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Differential Expression of ARIA Isoforms in the Rat Brain

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Summary

ARIA, heregulin, neu differentiation factor, and glial growth factor are members of a new family of growth and differentiation factors whose effects have been assayed on Schwann cells, skeletal muscle cells, and mammary tumor cell lines. To gain insight into their roles in the CNS, we studied the expression of ARIA in the rat brain. We found ARIA mRNA in all cholinergic neurons throughout the CNS, including motor neurons and cells of the medial septal nucleus and the nucleus basalis of Meynert. We also found that ARIA induces tyrosine phosphorylation of a 185 kDa protein in central and peripheral targets of these cholinergic neurons. ARIA mRNA, however, is not restricted to cholinergic neurons, suggesting that it may also play a role at other types of synapses. Its distribution in germinal layers of the telencephalon and cerebellum suggests that it may also play a role in the proliferation and/or migration of neuronal and glial precursor cells.

Introduction

ARIA is a protein purified from chicken brain on the basis of its ability to promote the synthesis of acetylcholine receptors (AChRs) in cultured skeletal muscle cells (Jessell et al., 1979; Usdin and Fischbach, 1986; Falls et al., 1993). This protein may play a role in promoting the initial accumulation of AChRs at developing neuromuscular junctions and also in maintaining the high AChR density characteristic of mature junctions. Like other growth and differentiation factors, ARIA probably produces biological effects beyond the one assayed in its original purification. We already know that ARIA increases the number of voltage-gated Na^+ channels in chick myotubes and myoblasts (Corfas and Fischbach, 1993a). Like AChRs, Na^+ channels are concentrated in the postsynaptic membrane of the neuromuscular junction (Beam et al., 1984; Flucher and Daniels, 1989). Since ARIA is purified from brain, it seems likely that it may influence the molecular architecture of interneuronal synapses. As a first step toward understanding the roles of this new factor in the nervous system, we have investigated the distribution of ARIA mRNA in the rat brain by in situ hybridization.

ARIA is a member of a family of proteins that includes glial growth factor (GGF), neu differentiation factor (NDF), and heregulin (HRG). GGF was purified on the basis of its ability to stimulate proliferation of Schwann cells (Brockes et al., 1980; Goodearl et al., 1993; Marchionni et al., 1993). NDF and HRG were purified as putative ligands for the neu/HER2 proto-oncogene receptor tyrosine kinase (Peles et al., 1992; Wen et al., 1992; Holmes et al., 1992). This 185 kDa transmembrane protein has been implicated in the pathogenesis of several types of adenocarcinomas (for review, see Singleton and Strickler, 1992). Members of this ligand family appear to be encoded by a single gene, but the gene is comprised of many exons, and a great variety of alternatively spliced messages have been detected (Marchionni et al., 1993; Wen et al., 1994). The picture is rather complex, and a common nomenclature has not yet emerged. Therefore, we will use the name ARIA for the molecule described in this paper.

Most of the isoforms appear to be synthesized as transmembrane precursor proteins. We assume that the extracellular portion of the protein is cleaved and released, constituting the soluble factor that we have detected in medium conditioned by motor neurons and by COS cells transfected with the ARIA clone (Corfas et al., 1993b; Falls et al., 1993). The extracellular part of the molecule contains an epidermal growth factor-like (EGF-L) domain near the putative transmembrane domain characterized in part by the position of six cysteine residues. It is important to note that members of the ARIA family of proteins do not activate the EGF receptor and that the sequence of the EGF-L is different from other EGF-L sequences (Peles et al., 1992; Holmes et al., 1992). In the ARIA family, the EGF-L sequence of about 50 amino acids is a critical part of the molecule. When expressed in bacteria, the EGF-L domain exhibits high affinity binding to appropriate target cell membranes and stimulates tyrosine phosphorylation of HER2/neu (Holmes et al., 1992; Wen et al., 1994). The EGF-L domain also stimulates AChR synthesis in cultured myotubes. The EGF-L domain is not the same in all isoforms. Based on sequence variation within the C-terminal portion of the EGF-L domain, it appears that this segment is encoded by three exons. The first splice site, which occurs at the fifth cysteine, distinguishes two classes named α and β (Holmes et al., 1992). The second splice site, which occurs a few codons after the sixth cysteine, distinguishes several subtypes within each class.

In this study, we have used two ARIA probes. The first one, an EGF-L probe, we assume is a pan-ARIA probe, since all known ARIA isoforms contain an EGF-L domain and EGF-L domains are identical over most of their length. In most isoforms described to date, the extracellular domain also contains an immunoglobulin-like (Ig-L) domain. This domain is encoded by a different set of exons than the EGF-L domain. The second probe directed against this domain was used to assay the coordinate expression of the EGF-L and Ig-L domains.

Early reports showed that the ARIA gene family is expressed in embryonic chick and mouse spinal cord motor neurons and in some cranial nerve motor nuclei (Falls et al., 1993; Marchionni et al., 1993; Orr-Urtreger et al., 1993). We have examined the expression of ARIA mRNA in the embryonic and postnatal chicken CNS (Corfas et al., 1993, Soc. Neurosci., abstract). However, many questions

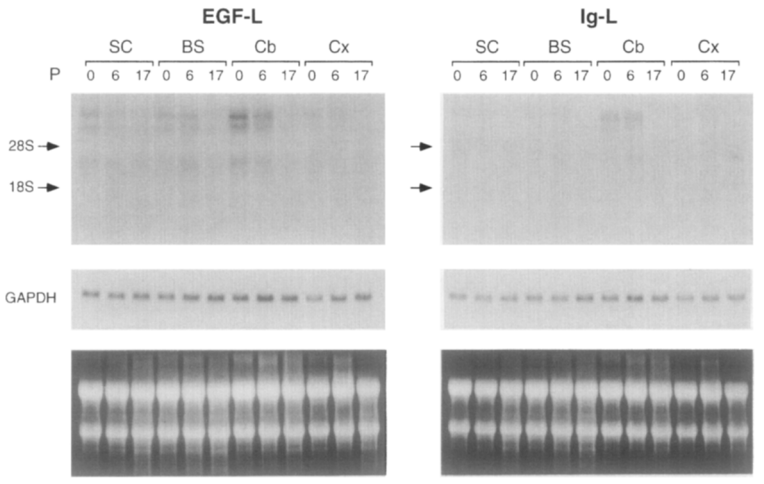

Figure 1. Northern Blot Analysis of ARIA mRNA in the Rat Brain

Total RNA (10 μg) were loaded in each lane. RNA was isolated from spinal cord (SC), brain stem (BS), cerebellum (Cb), and cortex (Cx) obtained from rats on P0, P6, and P17. Blots were probed with either the EGF-L or the Ig-L probe under high stringency conditions. The films were exposed for 95 hr at −70°C with two intensifying screens. The positions of the 28S and 18S ribosomal RNAs are indicated for reference. The ethidium-stained gels (lower panels) demonstrate that the amount and quality of the RNA was similar in all lanes. Filters were stripped and subsequently rehybridized to a human glyceraldehyde-3-phosphate dehydrogenase cDNA as an additional control for equivalent loading of samples.

regarding the role of ARIA in the brain can be better studied in the rodent, where the synaptic organization is more easily related to that of higher vertebrates. Therefore, we have isolated a clone for a form of ARIA expressed in the rat CNS and used it to study the distribution of ARIA mRNA by in situ hybridization in postnatal animals and in late-stage embryos.

Results

Cloning of a β1 Form of Rat ARIA

To study the distribution of ARIA mRNA in the rat CNS, a cDNA was amplified from postnatal day 20 (P20) rat spinal cord total RNA using the polymerase chain reaction (PCR) with primers derived from protein sequences N-terminal to the Ig-L region (sense) and from the transmembrane region (antisense). The product of the reaction was a 690 bp cDNA corresponding to about 70% of the presumed extracellular domain of the β1 isoform of the pro-ARIA protein. We have found that the β1 isoform is the most abundant in chick CNS. The original isoform isolated from a rat cell line, and the only one known at the time our experiments were initiated, contained an α motif (Wen et al., 1992). Additional isoforms have been isolated recently from a rat fibroblast cell line cDNA library, including one with a β1 EGF-L motif (Wen et al., 1994). Our clone, prB2-1, is identical at the nucleotide level to this rat β1 clone. It is 97% identical at the amino acid level to the human β1 (Holmes et al., 1992), and with only one gap, it is 81% identical to the original chick ARIA clone (12.7), which also contains a β1 motif (Falls et al., 1993).

Two independent probes were created from prB2–1 by digestion with Sph1. One contained the Ig-L domain (306 bp), whereas the other contained the EGF-L sequence (384 bp). Each fragment contained part of the spacer sequence between the EGF-L and Ig-L domains and were similar in G-C content. Preliminary studies of the ARIA/HRG/NDF/GGF gene structure indicate that the Sph1 site in prB2-1 is 15 nucleotides beyond the second exon of the Ig-L domain (Marchionni et al., 1993). Thus, only this portion, which amounts to 5% of the length of the probe, could hybridize to non-Ig-L domain–containing transcripts.

Analysis of ARIA mRNA in the CNS by Northern Blot

The expression of ARIA mRNA in spinal cord, brain stem, cerebellum, and cortex was analyzed by Northern blot in newborn (P0), 6-day-old (P6), and 17-day-old (P17) rats. With the EGF-L probe, three transcripts of approximately 12.0, 9.0, and 3.5 kb were detected in each region of the brain examined (Figure 1A). The 12.0 kb and 9.0 kb transcripts were more abundant than the 3.5 kb transcript. The strongest hybridization signal per microgram of RNA was observed in the cerebellum, and the cerebral cortex exhibited the lowest. The amount of all three transcripts declined with age between P0 and P17.

The decrease in ARIA mRNA concentration could be due either to a down-regulation of ARIA gene expression or to dilution of ARIA mRNA coincident with the appearance of glia or other non-ARIA-expressing cells. The latter is more consistent with the in situ hybridization results described below.

Unexpectedly, the signal observed with the Ig-L probe was only a small fraction of the EGF-L signal. Moreover, the Ig-L probe hybridized primarily to the 12.0 kb transcript (Figure 1B); the 9.0 and 3.5 kb bands were not detectable. The Ig-L signal was strongest in the cerebellum at each age examined, and it was least abundant in the spinal cord. This pattern is different from that characteristic of the EGF-L probe, which was highest in the cerebellum but lowest in the cerebral cortex. Another difference between the probes was evident in the time course. The signal detected by the EGF-L probe declined monotonically after birth, whereas the Ig-L signal did not decrease between P0 and P6. Identical results were obtained with a shorter probe confined to the amino-terminal part of the Ig-L domain (data not shown). Since the probes were uniformly labeled, the marked differences in the EGF-L and Ig-L Northern blots implies that many transcripts lack the Ig-L domain.

ARIA mRNA in Cholinergic Neurons

Spinal Cord

Previous studies of chick and mouse embryos show that ARIA mRNA is highly concentrated in spinal cord motor

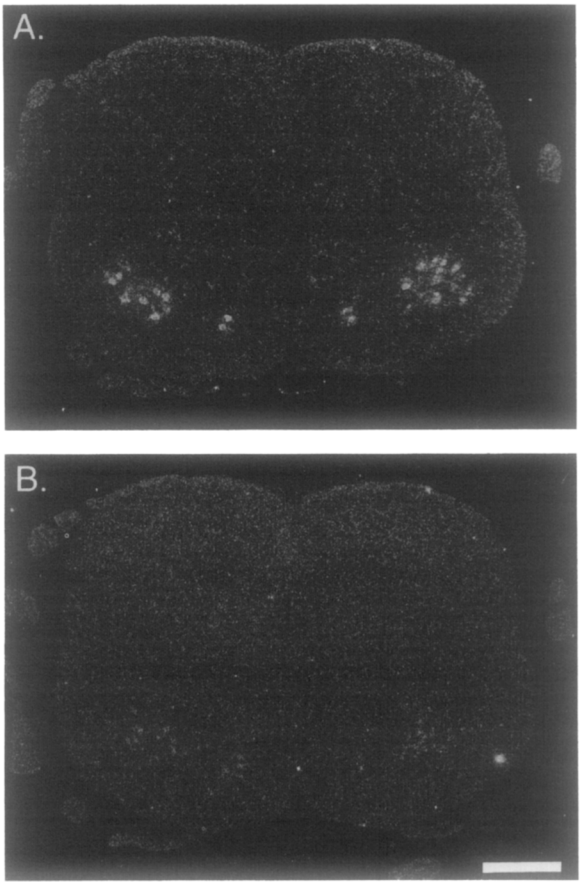

Figure 2. Expression of ARIA mRNA in the P17 Rat Spinal Cord

Autoradiographic localization of ARIA mRNA in transverse sections of lumbar spinal cord. (A) shows EGF-L probe; (B) shows Ig-L probe. Grains were visualized by dark-field illumination. Note the relatively faint but definite Ig-L signal in ventral horn motor neurons. Bar, 300 μm.

neurons (Falls et al., 1993; Marchionni et al., 1993; Orr-Urtreger et al., 1993). We have found that ARIA mRNA remains highly concentrated in spinal cord motor neurons after birth. Figure 2A shows a transverse section through the third lumbar spinal cord segment of a P17 rat that was hybridized with the EGF-L probe. Two groups of motor neurons are evident. The medial motor neuron pool innervates axial muscles, and the lateral pool innervates limb muscles. Both pools were labeled with the EGF-L antisense riboprobes. In counterstained sections, we did not observe any large cells that were not associated with grain clusters, and we conclude that all lumbar motor neurons express ARIA. Essentially the same results were obtained in sections of the cervical cord and thoracic cord, although our observations of the latter were more limited.

ARIA mRNA expression in motor neurons was not restricted to early postnatal stages, as the EGF-L probe generated an intense signal in spinal cord motor neurons of adult (lactating) rats. We also found ARIA mRNA in spinal cord motor neurons at E14, the earliest age examined (see below).

In agreement with the Northern blots, the Ig-L in situ hybridization signal was low as compared with the EGF-L signal (Figure 2B). However, despite the small number of grains, a clear signal above background was evident over ventral horn motor neurons. Once again, it appeared that most, if not all, motor neurons were labeled. The same striking disparity between the EGF-L signal and the Ig-L signal was evident at all ages examined. As noted above, the Ig-L probe extended beyond the sequences of the presumed Ig-L exons. Under our in situ hybridization conditions, it was unlikely that the extra 15 nucleotides contributed to the signal. In any case, our results provide an upper limit for the relative abundance of Ig-L-containing forms.

Preganglionic neurons in the intermediate column of thoracic spinal cord are also cholinergic. These neurons extend axons out of the spinal cord to innervate paravertebral and prevertebral sympathetic ganglia. Relatively large neurons in the intermediate column were labeled by the EGF-L probe. We did not confirm that the labeled cells were preganglionic neurons, but from their positions it seemed likely.

Every experiment was carried out simultaneously with antisense and sense probes. Sense probes gave no significant hybridization signal in any region; they are not depicted in any of the figures. In almost all experiments, parallel sections were hybridized with unrelated probes. These slides gave a different pattern of hybridization, serving also as controls.

Cranial Nerve Motor Nuclei

All cranial nerve motor nuclei that innervate striated muscle directly contained neurons that were labeled with both EGF-L and Ig-L probes. The oculomotor (III), trochlear (IV), motor nucleus of the trigeminal (V), abducens (VI), facial (VII), hypoglossal (XII), and nucleus ambiguus were heavily labeled by the EGF-L probe (Figures 3A1, 3B1, and 3C1). Consistent with Northern blot analysis of RNA extracted from this region, the Ig-L signal was less than the EGF-L signal, but the relative intensity of the two probes varied between nuclei (Figures 3A2, 3B2, and 3C2).

ARIA mRNA was also abundant in the dorsal motor nucleus of the vagus nerve (X; Figure 3C1). These cholinergic neurons do not innervate muscle directly; rather, they innervate parasympathetic ganglion neurons embedded in the walls of the viscera. ARIA mRNA was also abundant in the Edinger–Westphal nucleus, a group of neurons in the midbrain anterior and dorsal to III, that innervate parasympathetic ciliary ganglion neurons (data not shown). The presence of ARIA mRNA in brain stem and spinal cord preganglionic neurons suggests that ARIA may influence the expression of neuronal nicotinic receptors.

Other Cholinergic Nuclei in the CNS

ARIA mRNA was present in all basal forebrain cholinergic nuclei. The medial septal nucleus, both vertical and horizontal branches of the diagonal band of Broca, and the nucleus basalis of Meynert were heavily labeled with the EGF-L probe (Figures 4A–4D). In contrast to motor neurons in the spinal cord and brain stem, the Ig-L signal was identical to that of the EGF-L signal in the basal forebrain (cf. Figures 4A and 4B). ARIA mRNA was located in some of the largest cells in the nucleus basalis (Figure 4C), but we cannot claim that it is restricted to the cholinergic cells

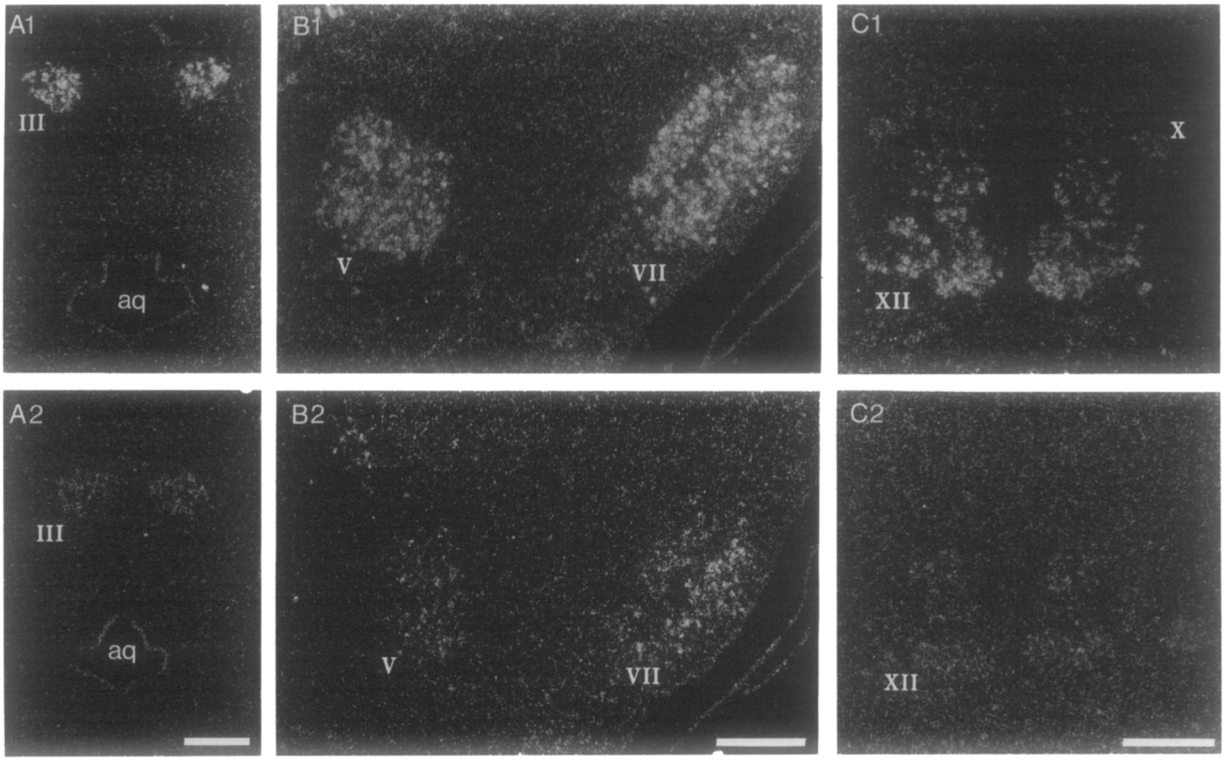

Figure 3. ARIA mRNA in Cranial Motor Nuclei

Sections of P6 rat brains hybridized either with the EGF-L probe (A1, B1, and C1) or the Ig-L probe (A2, B2, and C2). (A) shows coronal section through the mesencephalon; (B) shows para-sagittal section through the brainstem; (C) shows coronal section through the brainstem. III, oculomotor nucleus; V, motor trigeminal nucleus; VII, facial nucleus; X, dorsal motor nucleus of the vagus; XII, hypoglossal nucleus; aq, aqueduct. Bars, 250 μm.

in this nucleus. Two other groups of cholinergic neurons that project widely throughout the neocortex, the pedunculopontine and laterodorsal tegmental nuclei, were heavily labeled. It is worth noting that not all widely projecting neurons expressed ARIA. No label with either probe was detected in the locus coeruleus, the raphe nuclei, or the substantia nigra.

The medial habenula, a cholinergic nucleus in the dorsal thalamus, was heavily labeled with the EGF-L probe (Figure 4E). The adjacent lateral habenula, which is not cholinergic, was not labeled above background. Medial habenula neurons project to neurons in the interpeduncular nucleus that express AChRs. The striatum was labeled above background with the EGF-L probe, but the label was distributed rather diffusely with no obvious discrete clusters of grains (see Figure 6A).

Our survey was extensive, and all known cholinergic nuclei were included in sagittal or coronal sections. As summarized in Table 1, ARIA mRNA was found in all cholinergic nuclei.

Tyrosine Phosphorylation of p185 in Cholinoceptive Neurons

An early step in the action of ARIA on skeletal muscle is the induction of tyrosine phosphorylation of a 185 kDa transmembrane protein (p185), a putative ARIA receptor (Corfas et al., 1993b). In this study, we tested the effect of ARIA on p185 phosphorylation in neurons from the rat medial habenula, chick lumbar sympathetic ganglia, and chick ciliary ganglion. These cells are innervated by ARIA-expressing cholinergic neurons in the basal forebrain, the intermediate column of the thoracic spinal cord, and the Edinger–Westphal nucleus, respectively. In all three cases, ARIA treatment of cells grown in culture leads to tyrosine phosphorylation of p185 (Figure 5). However, this was not the case for every cell type. Neuronal cultures from rat cortex, cerebellar Purkinje cells, dorsal spinal cord, and chick dorsal root ganglia did not show p185 phosphorylation upon ARIA treatment (data not shown). The precise downstream effects of ARIA receptor activation remain to be determined.

ARIA mRNA Expression in Noncholinergic Neurons

ARIA mRNA was not restricted to cholinergic neurons. Figure 6 shows a low power view of a coronal section through the forebrain labeled with the EGF-L probe. In the hypothalamus, the supraoptic nucleus was labeled intensely. Other nuclei that project to the pituitary were also labeled (see Table 1). Conversely, we did not find a clear signal in hypothalamic nuclei that did not project to the

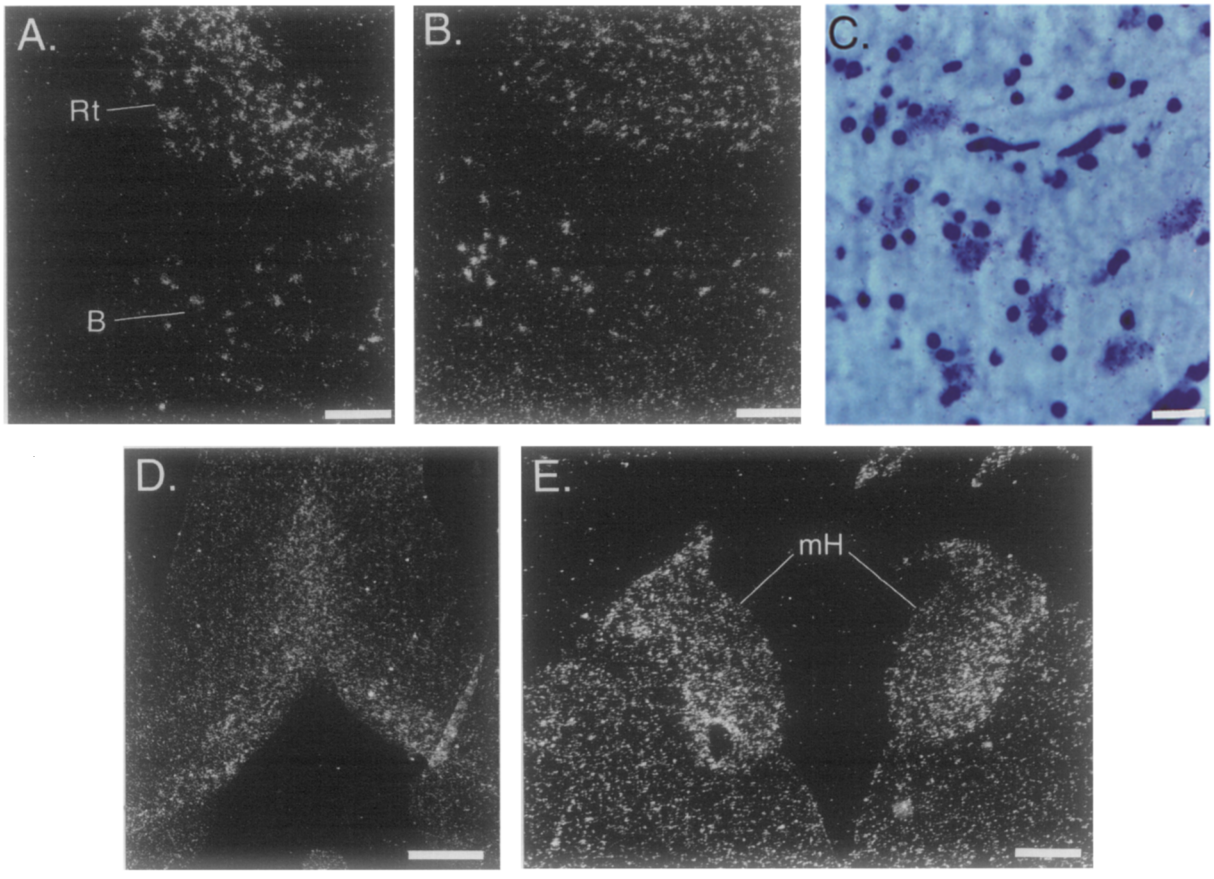

Figure 4. ARIA mRNA Expression in Forebrain and Thalamic Cholinergic Nuclei

(A), (B), and (C) are adjacent coronal sections through the basal forebrain of a P6 rat. Sections were hybridized with EGF-L probe (A) and Ig-L probe (B and C). Sections in (A) and (B) were visualized using dark-field illumination. Section in (C) was counterstained with Toluidine Blue and visualized with bright-field illumination. (D) shows coronal section through the basal forebrain of a P17 rat in the region of the medial septum and diagonal band of Broca hybridized with EGF-L probe. (E) shows coronal section through the epithalamus of a P17 brain hybridized with EGF-L probe. B, nucleus basalis of Meynert; mH, medial habenula nucleus; Rt, reticular thalamic nucleus (noncholinergic). Bars, 500 μm (A and B), 20 μm (C), 200 μm (D), 200 μm (E).

pituitary. In the hypothalamus, the intensity of the Ig-L signal was much less than that of the EGF-L signal.

GGF, a member of the ARIA/heregulin family, was purified from the bovine pituitary, but we did not detect a signal in the pituitary with either the EGF-L or the Ig-L probe. cDNAs were isolated from the pituitary using the PCR to amplify pituitary messages (Marchionni et al., 1993). However, the low abundance of the message in the pituitary is evident in the fact that only 1 in 10^7 clones were positive on screening a pituitary cDNA library (A. D. J. Goodearl, personal communication). Hypothalamic nuclei are, therefore, probably the major source of pituitary GGF.

The cerebral cortex was lightly labeled in most regions. A few regions, including the retrosplenial granular cortex and the perirhinal cortex, were more heavily labeled (Figure 6). The dentate gyrus and the CA3 region of the hippocampus were labeled, but the CA1 field was not. The transition between CA3 and CA1 was abrupt. Strong labeling was also observed in the hippocampal fissure.

Other noncholinergic nerve cell groups that were labeled with one or both ARIA probes are listed in Table 1. The mesencephalic part of the trigeminal nucleus and the reticular thalamic nucleus were particularly striking. The former is composed of neural crest–derived proprioceptive neurons, and the latter contains GABAergic, inhibitory neurons that project within the thalamus (Jones, 1985).

Subventricular Zone

Intense signals with the EGF-L and Ig-L probes were observed in the subventricular zone (SVZ) of the lateral ventricle in the telencephalon of P0, P6, and P17 rats. The subventricular zone is one of the germinal layers from which neurons and glia are generated (Angevine et al., 1969; Bayer and Altman, 1991). After birth, the SVZ gives rise principally to glia, particularly oligodendrocytes (Bayer and Altman, 1991), but recent data suggest that neuronal precursors are present at this time (Reynolds et al., 1992; Richards et al., 1992; Vescovi et al., 1993; Lois and Alvarez-Buylla, 1993).

At P6, ARIA mRNA was evident in the dorsal and lateral walls of the lateral ventricles, as well as in the extension of the SVZ into the olfactory bulb (Figure 7), a pathway for migrating neurons and glia (Luskin, 1993; Walsh and

Table 1. Regional Distribution of ARIA mRNA in the Brain

	EGF-L	Ig-L	ChAT
Rhombencephalon			
Cerebellum			
External granule cells layer (EGL)	++	+++	
Internal granule cells layer (IGL)	+++	++	
Medulla and pons			
Cranial motor nuclei (V, VI, VII, X, XII)	+++	+++	+
Nuc. ambiguus (Amb)	+	+	+
Dorsomedial trigeminal nuc. (DMSP5)	+	–	
Reticular nuclei (LRt, IRt)	+	+	+
Vestibular nuclei (MVe, SpVe)	++	++	+
Inferior olive (IO)	++	++	
Medioventral periolivary nuc. (MVPO)	+	+	
Pedunculopontine tegmental nuc. (PPTg)	++	++	+
Subpeduncular tegmental nuc. (SPTg)	+	+	
Ventral nuc. lateral lemniscus (VLL)	+	–	+
Pontine nuc. (Pn)	+++	+++	
Mesencephalon			
Cranial motor nuclei (III, IV)	+	+	+
Mesensephalic trigeminal nuc. (Me5)	+	+	
Edinger-Westphal nuc.	+	+	+
Superior colliculus, superficial gray layer (SuG)	++	++	
Nuc. optic tract (OT)	+	+	
Anterior pretectal nuc. (APTD, APTV)	+	+	
Olivary pretecal nuc. (OPT)	+	–	
Diencephalon			
Thalamus			
Medial habenulla (MHb)	++	–	+
Reticular thalamic nuc. (Rt)	++	++	
Entopeduncular nuc. (EP)	+	–	
Hypothalamus			
Supra optic nuc. (SON)	+++	–	
Paraventricular hypothalamic nuclei (V, MP, LM)	++	–	
Periventricular hypothalamic nuc. (Pe)	++	–	
Dorsomedial hypothalamic nuc. (DM)	+	–	
Telencephalon			
Basal Forebrain			
Medial septal nuc. (MS)	++	++	+
Horizontal limb of diag band (HDV)	++	++	+
Vertical diagonal band nuc. (VDB)	++	++	+
Nuc. basalis (B)	+++	+++	+
Ventral pallidum (VP)	+	+	+
Septofimbrial nuc. (SFi)	+	+	
Amygdaloid nuclei (MeA, BLA, LaDL)	+	+	+
Bed nuc. striata terminalis (BST)	+	+	
Caudate putamen (CPu)	+	+	+
Hippocampus			
Dentate gyrus (DG)	++	+	
CA3.	+	+	
Cerebral cortex	+	+	
Subventricular zone (SVZ)	+++	+++	

Distribution of ARIA mRNA-labeled neurons in postnatal rat brain. Nomenclature and abbreviations are those of Paxinos and Watson (1986). The relative density of labeling of the cells is classified as very intense (+++), intense (++), weak but above background (+), and not clear (–). Cells were classified as cholinergic based on the results of Butcher et al. (1992) and Lauterbon et al. (1993) using in situ hybridizations with ChAT probes. If a structure is not listed, it is not labeled by our criteria.

Cepko, 1993). The signal in the SVZ was even more intense at P0. The message was also present in the embryonic ventricular zone (VZ) and SVZ (see Figures 9C and 9D). Before birth, most of the precursors give rise to neurons (Bayer and Altman, 1991). These results raise the possibility that ARIA may play a role in the proliferation and/or migration of neuronal and glial precursors.

Cerebellum

Cells in the external granule cell layer of the rat cerebellum proliferate at a high rate during the first two postnatal weeks of life. During this period, postmitotic cells migrated inward along radial glia to form the internal granule cell layer. At P6, when the external and internal granule cell layers had approximately the same number of cells, the intensity and the width of the two regions labeled by the Ig-L probe were similar (Figure 8A). The EGF-L probe also labeled the external and internal granule cell layers, but its distribution was different from that of the Ig-L probe.

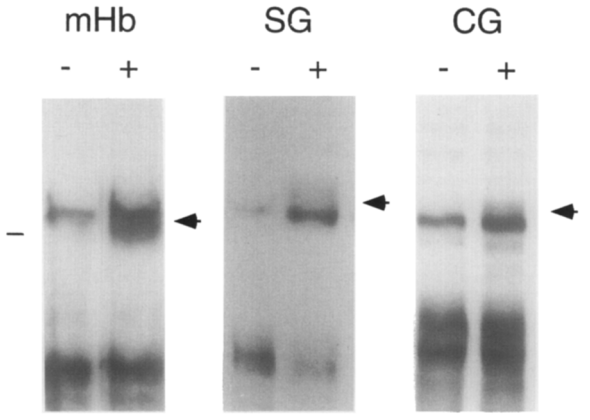

Figure 5. ARIA-Induced p185 Tyrosine Phosphorylation in Cholinoceptive Neurons

Phosphotyrosine immunoblots of neuronal cultures of rat medial habenula (mHb), chick lumbar sympathetic ganglia (SG), and chick parasympathetic ciliary ganglia (CG). Lysates of control untreated cells (–) and ARIA treated cells (+) were electrophoresed in 5% (mHb and SG) or 7% polyacrylamide gel electrophoresis gels. The arrowheads indicate the ARIA-induced phosphotyrosine band. The p185 bands in the SG and CG have the same apparent molecular weight as the bands previously observed in chick and rat muscle cells. In the medial habenula, the band has a slightly lower apparent molecular weight. The line on the left indicates the position of a 180 kDa molecular weight marker.

In addition to the diffuse signal, a subpopulation of very intensely labeled cells was evident in the internal granule cell layer (Figure 8B).

By P17, when the inward migration of granule cells was complete, the lg-L signal was no longer evident (data not shown). The diffuse EGF-L signal was also not evident at P17. However, a few intensely labeled cells were still evident at this time. These cells were located near the Purkinje cell layer, but it is clear that they do not correspond to Purkinje cells (Figure 8C); they may be Golgi or basket cells.

ARIA is not only expressed by cerebellar cells, but also by cells that project to the cerebellum. The inferior olive neurons are the source of climbing fibers that form synapses throughout the extensive Purkinje cell dendritic tree. The pontine nuclei neurons are the major source of

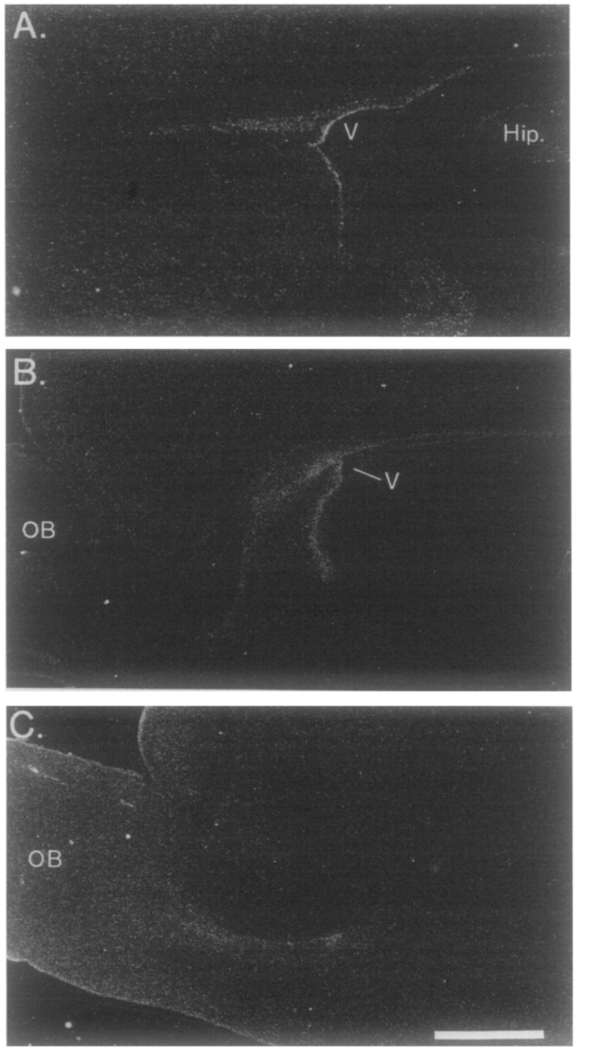

Figure 7. Expression of ARIA in the Subventricular Zone at P6

Parallel parasagittal sections through the telencephalon hybridized with the lg-L probe showing ARIA expression in the subventricular zone. (A) medial; (B) intermediate; (C) lateral. V, ventricle; Hip, hippocampus; OB, olfactory bulb. Bar, 900 μm.

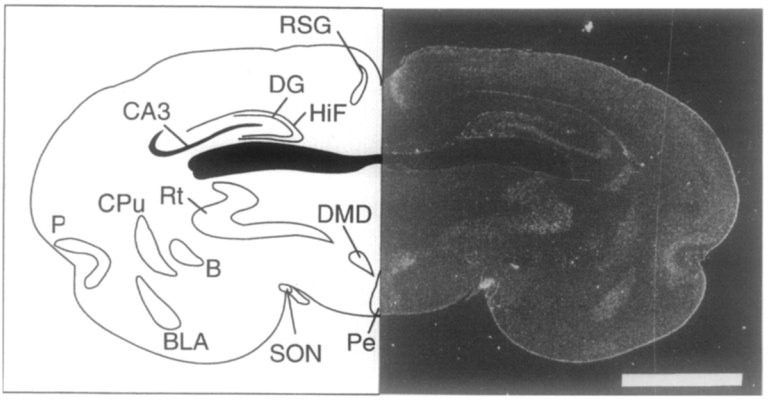

Figure 6. ARIA mRNA Expression in Noncholinergic Nuclei

Coronal section through the forebrain of a P6 rat hybridized with EGF-L probe. On the right is shown a dark-field view of the section; on the left, a schematic drawing indicating labeled structures. SON, supraoptic nucleus; DMD, dorsomedial hypothalamic nucleus; Pe, periventricular hypothalamic nucleus; BLA: basolateral amygdaloid nucleus; B, nucleus basalis of Meynert; CPu, caudate putamen (striatum); Rt, reticular thalamic nucleus; RSG, retrosplenial granular cortex; HiF, hippocampal fissure; DG, dentate gyrus of the hippocampus; P, perirhinal cortex. Note that the CA3 field of the hippocampus is labeled, whereas the CA1 field is not. Bar, 2 μm.

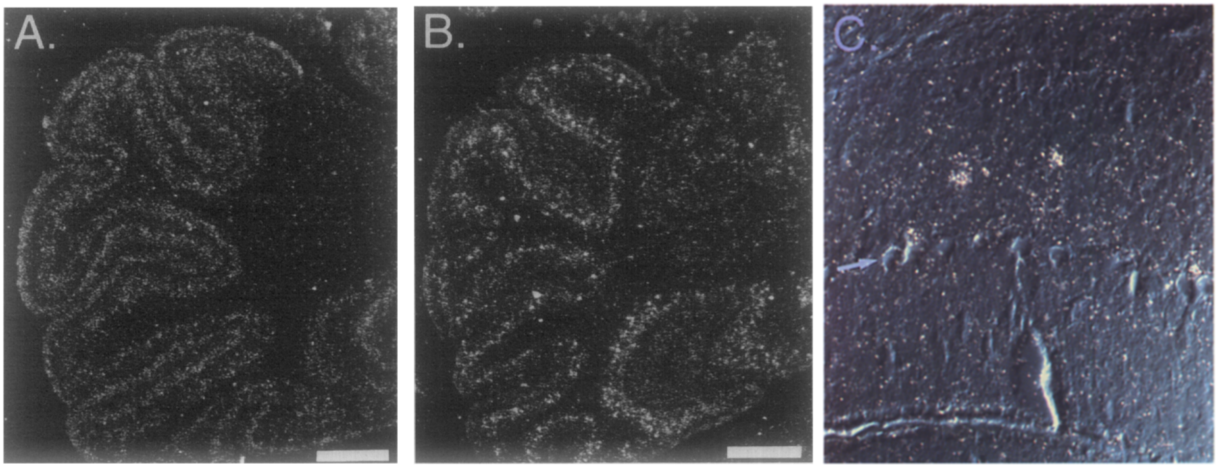

Figure 8. ARIA mRNA in the Cerebellum

Parasagittal sections through P6 cerebellum (A and B) and adult cerebellum (C) hybridized with either Ig-L probe (A) or EGF-L probe (B and C).
(A) The Ig-L probe labels the external and internal granule cell layers.
(B) The EGF-L probe also labels both granule cell layers, but some of the cells in the internal granule cell layer are more intensely labeled than others.
(C) A combination of dark-field and differential interference contrast illumination at high magnification shows that the cells labeled by the EGF-L probe are not in the Purkinje cell layer (one Purkinje cell is indicated by the arrow). Bars, 200 μm.

mossy fiber input to cerebellar granule cells. Both structures were heavily labeled with both probes (data not shown).

ARIA Expression in Embryonic Rats

We have studied the pattern of expression of ARIA mRNA in E14 and E17 rat embryos. As expected, the EGF-L probe labeled embryonic spinal cord motor neurons (Figure 9A). This is significant because it precedes the onset of neuromuscular junction formation in the rat, which is at E15, by one day (Dennis et al., 1981). As in the postnatal spinal cord, the intensity of the Ig-L label was far less than that of the EGF-L (Figure 9B). Cranial nerve motor nuclei were also labeled at this stage by the EGF-L probe (Figure 9C).

Dorsal root ganglia were heavily labeled (Figures 9A and 9B). In this case, the EGF-L probe labeled all cells, with a few more intensely labeled than the rest (Figure 9A). The Ig-L probe hybridized only to a subset of the neurons, but the intensity of label in these cells was comparable to that observed with the EGF-L probe (Figure 9B; see Orr-Urtreger et al., 1993). The cells labeled intensely with either probe were not obviously segregated within the ganglia. The paravertebral sympathetic ganglion chain (Figure 9E) and the superior cervical ganglion were also labeled. Sensory and autonomic ganglia have not yet been assayed in postnatal animals.

The germinal zone of the telencephalon was labeled by the ARIA probes in all embryos examined. At E14, when most of the cells of the developing telencephalon were in the VZ (Angevine, 1969), labeling was observed throughout the wall of the telencephalon (Figure 9C). At E17, when other cortical layers were evident, the signal was restricted to the germinal zone adjacent to the ventricle (Figure 9D). At these stages, most of the cells that withdraw from the VZ become neurons.

Another region that contains proliferating neuronal precursors is the nasal epithelium. As shown in Figure 9C, this structure was heavily labeled by ARIA probes.

At E17, the cerebellum is just beginning to bulge from the neural tube, and precursor cells destined to form the external granule cell layer have not yet migrated over the rhombic lip. At this stage, the EGF-L signal was concentrated in the core of the cerebellar anlage. No Ig-L signal was evident.

In the rat embryo, ARIA mRNA is not restricted to the nervous system. Others have reported mRNA in the gut, lung, and adrenal cortex (Orr-Urtreger, 1993; Meyer and Birchmeier, 1994). We have also found significant labeling in the heart, genital ridge, mesenchymal tissue, and skeletal muscle (tongue and limb). The signal intensity of the EGF-L and the Ig-L probes were equal at all nonneuronal sites.

Grains over the endocardium of the developing heart were particularly striking (Figure 9C). At higher power, it was evident that ARIA mRNA was prominent beneath the developing valves and extending into the endothelial lining of the major blood vessels (Figure 9E). Endocardial labeling persisted in postnatal animals.

Discussion

We believe that the EGF-L and Ig-L probes revealed the distribution of rat ARIA mRNA accurately because the two probes gave qualitatively similar results in most regions of the brain examined. Both the in situ hybridization and Northern blots showed that the Ig-L sequence, with few exceptions, was far less intense than the EGF-L signal in all regions tested. However, every structure labeled by the Ig-L probe was also labeled by the EGF-L probe. The EGF-L probe is probably the most general probe for the ARIA family, because this is the region of the molecule that is essential for all biological activities tested to date.

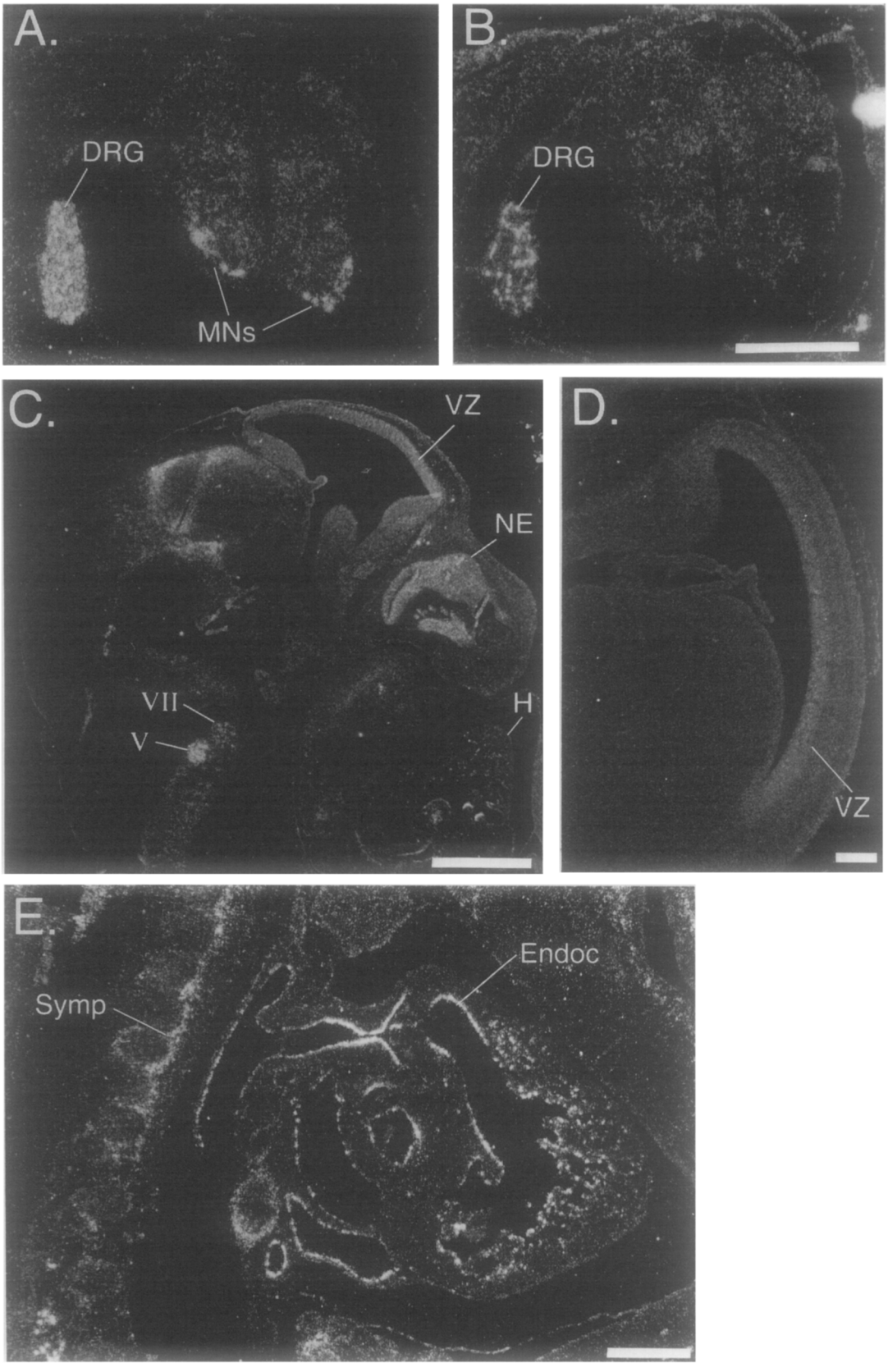

Figure 9. ARIA Expression in Rat Embryos

(A and B) Transverse sections through an E17 embryo at the level of the spinal cord hybridized with the EGF-L probe (A) or Ig-L probe (B).
(C) Parasagittal view of the head and upper trunk of an E14 embryo hybridized with EGF-L probe.
(D) Parasagittal section through the telencephalon of an E17 embryo hybridized with the EGF-L probe.
(E) Parasagittal section of an E17 embryo at the level of the heart.
MNs, spinal cord motor neurons; DRG, dorsal root ganglion; VZ, ventricular zone; NE, nasal epithelium; H, heart; V, motor trigeminal nucleus; VII, facial nucleus; Symp, sympathetic ganglion chain; Endoc, endocardium. Bars, 500 μm (A, B, and E); 250 μm (C and D).

Moreover, no isoforms of ARIA have been reported that lack an EGF-L domain.

The reduced Ig-L signal compared with the EGF-L implies that forms of ARIA must exist without the Ig-L domain. cDNAs have in fact been isolated from a rat brain stem library (D. Wen, personal communication) and from an embryonic chick brain library that encode ARIA-related cDNAs lacking an Ig-L domain (Y. Kuo and L. Role, personal communication; Rosen et al., unpublished data). The differences between the Ig-L and EGF-L probes were observed in each of the animals examined.

We did not examine single cells by double labeling, but the weight of our evidence indicates that individual neurons express both Ig-L-negative and Ig-L-containing transcripts. The most compelling indirect evidence is that essentially all spinal cord motor neurons were labeled with both probes. It will be important to determine the spectrum of transcripts expressed by particular types of neurons.

The fact that ARIA mRNA is present in E14 and E17 spinal cord motor neurons correlates in time with the initial accumulation of AChRs at developing neuromuscular junctions. The continued expression of the ARIA gene in P17 motor neurons correlates with the switch in AChR subunit composition that occurs during the first two weeks after birth. During this period, receptors containing γ subunits are replaced with ones containing ε subunits (Mishina et al., 1986; Gu and Hall, 1988). As noted above, ARIA promotes the expression of ε mRNA in cultured mouse myotubes (Martinou et al., 1991). It remains to be determined how a trophic factor or a related isoform can influence the expression of different genes at different times during development.

The level of ARIA mRNA remains high in adult motor neurons. This may seem surprising at first glance because the metabolic half life of AChRs increases dramatically after innervation (for review, see Salpeter and Loring, 1985). However, the size of the motor endplate and the total number of AChRs continues to increase after birth; the level of AChR synthesis may not decline at all. In situ hybridization studies have shown that AChR subunit mRNAs are abundant at mature neuromuscular junctions (Fontaine et al., 1988; Goldman and Staple, 1989). In addition to effects on AChR synthesis, a role for ARIA in expression of other synaptic proteins at mature junctions, as well as effects on perisynaptic Schwann cells, are under investigation.

It is likely that the ARIA gene is expressed in all cholinergic neurons. This generalization is based on our finding that ARIA mRNA is present in all nuclei that contain cholinergic neurons. One explanation for this remarkable correlation with the cholinergic phenotype is that ARIA is required for the formation and maintenance of cholinergic synapses between neurons. Perhaps the simplest hypothesis, based on studies of the neuromuscular junction, is that ARIA regulates the number of neuronal nicotinic receptors. Certainly the presence of ARIA in spinal cord and brain stem neurons that innervate autonomic ganglia is consistent with this suggestion. Early ablation of presynaptic inputs to the ciliary ganglion does, in fact, reduce the total number of AChRs (cf. Arenella et al., 1993 and Engisch and Fischbach, 1992). Medium conditioned by dorsal spinal cord neurons produces a large increase in ACh evoked currents recorded in chick sympathetic neurons (Gardette et al., 1991). Early experiments in our laboratory showed that partially purified ARIA can increase peak ACh currents in cultured ciliary ganglion neurons (Engisch, 1990). Our results showing ARIA-induced p185 phosphorylation in parasympathetic and sympathetic ganglion neurons are in agreement with these observations.

In the CNS in situ hybridization, immunohistochemical and electrophysiological experiments have shown that nicotinic AChRs are more abundant than previously suspected (for review, see Luetje et al., 1990). Although the role of nicotinic AChRs in synaptic transmission in the CNS remains unclear, many studies suggest actions on presynaptic terminals (Luetje at al., 1990). In any case, it is noteworthy that regions of high nicotinic AChR expression do receive input from ARIA-expressing cholinergic neurons in the basal forebrain and, in the case of the medial habenula, also respond to ARIA with p185 phosphorylation.

Basal forebrain cholinergic neurons project widely throughout the neocortex and the hippocampus in the rat, as well as in humans (Woolf, 1991), where they exert a profound effect on the membrane conductance of target neurons. This effect is due primarily to the activation of muscarinic AChRs.

In regard to muscarinic AChRs, it is interesting that the endocardium of embryonic and adult rats and chicks is heavily labeled by the ARIA probes. The function of this layer of endothelial cells is not yet clear. However, it has been suggested that it regulates the electrical properties of myocardium (for review, see Brutsaert, 1989) and contributes to the formation of the heart valves and septa. (Markwald et al., 1975). Thus, it is plausible that ARIA plays a role in the formation of the heart and its function in the adult. Early experiments in our laboratory showed that crude brain extract increased the percentage of ventricular myocardial cells that respond to ACh (Siegel and Fischbach, 1984). We have found that ARIA induces p185 phosphorylation in chick heart muscle cells in culture (G. C. and G. F., unpublished data). We are currently investigating the effect of ARIA on heart muscle muscarinic receptor expression. There is also no reason to ignore other types of ion channels as potential ARIA targets, since the ARIA gene is expressed in noncholinergic neurons in the postnatal rat brain.

Beyond the regulation of ion channel synthesis, our data suggest that ARIA may play a role in the proliferation or differentiation of neuronal and glial precursors. ARIA is expressed in ventricular germinal zones at a time (E14–E17) when the precursor cell populations give rise primarily to neurons, and at a later time (P6) when they give rise to glia. High levels of ARIA expression also coincide with high rates of proliferation and migration of granule cells in the cerebellum and in the dentate gyrus (Schlessinger et al., 1975). Regarding glial cells, ARIA does promote the maturation of oligodendrocyte progenitor cells (O2A) in vitro (Vartanian et al., 1994). A recent study also showed that GGF promotes the differentiation of neural crest cells into Schwann cells in vitro (Shah et al., 1994).

Multipotent progenitor cells exist in the adult rat brain

SVZ. Some of these cells when grown in vitro in the presence of EGF proliferate and differentiate into neurons and glia (Reynolds et al., 1992; Vescovi et al., 1993). Although ARIA does not activate the EGF receptor, it will be important to determine whether this family of ligands mimics or enhances the effects of EGF on neuronal precursors.

We have also found ARIA mRNA in proliferating and migrating neurons in the cerebellum. The results reported here are identical to those obtained in the chick using either an Ig-L probe or a full-length chick β1 probe (Corfas et al., 1993, Soc. Neurosci., abstract). Later, after the external granule cell layer is spent, ARIA EGF-L domain is heavily expressed in a subpopulation of cells located in the outer part of the internal granule cell layer. We are attempting to determine whether these cells are basket or Golgi neurons, which in humans have been shown to be cholinergic (De Lacalle et al., 1993). ARIA in the cerebellum may not only be provided by cerebellar cells, but also by inputs from the pontine nuclei and inferior olive.

Expression of the ARIA gene in the SVZ raises the possibility of a local paracrine or autocrine action. Self stimulation or an action on neighboring cells may also occur in autonomic ganglia or the medial habenula, where neurons express ARIA and nicotinic AChRs.

The next step in analysis of ARIA action in the CNS would be greatly facilitated by information regarding the ARIA receptor. Increasing evidence suggests that HER4, a recently cloned member of the EGF-receptor family (Plowman et al., 1993a), is the major p185 protein that is phosphorylated on tyrosine residues in response to binding by the ARIA family of ligands (Plowman et al., 1993b). We have, in fact, found that *HER4* mRNA is expressed in putative ARIA targets, including skeletal muscle, heart muscle, cells in the SVZ, and neurons that are innervated by ARIA-expressing cells.

Localization of ARIA gene expression has provided several new insights. The emerging picture of multiple ligands and multiple receptors is one that is characteristic of other types of polypeptide growth and differentiation factors, including the neurotrophin family and their corresponding receptor tyrosine kinases (for review, see Chao, 1992). Further progress will depend on identification and localization of specific ARIA isoforms, identification of ARIA receptors, and most important, the development of relevant bioassays.

Experimental Procedures

RNA Isolation and Northern Blot Analysis

Total RNA was isolated from various regions of the central nervous system of the rat at P0, P6, and P17 using the Ultraspec RNA isolation system according to the instructions of the manufacturer (Biotex Laboratories, Houston, TX). RNA concentration and estimates of purity were determined spectrophotometrically. RNA samples were fractionated on 1.3% agarose/2.2 M formaldehyde surface tension gels as described by Rosen et al. (1990). Ethidium bromide fluorescence was utilized as an aid to assess the equivalency of loading among different samples. Following electrophoresis, the samples were transferred to a charged nylon membrane (MagnaGraph, MSI, Westborough, MA). All blots were UV cross-linked using a Stratalinker (Stratagene, LaJolla, CA). Blots were hybridized using the rat ARIA cDNA fragments described below. Probe fragments were labeled by random oligonucleotide priming using a commercially available kit (Stratagene, LaJolla, CA). All hybridization and washing were done as described previously (Falls et al., 1993). Following washing, blots were exposed to Kodak XAR-5 film at −70°C using intensifying screens (DuPont Lightning Plus).

PCR and Subcloning

Oligonucleotides were synthesized based upon the sequence of rat NDF (Wen et al., 1992). The sense strand oligonucleotide (5′-CAGATTGAAAGAAATGAAGAGCC-3′) corresponds to codons spanning Pro-37 to Gln-45 of the proNDF sequence. The antisense strand oligonucleotide (5′-CACCACACACATGATGCCGAC-3′) corresponds to the codons spanning Val-256 to Val-262. Single-stranded cDNA was synthesized in a 50 μl reaction from 2 μg of P20 rat spinal cord total RNA using oligo$(dT)_{12-18}$ as primer and SuperScript H$^-$ Moloney Murine Leukemia Virus reverse transcriptase (Gibco-BRL, Gaithersburg, MD). Five microliters of the reverse transcription reaction was used as the template in a PCR using 1 μg of each of the primers described. PCR reactions were performed for 40 cycles of 94°C for 60 s, 50°C for 60 s, and 72°C for 90 s in a 100 μl volume containing 400 μM dNTPs, 1× Taq buffer, and 5 U of Taq DNA polymerase (Boehringer-Mannheim, Indianapolis, IN). PCR products were gel-purified and subcloned into the vector pCR-II using the TA cloning kit (Invitrogen, San Diego, CA). The sequence of the cloned inserts was determined by the dideoxy chain termination method using [α-^{32}P]dATP (NEN, Boston, MA) and Sequenase version 1.0 (U. S. Biochemicals, Cleveland, OH). Using vector-derived EcoRI restriction sites that flank the insert and an internal Sph I restriction site that falls within the center of the spacer domain (see text), two independent fragments corresponding largely to the Ig-L domain and the EGF-L domain were subcloned into pGEM-7Z to create two independent probes.

In Situ Hybridization

In situ hybridizations were performed as previously described (Sassoon and Rosenthal, 1993; Falls et al., 1993). Tissues were fixed in 4% paraformaldehyde in phosphate-buffered solution overnight at 4°C. For animals younger than 3 days post-partum, tissues were fixed by immersion. For older animals, intracardial perfusion was performed prior to dissection. After fixation, tissues were slowly dehydrated and embedded in paraffin. Serial sagittal or coronal sections (9 μm) were collected on gelatinized glass microscope slides. Hybridization with sense and antisense probes was carried out at 52°C for 18 hr in 50% deionized formamide, 0.3 M sodium chloride, 20 mM Tris-HCl (pH 7.4), 5 mM EDTA, 10 mM $NaPO_4$ (pH 8), 10% dextran sulfate, 1× Denhardt's solution, and 50 μg/ml total yeast RNA with 3.5 × 10^4 cpm/ml ^{35}S-labeled RNA probe under siliconized coverslips. Following hybridizations, coverslips were floated off in 5× SSC, 10 mM dithiothreitol at 50°C and washed in 50% formamide, 2× SSC, 10 mM dithiothreitol at 65°C. Slides were then rinsed in washing buffer, treated with RNAse A (20 μg/ml; Sigma), and washed at 37°C for 15 min in 2× SSC and then for 15 min in 0.1× SSC. Sections were dehydrated rapidly, processed for autoradiography using NTB-2 Kodak emulsion, exposed for 4 weeks at 4°C, and examined using both light- and dark-field illumination (Darklite, MVI, Avon, MA) under a dissecting microscope (SMZ-U, Nikon) or a compound microscope (Microphot, Nikon).

The templates for probe transcription used in this study corresponded to the EGF-L and the Ig-L domains of rat ARIA. Sense and antisense [α-^{35}S]UTP (>1000 Ci/mmol; New England Nuclear)-labeled RNA probes were generated by runoff transcription of the restriction-digested plasmid using T7 or SP6 RNA polymerase. Transcripts were degraded to an average length of 110 bp using alkaline hydrolysis (Sassoon and Rosenthal, 1993). Experiments with a probe directed to HER4 sequences were carried out simultaneously with slides containing adjacent tissue sections. The hybridization pattern was different than that obtained with the ARIA probes and thus served as an additional control. These results will be published elsewhere.

Identification of nuclei and other structures in the brain of postnatal rats was based on the atlas of Paxinos and Watson (1986). Structures in rat embryos were identified based on the atlas of Paxinos et al. (1991).

Cell Culture and ARIA Treatment

Medial habenulae were dissected from E18 Sprague-Dawley rats and incubated for 30 min at 37°C in dissociation medium (90 mM Na_2SO_4, 30 mM K_2SO_4, 5.8 mM $MgCl_2$, 0.25 mM $CaCl_2$, 1 mM HEPES, 1 mM

kynurenic acid) and 10 U/ml papain (Worthington, NJ). After rinsing in dissociation medium without enzyme, the habenulae were triturated gently, and the cells were transferred to culture medium (DMEM; 4% fetal calf serum, 1 × B27 supplements [GIBCO], 100 mM L-Gln, 1 × penicillin/streptomycin [GIBCO], 1 mM sodium pyruvate). Cells were plated in 24 well tissue culture plates coated with poly-D-lysine and laminin.

Chick lumbar sympathetic ganglia neurons were prepared as described by Role (1984) and plated on polyornithine coated 48 well dishes. Chick ciliary ganglion neurons were prepared as described by Cohen and Fischbach (1977) and plated on collagen coated 48 well dishes.

Three days after plating, cultures were treated with a dose of recombinant ARIA (Falls et al., 1993) that induced maximal response in AChR synthesis in skeletal muscle. Medial habenula cells were treated for 45 min. Sympathetic and ciliary ganglion neurons were treated for 30 min.

Phosphotyrosine Immunoblotting

Cells were treated with ARIA, washed with phosphate-buffered solution, solubilized in 40 μl of Laemmli sample buffer (2 ×), and heated to 95°C for 3 min. Aliquots were electrophoresed in 5% SDS-polyacrylamide gels, and transferred to polyvinylidene difluoride membranes (Millipore). The transferred proteins were probed with a monoclonal antibody raised against phosphotyrosine residues (mAb 4G10; kindly provided by Drs. B. Druker and T. Roberts, of the Dana Farber Cancer Institute). Bound antibody 4G10 was visualized with an HRP-conjugated goat anti-mouse IgG antibody (Boehringer Mannheim) and enhanced chemiluminescence (Amersham).

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