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A role for the acetylcholine receptor-inducing protein ARIA in oligodendrocyte development

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ABSTRACT **ARIA acetylcholine receptor-inducing activity protein, is a member of a family of ligands that includes the Neu differentiation factor, heregulin, and glial growth factor. These ligands all act through one or more receptor tyrosine kinases of ≈185 kDa. In some conditions these ligands promote proliferation, whereas in others they induce differentiation. ARIA was originally isolated from chick brain on the basis of its ability to induce synthesis of nicotinic acetylcholine receptors in skeletal muscle. In this paper we show that ARIA is expressed in the subventricular zone of the rat brain and that it enhances the development of oligodendrocytes from bipotential (O2A) glial progenitor cells. We have also found that ARIA induces tyrosine phosphorylation of a 185-kDa protein in O2A progenitor cells. ARIA does not increase bromodeoxyuridine incorporation by oligodendrocytes but is mitogenic when added to Schwann cells *in vitro*. Thus, ARIA accelerates the formation of oligodendrocytes *in vitro* and is expressed where it could exercise the same influence *in vivo*.**

Oligodendrocytes and Schwann cells form myelin in the central and peripheral nervous systems, respectively (for review, see ref. 1). While these cells share the capacity to make myelin, they differ in many respects. After migrating from the neural crest, Schwann cells proliferate within peripheral nerves to produce the requisite number of myelinating and nonmyelinating Schwann cells. Axons themselves are mitogenic to Schwann cells (2), and at least one known Schwann cell mitogen, glial growth factor, is produced by neurons in the anterior horns of the spinal cord and in dorsal root ganglia (3, 4). In contrast, forebrain oligodendrocytes arise from unipotential and bipotential progenitors located in the subventricular zone (SVZ), where they proliferate prior to migration and differentiation. Control of the requisite number of myelinating oligodendrocytes may involve a balance between survival and apoptosis of oligodendrocytes (5). Perhaps the most distinct difference between Schwann cells and oligodendrocytes is that whereas myelinating Schwann cells form a single internode, oligodendrocytes form one to ≈50 internodes. The molecular determinants of the number of internodes formed by an oligodendrocyte are unknown.

ARIA (acetylcholine receptor-inducing activity protein) stimulates the synthesis of nicotinic acetylcholine receptors and voltage-gated sodium channels in striated muscle (6, 7). However, ARIA was originally isolated from brain, where its function is unknown (6), and with *in situ* hybridization we have found that one of the most intense signals for ARIA mRNA within the brain is in the SVZ, the birthplace of oligodendrocyte progenitors. This suggested to us that ARIA might influence oligodendrocytes or their progenitors. To test this hypothesis we studied the effects of ARIA on the development of oligodendrocytes from glial progenitors (O2A cells) *in vitro*. O2A progenitors are bipolar, bipotential cells which can be isolated from the immature and the mature central nervous system (20, 22–24, 33). In the presence of serum, O2A progenitors develop into type II astrocytes, while in the absence of serum they develop into oligodendrocytes (22). Therefore, in the absence of serum this paradigm provides a simple assay to study the influence of growth factors and cytokines on glial development.

We examined the influence of ARIA on O2A cells and found that it induces properties characteristic of oligodendrocytes. Furthermore, exposure to ARIA doubles the number of oligodendrocytes that extend sheet-like processes. The effects of ARIA on oligodendrocyte progenitors appear to be mediated through a p185 receptor tyrosine kinase as evidenced by its phosphorylation upon treatment with ARIA similar to that seen in ARIA-treated muscle (8). In contrast to oligodendrocytes, Schwann cells respond to ARIA by increasing their mitotic rate. This is an expected result because ARIA, in addition to being homologous to the rodent Neu differentiation factor and human heregulin, is homologous to the bovine glial growth factor (GGF), a known Schwann cell mitogen (4, 9–12).

MATERIALS AND METHODS

Materials. The anti-galactosylcerebroside antibody O1 was generated by M. Schachner and colleagues (13), and the hybridoma was obtained from S. Pfeiffer (University of Connecticut, Farmington). Anti-bromodeoxyuridine, fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibodies, and FITC-conjugated goat anti-mouse antibodies were obtained from Sigma. ARIA or control solution was obtained from serum-free conditioned medium from COS cells transfected with the sense or antisense expression vector, respectively (5). All ARIA-containing conditioned media used in these experiments induced synthesis of acetylcholine receptor or induced tyrosine phosphorylation of an ≈185-kDa protein in the muscle cell line L6.

Isolation of Oligodendrocyte Progenitors. Oligodendrocyte progenitors were isolated from the forebrains of 1-day-old rats by a previously described method (14) with slight modifications (15). Cultures seeded with cells dissociated from forebrains at postnatal day 1 (P1) contain O2A progenitors, endothelial cells, microglia, neurons, and type II astrocytes all of which grow upon a monolayer of type I astrocytes. Following detachment of progenitors from the crude mixtures by a 15-hr 180-rpm shake, recovered cells, predominantly O2A progenitors, type II astrocytes, and microglia, were subjected to three successive incubations in 100-mm tissue culture dishes. This nonspecific panning enriched for O2A progenitor cells because astrocytes, endothelial cells, and microglia adhere more rapidly to tissue culture plastic

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Abbreviations: bFGF, basic fibroblast growth factor; FITC, fluorescein isothiocyanate; GGF, glial growth factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; P*n*, postnatal day *n*; PDGF, platelet-derived growth factor; SVZ, subventricular zone.

than do O2A progenitors. In addition, leucine methyl ester was added to the cells during panning, and similar to a previous report (15), this procedure reduced microglia to <0.2% of the plating mixture. Viable cells remaining in suspension were then plated onto polyornithine-coated 25-mm glass coverslips in Dulbecco's modified Eagle's medium (DMEM; GIBCO/BRL), plus 5% fetal bovine serum (day zero). After 3 hr plating medium was replaced with defined medium for oligodendrocytes (DM_{Oli}), which favors survival and growth of cells in the oligodendrocyte lineage. This medium contains DMEM plus 0.5% recrystalized bovine serum albumin, insulin (5 μg/ml), putrescine (100 μM), transferrin (50 μg/ml), selenium (5 ng/ml), triiodothyronine (30 nM), progesterone (20 nM), and D-biotin (10 ng/ml; Sigma).

Isolation of Schwann Cells. P1 rat sciatic nerves were digested sequentially with 0.1% collagenase and 0.25% trypsin plus 0.1% collagenase for 30 min, each followed by inactivation with fetal bovine serum (10%, vol/vol) (16, 17). Digested nerves were triturated 20 times with a Pasteur pipette, clumps were allowed to settle, and the supernatant was centrifuged at 900 × g for 4 min. Cells (5×10^6) were plated in one tissue culture dish of 10-cm diameter and treated for 3 days with 10^{-5} M 1-β-D-arabinofuranosylcytosine beginning on the day after plating, to reduce fibroblast contamination. After 7 days in culture, 85–95% of the cells were S100-positive. Cultured Schwann cells were replated onto polyornithine-coated cover glasses at a density of 30,000 cells per cm^2 and used directly for proliferation studies.

Immunocytochemistry and Survival Studies. Viability of cells was assessed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), which is cleaved by dehydrogenases of mitochondria to a water-insoluble purple formazan (18, 19). Three hours before the end of the incubation, the cultures were incubated with MTT to label living cells. Fifteen minutes prior to fixation, the cultures were incubated with the O1 antibody to label surface galactocerebroside. At the end of the incubation, cultures were fixed with 4% paraformaldehyde followed by FITC-conjugated second antibody. Viable oligodendrocytes were defined by colabeling with MTT staining and O1 immunoreactivity.

Immunoblotting and Identification of Phosphotyrosine Epitopes. Oligodendrocyte progenitors were stimulated to divide by treatment with basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF)-AA. To assay type II astrocytes, O2A progenitor cells were cultured in 5% fetal bovine serum. Cultures were exposed to control solution or ARIA for the times indicated. Reactions were terminated by aspirating the medium and adding lysis buffer (50 mM Tris/150 mM NaCl/1 mM EDTA/0.5% Triton X-100/0.5% Nonidet P-40/1 mM Na_3VO_4/1 mM phenylmethanesulfonyl fluoride, pH 8.0). Harvested lysates were triturated, incubated for 30 min at 4°C, and then preincubated with 30 μl of protein A-Sepharose for 1 hr. Insoluble material and that which nonspecifically bound to protein A-Sepharose were precipitated. Washed lysates were incubated overnight with anti-phosphotyrosine antibodies (Upstate Biotechnology, Lake Placid, NY), and antigen–antibody complexes were precipitated with protein A-Sepharose. Washed immunoprecipitates were denatured in loading buffer, resolved in SDS/5% polyacrylamide gels, and transferred to poly(vinylidene diflouride) membranes (Millipore). Phosphotyrosine epitopes were detected by sequential incubation of the blot with mouse anti-phosphotyrosine antibodies, peroxidase-conjugated anti-mouse Fab fragments, and chemiluminescence reagents and, finally, exposure to x-ray film.

***In Situ* Hybridization.** *In situ* hybridizations were performed as described (5). Brains were immersed in 4% paraformaldehyde in phosphate-buffered saline overnight at 4°C. After fixation, tissues were slowly dehydrated and embedded in paraffin. Coronal sections (9 μm) were collected on gelatin-coated glass slides. Hybridization with sense and antisense probes was carried out at 52°C for 18 hr in 50% deionized formamide/0.3 M NaCl/20 mM Tris·HCl, pH 7.4/5 mM EDTA/10 mM sodium phosphate/10% dextran sulfate/1× Denhardt's solution containing total yeast RNA (50 μg/ml) and ^{35}S-labeled RNA probe (3.5×10^4 cpm/μl) under siliconized coverslips. Slides were washed with 50% formamide/2× standard saline citrate (SSC)/10 mM dithiothreitol at 65°C, treated with RNase A, and then washed at 37°C for 15 min each in 2× SSC and 0.1× SSC. Sections were dehydrated rapidly, processed for autoradiography with NTB-2 Kodak emulsion, exposed for 4 weeks at 4°C, and examined by both light- and dark-field illumination.

Proliferation Studies. Oligodendrocyte progenitors or Schwann cells were cultured with ARIA, PDGF-AA (10 ng/ml; GIBCO/BRL), PDGF-AA plus bFGF (10 ng/ml), or a control solution (phosphate-buffered saline or control conditioned medium). On day 1, bromodeoxyuridine was added (5 μM) and on day 2 cells were fixed, incubated with a rabbit anti-bromodeoxyuridine antibody (Sigma) followed by a FITC-conjugated goat anti-rabbit antibody, and visualized with epifluorescence.

RESULTS

ARIA Promotes Differentiation of Oligodendrocytes. Oligodendrocyte progenitors were isolated from forebrains of P1 rats (14). Of the cells that can proliferate *in vitro*, brains from rats at this stage of development yield mostly glial progenitors, macroglia, microglia, and endothelial cells (15, 20). The isolates were depleted of microglia, astrocytes, and endothelial cells by differential adhesion to tissue culture plastic and treatment with leucine methyl ester. Cell suspensions enriched in progenitor were cells, then plated onto polyornithine-coated coverglasses (day 0) in the presence of serum-free medium conditioned by COS cells transfected withn an ARIA construct. As a control, cells were incubated with conditioned medium from COS cells transfected with an ARIA construct in the antisense orientation (control) or with unconditioned COS cell medium (6, 8). Serum promotes formation of type II astrocytes from O2A progenitors, and thus all experiments were performed in the absence of serum, except when generation of type II astrocytes was a goal.

O2A progenitors are characterized by bipolar morphology and A2B5 immunoreactivity, whereas oligodendrocytes have a characteristic multipolar morphology and surface galactocerebroside immunoreactivity (O1 positivity; refs. 21 and 22). *In vitro* the progenitors can develop into either type II astrocytes, which retain A2B5 binding and acquire glial fibrillar acidic protein (GFAP) immunoreactivity, or oligodendrocytes, which neither stain with the A2B5 antibody nor acquire GFAP immunoreactivity (22–24). Therefore, O1 positivity was taken as a measure of oligodendrocyte development. In addition to O1 staining, positive cells had at least one process longer than a cell diameter. After 48 hr, cultures treated with ARIA showed approximately twice as many O1-positive oligodendrocytes as did control cultures (Fig. 1*A*). Despite this increase in O1-positive cells with processes longer than one cell diameter, there was no increase in cell viability as assessed by MTT conversion.

The possibility exists that ARIA both induces proliferation of oligodendrocytes and decreases survival of other cell types. However, with ARIA treatment there was no detectable change in bromodeoxyuridine incorporation by oligodendrocytes or their progenitors (Fig. 1*B*). PDGF-AA and bFGF did stimulate bromodeoxyuridine incorporation by cells of the oligodendrocyte lineage. The simplest conclusion from the MTT conversion, bromodeoxyuridine incorporation, and immunocytochemical staining is that ARIA pro-

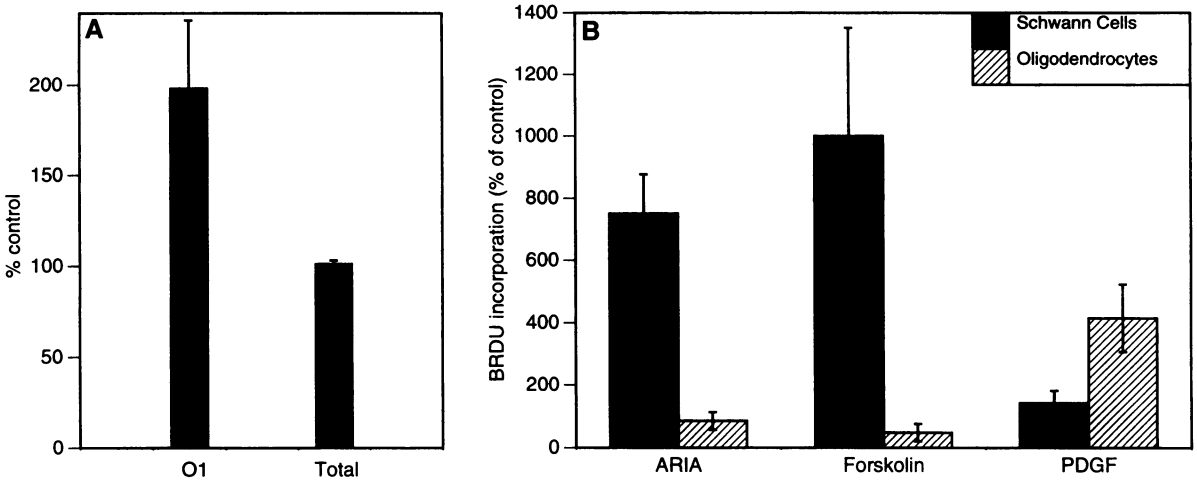

FIG. 1. Effect of ARIA on oligodendrocyte differentiation and survival. (*A*) Ratio of galactocerebroside-positive cells with processes greater than one cell diameter and total number of viable (MTT-positive) cells. Viable oligodendrocytes were identified by co-occurrence of MTT uptake with O1 immunoreactivity. Each point represents the mean and SD of three determinations performed in triplicate and is expressed as percentage of control. Raw numbers follow. O1- and MTT-positive cells: control, 16; ARIA, 31 cells per mm^2. Total MTT-positive cells: control, 67; ARIA, 68 per mm^2. For O1-MTT staining 20, and for MTT staining 200, 0.78-mm^2 fields were counted per experiment. (*B*) Mitogenic response of Schwann cells and oligodendrocytes to ARIA. Primary cultures of rat sciatic nerve Schwann cells were prepared by a modification of the method of Brocks *et al.* (17) Schwann cells or oligodendrocytes were plated onto polyornithine-coated glass coverslips and incubated with phosphate-buffered saline, control conditioned medium, ARIA-containing conditioned medium, forskolin (1 μM), or PDGF-AA (10 ng/ml) on day 0. On day 1, bromodeoxyuridine (BRDU, 1 μM, Boehringer Mannheim) was added, and on day 2, cultures were fixed and stained as described. Bromodeoxyuridine-positive nuclei were counted in 40 random nonoverlapping fields. Filled and hatched bars represent results from Schwann cells and oligodendrocytes, respectively. Results reported are the mean and SEM of three experiments performed in triplicate.

motes O2A progenitors toward oligodendrocyte differentiation rather than influencing survival or proliferation. ARIA stimulated incorporation of bromodeoxyuridine by Schwann cells 5-fold over controls. This was an expected result, as ARIA is homologous to GGF, a potent Schwann cell mitogen (3, 4, 12).

ARIA Stimulates Formation of Sheet-Like Process Outgrowth by Oligodendrocytes. More than 80% of oligodendrocytes in both ARIA and control cultures had spoke-like rather than sheet-like processes. However, the arborization of the spoke-like processes was more extensive in the ARIA-treated cultures than in controls. Furthermore, after 48 hr of ARIA treatment, oligodendrocytes had approximately twice as many sheet-like processes as did controls, and the sheets were larger (Fig. 2 *A* and *B*). This phenotype is characteristic of differentiated oligodendrocytes (20, 25, 26).

Stimulation of Tyrosine Phosphorylation of an ≈185-kDa Protein in O2A Progenitors by ARIA. ARIA induces tyrosine phosphorylation of one or more ≈185-kDa proteins thought to be homologous to members of the Neu/ErbB/HER family of receptor tyrosine kinases (8–11). We expanded the number of O2A progenitors in culture with PDGF-AA and bFGF (27) and from these derived three populations of cells to assay the effects of ARIA on protein tyrosine phosphorylation: (*i*) O2A progenitors maintained in a proliferating state by continuous exposure to PDGF-AA and bFGF, (*ii*) oligodendrocytes maintained in DM_{Oli} for 4 days, and (*iii*) type II astrocytes maintained in 5% fetal bovine serum for 4 days. The cultures were then treated with control solution or ARIA for 1 and 5 min. In O2A progenitors, ARIA induced tyrosine phosphorylation of a 185-kDa polypeptide that was evident at 1 min and more intense at 5 min (Fig. 3) but was not present at time zero. In cultures of oligodendrocytes, phosphorylated polypeptide at 185 kDa was present in untreated cells and ARIA treatment resulted in a more intense signal at this apparent molecular mass (Fig. 3).

To establish that ARIA was not acting on progenitor cells indirectly through activation of receptors on astrocytes, we examined type II astrocytes for the presence or absence of an ARIA-induced phosphorylation of a 185-kDa protein. The O2A progenitor and oligodendrocyte cultures contained ≈10% contaminating type II astrocytes. In cultures containing >90% type II astrocytes, there was little or no detectable tyrosine-phosphorylated polypeptide at 185 kDa. This suggests that ARIA acts directly on O2A progenitors and oligodendrocytes through a 185-kDa receptor tyrosine kinase as it does on muscle cells.

Localization of ARIA mRNA in the SVZ by *in Situ* Hybridization. In the forebrain oligodendrocytes arise from progenitors in the SVZ (29–31). We examined ARIA expression by *in situ* hybridization in sections of rat brains from embryonic day 17 to P6, using antisense RNA probes to the epidermal growth factor domain of ARIA. At P0, cells in the germinal matrix of the forebrain give rise predominantly if not exclusively to glia. Fig. 4 is a micrograph of a coronal section through the forebrain of a P0 rat. There was an intense signal for ARIA in SVZ, the tissue at the dorsal and lateral margins of the lateral ventricles (Fig. 4). The reactivity appeared in SVZ cells and the ependymal lining as well. A control, the sense RNA probe, did not elicit signal on equivalent sections. This result shows that ARIA is expressed in a region of the brain where oligodendrocytes are born and at a time when glial progenitors are undergoing differentiation.

DISCUSSION

In forming central and peripheral nervous system myelin, oligodendrocytes and Schwann cells respond to cues from neuronal and non-neuronal cells. Environmental factors that influence these predominantly postnatal events are important to our understanding both of early development and of regeneration following injury to the oligodendrocyte– or Schwann cell–myelin units. We observed ARIA mRNA in the SVZ of the postnatal rat brain and investigated the influence of this ligand on the development of rat oligodendrocytes *in vitro*.

We have shown that, *in vitro*, ARIA promotes differentiation of oligodendrocytes; it accelerates the formation of process-bearing, surface galactosylcerebroside-positive cells from galactosylcerebroside-negative bipolar glial progenitor

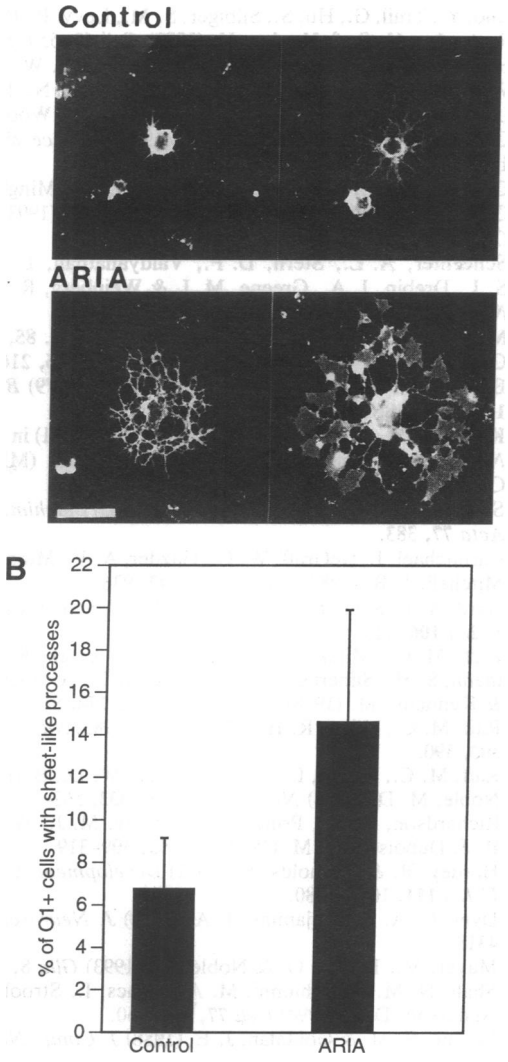

FIG. 2. (*A*) Morphology of cells in control and ARIA-treated cultures. Cultures were treated with a single dose of control solution or ARIA on day 0 and then fixed and stained as in Fig. 1 on day 2. Stained cells were viewed with epifluorescence on a Zeiss Axioskop and photographed with Ilford delta film. (Bar = 10 μm.) (*B*) Percentage of total O1-positive cells which have sheet-like processes. Results represent the mean and SD of two experiments performed in triplicate. Control, conditioned medium from COS cells transfected with the ARIA-antisense vector; ARIA, conditioned medium from COS cells transfected with the ARIA-sense vector.

cells. In serum-free medium containing survival factors (e.g., insulin-like growth factor I) and in the absence of mitogens, O2A progenitors will withdraw from the cell cycle and differentiate into oligodendrocytes (22–24,32). In our study, the effect of ARIA was to increase the rate of this transition. Another characteristic of some differentiated oligodendrocytes is formation of sheet-like processes; ARIA doubles the number of oligodendrocytes which extend these processes. These effects of ARIA on the rate of oligodendrocyte differentiation from progenitor cells and on morphology of oligodendrocytes in the absence of an effect on survival or proliferation are in contrast to the effects of ARIA or its homologue GGF on neural crest stem cells and Schwann cells. GGF was found to direct multipotent neural crest stem cells toward a Schwann cell rather than a neuronal fate (28). Schwann cells themselves proliferate in response to GGF (3, 4, 12).

Signaling by ARIA and homologous ligands from other species (Neu differentiation factor, heregulin, GGF) involves

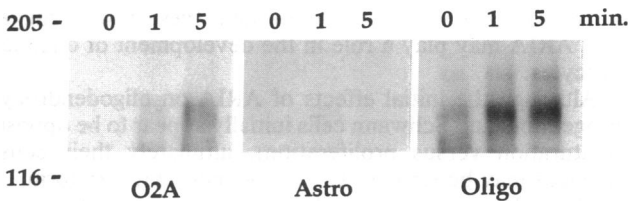

FIG. 3. Western blot of phosphotyrosine epitopes from cultures of O2A cells (*Left*), type II astrocytes (*Center*), and oligodendrocytes (*Right*) treated with ARIA. Lysates were immunoprecipitated with anti-phosphotyrosine antibodies and resolved by 5% PAGE. Proteins were transferred to Immobilon (Millipore) membranes, incubated with anti-phosphotyrosine antibodies, and detected with peroxidase-conjugated anti-mouse IgG Fab fragments and chemiluminescence reagents. Time of incubation (minutes) with ARIA is indicated on top, and apparent molecular mass (kilodaltons) at left.

binding to and activation of an ≈185-kDa receptor tyrosine kinase (4, 6–12). We have demonstrated that in O2A progenitors and in oligodendrocytes, ARIA stimulates the tyrosine phosphorylation of a 185-kDa protein. In cultures of O2A progenitors there is little or no tyrosine phosphorylation in unstimulated cells and a rapid induction of tyrosine phosphorylation following treatment with ARIA. In cultures of oligodendrocytes we find a low level of tyrosine phosphorylation at ≈185 kDa in unstimulated cells that is increased by treatment with ARIA. The phosphorylation in the absence of added ligand may represent constitutive receptor activation or synthesis of the ligand by cells in these cultures.

We do not see ARIA-induced tyrosine phosphorylation in cultures enriched >90% for type II astrocytes. Therefore, it is unlikely that the effects of ARIA on the development of cells in the oligodendrocyte lineage are mediated indirectly by type II astrocytes.

Neurogenesis is completed in the rat brain by the early postnatal period, and the SVZ at this time contains precursors which contribute to the oligodendrocyte lineage (29–31). Here we show ARIA mRNA within the SVZ of the P0 rat, and we have found a similarly intense signal in the SVZ of the P6 rat brain (34). Thus, ARIA is expressed in the developing rat nervous system at a time when oligodendrocyte differentiation is prominent and in a place where it is known to occur.

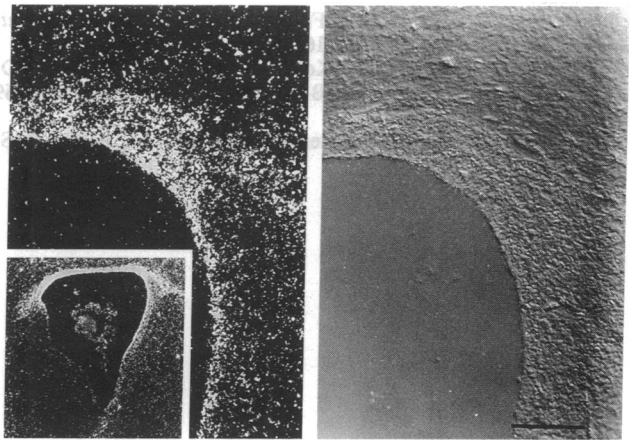

FIG. 4. *In situ* hybridization of ARIA in the SVZ of the P0 rat brain. (*Left*) Dark-field image shows *in situ* hybridization of a section from the forebrain of a P0 rat with an ^{35}S-labeled probe for the epidermal growth factor domain of the β-1 form of ARIA. The probe was designed so that it recognizes a sequence that is highly conserved among the members of the ARIA family. There is an intense signal from cells in the SVZ and there is also some labeling of a column of cells that may be migrating away from the SVZ. No signal was noted on an adjacent section with the ^{35}S-labeled sense probe (data not shown). (*Right*) Bright-field image. (Bar = 100 μm.)

Taken together with our *in vitro* data, these results suggest that ARIA may play a role in the development of oligodendrocytes.

Although the initial effects of ARIA on oligodendrocyte progenitors and Schwann cells initially appear to be opposite (maturation versus proliferation), ultimately their consequences may be similar. The oligodendrocyte can form 1 to ≈40 myelin internodes, and our preliminary evidence suggests that ARIA increases the number of processes extended by oligodendrocytes and therefore could allow them to contribute more internodes. However, each Schwann cell provides only one internode and to achieve more internodes it is necessary for the Schwann cells to increase in number, which they do in response to ARIA. Thus, through its effects on the myelin-forming cells, ARIA may enhance myelination in both the central and peripheral nervous systems, on one hand by driving precursor cells to form oligodendrocytes and oligodendrocytes to extend processes, and on the other hand by stimulating Schwann cells to proliferate. The opposite effects of ARIA on central and peripheral glia may provide a simple paradigm for comparing the divergent and convergent natures of the two systems.

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