Astroglia-derived retinoic acid is a key factor in glia-induced neurogenesis

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ABSTRACT **Astroglial cells are essential components of the neurogenic niches within the central nervous system. Emerging evidence suggests that they are among the key regulators of postnatal neurogenesis. Although astrocytes have been demonstrated to possess the potential to instruct stem cells to adopt a neuronal fate, little is known about the nature of the glia-derived instructive signals. Here we propose that all-*trans* retinoic acid, one of the most powerful morphogenic molecules regulating neuronal cell fate commitment, may be one of the glia-derived factors directing astroglia-induced neurogenesis. According to data obtained from several complementary approaches, we show that cultured astrocytes express the key enzyme mRNAs of retinoic acid biosynthesis and actively produce all-*trans* retinoic acid. We show that blockage of retinoic acid signaling by the pan-RAR antagonist AGN193109 prevents glia-induced neuron formation by noncommitted stem cells. Therefore, we provide strong *in vitro* evidence for retinoic acid action in astroglia-induced neuronal differentiation.—Környei, Z., Gócza, E., Rühl, R., Vörös, E., Orsolits, B., Szabó, B., Vágovits, B., Madarász, E. Astroglia-derived retinoic acid is a key factor in glia-induced neurogenesis. *FASEB J.* 21, 2496–2509 (2007)**

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Astroglial cells are among the major constituents of the stem cell microenvironment within the postnatal brain. Astrocytes residing in the subventricular zone (SVZ) and in the hippocampus (HC) function as either neurogenic stem cells or niche cells, and provide a suitable microenvironment for the long-term maintenance of neurogenetic capacity. Emerging evidence suggests that astrocytes can be the source not only of survival and growth-promoting factors, but also of signals directly affecting neuronal cell fate commitment.

A few glia-derived neurogenic signals have been identified (1). Glial cell line-derived factor (GDNF) significantly increases cell proliferation in the HC and seems to stimulate neurogenesis in the dentate gyrus (2, 3). Neurogenesin-1 (Ng1), a secreted astrocytic factor, promotes neuronal differentiation of adult neural stem cells by preventing adoption to glial fate by antagonizing BMP signaling (4). Neural stem cells expressing the nuclear receptor-related factor 1 (Nurr1) can be differentiated into dopaminergic neurons when cocultured with astrocytes derived from the ventral midbrain (5–7). This effect involves the glial secretion of Wnt-5a. Wnt-3 derived from hippocampal astrocytes stimulates Wnt/β-catenin signaling leading to neuronal differentiation of adult hippocampal stem cells (8).

In the present work we propose that all-*trans* retinoic acid, one of the major morphogenic molecules directing nervous system development, is among the astroglia-derived instructive factors. Retinoic acid (RA) is widely used as a potent inducer of neuronal differentiation by various multipotent cell populations (embryonic carcinoma cells, embryonic and neural stem cells) *in vitro*. The *in vitro* studies suggest that RA may function as a regulator of neural stem cell fate *in vivo*. This implication is supported by recent findings on retinoid signaling at sites of postnatal neurogenesis (9, 10). Exposure to exogenous retinoids or blocking RA signaling interferes with proliferation and differentiation within the neurogenic zones of the mature brain, such as the higher vocal center (HVC) in songbirds (11) or the SVZ and HC in mice (12, 13). Endogenous RA has recently been shown to be active within the adult stem cell niches, including the SVZ, the rostral migratory stream (RMS), and the HC (14–17).

Within the SVZ, most of the RA-responsive cells were identified as astrocytes (14), and we found a population of RA-activated astroglial cells within the hippocampi of RA reporter mice (18). Astrocytes in culture were shown to express RA receptors (19) and respond to exogenous RA (19–22). Despite the numerous descriptions of RA responsiveness by distinct astroglial populations, relatively few studies have indicated that post-

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natal astroglial cells may have the capacity for active RA synthesis (15, 23, 24).

We previously reported that astrocytes induce the neuronal differentiation of neuroectodermal stem cells by easily degradable, short range-acting secreted factors (25). The apparent similarities between RA and astroglia-induced neuron formation indicated that RA may be the factor responsible for the glial instructive effect. Based on the data shown below, we claim that *1)* cultured astroglial cells have the potential for active RA production and *2)* the astroglia-derived RA is one of the major inductive factors directing astroglia-induced neuron formation by different stem cell populations.

MATERIALS AND METHODS

Astroglial cultures

Astrocytes were isolated as described previously (25) from various brain regions of neonatal (P0-P3) wild-type (CD1 or FVB/N) or transgenic mice expressing eGFP under the control of the human GFAP promoter (26). In brief, meninges were removed and the tissue was minced by razor blades. The tissue pieces were subjected to enzymatic dissociation using 0.05% w/v trypsin and 0.05% w/v DNase I (Sigma-Aldrich, St. Louis, MO, USA) for 10 min at room temperature. The cells were plated onto poly-L-lysine (pLL) -coated plastic surfaces and were grown in minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS), 4 mM glutamine, and 40 μg/ml gentamicin (Sigma) in humidified air atmosphere containing 5% CO_2 at 37°C. The culture medium was changed on the first 2 days and every second or third day afterward. Confluent primary cultures were harvested by trypsinization and replated onto pLL-coated glass coverslips or into Petri dishes, according to the experimental design. Under these conditions, only a negligible contamination (<0.5% of the total population) of neurons (Tuj1+cells) was observed. Before coculture experiments, the mitotic activity of astrocytes was blocked with 10 μM cytosine arabinoside for 24 h.

NE-4C neuroectodermal stem cells

The NE-4C cells are derived from the fore- and midbrain vesicles of p53-deficient 9-day-old mouse embryos and were shown to display several neural stem cell properties (27, 25). The NE-4C cells were maintained in MEM supplemented with 5% FCS, 4 mM glutamine, and 40 μg/ml gentamicin in a CO_2 incubator at 37°C. For maintenance, subconfluent cultures were regularly split by trypsinization (0.05 w/v % trypsin in PBS) into poly-L-lysine-coated Petri dishes.

Embryonic stem cell line derivation and cultivation

The ES cell line R1 was established from (129/Sv×129/Sv-CP)F1 3.5 day blastocyst (28) and the CD1/EGFP ES cell line from the embryos of the TgN(GFPU)5Nagy transgenic strain. This strain is referred to as B5/EGFP in the primary publication (29). The B5/EGFP mice have been maintained on a CD1 background. RESGRO™ culture medium (THROMB-X, NV Biotechnological Development Company, Belgium) was used to obtain the CD1/EGFP ES cell line. Both R1 and CD1/EGFP ES cell lines gave high percent chimera newborns when aggregated with 8 cell-stage CD1 embryos using the protocol published by Nagy *et al.* (30). The newly established ES cell lines were kept on a primary embryonic mouse fibroblast feeder layer. The cells were passed in 1:4 every second day. ES cells were grown in KO-DMEM medium (Life Technologies, Inc., Gaithersburg, MD, USA) supplemented with 50 μg/ml streptomycin (Sigma), 50 U/ml penicillin (Sigma), 0.1 mM 2-mercaptoethanol (Sigma), 0.1 mM nonessential amino acids (Life Technologies), 1000 U/ml of leukemia inhibitory factor (ESGRO, Temecula, CA, USA), and 15% FCS (HyClone, Logan, UT, USA). Passages 8–10 from the newly established ES cell lines were used in our experiments.

Astroglia/stem cell cocultures

The parameters of coculture establishment were described in detail by Környei *et al.* (25). Briefly, in contact cocultures the stem cells were seeded on top of confluent monolayers of astroglial cells, allowing direct cell-to cell communication. In noncontact cocultures, the stem cells communicated with astrocytes only through the culture medium. The cocultures were maintained in serum-free MEM including 25% F12, 4 mM glutamine, ITS, and gentamicin (Sigma).

To obtain high efficiency neuron production by ES cells, we used ES-derived embryoid bodies (EB) in combination with the astrocytes. For differentiation of the EBs, the hanging drop method was used. Briefly, a day before preparation of the hanging drops, ES cells were passaged to the gelatin (0.1%) -coated Petri dishes (Greiner, Frickenhausen, Germany) in Dulbecco's modified Eagle's medium (KO-DMEM, Life Technologies) supplemented with 15% fetal calf serum (FCS, selected batches, HyClone), glutamax (Life Technologies, 100×), and 50 mM β-mercaptoethanol (Sigma). Cultivation medium (20 μl) containing 400 ES cells was placed on the lids of Petri dishes filled with phosphate-buffered saline (PBS). The EBs were grown in hanging drops for 24 h and subsequently plated onto astroglia monolayers in no more than 2 EB/cm^2 density.

Immunocytochemistry and determination of the neuron number

Both immunocytochemical stainings and determination of the neuron number were performed as described earlier (25). In the present work, antibodies to neuron-specific βIII-tubulin (ExBio, Praha, Czeh Republic) and titin (Development Studies Hybridoma Bank (DSHB), University of Iowa, IO, USA) were used in 1:2000 and 1:3 dilutions, respectively. The preparations were analyzed by a Zeiss Axiovert 200M microscope equipped with ApoTome. Averages and SD of neuron number were calculated from data obtained from three or four equally treated sister cultures. Experimental series were repeated at least three times.

HPLC

The astroglial cells were prepared from different areas of neonatal murine brains and maintained for 3 wk in serum-containing medium. Twenty-four hours before sample collection, the cells were washed with phosphate buffer several times and 1 μM retinol containing serum-free medium was added. After the incubation period, the medium was discarded and the cells were collected for sample preparation.

Sample preparation and HPLC-MS analysis were performed due to the method of Rühl (31). Briefly, 100 mg of the sample (if <100 mg of sample, methanol was added) was diluted with a 3-fold volume of isopropanol. The cells were

vortexed for 10 s, shaken for 6 min, and centrifuged at 13000 rpm in a Heraeus BIOFUGE Fresco at + 4°C. After centrifugation, the supernatants were dried in an Eppendorf concentrator 5301 (Eppendorf, Germany). The dried extracts were resuspended with 60 μl of methanol, vortexed, shaken, diluted with 40 μl of 60 mM aqueous ammonium acetate solution, transferred into the autosampler, and analyzed.

RT-PCR

Total RNA was isolated from astrocytes cultured for 3 wk using the High Pure RNA Isolation Kit (Roche, Nutley, NJ, USA) according to the manufacturer's protocol. Reverse transcription (RT) reactions were carried out using the Revert Aid First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD, USA). Total cDNA was subjected to PCR using the HotStarTaq kit (Qiagen, Valencia, CA, USA). Cycling conditions were 95°C for 15 min; 94°C for 30 s; 50°C for RALDH1, 51°C for RALDH2 and 56°C for RALDH3 for 40 s; 72°C for 1 min, for 40 cycles; and 72°C for 10 min using a Techne TC 512PCR System. The PCR products were run on ethidium bromide-containing agarose gel and visualized by UV *trans*-illumination. The following primers were used in this study:

RALDH1 5′gccagcagagcaaactcct3′, 5′tcgctcaacactccttttca3′ 399 bp;

RALDH2 5′acatcgatttgcagggagtc3′, 5′gtccaagtcagcatctgcaa3′ 496 bp;

RALDH3 5′cgaagagtgcgaaccagtta3′, 5′cttggtgaacttgacctcca3′ 117 bp (32).

PCR products were purified by eluting the bands from the agarose gel by using GenElute gel extraction column (Sigma). Automatic sequencing was performed on an ABI 3100 Genetic Analyzer (BIOMI Kft, Budapest, Hungary). A comparison of sequences with published GenBank sequences confirmed that the primers were specific for the given RALDH enzymes.

RA reporter bioassay

The F9 embryonal carcinoma cell line, stably transfected with the 1.8 kb promoter sequence of RARβ2 coupled to the *lacZ* gene, was used to measure active retinoids (33). The assay is appropriate for detection of all-*trans* RA, but will detect the 9-*cis,* 13-*cis,* and 4-*oxo* RA isomers as well (33). The F9 reporter cells were maintained in 10% FCS containing DMEM in the presence of 400 μg/ml G418. A day before the assay, F9 cell were seeded onto 24-well plates (100,000 cell/well). Astrocytes, P19, NE-4C, and ES cells were seeded on top of the F9 cells in 100–200,000 cell/well density. In some experiments the F9 reporter cells were passaged on top of astrocytic monolayers; 18 h later the cocultures were lysed and homogenized. The β-galactosidase activity of the homogenizates was determined by the chromogenic substrate ONPG (*o*-nitrophenyl β-D-galactopyranoside; Sigma). The optical density was measured at a wavelength of 420 nm.

Fluorescence-activated cell sorting

For FACS sorting experiments, we used total forebrains of P1 or P7 hGFAP-GFP transgenic mice. In older animals, the tissues were dissociated by MACS Neural Tissue Dissociation Kit (Miltenyi Biotec GmbH, Teterow, Germany) according to the protocol of the manufacturer.

The cell suspensions were first gated on forward scatter, then within this population based on GFP expression. Non-GFP-expressing astrocytes were used as negative control for background fluorescence. Flow cytometry was undertaken using a FACS Vantage flow cytometry analysis system (BD Biosciences, Pers, Hungary).

Cell viability

Different cell types grown in 96-well plates were subjected to various RAR antagonist concentrations for 48 h in serum-free medium. After the incubation period, the overall cell viability was determined according to the method by Mosmann (25, 34).

Time-lapse microscopy

Time-lapse recordings were performed on a computer-controlled Leica DM IRB inverted microscope equipped with 10×, 20 × objectives and an Olympus C4040z digital or an Olympus DP70 CCD camera. Cell cultures were kept at 37°C in a humidified 5% CO_2 atmosphere within custom-made microscope stage incubators (CellMovie; www.cellmovie.eu). Phase contrast images were acquired every 10 min for several days.

Retinoids

Both all-*trans* RA (Sigma) and the pan-RAR antagonist AGN193109 (Allergan Inc., Irvine, CA, USA; 35) stock solutions were dissolved in DMSO in a 10 mM concentration. All-*trans* RA was used in a final concentration of 100 nM. AGN193109 was added to the stem cell suspensions 5–45 min before establishment of the cocultures. The antagonist treatment was repeated daily until the end of the experiments. In experiments using retinoids or when collecting samples for retinoid determination, the cultures were carefully protected from light.

RESULTS

All-*trans* retinoic acid is detected in astrocytes by HPLC

To measure the RA content of astroglial cells, we performed LC-MS-MS analyses, described in detail by Rühl (31). All-*trans* RA was detected in all of the cultured astroglial populations derived from mesencephalon, cortex, cerebellum, or hindbrain (see **Table 1** and **Fig. 1**). The all-*trans* RA concentrations varied between 3 ng/g and 6.8 ng/g, a range compatible or even wider than those found in total brain extracts by different authors.[2] The highest all-*trans* RA values [6.8 ng/g and 4.7 ng/g] were measured from cortical astroglia samples.

In the chromatograms of the astroglia samples, many other not yet identified peaks can be seen (Fig. 1), indicating that other retinoid derivatives are also present in the astroglial cells. Note that there is a peak

[2] 1.6±0.3 ng/g in brains of adult mice (36); 1.6±0.1 ng/g in brains of adult mice; 2.3±0.3 ng/g in brains of 2–4 month mice (37) (data were calculated from pmol/ng values given in the original article).

TABLE 1. *Astroglial cells derived from different regions of neonatal murine brains contain all-trans RA in substantial amounts*[a]

	All-*trans* retinoic acid [ng/g]	
	Sample 1	Sample 2
Glia from mesencephalon	6.6	3.1
Glia from cortex	6.8	4.7
Glia from cerebellum	3	3.5
Glia from hindbrain	3.5	4
NE-4C neural stem cells	udl	1.5
NE-4C neural stem cells	0.23	udl

[a] Concentration of all-*trans* RA was measured by the LC-MS-MS method described in detail by Rühl (31). udl, under detection limit. See representative chromatogram in Fig.1. Quantification limit is at 0.2 ng/g.

coeluting with 13-*cis* RA in all glia samples. However, this peak elutes shortly before the standard 13-*cis* RA compound; therefore, it cannot definitely be identified as 13-*cis* RA. In the hindbrain glia samples, there is a peak coeluting with the 9-*cis* RA standard, but just in one of the two samples. We suggest (but are not sure) that this peak is actually 9-*cis* retinoic acid. Further research will show and prove the identity and quantity of this peak.

Astrocytes express mRNAs for the key enzymes in retinoic acid synthesis

Retinaldehyde-dehydrogenases (RALDHs), the key enzymes for RA synthesis, convert retinal into retinoic acid (reviewed in ref. 38). We tested the presence of the RALDH mRNAs in astroglial cells by RT-PCR. The data showed that RALDH1, RALDH2, and RALDH3 enzyme mRNAs were all present in cultured astroglial cells derived from either mesencephalon, hindbrain, hypothalamus, cerebellum, or cortex (**Fig. 2**).

In the lack of detailed quantitative analyses, the data obtained from a number of astroglial cultures did not provide evidence on quantitative differences in the RALDH mRNA expression between the distinct brain regions.

RA-sensitive bioassay demonstrates endogenous retinoic acid production by cultured astrocytes

To evaluate the direct biological activity of glial RA production, we used a bioassay based on the RA-inducible expression of the β-galactosidase enzyme (33). The β-galactosidase activity of the reporter F9 cells was either visualized by standard XGal staining (**Fig. 3*A, B***) or quantified by colorimetric assays from cell extracts using ONPG as substrate. The reporter cells responded to as low as 100 pM all-*trans* RA (Fig. 3*C*).

To check whether astrocytes actively produce RA, the F9 reporter cells were plated on top of astrocytic monolayers for 18 h. Both XGal staining and colorimetric assays revealed a reliable amount of RA in the cultures (Fig. 3*D, E*). In contrast, no significant RA production was observed by primary fibroblasts (Fig. 3*E*). The pan RAR antagonist AGN193109 prevented the astroglia-induced reporter cell activation (Fig. 3*E*).

To compare the RA production of astrocytes derived from different brain regions, we used cells isolated from either hypothalami, mesencephali, cortices, cere-

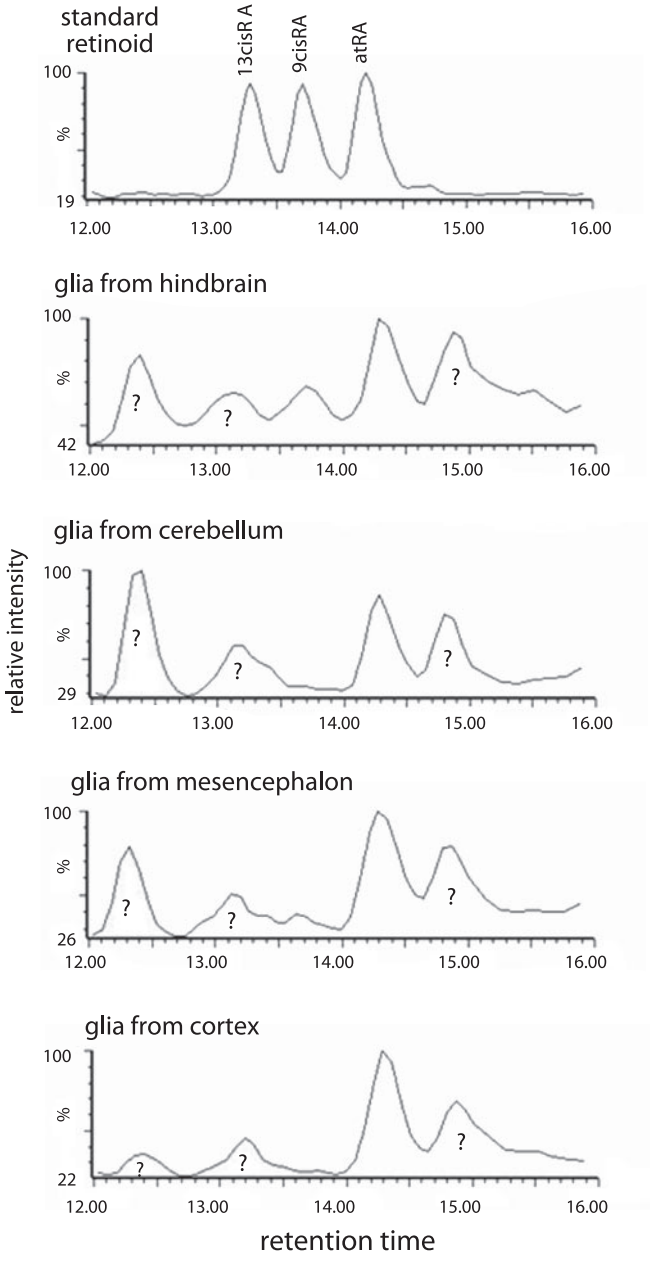

Figure 1. HPLC chromatogram of standards of RA isomers and astroglia samples derived from different brain regions. The chromatogram clearly demonstrates the presence of all-*trans* RA in all glia samples. Peaks with a comparable retention time of 13-*cis* RA and 9-*cis* RA in the hindbrain sample are close to the quantification level (0.2 ng/g), and not surely assignable. The presence of all-*trans* RA has also been confirmed by parallel UV detection. In the chromatograms, "?" marks representative peaks that could not definitely be identified.

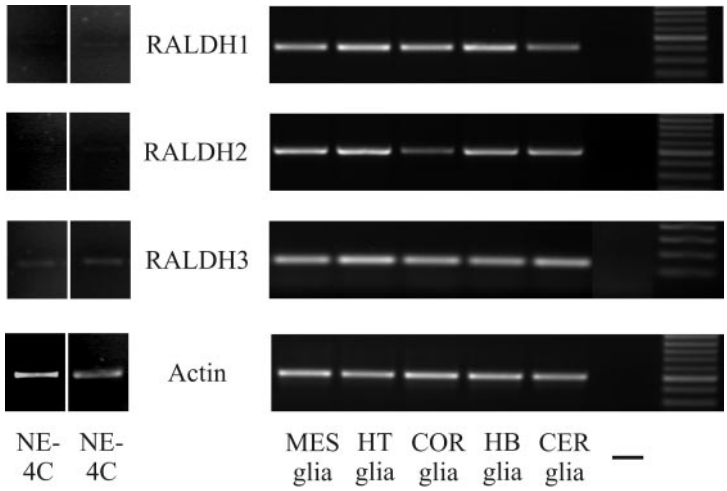

Figure 2. Retinaldehyde dehydrogenase 1, 2, and 3 enzyme mRNAs are expressed in astrocytes but can barely be detected in noninduced NE-4C neural stem cells. RT–PCR shows the expression of RALDHs in astroglial cells derived from different brain regions and cultured for 28 days. MES, mesencephalon; HB, hindbrain; HT, hypothalamus; CER, cerebellum; COR, cortex.

bella, or hindbrains of postnatal (P1-P3) mice. The astrocytes were cultured for 2 wk in serum-containing medium and plated on top of F9 reporter cells in equal cell numbers. During the incubation period (18 h), the astroglia/reporter cell cocultures were maintained in serum-free medium. The differences in F9 reporter cell activation by the distinct glial populations were not consistent if we compared the results of different experimental series. Therefore, we cannot state that astrocytes from any investigated brain area produce more RAR activators than the others. Figure 3*F* shows the results of a representative experiment. According to the calibration curve (Fig. 3*C*), astrocytes delivered RA for F9 cells in an amount comparable to 1–100 nM RA solutions, depending on the number of astrocytes (Fig. 3*F*).

To test whether astrocytes in the early postnatal brain also produce RA, pure astroglial preparations were isolated from transgenic mice expressing eGFP under the control of the human GFAP promoter (26) by FACS sorting. Cells sorted from newborn (P1) or 1-wk-old (P7) hGFAP-GFP mouse forebrains were seeded onto F9 reporter cells. By the end of the 18 h assay period, the sorted cells displayed typical flattened morphology (not shown), indicating viability. Surprisingly, freshly isolated astrocytes did not produce RA in detectable amounts (Fig. 3*G*). Astrocytes isolated from P1 forebrain and cultivated for another 7 days did produce a detectable amount of RA (Fig. 3*E*). To check a possible interference of the sorting procedure, cells were harvested and FACS-sorted from glial cultures derived from the forebrains of hGFAP-GFP mice. In contrast to freshly isolated cells, astrocytes cultured and selected by high GFP fluorescence after 2 wk *in vitro* growth produced RA sufficient to elicit reporter responses of F9 cells (Fig. 3*G*). The *in vivo* occurring low responsiveness of parenchymal astrocytes to RA was demonstrated by histological staining of brain sections of RARE reporter mice for β-galactosidase activity (**Fig. 4**). GFAP-positive cells with β-galactosidase activity were found only sporadically in the cortex (Fig. 4*B*), but were relatively abundant in the lateral ventricle wall (Fig. 4*A*; ref. 14) and in the hippocampus (Fig. 4*C–I*).

RA production by cultured astrocytes was also detected when the reporter cells were grown in noncontact cocultures with the glial cells (Fig. 3*H*). In such cocultures, no direct cell-to-cell adhesion was allowed between the reporter cells and the astrocytes. The data indicated that astroglial cells released RA in sufficient amounts to stimulate the target cells through the culture medium. Enhanced β-galactosidase activity, however, was not detected when F9 reporter cells were treated with glial conditioned medium (GCM). The GCM was freshly collected after incubating astrocytes for 24 h in serum-free medium, then immediately transferred from glial cultures to the reporter cells (Fig. 3*H*). These data are in accord with our previous findings showing that the presence of living astroglial cells can induce neuronal differentiation by neural stem cells, whereas glial CM has no direct inductive effect (25). It is likely that under serum-free conditions, RA is even more vulnerable to the physical moduli (*e.g.*, light, oxidation) than in the presence of protective serum factors (such as serum albumin or RBP).

Noncommitted stem cells do not produce retinoic acid in significant amounts

As a homogeneous population of noncommitted neural stem cells, we used the NE-4C immortalized neuroectodermal cell line. The NE-4C cells generate neurons upon induction with both all-*trans* RA (27) and astroglia-derived factors (25). Nondifferentiated NE-4C cells were harvested after 24 h incubation in 1 μM retinol containing serum-free medium. In contrast to astroglial cells, all-*trans* retinoic could not be detected in significant amounts in NE-4C neural stem cells by HPLC (Table 1). In three of the four NE-4C samples, the all-*trans* RA was under or very close to the detection limit of the system. In one of the samples, a low amount

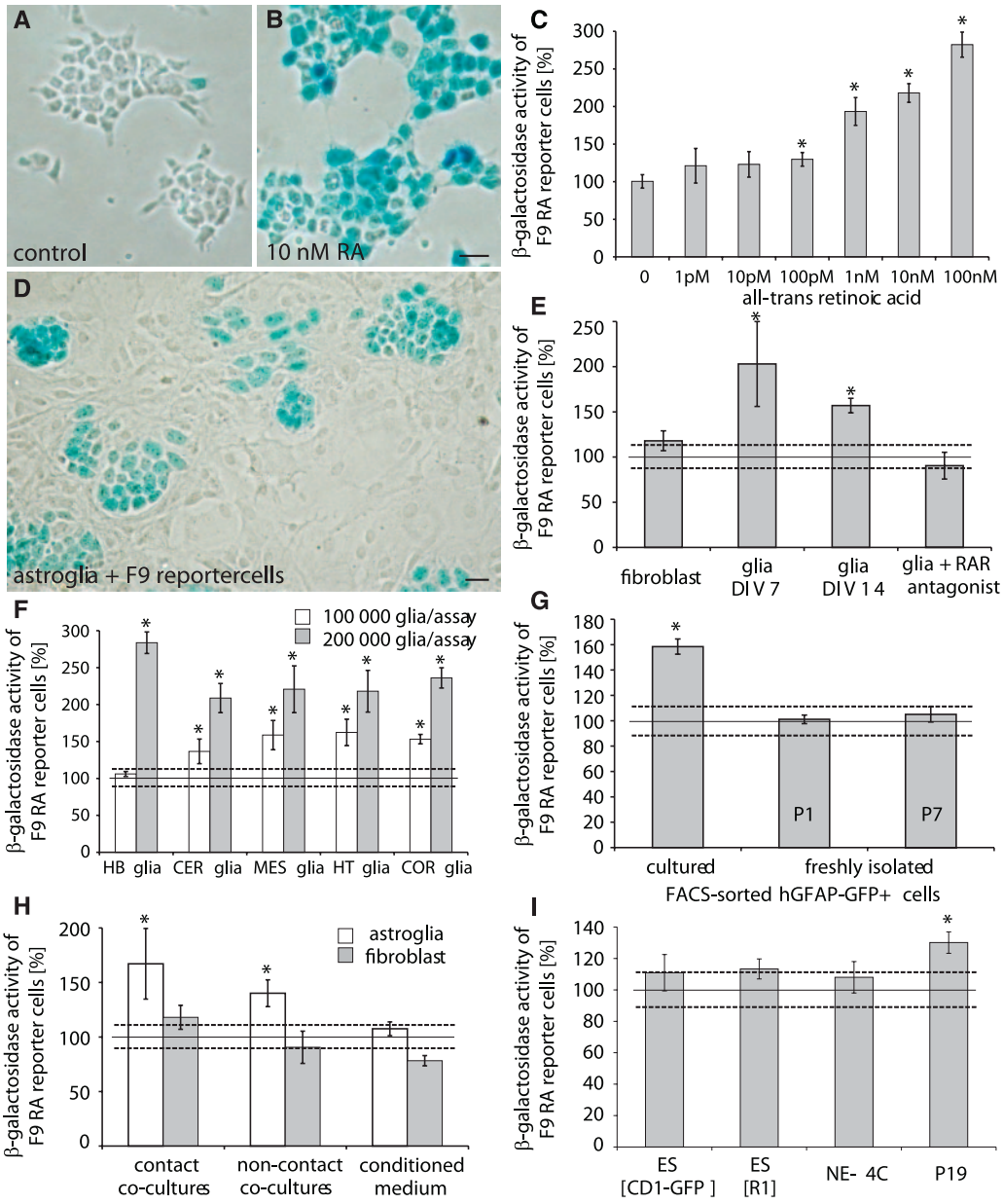

Figure 3. Astroglia-derived RA activates F9 RA-sensitive reporter cells carrying the RARβ2-lacZ reporter transgene. *A, B)* XGal staining of control and 10 nM RA-activated F9 reporter cells. *C)* β-Galactosidase activity in F9 reporter cells after treatment with different RA concentrations (n=4). The optical densities were determined in a colorimetric assay using ONPG as a substrate. *D)* F9 reporter cells form clusters on the surface of astrocytes and show strong X-Gal staining. *E–I)* β-Galactosidase activity of reporter cells incubated for 18 h with different cell types. Control F9 cells (100%) were incubated with medium only. Activity of the control and its range of SD are indicated by lines and dashed lines on each chart. *E)* The reporter cells are activated by forebrain (FB) astrocytes cultured for 7 or 14 days, but not by fibroblasts. The pan-RAR antagonist prevents glia-induced reporter cell response (n=6). *F)* All cultured astrocytic populations derived from different brain regions activate the RA reporter cells. The increase in β-galactosidase activity depends on the glia cell number used in the bioassay (n=6). *G)* FACS sorted, hGFAP-GFP-expressing astrocytes activate the reporter cells if they were previously maintained in culture for a prolonged period. Acutely isolated hGFAP-GFP astrocytes sorted from either P1 or P7 mouse forebrains and kept *in vitro* only for the 18 h of the assay do not activate reporter cells (n=3). *H)* Astrocytes produce RA both in contact and in noncontact cocultures with the F9 reporter cells. Treatment of the reporter cells with glial conditioned medium does not elevate their β-galactosidase activity (n=3). *I)* Neither NE-4C cells nor any of the two embryonic stem cell lines (R1 or CD1-GFP) produce detectable amounts of RA. A small increase of β-galactosidase activity can be seen in the presence of P19 cells (n=3). *Significant deviations from control values as calculated by a Student's t test. $P < 0.05$.

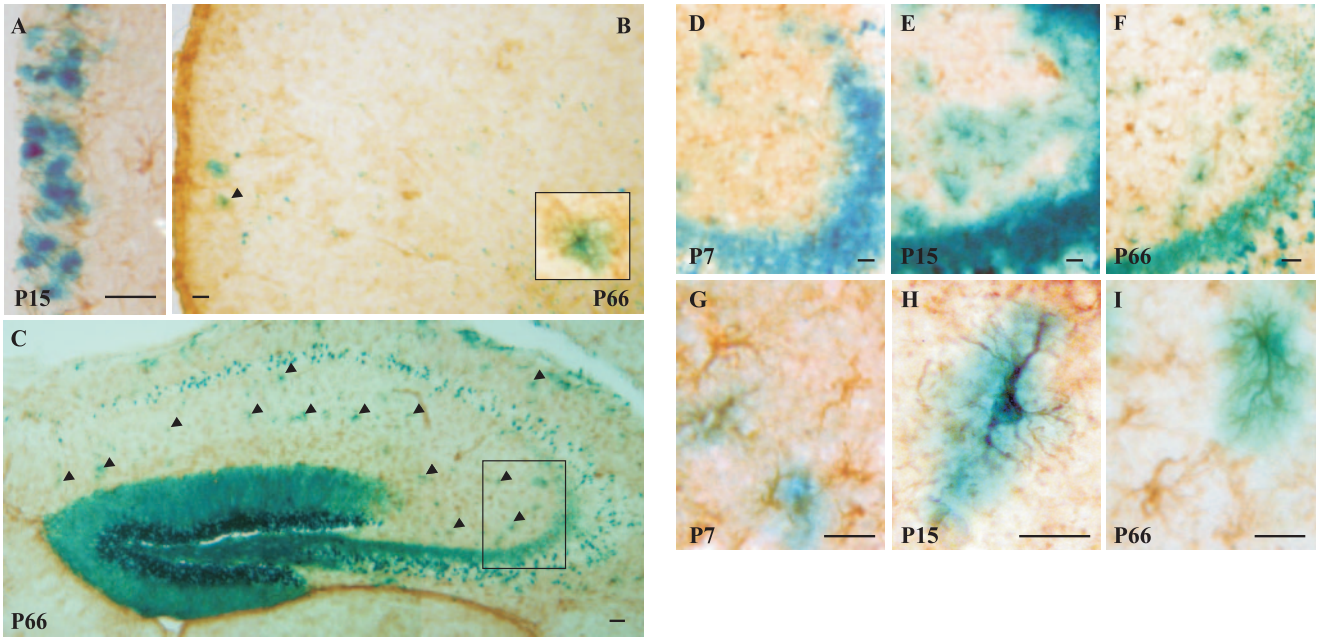

Figure 4. GFAP-positive cells with β-galactosidase activity were only sporadically found in the cerebral cortex, but were relatively abundant in the hippocampi of postnatal RA reporter mice carrying RARE-lacZ transgene (18). The RA-activated glial cells were identified by GFAP/ XGal double staining. *A)* XGal-labeled cells in the GFAP+ glia-rich dorsal wall of the lateral ventricle. *B)* RA-activated astrocytes can be rarely seen within the cerebral cortex. The arrowhead indicates a GFAP+/XGal+ cell, shown at higher magnification in the insert. *C)* In contrast to the cortex, many GFAP+/XGal+ double-labeled cells can be seen within the hippocampi of adult (P66) mice. Some are marked with arrowheads. The region marked by a rectangle is shown in panel *F. D–I)* Images taken with higher magnification show RA-responsive astrocytes in the hippocampi of reporter mice derived from different (P7, P15, and P66) postnatal ages. Scale bars: 25 μm."

[1.5 ng/g] of all-*trans* RA was found. We looked for RA production of stem cells by the RA reporter assay, too. The NE-4C cells did not induce β-galactosidase expression in the reporter cells (Fig. 3*I*).

In two independent NE-4C samples we could barely detect RALDH1 and RALDH2 mRNAs, but a faint band of RALDH3 mRNA was found in both samples (Fig. 2). We suppose that such a minimal level of RALDH3 is not sufficient to generate RA concentrations sufficient for activation of the reporter cells, but might explain the small all-*trans* RA content found in one of the four HPLC measurements.

The astroglia-induced neuron formation was also demonstrated in astroglia/embryonic stem cell (ES) cocultures (see below). Therefore, we determined RA production by the two ES cell lines (R1 and CD1-GFP) used in the experiments. As shown by the RA reporter assay, neither of the two lines produced any RA in their nondifferentiated state (Fig. 3*I*). However, some activation of the reporter cells occurred in the presence of P19 embryonic carcinoma cells.

Blocking retinoic acid signaling prevents astroglia-induced neuron formation

To prevent signaling through the RA receptors, we used the pan RAR antagonist AGN193109 (35). The stem cells were treated with the RAR antagonist for a few minutes before placing them into cocultures with astrocytes in order to prevent the immediate activation of RA-responsive genes. To make sure that the RAR antagonist itself does not affect stem cell viability, we determined the highest nontoxic concentrations of the drug. The results of the cell viability assays (34) revealed different sensitivities of various stem cell types for blocking of inherent RA signaling (**Fig. 5**). The relative viability of NE-4C neural progenitors decreased when cells were treated with 1 μM or higher doses of the pan-RAR antagonist for 48 h in serum-free medium (Fig. 5). The sensitivity of ES cells to RAR antagonism was much higher (100-fold) than that of the neuroectodermal cells. Both R1 and CD1-GFP ES cell clones responded to as little as 10 nM pan-RAR antagonist with a small, but significant, decrease in overall cell viability (Fig. 5). In the presence of 1 μM pan-RAR antagonist, ~50% of the ES cells died while the majority of NE-4C and P19 cells were unaffected. The overall viability of forebrain astrocytes did not decrease even in the presence of 1 μM pan-RAR antagonist (Fig. 5).

Antagonizing RA signaling hinders astroglia-induced neuron formation by neural stem cells

To examine whether RA signaling was involved in astroglia-induced neuron formation, NE-4C cells were treated with 100 nM RAR antagonist and put into cocultures with astrocytes. From the first day on, the

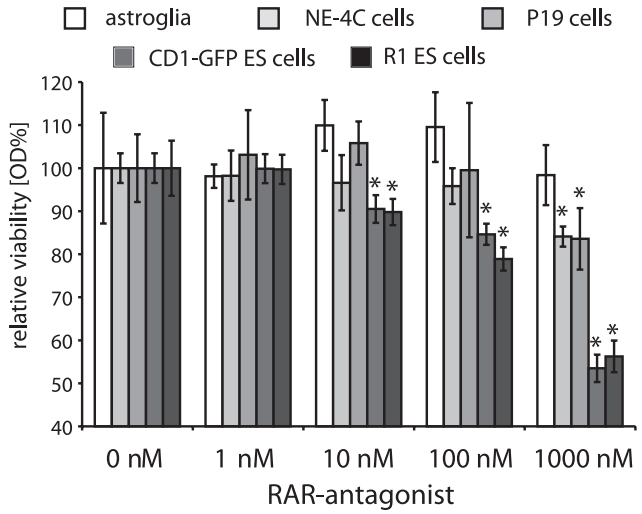

Figure 5. Relative viability of different cell types treated with the pan-RAR antagonist AGN193109 for 48 h in serum-free medium. 100% is the cell viability of untreated cells (n=8). *Significant deviations from control values calculated by a Student's t test. $P < 0.05$.

RAR antagonist was applied daily. The number of neurons was determined after 1 wk in preparations immunostained for neuron-specific tubulin. The RAR antagonist prevented the astroglia-induced neuron formation by NE-4C cells in a concentration-dependent manner (**Fig. 6*A***). The 100 nM RAR antagonist reduced the neuron number by 69 ± 8% in forebrain astroglia/NE-4C cultures (n=3). In cocultures with astrocytes purified from different brain regions, a similar high-rate decrease in neuron number could be observed, with variation between a 70 ± 3 and 88 ± 4% decrease (Fig. 6*B*). Daily treatment with the RAR antagonist, however, did not fully prevent neuron formation in any of the cocultures we tested.

RAR signaling is involved in the early steps of glia-induced neuronal differentiation

The astroglia environment also induced multitudinous neuronal differentiation by P19 embryonic carcinoma cells. Treatment with 100 nM RAR antagonist resulted in a 48 ± 11% decrease in P19-derived neuron number (Fig. 6*C*), but only if the antagonist treatment was started simultaneously with establishment of the coculture. If the RAR antagonist was applied 24 h later, no significant decrease in neuron number could be observed (Fig. 6*C*).

The schedule of neuron formation and maturation during glia-induced neurogenesis is similar to that seen in all-*trans* RA-treated monocultures of neuroectodermal NE-4C cells (**Fig. 7*A–H***). The first obvious morphological event of differentiation is the formation of compact cell assemblies starting ~ 30 h after exposure to either RA (Fig. 7*C*; Supplemental Movie 1) or glial factors (Fig. 7*E, G*; Supplemental Movie 2). Aggregate formation was previously shown to be indispensable for

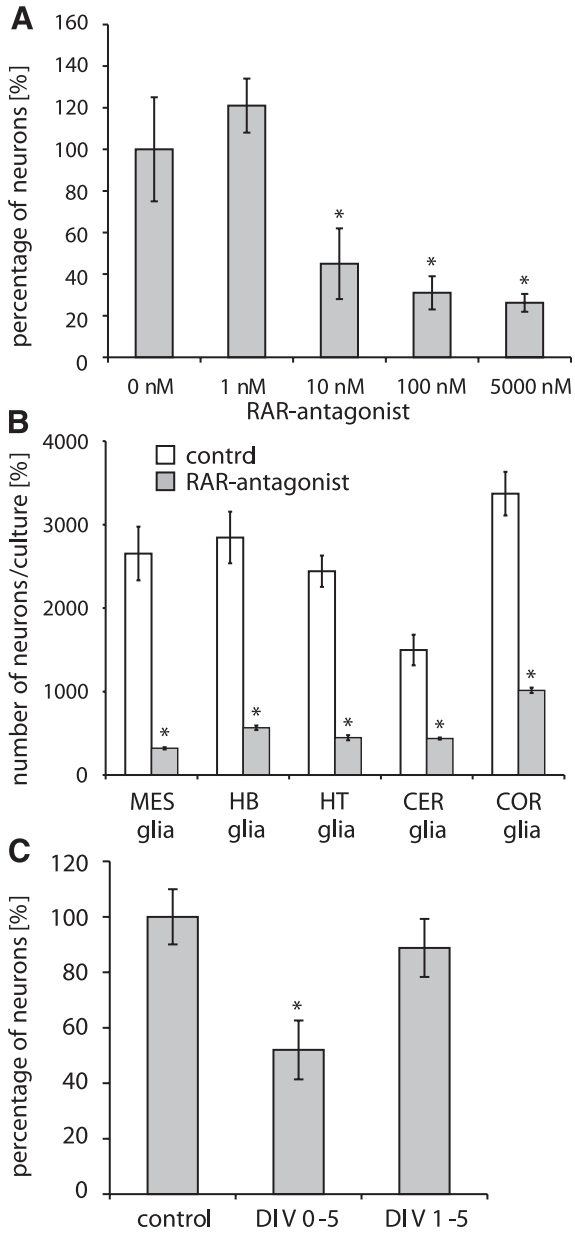

Figure 6. Blocking RA signaling prevents astroglia-induced neuron formation by neuroectodermal progenitors and embryonal carcinoma cells. The number of neurons was determined in astroglia/NE-4C or in astroglia/P19 cell cocultures immunostained for neuron-specific tubulin. *A)* The number of NE-4C cell-derived neurons in forebrain glia/NE-4C cell cocultures is reduced by daily RAR antagonist treatment in a concentration-dependent manner (n=6). *B)* A representative experiment illustrates the marked decrease in neuron number in cocultures of NE-4C cells with astrocytes purified from different brain regions. MES, mesencephalon; HB, hindbrain; HT, hypothalamus; CER, cerebellum; COR, cortex (n=3). *C)* The number of P19 neurons decreased upon daily treatment (DIV0–5) by the RAR antagonist in P19/astroglia cocultures. If the antagonist treatment was started only 24 h after establishment of the coculture (DIV1–5), no significant decrease in neuron number could be observed (n=4). *Significant deviations from control values, calculated by a Student's t test. $P < 0.05$.

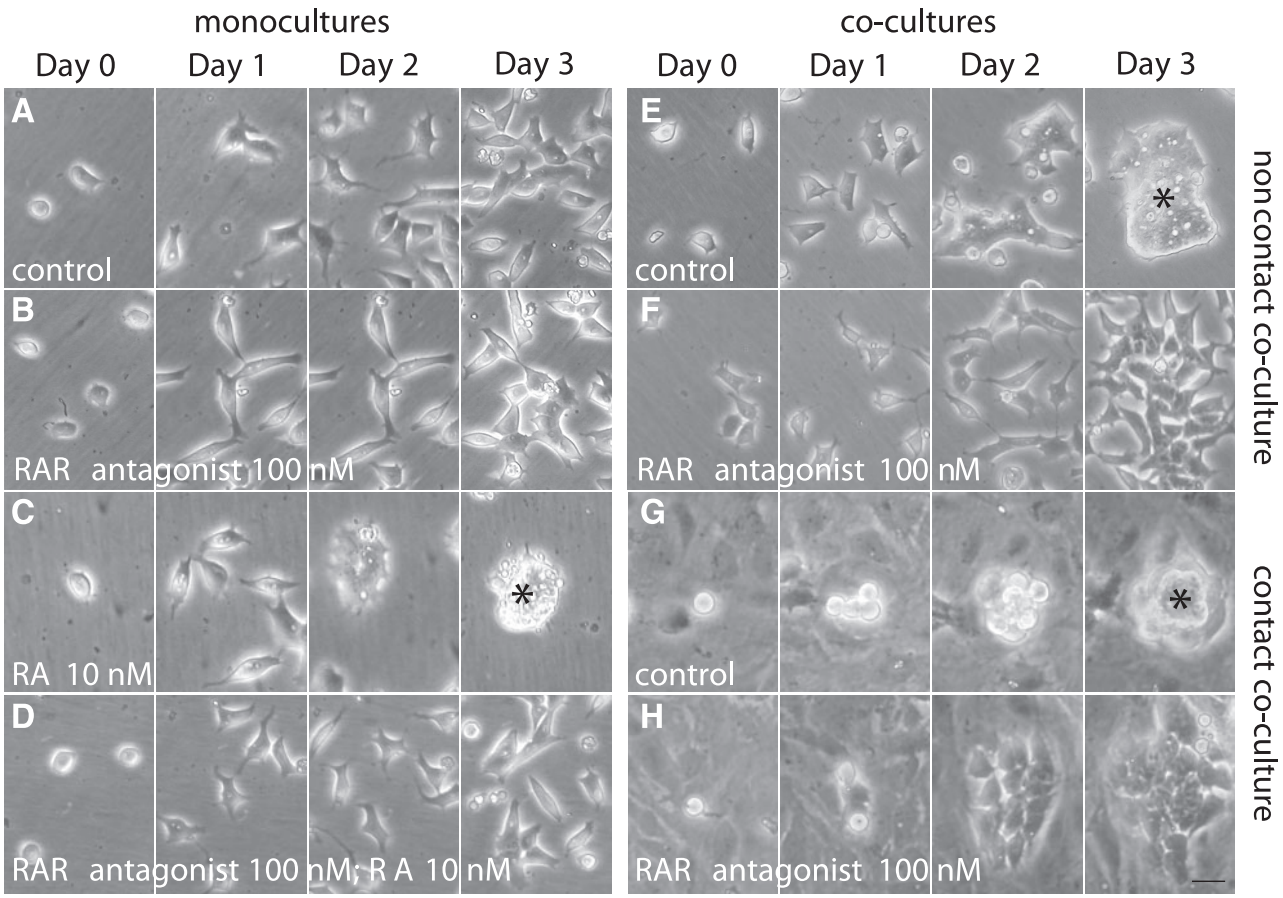

Figure 7. Blocking RA signaling prevents aggregate formation by NE-4C neuroectodermal progenitors. Time-lapse microscopic images show the development of NE-4C cells in monocultures (*A–D*) and in cocultures with astrocytes (*E–H*). *A–D)* NE-4C cells kept dividing for 3 days in monocultures in serum-free medium. In the presence of 10 nM retinoic acid, the cells formed compact aggregates. 100 nM pan-RAR antagonist prevented RA-induced aggregation if added before retinoic acid. *E–H)* In both noncontact (*E–F*) and contact cocultures (*G, H*) with astrocytes, NE-4C cells formed dense aggregates (*E, G*), reminiscent of those in RA-treated monocultures (*C*). The pan-RAR antagonist blocked the glia-induced aggregate formation (*F, H*). *Aggregates. Scale bar: 25 μm. See the related time-lapse movies (Supplemental Movies 1, 2).

the RA-induced neuronal differentiation of NE-4C cells (39, 40).

To see whether blocking RAR signaling interferes with this early step of neuronal differentiation, we treated the cultures with 100 nM pan-RAR antagonist and followed the development of the NE-4C cells by long-term time-lapse microscopy (Supplemental Movies 1, 2). The RAR antagonist did not interfere with the Motile behavior of the noninduced NE-4C cells, but prevented the aggregate formation in monocultures induced by 10 nM all-*trans* RA (Fig. 7*D*; Supplemental Movie 1). In astroglia/NE-4C cocultures, the RAR antagonist blocked the astroglia-induced aggregation (Fig. 7*F, H*, Supplemental Movie 2). These data indicate that astroglia-derived RA has an early impact on neuronal cell fate commitment of the progenitors.

Blocking RA signaling prevents astroglia-induced neuron formation by ES cells

Astroglia-induced neuron formation by ES cells was initiated by plating embryoid bodies (EBs) on the top of confluent astroglial monolayers. The EBs displayed similar average size, and the timing of their differentiation was comparable. The EBs kept growing on the top of astrocytes and developed into very large (up to visible, 1–2 mm large) structures in the cocultures (**Fig. 8*A–G***; Supplemental Movie 3*A*). Morphologically nondifferentiated cells did not leave the cell assemblies, but from the 4th or 5th day on intensive process outgrowth and outmigration of bipolar cells could be observed (Supplemental Movie 3*B*). The outmigrating cells and the network of the processes were identified by staining for neuron-specific tubulin (Fig. 8*H*; Supplemental Movies 3*A, B*) or neurofilament-M (not shown).

To investigate the role of RA signaling in glia-induced ES cell differentiation, we treated glia/ES cocultures with the pan-RAR antagonist AGN193109. To be sure that the drug penetrates into the thick, multilayered cell assemblies, we used concentrations 100-fold higher than the highest nontoxic concentration (1 nM) as determined by cell viability assays (Fig. 5). However, in cocultures with astrocytes, the daily

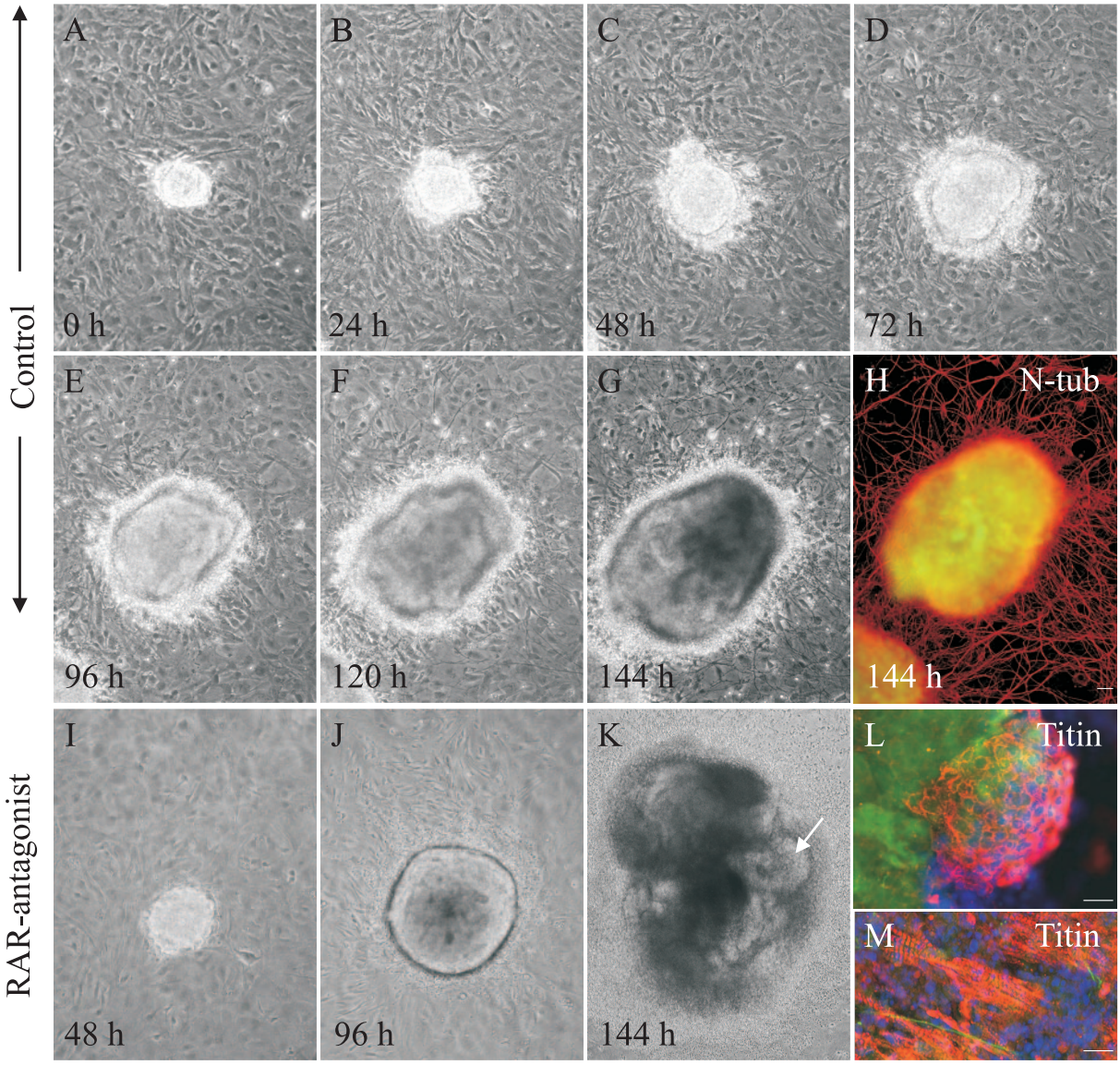

Figure 8. Embryonic stem cell-derived embryoid bodies differentiate into neurogenic aggregates in cocultures with astrocytes. Blocking RA signaling inhibits astroglia-induced neuron formation by the ES cells but initiates heart muscle type differentiation. *A–G)* Time-lapse images show the development of ES cell assemblies in astroglia/ES cell cocultures. An intense process outgrowth could be observed from the 90th h. *H)* N-tubulin immunostaining of the ES/glia coculture on the 6th day shows multitudinous neuronal differentiation by CD1-GFP ES cells. *I–K)* On daily treatment with the pan-RAR antagonist, large cell assemblies developed in the cocultures. No neuronal process outgrowth could be observed. *L, M)* In some antagonist-treated cocultures, the ES assemblies contained pulsating structures, which could be identified as heart muscle by immunostaining for titin. DAPI stains the cell nuclei. Scale bar: 50 μm. See the related time-lapse movies (Supplemental Movies 3, 4).

application of the 100 nM RAR antagonist did not result in ES cell loss. The size (area) of the individual ES cell aggregates did not decrease in either R1 ES/glia or CD1-GFP ES/glia cocultures after 6 days of RAR antagonist treatment compared with the untreated cocultures (**Fig. 9**). Moreover, the size distribution of the ES cell assemblies showed that more large aggregates (>0.4 mm^2) developed in the antagonist-treated cocultures than in the untreated ones. If we compared the total number of aggregates in control and 100 nM antagonist-treated cocultures, no significant decrease in aggregate number could be observed (altogether, 199 aggregates in control and 193 aggregates in 100 nM antagonist-treated cocultures; summarized data are obtained from three independent experiments using the two different ES cells lines).

Although the number of the ES cell assemblies was not decreased, significantly fewer aggregates contained neurons in the RAR antagonist-treated cocultures (altogether, 168 neurogenic aggregates in control and only 37 neurogenic aggregates in the presence of 100 nM antagonist). The percentages of neurogenic aggregates on treatment of the cocultures with 100 nM RAR antagonist are shown in **Table 2**.

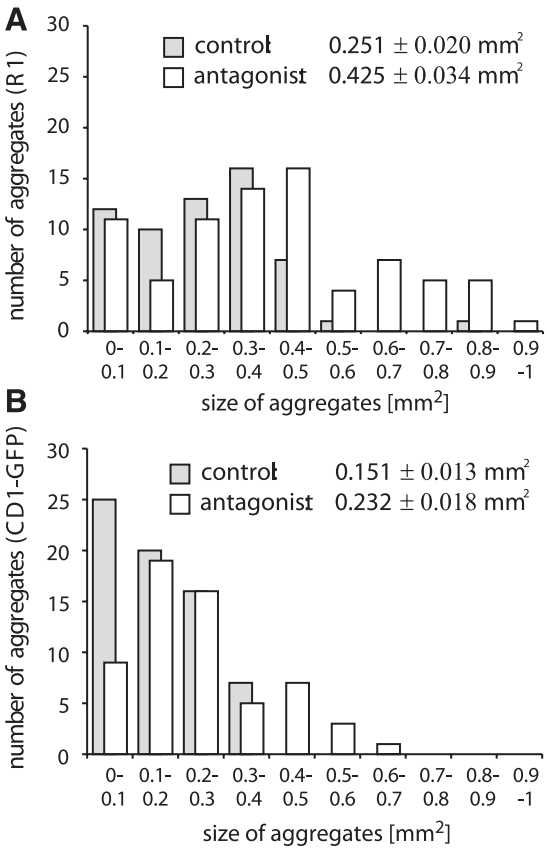

Figure 9. Size distribution of ES cell assemblies in control and 100 nM RAR antagonist-treated astroglia/ES cocultures. Areas of individual ES assemblies were determined on images taken on the 6th day of coculture. The average aggregate sizes and SES of the mean are indicated on the diagrams. The average sizes of both R1 (*A*) and CD1-GFP ES-derived aggregates (*B*) are significantly higher in RAR antagonist-treated cocultures than in the untreated ones. More large aggregates (>0.4 mm^2) developed in the antagonist-treated cocultures in both cases.

Blocking RA signaling enhances heart muscle type differentiation of embryoid bodies

As an unexpected finding, we observed ES cell-derived pulsating cell assemblies in the astroglia/ES cell cocultures treated with the RAR antagonist (supplemental movie 4). The pulsating tissues were usually parts of large ES cell assemblies (Fig. 8*K*) and were identified as heart muscle by staining for titin (Fig. 8*L, M*). The data indicate that blocking RAR signaling hinders neuronal but promotes an alternate heart muscle type differentiation.

DISCUSSION

In the past few years, several reports demonstrated that astroglial cells have the potential to induce neuron formation by noncommitted stem cells (25, 41–43). In the present work we tested our hypothesis that the astroglia-derived RA is one of the key signaling molecules responsible for the glia-induced neurogenesis.

Retinoic acid production is a common feature of cultured astrocytes

Our results obtained from experiments with the retinoid-sensitive bioassay demonstrated that cultured astroglial cells produce biologically active retinoids that can activate the RAR signaling pathway in the neighboring cells. We could not distinguish between the RA isomers by the applied bioassay, but HPLC analyses showed that all-*trans* RA was definitely present in the cultured astrocytes. The capacity of astrocytes to release RA indicated that retinaldehyde dehydrogenases, the key enzymes of RA synthesis, are functioning in the glial cells.

Significant differences between the RALDH profile of cerebellar (RALDH2,3) and cortical astrocytes (RALDH1,2) were shown by McCaffery *et al.* (24). In our experiments, RALDH1, RALDH2, and RALDH3 mRNAs were all present in all astroglia samples, and only minor differences could be observed between the expression levels of astrocytes derived from discrete brain regions. In agreement with these results, we could not detect reliable differences between the RA production of distinct astrocytic populations by the RA reporter assay. The HPLC analyses revealed some regional differences, with the cortical astroglia samples containing the most all-*trans* RA. However, the difference in RA content between cortical and other glia samples was only 2-fold, which would have little physiological impact. Based on these observations, we propose that the production of RA is a common feature of all neonatal astroglial populations maintained *in vitro* for prolonged periods (weeks).

In contrast to cultured astroglial cells, freshly isolated forebrain astrocytes did not produce RA in

TABLE 2. *Summary of data on astroglia-induced neuronal differentiation of two ES cell lines: R1 and CD1-GFP*[a]

	Percentages of neurogenic aggregates in astroglia/ES cocultures					
	Astroglia + R1 ES cells			Astroglia + CD1-GFP ES cells		
RAR antagonist	I.	II.	III.	I.	II.	III.
0 nM	85	71	89	86	60	100
100 nM	3	7	43	0	0	20

[a]In the control cultures (0 nM) most of the aggregates contained neurons, but the percentage of neurogenic aggregates was significantly reduced in the presence of 100 nM RAR antagonist. Data were obtained from I-II-III independent experimental series, where the number of the parallel cocultures was $n = 5$ (I), $n = 8$ (II), and $n = 4$ (III). The total aggregate number and the number of the neurogenic aggregates were determined in preparations fixed on the 6th day of coculture maintenance. The neurons were identified by staining for neuron-specific tubulin.

detectable amounts whether isolated from newborn or 1-wk-old old animals. There are several ways to explain the differences between the RA-producing capacity of cultured and freshly isolated astroglia. A possible explanation is that the *in vitro* conditions select for a subset of astrocytes with RA-producing activity, resulting in accumulation of a specific subpopulation during extended *in vitro* maintenance. A second possibility is that astrocytes within the postnatal forebrain parenchyme have limited access to RALDH enzyme substrates, including vitamin A. *In vitro*, however, the serum-containing medium provides a rich source of retinol that can be stored and used by the cultured glia. An alternative explanation may be that the isolated and *in vitro* maintained cells are released from a kind of inhibition blocking RA production *in vivo*. These hypotheses alone or in combination would also explain why we could see significant RA production by all the cultured astrocytic populations regardless of origin.

So far, we know very little about the *in vivo* retinoid metabolism and RA production of astrocytes. In the hippocampi of RA reporter mice (18), we could detect endogenous RA activity in cells with astroglial features. Such GFAP-expressing, RA-activated cells in the SVZ of adult mice were identified as slowly dividing neural precursors (14). Though RA exposure or blocking RAR signaling was shown to interfere with proliferation and differentiation within the neurogenic zones of the postnatal brain (11, 13), it is not known to what extent endogenous RA is contributing to the maintenance and/or induction of the RA-responsive populations, including GFAP+ cells. Another question is whether RA-responsive populations are identical to RA-producing cells. Disconnecting RA synthesis and RA responsiveness would not be a unique feature, as many sites of RA action in the forebrain do not colocalize with RA synthesis (44).

Glia-derived retinoic acid is a potent inducer of stem cell differentiation

As demonstrated earlier, the one cell-derived NE-4C neural stem cells gave rise to neurons if induced by all-*trans* RA or by the presence of astrocytes (25). Neuron formation was not impaired by a lack of functional p53 tumor suppressor protein (27), and these cells did not cause tumors if implanted into various tissues of adult or embryonic animals (45). On the basis of *in vitro* studies, NE-4C cells have been regarded as cloned embryonic neuroectodermal stem cells.

As described earlier (25), ES cells, if seeded individually onto glial monolayers, did not undergo neuronal differentiation within 2 wk. Embryoid body formation, however, rendered ES cells responsive to glia-derived instructive signals. The transition from a nonresponsive to a responsive state occurred within a 24 h period and seemed to depend on the formation of intercellular interactions. These observations indicate that differentiating ES cells have to reach a specific state to be able to respond to signals driving them further on the neural developmental pathway.

In glia/stem cell cocultures, blocking RA signaling inhibited neuron formation in predifferentiated ES aggregates. The data argue for a powerful role of glia-derived RA in inducing neuronal differentiation. Astroglial cells are known to produce factors such as FGF-2 (46) or LIF (47), which could counteract stem cell differentiation and support proliferation or maintenance of pluripotency (48). In cocultures of astrocytes with EBs, either the sensitivity of ES progenies to such factors changed, or their sensitivity to RA increased, such that RA could overcome the counteractions of other glia-derived signals.

RA was shown not only to induce neuronal differentiation of murine ES cells, but also to inhibit cardiomyogenesis (49). In accord with data on cardiomyocyte genesis in RAR antagonist-treated zebrafish embryos (50), a reduction in RA signaling caused the formation of contracting assemblies of muscle cells by ES cells. The data provide indirect evidence on the production of RA by cultivated astroglial cells and support the hypothesis that RA signaling creates a balance between noncardiac (neural) and cardiac identities of multipotent stem cells (50).

It is important to note that blocking RA signaling did not completely prevent glia-induced neuron formation in any type of coculture we tested. Although some residual RA activity cannot be ruled out, the role of other neural inducers must also be considered. Wnt signaling was shown to induce neuron formation in ventral midbrain glia/dopaminergic precursor or in hippocampal glia/hippocampal stem cell cocultures (7, 8). Blocking Wnt signaling, however, did not abolish astroglia-induced neuron formation in either case. These findings indicate that glia-derived RA and other glia-derived factors, such as Wnts, may complement each other's action. Their concurrent or successive inductive effect will result in neuronal cell fate commitment and determination of the neuronal phenotye.

Taken together, our data indicate that cultured astroglial cells produce RA in amounts sufficient to induce neuronal cell fate commitment by stem cells *via* signaling through RAR receptors. The present investigation suggests that astrocytes are a potential source of RA in the central nervous system. One of the main questions arising from our work is, under what physiological or pathological conditions can astrocytes produce RA *in vivo*? It is a question of whether this potential is limited to astrocytes residing in the neurogenic niches or parenchymal astrocytes in distinct regions and/or under certain conditions are also capable for active RA synthesis. To answer these questions, further analyses of glial retinoic acid production is required. FJ

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