UC Irvine
UC Irvine Previously Published Works

Title

Cell-specific regulation of agrin RNA splicing in the chick ciliary ganglion.

Permalink

https://escholarship.org/uc/item/4t31v4nv

Journal

Neuron, 12(4)

ISSN

0896-6273

Authors

Smith, M A

O'Dowd, D K

Publication Date

1994-04-01

Peer reviewed

Cell-Specific Regulation of Agrin RNA Splicing in the Chick Ciliary Ganglion

Martin A. Smith,* and Diane K. O'Dowd*†
*Department of Anatomy and Neurobiology
†Department of Developmental and Cell Biology
University of California, Irvine
Irvine, California 92717

Summary

Alternative splicing results in production of four agrin proteins (agrin$_0$, agrin$_8$, agrin$_{11}$, and agrin$_{19}$) with different AChR aggregating activities. However, the cellular origin of mRNAs encoding each agrin isoform remains unknown. Using single-cell PCR, we demonstrate that in the chick ciliary ganglion, nonneuronal cells express only mRNA encoding agrin$_0$, whereas neurons express one or any combination of agrin mRNAs. Moreover, significant differences were observed between the agrin mRNA profiles of ciliary and choroid neurons in the ganglion. The abundance of each agrin mRNA, the fraction of neurons expressing each transcript, and the combinations of transcripts expressed by neurons also change during development. Our results demonstrate that transcripts encoding agrin proteins with high AChR aggregating activity are expressed exclusively by neurons in the ciliary ganglion and that alternative splicing of agrin mRNA is regulated during development and in a cell-specific manner.

Introduction

Numerous lines of evidence support the hypothesis that the protein agrin mediates motor neuron-induced accumulation of acetylcholine receptors (AChRs) at the neuromuscular junction during development. For example, agrin induces clustering of AChRs on cultured skeletal muscle fibers (Nitkin et al., 1987), is expressed by embryonic motor neurons (Magill-Solc and McMahan, 1988; Tsim et al., 1992), and colocalizes with the earliest forming AChR clusters on embryonic limb muscle fibers (Fallon and Gelfman, 1989). However, agrin gene expression is not limited to somatic motor neurons. In situ hybridization studies have shown that parasympathetic neurons in the developing peripheral nervous system (Thomas et al., 1993) and virtually all major neuronal populations in the adult rat CNS (O'Connor et al., 1994) express agrin mRNA. In addition, many nonneuronal cells, including glia (Ruegg et al., 1992; O'Connor et al., 1994; Thomas et al., 1993) and muscle cells (Lieth et al., 1992), express agrin. These results suggest that agrin may play a much wider role in the nervous system than originally proposed.

In chicken, rat, and mouse, alternative splicing of exons encoding 8 and 11 amino acids gives rise to four distinct agrin mRNAs: agrin$_0$, agrin$_8$, agrin$_{11}$, and agrin$_{19}$ (Ferns et al., 1992; Rupp et al., 1992; Thomas et al., 1993). Expression studies aimed at understanding the structural basis of agrin's AChR aggregating activity have shown that inclusion or exclusion of these small exons has a dramatic impact on the AChR aggregating activity of the protein produced. Thus agrin$_8$, agrin$_{11}$, and agrin$_{19}$, derived from mRNAs containing either or both exons, are active in AChR aggregation assays, whereas agrin$_0$, lacking both the 8 and 11 amino acid sequences, has little or no aggregating activity (Ferns et al., 1992; Ruegg et al., 1992). Alternative splicing therefore may serve as an important mechanism for regulating expression of functionally distinct agrin proteins, for example in a cell-specific manner, during development and in the maturation of synapses.

Two lines of evidence support a differential pattern of alternative agrin mRNA splicing between neuronal and nonneuronal cells. First, polymerase chain reaction (PCR) analysis of RNA isolated from different tissues in embryonic chick has shown that, whereas agrin mRNA is expressed in brain, spinal cord, muscle, and sciatic nerve, transcripts encoding the active isoform agrin$_{11}$, are found only in brain and spinal cord, tissues which contain neuronal cell bodies (Ruegg et al., 1992). Second, analysis of acutely dissociated embryonic chick spinal cord cells using a cell fractionation technique has shown that the level of agrin$_{11}$ mRNA is positively correlated with enrichment for motor neurons, suggesting that agrin$_{11}$ is preferentially expressed by motor neurons over other cells in the spinal cord (Tsim et al., 1992). Together, these studies suggest that mRNAs encoding agrin proteins which have high activity in AChR aggregating assays have a neuron-specific pattern of expression. However, these studies did not examine the cell-specific expression of agrin$_8$ or agrin$_{19}$, nor could they address the possibility that multiple agrin isoforms might be expressed by a single cell or population of cells.

We have recently demonstrated that alternative splicing of agrin mRNA is regulated during development in the chick ciliary ganglion (Thomas et al., 1993). All four agrin mRNAs are expressed in the ganglion, and both the level of expression and pattern of alternative splicing change during development. The chick ciliary ganglion contains two populations of neurons, ciliary and choroid, as well as nonneuronal cells. In situ hybridization indicates that both ciliary and choroid neurons and nonneuronal cells in the ganglion express agrin. However, as in all other studies to date, in situ hybridization probes could not distinguish between different agrin mRNAs, preventing determination of the cellular origin of each agrin isoform. Based on the results of the studies described above, we predicted that mRNA encoding agrin proteins (agrin$_8$, agrin$_{11}$, and agrin$_{19}$) with high AChR aggregating activity would be expressed by neurons, whereas nonneuronal cells in the ganglion would express agrin$_0$ (Thomas et al., 1993). We sought to make a direct test of this hypothesis by determining the agrin mRNA profile of both

A

NN

N

N

B

N1

N2

2.0 nA
0.2 nA

4 ms

NN1

NN2

+20 mV
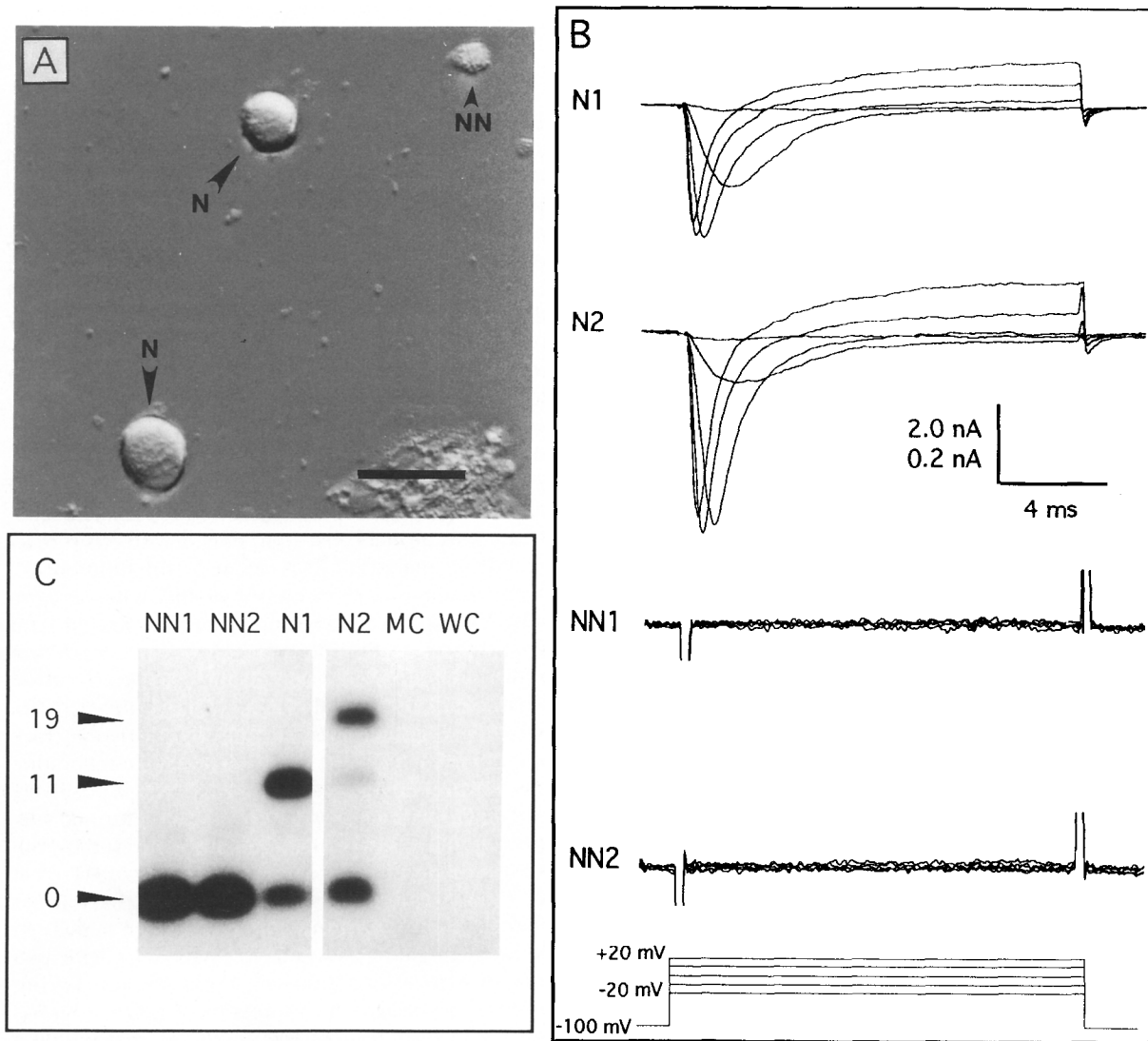-20 mV
-100 mV

C

NN1 NN2 N1 N2 MC WC

19

11

0

Figure 1. Morphological, Electrophysiological, and Agrin mRNA Profiles of Neuronal and Nonneuronal Ciliary Ganglion Cells

(A) Acutely dissociated cells prepared from E14 chick ciliary ganglia contain both neuronal cells (N) and nonneuronal cells (NN) that can be distinguished on the basis of cell body size and shape. Bar, 20 μm.

(B) Whole-cell voltage-clamp recordings demonstrate the presence of both inward and outward voltage-gated currents in neuronal cells (N1 and N2). The same stimulus paradigm does not activate any voltage-gated currents in two representative nonneuronal cells (NN1 and NN2). However, nonneuronal cells have high input resistances (2.2 and 2.9 GΩ for NN1 and NN2, respectively) compared with neurons (0.5 and 0.6 GΩ for N1 and N2, respectively). Note that the scale bar of 2 nA applies to the neuronal cells and 0.2 nA to the records from the nonneuronal cells.

(C) Composite film autoradiogram from a single experiment showing the PCR products amplified from RNA extracted from cells in (B). RNA PCR of cytoplasm harvested from nonneuronal cells, NN1 and NN2, results in amplification of a single product representing agrin$_0$. In contrast, amplification products of RNA from neurons N1 and N2 contain multiple agrin isoforms. The media control (MC), in which 2 μl of saline was aspirated into the patch pipette from the recording chamber, and the water control (WC), in which 2 μl of water was substituted for cell cytoplasm in the first strand cDNA synthesis and processed in parallel with the experimental lanes, are negative. Numbers to the left of the autoradiogram identify the agrin mRNAs represented in these cells.

neuronal and nonneuronal cells in the ganglion using a single-cell RNA PCR technique. The results of our studies show that nonneuronal cells in the ganglion express only agrin$_0$. In contrast, ganglionic neurons may express one or any combination of agrin mRNAs including agrin$_0$. The pattern of alternative splicing among single neurons appears to be regulated during development. Moreover, distinct patterns of agrin

mRNA expression were evident in ciliary and choroid neurons.

Results

Agrin mRNA Expression in Neurons and Nonneuronal Cells

Although in situ hybridization studies have shown

A

C 1 2 3 4 5 6 7 8 9 10 11

19 ►
11 ►
8 ►

0 ►

B

Relative Abundance (%)

100 — Non-Neuronal Cells (n=12)
Neurons (n=8 5)

75

50

25

0

0   8   11   19

C

Cells Expressing (%)

100

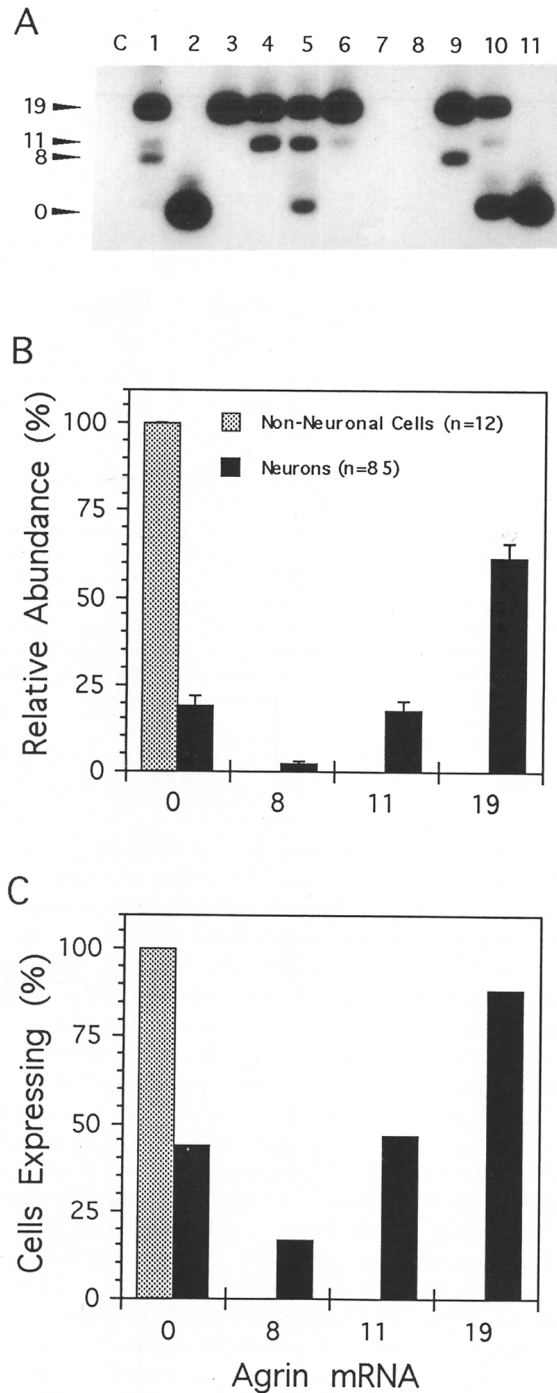75

50

25

0

0   8   11   19

Agrin mRNA

Figure 2. Cell-Specific Regulation of Alternative Splicing of Agrin mRNA

(A) Autoradiogram of a typical experiment showing the agrin mRNA profile of single cells dissociated from an E14 ciliary ganglion. With the exception of lane 2, each lane shows DNA amplified from a single identified neuron. Neurons express both multiple (e.g., cells 1, 4, 5, 9, and 10) and single (e.g., cells 3 and 11) agrin mRNAs. Based on its appearance, cell 2 was judged to be a nonneuronal cell, although no electrophysiological records were obtained for this particular cell to confirm its identification. Typical of other nonneuronal cells, however, this cell expressed only $agrin_0$. No product was amplified from neurons 7 and 8 or the water control, C. Numbers to the left of the autoradiogram identify the PCR product for each agrin isoform.

that both ciliary and choroid neurons, as well as nonneuronal cells in the ciliary ganglion, express agrin mRNA (Thomas et al., 1993), the specific forms of agrin expressed in different cell types have not been determined. We have used single-cell RNA PCR to analyze agrin gene expression in identified cells in the ciliary ganglion. Based on both their morphological appearance and electrophysiological properties, cell preparations acutely dissociated from embryonic day 14 (E14) ganglia contain both neurons and nonneuronal cells. Neuron cell bodies were typically round or elliptical in shape (Figure 1A), displayed voltage-gated sodium and potassium currents (Figure 1B), and in many instances could generate overshooting action potentials (e.g., see Figure 4). Whole-cell capacitance measurements ranged from 3.1 to 32.9 pF with a mean of 13.4 $\pm$ 0.7 pF (mean $\pm$ SEM; n = 91). In addition to neurons, a population of smaller, more irregularly shaped cells (Figure 1A) with a mean whole-cell capacitance of 3.8 $\pm$ 0.4 pF (mean $\pm$ SEM; n = 21) were also apparent. These cells never displayed voltage-gated sodium currents (Figure 1B) and were unable to fire action potentials. Although the majority of these cells also lacked voltage-gated potassium currents, 3 cells exhibited small outward currents (<100 pA) following large depolarizing pulses. Moreover, and consistent with their small size, these cells had relatively high input resistances compared with neurons, making it unlikely that these small cells might represent injured and shrunken neurons. Based on their appearance and electrophysiological properties, therefore, the small cells were classified as nonneuronal.

Following electrophysiological characterization of each cell, negative pressure was applied to the patch pipette, and the cell cytoplasm was aspirated into the pipette tip and transferred to a microfuge tube for reverse transcription. Aliquots of the first strand cDNA were subsequently amplified by PCR using nested primers that had previously been shown to amplify specifically four alternatively spliced agrin mRNAs ($agrin_0$, $agrin_8$, $agrin_{11}$, and $agrin_{19}$) present in RNA isolated from whole embryonic ciliary ganglia (Thomas et al., 1993). PCR products representing each of the four alternatively spliced agrin mRNAs expressed in the whole ganglion could also be resolved at the single-cell level (Figure 1C; Figure 2A). However, marked differences were apparent in the agrin mRNA profiles of different cell types and between individual cells. Cytoplasm harvested from neurons produced PCR products that

(B) The percent expression of each agrin mRNA isoform in individual E14 ciliary ganglion cells was determined using a phosphorimager. Values in this histogram represent an average for all neuronal or nonneuronal cells for which any PCR product was obtained. Whereas nonneuronal cells express only $agrin_0$, neurons can express any of the four agrin mRNAs. The fraction of neurons expressing each agrin mRNA is shown in (C). Histograms summarize the results from ten experiments similar to that shown in (A). Legend shows the total number of nonneuronal cells and neurons analyzed.

could include any permutation of the four agrin iso-forms (Figure 1C; Figure 2B). In contrast, $agrin_0$ was the only agrin mRNA that could be amplified from nonneuronal cells (Figure 1C; Figure 2B).

To investigate in more detail the differences in agrin mRNA expression among neurons, as well as between neurons and nonneuronal cells, we used a phosphor-imager to quantitate the agrin mRNA expression pro-files of cells acutely dissociated from E14 ganglia. Fig-ure 2A shows the results of a typical experiment in which RNA from single cells was amplified, with the cumulative data from ten independent experiments summarized in Figures 2B and 2C. Two measures were used to characterize the pattern of expression of agrin mRNA in ciliary ganglion cells. First, the percentage of total agrin mRNA represented by each agrin mRNA isoform in a single cell was determined and averaged over the total number of cells (neuron or nonneu-ronal) for which any PCR product was obtained (Fig-ure 2B). Second, the fraction of cells expressing a par-ticular agrin mRNA was calculated (Figure 2C). PCR products were obtained from 12 of 25 morphologically and electrophysiologically identified nonneuronal cells. Consistent with our initial results, $agrin_0$ was the only mRNA expressed in all 12 of these nonneuronal cells (Figures 2B and 2C). Moreover, in 6 additional cells identified as nonneuronal on the basis of their mor-phology alone, $agrin_0$ was also the only isoform ex-pressed. In contrast with the invariant agrin mRNA profile of the nonneuronal cells examined, various combinations of the four alternatively spliced agrin transcripts were evident in neurons (Figure 2A). PCR products were amplified from 85 of 91 electrophysio-logically identified neurons. Analysis of the mean rela-tive abundance of each agrin isoform in neurons for which PCR products were obtained shows that $agrin_{19}$ expression levels were at least 3 times higher than those of $agrin_0$, $agrin_8$, or $agrin_{11}$ (Figure 2B). In addi-tion to being the most abundant transcript, $agrin_{19}$ mRNA was the most frequently expressed mRNA, pres-ent in approximately 85% of neurons, compared with 45% for $agrin_{11}$ and $agrin_0$ and 15% for $agrin_8$.

We believe that the PCR reaction conditions used in this study provide a good estimate of the relative levels of agrin mRNA expression in individual cells. However, before coming to this conclusion, we con-sidered several alternative explanations. There was no correlation between the length of time elapsed after starting the electrophysiological recordings and har-vesting the cell's cytoplasm (approximately 15 min), or the time until cDNA synthesis was begun (0.25–4 hr) and the presence or relative abundance of the PCR products. Therefore, it seems unlikely that the ob-served pattern of agrin mRNA expression could be accounted for by a time-dependent loss of specific or low abundance agrin transcripts. Next, to rule out the possibility that differences in the pattern of agrin ex-pression between cells resulted from contamination between reagents or individual PCR reactions, two controls were routinely performed. First, an equiva-
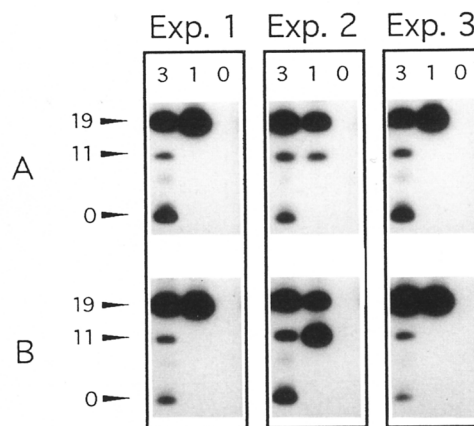

Figure 3. The Agrin mRNA Profile of Single Neurons Is Not a Result of Limiting Amounts of Template

Cytoplasm from 0 to 3 neurons (numbers) was pooled and re-verse transcribed. Subsequently, the cDNA was divided into two aliquots (A and B) and amplified by PCR as described, and the resulting agrin mRNA profiles were compared. In all cases, the overall pattern of agrin mRNA expression between sister amplifi-cations is similar. In some instances, for example amplification of cDNA from a single cell in experiment 2, differences in the relative abundance of specific mRNAs were evident.

lent amount of water was used in place of cell cyto-plasm during first strand cDNA synthesis. As a second control, an aliquot of the external recording medium was aspirated into the pipette and treated as a sham cell during the subsequent steps of cDNA synthesis and PCR amplification. At least one of these controls was included in all experiments, whereas most experi-ments included both. Data were only accepted for analysis in which such controls were negative.

Finally, we considered the possibility that a low agrin mRNA copy number in a single cell might be associ-ated with a probabilistic failure of the PCR amplifica-tion, resulting in underestimation of the relative abun-dance of a particular isoform or the number of cells in which it is expressed. If this was the case, we reasoned that amplification of aliquots of cDNA synthesized from a single cell would result in different products. Therefore, to test this possibility and establish the sensitivity of the technique, we performed the follow-ing experiment. Cytoplasm from 1 to 3 neurons was pooled in a single reverse transcription reaction, and the resulting cDNA was divided into two sister ali-quots that were amplified in separate PCR reactions. Comparison of the resulting autoradiograms (Figure 3) indicates that, although some variability in the relative abundance of different mRNAs was apparent, the over-all patterns of agrin mRNA expression observed in each sister aliquot was similar, regardless of the num-ber of cells pooled for the initial cDNA synthesis. We conclude therefore that the levels of expression in most ciliary ganglion cells are within the level of detec-tion by the techniques used here.
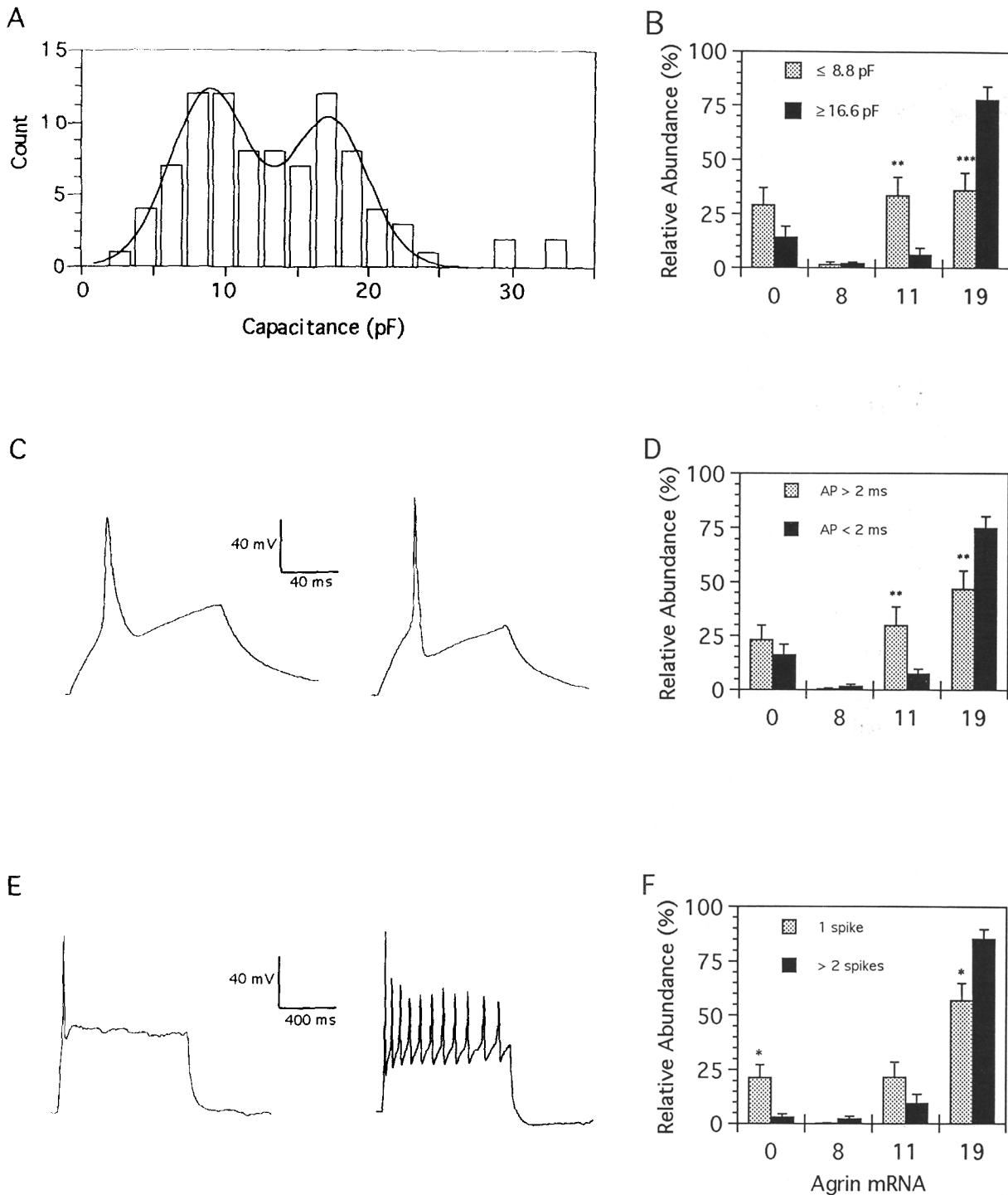
Figure 4. Ciliary and Choroid Neurons Exhibit Distinct Agrin mRNA Profiles

Neurons were classified as ciliary or choroid on the basis of three independent criteria, and their agrin mRNA profiles were compared.
(A) Capacitance histogram compiled from measurements of 91 electrophysiologically identified neurons. Histogram is fitted with a superimposed double-Gaussian distribution with a χ^2 value of 19.1, consistent with the presence of two normally distributed but overlapping populations representing small choroid and large ciliary neurons.
(B) The agrin mRNA profile of neurons to the left of the lower mode (choroid-like) and to the right of the upper mode (ciliary-like).
(C) Examples of two E14 neurons, one with a long duration (3.6 ms) action potential typical of choroid cells (left) and one with a short duration (1.4 ms) action potential characteristic of ciliary neurons (right). Action potentials were elicited in response to a 60 ms depolarizing current injection from a resting potential of −75 mV. Duration was measured at half-spike amplitude (threshold to peak).
(D) The average agrin mRNA profiles of neurons with long duration (2.69 ± 1.76 ms; mean ± SEM; n = 20) and those with action potential durations less than 2 ms (1.54 ± .03 ms; mean ± SEM; n = 30).
(E) Prolonged (600 ms) depolarizing current injection elicited a single action potential in an E14 neuron illustrated on the left, whereas the cell on the right fired a train of action potentials in response to an identical stimulus.
(F) Agrin profile of E14 neurons grouped according to the ability to produce only a single spike versus those that could fire three or more action potentials in response to a sustained depolarizing current injection. Note the similarity in agrin profiles of cells classified as ciliary or choroid on the basis of these three independent criteria.
Bars indicate SEM; *** p < .001, ** p < .01, * p < .05; Student's t test.

Ciliary and Choroid Neurons Exhibit Distinct Patterns of Alternative Splicing

The ciliary ganglion contains two populations of neurons, ciliary and choroid, that innervate distinct target muscles in the eye (Marwitt et al., 1971). However, criteria that have previously been used to distinguish these two population overlap. To investigate the possibility that alternative splicing of agrin mRNA is differentially regulated between ciliary and choroid neurons, we examined agrin mRNA expression in populations of neurons defined by three independent criteria: cell size, action potential duration, and firing pattern.

Previous studies have shown that ciliary neurons can be distinguished from choroid cells by their larger cell body diameter (Pilar et al., 1980) and capacitance (Dryer and Chiappinelli, 1985). Figure 4A shows a frequency histogram of capacitance measurements from E14 neurons (20 bins; 1.75 pF per bin). Attempts to fit this data with a single Gaussian distribution resulted in χ^2 values which led us to reject the hypothesis that a single population gave rise to this distribution. However, these data were well fitted by the sum of two Gaussian distributions with modes at 8.8 and 16.6 pF and χ^2 value of 19.1 (Figure 4A). To test the possibility that alternative splicing of agrin mRNA might differ between small choroid-like and large ciliary-like neurons, we analyzed the relative abundance of each agrin transcript in neurons with capacitances $\leqslant 8.8$ pF and compared this with the same measurement in neurons with capacitances $\geqslant 16.6$ pF. As shown in Figure 4D, small choroid-like neurons expressed significantly higher levels of $agrin_{11}$ mRNA (p < 0.01) but lower levels of $agrin_{19}$ (p < 0.001). $Agrin_8$ appears to be expressed at a similar low level in both populations of cells. Although a 2-fold difference was evident between the mean levels of $agrin_0$ expressed in ciliary- and choroid-like neurons, this difference was not significant (p < 0.113)

A recent report using conventional intracellular recording techniques suggests that ciliary neurons exhibit shorter duration action potentials and can sustain higher firing frequencies than choroid cells (Dryer, 1994). We also observed differences in action potential duration and spiking pattern of individual E14 neurons. Action potential duration ranged between 1 and 5 ms, and in our hands, approximately half of the neurons could generate multiple spikes in response to sustained depolarizing current, whereas only a single action potential could be elicited in the remaining cells (Figures 4B and 4C). As expected, neurons with short duration action potentials or multiple spiking neurons also tended to be large (data not shown). To determine whether the pattern of agrin mRNA expression was correlated with the ability to generate a short duration action potential, neurons were sorted into 2 bins, those with action potential durations < 2 ms (ciliary-like) and those with action potential durations > 2 ms (choroid-like). Similarly, to examine the relationship between agrin mRNA expression and action potential frequency, neurons were divided into two
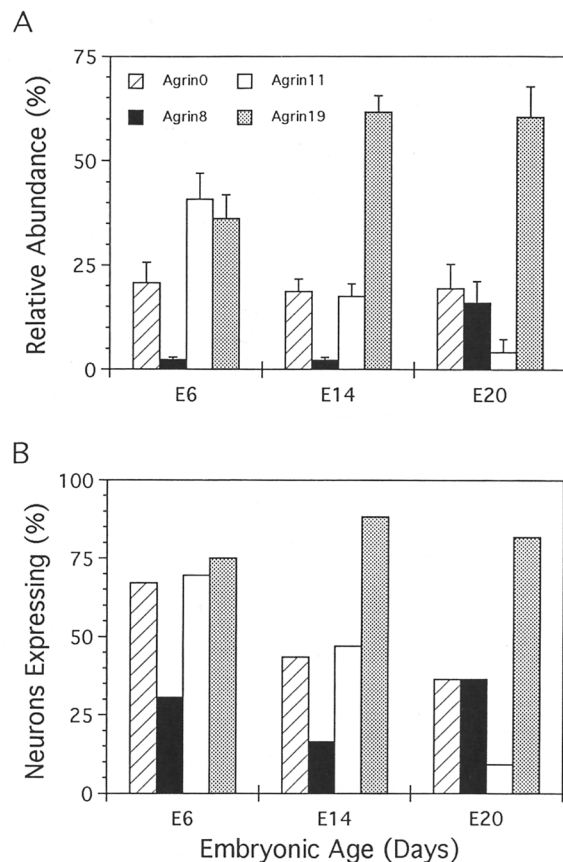

Figure 5. Regulation of Neuronal Agrin mRNA Expression during Development

Histograms showing the average relative abundance of each agrin mRNA in neurons (A) and the fraction of neurons expressing each transcript at different stages of embryonic development (B). Both the levels of expression and the percentage of neurons expressing each agrin isoform change during embryonic development. Bars indicate SEM.

groups, those able to fire three or more action potentials (ciliary-like) and those that fired only a single action potential (choroid-like) in response to prolonged current injection (Figures 4E and 4F). The pattern of agrin mRNA expression in these electrophysiologically defined populations of neurons shows a remarkable coincidence with the agrin mRNA profile obtained when neurons were sorted strictly on the basis of size. Thus, choroid-like neurons with long duration action potentials and low firing frequencies express significantly higher levels of $agrin_{11}$ and $agrin_0$ but lower levels of $agrin_{19}$. Together, these data suggest that ciliary and choroid neurons exhibit distinct patterns of agrin mRNA expression.

Agrin mRNA Expression during Development

The pattern of agrin RNA alternative splicing in the ciliary ganglion changes during development (Thomas et al., 1993). For example, $agrin_{11}$ is expressed in the ganglion by E6 but is absent at E14. To determine whether

Table 1. The Pattern of Alternative Splicing in Single Neurons Is Regulated during Development

E6		E14		E20	
mRNA	% of Neurons	mRNA	% of Neurons	mRNA	% of Neurons
0	0	0	3.5 (3)	0	6.1 (2)
8	0	8	0	8	3.0 (1)
11	14.0 (5)	11	5.9 (5)	11	3.0 (1)
19	16.8 (6)	19	29.4 (25)	19	39.4 (13)
0,8	8.3 (3)	0,8	0	0,8	6.1 (2)
0,11	0	0,11	1.2 (1)	0,11	0
0,19	0	0,19	9.4 (8)	0,19	9.1 (3)
8,11	0	8,11	1.2 (1)	8,11	0
8,19	0	8,19	4.7 (4)	8,19	15.2 (5)
11,19	27.9 (10)	11,19	14.1 (12)	11,19	3.0 (1)
0,8,11	2.8 (1)	0,8,11	0	0,8,11	0
0,8,19	5.6 (2)	0,8,19	5.9 (5)	0,8,19	12.1 (4)
0,11,19	11.1 (4)	0,11,19	20.0 (17)	0,11,19	3.0 (1)
8,11,19	0	8,11,19	1.2 (1)	8,11,19	0
0,8,11,19	14.0 (5)	0,8,11,19	3.5 (3)	0,8,11,19	0

At each stage in development examined, the number of neurons expressing each of 15 possible combinations of agrin mRNA was determined and expressed as a percentage of the total number of neurons for which a PCR product was obtained. Neurons for which no PCR product was obtained were excluded from the analysis. Results are ranked in order of increasing complexity of agrin mRNA profile. Numbers in parentheses represent number of neurons in each category.

the pattern of agrin mRNA expression observed in single cells could account for these observations, we analyzed mRNA harvested from neurons dissociated from early (E6), middle (E14), and late (E20) stage embryonic ganglia (Figure 5). Measurement of the relative abundance of agrin mRNA revealed that $agrin_0$ represents about 25% of all agrin transcripts at each stage of development studied. However, a developmental decrease in the fraction of neurons expressing $agrin_0$ suggests that, at later stages, $agrin_0$ represents a greater fraction of mRNA in cells which express it than earlier in development. Although the fraction of neurons expressing $agrin_8$ mRNA remained fairly constant, its relative abundance increased approximately 8-fold from 2% at E6 and E14 to 16% at E20, suggesting that the abundance of this isoform in single cells also increases during development. Marked changes were also evident in both the relative abundance and fraction of cells expressing $agrin_{11}$, which declined about 8-fold during development. Finally, whereas the fraction of neurons expressing $agrin_{19}$ remained fairly constant, the relative abundance of $agrin_{19}$ mRNA increased between E6 and E14 and was maintained at similar levels until E20.

We also analyzed the frequency with which combinations of agrin mRNAs were expressed (Table 1). With four distinct agrin isoforms, there are 16 possible combinations of alternatively spliced mRNAs that can be expressed. In 23 of 154 neurons (14.9%), no agrin products were detected; however, since we did not control for the efficiency of the cell cytoplasm-harvesting procedure or PCR reaction, these cells were not analyzed further. For those neurons in which one or more agrin mRNAs were amplified, our analysis demonstrates that, whereas all 15 remaining combinations were represented, the frequency of each combination varied with development (Table 1). At E6, the most frequently oc-

curring agrin profile was that containing both $agrin_{11}$ and $agrin_{19}$, representing 27.9% of the cells. In contrast, at E14 and E20, neurons containing only $agrin_{19}$ are the most abundant (29.4% and 39.4%, respectively). At E6 and E14, combinations of agrin mRNA that included $agrin_0$, $agrin_{11}$, or $agrin_{19}$ were more frequently observed than those including $agrin_8$, whereas at E20, combinations that included $agrin_{11}$ were the most rare.

Discussion

The observation that alternatively spliced agrin mRNAs encode proteins which differ in their AChR aggregating activity, coupled with agrin's widespread pattern of expression among neuronal and nonneuronal cells, has raised questions concerning the cellular origin of different agrin mRNAs. The results of our study provide direct evidence that, in the chick ciliary ganglion and perhaps throughout the nervous system, nonneuronal cells express only $agrin_0$, whereas neurons express mRNAs encoding $agrin_0$, $agrin_8$, $agrin_{11}$, and $agrin_{19}$. Moreover, no single combination of agrin mRNAs is excluded from the repertoire of agrin isoforms expressed by neurons in the ganglion, since during the period of embryonic development examined, neurons were observed that expressed as few as one, and as many as all four, agrin mRNAs.

Several studies have shown that, in developing central and peripheral nervous systems, agrin mRNA is present in both neuronal and nonneuronal cells (Rupp et al., 1991; Ruegg et al., 1992; Tsim et al., 1992; Thomas et al., 1993). Although agrin mRNA is widely expressed in the adult rat CNS, it is predominantly associated with neuronal perikarya, and levels of expression by glial cells are at or below the level of detection by in situ hybridization (O'Connor et al., 1994). The results of the present study demonstrating that embryonic

neurons express $agrin_0$ are consistent with the interpretation that in the adult rat CNS, neurons are the predominant source of all agrin mRNA, including $agrin_0$. Indeed, in preliminary experiments, application of the single-cell techniques employed in the present study have revealed that $agrin_0$ is the most abundant mRNA in some neurons dissociated from postnatal day 8 mouse somatosensory cortex (unpublished data).

During development, agrin released from motor nerve terminals induces the accumulation of AChR at the motor endplate (for review see Nastuk and Fallon, 1993). The ciliary ganglion is a parasympathetic ganglion that contains two population of motor neurons which innervate distinct muscle targets in the eye. Whereas choroid neurons form en passant–type synapses with their smooth muscle targets, which are typical of autonomic neurons (Meriney and Pilar, 1987), ciliary neurons form endplates on striated muscle fibers in the eye (Pilar et al., 1981). Although both classes of neurons in the ganglion express agrin mRNA (Thomas et al., 1993), given the marked differences in the degree of organization of the synaptic terminations of ciliary and choroid neurons, we sought to determine whether agrin gene expression between these two types of neurons might differ. With the exception of the tissues they innervate, other single criteria used to distinguish between ciliary and choroid neurons identify overlapping populations of cells. Therefore, we examined the agrin profile of ciliary and choroid neurons identified on the basis of three independent criteria: cell soma size, action potential duration, and firing pattern.

Previous studies have shown that ciliary neurons are, on average, larger than choroid neurons (Pilar et al., 1980; Dryer and Chiappinelli, 1985). Consistent with this observation, frequency distribution histograms of capacitance measurements in cells at E14 suggested the presence of overlapping populations of large and small neurons. Comparison of their agrin profiles showed that, on average, large neurons express lower levels of $agrin_0$ and $agrin_{11}$ but greater amounts of $agrin_{19}$. It has also been reported that the spike duration of ciliary neurons is typically half that of choroid cells (Dryer, 1994). We found that cells with short duration action potentials expressed significantly more $agrin_{19}$ and less $agrin_{11}$, similar to the agrin profile of large neurons. Finally, it has been reported that with direct injection of current, ciliary neurons fire at higher frequency than choroid cells (Dryer, 1994). In the present study, we found that, like large neurons and neurons with short duration action potentials, the agrin profile of repetitively firing cells was characterized by lower levels of $agrin_0$ and $agrin_{11}$, but higher expression of $agrin_{19}$. Together, these data suggest that, on the basis of several independent criteria, ciliary and choroid neurons exhibit a quantitatively distinct pattern of agrin gene expression. Despite the quantitative differences revealed by this analysis however, qualitatively, the average agrin mRNA profile of all neurons examined is remarkably similar, consis-

tent with the observation that in cell culture all neurons in the ganglion are competent to establish functional synaptic contacts on skeletal muscle fibers at which AChRs accumulate (Role et al., 1987).

One alternative explanation for the differences in agrin mRNA expression profiles between the populations of cells defined above are that small cells, those with long duration action potentials and those that are unable to fire repetitively, are simply less mature than their counterparts. Several observations argue against this interpretation. First, previous studies have shown that the largest increases in ionic conductances underlying the action potential in ciliary ganglion neurons occur between E7 and E13 (Dourado and Dryer, 1992), suggesting that the electrophysiological properties of ciliary and choroid neurons are mature by E14. Second, in the whole ganglion, $agrin_8$ expression is associated with relatively late embryonic stages (Thomas et al., 1993). However, both populations of E14 neurons identified in the present study express a similar low level of $agrin_8$ and, based on this measure of maturity, are at a similar stage of development. Finally, somatostatin has been shown to be preferentially expressed in choroid neurons (Epstein et al., 1988). In preliminary studies (unpublished data), we amplified cDNA from individual neurons for both agrin and somatostatin mRNA. Despite the difference in age, E20 somatostatin positive neurons exhibited an agrin mRNA profile similar to that seen in small cells at E14.

Agrin gene expression in the ciliary ganglion is regulated during development (Thomas et al., 1993). The results of the present study demonstrate that some of the changes in agrin mRNA isoform expression observed in the whole ganglion are mirrored by changes at the single-cell level. For example, in the whole ganglion, $agrin_{11}$ is most abundant at E6 but declines to a level below detection by E14. In single neurons, the relative abundance of $agrin_{11}$ is at the highest level at E6 and declines during subsequent development, a change that is compounded by a decline over a similar time course in the number of neurons containing $agrin_{11}$. In contrast, other aspects of agrin expression in the whole ganglion do not appear to have a simple relationship with the pattern of expression observed in single neurons. For example, $agrin_{19}$ expression peaks in the ganglion at E10, whereas our single-cell analysis of dissociated cells indicates that $agrin_{19}$ is expressed by the majority of neurons and at relatively high abundance throughout development. Moreover, although changes in $agrin_8$ expression at the single-cell level were generally consistent with those observed in the whole ganglion, with the highest levels of expression being observed in late stage neurons, the single-cell data predict the peak in $agrin_8$ expression to be both smaller relative to $agrin_{19}$ and later in development than seen in the whole ganglion. Two possible explanations could account for this apparent discrepancy. First, the acutely dissociated cell preparations may become increasingly skewed toward the exclusion of cells, perhaps ciliary neurons which are larger and

may be more easily damaged, that express greater amounts of $agrin_8$ and proportionately less $agrin_{19}$. We believe this unlikely, as it has been reported (Margiotta and Gurantz, 1989) that, at E14, approximately 75% of all neurons are recovered using an identical dissociation technique. Furthermore, the range of cell capacitances observed among neurons at E14 suggests that we sampled from a broad population of neurons.

More intriguing is the possibility that $agrin_8$ mRNA may be partitioned in a region of the cell which is not readily sampled when harvesting cell cytoplasm. Partitioning of maternal mRNA within oocytes is necessary for normal development (Jeffery, 1985). In the nervous system, it has been shown that polyribosomes are present at the base of synaptic spines, and there is accumulating evidence to suggest that specific mRNAs are preferentially localized in dendrites (for review see Steward and Banker, 1992). Skeletal muscle fibers also express agrin that is colocalized at nerve-induced AChR clusters (Lieth et al., 1992; Lieth and Fallon, 1993), and agrin originating from the postsynaptic cell may play a role in synapse formation. In the adult ganglion, the most richly innervated regions on both ciliary and choroid neurons are short processes or pseudodendrites that begin to appear as early as E11 during development (Landmesser and Pilar, 1974; Jacob and Berg, 1983; Jacob, 1991). This raises the possibility that $agrin_8$ is preferentially localized in the pseudodendrites either which may be lost during the dissociation procedure or from which it is difficult to harvest the cell cytoplasm and RNA. As antibodies that are specific for each agrin isoform become available, it will be of interest to determine whether different agrin proteins have nonuniform distributions and preferential localizations at synaptic sites on ciliary ganglion neurons.

Studies of agrin gene expression have shown that mRNA encoding $agrin_0$ is the most abundant agrin transcript in the chick ciliary ganglion and appears to be strongly regulated during development (Thomas et al., 1993). Similar patterns of $agrin_0$ expression and developmental regulation have also been reported in the CNS (Hoch et al., 1993; O'Connor et al., 1994). The results of the present study clearly demonstrate that, not only do neurons in the ciliary ganglion express $agrin_0$ mRNA, but that the pattern of neuronal expression is regulated during development. However, we do not believe that the decrease during development in the fraction of neurons expressing $agrin_0$ contributes to its decline in the whole ganglion. Our results show that upregulation of $agrin_0$ mRNA in those neurons which express it serves to maintain the relative abundance of neuronal $agrin_0$ at a constant level during development. Because the majority of cells in the ganglion are nonneuronal cells which only express $agrin_0$, it seems likely that the steep decline in $agrin_0$ which occurs in the whole ganglion following synapse formation (Thomas et al., 1993) reflects a decrease in the levels of agrin expression by nonneuronal cells. The identity of the cellular signals that control agrin

gene expression in both neuronal and nonneuronal cells in the ganglion remains to be established.

Experimental Procedures

Cell Preparation

Cells were dissociated from ciliary ganglia of embryonic chicks and plated onto glass coverslips by a modification of the procedure previously described by Margiotta and Gurantz (1989). Briefly, four to six ganglia were dissected out into ice-cold calcium-free saline, trimmed of adhering connective tissue and nerve roots, and cut into halves or quadrants, depending on age. Ganglia were subsequently incubated in 2 ml of calcium-free saline containing 0.75 mg/ml collagenase A (Boehringer Mannheim) for 45 min at 37°C, then washed 4 times in 4 ml of saline containing 5.4 mM $CaCl_2$ and 10% heat-inactivated horse serum, and resuspended in 200 µl of the same solution, and cells were dissociated by trituration through a fire-polished pipette. Dissociated cells were plated on 18 mm square poly-D-lysine-coated glass coverslips (Margiotta and Gurantz, 1989) at a density of one ganglion per coverslip in plastic tissue culture dishes and allowed to recover for 1 hr in a 5% CO_2 atmosphere at 37°C, and then the dish was flooded with additional saline.

Electrophysiological Recording

Coverslips with dissociated cells were transferred to a recording chamber mounted on a Nikon TMS inverted microscope, and cells were visualized with Hoffman optics at a magnification of 600x. The cells were bathed in an external medium containing 140 mM NaCl, 4 mM $MgCl_2$, 5 mM HEPES, 1 mM $CaCl_2$, and 3 mM KCl (pH 7.2). Electrophysiological recordings were obtained using the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981). Recording pipettes were unpolished and had resistances between 0.5 and 4 MΩ when filled with the internal solution consisting of 20 mM KCl, 120 mM potassium gluconate, 10 mM HEPES, 1.1 mM EGTA, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 2 mM ATP (pH 7.2). Following formation of a high resistance seal, the capacitance associated with the electrode and patch was subtracted prior to rupture of the patch. Upon breaking into the cell, whole-cell capacitance was determined by integrating the area under the capacitative transient in a current record obtained by averaging the current responses elicited by ten depolarizing voltage steps from −70 to −60 mV. Data were collected and analyzed using a List EPC-7 patch-clamp amplifier, a Dell 386 computer, and pCLAMP software (Version 5.5.1; Axon Instruments). All recordings were performed at room temperature.

Cell RNA Harvesting and PCR Amplification

At the end of the electrophysiological recording, negative pressure was applied to the back of the pipette, and the contents of the cell were aspirated into its tip (Lambolez et al., 1992). The tip of the pipette was then broken against the side of a microcentrifuge tube, and approximately 2 µl of the pipette's contents were expelled and rapidly mixed into 7.5 µl of ice-cold first strand cDNA synthesis mixture (0.67 mM dNTPs [Pharmacia], 6.7 µM random hexanucleotide primers [Pharmacia], 10 U of RNasin [Promega] in 1.3x reverse transcriptase buffer [GIBCO-BRL]) and stored on ice for up to 4 hr. First strand cDNA was synthesized by addition of 100 U of Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL) to bring the mixture to a final volume of 10 µl, followed by incubation at 37°C for 1 hr. The reaction was terminated, and DNA-RNA hybrids were denatured by incubation at 90°C for 10 min.

To examine the relative abundance of agrin mRNA in identified cells, cDNA from the first strand synthesis reaction was subjected to two rounds of amplification by PCR using nested primers flanking a region of alternative splicing located at amino acid 1745 in the sequence predicted by the chick agrin clone CBA-1 (Tsim et al., 1992), as previously described (Thomas et al., 1993). In the first round of amplification, 5 µl of each cDNA reaction mixture was amplified in a 50 µl reaction for 35 cycles (94°C, 30s; 58°C, 1 min; 72°C, 1.5 min; 72°C, final extension step of 6

min) in a DNA thermal cycler (PCR 9600; Perkin-Elmer Cetus) using the forward and reverse primer pair F1/R1 whose 5' ends correspond to nucleotides 5196 and 5607, respectively, of the chick agrin clone CBA-1 (Tsim et al., 1992). Aliquots of each first round PCR were diluted 1000-fold and reamplified for 35 cycles (94°C, 30s; 54°C, 1 min; 72°C, 1.5 min; 72°C; final extension step of 6 min) using a second primer pair F2-R2 whose 5' ends correspond to nucleotides 5377 and 5499 of CBA-1, respectively. Products of the second round of PCR amplification were labeled at their 5' ends by including approximately 2×10^5 cpm of F2 labeled with ^{32}P using T4 kinase in each reaction (Sambrook et al., 1989). Twenty microliters of the amplified reaction mixture was fractionated by electrophoresis on an 8% polyacrylamide gel, and the relative levels of each PCR product were determined by analysis on a Bio-Rad phosphorimager using the PhosphorAnalyst (Bio-Rad) software package.

PCR Primers Used in the Study

The following primers were used: F1, CGTTGAAATCTCCACTTT-CCGTG; R1, CAGGTCATACATCATCTGCACGA; F2, TTTGATGG-TAGGACGTACATGGA; R2, TTTGATGCTCAGCTCAAAGTGGT.

References

Dourado, M. M., and Dryer, S. E. (1992). Changes in the electrical properties of chick ciliary ganglion neurones during embryonic development. J. Physiol. 449, 411–428.

Dryer, S. E. (1994). Neurotransmitter receptors and ionic channels of chick ciliary ganglion: biophysics, pharmacology, and regulation of their expression during embryonic development. Prog. Neurobiol., in press.

Dryer, S. E., and Chiappinelli, V. A. (1985). Properties of choroid and ciliary neurons in the avian ciliary ganglion and evidence for substance P as a neurotransmitter. J. Neurosci. 5, 2654–2661.

Epstein, M. L., Davis, J. P., Gelman, L. E., Lamb, J. R., and Dahl, J. L. (1988). Cholinergic neurons of the chicken ciliary ganglion contain somatostatin. Neuroscience 25, 1053–1060.

Fallon, J. R., and Gelfman, C. E. (1989). Agrin-related molecules are concentrated at acetylcholine receptor clusters in normal and aneural developing muscle. J. Cell Biol. 108, 1527–1535.

Ferns, M., Hoch, W., Campinelli, J. T., Rupp, F., Hall, Z. W., and Scheller, R. H. (1992). RNA splicing regulates agrin-mediated acetylcholine receptor clustering activity on cultured myotubes. Neuron 8, 1079–1086.

Hamill, O. P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F. J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflügers Archv. 391, 85–100.

Hoch, W., Ferns, M., Campanelli, J. T., Hall, Z. W., and Scheller, R. H. (1993). Developmental regulation of highly active alternatively spliced forms of agrin. Neuron 11, 479–490.

Jacob, M. H. (1991). Acetylcholine receptor expression in developing chick ciliary ganglion neurons. J. Neurosci. 11, 1701–1712.

Jacob, M. H., and Berg, D. K. (1983). The ultrastructural localization of α-bungarotoxin binding sites in relation to synapses on chick ciliary ganglion neurons. J. Neurosci. 3, 260–271.

Jeffrey, W. R. (1985). Specification of cell fate by cytoplasmic determinants in ascidian embryos. Cell 41, 11–12.

Lambolez, B., Audinat, E., Bochet, P., Crépel, F., and Rossier, J. (1992). AMPA receptor subunits expressed by single Purkinje cells. Neuron 9, 247–258.

Landmesser, L., and Pilar, G. (1974). Synapse formation during embryogenesis on ganglion cells lacking a periphery. J. Physiol. 241, 715–736.

Lieth, E., and Fallon, J. R. (1993). Muscle agrin: neural regulation and localization at nerve-induced acetylcholine receptor clusters. J. Neurosci. 13, 2509–2514.

Lieth, E., Cardasis, C. A., and Fallon, J. R. (1992). Muscle-derived agrin in cultured myotubes: expression in the basal lamina and at induced acetylcholine receptor clusters. Dev. Biol. 149, 41–54.

Magill-Solc, C., and McMahan, U. J. (1988). Motor neurons contain agrin-like molecules. J. Cell Biol. 107, 1825–1833.

Margiotta, J. F., and Gurantz, D. (1989). Changes in the number, function, and regulation of nicotinic acetylcholine receptors during neuronal development. Dev. Biol. 135, 326–339.

Marwitt, R., Pilar, G., and Weakley, J. N. (1971). Characterization of two cell populations in the avian ciliary ganglion. Brain Res. 25, 317–334.

Meriney, S. D., and Pilar, G. (1987). Cholinergic innervation of the smooth muscle cells in the choroid coat of the chick eye and its development. J. Neurosci. 7, 3827–3839.

Nastuk, M. A., and Fallon, J. R. (1993). Agrin and the molecular choreography of synapse formation. Trends Neurosci. 16, 72–76.

Nitkin, R. M., Smith, M. A., Magill, C., Fallon, J. R., Yao, M. Y.-M., Wallace, B. G., and McMahan, U. J. (1987). Identification of agrin, a synaptic organizing protein from Torpedo electric organ. J. Cell Biol. 105, 2471–2478.

O'Connor, L. T., Lauterborn, J. C., Gall, C. M., and Smith, M. A. (1994). Localization and alternative splicing of agrin mRNA in adult rat brain: transcripts encoding isoforms that aggregate acetylcholine receptors are not restricted to cholinergic regions. J. Neurosci. 14, 1141–1152.

Pilar, G., Landmesser, L., and Burstein, L. (1980). Competition for survival among developing ciliary ganglion cells. J. Neurophysiol. 43, 233–254.

Pilar, G., Tuttle, J., and Vaca, K. (1981). Functional maturation of motor nerve terminals in the avian iris: ultrastructure, transmitter metabolism and synaptic reliability. J. Physiol. 321, 175–193.

Role, L. W., Roufa, D. G., and Fischbach, G. D. (1987). The distribution of acetylcholine receptor clusters and sites of neurotransmitter release along chick ciliary ganglion neurite-myotube contacts in culture. J. Cell Biol. 104, 371–379.

Ruegg, M. A., Tsim, K. W. K., Horton, S. E., Kröger, S., Escher, G., Gensch, E. M., and McMahan, U. J. (1992). The agrin gene codes for a family of basal lamina proteins that differ in function and distribution. Neuron 8, 691–699.

Rupp, F., Payan, D. G., Magill-Solc, C., Cowan, D. M., and Scheller, R. H. (1991). Structure and expression of a rat agrin. Neuron 6, 811–823.

Rupp, F., Özçelik, T. H., Linial, M., Peterson, K., Francke, U., and Scheller, R. (1992). Structure and chromosomal localization of the mammalian agrin gene. J. Neurosci. 12, 3535–3544.

Sambrook, J. M., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

Steward, O., and Banker, G. A. (1992). Getting the message from the gene to the synapse: sorting and intracellular transport of RNA in neurons. Trends Neurosci. 15, 180–186.

Thomas, W. S., O'Dowd, D. K., and Smith, M. A. (1993). Developmental expression and alternative splicing of chick agrin RNA. Dev. Biol. 158, 523–535.

Tsim, K. W. K., Ruegg, M. A., Escher, G., Kröger, S., and McMahan, U. J. (1992). cDNA that encodes active agrin. Neuron 8, 677–689.