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Developmental Expression and Alternative Splicing of Chick Agrin RNA

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Agrin is a synaptic basal lamina protein that has been proposed to mediate motor neuron-induced clustering of acetylcholine receptors during development of the neuromuscular junction. The chick ciliary ganglion is a parasympathetic ganglion that contains motor neurons that project to striated and smooth muscle targets in the eye. We have examined agrin gene expression in the chick ciliary ganglion during normal embryonic development using *in situ* hybridization and quantitative PCR techniques. Ganglia were specifically labeled by antisense agrin cRNA probes and the density of labeling changed during development. Hybridization was most intense in sections of ganglia obtained from embryos before embryonic Day 15 (E15), declining to relatively low levels by hatching at E20. Throughout embryonic development labeling was associated with glial cells, in addition to both ciliary and choroid neurons. Measurement of agrin mRNA levels by competitive PCR showed that agrin gene expression in the ganglion increased dramatically between E8 and E10, was sustained at high levels from E10 to E14, and declined thereafter. This time course is coincident with the period of synapse formation between ganglionic neurons and their peripheral targets. Previous studies in chick CNS have shown that alternative RNA splicing of a single exon encoding 11 amino acids gives rise to an active and an inactive agrin protein. Our analysis of RNA isolated from chick ciliary ganglia demonstrated that a second, previously uncharacterized exon encoding 8 amino acids can also be spliced into the same region. Alternative splicing of both the 8- and the 11-amino-acid exons results in expression of four distinct agrin transcripts in the ganglion. Changes in the level of total agrin mRNA in the ganglion reflect developmentally regulated changes in the levels of these alternatively spliced agrin isoforms. These results demonstrate that agrin is expressed in autonomic motor neurons of the peripheral nervous system and support a wider role for agrin as a synaptogenic protein, not limited to spinal motor neurons.

INTRODUCTION

Regulation of gene expression is an important means of specifying regional differences and connectivity in the developing nervous system. While some genes are regulated by signals intrinsic to the cell, both retrograde and anterograde signals have also been shown to play a role in controlling levels of neuronal gene expression during normal development. At the vertebrate neuromuscular junction, differentiation is coordinated through signals exchanged between motor neurons and the muscle fibers they innervate. For example, prior to innervation AChR[2] are present at low density and randomly distributed over the muscle fiber's surface. Innervation leads to a rapid increase in the density of AChR at the region of nerve contact and a concomitant decrease in the density of receptors in the extrajunctional membrane (Dennis, 1981). Studies pioneered by Lømo and colleagues (Lømo and Westgaard, 1976) have demonstrated that loss of extrajunctional AChR is the result of blockade of receptor synthesis by synaptically mediated muscle activity (Goldman *et al.*, 1988). However, even in the presence of drugs that block neuromuscular transmission a high concentration of AChR accumulates at the site of nerve-muscle contact (Cohen, 1972). Thus, in addition to nerve-evoked muscle activity, other neural influences must also play an important role in promoting the accumulation of AChR at the developing neuromuscular synapse.

Several molecules present in nervous tissue, including agrin, ARIA, and calcitonin gene-related peptide, have been proposed to play a role in motor neuron-induced accumulation of AChR at the neuromuscular junction (Froehner, 1993). Agrin, a component of the synaptic basal lamina (Reist *et al.*, 1987), is the most well-characterized of these putative anterograde signaling proteins.

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[2] Abbreviations used: AChR, acetylcholine receptor(s); bp, base pair; SSC, saline sodium citrate; E, embryonic day; EDTA, ethylenediaminetetracetic acid; PCR, polymerase chain reaction.

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Agrin is expressed by embryonic motor neurons (Magill-Solc and McMahan, 1988; Rupp *et al.*, 1991; Tsim *et al.*, 1992) and transported down motor nerve axons (Magill-Solc and McMahan, 1990). Moreover, it has been demonstrated that agrin released from neurons induces the formation of high-density AChR aggregates on cultured muscle cells (Reist *et al.*, 1992) and is colocalized with the earliest forming receptor clusters (Cohen and Godfrey, 1992). Based on these observations it has been proposed that agrin is the neuronal agent that triggers aggregation of AChR at developing neuromuscular junctions (McMahan, 1990).

Marked increases in size and complexity of AChR aggregates characterize the early stages of neuromuscular synapse formation (Slater, 1982; Smith and Slater, 1983; Dahm and Landmesser, 1991). In contrast, changes in the number and pattern of AChR clusters are rarely observed at mature synaptic sites (Slater, 1982; Balice-Gordon *et al.*, 1990). If agrin released from motor nerve terminals drives organization of AChR clusters at the neuromuscular junction, then two possible mechanisms could account for changes in AChR distribution during development. On one hand agrin expression might be regulated during development. For example, high levels of agrin would be produced during phases of active neuromuscular synapse formation, and lower levels associated with maintenance of the mature postsynaptic apparatus. Alternatively, the sensitivity of the muscle fiber to agrin might be developmentally regulated, perhaps through changes in agrin receptor number or expression of different agrin receptor isoforms. Developmental changes in the sensitivity of muscle cells to agrin will be difficult to address until the agrin receptor protein is purified or the gene cloned. However, following cloning of agrin in several species, it is now feasible to examine the regulation of agrin gene expression during synapse formation (Rupp *et al.*, 1991; Ruegg *et al.*, 1992; Smith *et al.*, 1992; Tsim *et al.*, 1992).

In this report we have analyzed agrin gene expression in the chick ciliary ganglion. The ciliary ganglion consists of two populations of cholinergic motor neurons that innervate distinct structures in the eye (Marwitt *et al.*, 1971; Pilar and Tuttle, 1982). Ciliary neurons form focal synaptic contacts with striated muscle fibers in the iris and ciliary body (Pilar *et al.*, 1981). On the other hand, choroid neurons innervate smooth muscle cells of the choroid vasculature through a network of undirected *en passant* synapses (Meriney and Pilar, 1987). In addition to the fact that at least some neurons in the ciliary ganglion normally innervate striated muscle, agrin expression in the ganglion is predicted by the observation that all ganglionic neurons are competent to form functional synapses with chick myotubes in cell culture at which AChR are clustered (Role *et al.*, 1985; Role *et al.*, 1987). The organization of the ciliary ganglion therefore provides a unique opportunity to study the regulation of agrin gene expression during synaptogenesis. Our study demonstrates that both populations of motor neurons in the ganglion express agrin mRNA. Changes in the levels of expression and the pattern of alternative agrin RNA splicing during embryonic development suggest a possible role for agrin in synapse formation between autonomic neurons and their peripheral targets.

MATERIALS AND METHODS

RNA Isolation and First Strand cDNA Synthesis

Total RNA was isolated by the single-step method of Chomczynski and Sacchi (1987) from pools of 2–10 E18 spinal cords or E18 optic lobes, and 50–125 embryonic ciliary ganglia, depending on age, and the concentration was determined by measuring absorption at 260 nm (Sambrook *et al.*, 1989). cDNA templates for amplification by PCR were synthesized by reverse-transcription of total RNA (10 ng/μl) using Moloney's murine leukemia virus reverse transcriptase (10U/μl; Gibco-BRL) with random hexanucleotide primers (5 pmole/μl; Pharmacia-LKB) and dNTPs (0.5 m*M*; Pharmacia-LKB) for 1 hr at 37°C in the buffer supplied by the manufacturer.

In Situ Hybridization

cDNA templates. Templates for synthesis of cRNA probes used for *in situ* hybridization studies were generated by subcloning PCR amplification products of E18 chick optic lobe and spinal cord RNA as described by Kawasaki (1990). Two pairs of oligonucleotide primers were synthesized, F1/R1 and F2/R2, whose 5′ ends correspond to nucleotides 3349/3681 and 3874/4372, respectively, of the sequence of the chick agrin clone CBA-1 (Tsim *et al.*, 1992). Forward primers contained the recognition sequence for *Eco*RI at their 5′ end, while reverse primers contained the sequence for *Hin*dIII. First-strand cDNA from 5 μg of total RNA was amplified in a DNA thermal cycler (Ericomp) with *Taq* polymerase (Promega) in the manufacturer's buffer for 35 cycles (1.5 min, 94°C; 2 min, 55°C; 3 min, 72°C; final extension 7 min, 72°C). PCR products were subsequently restricted with *Eco*RI and *Hin*dIII and following gel purification subcloned into pBluescript (Stratagene), and their identity was verified by sequencing. Plasmid pCA1 contains the F1/R1 primed insert, and pCA2 contains the F2/R2 primed insert.

Tissue preparation. Ciliary ganglia, isolated from White Leghorn chick embryos (Chino Valley Ranchers),

were fixed overnight in cold 4% paraformaldehyde in phosphate buffer (80 m*M* Na_2HPO_4, 50 m*M* NaH_2PO_4, pH 7.2). Ganglia were cryoprotected by stepwise equilibration in 5, 10, 20, and 30% sucrose dissolved in phosphate buffer. Spinal cords were fixed for 1 h in cold 4% paraformaldehyde and cryoprotected by equilibration in 20% sucrose in phosphate buffer. Fixed tissues were subsequently embedded in O.C.T. (Miles, Inc.) and stored at −80°C. Twelve-micrometer frozen sections were cut on a cryostat (Reichert), mounted onto Vectabond (Vector Labs)-coated slides, and postfixed for 5 min in cold 4% paraformaldehyde in phosphate buffer.

Hybridization. *In situ* hybridization using sense and antisense ^{35}S-labeled agrin cRNA probes was performed essentially as described by Simmons *et al.* (1989) Tissue sections were prehybridized in 100 m*M* Tris buffer (pH 8.0) containing 50 m*M* EDTA and 0.5% Triton X-100, for 30 min at room temperature, dehydrated in ethanol, and air dried. Sections were hybridized with 10^7 cpm/ml agrin cRNA probe for 17 hr at 60°C, in a solution containing 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02 mg/ml bovine serum albumin fraction V, 50% deionized formamide, 20% dextran sulfate, 300 m*M* NaCl, 1 m*M* EDTA, 0.5 mg/ml yeast transfer RNA, and 10 m*M* dithiothreitol in 10 m*M* Tris, pH 8.0. Following hybridization the sections were transferred through five changes of 4× SSC (600 m*M* NaCl, 60 m*M* Na Citrate, pH 7.0), over a period of 40 min, followed by treatment with ribonuclease A (0.02 mg/ml in 500 m*M* NaCl, 1 m*M* EDTA, 10 m*M* Tris, pH 8.0) for 30 min at 37°C and washing in descending concentrations of SSC to a final stringency of 0.1× SSC at 70°C for 30 min. Sections were dehydrated in ethanol, air dried, and exposed to nuclear track emulsion (Kodak NTB2, 1:1 with H_2O) for 1–2 weeks at 4°C, and [^{35}S]cRNA hybridization was evaluated autoradiographically.

Polymerase Chain Reactions

Competitive PCR. Levels of agrin mRNA in total RNA isolated from ciliary ganglia obtained at different stages of embryonic development were determined by competitive PCR, essentially as described by Gilliland and colleagues (1990). The plasmid pCA2 contains a chick agrin PCR fragment, corresponding to nucleotides 3874–4350 of CBA-1 (Tsim *et al.*, 1992), that includes a unique *Kpn*I restriction site. The competitive template (plasmid $pCA2_{Bam}$) was derived from pCA2 by mutation of the *Kpn*I restriction site to a *Bam*HI site using the recombinant PCR method of Higuchi *et al.* (1988). Competitive PCR was performed essentially as described by Gilliland *et al.* (1990). For each determination, cDNA from 100 ng of total RNA was coamplified for 40 cycles in a DNA thermal cycler (Perkin-Elmer, Cetus) (1 min, 94°C; 2 min, 60°C; 3 min, 72°C with a final extension step of 10 min, 72°C) with different amounts (1–100 pg) of $pCA2_{Bam}$ using the forward and reverse primer pair F3/R3, flanking the natural *Kpn*I or engineered *Bam*HI restriction sites in the target agrin cDNA or $pCA2_{Bam}$, respectively. PCR products were labeled by addition of 2×10^5 cpm of primer F3 to the PCR reaction mixture previously labeled with ^{32}P using T4 kinase (Sambrook *et al.*, 1989). Five percent of the amplified reaction mixture was subjected to >10-fold overdigestion with *Bam*HI and resolved by electrophoresis on a 6% polyacrylamide gel (Sambrook *et al.*, 1989). The amount of PCR product synthesized from the native agrin cDNA and competing $pCA2_{Bam}$ templates in each reaction was determined by computer-assisted densitometry of gel autoradiograms (MCID, Imaging Research Inc.). The amount of agrin cDNA was determined by linear estimation from the relationship between the ratio of $pCA2_{Bam}$ and agrin cDNA PCR products expressed as a function of the amount of $pCA2_{Bam}$ added to the reaction.

PCR analysis of alternative RNA splicing. To examine levels of alternatively spliced agrin mRNA, two rounds of PCR amplification using pairs of nested primers were used. First-strand cDNA, synthesized by random priming from 100 ng of total RNA, was amplified for 35 cycles (94°C, 1 min; 55°C, 2 min; 72°C, 3 or 10 min for the final extension) with primers F4/R4 whose 5′ ends correspond to 5196 and 5607 nucleotides, respectively, of CBA-1 (Tsim *et al.*, 1992). Aliquots of the first-round PCR were diluted 2000-fold and reamplified under the same conditions using a second primer set F5/R5 located internally to F4/R4 and beginning at nucleotides 5377 and 5499 of CBA-1, respectively. PCR products were labeled by inclusion of 2×10^5 cpm of F5 labeled with ^{32}P using T4 kinase (Sambrook *et al.*, 1989). Ten to 20% of the second-round amplified reaction mixture was analyzed by electrophoresis on an 8% polyacrylamide gel. The relative levels of each PCR product were determined by computer-assisted densitometry of the resulting autoradiograms.

PCR primers used in this study.

F1 5′-TAGAATTCAGAGCAGCGCTGTCCGTGTC
R1 5′-ATAAGCTTGCAGGTGCTGCCAGCTTGGC
F2 5′-TAGAATTCCCATCAGGTACTTCATCCCC
R2 5′-ATAAGCTTTCTCCCTCAGGTCGTACATC
F3 5′-TCGTGGCAAGGACTTCATCTCC
R3 5′-TCGTACATCTGGTTGTTCACATCC
F4 5′-CGTTGAAATCTCCACTTTCCGTG
R4 5′-CAGGTCATACATCATCTGCACGA
F5 5′-TTTGATGGTAGGACGTACATGGA
R5 5′-TTTGATGCTCAGCTCAAAGTGGT

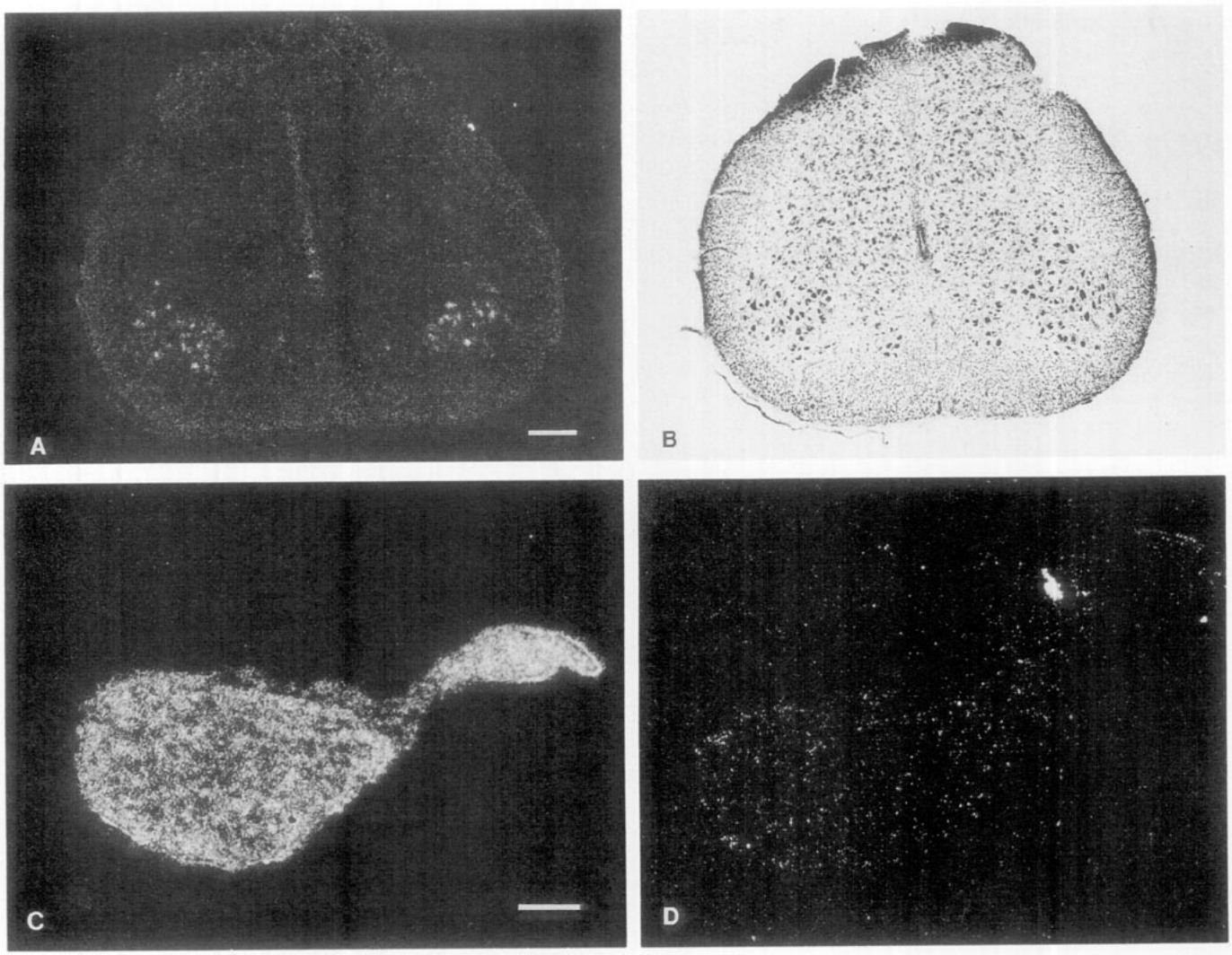

FIG. 1. Agrin mRNA is expressed in the chick ciliary ganglion. ^{35}S-labeled antisense and sense agrin cRNA probes were hybridized to 12 μm frozen tissue sections. (A) Dark-field photomicrograph of a section through an E19 spinal cord, hybridized with an antisense probe, reveals discrete labeling in the ventral horn. (B) Bright-field photomicrograph of an adjacent section stained with toluidine blue shows labeling associated with the large cell bodies of motor neurons in the ventral horn. (C) Hybridization of an antisense agrin probe to an E12 ciliary ganglion results in the appearance of silver grains throughout the ganglion and in a nerve root. (D) An adjacent section hybridized with the sense probe shows low levels of nonspecific hybridization. Bar, 200 μm in A, B; 100 μm in C, D.

RESULTS

Localization of Agrin mRNA in the Developing Ciliary Ganglion

For the *in situ* hybridization studies, ^{35}S-labeled antisense and sense cRNA agrin probes, representing nucleotides 3349–3681 of chick agrin (Tsim *et al.*, 1992), were synthesized from the template pCA1. Previous studies using *in situ* hybridization have shown that agrin mRNA is expressed at high levels in spinal motor neurons (Rupp *et al.*, 1991; Smith *et al.*, 1992; Tsim *et al.*, 1992). Therefore, to test its specificity, we examined the hybridization pattern of pCA1 to sections of E10 and E19 chick spinal cord. Cells located in the ventral horns of the spinal cord exhibited the highest levels of hybridization, with lower levels of labeling evident throughout the remaining gray matter of the cord (Fig. 1A). Analysis of adjacent sections stained with toluidine blue (Fig. 1B) indicated that labeling in the ventral horns was localized within the large, basophilic cell bodies of spinal motor neurons. Similar results were also obtained with a second probe, synthesized from pCA2, a plasmid representing a different region of chick agrin (nucleotides 3874–4372, data not shown). Both probes therefore, exhibited a pattern of expression in the spinal cord identi-

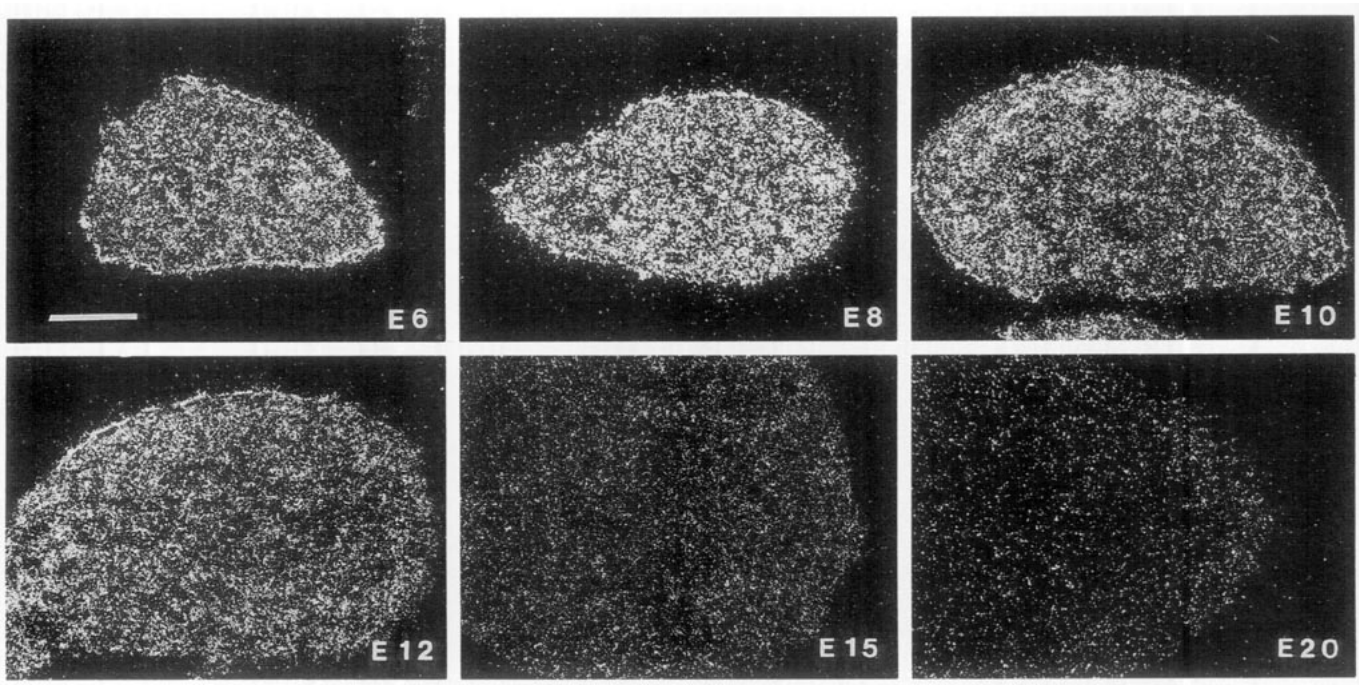

FIG. 2. Agrin mRNA expression changes during development in the ciliary ganglion. Dark-field photomicrographs of emulsion autoradiograms of 12-μm frozen sections through ciliary ganglia hybridized with an antisense agrin cRNA. The highest silver grain densities are seen at early stages (E6-E12) and labeling density declines as development progresses. All micrographs were obtained using a constant film exposure from a single experiment, in which sections were processed in parallel, to permit comparisons of grain densities. Bar, 100 μm.

cal to that described for other agrin cRNA probes (Rupp *et al.*, 1991; Smith *et al.*, 1992; Tsim *et al.*, 1992).

To determine whether agrin mRNA is expressed by cells in the chick ciliary ganglion, sections of E12 ciliary ganglia were hybridized to antisense and sense cRNA probes derived from pCA1. In contrast to the pattern of labeling in the spinal cord, labeling in the ciliary ganglion was relatively homogeneous throughout the ganglion and was also present in the afferent and efferent nerve roots emerging from it (Fig. 1C). Similar results were also obtained using cRNA probes derived from pCA2 (data not shown) and we therefore concluded that agrin mRNA is expressed in the ciliary ganglion.

The chick ciliary ganglion has been used to study cell–cell interactions during neuromuscular synapse formation (Role *et al.*, 1985; Role *et al.*, 1987). Our observation

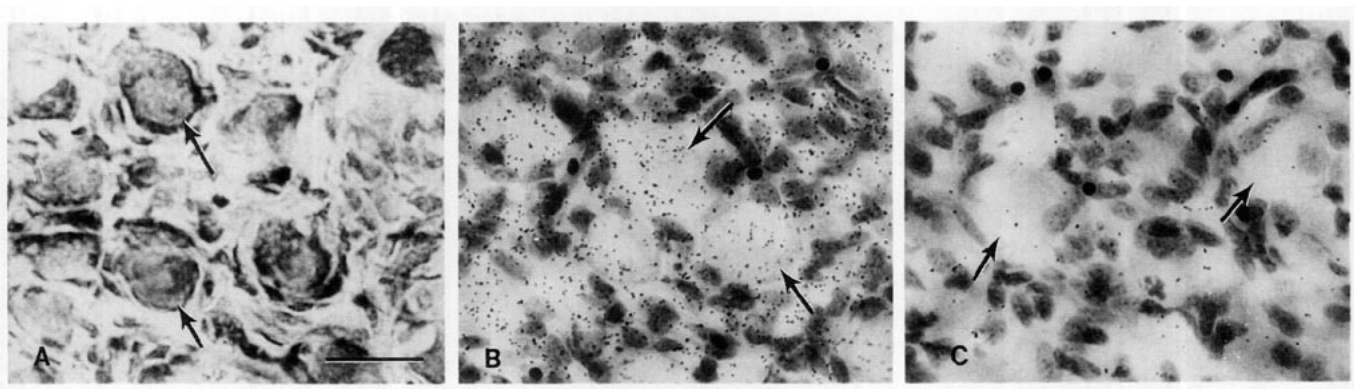

FIG. 3. Agrin mRNA is localized in both neurons and nonneuronal cells. The cellular localization of silver grains is illustrated in high magnification, bright-field photomicrographs of sections through an E15 ciliary ganglion (A) stained with cresyl violet in which large, darkly stained neuronal somata (arrows) and smaller, surrounding nonneuronal cells are visible. (B) Adjacent section processed for *in situ* hybridization with an antisense agrin cRNA probe shows grains distributed over both large neuronal cell bodies and the smaller nonneuronal cells. (C) Next adjacent section, hybridized with the sense control probe. Only a low level of nonspecific labeling is observed. Bar, 25 μm.

that ciliary ganglia from E12 embryos express agrin mRNA suggested that the ganglion might also be useful in understanding the cellular interactions that regulate neuronal agrin gene expression. We therefore performed a detailed analysis of the pattern of agrin mRNA expression in the ciliary ganglion during development. Figure 2 shows the results of a single experiment in which sections from E6 through E20 ciliary ganglia were hybridized with the antisense agrin cRNA probe derived from pCA1. Sections for *in situ* hybridization were processed in parallel, and photomicrographs made using fixed exposures, to facilitate comparison between sections from different developmental stages. The highest levels of labeling were seen in sections of ganglia obtained from embryos before E15 and labeling declined to relatively low levels by hatching at E20. Similar results were also seen in two other experiments in which 4–10 ganglia each from E6, E8, E10, and E20 or E8, E12, E15, and E20 were processed under identical conditions.

Labeling with antisense agrin probes showed a uniform distribution in the ciliary ganglion at all developmental stages examined (Fig. 2). To determine whether hybridization was specifically associated with the neurons in the ganglion, we assessed the histological organization of the tissue in Nissl-stained sections. Neurons were identified as large, round, intensely basophilic cells (Fig. 3A). Adjacent sections were hybridized with an antisense agrin probe and counterstained with toluidine blue. Although RNAase treatment associated with the *in situ* hybridization procedure reduces the basophilic nature of the neurons due to a loss of Nissl substance, silver grains were clearly located over the large, round, neuronal somata (Fig. 3B). Serial sections through the ganglion revealed labeling associated with all neuronal profiles, demonstrating that both populations of motor neurons in the ganglion, choriod and ciliary, express agrin mRNA.

Two lines of evidence indicate that nonneuronal cells in this system also express agrin mRNA. First, sections through afferent and efferent nerve roots of the ganglion showed high levels of hybridization with antisense agrin probes (Fig. 1C). Since nerve roots lack neuronal somata and are composed primarily of Schwann cells, this suggests that Schwann cells express agrin mRNA. Second, in sections through the ganglion, silver grains were associated with populations of presumptive glial cells that occupy the spaces between neuronal somata, suggesting that in addition to neurons, nonneuronal cells within the ganglion also express agrin mRNA during embryonic development.

Quantitation of Developmental Changes in Agrin mRNA

Our *in situ* hybridization studies demonstrate that the density of agrin mRNA in the ciliary ganglion de

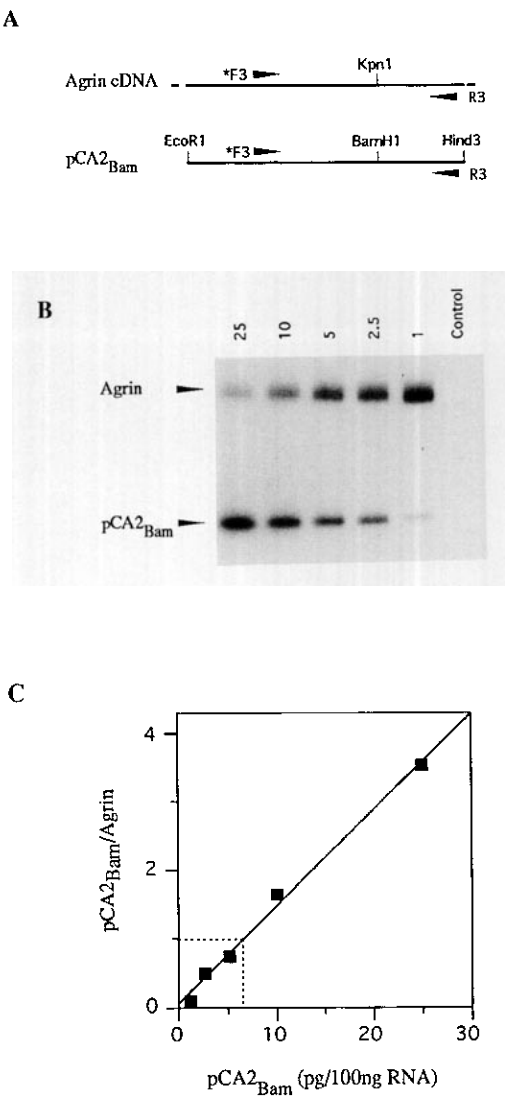

FIG. 4. Quantitation of agrin mRNA expression in the ciliary ganglion by competitive PCR. (A) Restriction map showing location of natural *Kpn*I and engineered *Bam*HI restriction sites in agrin and $pCA2_{Bam}$, respectively, together with primers F3 and R3 used in competitive PCR reactions. (B) In a typical experiment cDNA synthesized from 100 ng of E18 ciliary ganglion RNA was coamplified with picogram amounts of $pCA2_{Bam}$ as indicated above the lanes. PCR products were labeled at their 5′ end by incorporation of the ^{32}P-labeled forward primer (*F3). After restriction with *Bam*HI, PCR products were resolved by gel electrophoresis and visualized by autoradiography. The amount of product derived from the native agrin template increases as the concentration of competing template decreases. The control lane contains an equivalent amount of reaction mixture from a PCR performed in the absence of either template. (C) The yield of reaction product synthesized from each template was determined by densitometry, and the amount of agrin mRNA/100 ng total RNA (dotted lines) was estimated from a plot of the ratio of $pCA2_{Bam}$/agrin cDNA product expressed as a function of $pCA2_{Bam}$ concentration.

clines during embryonic development. However, developmental changes in the size of the ganglion and the number and size of neurons and glial cells it contains make estimates of the absolute amount of agrin mRNA/ganglion from density measurements of *in situ* hybridization autoradiograms unreliable. We therefore used competitive PCR (Gilliland *et al.*, 1990) to quantify changes in agrin gene expression during development. A competitive template, $pCA2_{Bam}$, was constructed by introducing a single base pair mutation that transformed a unique *Kpn*I restriction site in agrin into a *Bam*HI site. (Fig. 4A). This manipulation enabled us to distinguish by size, PCR products generated either from cDNA reverse-transcribed from the endogenous agrin mRNA or from the competitive template $pCA2_{Bam}$, following digestion with *Bam*HI and resolution by gel electrophoresis. The amplified products representing endogenous agrin mRNA and $pCA2_{Bam}$ were visible in autoradiograms as 339- and 204-bp bands, respectively (Fig. 4B).

Under the conditions used in these experiments only products amplified from the endogenous agrin template or $pCA2_{Bam}$ were evident in the autoradiograms, indicating that competition for the primers was between these two templates only. Steps were also taken to avoid or detect several other sources of potential error. First, since incomplete digestion of $pCA2_{Bam}$ products could result in overestimation of agrin cDNA levels, we determined the conditions for restriction digests that resulted in complete digestion of products from 100 pg of $pCA2_{Bam}$ amplified alone, the maximal amount of $pCA2_{Bam}$ used in the competitive PCR assays. Formation of heterodimers between amplified products has also been reported as a possible source of error in competitive PCR (Gilliland *et al.*, 1990). Double digestion of PCR products with both *Kpn*I and *Bam*HI resulted in the appearance of a single 204-bp band in autoradiograms, indicating that all products contain either a *Kpn*I or a *Bam*HI restriction site (data not shown). Thus, heterodimeric products were below the level of detection or absent from this system. Finally, a control in which RNA was replaced by an equivalent amount of water during first-strand cDNA synthesis was included in all experiments to monitor contamination between reagents. Autoradiograms were analyzed only if there were no detectable PCR products in this control lane.

As an example, an autoradiogram of a single determination of the amount of agrin mRNA present in 100 ng of total RNA isolated from E18 ciliary ganglia is shown in Fig. 4B. The amount of PCR product synthesized from each template was determined by computer-assisted densitometry, and the ratio of $pCA2_{Bam}$:agrin cDNA product plotted as a function of $pCA2_{Bam}$ concentration

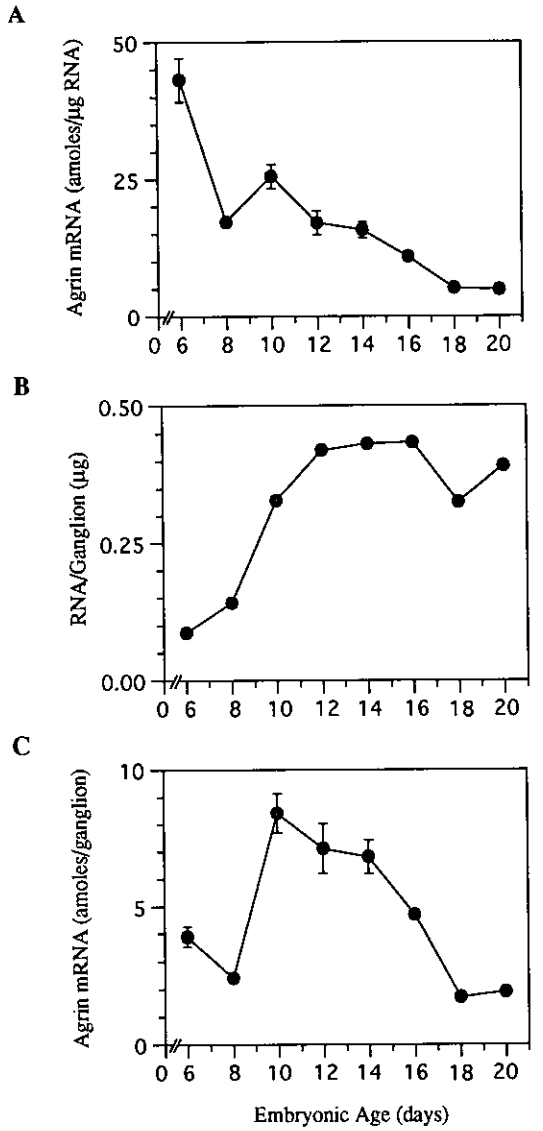

FIG. 5. Agrin mRNA levels change in the chick ciliary ganglion during development. (A) Based on the molecular weight of the competitive template $pCA2_{Bam}$, agrin mRNA concentrations, expressed in attomoles/μg of total RNA, at different stages of development were obtained by competitive PCR analysis described in Fig. 4. (B) Total RNA per ganglion was estimated from the yields of RNA isolated from pools of ganglia at the indicated developmental ages. (C) The amount of agrin mRNA expressed in attomoles per ganglion at each developmental stage was determined by normalizing the competitive PCR data in (A) to the amount of total RNA/ganglion in (B). The level of agrin mRNA in the ganglion increases dramatically between E8 and E10 and is sustained through to E14, coincident with the period of synapse formation between ganglionic neurons and their targets in the periphery. Bars in (A) and (C) indicate SEM where the error is larger than the symbol. Each data point represents the mean of four determinations except for E16 and E18 where $n = 3$.

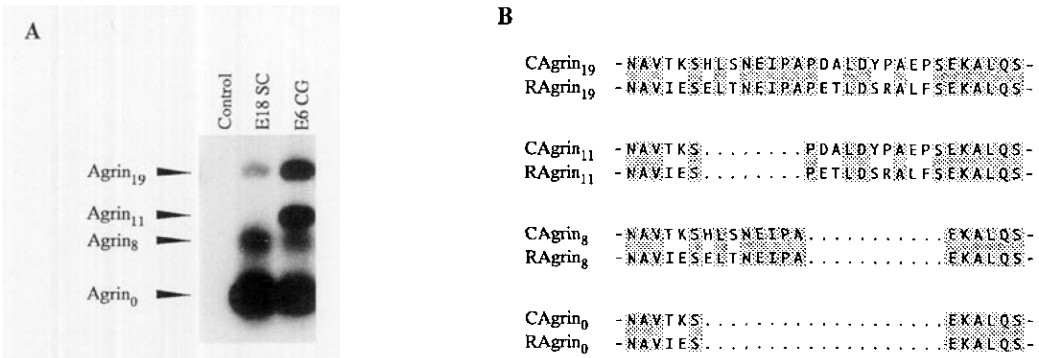

FIG. 6. Alternative RNA splicing gives rise to four agrin transcripts in chick ciliary ganglia. (A) First-strand cDNA, synthesized from RNA isolated from E18 spinal cords and E6 ciliary ganglia, was amplified by two rounds of PCR using nested primers flanking the insertion point of exon B identified in the chick agrin clone CBA-1 (Tsim *et al.*, 1992). The control lane represents a reaction in which RNA was omitted from the first-strand cDNA synthesis. Resolution of reaction products by gel electrophoresis followed by autoradiography reveals four alternatively spliced agrin mRNAs. (B) The amino acid sequence of chicken agrin (CAgrin) isoforms and comparison of homologous regions present in rat agrin (RAgrin). Identical residues are shaded.

(Fig. 4C; $r = 0.993 \pm 0.002$, mean correlation coefficient for all experiments ± SEM, $n = 30$). The amount of agrin mRNA/100 ng of total RNA was determined mathematically by linear estimation for a ratio of $pCA2_{Bam}$:agrin cDNA products equal to one.

Based on the molecular weight of the competing template, the competitive PCR experiments allowed us to determine the molar concentration of agrin mRNA/μg total RNA extracted from ciliary ganglia at different embryonic stages (Fig. 5A). These data demonstrate that the concentration of agrin mRNA was highest at E6, the earliest time point examined. There did not, however, appear to be a simple linear decline in the concentration of agrin message as a function of developmental age. A 2.5-fold decrease between E6 and E8 was followed by a transient increase in the concentration of agrin message at E10, returning by E12 to levels of expression similar to that at E8. Agrin mRNA expression decreased from E14 to its lowest level at E18, where it remained until at least E20. Over the entire period of development the concentration of agrin mRNA decreased approximately 10-fold.

To provide a more direct comparison with the results of our *in situ* hybridization studies, we used the competitive PCR data on agrin mRNA concentration to determine the amount of agrin mRNA expressed per ciliary ganglion during development. The amount of total RNA per ganglion at each stage in development was calculated from the yield of RNA extracted from pools of 50–125 ganglia. The amount of RNA per ganglion was lowest at E6, increased markedly between E6 and E12, and remained at this level until E20 (Fig. 5B). Despite the observation that the concentration of agrin mRNA/μg total RNA was highest at E6, when the amount of agrin mRNA was normalized on a per ganglion basis, it was apparent that there were relatively few copies of agrin mRNA in the ganglion at this time (Fig. 5C). Between E8 and E10, however, the amount of agrin mRNA/ganglion increased almost fourfold, with high levels of expression maintained up until E14 (Fig. 5C). This is consistent with the high density of labeling observed by *in situ* hybridization over this same period, a time when the ganglion increases markedly in diameter (Fig. 2) and the number of neurons in the ganglion decreases by approximately 50% due to naturally occurring neuronal cell death (Landmesser and Pilar, 1974a). In line with the results of *in situ* hybridization studies, levels of agrin mRNA/ganglion decrease during subsequent development.

Alternative Splicing of Agrin RNA

Studies in both rat and chicken (Ferns *et al.*, 1992; Tsim *et al.*, 1992; Ruegg *et al.*, 1992) have shown that through alternative RNA splicing, a single agrin gene is responsible for the synthesis of a family of proteins whose individual members differ in their ability to induce AChR aggregation in cultured myotubes. In chick, attention has focused on a region referred to as exon B that encodes an 11-amino-acid insert present only in the chick agrin protein that induces AChR aggregation (Ruegg *et al.*, 1992). To determine which forms of agrin are expressed by cells in the ciliary ganglion, RNA from E6 ganglia and E18 spinal cord was subjected to two rounds of amplification by PCR using nested primers flanking exon B. Based on the electrophoretic mobility of the PCR products, agrin transcripts that include exon B were present in E6 ciliary ganglia, while transcripts

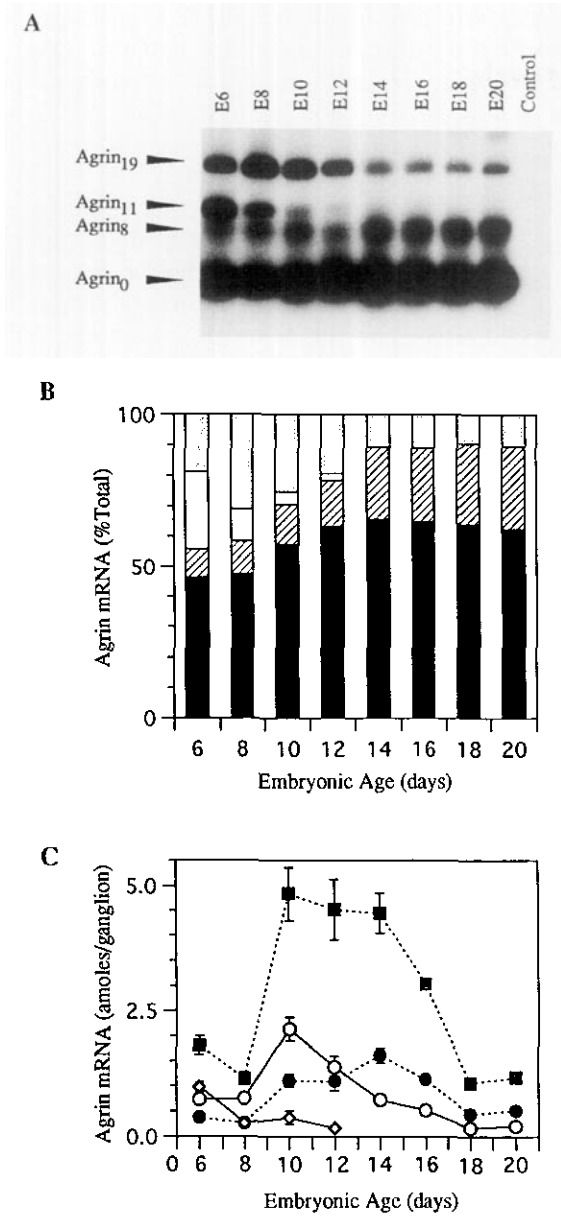

FIG. 7. Levels of alternatively spliced agrin mRNA change during development. First-strand cDNA synthesized from RNA isolated from embryonic ciliary ganglia was subject to two rounds of amplification using nested primers. (A) In a typical experiment an autoradiogram exposed overnight shows that at each developmental stage the relative levels of expression of the different agrin isoforms are distinct. For example, at E6, $agrin_{11}$ represents a relatively large fraction of the total agrin present compared to a much smaller fraction at E12. (B) From short exposure (3–5 hr) autoradiograms the level of expression of each transcript was determined by densitometry and expressed as a fraction of total agrin mRNA. The histogram summarizes results of three experiments. $Agrin_0$, filled bars; $agrin_8$, cross-hatched bars; $agrin_{11}$, open bars; $agrin_{19}$, stippled bars. (C) To determine absolute levels of each agrin mRNA per ganglion as a function of development, the percentage of expression of each agrin transcript was normalized to the amount of total agrin mRNA (i.e., all transcripts, Fig. 5) expressed per ganglion at each age. As illustrated in the graph, each agrin isoform exhibits a distinct pattern of expression during embryonic development. $Agrin_0$, filled squares; $agrin_8$, filled circles; $agrin_{11}$, open diamonds; $agrin_{19}$, open circles. Bars show SEM, $n = 3$ for each time point.

lacking exon B were found in both E6 ganglia and E18 spinal cord (Fig. 6A). In addition, two other products of the amplification reaction were evident, suggesting the presence of previously uncharacterized chick agrin transcripts. Based on electrophoretic mobility, one product predicted an mRNA containing a single exon encoding an 8-amino-acid insert. A higher molecular weight band was also observed, consistent with an mRNA including both exons encoding the 8- and 11-amino-acid inserts.

To confirm their identity, DNA from each of the four bands amplified from ciliary ganglion RNA was ligated into a cloning vector, and three to six clones from each ligation were sequenced. The alignment of the predicted amino acid sequences encoded by chick agrin mRNA is shown in Fig. 6B, together with a comparison of the homologous regions recently identified in embryonic rat spinal cord (Ferns *et al.*, 1992). In line with the nomenclature proposed by Ferns *et al.* (1992), we refer to the four chick agrin splicing variants as $agrin_0$ (exclusion of both exons), $agrin_8$ (inclusion of a 24-nucleotide exon encoding 8 amino acids), $agrin_{11}$ (inclusion of a 33-nucleotide exon encoding 11 amino acids), and $agrin_{19}$ (inclusion of both exons). Of the two exons, the 8-amino-acid sequence present in both $agrin_8$ and $agrin_{19}$ was the most highly conserved (75% amino acid identity for $agrin_8$ compared to 45% for $agrin_{11}$) between chick and rat.

Developmental Regulation of Alternative Agrin RNA Splicing

The observation that several forms of agrin mRNA are expressed in E6 ciliary ganglia led us to question whether the levels of alternatively spliced variants might change during development. Accordingly, we applied PCR amplification using nested primers to RNA isolated from ciliary ganglia of different embryonic ages. As can be seen in the autoradiogram from a typical experiment (Fig. 7A), the results of this study confirmed our earlier observation that four distinct agrin mRNAs are expressed in the ganglion. In addition, these experiments revealed that the levels of expression of each transcript changed during development. For example, expression of $agrin_{11}$ appeared to be restricted to early stages of development while the relative abundance of $agrin_8$ increased with embryonic age.

To quantify these changes we used densitometry to determine the fraction of total agrin mRNA that each alternatively spliced isoform represented at different developmental stages. Since high levels of $agrin_0$ expression at all developmental stages could saturate the film and thus result in underestimation of $agrin_0$ and overestimation of the contribution of less abundant agrin isoforms, density measurements were obtained from autoradiograms exposed for the shortest period of time (3–5 hr at room temperature) consistent with being able to unambiguously visualize all four transcripts. Measurements taken from the same autoradiograms following longer exposures (12–15 hr at −70°C), as in Fig. 7A, resulted in a 10% reduction (10 ± 1.8%, mean ± SEM, $n = 3$) in the estimated relative abundance of $agrin_0$ expression compared to values obtained for shorter exposures. However, there was no change in the developmental profile overall. Errors associated with nonlinearity in film autoradiograms in the data acquired from shorter exposures are likely to be even lower.

Cumulative data from three experiments similar to that shown in Fig. 7A is presented in Fig. 7B. At E6 and all subsequent stages, $agrin_0$ was the most abundant transcript in the ganglion. The level of $agrin_0$ mRNA increased from 40% of total agrin mRNA at E6–8 to 60% at E14, where it was maintained through to E20. A similar trend, although at a much lower level, was also evident in the pattern of $agrin_8$ expression. In contrast, the relative abundance of $agrin_{11}$ mRNA was highest in E6 ganglia and declined thereafter, to being undetectable in RNA from E14 ganglia, even following longer exposure of the film autoradiograms (Fig. 7A). The expression of $agrin_{19}$ also exhibited a unique pattern, rising to a peak at E10 and declining to a low level at E14 that was subsequently maintained during the remainder of embryonic development.

Potential differences in amplification efficiency between PCR reactions make direct comparison between different developmental stages unreliable. However, knowing the total amount of agrin mRNA/ganglion and the fraction of total agrin mRNA represented by each isoform, we were able to calculate the absolute amounts of each agrin transcript/ganglion at different developmental ages (Fig. 7C). Changes in the level of $agrin_0$ mRNA/ganglion exhibited a developmental course similar to that of total agrin mRNA/ganglion, as expected given that $agrin_0$ is the most abundant transcript in the ciliary ganglion at all developmental stages examined. Levels of $agrin_8$ mRNA were highest between E10 and E16 and were quite low before and after this period. The highest level of expression of $agrin_{11}$ was observed at E6. $Agrin_{11}$ expression subsequently fell to a low level by E8 that was maintained until E12. By E14, $agrin_{11}$ mRNA was below the limit of detection. $Agrin_{19}$ levels increased markedly from E8 to E10, followed by a steady decline to E18.

DISCUSSION

In light of agrin's proposed role at the neuromuscular junction recent studies have examined agrin gene expression in CNS motor neurons (Rupp *et al.*, 1991; Ferns *et al.*, 1992; Ruegg *et al.*, 1992; Smith *et al.*, 1992; Tsim *et al.*, 1992). In this report we focus our attention on the chick ciliary ganglion, a parasympathetic ganglion that contains two distinct populations of motor neurons (Marwitt *et al.*, 1971; Pilar and Tuttle, 1982). We demonstrate that in addition to motor neurons in the CNS, both populations of autonomic motor neurons in the ciliary ganglion also express agrin. We further show that alternative RNA splicing results in expression of four agrin mRNAs in the ciliary ganglion and that each isoform exhibits a distinct pattern of expression during development that can be correlated with synapse formation in the periphery. These results suggest that in addition to its proposed role in synapse formation between spinal motor neurons and skeletal muscle (McMahan, 1990), agrin may also play a role in synapse formation between autonomic neurons and their target tissues.

Previous studies have identified two neuronal cell populations in the ciliary ganglion which have different peripheral targets. Ciliary neurons project to striated muscle fibers in the iris and ciliary body while choroid neurons innervate smooth muscle targets in the eye (Marwitt *et al.*, 1971). Ciliary and choroid neurons are also located in different regions of the ganglion; ciliary neurons are most densely packed in the dorsolateral part of the ganglion while choroid neurons are situated ventromedially (Marwitt *et al.*, 1971; Landmesser and Pilar, 1972; Pilar *et al.*, 1980). In serial sections through the ganglia we found no evidence, by *in situ* hybridization, for a significant population of unlabeled cells, suggesting that agrin is expressed in both ciliary and choroid neurons. Indeed the homogeneous distribution of labeling is consistent with the interpretation that agrin mRNA is also expressed by nonneuronal cells in the ganglion as well as in neurons. Supporting this conclusion is the observation of labeling in tissue surrounding neuron cell bodies and in ganglionic nerve roots that contain glial cells and axons but from which neuronal somata are absent.

cDNAs representing $agrin_0$ and $agrin_{11}$ mRNAs expressed in the ciliary ganglion have previously been isolated from embryonic chick brain libraries (Ruegg *et al.*,

1992; Tsim *et al.*, 1992). We report the characterization of two additional chick agrin mRNAs, $agrin_8$ and $agrin_{19}$, homologous to agrin transcripts recently identified in embryonic rat spinal cord (Ferns *et al.*, 1992). In expression studies, when tested against chick myotubes, rat $agrin_8$, $agrin_{11}$, and $agrin_{19}$ are active, while $agrin_0$ exhibits little or no activity in AChR aggregating assays (Ferns *et al.*, 1992). The fact that both neurons and glial cells in the ganglion express agrin mRNA raises the issue of what cellular pattern of expression the different transcripts in the ganglion have. Using PCR, Ruegg *et al.* (1992) have shown that $agrin_{11}$ is present in nervous tissues but not muscle of embryonic chicks. Furthermore, RNA isolated from optic or sciatic nerve contains only $agrin_0$ while retina and dorsal root ganglia express both $agrin_{11}$ and $agrin_0$ mRNA (McMahan *et al.*, 1992), evidence that neurons, not glial cells, express the active agrin isoform. Based on these results we would predict that in the chick ciliary ganglion, neurons express $agrin_{11}$ and the other active isoforms, $agrin_8$ and $agrin_{19}$, while nonneuronal cells, probably representing Schwann and satellite cells, express $agrin_0$. However, the possibility that $agrin_0$ is also expressed by neurons in the ganglion remains to be tested.

Consistent with agrin's proposed role as a synaptogenic protein, agrin mRNA is present in ventral horn neurons of E5–6 chick spinal cord, a time when motor neurons first establish functional contacts with limb muscles (Tsim *et al.*, 1992). In the present study we show that agrin mRNA is also expressed in the ciliary ganglion at the time contact is established and synapses are formed between the ganglionic motor neurons and their peripheral targets. At E6, ganglionic neurons have begun to reach their targets but they do not begin to establish functional synaptic contacts until after E8. By E6, significant levels of all four agrin mRNA isoforms are detectable in the ganglion. Thus, muscle fibers could be exposed to agrin released from ingrowing growth cones and induce AChR aggregation before the formation of any closely apposed neuromuscular contacts. This possibility is consistent with the finding that the first high-density clusters of AChR in developing chick wing muscle are not found in close association with neuronal growth cones or nerve terminals (Smith and Slater, 1983; Dahm and Landmesser, 1991).

Active synapse formation between ganglionic neurons and their peripheral targets occurs during E8–E14 (Landmesser and Pilar, 1974b). When examined by *in situ* hybridization, the density of labeling in the ganglion is relatively high but does not appear to change significantly during this period. Coincident with this wave of synapse formation, however, dramatic increases take place in the size of both neuronal somata and the ganglion as a whole (Landmesser and Pilar, 1974a). Thus, in order to maintain the constant density of silver grains observed in the dark-field micrographs over this period of embryonic development, the levels of agrin mRNA in the ganglion must increase. Indeed, quantitation of changes in agrin mRNA expression by competitive PCR revealed a marked increase in agrin mRNA levels in the ganglion between E8 and E10, with a high level maintained until E14. The correlation in the temporal pattern of agrin expression in the ciliary ganglion and synapse formation between ganglionic neurons with peripheral targets suggests that in addition to its proposed role in synaptogenesis by spinal motor neurons, agrin plays an important role in the peripheral nervous system during synapse formation between autonomic neurons and their target tissues.

We have also shown that the levels of expression of different agrin transcripts change during development. These data suggest that functional differences between agrin proteins are important for specific aspects of nerve–muscle interaction during synapse formation. In support of this idea, quantitative differences in AChR aggregating activity of different agrin isoforms have been demonstrated such that $agrin_8 > agrin_{19} > agrin_{11}$ (Ferns *et al.*, 1992). In contrast, $agrin_0$ appears to have little or no receptor clustering activity when assayed on chick myotubes (Ferns *et al.*, 1992; Ruegg *et al.*, 1992). Agrin mRNA expression profiles obtained in our studies show that each of the active isoforms peaks in ascending order of activity as development proceeds. Thus, the highest levels of $agrin_{11}$ occur at E6, $agrin_{19}$ peaks at E10 and $agrin_8$ is maximal at E14. How might this temporal pattern of expression of agrin isoforms contribute to synapse formation? One possibility is that spatial stability of the postsynaptic apparatus depends on the agrin protein that induces it. The adult neuromuscular junction is an extremely stable structure (Balice-Gordon *et al.*, 1990) such that even following denervation, the distribution of AChR remains unchanged over relatively long periods of time (Dennis, 1981). During development, however, neuromuscular synapses undergo extensive and coordinated modeling of both pre- and postsynaptic components (Slater, 1982; Smith and Slater, 1983; Dahm and Landmesser, 1991). A degree of spatial plasticity would seem to be desirable in a rapidly growing system, and perhaps this is accomplished by expression of different agrin isoforms during development. The trend in our data predicts that at mature neuromuscular synapses $agrin_8$, the most potent isoform in AChR clustering assays (Ferns *et al.*, 1992), would predominate. This prediction could be tested as antibodies specific for each agrin protein become available.

Changes in levels of agrin mRNA in the ciliary gan-

glion during development reflect changes in relative levels of alternatively spliced mRNA isoforms. Although the mechanisms underlying these changes are unknown, one possibility is that the pattern of expression we observe reflects the execution of a program of agrin gene expression that is intrinsic to cells. Agrin mRNA is already present by E6 and may be expressed in cells in the ganglion independent of cellular interactions. However, the apparent coordination of agrin mRNA expression with synapse formation between ganglionic neurons and their peripheral target tissues makes this possibility seem unlikely and instead supports the idea of target tissues influencing agrin expression. Previous studies have shown that neurons in the ganglion are directly influenced by target-dependent interactions. Survival of ganglionic neurons is target dependent (Landmesser and Pilar, 1974a) and postganglionic axotomy in hatchling chicks results in a decrease in levels of AChR subunit mRNA (Boyd *et al.*, 1988).

In summary, our findings demonstrate that alternative RNA splicing of the agrin gene results in the expression of four distinct agrin mRNAs in the ciliary ganglion. Each agrin isoform exhibits a unique, developmentally regulated pattern of expression in the ganglion. The temporal correlation between high levels of agrin mRNA expression in the ciliary ganglion and synapse formation of ganglionic neurons with peripheral targets strongly supports a more generalized role for agrin as a synaptogenic protein, not limited to spinal cord motor neurons. These results, together with the accessibility of the ganglion, its targets, and inputs, establish the ciliary ganglion as an attractive system in which to examine factors involved in the regulation of agrin gene expression.

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