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Agrin Gene Expression in Ciliary Ganglion Neurons Following Preganglionic Denervation and Postganglionic Axotomy

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Agrin is an extracellular matrix protein that has been implicated as a synaptogenic agent in the peripheral and central nervous systems. Both the level of expression and pattern of alternative splicing of agrin mRNA are developmentally regulated. As a step toward identifying signals important in regulating agrin gene expression in neurons, we examined the effects of postganglionic axotomy or preganglionic denervation on agrin mRNA levels and alternative splicing in ciliary ganglia of posthatch chicks. In comparison to unoperated age-matched controls, *in situ* hybridization with a pan-specific agrin cRNA probe demonstrated a significant decrease in neuronal agrin mRNA expression as a result of axotomy. Reverse transcription-polymerase chain reaction analysis demonstrated that axotomy also resulted in changes in the pattern of alternative splicing of agrin mRNA. Underlying these changes are decreases in the molar amounts of transcripts encoding the neuron-specific isoforms agrin8 and agrin19, homologous to rat agrin proteins that have high AChR aggregating activity. Similar, but less dramatic changes in agrin expression following axotomy were also observed in unoperated neurons on the contralateral side. In contrast, the only significant change in agrin gene expression following ganglionic denervation was a small decline in the relative abundance of agrin 8 mRNA in operated versus unoperated age-matched control ganglia. Major changes in agrin gene expression following axotomy but not denervation are consistent with the notion that agrin synthesized by ganglionic neurons exerts its effects in the periphery rather than at synapses formed between ciliary ganglion neurons and their preganglionic input. These data suggest that the pattern of alternative splicing and the absolute amount of agrin mRNA in ciliary ganglion neurons may be regulated by target tissue interactions. © 1995 Academic Press, Inc.

INTRODUCTION

An early event in the development of the neuromuscular junction is accumulation of AChR² at the site of

nerve-muscle contact. Initially, a high-density region of AChR appears as a patch of receptors that extends beyond the point of nerve-muscle contact. However, as development proceeds, AChR become increasingly restricted in the muscle fiber membrane to the region in immediate contact with the motor nerve terminal. During this same period, the shape of the receptor patch also changes, mirroring changes in the morphology of the maturing nerve terminal. Numerous studies have shown that these and other aspects of the spatial and temporal differentiation of the neuromuscular junction are coordinated during development by signals exchanged between motor nerves and the skeletal muscle fibers they innervate (reviewed in Hall and Sanes, 1993; Connor and Smith, 1994).

A large body of evidence suggests that agrin, a neurally derived protein, directs the accumulation of AChR at the neuromuscular junction (reviewed in McMahan, 1990; Bowe and Fallon, 1995). Moreover, the observation that agrin mRNA is also present in the brains and peripheral ganglia of chicks and rodents suggests a broader role for agrin as a synaptogenic protein not limited to spinal motor neurons (Rupp *et al.*, 1991; Hoch *et al.*, 1993; Thomas *et al.*, 1993; Ma *et al.*, 1994; O'Connor *et al.*, 1994). Previous studies have shown that alternative splicing of exons encoding 8 and 11 amino acids at a region referred to as B in chick (McMahan *et al.*, 1992; Ruegg *et al.*, 1992; Tsim *et al.*, 1992) or z in rodent (Ferns *et al.*, 1992; Hoch *et al.*, 1993) gives rise to agrin proteins (agrin0, -8, -11, and -19) that differ in their AChR aggregating activity (Ferns *et al.*, 1992; Ruegg *et al.*, 1992; Tsim *et al.*, 1992).

Analysis of the agrin mRNA profiles of single chick ciliary ganglion cells (Smith and O'Dowd, 1994) has demonstrated that agrin8, -11, and -19 mRNAs are expressed only by neurons. The chick agrin11 form has been shown to have high AChR aggregating activity (Ruegg *et al.*, 1992; Tsim *et al.*, 1992) and based on studies of the homologous isoforms in rat (Ferns *et al.*, 1992), chick agrin8 and -19 are likely to have high activity also. In contrast, agrin0 mRNA, which lacks both the 8-

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² Abbreviations used: RT-PCR, reverse transcription-polymerase chain reaction; PCR, polymerase chain reaction; AChR, acetylcholine receptor; E, embryonic day; P, posthatch day.

and 11-amino-acid alternatively spliced exons and encodes a protein with low AChR aggregating activity (Ferns *et al.*, 1992; Ruegg *et al.*, 1992; Tsim *et al.*, 1992), is present in neurons and nonneuronal cells (Smith and O'Dowd, 1994). Moreover, in the chick ciliary ganglion, a parasympathetic ganglion that innervates muscle fibers in the eye, agrin gene expression and alternative splicing are regulated such that the levels of agrin mRNA encoding agrin proteins with the greatest AChR aggregating activity are highest during synapse formation with the target tissue (Thomas *et al.*, 1993; Smith and O'Dowd, 1994). Observation of a complex pattern of agrin mRNA expression in developing rat brain and spinal cord (Hoch *et al.*, 1993), similar to that seen in the chick ciliary ganglion, suggests that the ordered expression of the different agrin isoforms is functionally important and subject to regulatory control. While in principle, signals regulating agrin gene expression may be purely intrinsic to the cell, the complexity and timing of agrin gene expression in the nervous system suggest that some extrinsic signal is also likely to play a role. We have begun a series of experiments aimed at identifying these signals.

The accessibility of the chick ciliary ganglion to surgical manipulation provides a unique opportunity to examine the role of cell-cell interactions in regulating agrin gene expression (for example see Jacob and Berg, 1987; Boyd *et al.*, 1988; McEachern *et al.*, 1989). For these studies preganglionic inputs or postganglionic connections with target tissues were surgically transected in ciliary ganglia of newly hatched chicks. Whereas loss of afferent input to the ganglion has relatively little effect on agrin gene expression, both the level of agrin mRNA and pattern of alternative splicing in ganglionic neurons are strongly influenced by postganglionic axotomy, suggesting a role for target tissues in regulating neuronal agrin gene expression.

MATERIALS AND METHODS

Surgical Procedures

The experimental paradigm was essentially the same as that used previously to examine the role of afferent input and target dependence of neurotransmitter receptor expression in chick ciliary ganglion neurons (Jacob and Berg, 1987; Boyd *et al.*, 1988; Jacob and Berg, 1988; McEachern *et al.*, 1989). Surgeries were performed on P2-4 White Leghorn chicks, as described (Jacob and Berg, 1987). Briefly, chicks were anesthetized with methoxyflurane followed by sodium pentobarbital and the ciliary ganglion was exposed. For preganglionic denervation, the single input from the Edinger-Westphal (accessory oculomotor) nucleus to the ciliary ganglion was cut with iridectomy scissors. For postganglionic axotomy, the ciliary and choroid nerves emerging from the

ganglion were transected. Denervation or axotomy was performed unilaterally. Two criteria were used to establish the success of the surgery. First, the lack of a pupillary light reflex following recovery and, second, verification at the time ganglia were harvested that the nerves had remained severed. Ipsilateral operated and contralateral unoperated ganglia were isolated either 5 days following axotomy or 10 days after denervation, a time suggested by previous studies (Jacob and Berg, 1987) as one where measurable changes in agrin gene expression might be expected and response to injury minimized. Ganglia from age-matched (P8 for axotomy and P13 for denervation) unoperated control animals were collected similarly. Ganglia harvested as a source of RNA for use in PCR experiments were dissected free of connective tissue and nerve roots removed, quick frozen in liquid nitrogen, and stored at -80°C . Tissue to be analyzed by *in situ* hybridization was fixed and sectioned at $12\text{ }\mu\text{m}$ as described (Thomas *et al.*, 1993).

In Situ Hybridization

In situ hybridization was performed as described (Thomas *et al.*, 1993), using pan-specific ^{35}S -labeled anti-sense and sense agrin cRNA probes synthesized from a linearized cDNA template, pCA1, corresponding to nucleotides 3349-3681 of the chick agrin clone CBA-1 (Ruegg *et al.*, 1992; Tsim *et al.*, 1992). Hybridizations were performed using a probe concentration of 10^7 cpm/ml. Emulsion autoradiograms were exposed for a period of 1-3 weeks at 4°C . Agrin mRNA expression in ganglionic neurons was quantified by counting silver grains over neuronal somata visualized at $600\times$ magnification and the diameter of each neuron determined using a calibrated ocular micrometer. For each experiment, silver grains over neuronal profiles hybridized to the antisense probe were counted and grain densities determined based on soma diameter. Nonspecific binding was assessed by counting silver grains over neurons in alternate sections cut from the same tissue but hybridized to the sense control probe. Subsequently, the average grain density for neurons hybridized to either the antisense or sense probe was determined for each condition. Specific hybridization was defined as the difference in grain density observed between the antisense and sense probes. Levels of nonspecific hybridization were low (approximately 20% of total) and similar across all conditions. All counts were performed blind with respect to experimental manipulation. Ganglia from several (but not all) experimental and control conditions were analyzed within a single experiment.

Polymerase Chain Reactions

Total RNA was isolated by the single-step method (Chomczynski and Sacchi, 1987) from a pool of 10-12 cil-

iary ganglia for each condition using TRI Reagent (Molecular Research Center). RNA concentrations were determined by measuring absorption at 260 nm (Sambrook *et al.*, 1989).

The relative abundance of alternatively spliced agrin mRNAs was determined in parallel experiments for each pool of RNA by PCR. cDNA equivalent to 50–100 ng of total RNA was subject to two rounds of amplification using nested primers, F4/R4 and F5/R5, that flank region B of chick agrin (Ruegg *et al.*, 1992; Tsim *et al.*, 1992; see listing of primer sequences below). Reaction conditions and specificity of the primers have been described elsewhere (Thomas *et al.*, 1993). PCR products, radiolabeled by inclusion of approximately 2×10^5 cpm of ^{32}P -labeled forward primer in the reaction mixture, were resolved by electrophoresis on an 8% polyacrylamide gel and the relative abundance of each determined by analysis on a phosphorimager (Molecular Dynamics). Experiments were accepted for analysis only if the control lane, in which water was substituted for RNA during first-strand cDNA synthesis, was negative.

To determine the absolute amounts of each alternatively spliced agrin mRNA, we first measured the molar concentration of all agrin mRNAs/ μg of total RNA in each pool by using competitive PCR and the competing template pCA2_{Bam} as described (Thomas *et al.*, 1993). Subsequently, the molar concentration of each alternatively spliced agrin transcript was calculated based on its relative abundance. Serial dilutions of pCA2_{Bam} were made in TE with 0.5 $\mu\text{g}/\mu\text{l}$ yeast tRNA as carrier. For each determination, cDNA from 50 ng of total RNA was coamplified with different amounts (0.005–10 pg) of pCA2_{Bam} for 40 cycles using the primer pair F3/R3 in a reaction volume of 50 μl . Reaction products were labeled by inclusion of approximately 2×10^5 cpm of ^{32}P -labeled primer F3. Subsequently 2 μl of each PCR reaction was subjected to a >10-fold overdigestion with *Bam*HI and the products were separated by electrophoresis on a 6% polyacrylamide gel. Product yields from the native agrin template and competing template, pCA2_{Bam}, were analyzed using a phosphorimager (Molecular Dynamics) and the amount of agrin mRNA was determined by linear estimation.

PCR Primers Used in This Study

We used: F3, 5'-TCGTGGCAAGGACTTCATCTCC; R3, 5'-TCGTACATCTGGTTGTTACATCC; F4, 5'-CGT-TGAAATCTCCACTTCCGTG; R4, 5'-CAGGTCATAC-ATCATCTGCACGA; F5, 5'-TTTGATGGTAGGACGT-ACATGGA; R5, 5'-TTTGATGCTCAGCTCAAAGTGGT.

RESULTS

Cellular Localization and Levels of Agrin mRNA in Denervated and Axotomized Ciliary Ganglia

The complex pattern of agrin mRNA expression apparent in the chick ciliary ganglion during development

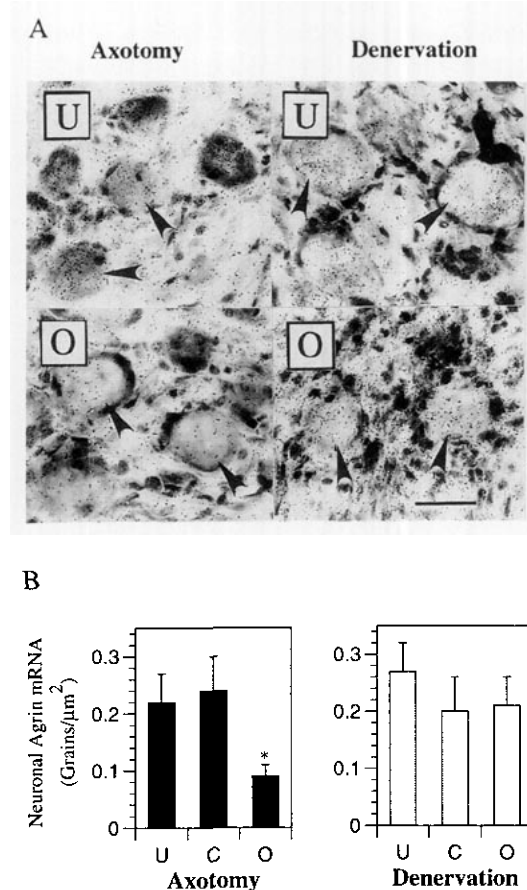


FIG. 1. Localization and quantitation of agrin mRNA in ganglionic neurons following axotomy and denervation. Ciliary ganglia were harvested from age-matched unoperated control chicks (U) and the unoperated contralateral (C) and operated (O) sides of chicks following axotomy or denervation as described under Materials and Methods. (A) Bright-field photomicrograph of emulsion autoradiograms from unoperated control, axotomized, and denervated ciliary ganglia processed for *in situ* hybridization with an antisense agrin cRNA probe and stained with toluidine blue. Silver grains are located over neuronal somata (arrowheads) and nonneuronal cells throughout the sections. Scale bar, 30 μm . (B) Silver grains over neuronal profiles were counted as described under Materials and Methods and the mean grain densities/neuron plotted for each condition. Neurons in axotomized ganglia have significantly fewer silver grains than those in age-matched unoperated controls ($P < 0.05$, Student's *t* test). No effect on silver grain density over neurons was observed following denervation. Bar charts summarize results of 12–59 neurons from 5–8 ganglia/condition. Bars show SEM $n = 3$ –4 experiments depending on condition.

suggests that cell-cell interactions may play a role in regulating agrin gene expression in neurons (Thomas *et al.*, 1993). As a step toward testing this hypothesis we used a pan-specific agrin probe and *in situ* hybridization to examine the levels of agrin mRNA expressed in ganglionic neurons following denervation or axotomy. As illustrated in Fig. 1A, silver grains are seen throughout sections from axotomized, denervated, and unoperated age-matched (P8 and P13) control ganglia, indicating that agrin mRNA is present in both neurons and non-

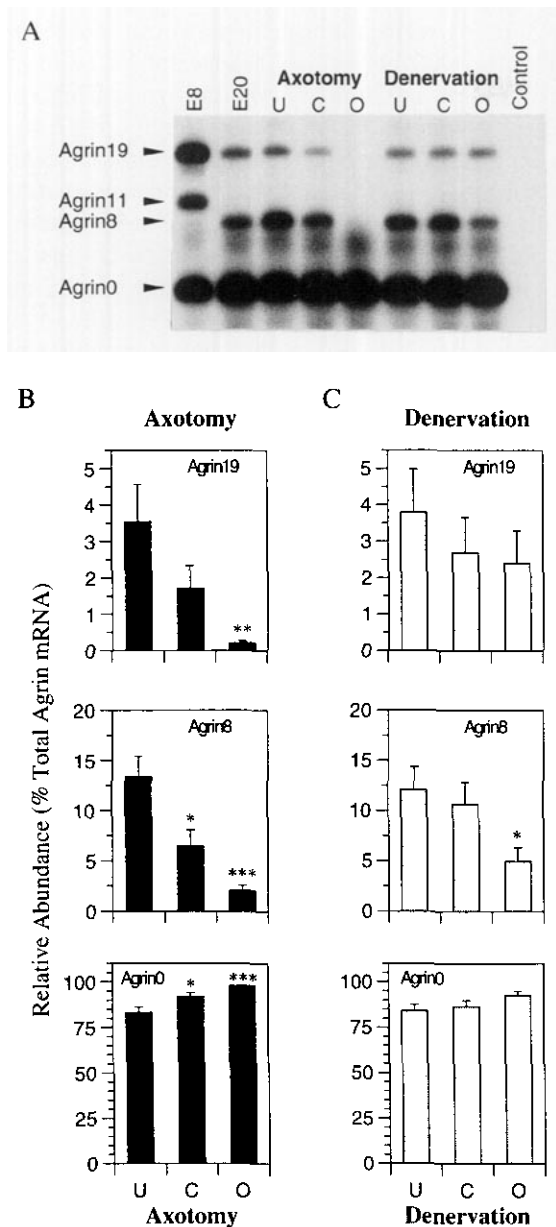


FIG. 2. The relative abundance of alternatively spliced agrin mRNAs in ciliary ganglion RNA is dramatically altered following axotomy. Ciliary ganglion RNA isolated from age-matched unoperated control ganglia (U), unoperated ganglia contralateral to the operated side (C), or operated ganglia (O) either 5 days after axotomy or 10 days following denervation was amplified by RT-PCR using nested primers flanking the region of alternative splicing that gives rise to four agrin proteins (agrin0, -8, -11, and -19) that differ in their AChR aggregating activity. (A) Autoradiogram of a typical experiment in which the PCR products obtained from E8 and E20 ciliary ganglion RNA have been included for comparison and to provide size markers identifying different agrin transcripts. Note the marked decline in relative abundance of the neuron-specific agrin mRNAs, agrin8 and -19, in RNA isolated from axotomized ganglia. Some decline in the relative abundance of agrin8 mRNA is also apparent following denervation. No PCR products were present in the control lane in which RNA was omitted from the first-strand cDNA synthesis. Based on its restriction map and its inconsistent appearance across different experiments the band below agrin8 is artifactual. (B) In comparison to their age-matched unoperated controls, significant declines in the relative abun-

neuronal cells in each condition. This pattern of cellular localization is similar to that seen in embryonic ciliary ganglia (Thomas *et al.*, 1993). To determine whether either of the surgical manipulations resulted in changes in agrin mRNA levels in neurons, we performed silver grain counts on sections counterstained with toluidine blue in which neurons were distinguished from nonneuronal cells based on size and morphology. Only neurons with no evidence of damage from tissue processing were accepted for analysis. Neurons located at the edges of ganglia were also excluded. Whereas no effect was apparent on the levels of hybridization 10 days following denervation, neurons 5 days after axotomy showed a significant twofold decrease in silver grain density compared to those in either age-matched control or contralateral ganglia (Fig. 1B). The silver grain density over the tissue between neurons was similar in all conditions, suggesting that the experimental procedures had no effect on the level of agrin mRNA in nonneuronal cells; however, we did not quantitate this further.

Previous studies have identified two populations of neurons in the ganglion, ciliary and choroid, that innervate different structures in the eye and can be distinguished by soma diameter and location (Marwitt *et al.*, 1971; Landmesser and Pilar, 1972; Pilar *et al.*, 1980). We have shown that embryonic ciliary and choroid neurons can also be distinguished by their agrin mRNA expression profile (Smith and O'Dowd, 1994), raising the possibility that axotomy might affect ciliary and choroid neurons differentially. The homogeneous distribution of grains within a single ganglion under all conditions and the finding that there was no apparent correlation between silver grain density and neuron cell body diameter suggested that the axotomy induced decrease in agrin mRNA expression is similar in both ciliary and choroid neurons (data not shown).

Alternative Splicing of Agrin RNA Is Altered Following Axotomy and Denervation

The *in situ* hybridization experiments using a pan-specific agrin probe indicated that axotomy induces a significant decrease in agrin mRNA expression in neurons. To determine whether the levels of specific agrin

dance of agrin8 and agrin19 mRNAs were accompanied by an increase in agrin0 mRNA in RNA from axotomized ganglia. Similar but smaller changes in alternative splicing of agrin8 and agrin0 mRNA were also apparent in RNA from ganglia contralateral to the axotomized side. (C) In contrast to axotomy, denervation has a much smaller effect on alternative splicing of agrin mRNA. Only in the case of agrin8 mRNA in RNA from denervated ganglia was a decline observed when compared to the age-matched unoperated controls. Bars show SEM, $n = 6$ experiments. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, Student's *t* test comparison with age-matched control.

transcripts might be differentially regulated under these conditions, we examined the relative abundance of each agrin mRNA isoform (agrin0, -8, -11, and -19) using RT-PCR with nested primers flanking the region of alternative splicing. An autoradiogram from a typical experiment is shown in Fig. 2A. The agrin mRNA profile of age-matched unoperated control ganglia is similar to that of late stage (E20) embryonic ganglia; agrin0 is the most abundant transcript, agrin8 and -19 are expressed at lower but significant levels, and agrin11 is below detection. However, striking differences were apparent in the agrin mRNA profile obtained from axotomized ganglia; both agrin8 and agrin19 mRNAs were reduced to barely detectable levels. A smaller decline in agrin8, and -19 was also apparent in the contralateral ganglion following axotomy compared to age-matched unoperated controls. With the exception of a small decrease in the relative abundance of agrin8 mRNA, denervation appears to have relatively little effect on alternative splicing.

To quantify these changes, we used a phosphorimager to determine the relative abundance of each agrin transcript, defined as the fraction of total agrin mRNA represented by each alternatively spliced agrin mRNA. The cumulative data for six experiments similar to that shown in Fig. 2A are presented in Fig. 2B. When compared with RNA isolated from unoperated age-matched control ganglia, the relative abundance of agrin19 and agrin8 mRNA in axotomized ganglia declined 16 ($P \leq 0.01$, Student's *t* test)- and 6-fold ($P \leq 0.001$), respectively. Declines in neuron-specific mRNAs were concomitant with a 1.2-fold increase ($P \leq 0.001$) in the relative abundance of agrin0. A similar trend, but of smaller magnitude, was also evident on the side contralateral to the axotomy where the relative abundance of agrin19 and -8 declined approximately 2-fold and agrin0 increased 1.1-fold compared to the age-matched control. Statistical analysis indicated that changes in the relative abundance of agrin8 and agrin0 were significant at the $P \leq 0.05$ level. However, despite this decline the relative abundance of the neuron-specific agrin mRNAs, agrin8 and -19, was still greater ($P \leq 0.05$) in RNA isolated from contralateral unoperated ganglia than in RNA from the axotomized side. In contrast, the only statistically significant effect of denervation was a 2-fold decline ($P \leq 0.05$) in the relative abundance of agrin8 mRNA in RNA isolated from operated ganglia.

Absolute Levels of Agrin mRNAs Are Altered by Axotomy

To determine the effects of axotomy or denervation on the absolute amounts of alternatively spliced agrin mRNAs, we employed a competitive PCR strategy to measure the concentration of total agrin mRNA. From

these data we were able to calculate the molar amount of each alternatively spliced transcript based on its relative abundance (Thomas *et al.*, 1993). As illustrated in Fig. 3A, the concentration of total agrin mRNA isolated from axotomized ganglia was significantly decreased by approximately 2-fold relative to the unoperated age-matched controls. Axotomy induced declines in total agrin mRNA appear to be due in part to 11- and 28-fold decreases in molar amounts of agrin8 and -19 mRNA, respectively (Fig. 3A). No significant change in the absolute amount of agrin0 mRNA was detected. Changes were also detected in the molar amounts of agrin mRNAs present in RNA isolated from unoperated ganglia contralateral to the axotomized side (approximately 4-fold declines in agrin8 and -19). The molar concentrations of agrin8 and -19 mRNA in RNA from contralateral ganglia were 3- to 7-fold greater ($P \leq 0.001$) than in RNA from axotomized ganglia.

Consistent with the results of the *in situ* hybridization study, no marked changes in the concentration of total agrin mRNA or molar amounts of alternatively spliced transcripts was observed following denervation (Fig. 3B). However, due to the relatively large SEM associated with our estimate of the total agrin mRNA concentration in RNA isolated from unoperated age-matched control ganglia, it is possible that small changes caused by denervation could have been obscured.

DISCUSSION

We have examined two possible sources of cellular interaction, afferent input and contact with target tissues, that might play a role in regulating agrin gene expression in the ciliary ganglion. The decrease in total agrin mRNA in ciliary ganglion neurons measured by *in situ* hybridization, together with changes in the relative abundance of the alternatively spliced agrin mRNAs and decline in the molar concentration of the neuron-specific mRNAs encoding agrin8 and -19, suggest that neuronal agrin gene expression is influenced by contact with target tissues. The importance of target tissues as a regulator of agrin gene expression is further supported by the observation that agrin transcript levels in the ganglion increase dramatically at the time neurons first make contact and begin to form synaptic connections with their targets in the eye (Thomas *et al.*, 1993).

Levels of nicotinic AChR (Jacob and Berg, 1987), mRNA encoding the $\alpha 3$ -subunit of the neuronal nicotinic receptor (Boyd *et al.*, 1988), α -bungarotoxin binding sites (Jacob and Berg, 1987), and GABA sensitivity (McEachern *et al.*, 1989) decline following denervation. In contrast, with the exception of a small decrease in the relative abundance of agrin8 mRNA, denervation had little effect on agrin gene expression in ciliary ganglion neurons. Thus, while denervation perturbs proteins that

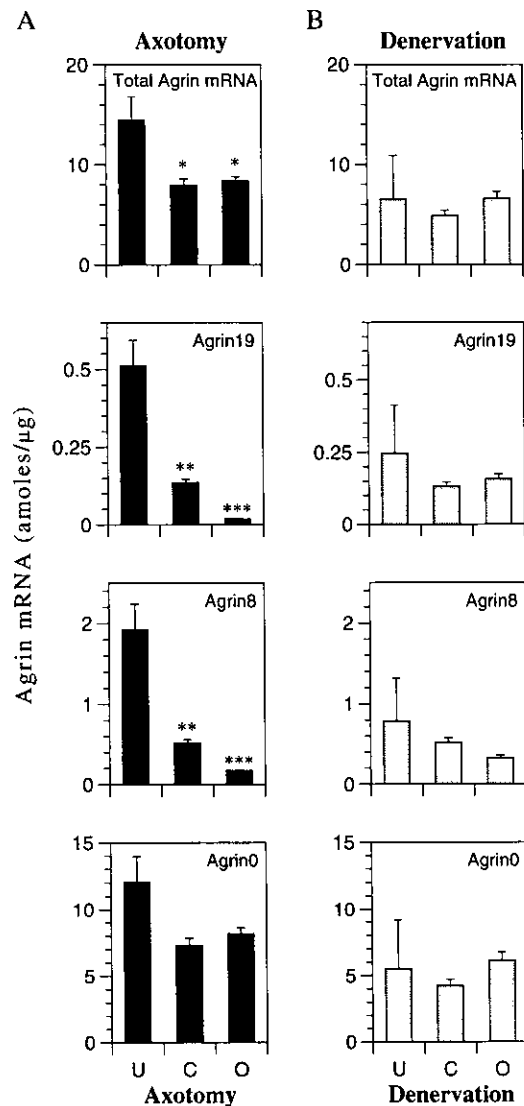


FIG. 3. The concentration of total agrin mRNA and molar amounts of neuron-specific agrin transcripts are altered by axotomy. Pools of RNA from age-matched unoperated control ganglia (U), unoperated ganglia contralateral to the operated side (C), or operated ganglia (O) following axotomy or denervation and previously analyzed for the relative abundance of alternatively spliced agrin mRNAs were subject to competitive PCR to determine the molar concentration of total agrin mRNA (i.e., agrin0, +8, +11, +19 mRNA) in each. The molar amount of each alternatively spliced transcript was calculated based on the relative abundance of each agrin mRNA (Fig. 2) and concentration of total agrin mRNA under each condition. (A) Compared to the age-matched controls, agrin mRNA concentration is significantly decreased in both axotomized ganglia and unoperated contralateral ganglia following axotomy. Underlying the decrease in the concentration of total agrin mRNA are significant declines in molar amounts of neuron-specific mRNAs encoding agrin19 and agrin8. (B) In contrast, no significant change was observed in the concentration of total agrin mRNA or any of the alternatively spliced transcripts following denervation. Bars show SEM, $n = 3-5$ determinations for each condition. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, Student's t test comparison with age-matched control.

are likely to play a role at synapses onto ganglionic neurons, it seems to be of less importance in regulating expression of agrin, a protein that seems likely to exert its effects at synapses made between ciliary ganglion neurons and their peripheral targets. Thus, signals regulating agrin gene expression may be distinct from those modulating other neuronal proteins.

Previous studies have shown that by 5 days following axotomy about 40% of the neurons in the ganglion die (Jacob and Berg, 1987), raising the possibility that axotomy-induced decreases in agrin expression are part of a global decline in mRNA levels or other nonspecific response to injury. Several lines of evidence however, suggest that this is unlikely. First, in late stage embryonic ganglia single-cell RT-PCR analysis has shown that greater than 90% of neurons express agrin8 and/or -19 and that these neuron-specific mRNAs make up the bulk of all agrin mRNA in these cells (Smith and O'Dowd, 1994). Although we cannot rule out the possibility, the widespread expression of neuron-specific agrin mRNAs within ganglionic neurons makes it unlikely that the decline in agrin8 and -19 reflects loss of a specific neuronal population that might express disproportionate amounts of these transcripts. Second, assuming that all neurons express similar amounts of agrin8 and -19, the >90% decrease in these specific agrin mRNAs is substantially larger than that predicted by neuronal death alone. Third, our PCR-based estimates of agrin mRNA expression were not determined on a per ganglion basis but rather on a fixed amount of RNA. Last, both light and electron microscopic studies suggest that surviving neurons are normal (Jacob and Berg, 1987, 1988). As a precaution against introducing any bias in our data from unhealthy or damaged neurons, grain density measurements of agrin expression levels were made only over neuronal profiles with clearly defined intact somata. Additionally, other studies have shown that expression of β -actin mRNA (Boyd *et al.*, 1988) and β -tubulin mRNA (M. S. Levey and M. H. Jacob, personal communication) are unaffected by the surgical procedure.

Whereas gliosis is a well-characterized response of astrocytes and microglia to injury in the CNS, we know of no evidence that significant levels of nonneuronal cell (Schwann and satellite cell) proliferation or migration occur in the ciliary ganglion following axotomy. To the contrary, previous electron microscopic studies show that both neurons and nonneuronal cells in axotomized ganglia are normal (Jacob and Berg, 1988). Moreover, barring the unlikely possibility that axotomy-induced changes in neurons are exactly offset by those in nonneuronal cells, measurements of ganglionic protein and levels of β -actin and β -tubulin mRNA, which would be sensitive to changes in nonneuronal cells, are also unaffected by the surgical procedure. Similarly, given that

nonneuronal cells in the ganglion are both more abundant than neurons and express only agrin0 mRNA (Smith and O'Dowd, 1994) proliferation of glial cells would be expected to result in an increase in agrin0 mRNA. However, the molar amounts of agrin0 actually declined following axotomy, arguing against a significant level of gliosis. Finally, the *in situ* hybridization studies quantify changes in agrin mRNA specifically in neurons and are independent of a contribution by non-neuronal cells. Taken together, these results indicate that it is unlikely that axotomy-induced changes in agrin gene expression could be accounted for by nonspecific effects including neuronal cell death or gliosis.

Ganglionic axotomy has previously been reported to result in a bilateral decrease in GABA sensitivity in chick ciliary ganglion neurons (McEachern *et al.*, 1989). We also noted changes in the relative abundance and molar concentration of neuronal agrin mRNAs in unoperated ganglia contralateral to the axotomy. Together these findings suggest that in addition to direct effects of axotomy, an additional mechanism modulating gene expression in the contralateral ganglion exists. One possibility is that the anesthetic itself caused changes in agrin gene expression, although we found no evidence of such an effect in ganglia contralateral to the denervated side. A second possibility is that axotomy results in the production of a factor or factors, perhaps from the denervated target tissues in the eye, that act systemically. In this regard it would be interesting to learn whether agrin expression in other nervous tissues is similarly affected. Finally, injury to a motor nerve has been shown to specifically induce sprouting and synapse formation by the intact nerve innervating the homologous muscle on the opposite side of the body (reviewed in (Rotshenker, 1988). Thus agrin, and perhaps other genes, may be modulated by a transneuronal pathway. Regardless of the mechanism, however, it is clear that both the level of expression and alternative splicing of agrin mRNA can be regulated even in neurons whose synaptic contacts with the periphery are intact. However, the fact that alternative splicing and molar concentration of neuron-specific agrin mRNAs in axotomized ganglia were significantly below those of the unoperated contralateral ganglia suggests that agrin gene expression in operated ganglia is subject to an additional influence above that affecting the unoperated contralateral controls.

The results of this study suggest that the maintenance of normal levels of agrin expression by ciliary ganglion neurons depends on contact with the target tissues in the eye. Growth and maturation of ciliary ganglion neurons is known to be influenced by soluble factors present in target tissues, including ciliary neurotrophic factor (Barbin *et al.*, 1984; Halvorsen and Berg, 1989), growth-promoting activity (Nishi and Berg, 1981; Leung *et al.*,

1992), choline acetyltransferase-stimulating activity (Nishi and Berg, 1981), and activin (Coulombe *et al.*, 1993). Other studies have shown that contact with muscle membrane remnants (Tuttle, 1983) may also play a role in supporting survival and differentiation of ciliary ganglion neurons. Cell culture experiments are currently underway to determine whether these or other target-dependent cellular interactions regulate agrin gene expression in ganglionic neurons.

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REFERENCES

- Barbin, G., Manthorpe, M., and Varon, S. (1984). Purification of the chick eye ciliary neuronotrophic factor. *J. Neurochem.* **43**, 1468-1478.
- Bowe, M. A., and Fallon, J. F. (1995). The role of agrin in synapse formation. *Annu. Rev. Neurosci.* **18**, 443-462.
- Boyd, R. T., Jacob, M. H., Couturier, S., Ballivet, M., and Berg, D. K. (1988). Expression and regulation of neuronal acetylcholine receptor mRNA in chick ciliary ganglia. *Neuron* **1**, 495-502.
- Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159.
- Connor, E. A., and Smith, M. A. (1994). Retrograde signaling in the formation and maintenance of the neuromuscular junction. *J. Neurobiol.* **25**, 722-739.
- Coulombe, J. N., Schwall, R., Parent, A. S., Eckenstein, F. P., and Nishi, R. (1993). Induction of somatostatin immunoreactivity in cultured ciliary ganglion neurons by activin in choroid cell-conditioned medium. *Neuron* **10**, 899-906.
- 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.
- Hall, Z. W., and Sanes, J. R. (1993). Synaptic structure and development: The neuromuscular junction. *Neuron* **10**(Suppl.), 99-121.
- Halvorsen, S. W., and Berg, D. K. (1989). Specific down-regulation of the α -bungarotoxin binding component on chick autonomic neurons by ciliary neuronotrophic factor. *J. Neurosci.* **9**, 3673-3680.
- Hoch, W., Ferns, M., Campinelli, 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., and Berg, D. K. (1987). Effects of preganglionic denervation and postganglionic axotomy on acetylcholine receptors in the chick ciliary ganglion. *J. Cell Biol.* **105**, 1847-1854.
- Jacob, M. H., and Berg, D. K. (1988). The distribution of acetylcholine receptors in chick ciliary ganglion neurons following disruption of ganglionic connections. *J. Neurosci.* **8**, 3838-3849.
- Landmesser, L., and Pilar, G. (1972). The onset and development of transmission in the chick ciliary ganglion. *J. Physiol.* **222**, 691-713.
- Leung, D. W., Parent, A. S., Cachianes, G., Esch, F., Coulombe, J. N., Nikolics, K., Eckenstein, F. P., and Nishi, R. (1992). Cloning, expression during development, and evidence for release of a trophic factor for ciliary ganglion neurons. *Neuron* **8**, 1045-1053.
- Ma, E., Morgan, R., and Godfrey, E. W. (1994). Distribution of agrin mRNAs in the chick embryo nervous system. *J. Neurosci.* **14**, 2943-2952.

- Marwitt, R., Pilar, G., and Weakley, J. N. (1971). Characterization of two cell populations in the avian ciliary ganglion. *Brain Res.* **25**, 317-334.
- McEachern, A. E., Jacob, M. H., and Berg, D. K. (1989). Differential effects of nerve transection on the ACh and GABA receptors in chick ciliary ganglion neurons. *J. Neurosci.* **9**, 3899-3907.
- McMahan, U. J. (1990). The agrin hypothesis. *Cold Spring Harbor Symp. Quant. Biol.* **55**, 407-418.
- McMahan, U. J., Horton, S. E., Werle, M. J., Honig, L. S., Kröger, S., Ruegg, M. A., and Escher, G. (1992). Agrin isoforms and their role in synaptogenesis. *Curr. Opin. Cell Biol.* **4**, 869-874.
- Nishi, R., and Berg, D. K. (1981). Two components from eye tissue that differentially stimulate the growth and development of ciliary ganglion neurons in cell culture. *J. Neurosci.* **1**, 505-513.
- 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.
- Rotshenker, S. (1988). Multiple modes and sites for the induction of axonal growth. *Trends Neurosci.* **11**, 363-366.
- Ruegg, M. A., Tsim, K. W. K., Horton, S. E., Kröger, S., 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.
- Sambrook, J. M., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Smith, M. A., and O'Dowd, D. K. (1994). Cell-specific regulation of agrin RNA splicing in the chick ciliary ganglion. *Neuron* **12**, 795-804.
- 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.
- Tuttle, J. B. (1983). Interaction with membrane remnants of target myotubes maintains neurotransmitter sensitivity of cultured neurons. *Science* **220**, 977-979.