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Activin A and Follistatin Expression in Developing Targets of Ciliary Ganglion Neurons Suggests a Role in Regulating Neurotransmitter Phenotype

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Summary

The avian ciliary ganglion contains choroid neurons that innervate choroid vasculature and express somatostatin as well as ciliary neurons that innervate iris/ciliary body but do not express somatostatin. We have previously shown in culture that activin A induces somatostatin immunoreactivity in both neuron populations. We now show in vivo that both targets contain activin A; however, choroid expressed higher levels of activin A mRNA. In contrast, follistatin, an activin A inhibitor, was higher in iris/ciliary body. Iris cell-conditioned medium also contained an activity that inhibited activin A and could be depleted with anti-follistatin antibodies. These results suggest that development of somatostatin is limited to choroid neurons by differential expression of activin A and follistatin in ciliary ganglion targets.

Introduction

The influence of target tissues on the neurotransmitter phenotype of developing neurons has been well established (reviewed in Landis, 1990; Dryer, 1994; Nishi, 1994). For example, in the developing sympathetic nervous system, noradrenergic sympathetic neurons switch to a cholinergic phenotype upon contact with the sweat gland target cells in the rat footpad (Leblanc and Landis, 1986; Landis, 1994). This transition is regulated by a diffusible target-derived factor (Schotzinger and Landis, 1988; Stevens and Landis, 1990; Habecker et al., 1995). Neural plasticity of a similar type regulated by exogenous molecules has been demonstrated in vitro. Sympathetic neurons have been shown to undergo an adrenergic to cholinergic switch when cocultured with cardiac myocyte–conditioned medium (Furshpan et al., 1976; Weber, 1981; Potter et al., 1986). This effect has been shown to be due to the presence of leukemia inhibitory factor (Fukada, 1985; Yamamori et al., 1989) in the conditioned medium. Addition of ciliary neurotrophic factor (CNTF) to cultures of sympathetic neurons also induces an adrenergic to cholinergic switch (Saadat et al., 1989; Ernsberger et al., 1989). However, transgenic mice deficient for leukemia inhibitory factor were found to have normal sweat gland innervation and function (Rao et al., 1993). In addition, vasoactive intestinal peptide immunoreactivity and choline acetyltransferase (ChAT) activity in the footpad sweat glands of transgenic mice deficient for CNTF were unchanged compared with wild type (Masu et al., 1993). These results suggest that these factors are not essential to the regulation of neurotransmitter phenotype in vivo. Although evidence demonstrates that target-derived factors are important to the development of neurotransmitter phenotype, there is limited understanding of the specific molecules that regulate the processes in the developing embryo (reviewed in Patterson and Nawa, 1993).

We have used the avian ciliary ganglion to investigate the effect of target-derived molecules on the regulation of neurotransmitter phenotype. In the ciliary ganglion, two distinct neuronal populations innervate separate targets in the eye (Marwitt et al., 1971). The ciliary neurons innervate the striated muscle of the iris and ciliary body to regulate pupilary dilation and lens accommodation. The choroid neurons innervate the smooth muscle surrounding the arterial vasculature of the choroid layer and aid in oxygenation of the eye. These neuronal populations have the same neural crest derivation (Narayanan and Narayanan, 1978), receive preganglionic input from the accessory oculomotor nucleus (Narayanan and Narayanan, 1976), and synthesize acetylcholine as the primary neurotransmitter (Burt and Narayanan, 1976; Chiappinelli et al., 1976; Coulombe and Bronner-Fraser, 1990). However, only the choroid neurons express the neuromodulatory peptide somatostatin (Epstein et al., 1988; De Stefano et al., 1993), which has been shown to regulate acetylcholine release (Guillemin, 1976; Gray et al., 1990).

Neurons isolated from the ciliary ganglion and maintained in culture have been used to characterize the molecules that regulate somatostatin expression. Previous studies showed that choroid muscle cells will induce somatostatin expression in ciliary ganglion neurons in vitro. The ability of ciliary neurons to express somatostatin in culture was shown by retrograde labeling of the neuron cell bodies before placing them in culture (Coulombe and Nishi, 1991). The choroid cell–derived, somatostatin-stimulating activity was shown to be due to activin A (Coulombe et al., 1993).

Activin A has been implicated as a regulator in many different physiological and developmental processes (for review, see Ying, 1989). Activin A is a homodimer of the inhibin βA chain (Mason et al., 1985) and was originally described as having a stimulatory effect on the release of follicle-stimulating hormone from the pituitary gland. The action of activin A in vivo is negatively regulated by follistatin (Ling et al., 1985; Nakamura et al., 1990), an activin A–binding protein (Kogawa et al., 1991; Sumitomo et al., 1995). During early embryonic development in Xenopus, the action of the more widely distributed activin A is regulated by localized expression of follistatin (Hemmati-Brivanlou and Melton, 1992, 1994a, 1994b). Exogenous activin A has been shown in vitro to promote survival of some neurons (Schubert et al., 1990) and to increase mRNA levels for several neuropeptides in sympathetic neurons (Fann and Patterson, 1994).

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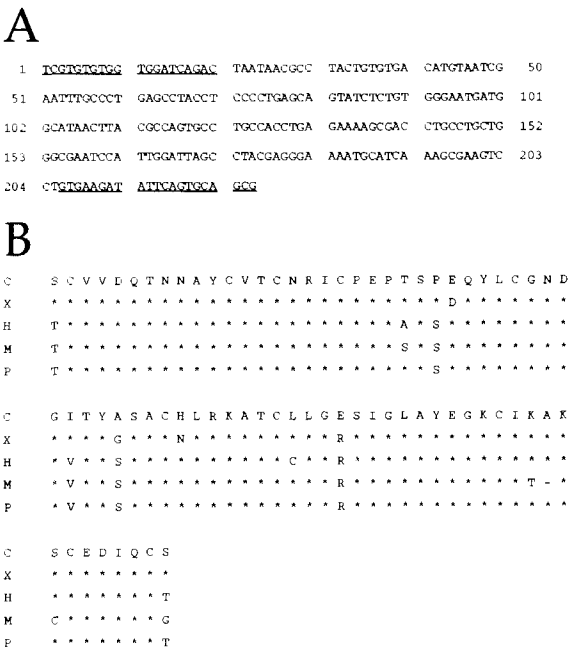

Figure 1. Chick Follistatin Fragment Sequence and Amino Acid Comparison with other Species

(A) The 223 bp fragment of chick follistatin was cloned by RT–PCR and sequenced. Primer regions were underlined.

(B) A comparison of the amino acid translation of the chick fragment (C) against human (H), pig (P), Xenopus (X), and mouse (M) follistatin showed relative conservation at the amino acid level. The accession number for chick follistatin is U34589.

We have examined whether the differential expression of activin A and follistatin in targets innervated by ciliary ganglion neurons results in the differential induction of somatostatin expression. Our results suggest that sufficient activin A is available at the choroid layer to induce somatostatin in choroid neurons, but the presence of excess follistatin in the iris prohibits an induction of somatostatin in the ciliary neurons.

Results

Chicken-Specific Probes for Follistatin and Activin A

To investigate the expression of activin A and follistatin mRNA, chicken-specific probes were obtained. Cloning of follistatin from several species has shown that there is one gene product (Shimasaki et al., 1988a, 1988b; Albano et al., 1994) that encodes several protein isoforms arising from alternatively spliced mRNA and posttranslational modifications (reviewed in Sugino et al., 1993). A cDNA fragment of follistatin was amplified from embryonic chick ovary RNA using reverse transcriptase–polymerase chain reaction (RT–PCR). The primers were designed based on a conserved region of cloned follistatin sequences from other species (Hemmati-Brivanlou and Melton, 1994b; Shimasaki et al., 1988a, 1988b). The PCR product was ligated into the pCRII vector and sequenced (Figure 1A). Riboprobes generated from this construct were designed to recognize all mRNA splice variants described. Comparison of the deduced amino acid sequence encoded by this fragment with follistatin proteins from other species revealed a high degree of conservation (Figure 1B). A full-length cDNA clone of chick activin A (inhibin βA) was obtained from Dr. Patricia Johnson (Chen and Johnson, 1994, Biol. Reproduct., abstract). The region used for activin A probe synthesis was not homologous to the inhibin α transcript or the inhibin βB transcript.

Multiprobe RNase Protection Assay

To compare both activin A and follistatin mRNA transcript levels in the same sample, a multiprobe RNase protection assay was used (Qian et al., 1993). To determine the sensitivity of this assay, the linear range of detection was determined for each of the probes. Activin A and follistatin mRNA levels were measured in the same sample. mRNA levels of a constitutively expressed gene, chick ribosomal binding protein S17 (CHRPS; Trüeb et al., 1988), were measured as an internal loading control. Total RNA from embryonic day 17 (E17) chick ovary was used to establish multiprobe assay conditions, as both of these proteins are known to be present within this tissue (Mason et al., 1985; Vale et al., 1986). Probe sizes were chosen to prevent overlap in the protected fragments. To demonstrate the sensitivity of this assay, protected bands for activin A, follistatin, and CHRPS were detected in RNA from embryonic ovary (Figure 2, left). Unprotected probe (Figure 2, right) showed the predicted size prior to processing. No protected fragments were detected when yeast tRNA was used in place of tissue RNA. Chick genomic DNA was also negative (data not shown). Relative intensity measurements of the signal from the protected fragments were obtained from a scanned phosphorimage. The signal increased with increasing amounts of total RNA, and the signals were linear for all three probes tested (Figure 2B). In subsequent experiments, we chose conditions from tissues that would yield signals within the linear range of sensitivity of this assay.

Activin A and Follistatin mRNA in the Developing Iris and Choroid

If activin A and follistatin regulate the expression of somatostatin in ciliary ganglion neurons in vivo, mRNA for these factors should be detectable in the targets of the ciliary ganglion during development. E9–E16 encompass a critical period of development during which both the iris/ciliary body (Pilar et al., 1980, 1987) and choroid (Meriney and Pilar, 1987) tissues mature as they become innervated. In particular, somatostatin expression increases during this period until all the choroid neurons express the neuropeptide at E14 (Smet and Rush, 1993). RNase protection analyses of RNA from E9–E16 iris and E9–E16 choroid were done in parallel to compare RNA levels for activin A and follistatin (Figure 3A). Analysis of ciliary body mRNA was not performed because isolation of uncontaminated ciliary body separate from neural retina and lens epithelium is difficult to obtain. Follistatin and activin A transcripts in the iris increased from E9 to E16 (Figure

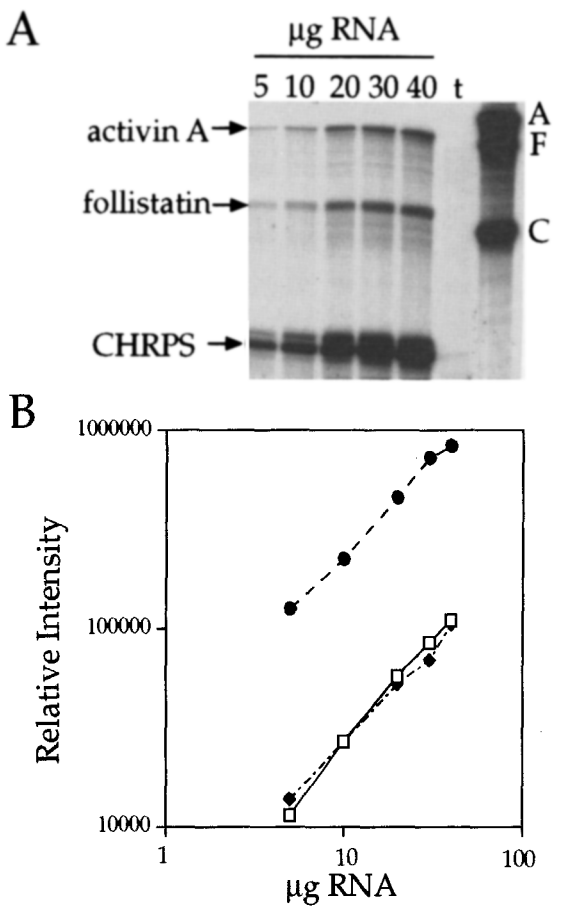

Figure 2. Multiprobe RNase Protection Assay Standard Curve

(A) An autoradiograph of 5–40 μg of E17 chick ovary total RNA hybridized with activin A, follistatin, and CHRPS probes. All three protected fragments (arrows) were visible in each sample. Unprotected probe (A, F, and C) ran higher than the protected fragments. The tRNA negative control (t; 20 μg of RNA) had no protected fragments.

(B) A logarithmic plot of the scanned phosphorimage showed a linear increase in signal intensity relative to increases in RNA concentration ($r = 0.999$ for activin A; $r = 0.992$ for follistatin; $r = 0.994$ for CHRPS). This is a representative experiment from four repetitions that show similar results. Circles, CHRPS; squares, activin A; diamonds, follistatin.

3B). In contrast, follistatin mRNA levels in choroid remained low relative to the iris through all ages tested. Activin A mRNA was high from E9 to E14 but dropped at E16 (Figure 3C). These results indicate a striking difference in mRNA expression for activin A and follistatin between the two target tissues of the ciliary ganglion neurons. The observations that follistatin mRNA levels in the choroid were lower than those in the iris, while the level of activin A was higher in the choroid than in the iris, support the hypothesis that differential expression of these factors in the two targets leads to a differential expression of somatostatin in the neurons that innervate them.

Immunolocalization of Activin A and Follistatin to Target Tissues of Ciliary Ganglion Neurons

To demonstrate that expression of activin A and follistatin protein correlates with their respective mRNA expression

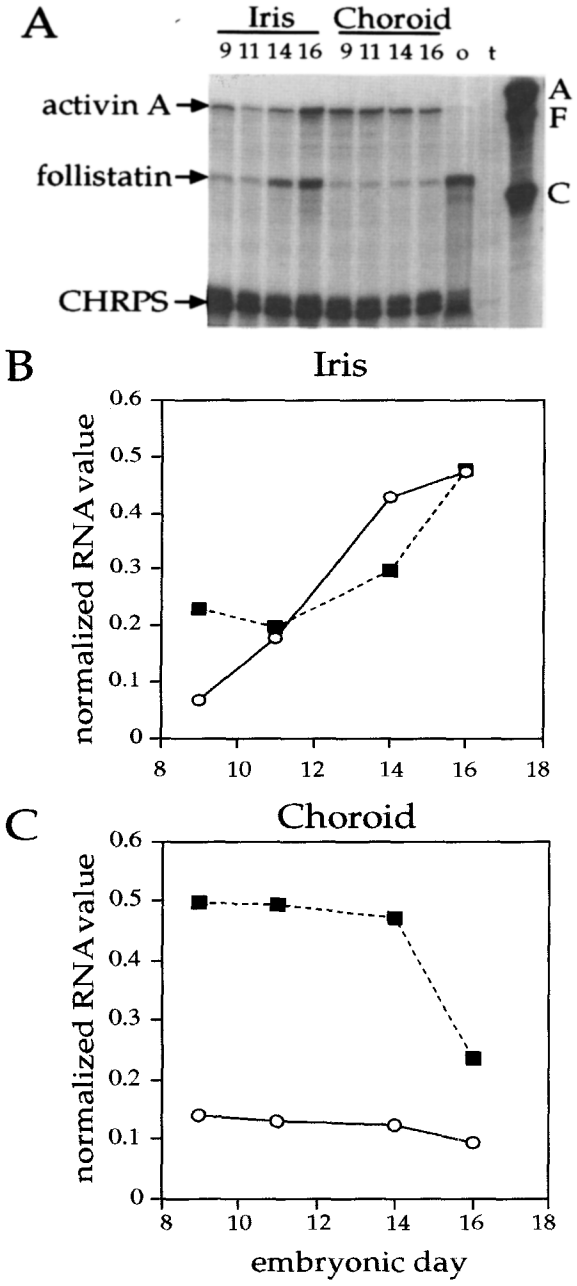

Figure 3. Follistatin and Activin A in Iris and Choroid Tissue during Development

(A) An autoradiograph of 20 μg each of E9–E16 iris and E9–E16 choroid total RNA after protection with activin A, follistatin, and CHRPS probes showed all three protected fragments (arrows) were visible in each sample. Unprotected probe (A, F, and C) ran slightly higher than the protected fragments. Adult ovary was run as a positive control (o; 20 μg of total RNA). The tRNA negative control (t; 20 μg) had no protected fragments.

(B and C) Normalized RNA values for activin A and follistatin were determined as a ratio of relative signal intensity for each probe over the relative intensity for CHRPS, within the same sample. Follistatin and activin A mRNA in the iris increased from E9 to E16 (B). Follistatin in the choroid remained static, while activin A was high from E9 to E14 and dropped at E16 (C). This is a representative experiment from four repetitions showing similar results. Squares, activin A; circles, follistatin.

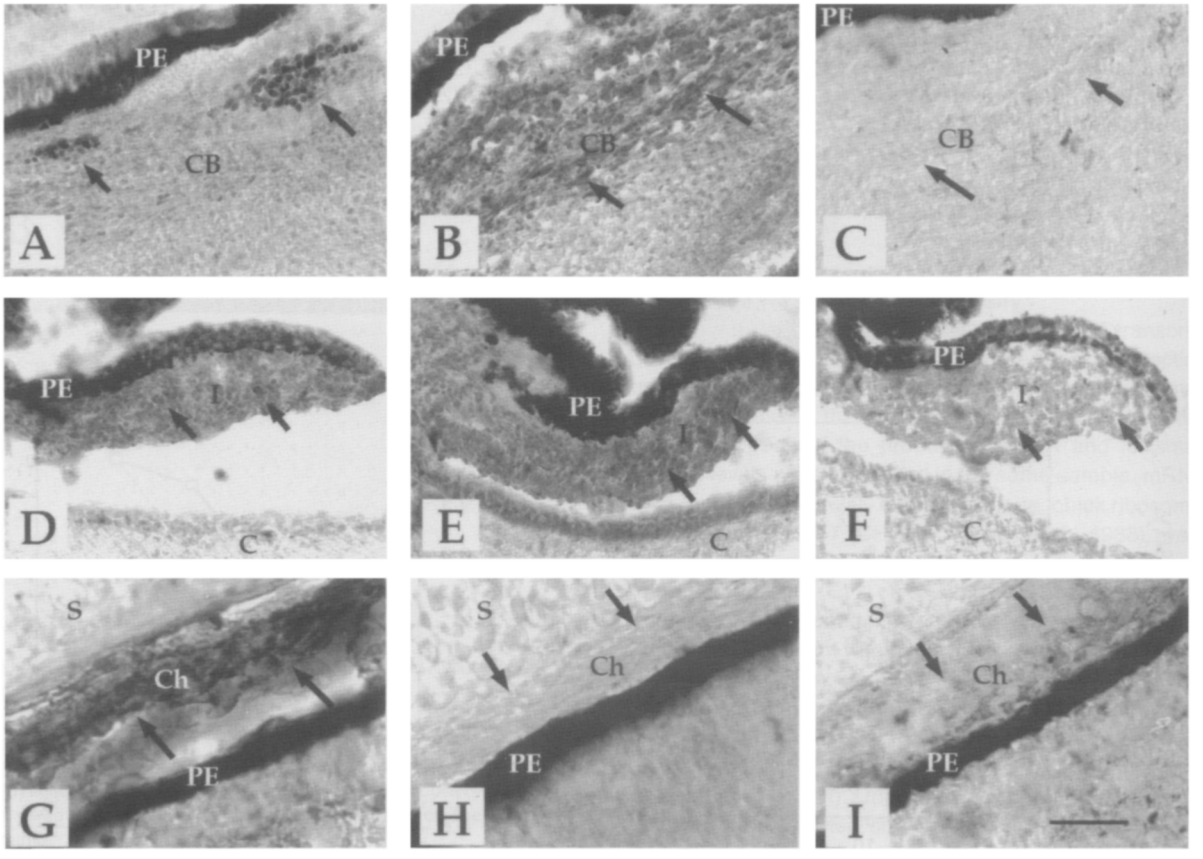

Figure 4. Activin A and Follistatin Immunolocalization in Ciliary Ganglion Neuron Targets In Vivo

Cryostat sections of E11 ciliary body (A–C) and iris (D–F) show double peroxidase anti-peroxidase staining for activin A (A and D, arrows) and follistatin (B and E, arrows). Sections of E12 choroid reveal strong staining for activin (G, arrows) but not for follistatin (H). Normal rabbit serum staining was included as a control. Arrows indicate comparable target areas (C, F, and I). Pigment epithelium staining could not be assessed owing to high pigment granule content in these cells. C, cornea; CB, ciliary body; Ch, choroid layer; I, iris stroma; PE, pigmented epithelium; S, sclera. Bar, 700 μm (A–F), 450 μm (G–I).

patterns, antisera produced against activin A (Vaughan et al., 1989; Kokan-Moore et al., 1991) and follistatin (Sugawara et al., 1990; Petraglia et al., 1994) were used to immunolocalize these proteins to targets of ciliary ganglion neurons. Activin A immunoreactivity was first detected at E11 in the developing ciliary body (Figure 4A) and iris (Figure 4D). In the choroid layer, activin A immunoreactivity could be seen at E12 (Figure 4G), the earliest age at which reliable histology could be obtained. Follistatin immunoreactivity was also observed at E11 in the musculature of the ciliary body (Figure 4B) and the iris stroma (Figure 4E); however, at E12 in the choroid layer, follistatin immunoreactivity was not detectable (Figure 4H). Specificity of the activin A and follistatin immunostaining was assessed with normal rabbit serum (Figures 4C, 4F, and 4I). In addition, preabsorption of anti-activin A with the peptide used to generate the antiserum displayed no immunoreactivity above background on adjacent sections (data not shown).

Iris Cell–Conditioned Medium Inhibits Somatostatin Induction in Ciliary Ganglion Neurons

The biological role of activin A and follistatin expression in the choroid and iris was investigated using cells derived from these target tissues in a bioassay that measured their effects on somatostatin induction in ciliary ganglion neurons. To confirm that cultured choroid and iris cells expressed activin A and follistatin, levels of mRNA were measured using RNase protection. Follistatin mRNA levels were similar in both iris and choroid cells in culture (Figure 5). Activin A was approximately 4-fold higher in the choroid cells relative to the iris cells. These relative mRNA levels for activin A and follistatin were similar to the ratios seen at E11 in the targets in vivo (see Figure 4). Synthesis of activin A and follistatin protein was assessed using immunocytochemistry (Figure 6). Activin A immunoreactivity was found as punctate perinuclear staining in multiple cell types of iris cultures (Figure 6A). Similar punctate staining was found in choroid smooth muscle cells (Figure 6D). Follistatin immunoreactivity was most dramatic in iris culture myotubes (Figure 6B) but was weak in choroid smooth muscle cells (Figure 6E). As a control, choroid and iris cultures were stained after replacing the primary antiserum with normal rabbit serum (Figures 6C and 6F).

To determine the ability of iris cell–conditioned medium (ICM) to inhibit the induction of somatostatin, we used the same bioassay that was previously used to identify activin A as an inducer of somatostatin expression in ciliary gan-

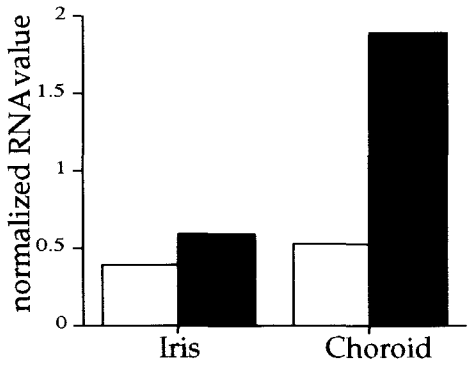

Figure 5. Follistatin and Activin A in Iris and Choroid Cells in 6-Day-Old Cultures

Total RNA (10 μg) from cells in culture was hybridized to activin A, follistatin, and CHRPS probes. The histogram of the normalized RNA values from this protection are shown for activin A (closed bars) and follistatin (open bars). RNA values were obtained using the ratio of the relative intensity of probe signal to the relative intensity of the loading control, CHRPS. The follistatin RNA value was similar in the iris and choroid cells in culture. In contrast, the activin A value was approximately 4-fold higher in the choroid cells than in the iris cells. One representative experiment out of four repetitions with similar results is shown.

glion neurons (Coulombe et al., 1993). Serum-free conditioned medium from iris-derived cultures (ICM) that contained myotubes was concentrated and added to E8 ciliary ganglion neurons cultured in the presence or absence of 10 ng/ml recombinant activin A. This dose of activin A was previously demonstrated to be within the linear range for somatostatin induction (Coulombe et al., 1993). ICM significantly reduced ($p < .05$) the percentage of neurons that express somatostatin immunoreactivity when compared with neurons grown in control medium (Figure 7A). To investigate whether this inhibition of somatostatin immunoreactivity was mediated by the secretion of follistatin by iris cells, ICM was immunodepleted with an anti-follistatin antiserum (Sugawara et al., 1990). Immunodepletion significantly restored somatostatin induction in the presence of activin A ($p < .05$ as compared with ICM treatment). Immunodepleted ICM in the absence of activin A did not stimulate somatostatin induction, suggesting that activin A may be bound to follistatin and removed with follistatin immunodepletion. Quantitation of activin A and follistatin protein levels in ICM was not analyzed. To control for deleterious effects of ICM on ciliary ganglion neurons, neuronal survival and ChAT activity were assayed. Neither survival nor ChAT activity was reduced in the presence of ICM (Figures 7B and 7C). The bioassay results, together with the different expression patterns of activin A and follistatin, support our hypothesis that activin A signaling in the iris is selectively inhibited by follistatin protein and regulates somatostatin phenotype in ciliary ganglion neurons.

Discussion

In the current study, we have investigated the in vivo distribution of activin A and follistatin and the in vitro potential of ICM to modulate somatostatin induction. Previous work from our laboratory has shown that activin A from choroid cell–conditioned medium induces somatostatin expression in ciliary ganglion neurons (Coulombe et al., 1993). Using an RNase protection assay, we have now demonstrated that activin A mRNA is present in both target tissues of the ciliary ganglion neurons but is higher in the choroid than in the iris. In contrast, follistatin mRNA is higher in the iris. In agreement with these results, immunostaining for activin A and follistatin indicates that activin

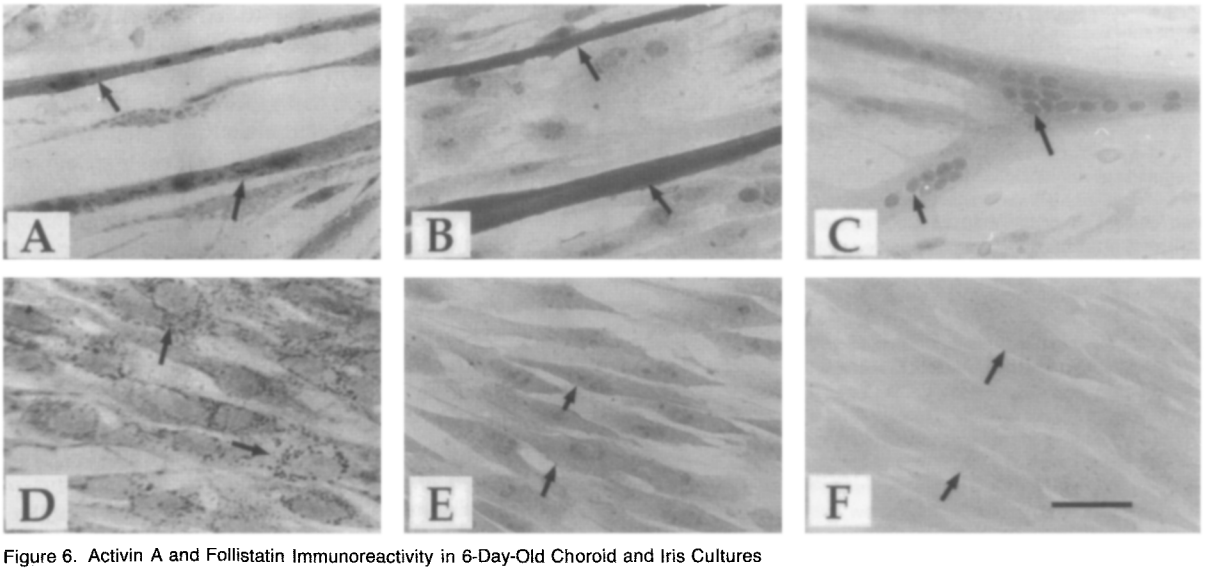

Figure 6. Activin A and Follistatin Immunoreactivity in 6-Day-Old Choroid and Iris Cultures

Iris cultures (A–C) show punctate, perinuclear staining for activin A in multiple cell types, including multinucleated myotubes (A, arrows). Choroid (D–F) cultures reveal similar punctate staining patterns (D, arrows). Follistatin immunoreactivy (B and E) is most dramatic in iris-derived myotubes (B, arrows). Normal rabbit serum staining was included as a control (C and F). Nuclei in myotubes showed nonspecific staining as assessed with the normal rabbit serum (C, arrows), while choroid cells were negative (F, arrows). Bar, 75 μm.

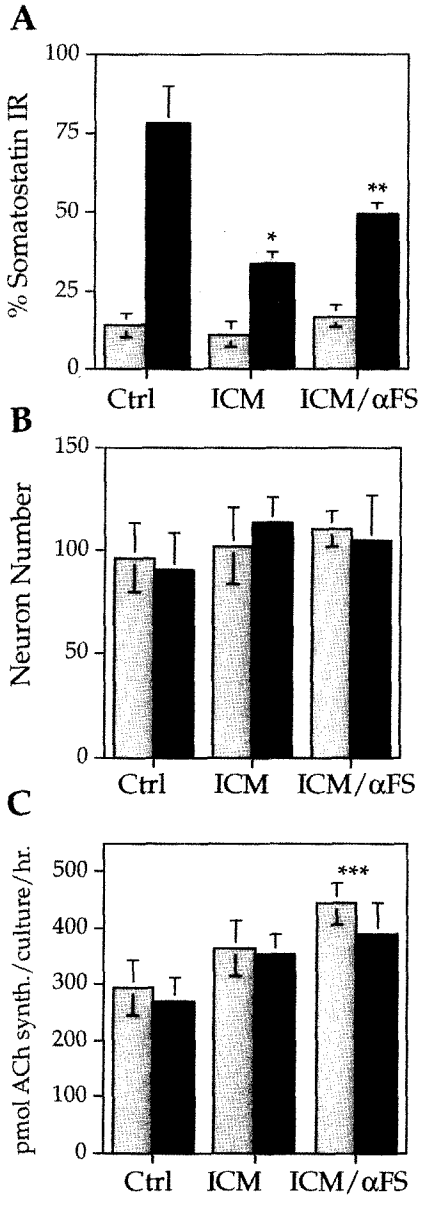

Figure 7. Somatostatin Induction, Neuron Survival, and ChAT Activity in Cultured Ciliary Ganglion Neurons

Percentage of somatostatin immunoreactivity (A), neuron survival (B), and ChAT activity (C) were measured in ciliary ganglion neurons cultured for 4 days in control medium (Ctrl), iris cell–conditioned medium (ICM), or ICM that had been immunodepleted with an antibody specific for follistatin (ICM/αFS). Ciliary ganglion neuron cultures from each experimental condition were maintained with 10 ng/ml activin A (closed bars) or without activin A (gray bars). The values indicated for each column are the mean of three different cultures from one representative experiment. Error bars represent SD. Single asterisk, significantly differs from control with activin A; double asterisk, significantly differs from ICM with activin A; triple asterisk, ICM/αFS with and without activin ChAT activity significantly differs from control with or without activin A. Significance at $p < .05$ by one-way ANOVA.

A is present in both choroid and iris, while follistatin is detectable only in the iris during the period of somatostatin induction. Moreover, the immunohistochemical results have localized both proteins to the iris/ciliary body and the choroid layer, regions that contain the target cells for ciliary and choroid neurons. As previously noted, iris- and choroid-derived cells in culture produce factors that can influence transmitter phenotype. Choroid cell–conditioned medium induces somatostatin and contains activin A, which alone can induce somatostatin (Coulombe et al., 1993). We have now shown that ICM inhibits somatostatin induction and that this inhibition is significantly relieved by immunodepletion of ICM with anti-follistatin antibodies. A model that accounts for these findings is that activin A produced in the choroid layer induces somatostatin in the choroid neurons, but activin A signaling in the iris and ciliary body is blocked by local production of follistatin.

Several additional observations led to other areas of inquiry. The increasing expression of activin A and follistatin in the iris correlates with increasing neuronal innervation in this target tissue. Although these studies do not address whether innervation may regulate activin A and follistatin expression, such regulation of target-derived factors has been demonstrated in sympathetic innervation of rodent sweat glands. Production of sweat gland factor activity, which induces the noradrenergic to cholinergic switch, is dependent on proper innervation (Habecker and Landis, 1994; Habecker et al., 1995). While cultures of choroid and iris cells produce activin A and follistatin independently of innervation in vitro (see Figures 5 and 6), the sources of these cultures are innervated at the time of removal. In addition, there is a decrease in activin A mRNA levels seen consistently at E16 in the choroid (see Figure 3). The dependence of ciliary ganglion neurons on activin A for maintenance of somatostatin expression has been shown up to E14 in vitro (Coulombe and Nishi, 1991). Potentially, this decrease at E16 reflects a loss of dependence on activin A once the adult neurotransmitter phenotype has been established. Finally, ChAT activity consistently increased in ciliary ganglion cultures treated with ICM or ICM that had been depleted with anti-follistatin antibodies (Figure 7C), indicating that follistatin is not regulating ChAT activity. Although a target-derived ChAT induction in ciliary ganglion neurons has been demonstrated previously (Nishi and Berg, 1979; Tuttle et al., 1983), factors that affect ChAT activity remain to be purified.

The identification of follistatin transcripts and immunoreactivity in the developing iris is complemented by the appropriate biological activity in ICM observed in the somatostatin induction assay. The significant but partial relief of inhibition of somatostatin induction by follistatin-immunodepleted ICM (see Figure 7A) demonstrates the presence of follistatin. The lack of complete relief of inhibition could be explained by several alternatives. One plausible explanation is that the antibodies used for the immunodepletion were generated against porcine follistatin and thus may not be able to recognize all the possible isoforms of chicken follistatin. Another possibility is that other inhibitors of somatostatin induction may be present in the ICM. Interestingly, we do not see induction of somatostatin in immunodepleted ICM in the absence of exogenous activin A. This may indicate that secreted activin A is associated

with follistatin and is removed upon immunodepletion of ICM with follistatin-specific antibodies. This possibility is consistent with our model of iris-derived follistatin as an inhibitor of somatostatin induction in ciliary neurons.

Activin A and follistatin may not be the only regulators of somatostatin expression. Activin A signals through a receptor serine/threonine kinase complex expressed in several isoforms (reveiwed in Vale et al., 1991; Mathews, 1994). Regulation may occur through expression of different levels or isoforms of either the type I or type II receptors on the choroid and ciliary neurons. Posttranslational processing has also been shown to regulate activity of several members of the transforming growth factor β superfamily (reviewed in Kingsley, 1994), and such processing may occur for activin A in this system by regulating its availability in an active form at the synapse. The inhibins, which share a common subunit with the activins, have been shown to block activin A function in some systems (Yu et al., 1987; Ying, 1989). However, inhibin A was unable to affect somatostatin expression in our in vitro assay, suggesting that it does not function in the establishment of neurotransmitter phenotype in the ciliary ganglion (Coulombe et al., 1993). Our activin A reagents are specific for inhibin βA but do not distinguish among family members that contain the common βA subunit. Thus, our results do not rule out a role for other βA-containing proteins. Interestingly, the partial inhibition seen with follistatin-immunodepleted ICM (Figure 7A) suggests that other factors, in addition to follistatin, may act to inhibit somatostatin expression. Finally, other molecules may be required to make cells competent to respond to activin signaling. Such a requirement has been demonstrated in the fibroblast growth factor–dependent, activin-mediated induction of mesoderm in early Xenopus development (Cornell and Kimelman, 1994; LaBonne and Whitman, 1994).

Although these possibilities are intriguing, we do not think they detract from the model we propose based on the results of this study: that limited expression of somatostatin in the neurons of the ciliary ganglion results from a differential expression of activin A and its inhibitory binding protein, follistatin, in the distinct targets of these neurons. Our results show a unique role for the antagonistic functions of endogenous activin A and follistatin in the regulation of neurotransmitter phenotype during development. This study bridges the gap between an in vitro demonstration of neurotransmitter phenotype regulation and the appropriate temporal and spatial in vivo expression of the molecules.

Experimental Procedures

Tissue Collection and Cell Culture

Iris Cell Cultures

E11 chick irises were isolated in Earle's balanced salt solution (GIBCO–BRL, Grand Island, NY) by removing the overlying cornea and cutting inside the iris/ciliary body boundary. Iris stroma was separated from the pigmented epithelium and mechanically dissociated in modified Puck's glucose solution by trituration through a reduced bore Pasteur pipette. Following dissociation, cells were filtered through 130 mm^2 polyamide nylon mesh (Tetko, Inc., Briarcliff Manor, NY) and plated at 5×10^4 cells/ml. Iris cultures were grown for 48 hr on polymerized, collagen-coated 24-well tissue culture plates (Linbro; Flow Laboratories, Inc., McLean, VA) in 500 μl of L15 plus 10% horse serum. Myotubes from the iris stromal cultures were allowed to differentiate by replacing serum-containing medium with serum-free medium. Serum-free L15 was supplemented with 2.5 mg/ml bovine serum albumin, 25 mg/ml ovotransferrin, 30 nM selenium, and 2.5 mg/ml insulin (Sigma Chemical Co., St. Louis, MO). Both serum-containing and serum-free L15 included 6 mg/ml glucose, 20 U/ml penicillin, 2 mM glutamine, and 2 mg/ml streptomycin (GIBCO–BRL).

Choroid Cultures

Choroid cells were prepared as previously described (Coulombe and Nishi, 1991). Cells were plated on tissue culture dishes coated with rat tail collagen and grown in modified L15 medium supplemented with 10% chick serum and penicillin/streptomycin/glucose, as described above, for 2 days. Cells were transferred to serum-free conditions supplemented as described for iris cultures.

Constructs

A fragment of chick ribosomal protein S17 (CHRPS) of 361 bp was obtained by RT–PCR (Beverly, 1991) using the primer pair based on the published sequence (Trüeb et al., 1988): forward, 49–67, and reverse, 389–409, with cycling conditions of 93°C (melting), 54°C (annealing), and 72°C (extension), for 1 min each (30 cycles) with 1 U of *Taq* DNA polymerase per reaction (Promega, Madison, WI). The fragment was subcloned into pBluescript SK+/– (Stratagene, La Jolla, CA). A fragment of chick follistatin was obtained by RT–PCR from E17 ovary using Xenopus primers from a region conserved between Xenopus and human follistatin sequences (Hemmati-Brivanlou and Melton, 1994b). The primers recognized an equivalent region in the rat follistatin gene that crossed the exon 4/5 boundary (Shimasaki et al., 1989). In chick, there does not appear to be an exon boundary in the region covered by the primers, as no shift in size of the 223 bp product was observed when the primers were used to amplify genomic DNA. The 223 bp fragment was generated with the primer pair from the published sequence: forward, 639–658, and reverse, 842–862, with the same amplification conditions used for CHRPS. The chick follistatin was cloned into the pCRII vector (Invitrogen, San Diego, CA). The sequence of the inserts was confirmed by Sequenase sequencing analysis. A full-length chick inhibin βA cDNA in pBluescript was a generous gift from Dr. Patricia Johnson, Cornell University, Ithaca, NY.

Template and Probe Production

Templates for riboprobe production were generated by linearizing constructs in the multiple cloning site or within the insert to generate blunt ends or 5′ overhangs. Linearized templates were treated with 50 μg/ml proteinase K, 1% SDS for 30 min at 37°C. The solution was extracted with an equal volume of phenol:chloroform:isoamyl alcohol in a ratio of 25:24:1. The aqueous phase was precipitated with 0.1 vol of 3M sodium acetate (pH 7.0) and 2.5 vol of 100% ethanol and kept at −20°C for at least 1 hr. Pellets were spun and washed one time with 70% ethanol. The DNA was resuspended in H_20 for storage at −20°C. Riboprobes were generated in the following reaction mix: 12.5 μM cold rCTP (250 μM cold rCTP for CHRPS), 1 × transcription buffer, 10 mM dithiothreitol, 10 U of RNasin, 500 μM rUTP, 500 μM rATP, 500 μM rGTP, 750 ng of template, 25 μCi of [^{32}P]rCTP (3000 Ci/mmol), and 10 U of bacterial RNA polymerase. The free nucleotides and the RNasin were purchased from Promega, and the remaining reagents were from GIBCO–BRL. The mix was incubated for 1 hr at 37°C, and the remaining template was removed by digestion with 10 U of RNase-free DNase I. Proteinase K treatment and extraction were as described for template production. The probes were precipitated with 1 μl of 10 mg/ml glycogen plus sodium acetate and ethanol, as before. Probes were routinely generated with activity greater than 10^7 dpm prior to purification. Full-length probes were purified essentially as described (Gilman, 1991), with the following modification: the gel band was crushed with a tube pestle and extracted in 500 μl of 0.3 M NaCl, 0.5% SDS, 10 mM Tris (pH 7.5) for 45 min on a rocker at 37°C. Purified probes were suspended in 40 μl of hybridization buffer (40 mM PIPES [pH 6.7], 0.4 M NaCl, 1 mM EDTA) and combined in the hybridization mix at 10^5 dpm each for activin A and follistatin and 10^4 dpm for CHRPS.

RNase Protection Assay

Total RNA was obtained from embryonic tissue or cells in culture using acid phenol extraction (Chomczynski and Sacchi, 1987). Purified pellets were resuspended in hybridization buffer. Total RNA and probes were mixed and hybridized 15–18 hr at 42°C. Protection and processing were done as described (Gilman, 1991). Protected fragments for activin A, follistatin, and CHRPS were 410, 223, and 122 bp, respectively. The unprotected probes ran 20–70 bases higher than the protected fragments. Fragments were run on 6% acrylamide/8 M urea/0.5× TBE gels. Dried gels were exposed to autoradiographic film (X-omat; Imaging Products International, Simi Valley, CA) and phosphorimager screens (Molecular Dynamics, Sunnyvale, CA).

Analysis of mRNA Levels

The phosphorscreen image was scanned using Scanner Control SI (Molecular Dynamics) at 200μ resolution. Relative signal intensity from the scanned phosphorimage was determined using the IPLab Gel software (Signal Analytics, Vienna, VA) to generate the values for protected fragments. Relative intensity for activin A and follistatin mRNA was expressed as a ratio to the relative intensity of CHRPS mRNA to obtain the RNA value. Normalization to the CHRPS loading control allowed for comparison between samples and eliminated variation in sample handling.

Preparation of ICM

Iris cultures were differentiated in serum-free L15 for 3 days to allow large multinucleated myotubes to form. Cultures were then fed with fresh serum-free L15, and ICM was collected 48 hr later. ICM was centrifuged at 1000 × g at 4°C and concentrated 6-fold in a 10 kDa-cutoff Centriprep filter unit (Amicon, Beverly, MA). Concentrated ICM was sterile filtered through a 0.22 μm filter and stored at −80°C prior to use in the somatostatin induction assay.

Bioassay for Somatostatin Induction

Neuronal Culture

Somatostatin expression was assayed by immunoreactivity in cultured E8 ciliary ganglion neurons as previously described (Coulombe and Nishi, 1991). In brief, ciliary ganglia were dissociated and plated at a density of one ganglion per well into drilled 35 mm^2 tissue culture dishes. Cut (1.5 cm^2) Petriperm biomembrane (Heraeus Instruments, Inc., South Plainfield, NJ) had been glued under the 1 cm hole, and the resulting well was coated with poly-D-lysine (100 mg) and then laminin (2 mg) prior to plating. Neurons were grown in the presence of 1 ng/ml recombinant growth-promoting activity (Finn and Nishi, submitted) to promote survival (Eckenstein et al., 1990). Growth-promoting activity has no somatostatin induction capacity in this assay (Coulombe et al., 1993). Dose responses (0–50 ng/ml) of recombinant human activin A (Genentech, San Francisco, CA) were tested in the presence of 2× concentrated serum-free L15 or 2× concentrated ICM for 5 days in a volume of 200 μl. Medium was changed every 36 hr.

Immunocytochemistry

After the 5 day culture in the described medium, neurons were processed for double peroxidase-antiperoxidase immunoreactivity (Vacca et al., 1980) using a rabbit anti-somatostatin antiserum (INC Star Corp., Stillwater, MN) as described (Coulombe and Nishi, 1991) with some modifications. Cultures were incubated with goat anti-rabbit antiserum (Sternberger Immunochemicals, Baltimore, MD) at a dilution of 1:125 in blocking solution, and Activity Select peroxidase-rabbit antiperoxidase complex (Sternberger Immunochemicals) was used at 1:200 in blocking solution. Blocking solution consisted of 10% horse serum, 5% chick serum, 2% lamb serum, 1% Triton X-100 in PBS. Normal rabbit serum staining was performed as a control for nonspecific immunoreactivity.

Immunodepletion

Immunodepletion was accomplished by repeatedly passing 10 ml of ICM through a 1 ml Hi-trap protein G column (Biorad, Hercules, CA) to which 50 μl of anti-follistatin antiserum (Sugawara et al., 1990) had been bound. Antibody binding to the column was monitored by the absence of immunoreactivity on cultured iris myotubes with the flowthrough.

Immunoreactivity Scoring

The number of neurons containing somatostatin immunoreactivity (SOM IR) was viewed under a Zeiss microscope with a 40× objective using bright-field optics. A neuron was defined as an ovoid cell with an axonal process at least two cell diameters long. SOM IR was scored positive if the cytoplasm contained punctate dark brown diaminobenzidine reaction product. Ten fields of view were scored for SOM IR–positive neurons and total neurons for each well. Three wells for each condition tested were included in each experiment. Percentage of SOM IR was determined by summing the SOM IR–positive neurons per well and dividing by the total neurons per well. Mean percentage of SOM IR and SD were determined using percentage of SOM IR from each well from one representative experiment. A one-way ANOVA followed by Scheffe's multiple range test was used to assess the statistical significance of the results.

Determination of Neuronal Survival and ChAT Activity

Neuronal survival per culture was determined by adding the number of neurons in ten fields of view at 400×. Reported values were the average of three cultures from one representative experiment. A one-way ANOVA followed by Scheffe's multiple-range test was used to assess the statistical significance of the results. ChAT activity was determined as described (Coulombe and Nishi, 1991).

Activin A and Follistatin Immunolocalization

Antibodies

Rabbit anti-follistatin antiserum (Sugawara et al., 1990) was a generous gift from Dr. Shunichi Shimasaki, The Whittier Institute, La Jolla, CA. Rabbit anti-inhibin βA antiserum and activin A peptide$^{81-113}$ (Vaughan et al., 1989) were generous gifts from Dr. Wylie Vale, The Salk Institute, La Jolla, CA.

Cultured Cell Immunohistochemistry

Iris or choroid cell cultures were established on collagen-coated glass coverslips and maintained for 6 days. On day 6, cultures were fixed in fresh Zamboni's fixative for 20 min and washed in PBS. Cultures were then incubated at 4°C overnight in blocking solution. Polyclonal rabbit anti-inhibin βA or anti-follistatin antiserum diluted 1:500 in blocking solution was then incubated for 2 hr at 25°C. Antibody localization was detected using single peroxidase-antiperoxidase staining. Specificity was controlled for by comparing staining in the absence of a primary antibody or incubating with normal rabbit serum instead of primary antibody. Anti-inhibin βA specificity was further tested by preabsorbing 15 μl of the antiserum with 100 μg of the activin peptide$^{81-113}$ used to generate the antiserum.

Cryostat Section Immunohistochemistry

Whole eyes from E11 and E12 chick embryos were removed and fixed in fresh Zamboni's fixative for 30 min at 25°C. Histologically reliable cryostat sections of choroid younger than E12 could not be obtained. The eyes were then washed three times with 30 min incubations in PBS prior to equilibration in OCT embedding medium (TissueTek, Elkhart, IN). Sections (12 μm) were cut on a Leica Jung Frigocut 2800N cryostat. Sections were mounted on gelatin-coated glass slides and processed for double peroxidase anti-peroxidase staining. Specificity controls were performed as for culture staining.

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