 ng/ml CNTF (+) or vehicle control (-) for an additional 2 d either in the continued presence of RA (Pre/Co) or in the absence of RA (Pre). Alternatively, the cultures were co-treated on DIV2 with RA and CNTF (Co) for 2 d. **(A)** Pre-incubation of RA reduces the number of CNTF-induced GFAP⁺ cells **(B)** Pre-incubation of RA reduces CNTF-induced GFAP protein expression analyzed by Western blotting.

Figure 4. A 24 hr exposure to RA leaves non-astroglial lineages unaffected in E13 cortical progenitors

E13 cortical progenitor cells were cultured in bFGF- and EGF- containing medium throughout the experiment. (A) RA does not significantly reduce thymidine incorporation. Cells were treated on DIV1 with 0–1 μM RA for 24 hr. [³H] thymidine was added to the medium for the last 4 hr. (B) RA does not significantly reduce the percentage of BrdU⁺ cells. Cells were treated on DIV1 with 0–1 μM RA for 24 hr. BrdU (10 μM) was added to the medium for the last 4 hr. The image is a representative fluorescence photomicrograph of BrdU staining. (C) Representative fluorescence photomicrographs of E13 cortical progenitors stained with MAP2 (red), a marker for mature neurons. Cells were treated with vehicle control DMSO or 1 μM

RA on DIV1 for 24 hr. The medium was then changed to remove RA and cells were cultured for another 7 d. Scale bar, 20 μm . **(D)** Quantification of data in panel C demonstrating that exposure to 0–1 μM RA for 24 hr does not change the number of MAP2⁺ neurons 7 d later. **(E)** Exposure to 0–1 μM RA for 24 hr does not change the number of newly generated, βIII tubulin⁺ neurons determined 7 d later. Cells were cultured and treated as in (C). The image is a representative fluorescence photomicrograph of βIII tubulin staining. **(F)** A 24 hr exposure to 0–1 μM RA does not change the level of βIII tubulin protein expression determined 7 d later by Western analysis. Cells were cultured and treated as in (C). **(G)** A 24 hr RA exposure does not change the protein expression of nestin, βIII tubulin, or PLP determined 7 d later by Western analysis. Cells were cultured and treated as in (C).

Figure 5. More E13 cortical progenitors remain in a proliferative state when cultures were co-treated with CNTF and RA than with CNTF alone

E13 cortical progenitor cells were cultured in bFGF- and EGF- containing medium throughout the experiment. Cells were pre-treated with RA for 1 d followed by 3 d CNTF treatment in the continued presence of RA. (A) A representative fluorescence photomicrograph and quantification of Olig2⁺ cells. (B) A representative fluorescence photomicrograph and quantification of NG2⁺ cells. The differences in the number of Olig2⁺ and NG2⁺ cells among various treatment groups were not statistically significant. (C) Quantification of MAP2⁺ cells. (D) Quantification of apoptotic cells by nuclear condensation and/or fragmentation. (E) A representative fluorescence photomicrograph and quantification of active caspase 3⁺ cells.

(F) A representative fluorescence photomicrograph and quantification of TUNEL⁺ cells. **(G)** Percent of BrdU⁺ cells. Cells were pulsed with 20 μ M BrdU for 2 hr immediately before fixing.

Figure 6. RA inhibits CNTF-induced astroglialogenesis in E13 cortical progenitors even when bFGF/EGF were removed during CNTF treatment

E13 cortical progenitors were initially cultured in bFGF- and EGF- containing medium. Cells were treated with RA on DIV1 for 1 d, followed by 3 d CNTF treatment in the continued presence of RA but in bFGF- and EGF-free medium. **(A)** RA inhibits CNTF induction of cells expressing GFAP. **(B and C)** RA does not alter the number of cells expressing βIII tubulin (B) or NG2 (C). **(D)** Percent of nestin⁺ cells. **(E)** Percent of BrdU⁺ cells. Cells were pulsed with 20 μM BrdU for 2 hr immediately before fixing.

Figure 7. RA inhibition of astrogliogenesis of E13 cortical progenitors requires RAR function and can be mimicked by agonists of RAR α and RAR β , but not RAR γ

E13 cortical progenitor cells were cultured in bFGF- and EGF-containing medium throughout the experiment. (A) Expression of a dominant negative RAR blocks the inhibitory effect of RA on CNTF-induced astrogliogenesis. Freshly dissociated cells were transfected with an expression vector encoding a dominant-negative RAR (RAR-DN) or the vector control.

Twenty-four hours after plating, the cells were treated with 1 μ M RA or vehicle control DMSO for 24 h, followed by stimulation with 50 ng/ml CNTF for another 2 d in the continued presence of RA. Cells were then fixed and the number of GFAP⁺ astroglial cells was analyzed for the total cell population. (B) Treatment with a pan-RAR agonist elicits a similar effect as RA on CNTF-induced GFAP expression. Cells were incubated on DIV1 with 1 or 10 nM of pan-RAR agonist TTNPB, or vehicle control (-) for 24 hr. RA (1 μ M) was used as a positive control. Cells were then stimulated with 50 ng/ml CNTF for 2 d in the continued presence of RA and GFAP expression was analyzed by Western blotting. (C) Treatment with Am580, a RAR α -specific agonist, inhibits CNTF-mediated induction of GFAP⁺ cells. Cells were treated on DIV1 with vehicle control, 1 μ M RA, 1 or 10 nM Am580 for 24 hr, followed by 50 ng/ml CNTF treatment for another 2 d in the continued presence of RA. The number of GFAP⁺

astroglial cells was analyzed. **(D)** Treatment with Am580 suppresses CNTF-induction of GFAP-protein expression analyzed by Western blotting. Cells were treated as in panel C. The relative GFAP protein expression (%) was normalized to β -actin loading control. **(E)** Treatment with BMS 213309, a RAR β -specific agonist, suppresses CNTF-induction of GFAP-protein expression analyzed by Western blotting. Cells were treated similar to panel C. **(F)** Treatment with CD437, a RAR γ -specific agonist, has no effect on CNTF-induction of GFAP-protein expression. Cells were treated similar to panel C.

Figure 8. RA inhibition of CNTF-induced astroglialogenesis in E13 cortical progenitors is independent of STAT3-signaling

E13 cortical progenitor cells were cultured in bFGF- and EGF- containing medium throughout the experiment. **(A)** A 24 hr treatment with RA does not affect the transcription of CNTF receptor α (CNTFR- α). Cells were treated on DIV1 for 24 hr with 0–1 μ M RA. The expression of CNTFR- α was analyzed by RT-PCR. **(B)** A 24 hr treatment with RA does not affect the protein expression of gp130 analyzed by Western blotting. **(C)** A 24 hr RA treatment does not interfere with CNTF-induced STAT3 phosphorylation. Cultures were pre-treated on DIV1 with DMSO (–) or 1 μ M RA (+) for 24 hr, and then stimulated with 50 ng/ml CNTF for 20, 45, or 90 min in the continued presence of RA. Cell lysates were analyzed for phospho-STAT3 (p-STAT3) by Western blotting. Total STAT3 was used as a loading control. **(D)** RA does not interfere with CNTF-induced, STAT3-mediated transcription. Freshly dissociated E13 cortical progenitors were transfected with a STAT3-luciferase reporter. Cells were treated on DIV1 with 1 μ M RA for 24 hr, and then stimulated with 50 ng/ml CNTF for another 24 hr in the continued presence of RA.

Figure 9. RA promotes astrogliogenesis of E17 cortical progenitors

E17 cortical progenitor cells were cultured in bFGF- and EGF- containing medium as for E13 cultures to stimulate cortical progenitor cell proliferation. bFGF and EGF were included throughout the experiments except for panel C, in which bFGF and EGF were removed during the period of CNTF treatment. (A) RA synergizes with CNTF to induce GFAP protein expression in E17 cortical progenitors. Cells were pre-treated on DIV1 with 0–1 μ M RA for 24 hr prior to stimulation with 50 ng/ml CNTF for 2 d in the continued presence of RA. GFAP expression was analyzed by Western blotting. (B) RA synergizes with CNTF to increase the number of GFAP⁺ astrocytes generated from E17 cortical progenitors. Cells were treated as in panel A. (C). RA also synergizes with CNTF to increase the number of GFAP⁺ astrocytes

generated from E17 cortical progenitors when bFGF/EGF were removed from the medium during CNTF treatment. Cells were treated as in panel A except that bFGF and EGF were removed from the medium during CNTF treatment period. **(D)** Dose-dependent induction of astrogliogenesis by RA alone in E17 cortical progenitors. Cells were treated on the day of plating with 0–1 μ M RA for 6 d and the number of GFAP⁺ cells was analyzed. **(E)** Dose-dependent induction of GFAP protein expression by RA alone in E17 cortical progenitors. Cells were treated as in panel D and GFAP protein expression was analyzed by Western blotting. **(F)** A pan-RAR antagonist suppresses CNTF induction of GFAP expression. Cells were treated on the day of plating with 50 nM BMS 189453, a pan-RAR antagonist. Twenty-four hours later, cells were stimulated with 50 ng/ml CNTF for 6 d in the continued presence of BMS 189453.

Figure 10. Activation of RAR α and RAR γ , but not RAR β , promotes astroglialogenesis in E17 cortical progenitors

E17 cortical progenitor cells were cultured in bFGF- and EGF- containing medium throughout the entire experiment. (A) RAR α -specific agonist increases GFAP protein expression analyzed by Western blotting. Cells were treated with 0–100 nM Am580 on the day of plating for 6 d. (B) RAR α -specific agonist and CNTF synergistically induce GFAP expression. Cells were treated with 0–10 nM Am580 on DIV1 for 24 hr followed by 50 ng/ml CNTF after another 2 d in the continued presence of Am580. Treatment with 1 μ M RA was used as a positive control. (C) Both RAR α and RAR γ agonists induce GFAP expression. On the day of plating, E17 cortical progenitors were treated for 6 d with vehicle control (–), 1 μ M RA, 100 nM Am580, or 100 nM CD437, a RAR γ -specific agonist. (D) RAR γ -specific agonist and CNTF synergistically induce GFAP expression. Cells were treated with 0–100 nM CD437 on DIV1 for 24 hr cells followed by 50 ng/ml CNTF after another 2 d in the continued presence of CD437. Treatment with 1 μ M RA was used as a positive control. (E) RAR β -specific agonist does not affect GFAP protein expression. E17 cortical progenitors were treated with 0–60 nM BMS 213309, a RAR β -specific agonist, on the day of plating for 6 d. (F) A comparison of the effect of RAR β , RAR γ agonist and RA on GFAP expression. Cells were treated on the day of plating with vehicle control (–), 1 μ M RA, 60 nM BMS 213309, or 100 nM CD437 for 6 d.

Figure 11. Activation of RAR β may be responsible for RA inhibition of astrogliogenesis at E13
(A) Semi-quantitative RT-PCR analysis of the expression of RAR α , RAR β and RAR γ , the three isoforms of RAR, in E13 and E17 cortical cultures on DIV1. β -Actin was used as an internal control. **(B)** RA inhibition of CNTF-induced GFAP protein expression at E13 requires the function of RAR β . E13 cortical progenitor cells were cultured in bFGF- and EGF- containing medium throughout the entire experiment. On DIV1, E13 cortical progenitors were pre-treated with LE135, a RAR β -specific antagonist for 1 hr prior to RA treatment (1 μ M, 24 hr). The cells were then stimulated with 50 ng/ml CNTF for an additional 2 days. GFAP expression was analyzed by Western blotting.

Figure 12. bFGF-responsive progenitors isolated from E13 and E17 respond differently to RA
Cells were isolated from E13 or E17 cortex and cultured in the same bFGF-only medium throughout the entire experiment. Cells were treated with vehicle control DMSO or 1 μ M RA on DIV1 for 24 hr, followed by vehicle control or 50 ng/ml CNTF treatment for 2 d in the continued presence of RA. **(A)** RA inhibits CNTF-induced astrogliogenesis in bFGF-responsive E13 cortical progenitors. **(B)** RA has no effect on apoptosis in bFGF-responsive E13 cortical progenitors. **(C)** RA potentiates CNTF-induced astrogliogenesis in bFGF-responsive E17 cortical progenitors. **(D)** RA has no effect on apoptosis in bFGF-responsive E17 cortical progenitors.

Figure 13. E13 bFGF-responsive cortical progenitors that have been cultured for 4 d resemble progenitors isolated from E17 cortex in their RA responsiveness
 E13 cortical progenitors were cultured for 4 d in the bFGF-containing medium. Cells were then re-suspended and re-plated in bFGF-containing medium. One day later, cells were treated with 1 μ M RA or vehicle control for 24 hr followed by 50 ng/ml CNTF treatment for another 3 d in the continued presence of bFGF and RA. **(A)** Percent of GFAP⁺ cells. **(B)** Percent of S100 β ⁺ cells.