Activin A and follistatin control expression of somatostatin in developing ciliary ganglion neurons

by

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CERTIFICATE OF APPROVAL

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This dissertation is dedicated to my parents,
Barbara Ann Earwicker and Harley James Earwicker,
and to my husband, Tristan Darland.

I could not have accomplished this goal without their
continuous support and encouragement.
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True science teaches, above all, to doubt and be ignorant.

Miguel de Unamuno, *The Tragic Sense of Life*, 1913

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Abstract

The influence of target tissues on the differentiation of innervating neurons has been established as an integral component of neuron development. One important outcome of the neuron/target interactions is establishment of neurotransmitter phenotype. This can be defined as the expression of a primary small molecule transmitter in addition to any modulatory neuropeptides. The current study addresses the issue of target-dependent control of neuropeptide expression in avian ciliary ganglion neurons in vivo. The ciliary and choroid neurons within this ganglion use acetylcholine as their primary transmitter, but only the choroid neurons express somatostatin. Because the neuron populations innervate distinct ocular targets, one contributing factor for this difference in peptide expression may be different influences from the separate target tissues. Previously published data indicates that neither population of neurons expresses somatostatin in vitro unless cultured with target cells derived from the choroid layer or conditioned medium from these cells. These target cells have been shown to produce a somatostatin-stimulating activity, activin A. The current study provides evidence that activin A is differentially expressed in the two targets, with high levels in the vascular smooth muscle innervated by the choroid neurons and relatively low levels in the striated muscle of the iris/ciliary body innervated by the ciliary neurons. This expression is observed during the window of development when somatostatin expression is induced in the choroid neurons. Interestingly, a potent inhibitor of activin A, follistatin, is also differentially expressed in the targets, but in an opposite pattern to that of activin A. Conditioned medium from iris cells in culture can inhibit the somatostatin-inducing effect of activin A. This inhibition is likely due to follistatin produced by the iris cultures, because pretreatment of the conditioned medium with follistatin specific antibodies results in a relief of inhibition. Choroid cells placed in culture differentiate into smooth muscle and express activin A and low levels of follistatin concomitant with cellular differentiation. Treatment of the choroid cells with the pleiotrophic cytokine, TGFβ, increases differentiation with a corresponding increase in activin A levels. Inhibition of differentiation with either the proliferation-inducing cytokine, FGF2, or a blocking antibody specific for TGFβ results in reduced smooth muscle characteristics and inhibition of activin A expression. Taken together, these data lead to the hypothesis that activin A from the developing choroid layer induces somatostatin in the choroid neurons, but that follistatin from the iris/ciliary body inhibits activin A and prevents somatostatin expression in the ciliary neurons. This hypothesis was tested directly in vivo by manipulating somatostatin levels with

application of exogenous activin A and follistatin to developing embryos. Intraocular injections of activin A increased somatostatin positive neurons 39% over controls. In contrast, injection of follistatin reduced somatostatin positive neurons by 23%. An additional method of delivery, application of follistatin to the chorioallantoic membrane, reduced somatostatin positive neurons by over 50% relative to controls. These data support a role for activin A in the induction of somatostatin in choroid neurons in normal development. Furthermore they indicate that selective expression of the activin A inhibitor, follistatin, is a key component in control of peptide expression in ciliary ganglion neurons via the mechanism of localized inhibition of activin A.

Introduction

I. Historical Perspective

It has become increasingly apparent over the last several decades that the development and differentiation of the peripheral nervous system is to a great degree influenced by the target tissues that the neurons innervate. Even early in the study of neuroembryology, the importance of the target on neuron development was appreciated. Based upon his extensive observations of neuroanatomical structures S. Ramon y Cajal suggested the idea of synaptic pruning at the site of contact with the target: "There is no doubt that, at first, many imperfect connections are formed...But that these incongruences are progressively corrected, up to a certain point, by two parallel methods of rectification. One of these occurs in the periphery..." (Ramon y Cajal as quoted in Hamburger, 1975). An early study that emphasized the importance of the target in neuronal development involved the transplant of forelimb primordia to more caudal regions in the developing salamander (Detwiler, 1920). Although this study was undertaken to address the behavior of axons growing into a foreign environment, Detwiler made note of the fact that the ganglia remaining at the transplant source were hypoplastic and the ganglia at the site of additional peripheral tissue were hyperplastic. This issue of target effects on neuronal development was further explored by Hamburger in his study of the effects of wing ablation on the growth and development of spinal ganglia which also showed a distinct hypoplasia in the absence of target tissue (Hamburger, 1934). In a fruitful collaboration, Levi-Montalcini and Hamburger described in detail the proliferation and degeneration of the spinal ganglia under normal growth conditions and in the absence of target influences (Hamburger and Levi-Montalcini, 1949). These studies preface much of the work on the phenomenon of naturally occurring cell death that is continuing today.

Although the studies described above emphasize the importance of the peripheral targets on the survival of innervating neurons, they do not address any additional effects on the differentiation of the neurons. Some aspects of this issue were first examined in the differentiation of the autonomic derivatives of the neural crest, specifically on the neurotransmitter repertoire expressed by the cells. The neurotransmitter expressed by a given neuron is an integral component of its signalling specificity. Expression of the synthetic and degradative enzymes involved in production and clearing of the transmitter, expression of the transmitter itself and of any additional neuromodulatory

peptides constitutes the neurotransmitter phenotype of the neurons. To begin to address how neurotransmitter phenotype might be established in developing neurons, Le Douarin and colleagues used chick/quail chimaera transplant techniques with neural crest tissue that gives rise to the majority of peripheral nervous tissue. They demonstrated that quail neural tube/crest tissue from the trunk region lost its normal adrenergic phenotype when transplanted into the vagal tube region (Le Douarin and Teillet, 1974). The vagal region normally receives primarily cholinergic innervation and the trunk neurons that developed in this novel site developed cholinergic traits. Additional studies with transplants of cholinergic sections of the Remak ganglion in chick show that the neurotransmitter characteristics of the ganglia can be reversed when the ganglion is transplanted to trunk regions and innervates targets that normally receive only adrenergic input (Douarin, 1980). It is important to note that the transition to expressing adrenergic characteristics in this system took place after the cholinergic expression had been specified prior to transplant, indicating that reversal of phenotype can occur in the presence of influencing factors from the target. These and other studies describe the importance of the target on the establishment of the neurotransmitter expressed by the neurons, but do not reveal what target-derived factors may be responsible for this component of neuronal differentiation.

The framework for many of the basic biological questions involved in neuronal development was laid down by the early transplant studies, the results of which guide current efforts to describe specific target-derived factors that control these events and the mechanisms by which they occur. The first factor that was tracked down based upon its ability to support neuronal survival was nerve growth factor (Levi, 1983). Interestingly, this factor was shown to have specific effects not only on neuronal survival but on increasing both ganglion volume and tyrosine hydroxylase expression in axotomized rat sympathetic ganglia (Levi et al., 1975). These data suggest that this factor, in particular, can have multiple effects on developing neurons. Progress in identifying specific target-derived factors that affect neuronal development, such as NGF, has been aided tremendously with the use of tissue culture systems that allow for direct assay of a variety of neuronal characteristics. The techniques that allow neuronal growth separate from their in situ environment were first described by Harrison in his study examining the formation of the nerve fiber and including an early description of the growth cone (Harrison, 1910). The methods used for culturing neurons in vitro have been refined considerably since that time, but the basic premise remains the same.

II. Target-derived factors that mediate neuronal differentiation and neurotransmitter phenotype

The focus here is on those factors which have been proposed to function in the establishment of neurotransmitter phenotype in developing neurons. Many culture studies undertaken to resolve issues of target-derived factors controlling neuronal differentiation have utilized extracts of a variety of peripheral targets to control development of neurons in culture (Patterson et al., 1976; Patterson and Chun, 1977a; Fukada, 1980; Nishi and Willard, 1988; Nawa and Sah, 1990). For instance an adrenergic to cholinergic shift in transmitter phenotype, reminiscent of the transplantation studies with the Remak ganglia, has been described for rat sympathetic neurons in culture (Mains and Patterson, 1973). When rat sympathetic neurons are grown in microculture in the presence of cardiac myocytes, neurons with adrenergic and cholinergic properties are observed as well as neurons with dual characteristics (Furshpan et al., 1976). Further investigation of this cardiac-conditioned medium effect on neurotransmitter phenotype indicated that it is most likely due to the cytokine leukemia inhibitory factor (LIF) produced by the myocytes (Fukada, 1985; Yamamori et al., 1989). Addition of another cytokine, ciliary neurotrophic factor (CNTF), to sympathetic neurons can also promote an adrenergic to cholinergic switch in phenotype (Saadat et al., 1989; Ernsberger et al., 1989). Study of the effects of these factors in neurons isolated from their normal environment has contributed significantly to our understanding of the establishment of neurotransmitter phenotype as well as the plasticity of this neuronal characteristic.

Additional information on the importance of peripheral tissue-derived factors that control neurotransmitter phenotype can be gleaned from a series of studies addressing these issues directly in the developing animal. Sympathetic neurons of the superior cervical ganglion are catecholaminergic. A significant portion express neuropeptide Y (NPY) (Elfvin et al., 1993). After postganglionic axotomy there is a drastic change in transmitter expression with a downregulation of catecholaminergic traits and a loss of NPY immunoreactivity and a corresponding upregulation of cholinergic characteristics, including vasoactive intestinal peptide (VIP), galanin and substance P immunoreactivity (reviewed in Zigmond and Sun, 1997). LIF has been proposed to mediate a large component of this post-axotomy transition. This hypothesis is supported by studies using a function blocking LIF antibody to inhibit the changes in VIP (Sun et al., 1994). In studies that address the role of target effects on neurotransmitter phenotype during

development rather than post-injury, Landis and co-workers have described a transition from noradrenergic to cholinergic in sympathetic neurons that innervate the rat footpad sweat gland (Landis, 1990; Schotzinger et al., 1994; Habecker et al., 1997). In the absence of peripheral target, when associated with an inappropriate target, or when cultured alone, these neurons do not undergo an alteration in transmitter expression. However, if cultured with sweat gland cells or their conditioned medium and in association with the appropriate target they undergo a full switch to a cholinergic cell type with few noradrenergic traits remaining (Schotzinger and Landis, 1988; Stevens and Landis, 1990; Habecker et al., 1995). Several candidate molecules have been proposed to mediate this transition, CNTF and LIF among them, however they are not the likely in vivo mediators (Rohrer, 1992; Rao et al., 1993). Efforts are underway to isolate the sweat gland factor (SGF), but its exact nature is unclear to date. The previously described studies have contributed much to our understanding of the establishment of neurotransmitter phenotype in developing neurons, however the complexities of the sympathetic nervous system and the elusive nature of the factors involved in specification remain as hurdles for further research.

III. The avian ciliary ganglion and its ocular targets

One system that lends itself well to examining the effects of target-derived factors on the development and differentiation of innervating neurons is the avian ciliary ganglion and its targets in the eye. Several characteristics of this ganglion make it ideal for these studies, not least among them its accessibility and simplicity. The parasympathetic ciliary ganglion is located immediately subjacent to the optic nerve and contains two distinct populations of neurons (Marwitt et al., 1971). The neurons have a similar embryonic derivation from the neural crest (Narayanan and Narayanan, 1978) and both populations receive cholinergic input from the accessory oculomotor nucleus, which is the avian equivalent of the Edinger-Westphal nucleus (Narayanan and Narayanan, 1976). In addition both utilize acetylcholine at synaptic contacts with the target. Their common characteristics diverge at this point. The two neuronal populations innervate distinct targets in the periphery: ciliary neurons innervate the striated muscle of the iris and ciliary body which are involved in pupilary constriction and accomodation (Pilar et al., 1987) and choroid neurons innervate the vascular smooth muscle of the choroid layer to promote constriction of the blood vessels and subsequent oxygenation of the eye (Meriney and Pilar, 1987). The neurons have disparate sizes and locations within the ganglion with the ciliary neurons being approximately 25-40 μm in diameter and

having a predominantly central location. The choroid neurons, on the other hand, are 15-25 μm in diameter and are found in the perimeter of the ganglion (Marwitt et al., 1971; Epstein et al., 1988; DeStefano et al., 1993). Preganglionic endings on the ciliary neurons form calyces, while those on the choroid neurons form bouton-like endings. An additional distinction between the two neuron types which is of concern here is that the choroid neurons express the co-transmitter, somatostatin, while the ciliary neurons have no known neuropeptides (Epstein et al., 1988; DeStefano et al., 1993). Despite the many similarities they share early in development, these two populations become quite distinct in form and function as they innervate their targets and differentiate.

IV. Establishment of neurotransmitter phenotype in the ciliary ganglion

One hypothesis to explain the difference in neurotransmitter phenotype in the ciliary ganglion neurons is that target-derived factors mediate the differential expression of somatostatin. Ciliary ganglion neurons are readily grown in culture (Nishi and Berg, 1979; Kato and Rey, 1982), but do not express somatostatin when grown alone. However, when cultured with cells derived from the choroid layer or medium conditioned by these cells, somatostatin expression is induced in the neurons (Coulombe and Nishi, 1991). These data indicate that the target cells produce a soluble, diffusible factor that controls peptide expression in ciliary ganglion neurons. Moreover, retrograde labelling of ciliary neurons followed by dissociation of the ganglion and growth in culture revealed that in the presence of choroid conditioned medium, dil labelled neurons were somatostatin positive (Coulombe and Nishi, 1991). This is an important point because it demonstrates that both populations readily respond to the Somatostatin-Stimulating Activity (SSA) in the choroid-conditioned medium. This is particularly intriguing because only the choroid neurons normally produce somatostatin in vivo.

Several pieces of evidence point to a candidate molecule for SSA produced by choroid target cells (Coulombe et al., 1993). Activity was retained in a sizing column fraction at approximately 30-40 kD and several proteins in this size range were tested for their ability to duplicate SSA. Only activin A was able to induce somatostatin expression in ciliary ganglion neurons. Activin A message was detectable in choroid cultures and protein was observed in choroid-conditioned medium with western analysis. Finally, the choroid-conditioned medium was tested in a hemoglobin induction assay that is

specific for activin. Not only did the conditioned medium function similarly to activin A tested in parallel, but it also could be inhibited with preincubation of the medium with follistatin, a potent inhibitor of activin A (Coulombe et al., 1993). Cumulatively these data point to activin A as a candidate molecule for somatostatin induction in ciliary ganglion neurons.

V. Characteristics of activin A

Activin A is a member of the activin subfamily in the larger TGFβ superfamily of pleiotrophic cytokines (reviewed in Kingsley, 1994). It is most closely related to the inhibins and indeed shares subunit homology with this protein (Figure 1, top panel). The activins are homo- or heterodimers of the inhibin βA, βB or βC chains, which are approximately 25-30 kD in size (Mason et al., 1985; Vale et al., 1991; Hotten et al., 1995)(Figure1, bottom panel). They were originally described for their ability to activate follicle stimulating hormone release from the anterior pituitary in opposition to the inhibitory effects of inhibin family members (Vale et al., 1991). It is interesting to note that the inhibins show no function in the somatostatin induction assay and do not suppress the effects of activin A in this assay despite the β subunit that they share in common (Coulombe et al., 1993). The activin family signals through a serine/threonine kinase receptor complex, similar to several other members of the TGFβ superfamily (Mathews, 1994; Josso and di Clemente, 1997). The complex contains a Type II subunit that is thought to bind the activin A ligand in order to promote association with and phophorylation of the Type I subunit (ten Dijke et al., 1996). Complementation studies with function blocking Type I subunits indicate that the signalling complex is likely a heterotetramer (Weis-Garcia and Massague, 1996). The intracellular components of activin A signalling are still under heavy investigation, however the SMAD proteins (*sma* from C. elegans plus MAD, mothers against decapentaplegic from *Drosophila*) have been proposed to function in the pathway (recently reviewed in Massague, 1996; Mehler et al., 1997).

Activin A has been demonstrated to exert numerous effects in several systems (Ying, 1989). In addition to its role in regulating follicle-stimulating hormone release, it has been reported to be involved in mesoderm induction in the early Xenopus embryo (Hemmati-Brivanlou and Melton, 1994; Hemmati-Brivanlou and Melton, 1997), to support survival in P19 teratocarcinoma cells (Schubert et al., 1990), to be a component of granulosa cell differentiation (Yokota et al., 1997) as well as erythroid cell differentiation (Yu et al., 1987) and more recently to promote smooth muscle

development in the embryonic avian iris (Link and Nishi, 1998b). The variety and range of activin A effects reflect the cell type specific response to this factor, yet together they implicate activin A as a potent regulator of growth and differentiation.

VI. Goals of the study

The current study seeks to address the questions of how neurotransmitter phenotype is established in ciliary ganglion neurons in vivo and what molecule(s) may be involved. The hypothesis is that target-derived activin A induces somatostatin expression in choroid neurons of the ciliary ganglion. In order to establish that particular factors control this biological function in vivo, there are a number of criteria that must be met. First the factor must mediate function on an appropriate cell type in vitro. Second, the factor must then be demonstrated in vivo with the correct temporal and spatial pattern to mediate function. Third, overexpression of the factor should exert the predicted effect in vivo, similar to the effect in vitro. Fourth, and most importantly, blocking the factor in vivo should inhibit the effect.

Previous studies that are the foundation of the current research clearly established that activin A induces somatostatin expression in ciliary ganglion neurons (Coulombe and Nishi, 1991; Coulombe et al., 1993). Thus, the first criteria has been met. The studies described in the following pages pursue fulfillment of the remaining criteria for establishing activin A as a component of the in vivo mechanism for inducing somatostatin expression in the ciliary ganglion neurons. In addition, a novel mechanism for establishing neurotransmitter phenotype will be proposed that involves not only differential expression of the inducer, activin A, but also localized expression of an inhibitor, follistatin, to control selective expression of somatostatin in ciliary ganglion neurons.

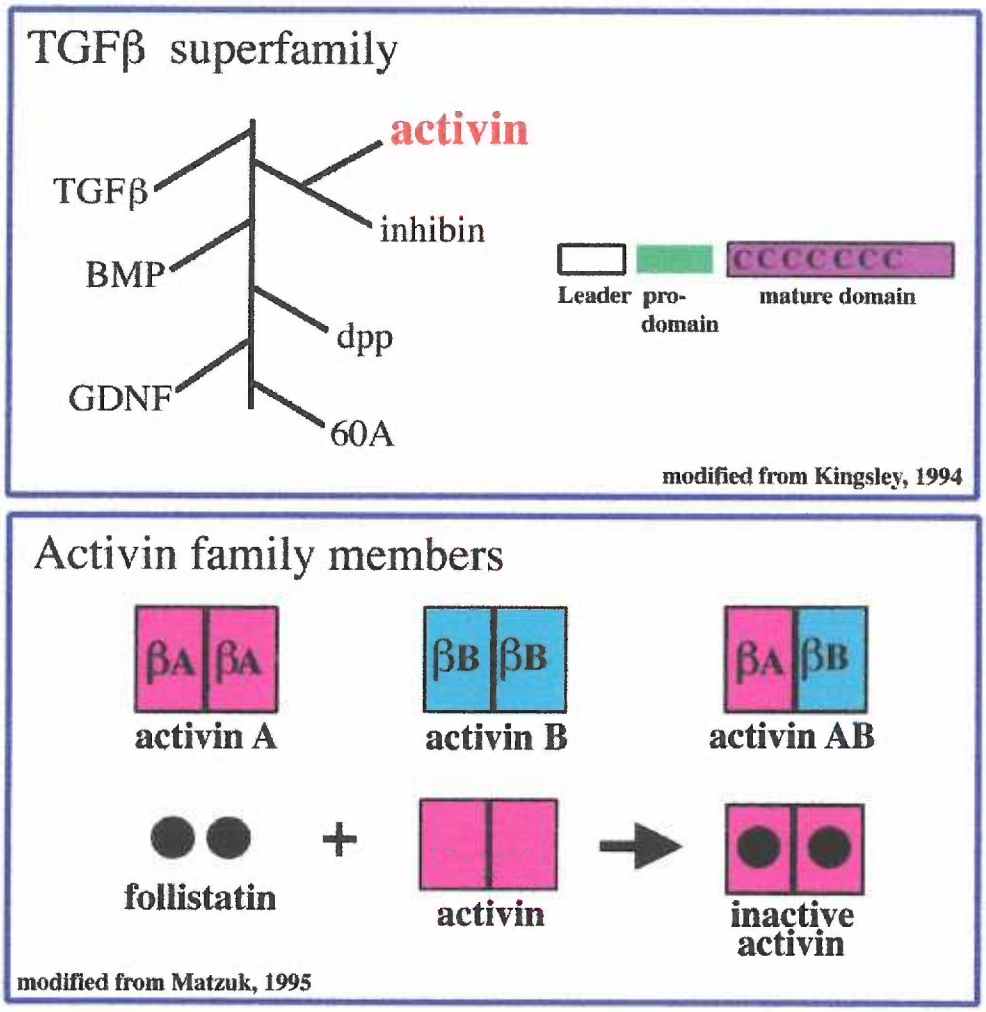

Figure 1. Characteristics of activin A

Activin A is a member of the larger TGFβ superfamily of pleitrophic cytokines (top panel). TGFβ family members have been described in nearly every species examined. Their common structural domains include an N-terminal leader sequence, a pro domain that must be cleaved for activation and a series of conserved cysteines that are thought to be involved in intramolecular folding and in the dimer association required for function. The activins are most closely related to the inhibins and share a common β subunit. Three β chains that can associate in a variety of combinations to form the activin family members have been described so far: βA, βB and βC (bottom panel). For simplicity only the βA and βB chains are shown. A potent inhibitor of activin action is follistatin. This protein binds to the β chain subunits and prevents activin association with the signalling receptor. Together, activin A and follistatin have been shown to regulate a number of developmental and physiological processes.

Materials and Methods

Tissue collection and cell culture

Iris cell cultures :

E11 chick irises were isolated in Earle's balanced salt solution (EBSS; 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 the stroma was 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 x 10^4 cells per ml. Iris cultures were grown for 48 hours on polymerized collagen coated 24 well tissue culture plates (Linbro; Flow Laboratories, Inc., McLean, VA) in 500 µl of L-15-CO_2 (Mains and Patterson, 1973) + 10% horse serum. Myotubes from the iris stromal cultures were allowed to differentiate by replacing serum containing medium with serum free medium. Serum free L-15-CO_2 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 and serum free L-15-CO_2 included 6 mg/ml glucose, 20 U/ml penicillin, and 2 mg/ml streptomycin (Sigma Chemical Co.).

Choroid Cultures :

Choroid cells were prepared as previously described (Coulombe and Nishi, 1991). In brief, the choroid layer was microdissected away from the retina and sclera and the retinal pigmented epithelial layer was daubed off with cotton. Cells were exposed to 0.8% trypsin at 37°C for fifteen minutes. Trypsin was inactivated with 10% serum containing medium. A single cell suspension was made with gentle trituration. Cells were plated on tissue culture dishes coated with rat tail collagen purified as described (Bornstein, 1958) and grown in modified L-15 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.

Preparation of iris conditioned medium

Iris cultures were differentiated in serum free L-15-CO_2 for 3 days to allow large multinucleated myotubes to form. Cultures were then fed with fresh serum free L-15-CO_2 and conditioned medium was collected 48 hours later. Iris conditioned medium (ICM) was centrifuged at 1000 x g at 4°C and concentrated 6 fold in a 10 kD-cut off

Centriprep filter unit (Amicon, Beverly, MA). Concentrated iris conditioned medium was sterile filtered through a 0.22 micron filter and stored at -80°C prior to use in the somatostatin induction assay.

Addition of cytokines to choroid cultures

When the cells were switched to serum free medium on day 2, growth factors were added at the following concentrations: recombinant human transforming growth factor β1 (TGFβ1) 10 ng/ml or 1 ng/ml, as indicated (Lot # GO98AD, Genentech, San Francisco, CA) and recombinant bovine fibroblast growth factor 2 (FGF2), 1 ng/ml (gift from Dr. Felix Eckenstein). Recombinant FGF2 was indistinguishable from native FGF2 in several bioassays (Eckenstein et al., 1990).

RNase protection assay

Constructs

A fragment of chick ribosomal protein S17 (CHRPS) of 361 base pairs 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), one minute each (30 cycles) with one unit/reaction of Taq DNA polymerase (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 embryonic day 17 (E17) ovary using Xenopus primers from a region conserved between Xenopus and human follistatin sequences (Hemmati-Brivanlou et al., 1994). 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 base pair product was observed when the primers were used to amplify genomic DNA. The 223 base pair 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 pCR™II vector (Invitrogen, San Diego, CA). The sequence of the inserts was confirmed by Sequenase sequencing analysis. A full length chick activin 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 (activin βA) to generate blunt ends or 5' overhangs. Linearized templates were treated with 50 µg/ml proteinase K and 1% SDS, 30 minutes 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 10% volume of 3M sodium acetate, pH 7.0 and 2.5 volumes of 100% ethanol and kept at -20°C for at least one hour. Pellets were spun and washed one time with 70% ethanol. The DNA was resuspended in H_2O for storage at -20°C. Riboprobes were generated in the following reaction mix: 12.5 µM cold rCTP (250 µM cold rCTP for CHRPS); 1X transcription buffer, 10 mM DTT, 10 units RNasin, 500 µM each rUTP, rATP, rGTP; 750 ng template; 25 µCi ^{32}P rCTP, 3000 Ci/mMole (New England Nuclear, Boston, MA) and 10 units 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 one hour at 37°C and the remaining template was removed by digestion with 10 units 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 0.3M NaCl, 0.5% SDS and 10 mM Tris, pH 7.5 for 45 minutes 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, 1mM EDTA) and combined in the hybridization mix at 100,000 dpm each for activin A and follistatin and 10,000 dpm for CHRPS.

RNase Protection

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 hours at 42°C. Protection and processing were done as described (Gilman, 1991). Protected fragments for activin A, follistatin and CHRPS were 410 bp, 223 bp and 122 bp, respectively. The unprotected probes ran 20-70 bases higher than the protected fragments. Fragments were run on 6% acrylamide/8M urea/0.5X TBE gels along with radiolabelled standards ranging from 100 to 500 bp (Ambion, Austin, TX). 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 were 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. For some experiments, fold changes relative to control levels were determined by attributing a value of 1 to control conditions (four day, serum free cultures). Statistical analyses were done with Statistica (StatSoft, Inc. Tulsa, OK) using the T-test for independent variables comparing absolute and ratio values. All values compared had a normal distribution. P values are listed in the text.

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). Briefly, ciliary ganglia were dissociated and plated at a density of 1 ganglion per well into drilled 35 mm^2 tissue culture dishes. Petriperm™ biomembrane (Heraeus Instruments, Inc., South Plainfield, NJ) was cut to 1.5 cm^2 and glued under the 1 cm hole and the resulting well was coated with poly-D-lysine (0.5 mg/ml) and then laminin (1 mg/ml) prior to plating. Neurons were grown in the presence of 1 ng/ml recombinant Growth Promoting Activity (GPA; Eckenstein et al., 1990; Finn and Nishi, 1996; Finn et al., 1998) to promote survival. GPA has no somatostatin induction capacity in this assay (Coulombe et al., 1993). Dose responses (0-50 ng/ml) to recombinant human activin A (Genentech, San Francisco, CA) were tested in the presence of 2X concentrated serum free L-15 or 2X concentrated iris conditioned medium for 5 days in a volume of 200 μl. Medium was changed every 36 hours.

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 the following 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 non-specific 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 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 flow through.

Immunoreactivity scoring

The number of neurons containing somatostatin immunoreactivity (SOM IR) was viewed under a Zeiss microscope with a 40X objective using bright field optics. Neurons were 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. Percent SOM IR was determined by summing the SOM IR positive neurons per well and dividing by the total neurons per well. Mean percent SOM IR and the standard deviation (SD) were determined using percent 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 choline acetyltransferase activity

Neuronal survival per culture was determined by adding the number of neurons in ten fields of view at 40X. Reported values were the average of 3 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. Choline acetyltransferase activity was determined as described (Coulombe and Nishi, 1991). In brief, cultures were washed with modified Puck's saline solution and extracted in the following solution: 0.05 M sodium phosphate buffer (pH 7.5) containing 0.2 M NaCl,

5 mg/ml bovine serum albumin, 0.5% Triton X-100, 10 mM choline chloride, 0.2 mM eserine sulfate, 10 mM EDTA and 5 mM dithiothreitol. Homogenate (25 µl) was incubated at 37°C for 30 minutes in combination with 5 µl ^{3}H-acetylCoA and the reaction was terminated with addition of PBS. Labelled acetylcholine was separated from the substrates using 0.2 ml of 5 mg/ml tetraphenyl boron in 4 ml of toluene-based scintillation fluid.

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$^{81\text{-}113}$ (Vaughan et al., 1989) were generous gifts from Dr. Wylie Vale, The Salk Institute, La Jolla CA.

Cultured Cell Immunohistochemisrty

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 minutes 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 were then incubated for 2 hours at 25°C. Antibody localization was detected using single peroxidase-antiperoxidase (PAP) staining. Specificity was controlled for by comparing staining in the absence of a primary antibody or incubating with normal rabbit serum instead of primary. Anti-inhibin βA specificity was further tested by preabsorbing 15 ml of the antiserum with 100 mg of the activin peptide$^{81\text{-}113}$ 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 minutes 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 minute incubations in PBS prior to equilibration in O.C.T. 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 PAP staining. Specificity controls were performed as for culture staining.

Smooth muscle marker immunolocalization

Cultured cells or choroid layer tissue were fixed for 30 minutes at 25°C in 4% paraformaldehyde plus 15% picric acid in 0.2M sodium phosphate buffer, pH 7.3. Cultures and choroid layer whole mounts were blocked in 0.5M NaCl, 10% horse serum, 10% chick serum, 2% lamb serum and PBS at 4°C for 24 hours. Primary antibody incubations were overnight at 4°C. Bound antibody was visualized using double PAP immunostaining (Vacca et al., 1980) with goat anti-rabbit or mouse IgG (Sternberger Immunochemicals, Baltimore, MD) diluted 1:150 in blocking solution followed by incubation with rabbit or mouse PAP (Sternberger Immunochemicals) diluted 1:300 in blocking solution, essentially as described previously (Coulombe and Nishi, 1991). Monoclonal antibodies were all obtained from Sigma and were diluted in blocking solution as follows: anti-smooth muscle specific actin (SMSA), 1:500; anti-desmin, 1:50; anti-myosin light chain kinase (MLCK), 1:1000; anti-caldesmon, 1:800; anti-calponin, 1:1000. The polyclonal antisera against chick muscarinic acetylcholine receptors (AChR) m2, m3 and m4 (McKinnon and Nathanson, 1995) were a generous gift from Dr. Neil Nathanson (University of Washington, Seattle) and were diluted 1:200, 1:1000 and 1:500, respectively. Normal mouse and normal rabbit serum diluted 1:1000 were used as controls for staining specificity. Cell counts were done after Hoechst dye nuclear staining (10 μg/ml solution, Sigma) and visualization using a DAPI filter. For the comparison of TGFβ1 and FGF2 effects on smooth muscle marker expression, the cells were scored as: -, no immunoreactive (IR) cells; +, 1-20% cells IR; ++, 21-40% cells IR; +++, 41-60% cells IR; ++++, 61-80% cells IR; +++++, 81-100% cells IR.

TGFβ mRNA detection and blocking

RT/PCR for Detection of TGFβ mRNA

Total RNA from E14 choroid, iris, ciliary ganglion and six day choroid cultures was purified (Chomczynski and Sacchi, 1987) and used to generate cDNA according to Current Protocols (Beverly, 1991) using Superscript II RNA polymerase (Gibco/BRL). CHRPS specific primers (Darland et al., 1995) amplified a band from the cDNA reaction as a positive control. PCR primers were designed against the conserved region of the TGFβ family members that codes for the mature protein domain (Jakowlew et al., 1988; Jakowlew et al., 1988; Jakowlew et al., 1990) so that all TGFβ isoforms could be detected. The primer pair sequence was taken from the published sequence for chick TGFβ3 (Jakowlew et al., 1988), and should recognize the different messages for TGFβ proteins. The forward primer (nts 990-1013) was 5' GCGTCCTCTTTACATTGACTTCCG3' and the reverse primer (nts 1233-1254) was

5' CACCATATTGGAGAGCTGCTCC 3'. Cycling conditions with Taq DNA Polymerase (Promega, Madison, WI) were 93°C, melting; 56°C, annealing; 72°C, extension for one minute each, times 30 cycles with 1 mM dNTP mix and 2 mM $MgCl_2$ for optimal band resolution. The negative control reaction had no reverse transcriptase in the cDNA synthesis step.

TGFβ Blocking Assay

The TGFβ blocking antibody (R&D Systems, Minneapolis, MN) was used at 50 μg/ml, a concentration which we determined to have maximum blocking capacity in our assay system (data not shown). The antibody was added to the culture medium when the cells were switched from serum-containing to serum-free conditions and fresh antibody was added 24 hours later. The cells were grown in the presence of antibody for 48 hours prior to collection. An anti-laminin polyclonal antisera (1:1000; Gibco/BRL) was used to control for effects due to culturing the choroid cells in the presence of antibody. An additional control involved culturing 1 ng/ml of TGFβ1 plus 50 μg/ml of the blocking antibody to determine blocking capability in the presence of exogenous TGFβ1. Cells were fixed and stained for SMSA immunoreactivity as described above. Statistical analyses were done with Statistica (StatSoft, Inc. Tulsa, OK) using the T-test for independent variables comparing absolute and ratio values. All values compared had a normal distribution. P values are listed in the text.

Manipulation of somatostatin levels in vivo

Intraocular and systemic delivery of activin A and follistatin

Embryonic day 3 (E3) chicken embryos (White Leghorn, XL and HN breeds, Oregon State University Poultry Science Department, Corvallis, OR) were cracked into shell-less culture as previously described (Dunn, 1974; Finn et al., 1998). E10 or E11 embryos were given intraocular injection of 300 ng of recombinant human activin A (lot #15365-36, National Hormone and Pituitary Program, distributor, McKesson BioServices, Rockville, MD) or follistatin (lot #B3904, National Hormone and Pituitary Program) in L15-CO_2 medium with 2.5 mg/ml bovine serum albumin (Sigma Chemical Co., St. Louis, MO), and 0.25% fast green dye in a final volume of 2 μl. Control animals were given vehicle alone. Animals injected earlier than E10 had a high fatality rate due to injection-induced hemorrhage. Using a drawn capillary pipette, one eye was injected through Schlemm's canal behind the lens and onto the vitreous body with the remaining eye as uninjected control. The ciliary ganglia and eyes were collected after four days further development. In some embryos follistatin was applied

to the chorioallantoic membrane (CAM). For this approach E3 embryos were windowed as described (Finn et al., 1998) and at E7 were given 1 μg of follistatin or vehicle alone in a final volume of 3μl. Daily deliveries were repeated until E14 when ciliary ganglia and eyes were collected.

Immunohistochemistry of ciliary ganglion neurons and target tissues

Tissue was fixed in Zamboni's solution (4% paraformaldehyde, 15% picric acid in 0.1 M sodium phophate buffer, pH 7.2) for 30 minutes at 23°C, washed with phosphate buffered saline solution (PBS, pH 7.2) and saturated with sucrose to 30%. The tissue was imbedded in Tissue Tek O.C.T. compound (Sakura Finetek U.S.A., Inc., Torrance, CA) and 10 μm frozen sections were cut. Sections were blocked with 10% horse serum, 0.5 M NaCl, 0.5% Triton X-100, 0.1% sodium azide in PBS, pH 7.2. Ciliary ganglia were stained for somatostatin using a rat monoclonal antibody (Accurate Chemical and Scientific Corporation, Westbury, NY) diluted 1:100 in blocking solution without detergent. Normal rat serum was diluted 1:500 as a negative control. Endogenous peroxidase was inactivated with 30% ethanol, 10% peroxide in PBS. Secondary antibodies, goat anti-rat IgG (1:150, Sternberger Monoclonals, Baltimore, MD) and rat IgG coupled to a PAP complex (1:300, Sternberger), were diluted in 10% horse serum in PBS. Sections were processed with the double PAP method (Vacca et al., 1980) using diamobenzidine as a substrate with 0.03% NiCl for signal enhancement. A 0.5% Eosin Y plus 0.2% glacial acetic acid solution (Sigma) was used to counterstain. Sections were dehydrated in ethanol, xylene treated and set in Pro-Texx* Mounting Medium (Baxter Diagnostics, Deerfield, IL). Target tissues were stained for SMSA (1:500) in blocking solution. Normal mouse serum (1:500) was used as a negative control. Endogenous peroxidase was inactivated as above. Secondary antibodies were goat anti-mouse IgG (1:200) and mouse IgG coupled to PAP (1:400) diluted in blocking solution with 0.1% Triton X-100 and without azide. Sections were processed as above, but with a single round of PAP.

Counting methods and statistical analyses, in vivo studies

Percent somatostatin values represent the number of positive neurons over the total number of neurons determined in every fifth section to give a representative count through the ganglia. The number of positive neurons and total neurons observed were multiplied by five to obtain the values listed in the text and Tables I and II. Cells with large round soma were scored as neurons. Non-neuronal cells surrounding the neurons in the ganglion were distinguishable by their flat, crescent shape. A blue filter

was used to increase the contrast between the dark PAP reaction product and the pink eosin counterstain. A neuron was scored as immunoreactive for somatostatin if granular reaction product could be detected in the soma. A neuron was scored as negative if reaction product was not detected. Counts were determined without reference to treatment conditions. Comparisons of control versus test conditions were done with Statistica (Statsoft, Inc., Tulsa, OK) using a standard T-test for both absolute and ratio values. For determining cell body diameter, nuclei were stained with hematoxylin solution (0.75%, Sigma) and dehydrated by standard methods prior to mounting in Pro-Texx*. The diameter of ovoid cells with large round pericentric nuclei were measured relative to a grid calibrated to a slide micrometer.

RNase protection analysis of treated eye mRNA

Total RNA was purified from whole eye tissue from the CAM application experiments as described previously and processed for protection of activin A, follistatin and CHRPS mRNA.

CHAPTER 1

Developmental expression of activin A and follistatin in targets of ciliary ganglion neurons suggests a function in the regulation of neurotransmitter phenotype

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Summary

The avian ciliary ganglion contains choroid neurons that innervate choroid vasculature and express somatostatin and 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 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 co-cultured 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 (LIF; 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 LIF were found to have normal sweat gland innervation and function (Rao et al., 1993). In addition vasoactive intestinal peptide immunoreactivity and choline acetyltransferase activity in the footpad sweat glands of transgenic mice deficient for CNTF were unchanged compared to wildtype (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; DeStefano 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 (reviewed in 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 (FSH) 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; Hemmati-Brivanlou and Melton, 1994a and b). 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).

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., 1988; Shimasaki et al., 1989; Albano et al., 1994) that encodes several protein isoforms arising from alternatively spliced mRNA and post-translational 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 (Shimasaki et al., 1988; Shimasaki et al., 1989; Hemmati-Brivanlou et al., 1994). The PCR product was ligated into the pCR^{TM}II 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, 1996). The region used for activin A probe synthesis was not homologous to the inhibin α transcript or the activin βB transcript.

Multiprobe RNase Protection Assay

In order 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). In order 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. Messenger RNA levels of a constitutively expressed gene, chick ribosomal protein S17 (CHRPS; Trüeb et al., 1988), were measured as an internal loading control. Total RNA (15-40 μg) 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 2A, left). Unprotected probe (Figure 2A, 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. Linear regression analysis of the relative intensity values for each set of protected fragments gave r values of 0.994, 0.999 and 0.992 for CHRPS, activin A and follistatin, respectively (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, then mRNA for these factors should be detectable in the targets of the ciliary ganglion during development. Embryonic days 9-16 encompass a critical period of development during which both the iris/ciliary body (Pilar et al., 1980; Pilar et al., 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 total RNA (20 μg) 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-E16 (Figure 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-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 supports 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 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 (Figure 4C, F, and I). In addition preabsorption of the 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 four-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 (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 barely detectable 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 (Figure 6C and F).

To determine the ability of iris cell conditioned medium 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 ganglion neurons (Coulombe et al., 1993). Serum-free conditioned medium from iris-derived cultures (iris conditioned medium, ICM) which 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 ($p<0.05$) the percentage of neurons that express somatostatin immunoreactivity when compared to 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 a follistatin antiserum (Sugawara et al., 1990). Immunodepletion significantly restored somatostatin induction in the presence of activin A ($p<0.05$ as compared to 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 choline acetyltransferase (ChAT) activity were assayed. Neither survival nor ChAT activity were reduced in the presence of ICM (Figure 7B and C). 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 that activin A controls somatostatin phenotype in choroid neurons.

Discussion

In the current study we have investigated the in vivo distribution of activin A and follistatin and the in vitro potential of iris conditioned medium to modulate somatostatin induction. Previous work from our laboratory has shown that activin A from choroid 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 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 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 which 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 conditioned medium induces somatostatin and contains activin A, which alone can induce somatostatin (Coulombe et al., 1993). We have now shown that iris conditioned medium inhibits somatostatin induction and that this inhibition is significantly relieved by immunodepletion of the conditioned medium with 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 lead 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 (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 (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 which had been depleted with 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 iris conditioned medium observed in the somatostatin induction assay. The significant but partial relief of inhibition of somatostatin induction by follistatin immunodepleted ICM (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, thus they 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. Post-translational processing has also been shown to regulate activity of several members of the TGFβ 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 (Ying, 1989; Yu et al., 1987). However, inhibin βA 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 sub-unit. 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 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 spacial in vivo expression of the molecules.

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A

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  1    TCGTGTGTGG  TGGATCAGAC  TAATAACGCC  TACTGTGTGA  CATGTAATCG   50
 51    AATTTGCCCT  GAGCCTACCT  CCCCTGAGCA  GTATCTCTGT  GGGAATGATG  101
102    GCATAACTTA  CGCCAGTGCC  TGCCACCTGA  GAAAAGCGAC  CTGCCTGCTG  152
153    GGCGAATCCA  TTGGATTAGC  CTACGAGGGA  AAATGCATCA  AAGCGAAGTC  203
204    CTGTGAAGAT  ATTCAGTGCA  GCG
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B

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C      S C V V D Q T N N A Y C V T C N R I C P E P T S P E Q Y L C G N D
X      * * * * * * * * * * * * * * * * * * * * * * * D * * * * * * *
H      T * * * * * * * * * * * * * * * * * * * * * A * S * * * * * * *
M      T * * * * * * * * * * * * * * * * * * * * * S * S * * * * * * *
P      T * * * * * * * * * * * * * * * * * * * * * * S * * * * * * * *

C      G I T Y A S A C H L R K A T C L L G E S I G L A Y E G K C I K A K
X      * * * * G * * * N * * * * * * * * R * * * * * * * * * * * * * *
H      * V * * S * * * * * * * * * * C * * R * * * * * * * * * * * * *
M      * V * * S * * * * * * * * * * * * R * * * * * * * * * * T - * *
P      * V * * S * * * * * * * * * * * * R * * * * * * * * * * * * * *

C      S C E D I Q C S
X      * * * * * * * *
H      * * * * * * * T
M      C * * * * * * G
P      * * * * * * * T
```

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 are 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.

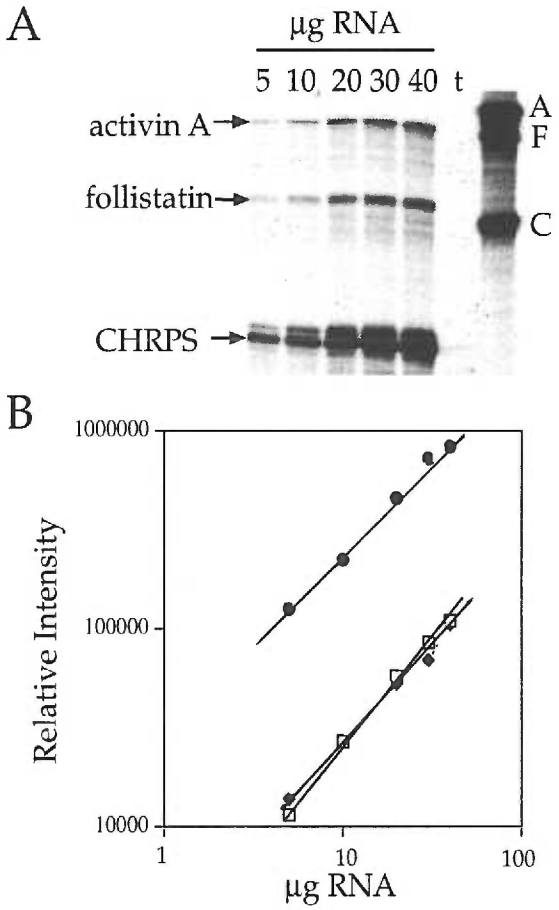

Figure 2. Multiprobe RNase Protection Assay (RPA) standard curve. (A) An autoradiograph of 5-40 µg of E17 chick ovary total RNA hybridized with activin A, follistatin and CHRPS probes. All three protected fragments (arrows) were visible in each sample. Unprotected probe (A, F and C) ran higher than the protected fragments. The tRNA negative control (t; 20 µg RNA) had no protected fragments. (B) Linear regression analysis of relative intensity from 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 and $r = 0.994$ for CHRPS). Symbols represent CHRPS (circles), activin A (squares), follistatin (diamonds). This was a representative experiment from four repetitions which showed similar results.

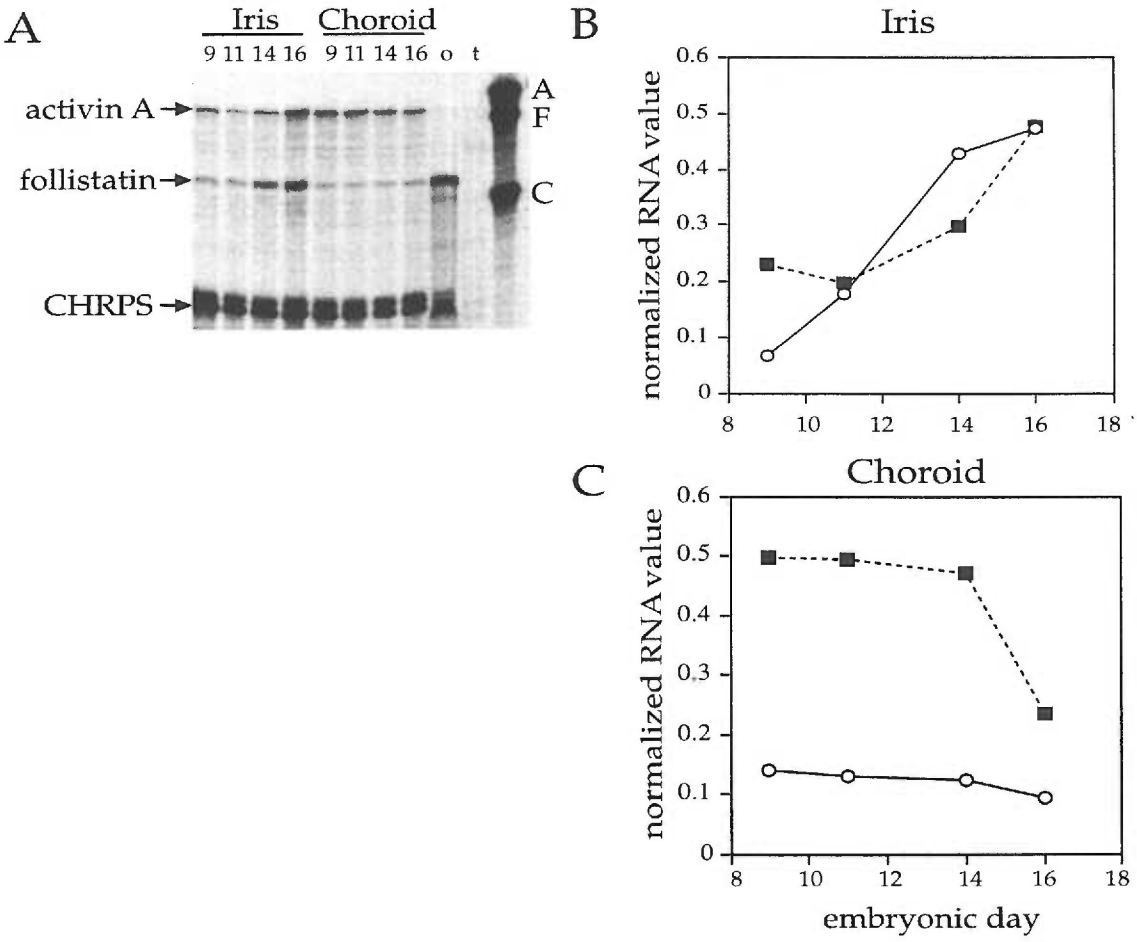

Figure 3. Follistatin and activin A in iris and choroid tissue during development.

(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) 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-E16. Follistatin in the choroid remained static, while activin A was high from E9-E14 and dropped at E16. Symbols respresent activin A (squares), follistatin (circles). This is a representative experiment from four repetitions showing similar results.

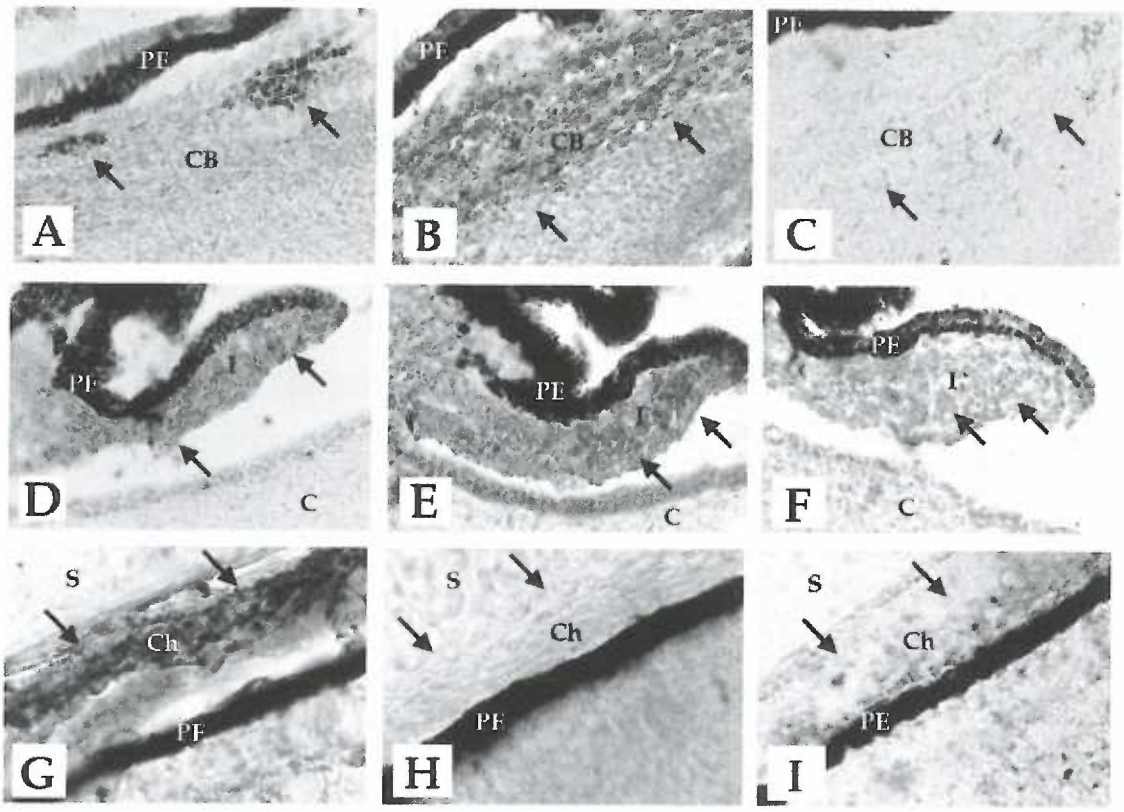

Figure 4. Activin A and follistatin immunolocalization in ciliary ganglion neuron targets in vivo.

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

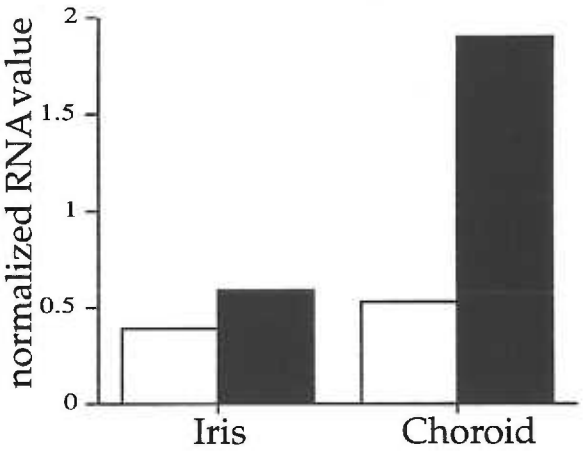

Figure 5. Follistatin and activin A mRNA in 6 day choroid and iris cultures

Total RNA (10 μg) from cells in culture were hybridized to activin A, follistatin and CHRPS probes. The histogram of the normalized RNA values from this protection are shown for activin A (filled columns) and follistatin (open columns). RNA values were obtained using the ratio of 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 four fold higher in the choroid cells than in the iris cells. One representative experiment out of four repetitions with similar results is shown.

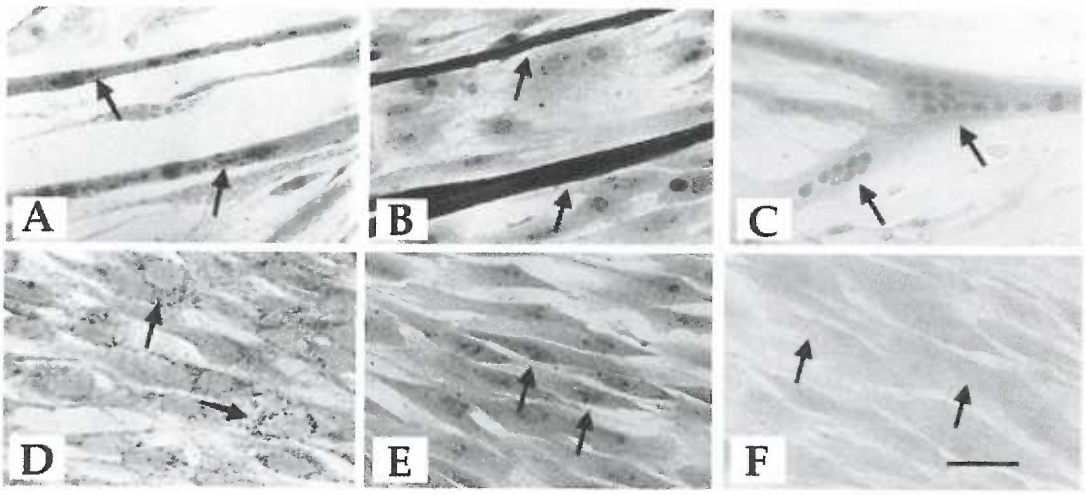

Figure 6. Activin A and follistatin immunoreactivity in 6 day choroid and iris cultures

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

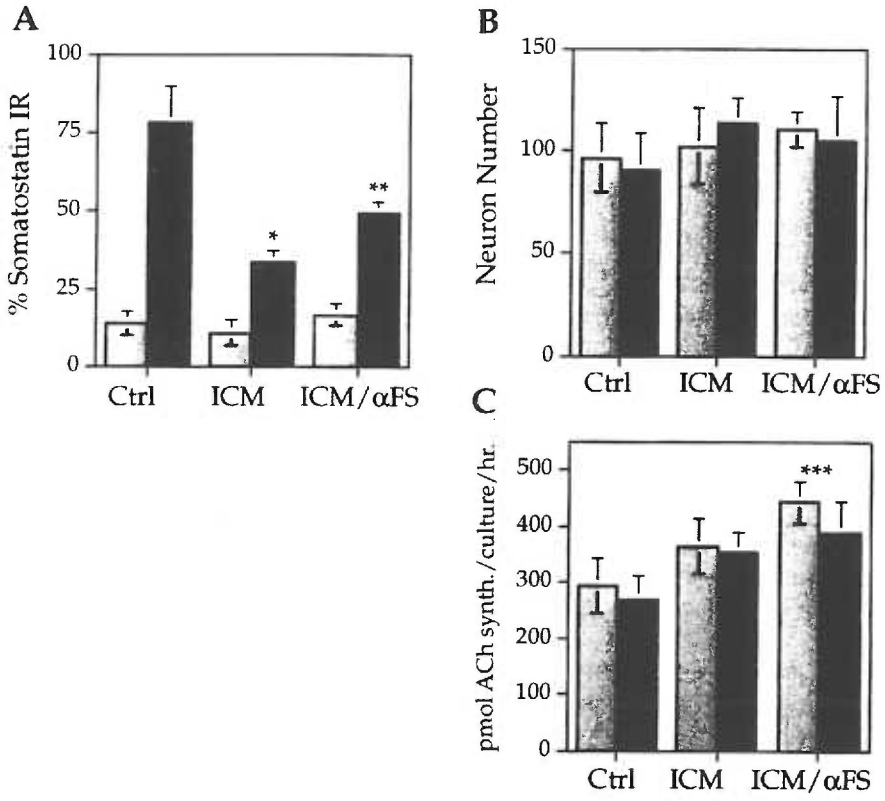

Figure 7. Somatostatin induction, neuron survival, and ChAT activity in cultured ciliary ganglion neurons.

(A) Percent somatostatin immunoreactivity, (B) neuron survival, and (C) choline acetyltransferase activity in E8 ciliary ganglion neurons cultured for 4 days in control medium (ctrl), iris cell conditioned medium (ICM), or ICM which 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 (filled columns) or without activin A (stipled columns). The values indicated for each column are the mean of three different cultures from one representative experiment. Error bars represent SD. * : Significantly differs from control with activin A. ** : Significantly differs from ICM with activin A. *** : ICM/αFS with and without activin ChAT activity significantly differs from control with or without activin A. Significance at $p<0.05$ by one-way ANOVA.

CHAPTER 2

Activin A and follistatin in choroid smooth muscle cells: differentiation-dependent expression of target-derived regulators of neuropeptide phenotype

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Summary

Considerable evidence exists to indicate that targets are an important source of factors that influence neuronal development, however, less is known about the regulation of expression of these target-derived factors. One system in which these issues can be examined involves the choroid neurons of the avian ciliary ganglion and their smooth muscle targets in the choroid layer. We have previously shown that expression of somatostatin in choroid neurons is induced by activin A, which is expressed by the vascular smooth muscle. We have hypothesized that activin A is a retrograde effector of neuropeptide expression. In order to study the molecular and cellular interactions necessary for expression of activin A, we have established a serum-free culture system in which dedifferentiated cells from embryonic day 14 choroid layer recapitulate the pattern of smooth muscle differentiation. These cells develop expression of several proteins specific for smooth muscle, such as smooth muscle specific actin, calponin, smooth muscle specific myosin light chain kinase and the muscarinic acetylcholine receptor, subtype m2. Expression of smooth muscle specific markers was accelerated by TGFβ, inhibited by FGF2 and a pan-specific TGFβ blocking antibody. Under all conditions, activin expression was regulated in parallel with the differentiation of the smooth muscle cell phenotype. The expression of follistatin, which antagonizes activin A action, was unaffected by FGF2 and only slightly increased by TGFβ. These results are consistent with the hypothesized role of activin A in the choroid as a retrograde effector of peptide phenotype. They indicate that target-derived activin A expression is independent of continuous innervation and co-culture with endothelial cells. Moreover, they reveal that target-derived activin A expression is controlled coincident with the differentiation of the target cells themselves.

Introduction

Target tissues produce retrograde signals that have significant impact on the growth and differentiation of the neurons that innervate them during development. Such target-derived effectors can support neuronal survival during the period of naturally occurring cell death, a role that has been substantially demonstrated for the members of the neurotrophin family (Oppenheim, 1991; Ip and Barde, 1996). Nerve growth factor (NGF), for example, is induced in the peripheral targets of the trigeminal neurons upon innervation and increases coincident with the period of innervation (Davies et al., 1987). In contrast to NGF, brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) are expressed in the trigeminal targets prior to innervation and are reduced thereafter and have only a transient role in supporting neuronal survival (Buchman and Davies, 1993). In addition to survival, neurons can be dependent on their targets for other aspects of their development such as the establishment of the neurotransmitter phenotype that a neuron manifests. This has been carefully studied in the sympathetic innervation of the rat footpad sweat gland where the neurons undergo a developmental switch from noradrenergic to cholinergic under the influence of target-derived sweat gland factor (SGF; Landis, 1990; Habecker et al., 1997). Interestingly, the target expression of SGF is another instance where innervation induces expression of a retrograde factor that controls neuronal development (Habecker et al., 1995). How production of these target-derived factors is regulated thus becomes an integral component of the establishment of appropriate connections in the developing nervous system.

Target-derived retrograde signals also have been described in the ocular targets of the parasympathetic avian ciliary ganglion. Ciliary ganglion neurons undergo a period of target-dependent cell death during development (Landmesser and Pilar, 1976a). A target-derived molecule, CNTF, has been proposed as the limiting neurotrophic factor that supports the surviving neurons in vitro and in vivo (Eckenstein et al., 1990; Finn and Nishi, 1996; Finn et al., 1997). In addition to supporting neuronal survival, target derived factors have been proposed to regulate the expression of potassium channels in the developing neurons. The calcium-dependent potassium current ($I_{K^+[Ca^{+2}]}$) and the transient current (I_A) are both expressed in the neurons in vivo after contact with the targets has been established and are only expressed in vitro if the neurons are co-cultured with target cells (Dourado and Dryer, 1992; reviewed in Dryer, 1994). Target-

mediated neuronal survival and differentiation are important components in this developing system.

One additional aspect of ciliary ganglion development which is thought to be regulated by target-derived molecules is the establishment of neuropeptide phenotype (Coulombe and Nishi, 1991). The ciliary ganglion consists of two populations of cholinergic neurons which have differential expression of the co-transmitter, somatostatin (Epstein et al., 1988; De Stefano et al., 1993). The choroid neurons innervate the vascular smooth muscle in the choroid layer and express somatostatin, while the ciliary neurons innervate the iris/ciliary body and do not express somatostatin. Two proteins have been proposed to control this differential expression of neuropeptide: activin A and follistatin. Activin A induces somatostatin in ciliary ganglion neurons and follistatin inhibits activin A action (Coulombe et al., 1993; Darland et al., 1995). Activin A and follistatin have been proposed to control the expression of somatostatin in developing neurons based upon their differential expression in the targets (Darland et al., 1995). The iris/ciliary expresses both activin A and follistatin, but the choroid layer expresses predominantly activin A (Darland et al. 1995). Thus, selective expression of the inhibitor, follistatin, is thought to control availability of the inducer, activin A, in the establishment of neuropeptide phenotype in developing ciliary ganglion neurons.

Although target-derived factors that influence ciliary ganglion neuron development have been described, little is known about the regulation of these factors within the developing targets themselves. For instance, it is unclear at what stage of choroid development the expression of activin A is initially induced in the target and whether this expression is dependent upon neuronal innervation. The choroid layer is of particular interest because it develops as a vascular structure in concert with the establishment and differentiation of the choroid neuron innervation from the ciliary ganglion. Message for activin A and follistatin have been detected in the choroid layer as early as embryonic day 9 (E9), which is slightly after the beginning of somatostatin induction in the ciliary ganglion neurons at E8 (Darland et al., 1995). Activin A levels are considerably higher than follistatin, consistent with the somatostatin inducing ability of the choroid target. At this point the choroid consists of primary blood vessels which are growing throughout the layer, around the circumference of the eye and undifferentiated mesenchymal cells which will contribute to the developing vessel system (Meriney and Pilar, 1987). Innervation takes place on the smooth muscle cells

beginning at E6 and functional chemical synapses have been detected as early as stage 36, approximately E9 (Meriney and Pilar, 1987).

These data lead to the question of how initial production of activin A and follistatin are induced in the smooth muscle. Is this expression an integral component of the normal differentiation program of vascular smooth muscle cells or is it a result of contact with endothelial cells? An additional question arises as to what are the effects of neuron/target interactions and the concomitant differentiation of these two cell types in the integrated form and function of the developing vessel network in the choroid layer. The data we present here focus on the question of when activin A and follistatin are expressed in the choroid target cells relative to differentiation of these vascular smooth muscle cells. We have developed a culture system of choroid-derived cells that recapitulates in vitro the development of the vascular smooth muscle in the choroid target. We demonstrate that TGFβ autocrine signalling is a component of choroid smooth muscle differentiation. Moreover, we present evidence that activin A expression in E14 choroid cultures is dependent upon smooth muscle differentiation and is independent of continuous innervation.

Results

Smooth muscle specific proteins in the choroid layer in vivo

In order to determine which markers of smooth muscle are normally expressed in the vasculature of the eye, embryonic day 14 (E14) choroid layers were stained as whole mounts with a series of muscle-specific antibodies. Immunoreactivity for SMSA (Skalli et al., 1986; Fig. 1A) was detected around the blood vessels and interspersed among them. The staining pattern clearly outlined the vasculature. Immunoreactivity for smooth muscle myosin light chain kinase (MLCK, Fig. 1B), a Ca^{+2}/calmodulin binding protein (Olson et al., 1990), and calponin (Fig. 1C), a smooth muscle F-actin binding protein (Frid et al., 1992) were also detected in the smooth muscle cells surrounding the vasculature. Immunoreactivity for the muscarinic acetylcholine receptor (AChR), subtype m2 (Habecker et al., 1993; Fig. 2D) showed dark reaction product in a longitudinal array of staining, running parallel with the length of the capillaries and veins. The m2 receptor is the predominant isoform expressed in smooth muscle cells (Eglen et al., 1994). The m4 receptor is expressed at very low levels in vascular smooth muscle and the m3 receptor is absent; neither were examined in choroid whole mounts. Normal mouse and rabbit serum negative control conditions showed no immunoreactivity (data not shown). The immunoreactivity for the muscle markers shows the localization of smooth muscle within the choroidal vasculature.

Development of choroid smooth muscle in culture

In order to examine the in vitro progression of differentiation in the choroid-derived cells, we monitored the time course of expression of SMSA immunoreactivity in cultures derived from E14 choroid layer. Expression of SMSA was negative shortly after the cells were placed in culture and then increased until essentially all the cells were positive for SMSA by day six in culture (Fig. 2). The cells reached confluence by day 5 in culture. The cells were switched to serum-free conditions after 48 hours. If the cells were maintained in chick serum instead of being switched to serum-free conditions, expression of SMSA was repressed (data not shown). The choroid layer-derived smooth muscle cell population was nearly homogeneous, with less than 1% of the cells unstained for SMSA. The remaining unstained cells were likely endothelial and retinal pigmented epithelial cells based upon cell morphology and pigment granule content, respectively (data not shown).

In order to determine whether the choroid cells expressed markers in addition to SMSA, cultures were tested for immunoreactivity to several smooth muscle specific proteins. Cultures were analyzed after six days when essentially all cells have acquired immunoreactive staining for SMSA. The choroid cells showed positive immunoreactivity for SMSA in a pattern showing the cytoskeletal architecture (Fig. 3A). Approximately 50-60% of the cells were immunoreactive for smooth muscle MLCK (Fig. 3B). Positive immunoreactivity for the muscle intermediate filament protein, desmin (Debus et al., 1983; data not shown), and calponin (Frid et al., 1992; Fig. 3C), was detected in approximately 20-30% of the cells in culture. Muscarinic AChR m2 immunoreactivity in a punctate staining pattern was observed in all cultured cells (Fig. 3D). In contrast m3 was not detectable and only low levels of positive immunoreactivity for m4 were observed. This expression pattern of receptor subtypes in the choroid cells coincides with data published for other smooth muscle cell types (Eglen et al., 1994). Negative control monoclonal and polyclonal antibodies had background level staining (data not shown).

Expression of activin A and follistatin in choroid cultures

In order to determine when the neuropeptide regulators, activin A and follistatin, were expressed in choroid smooth muscle cells relative to their state of differentiation, we examined the time course of expression of activin and follistatin. Activin A and follistatin mRNA were essentially below the detectability of our assay at 2 days in culture (data not shown) before the cells were switched to serum free conditions. Levels of mRNA quantified by RNase protection assay (RPA) for activin A and its inhibitor, follistatin, increased in culture (Figs. 4A and 4B) with a time course that was similar to that observed for SMSA protein (compare to Fig. 3). Message levels peaked for activin A and follistatin at 6 days and dropped after this point as the cells became quiescent. The relative mRNA levels for activin A were approximately 7-fold higher than follistatin at the time of peak expression, consistent with in vivo expression levels previously described (Darland et al., 1995). The graph shown is representative of 6 experiments of similar time course series. Peak expression was sometines observed at 7 days in culture, but never prior to confluence at 5 or 6 days.

Cytokine effects on choroid cell differentiation

The time course of activin A and follistatin expression correlated closely with the timing of SMSA expression as the choroid cells differentiated in culture. In order to determine whether activin A and follistatin expression were dependent on choroid cell

differentiation we first needed to manipulate the differentiation state of the cells in culture. Consequently, we tested a number of cytokines expressed in the eye for their ability to affect the differentiation of choroid smooth muscle at four days in culture. This time point was chosen so that effects on SMSA above or below control culture levels could be determined. Leukemia inhibitory factor, interleukin-1α and activin A had no apparent effect on differentiation as indicated by expression of SMSA and cell number (data not shown). A striking effect was observed with TGFβ1 treatment, which induced high levels of SMSA, relative to controls (Figure 5A; $p < 0.001$, T-test). Dose response curves indicated that 10 ng/ml was a saturating concentration, with 1 ng/ml of TGFβ1 giving a half-maximal dose (data not shown). In contrast to the TGFβ effect, FGF2 increased the total cell number of the cultures (Fig. 5B; $p = 0.02$, T-test), and had a repressive effect on SMSA immunoreactivity (Fig. 5A; $p = 0.12$, T-test), even at low doses. The FGF2 dose used for this comparison was sub-saturing, because higher levels resulted in excessive proliferation leading to cell detachment from the substratum. Expression of SMSA in these four day culture control conditions were consistent with the levels seen at four days in the time course assay (compare to Fig. 3). There was a distinct morphological change in cell shape in the cultures treated with TGFβ1 versus control conditions. The control cultures (Fig. 5C) and the FGF2 cultures (data not shown) had an elongated, myofibroblast-like phenotype. However, the choroid smooth muscle cells exposed to exogenous TGFβ1 had a flat morphology (Fig. 5D). The morphological changes in choroid cells treated with TGFβ1 are similar to the cellular hypertrophy observed in rat aortic smooth muscle cultures treated with TGFβ1 (Owens et al., 1988). TGFβ1 upregulates deposition of extracellular matrix proteins (Roberts et al., 1986; Ohji et al., 1993) and their integrin receptors (Heino et al., 1989), suggesting that the flatter appearance of the cells is reflective of an increase in adhesivity.

We focused our efforts on TGFβ1 and FGF2 effects on differentiation to determine if any other smooth muscle proteins in addition to SMSA were affected by these cytokines. Four day choroid cultures were exposed to control, TGFβ1 or FGF2 treatment then stained with antibodies to several muscle proteins (Table I, see Methods for scoring). The cultures were stained at day four, when about 30-40% of the cells expressed SMSA. This allowed for observation of differences in cytokine-induced effects above or below control levels. Desmin and MLCK staining increased in the cultures treated with TGFβ1 by approximately 50%. Staining for smooth muscle caldesmon, a calmodulin-binding protein (Frid et al., 1992) was not detectable in

control or FGF2 treated cultures, but was detectable in approximately 10% of the cells treated with TGFβ1. Calponin staining increased markedly such that nearly all cells appeared positive after exposure to TGFβ1. A similar result was seen with SMSA staining (see also staining in Fig. 5D). Staining for the muscarinic AChR m4 increased slightly in the TGFβ1 treated cells. The staining for the m2 receptor subtype is normally high in these smooth muscle cells, but was darker and more abundant in the TGFβ1 treated cells. In all cases, FGF2 suppressed expression of muscle proteins below control levels. Negative control antibodies showed no staining above background in all conditions tested. TGFβ1 treatment produced effects comparable to induction of differentiation, while FGF2 seemed to promote proliferation at the expense of differentiation.

If activin A and follistatin expression are dependent upon smooth muscle cell differentiation, then accelerating or inhibiting the differentiation state of the cells should have a corresponding effect on activin A and follistatin expression. Analysis of message levels for activin A and follistatin by RPA indicated that their expression in choroid cultures was significantly increased by TGFβ1 (Fig. 6). RNA values for activin A and follistatin obtained by RPA were normalized to the internal loading control, CHRPS. Fold change in RNA value was obtained by comparison with the control value. Activin A mRNA (Fig. 6B) increased nine-fold in choroid smooth muscle cells treated with TGFβ1 (p value <0.001, significantly different from control or FGF2 treated cultures, T-test). Follistatin mRNA levels (Fig. 6B), normally low in choroid cells, showed a two-fold increase in the presence of TGFβ1 (p value <0.001, significantly different from control or FGF2 treated cultures, T-test). In contrast, FGF2 treatment did not alter activin or follistatin mRNA levels significantly. The effect of TGFβ1 on mRNA levels was reflected at the protein level in the choroid cells as well. Immunostaining for follistatin is normally low in the choroid cells and did not increase detectably in the presence of TGFβ1 (data not shown). However, the TGFβ1 treated choroid cells showed a marked increase in immunoreactivity for activin A (Fig. 6D), with a higher density of reaction product (arrows in D), compared to control treated cultures stained for activin A (Fig. 6C).

TGFβ expression in choroid cultures

Since choroid cells in serum free medium without TGFβ1 differentiated to some degree (see Table I), we tested whether TGFβs were endogenously expressed and thus acting as autocrine factors. Using RT/PCR with pan-TGFβ primers, TGFβ mRNA was

detected in choroid cultures and in isolated choroid layer (Fig. 7). TGFβ mRNA was also detected in the iris anlage, which parallels previously published data for TGFβ1 and β2 mRNA in mammalian species (Tripathi et al., 1993). Interestingly, the ciliary ganglion, which contains the neurons that innervate the iris and choroid in chicken, was strongly positive for TGFβ. Amplification of mRNA product with these primers indicates the presence of mRNA encoding the conserved mature domain of the TGFβ family, although the specific TGFβ subtype is not indicated. The PCR product obtained with these primers was subcloned and sequenced to confirm RT/PCR specificity. The sequence of the subcloned fragment showed 98% homology to TGFβ3 described for chicken (data not shown).

Since TGFβ1 strongly accelerated choroid smooth muscle differentiation (Fig. 5 and Table I) and TGFβ message could be localized to target tissues in vivo and to choroid cells in vitro (Fig. 7), we wanted to determine if blocking TGFβ action in the cultures would prevent differentiation. A pan-specific blocking antibody against TGFβ proteins was used to test the role of TGFβ in choroid smooth muscle differentiation. Choroid-derived cells treated with antibody had a greater than 50% reduction in SMSA positive cells, relative to controls (Fig. 8A; $p < 0.001$, T-test) as well as an absence of activin A protein as measured by immunoreactivity (data not shown). Treatment with 1 ng/ml of TGFβ1 alone resulted in the expected increase in SMSA positive cells over control levels, consistent with a sub-saturating dose ($p = 0.1$ relative to control, T-test). The blocking antibody also partially inhibited the differentiation inducing effect of exogenous TGFβ1 when the antibody was added to the cultures together with the cytokine ($p = 0.009$, compared to both control and TGFβ1 treated cultures, T-test). In the presence of the TGFβ blocking antibody there was no significant effect on total cell number (Fig. 8B; $p = 0.79$, T-test), although there was a consistent if not significant decrease in cell number in the presence of TGFβ1 ($p = 0.2$ relative to control, T-test). Cultures were collected at four days, prior to complete differentiation so that increased or decreased changes in SMSA expression, relative to control values, could be readily determined. Treatment of the cells with a control antibody to laminin showed no effect on SMSA expression or total cell number relative to controls (data not shown).

Discussion

The data presented here describe the differentiation of choroid-derived cultures and the subsequent expression of activin A and follistatin. We find that the choroid cells express several proteins indicative of a smooth muscle phenotype and that expression of these markers is enhanced in the presence of TGFβ1. Expression of activin A and follistatin correlates with the acquisition of smooth muscle characteristics and is correspondingly enhanced with TGFβ1 treatment. Blocking TGFβ in the choroid cells with a specific antibody prevents progression of differentiation to the smooth muscle phenotype. Expression of activin A and follistatin is independent of continued innervation in these smooth muscle cultures taken from post-innervation choroid layer in vivo. Cumulatively, these data indicate that expression of the target-derived factor, activin A, that controls neuropeptide phenotype in ciliary ganglion neurons is dependent upon the differentiation of the vascular smooth muscle target cells themselves.

Vascular smooth muscle differentiation has been studied in other systems and in general involves a proliferative phase of cell growth followed by a differentiation phase with expression of contractile proteins and production of extracellular matrix proteins as the cells associate with forming vessels (Schwartz et al., 1986; Roberts et al., 1986; Folkman et al., 1992; Nehls et al., 1994). Arterial smooth muscle cells placed in culture normally undergo dedifferentiation characterized by an early loss of smooth muscle characteristics including loss of contractility and loss of expression of late stage markers of smooth muscle differentiation (Chamley-Campbell et al., 1981; Kocher et al., 1985; Rothman et al., 1992). Some of these traits are reacquired over time in culture as the cells progress through a fibroblast-like proliferative stage to a more differentiated state which parallels normal progression of differentiation in mesenchymal cells recruited to a smooth muscle fate in neovascularization. The choroid smooth muscle cells follow a similar pattern of dedifferentiation followed by redifferentiation in culture. In several vascular smooth muscle populations, SMSA expression has been correlated with differentiation state (Kocher et al., 1985; Owens et al., 1986; Pauly et al., 1992; Nicosia et al., 1995). Although this protein is not exclusively expressed in smooth muscle cells (Ronnov-Jessen et al., 1993; Kurosaka et al., 1995), it is the first to be re-expressed as the cells differentiate in culture (Owens et al., 1986; Skalli et al., 1989). Our results of SMSA expression in choroid cells coincide with published data for other smooth muscle derived cell types (Pauly et al., 1992; Rothman et al., 1992). Most studies examining smooth muscle differentiation

use predominantly this marker to assess differentiation (Kocher et al., 1985; Schwartz et al., 1986; Duband et al., 1993; Shanahan et al., 1993). We have used this as well as late stage markers of muscle phenotype to determine the extent of differentiation in our population. We observed expression of cytoskeletal associated proteins SMSA, MLCK, calponin and calmodulin as well as the muscarinic AChR in the choroid cells which indicates not only a smooth muscle phenotype, but also an advanced stage of differentiation.

Treatment of the choroid cultures with TGFβ1 accelerates differentiation and pushes the cells further into the smooth muscle phenotype. Previous studies with TGFβ1 and vascular smooth muscle cells have used primarily SMSA as an indicator for TGFβ1 effects. Rather than relying on a single marker, we expanded this analysis to include several proteins indicative of a smooth muscle phenotype and showed that TGFβ1 is able to upregulate essentially all proteins examined. TGFβ1 increased immunoreactivity for SMSA in a number of vascular smooth muscle cultures (Björkerud et al., 1991; Verbeek et al., 1994) and we saw a similar effect in the choroid cells. In addition, several smooth muscle markers were upregulated in the presence of TGFβ1, including caldesmon which is normally detected only in mature muscle in vivo and not in cultures (Frid et al., 1992). One important observation about the differentiation induced by TGFβ1 treatment is that essentially all cells expressed SMSA, calponin and the m2 AChR, while other markers tested showed only a partial population response. Longer exposure to TGFβ1 might allow all cells to achieve the same degree of differentiation. Additionally, it may be that TGFβ1 alone is insufficient to induce 100% expression of the proteins such as caldesmon, desmin or MLCK. Co-culture with endothelial cells may provide additional signals for further differentiation. One other possibility is that our cultures represent a mixed population of smooth muscle cells that are differentially responsive to TGFβ1 as has been proposed for smooth muscle cells from the tunica media of elastic arteries (Topouzis et al., 1996), vascular smooth muscle cells (D'Amore, 1993) or the variable microvascular pericyte populations (Nehls et al., 1991).

In contrast to the effects seen with TGFβ1 treatment, FGF2 significantly inhibited the differentiation of the choroid cells in culture. We saw a marked absence of smooth muscle proteins in cultures grown in the presence of this cytokine. FGF2 has been shown to promote vascular smooth muscle cell migration (Bilato et al., 1995) and choroidal angiogenesis (Soubrane et al., 1994). In addition, when brain microvessel

pericytes, smooth muscle-like cells derived from the capillary walls, are treated with FGF2 they show a low level of SMSA expression similar to what we observed in our cultures (Verbeek et al., 1994). Our study suggests that in the choroid smooth muscle cells, the differentiation-suppressing effects extend beyond SMSA to encompass several markers of smooth muscle differentiation, including the mAChR. We did observe an increase in cell numbers in FGF2 treated cultures and found that at saturating doses the cells proliferated to such an extent that they contracted and peeled off the substratum. Our data suggest that FGF2 is involved in the early phase of myfibroblast proliferation prior to onset of differentiation, and that a balance ensues between proliferative versus differentiative cues as the cells grow in culture.

An additional approach to inhibit differentiation in choroid smooth muscle cells involved using a TGFβ1 pan-specific blocking antibody to block potential autocrine stimulation. Many cells in culture are able to produce mRNA and protein for at least one of the TGFβ family members, however not all are able to produce active forms of the molecule (Roberts et al., 1990; Pfeffer et al., 1994). The fact that we see a functional block of differentiation with TGFβ blocking antibody treatment suggests that not only is TGFβ produced by the choroid cells in culture, but it is in an active form normally participating in autocrine regulation of differentiation in these cells. The blocking antibody we used does not distinguish among the three forms of TGFβ described for muscle cells, but blocks all three with similar efficacy. We did not observe a complete inhibition of differentiation, but this could be due to antibody inefficiency or to TGFβ1 bound to the extracellular matrix that is active, but unavailable for inhibition by the antibody. An additional explanation is that more than one molecule is involved in the differentiation process and the partial inhibition of differentiation reflects blockade of only the TGFβ component. TGFβ1 has a positive regulatory effect on its own production (Rasmussen et al., 1995) so that treatment of the choroid cells with exogenous TGFβ1 may initiate an autocrine loop that pushes the cells toward rapid acquisition of a differentiated phenotype. In support of this we were able to detect TGFβ1 message in choroid cultures as well as in the choroid layer itself. TGFβ1 has been detected in several ocular tissues (Saha et al., 1992; Tripathi et al., 1991) and we are able to detect message for the conserved domain of the TGFβ family in the choroid layer as well as the choroid cells in culture. We have not localized the specific isoform(s) expressed in the choroid layer, but the mRNA signal does suggest that TGFβ family members are expressed locally.

It is interesting to note that in all circumstances where the differentiation state of the choroid cells was manipulated, activin A expression and to a certain degree follistatin expression, paralleled this differentiation . Activin A levels were much higher than follistatin under all conditions tested which is consistent with their role as neuropeptide regulators in ciliary ganglion neurons. Several markers of the smooth muscle phenotype were detectable in our cultures and activin A and follistatin message were at their peak when the markers were fully expressed. Indeed, with the TGFβ1-induced increase in differentiation, we saw a corresponding induction of both activin A and follistatin message. In contrast when the differentiation of the cells was inhibited with FGF2 to sustain an undifferentiated, proliferative state, we saw elimination of nearly all smooth muscle proteins as well as an absence of activin A protein. An additional method of inhibiting differentiation utilized a pan-TGFβ blocking antibody. In the presence of this inhibitory antibody, smooth muscle proteins and activin A were significantly reduced. Cumulatively these data indicate that expression of activin A and follistatin are closely tied to the differentiation state of the cells. More importantly blocking progression of differentiation in choroid cultures prevents expression of activin A which indicates that target differentiation is required for the production of this protein that controls neuropeptide phenotype in choroid neurons.

One important component of the target expression of neurodifferentiation molecules is the influence of the neurons themselves on target development and on subsequent expression of target-derived factors. An example of this has been described for the sympathetic innervation of the rat footpad sweat gland (Landis, 1991; Schotzinger and Landis, 1994; Habecker et al., 1997). As the sweat gland cells develop, they produce sweat gland factor (SGF) that initiates a phenotypic switch from noradrenergic to cholinergic in the innervating neurons. SGF is dependent upon innervation for induction of its expression in the target tissue. This has been demonstrated in vitro and in vivo (Habecker and Landis, 1994; Habecker et al., 1995). It is interesting to note that differentiation of the choroid cells in culture and subsequent expression of the neuroeffectors, activin A and follistatin, is independent of continued innervation. The cultures are derived from E14 choroid layer, which is past the development point where neurons contact the smooth muscle target . In contrast, cultures derived from E5 choroid layer consist of a mixed population of endothelial-like, pigmented epithelium-like and mesenchymal cells, which is distinct from the E14-derived cultures. However the E5 cultures express low, but detectable levels of activin A (data not shown), even though there is no evidence to indicate that the choroid layer is innervated at this point

and detectable neuronal processes are not observed until E6 (Meriney and Pilar, 1987). These data point to the induction of activin A expression in the choroid layer as innervation independent. In addition, there are less than 1% contaminating endothelial cells present in the choroid cultures, suggesting that expression of activin A and follistatin are not induced by direct co-culture with endothelial cells.

In summary our data indicate that expression of activin A and follistatin, proteins that control neuropeptide phenotype in ciliary ganglion neurons, is coordinated with the differentiation of the choroid smooth muscle cells that produce them. These data suggest a model for how neuron/target interactions may occur in the developing ciliary ganglion and its ocular target, the choroid layer. Mesenchymal cells in the choroid layer differentiate in a TGFβ-dependent fashion and participate in the formation of new blood vessels as the choroid layer develops. As differentiation proceeds the smooth muscle cells produce high levels of activin A and low levels of the inhibitor follistatin. When the choroid neurons contact the smooth muscle cells around the blood vessels they receive not only trophic support (Finn et al., 1997), but neurodifferentiation signals. The activin A produced by the differentiated smooth muscle cells induce somatostatin expression in the choroid neurons which are able to manifest their full, functioning phenotype under the influence of the target. This view of neuron/target interactions in the developing choroid layer fits well with the model of choroid layer blood vessel formation proposed by D'Amore (D'Amore, 1994). The current study suggests an added dimension of retrograde factors produced by the smooth muscle cells that affect the growth and differentiation of the innervating neurons. However additional questions remain, for example, does innervation by the neurons promote association of later stages of blood vessel formation? Do post-injury blood vessel growth and reinnervation share similar mechanisms with the initial development of the system? If so, is neuropeptide phenotype regulated in a similar manner? The data presented here have laid the foundation for addressing these questions and contribute to the understanding of how target-derived signals that affect neurodifferentiation can be regulated in developing target cells.

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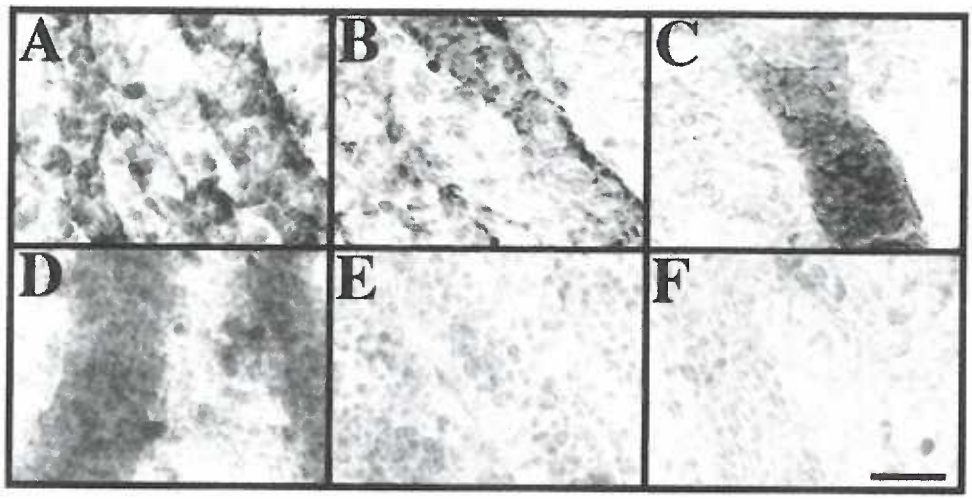

Figure 1. Expression of smooth muscle markers in isolated choroid whole mounts.

Choroid layers were collected from embryonic day 14 embryos and the RPE was removed. Whole mounts were stained for (A) SMSA, (B) MLCK, (C) calponin and (D) muscarinic AChR, m2 subtype using PAP immunohistochemistry. Normal rabbit serum (E) and normal mouse serum (F) negative controls are shown for comparison. Bar is equal to 50 μm.

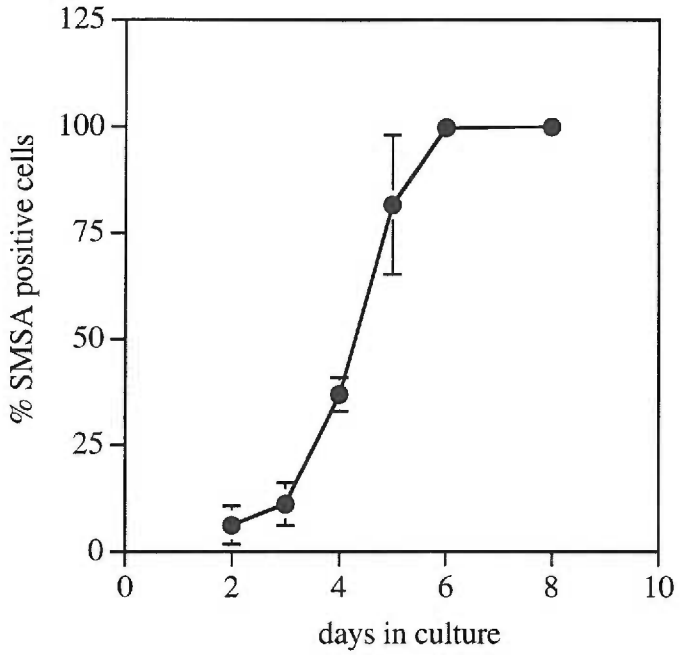

Figure 2. Time course of SMSA expression in choroid-derived cultures. E14 choroid cells were grown in chick serum containing medium for two days and then switched to serum free conditions. Cells were confluent by day 5 in culture. Cultures were fixed and stained for SMSA immunoreactivity. The percentage value represents the number of immunoreactive cells divided by the total cell number counted in 5 fields of view per dish. Total cell number counts were determined after staining the nuclei with Hoechts. Each point represents the mean of 5-10 dishes pooled from at least three different experiments, with error bars representing the standard deviation from the mean.

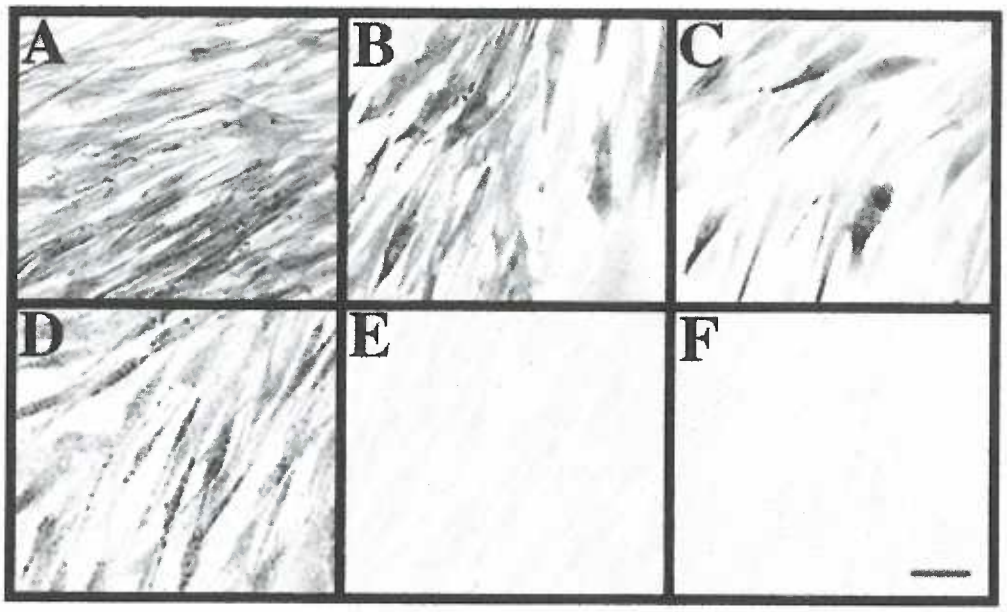

Figure 3. Expression of smooth muscle markers in choroid-derived cultures.

Cultures were grown for two days in serum then switched to serum free conditions for 4 days as in Fig. 2. Cultures were fixed and stained for (A) SMSA, (B) MLCK, (C) calponin and (D) muscarinic AChR, m2 subtype immunoreactivity. Normal rabbit serum (E) and normal mouse serum (F) negative controls are shown for comparison. Bar is equal to 25 μm.

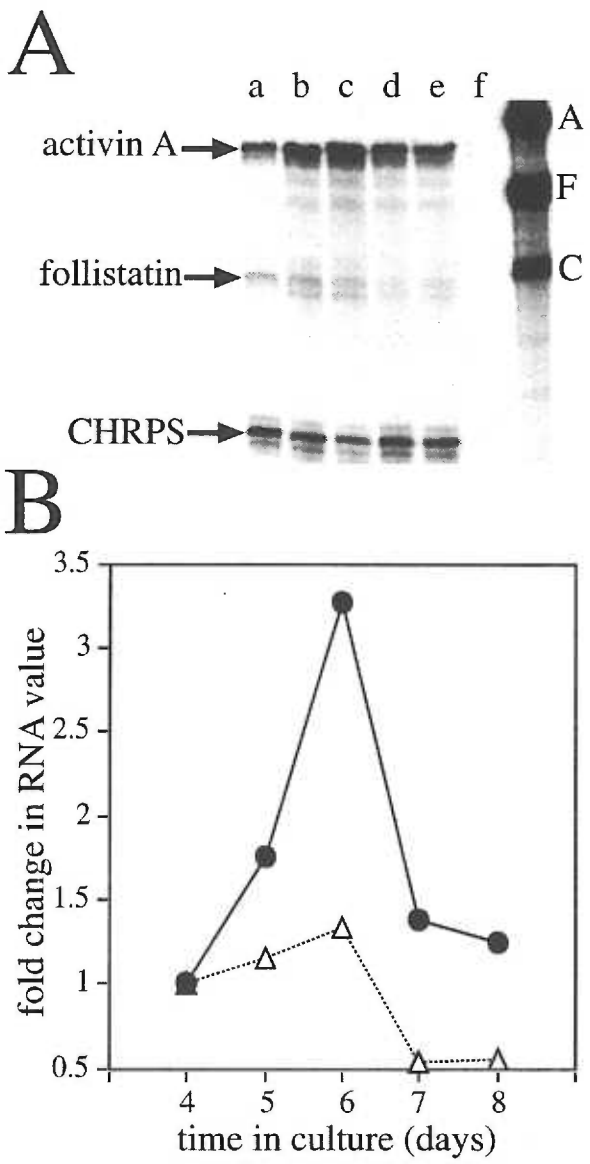

Figure 4. Activin A and follistatin expression in choroid cultures. (A) RPA of activin A and follistatin where arrows indicate the protected fragments for activin A, follistatin and CHRPS (internal loading control). Lanes a through e contain 20 μg of total RNA from cultures collected from four to eight days. Lane f contains tRNA as a negative control and the unprotected probes are to the right. (B) RNA values for activin A (closed circles) or follistatin (open triangles) obtained by normalization to the internal loading control, CHRPS. Message levels for both peak at days in culture, although follistatin is barely detectable. This is a representative experiment of 4 repeats.

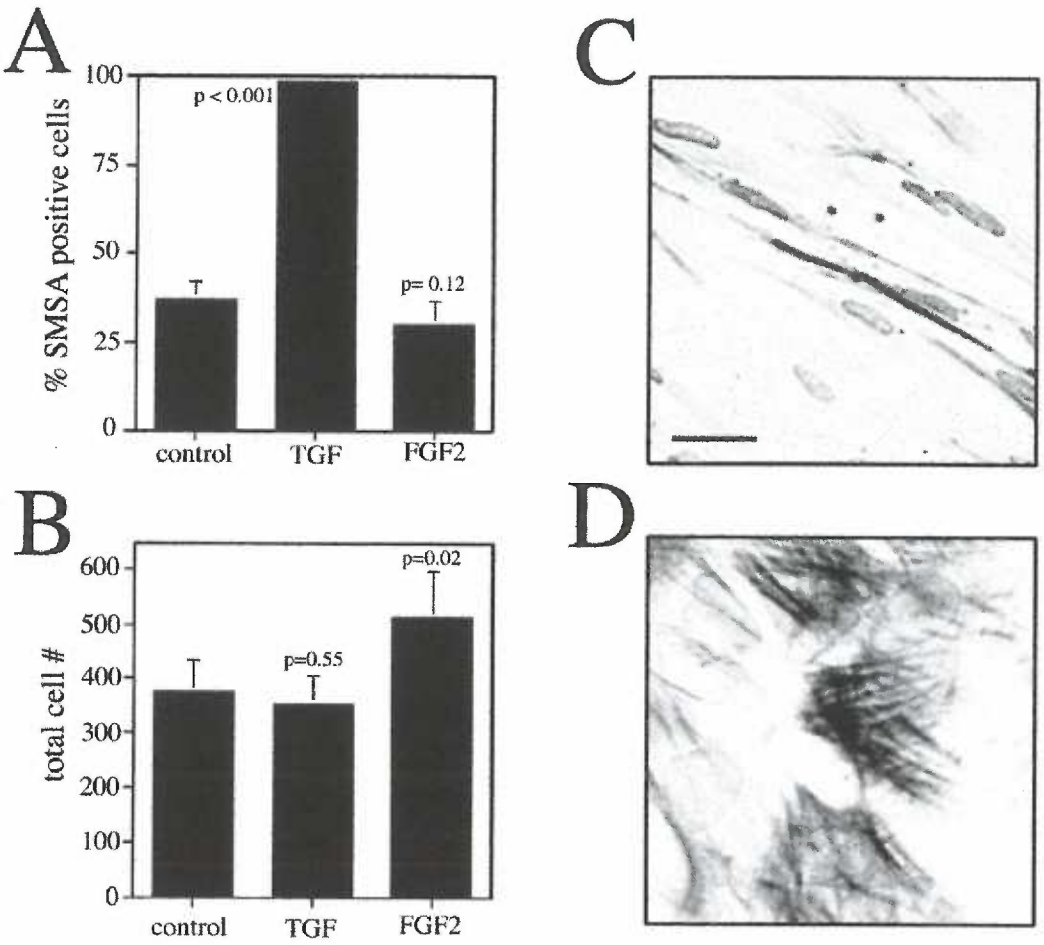

Figure 5. TGFβ1 enhances and FGF2 suppresses expression of SMSA in choroid cultures.

The percentage of SMSA positive cells (A) and the total cell number (B) is shown for choroid cultures grown two days in serum then two days in serum-free conditions with either 10 ng/ml of TGFβ1 or 1 ng/ml FGF2. The cells were fixed and stained for SMSA. The percentage value represents the number of immunoreactive cells divided by the total cell number counted in 5 fields of view per dish. Total cell number counts were determined after staining the nuclei with Hoechts. The values represent the mean of at least 6 replicates pooled from three different experiments. Error bars represent the standard deviation from the mean and P values represent statistical difference relative to control. Sister cultures were fixed and stained for SMSA to reveal the morphology changes seen under serum free (C) and TGFβ1 treated (D) conditions. Bar equals 50 μm.

TABLE I. Immunohistochemical staining ofsmooth muscle markers in choroid-derived cells grown in control, TGFβ1 (10 ng/ml) or FGF2 (1 ng/ml)

marker	control	TGFβ1	FGF2
desmin	+	+++	-
MLCK	+	+++	-
caldesmon	-	+	-
calponin	+	+++++	-
α-SMSA	++	+++++	+
AChR m4	+	++	-
AChR m2	++++	+++++	+

TGFβ1, transforming growth factor β 1; FGF2, fibroblast growth factor, 2; MLCK, myosin light chain kinase; α-SMSA, smooth muscle specific α actin; AChR m2 or m4, acetylcholine receptor subtypes

- , no positive cells; +, 1-20% positive; ++, 21-40% positive; +++, 41-60% positive; ++++, 61-80% positive; +++++, 81-100% positive

Cultures were grown for 2 days in CS containing medium, then switched to SF medium +/- cytokines. Cultures were fixed 48 hours later and stained for smooth muscle markers

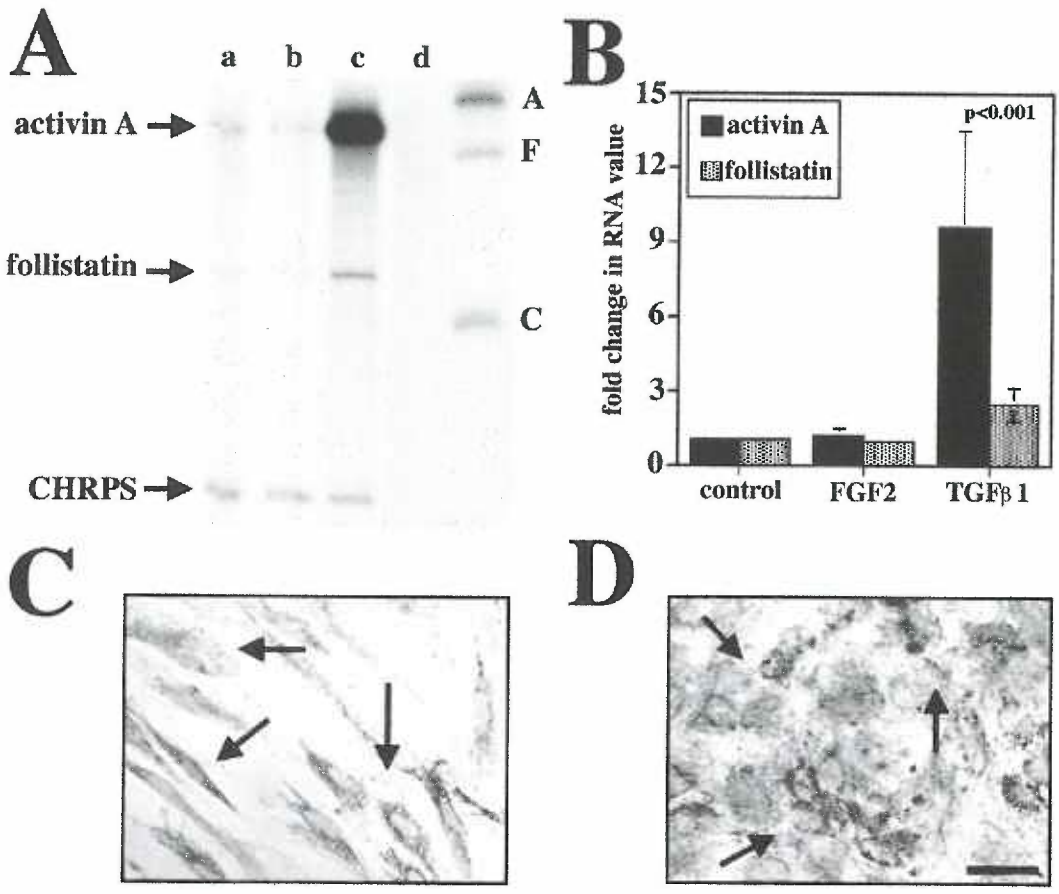

Figure 6. TGFβ1 increases activin A mRNA in choroid cultures. RPA of choroid cultures where arrows indicate the protected fragments for activin A, follistatin and the internal loading control, CHRPS (A). Lanes a-d contain total RNA purified from a, control conditions; b, 1 ng/ml FGF2 treated cultures; c, 10 ng/ml TGFβ1 treated cultures; d, tRNA as a negative control. Lane e contains unprotected probes. Bar graph of the normalized values for activin A (closed bars) and follistatin (stippled bars), where control levels of each are given the value of 1 (B). Fold changes in RNA are the mean value pooled from four independent experiments. Error bars represent the standard deviation from the mean. Lower panels show sister cultures (5 days) grown in control (C) or 10 ng/ml TGFβ1 (D), fixed and stained for activin A immunoreactivity. Bar equals 25 μm.

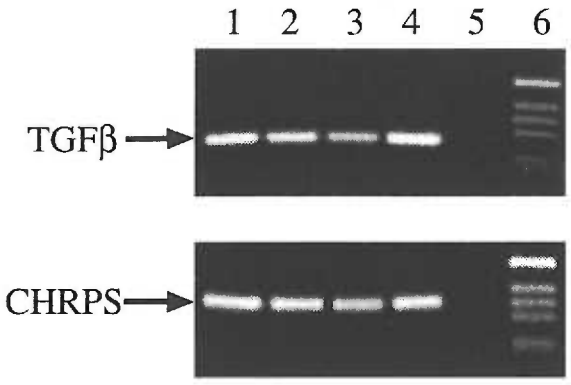

Figure 7. Expression of TGFβ mRNA in ocular tissues.
RT/PCR detection of TGFβ mRNA in lane 1, E14 iris; lane 2, E14 choroid; lane 3, E14 ciliary ganglion; lane 4, 6 day choroid cultures; and lane 5, reverse transcriptase minus negative control. The data is representative of three repeats with similar results. The PCR product was subcloned and sequenced to confirm primer specificity.

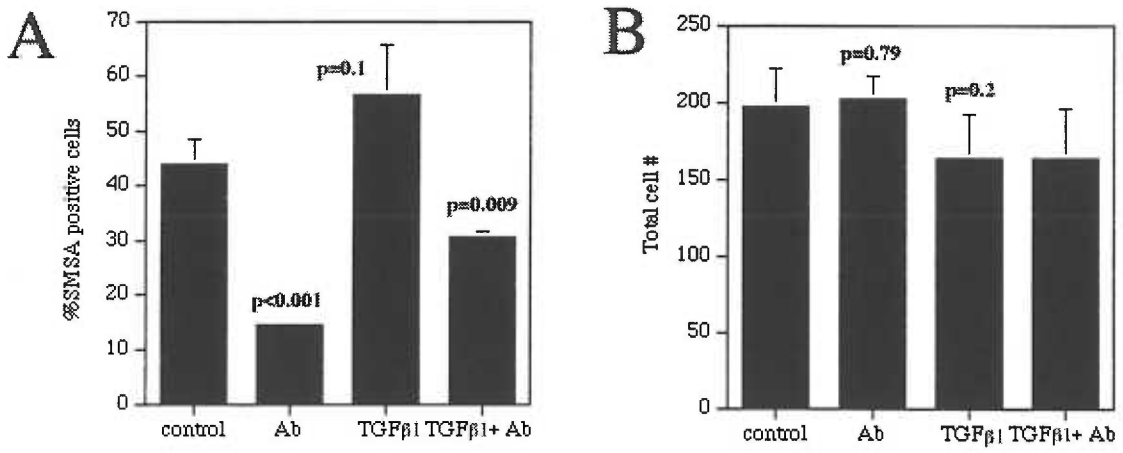

Figure 8. Blocking TGFβ action suppresses choroid cell differentiation. The percentage of SMSA positive cells (A) and the total cell number (B) is shown for choroid cultures grown for two days in serum then switched to serum free medium plus or minus antibody. The pan-specific blocking antibody (50 μg/ml) and TGFβ1 (1 ng/ml) were added daily to serum free medium. The cells were fixed and stained for SMSA. The percentage value represents the number of immunoreactive cells divided by the total cell number counted in 5 fields of view per dish; total cell number counts were determined after staining the nuclei with Hoechts. Bars represent the mean of three replicates from a single representative experiment (out of three). Error bars represent the standard deviation from the mean.

CHAPTER 3

Activin A and follistatin control expression of somatostatin in the ciliary ganglion in vivo.

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Summary

An important developmental question concerns whether neurotransmitter phenotype is an inherent property of neurons or is influenced by target tissues. This issue can be addressed in the avian ciliary ganglion (CG) which contains two cholinergic populations, ciliary and choroid neurons, which manifest differential expression of the co-transmitter, somatostatin. One explanation for this distinction is that the neurons receive different retrograde signals from the separate targets they innervate. Both populations are capable of expressing somatostatin when cultured with choroid target cells, choroid-cell conditioned medium or activin A. Activin A and its inhibitor, follistatin, have differential expression patterns in target tissues such that activin A is in excess in the choroid, innervated by somatostatin-positive choroid neurons, but follistatin is in excess at iris/ciliary body, innervated by somatostatin-negative ciliary neurons. The present study tests the hypothesis that combined effects of target-derived activin and follistatin are responsible for selective expression of somatostatin in choroid neurons. Intraocular injection of activin A or follistatin (300 ng injected at E10/E11) resulted in a 39% increase or a 23% decrease, respectively, in somatostatin positive neurons relative to controls. Chorioallantoic membrane application of follistatin (1 μg daily from E7-E13) reduced somatostatin positive neurons by 54%. Neuron number and target tissue development were unaffected by treatment. These data underscore the importance of target-derived factors in controlling neurotransmitter phentoype. In addition, they suggest a novel mechanism for this process in vivo: selective expression of inhibitor, follistatin, in the targets regulates availability of somatostatin-stimulating factor, activin A.

Introduction

Retrograde signalling from target to neuron is an important component of neuronal differentiation and development. For example, target-derived neurotrophic factors such as nerve growth factor directly influence neuronal survival in several central and peripheral nerve populations during the period of developmental cell death (reviewed in Oppenheim, 1991; Ip and Yancopoulos, 1996). In addition target effects on neuronal development have been demonstrated at the level of potassium channel composition in developing ciliary ganglion neurons (Dryer, 1994). Another aspect of neuronal signalling that is influenced by the target is neurotransmitter phenotype. The primary neurotransmitter and any peptide co-transmitters that a neuron produces are components of the chemical language that a neuron uses to communicate with its target. In a series of studies Landis and coworkers have shown that sympathetic neurons innervating sweat glands in the rat footpad undergo a transition from noradrenergic to cholinergic-phenotype under the influence of a target-derived factor (Schotzinger and Landis, 1994; Habecker et al., 1997).

The avian ciliary ganglion is an ideal system in which to examine the influence of retrograde signals on neuronal differentiation, specifically with regard to neurotransmitter phenotype. This parasympathetic ciliary ganglion consists of two populations of neurons: choroid neurons that innervate vascular smooth muscle and ciliary neurons that innervate the striated muscle in iris/ciliary body (Marwitt et al., 1971). Both populations utilize acetylcholine as their primary small molecule transmitter, but only the choroid neurons express the neuropeptide somatostatin as a co-transmitter (Epstein et al., 1988; De Stefano et al., 1993). Somatostatin expression in choroid neurons is not reliably detected until embryonic day 8 (E8), after contact with the target has been established (Meriney and Pilar, 1987; Smet and Rush, 1993). The full complement of somatostatin-positive neurons is observed at E14. This difference in peptide expression may stem from distinct retrograde signals produced by the separate targets.

Previous studies from our laboratory have suggested that ciliary ganglion targets do, indeed, produce retrograde signals that control neuropeptide expression in the neurons. When E8 ciliary ganglion neurons are placed in culture, somatostatin expression is not detected unless the neurons are co-cultured with choroid-derived smooth muscle or with conditioned medium from the smooth muscle (Coulombe and Nishi, 1991).

Interestingly, ciliary neurons can also be induced to express somatostatin by the choroid conditioned medium, indicating that the somatostatin-stimulating activity is instructive rather than permissive. The somatostatin-stimulating activity from choroid-conditioned medium was later identified as activin A (Coulombe et al., 1993). Messenger RNA and protein for activin A have been detected in both targets (Darland et al., 1995). However an endogenous inhibitor of activin A, follistatin, is detected predominantly in the iris/ciliary body target. These studies also demonstrated that activin A and follistatin are expressed in a temporal and spatial manner that is consonant with a role for these factors in regulating somatostatin neuropeptide expression in ciliary ganglion neurons (Darland, et al., 1995).

These data suggest the hypothesis that a balance between the levels of activin A and follistatin in the targets regulate neuronal somatostatin expression. Specifically, excess activin A from the choroid layer induces somatostatin in the choroid neurons, but the presence of follistatin from the iris-ciliary body prohibits expression of somatostatin in the ciliary neurons by blocking activin A. Activin A and follistatin can control somatostatin expression in cultured ciliary ganglion neurons and are expressed at the right time and place to mediate this function in vivo; however, it is important to establish their role as regulators of neurotransmitter phenotype in the normal developmental context. In the current study we address these issues by manipulating the neuropeptide phenotype in ciliary ganglion neurons in vivo.

Results

Detection of somatostatin in developing ciliary ganglia

Somatostatin expression was assayed by immunohistochemistry using a highly sensitive double peroxidase/anti-peroxidase (PAP) detection method. The immunoreactive choroid neurons were generally 10-15 μm in diameter and predominantly located within the perimeter region of the ganglion. They had dark, punctate reaction product that generally filled the cell body and surrounded the nucleus (arrowheads in Figure 1A,B). In contrast, the neurons that had no reaction product were 20-30 μm in diameter. These larger ciliary neurons were located predominantly in the center of the ganglion (arrows, Figure 1A,C). A higher magnification of choroid neurons darkly stained for somatostatin (arrowheads in 1B) were compared with ciliary neurons that were negative for the reaction product (arrows, 1C). Comparative staining with normal rat serum showed no reaction product (Figure 1D).

In order to deliver exogenous activin A and follistatin to developing embryos we adopted the shell-less technique of growing chick embryos. This ex-ovo paradigm allows for direct manipulation of the embryos as they grow (Dunn et al., 1981; Finn et al., 1997 in press). Embryos are cracked into culture tripods at embryonic day 3 (E3) and develop at a rate similar to that of intact embryos. In order to ensure that somatostatin expression was unaltered during growth under these conditions, we determined the developmental time course of somatostatin expression in a the shell-less culture system. Ganglia from the shell-less equivalent of E8 to E15 embryos were stained for somatostatin and scored for the percentage of positive neurons relative to the total number of neurons scored (Figure 2). We observed an increase in somatostatin expression correlated with developmental age. At E8, less than 10% of the neurons are immunoreactive for somatostatin. The two neuron populations in the ganglion are of a similar size at this stage of development. This corresponds to the beginning of the cell death period in the ganglion (Landmesser and Pilar, 1974b) which overlaps with the induction of somatostatin expression (Smet and Rush, 1993). Cell death is essentially complete at E14 at which point we observed that approximately 50% of the neurons were immunoreactive for somatostatin. By this period of development the ciliary and choroid neurons were distinguishable by the collective criteria of soma size, location within the ganglia and presence or absence of somatostatin. These distinguishing characteristics are maintained through adulthood (DeStefano et al., 1993).

Intraocular injection of recombinant activin A and follistatin

If activin A induces somatostatin in ciliary ganglion neurons in vivo and follistatin blocks this induction, then altering the balance of these factors during development should result directly in changes in somatostatin expression levels in the neurons. Aqueous humor clears rapidly from the anterior and posterior chambers of the eye, however injection behind the lens prevents rapid clearing via this outflow system (Millar and Kaufman, 1995). We therefore altered local levels of activin A or follistatin by injecting exogenous recombinant protein onto the vitreous body behind the lens. Preliminary tests with this injection technique showed that fast green tracking dye was retained on the vitreous body for greater than 24 hours post-injection (data not shown). Recombinant activin A (300 ng) or follistatin (300 ng) was injected into E10 or E11 eyes. The embryos were allowed to develop 4 additional days and the ciliary ganglia were collected and examined for somatostatin immunoreactivity. In order to determine if the injection method itself altered neuron number or percent somatostatin positive neurons, a series of control injected eyes were compared with uninjected eyes from the same animal. Ganglia from control injected eyes had 4929$\pm$611 neurons with 42.9$\pm$3% somatostatin-positive, while ganglia from uninjected eyes had 5303$\pm$723 neurons with 49.3$\pm$7% somatostatin-positive (n=4 for both). Since there was a slight, though not statistically significant reduction in both neuron number and somatostatin positive cells (p=0.46, neuron number; p=0.13, % positive), all injected eyes were compared to the injection controls rather than the internal uninjected eye.

Table I shows the results of this injection series. The percentage of somatostatin positive neurons observed in control injected ganglia was 47.7%$\pm$6. In contrast, the activin A treated ganglia had 66.4$\pm$7.5% somatostatin positive cells, an increase of 39% relative to control levels (see Figure 3). Substantial data exists to indicate that only choroid neurons, which comprise approximately 50% of the neuronal population in the ganglia, express somatostatin (Epstein et al., 1988; De Stefano et al., 1993). Presumably, this increase in somatostatin-positive neurons represents an induction of somatostatin expression in the ciliary neurons, where exogenous activin A has overcome the inhibition of locally expressed follistatin. Control injected ganglia have somatostatin staining patterns similar to untreated ganglia (compare Figure 4A/B with Figure 1). In ganglia injected with activin A, there was an increase in somatostatin-positive neurons, most notably in the central regions of the ganglia where somatostatin

positive neurons are normally rarely detected (arrows, Figure 4C/D). Higher magnification showed diffuse reaction product within the soma (Figure 4D), relative to the dense staining seen in the smaller choroid neurons. This may be due to the larger size of the ciliary neurons or to a less efficient production of somatostatin in response to the inductive signal of activin A. An opposite result was obtained with injection of the inhibitor, follistatin. In the presence of inhibitor, only 36.8±10.7% somatostatin-positive neurons were detected. This is a decrement of 23% relative to controls (see Figure 3). In these ganglia, many of the small perimeter neurons, which are normally positive for somatostatin, showed no reaction product (arrowheads, Figure 4E/F).

Chorioallantoic membrane delivery of follistatin

In order to increase availability of follistatin to the terminals of choroid neurons, we also delivered follistatin systemically by application to the chorioallantoic membrane (CAM). The intraocular injection method for follistatin probably allowed only limited access of the more anterior choriod neuron terminals to exogenous protein due to reduced diffusion through the vitreous body. Because the choroid layer is a highly vascularized sheath, we sought to capitalize on its intimate connection with the developing circulatory system. Follistatin delivered via this method should have increased accessibility to the terminals of choroid neurons which innervate the vascular smooth muscle of the choroid layer. This method has been used successfully to deliver neurotrophic factors to several autonomic neuron populations during the period of developmental cell death (Oppenheim et al., 1991; Finn et al., 1997, in press). From E7 to E13, 1μg of recombinant follistatin was applied daily onto the CAM. With a standard egg volume of 50 ml, this constitutes approximately 20ng/ml of follistatin per day. Even if diffusion is only 50% efficient, this level is within the range of effective dose for blocking activin A effects in vitro (Darland et al., 1995; Link and Nishi, 1997, submitted). The systemic delivery of follistatin had no apparent effect on gross development of the embryo. There was no statistical difference in the number of neurons per ganglion between control and untreated CAM embryos (control, 6688±1817; untreated, 7138±1435; p= 0.64). Similarly, no difference in the percent of positive neurons was observed (control, 52.2±4%; untreated, 49.1±2%; p=0.11) indicating that the windowing and CAM application do not affect somatostatin expression or neuron number (n=6 for both). There was no apparent effect of treatment on mean cell body diameter among untreated, control, or follistatin CAM

application ganglia (data not shown). Follistatin-treated ganglia were collected at E14, sectioned and examined for somatostatin immunostaining.

Table II lists the results of CAM application of follistatin. Control ganglia had 1276±271 neurons with 54±4% somatostatin positive. In striking contrast, the follistatin treated ganglia had 1228±280 neurons with only 25±9% somatostatin-positive, representing a 54% reduction in the percentage of somatostatin-positive neurons relative to control (see Figure 3). Clusters of small choroid neurons in the perimeter showed little to no reaction product (Figure 4E/F). Neurons with detectable perinuclear staining remained, but they were often among a cluster of small, negative neurons in the perimeter (arrowheads, Figure 4F). A separate set of animals given 750 ng/day of follistatin showed a 37.5% reduction in somatostatin levels in the ganglia relative to controls, but with high variability (control = 53.8±3.7% somatostatin positive neurons; follistatin treated = 33.6±17%, data not shown). The difference in magnitude of effect with the lower protein level suggested that the inhibitory effect of follistatin given via the CAM was dose-dependent.

Development of iris-ciliary body and choroid target tissues in the presence of exogenous activin A and follistatin

In order to determine if ciliary ganglion targets were affected by CAM follistatin treatment, we examined iris/ciliary body and choroid layer for normal development. Whole eyes from E14 CAM application were collected for immunohistochemical analysis and 10 μm cross-sections were cut through the full diameter of the lens and stained for the smooth muscle marker, smooth muscle specific actin (SMSA) (Figure 5). At E14 the iris is a mixture of smooth and striated muscle reflecting the progressive transition to a predominantly striated tissue later in development (Link and Nishi, submitted, 1997). No change in pigmented epithelium (PE) or stromal (ST) smooth muscle staining pattern in follistatin-treated embryos (Figure 5B) was detectable relative to controls (Figure 5A). Smooth muscle staining in the ciliary body was similar in control and treated tissues (data not shown). The vascular choroid layer, situated between the retinal pigmented epithelium (RPE) and the sclera (SC), showed normal blood vessel staining in both control (Figure 5D) and follistatin (Figure 5E) CAM embryos. Some swelling and disruption of the RPE layer was noted in some but not all of the follistatin treated eyes. Normal mouse serum staining of each target was shown for comparison (Figure 5C, F). One eye from each animal was processed for total

RNA purification. Activin A and follistatin levels were unchanged in whole eyes of control versus treated animals (data not shown). This indicated that there was no gross upregulation or reduction of message for endogenous activin A or follistatin in response to altering exogenous levels.

Discussion

In the current study, we tested the target-derived factors activin A and follistatin for their ability to control somatostatin expression levels in the developing ciliary ganglion in vivo. With intraocular activin A injections we observed an increase in the percentage of ciliary ganglion neurons that expressed somatostatin. In contrast, follistatin application with two different methods significantly reduced the percentage of ciliary ganglion neurons that expressed somatostatin. These data suggest a novel mechanism for regulating neurotransmitter phenotype in vivo, where expression of peptide levels is regulated not by varying levels of the inducing activity, but by selective expression of an inhibitor. Moreover, these data describe a novel in vivo function for activin A and follistatin in the target-dependent regulation of neuronal differentiation.

Activin A and follistatin have been shown to have opposing effects in a number of in vivo systems. This opposition is mediated via the ability of follistatin to bind to activin A, thereby inhibiting the interaction between activin A and its serine/threonine kinase receptor (Kogawa et al., 1991; Sumitomo et al., 1995; deWinter et al., 1996). Activin A often functions as an inducer of specific phenotypes. For example, in early Xenopus development, activin A can induce the induction of mesoderm (Hemmati-Brivanlou and Melton, 1992; LaBonne and Whitman, 1994). However, activin A also acts in the mature animal to regulate the release of follicle stimlulating hormone from the anterior pituitary (Ling et al., 1985; Nakamura et al., 1990). In both cases, follistatin inhibits the effect of activin A. This pattern of antagonistic roles for these cytokines is reinforced with the data presented here. A model for the possible function of activin A and follistatin in regulating transmitter phenotype is presented in Figure 6. The vascular smooth muscle cells of the choroid layer express the somatostatin-inducing molecule, activin A, and do not express significant levels of the activin A inhibitor, follistatin. The excess of inducer results in somatostatin expression in the choroid neurons that innervate the choroid smooth muscle cells. Activin A is also expressed in the iris/ciliary body target, however enough follistatin appears to be expressed in the striated muscle target cells to prohibit induction of somatostatin expression within the innervating neurons. This "balance hypothesis" can account for the differences in neuropeptide expression observed in the developing ganglion. It is important to note that although activin A induces somatostatin in this developing system, it is the selective and localized expression of the inhibitor, follistatin, that determines the phenotypic outcome in the neurons.

Our injection and CAM application studies were undertaken in an attempt to shift the balance of inducer and inhibitor present in the system in order to alter somatostatin levels in the ciliary ganglion neurons. Increasing activin A protein levels by injection evoked a significant alteration in the profile of neurons expressing the neuropeptide somatostatin. The observed increase in somatostatin positive neurons was likely due to an upregulation of neuropeptide in the ciliary neurons, which normally do not express this co-transmitter. The staining method used in this study did not give a quantitative analysis of peptide levels within the individual cells, so it remains unclear whether the choroid neurons --normally positive for somatostatin-- have increased peptide expression in response to exogenous activin A. It is apparent that with injection of activin A, however, almost 40% of the neurons that are somatostatin-negative under control conditions are able to express somatostatin in the presence of this inducer. The total number of neurons observed in treated conditions was not significantly different from injected controls. Therefore, the differences in somatostatin expression observed under the influence of activin A or follistatin cannot be due to the differential loss or survival of either the choroid or ciliary neurons, respectively. The percentage of somatostatin-positive neurons for control and untreated ganglia is consistent with previously published data (Smet and Rush, 1993). There is no evidence that activin A promotes survival of either population of ciliary ganglion neurons (Coulombe et al., 1993), although there has been a report of a survival effect mediated by activin A on P19 cells (Schubert et al., 1990). The fact that not all the neurons in the ganglion were capable of responding to the inductive effects of activin A may be due to the fact that levels of activin A achieved by anterior injection are insufficient to overcome the inhibiting signal of follistatin from the iris-ciliary body. Thus the balance of inductive versus inhibitory signals may have been shifted, but not to a degree that allows for all neurons to respond.

The activin A inhibitor, follistatin, also affected neuropeptide expression in the ciliary ganglion by reducing the percent of somatostatin-positive neurons, in direct contrast to the effect of activin A. This reduction was observed with two different application methods. Presumably, exogenously applied follistatin was acting on choroid neurons to block the somatostatin-inducing activity of activin A. Interestingly, the two methods used to deliver follistatin had different degrees of efficacy. Follistatin applied to the vascular CAM had a greater impact in reducing somatostatin-expressing cells than did intraocular injection. One explanation is that the anterior injection of follistatin limited

the accessibility of the inhibitor to the choroid neurons. In addition, the injections were delivered at E10/11, which is midway through the somatostatin induction period in the ganglia. A portion of the choroid neuron population may already have been directed toward somatostatin induction by this time and thus were resistant to additional follistatin. It is interesting to note that activin A or follistatin injections given later in development, at E12 or E13, were substantially less effective (data not shown), suggesting that there is a relatively narrow window of time during which the co-transmitter phenotype is not completely established and the neurons are still more responsive to exogenous cues. In contrast, the CAM application method initiated exogenous follistatin at E7, the beginning of the normal period for somatostatin induction in vivo. It also utilized the embryonic circulatory system, of which the vascular choroid layer is an integral component. The choroid neurons innervating the smooth muscle cells of this layer would be readily exposed to the inhibitor at a relatively early time in development.

The striking reduction in somatostatin expression observed with the CAM application method reflects the ability of follistatin to exert is inhibitory effect on an endogenous factor that regulates somatostatin expression at the choroid neuron target. The endogenous inducer of somatostatin expression which was inhibited by follistatin is likely to be activin A. Both ciliary and choroid neurons express the Type IIA serine/threonine kinase receptor for activin A (Kos and Coulombe, 1996) and are able to respond in vitro by expressing somatostatin (Coulombe and Nishi, 1991). Follistatin demonstrates strong binding to and potent inhibition of activin A (Nakamura et al., 1990; Sumitomo et al., 1995). It has been shown to associate with other proteins as well, in particular, members of the bone morphogenetic protein (BMP) family, albeit with a much lower affinity than to activins (Yamashita et al., 1995; Hogan, 1996; Fainsod et al., 1997). In fact, activin A, BMP2 and BMP6 have been shown to alter message levels for neuropeptides expressed by sympathetic neurons in culture as detected by RT/PCR analysis (Fann and Patterson, 1994). Interestingly, the pattern of peptide expression induced by activin A was similar to that of the BMPs, but did not show complete overlap. The distinct responses to the different inducing agents were most notable when the neurons were cultured in depolarizing conditions. Although we cannot completely rule out a role for BMPs in determining peptide phenotype in ciliary ganglion neurons, activin A is the most likely candidate as the endogenous mediator of somatostatin phenotype due to its distinctive expression pattern

in the targets, the response of the neurons to expressing peptide in culture and to the potent inhibition noted in vivo with exogenous follistatin application.

When and how neurotransmitter phenotype is established in neurons and what factors might be responsible for this aspect of neuronal differentiation are fundamental questions in developmental biology. Much of the pioneering work examining plasticity with regard to neurotransmitter phenoytpe was initiated either with chick/quail transplantation studies designed to test the effect of the local environment on neuronal differentiation (reviewed in Le Douarin, 1980) or with sympathetic neurons cultured in conditioned medium from a variety of sources (reviewed in Patterson, 1978). As a result of these studies, some candidate molecules have been described. Among these are Leukemia Inhibitory Factor (LIF), which has been shown to play a role in the changes in neuropeptide expression observed in axotomized sympathetic ganglia (reviewed in Zigmond and Sun, 1997). In this system, a majority of the neuropeptide Y expressing neurons switch to a predominantly vasoactive intestinal peptide and galanin positive phenotype after axotomy. The source of LIF in this case however, does not appear to be the targets of the sympathetic neurons, but rather the surrounding Schwann cells, satellite cells or both.

Another example of neurotransmitter plasticity occurs in the innervation of the rodent footpad sweat glands, which involves a developmental rather than an injury-induced change in phenotype. The sympathetic neurons that innervate the sweat gland undergo a noradrenergic-to-cholinergic switch in the repertoire of primary transmitters and co-transmitters that are expressed (reviewed in Schotzinger et al., 1994; Habecker et al., 1997). This conversion has been clearly demonstrated to be dependent on a retrograde, target-derived signal, although the identity of the sweat gland factor has been elusive. Interestingly, the sweat gland factor appears to be induced in response to neuronal input at the time of innervation (Habecker et al., 1995). In contrast, both the choroid smooth muscle and the iris/ciliary body muscle express activin A and follistatin in culture independent of innervation (Darland et al., 1995). This indicates that either innervation was not required for expression of these factors or that specification of the muscle had occurred prior to collection for growth in culture.

In summary, these in vivo changes in somatostatin neuropeptide levels induced by specific factors, activin A and follistatin, reflect the plasticity of this aspect of the developmental program that regulates the differentiation of ciliary ganglion neurons.

Several questions remain as to whether a change in neurotransmitter phenotype under these varying conditions--excess inducer or inhibitor--can be considered an alteration in overall neuronal differentiation that reflects a behavioral or lineage change similar to that seen in the sympathetic neurons innervating the rodent sweat gland. Can these neuropeptide/transmitter changes that have now been described for multiple factors in several neuron populations be induced to alter neuron signalling and subsequent firing characteristics? Answers to these and other questions concerning neuronal differentiation, retrograde signalling from the target and the establishment of specific connections remain to be elucidated. The data from this study establish a base for addressing these questions in the developing ciliary ganglion. The effects of activin A and follistatin on somatostatin expression in vivo shed light on the question of whether neurotransmitter phenotype is under environmental or autonomous influence. The current data indicate that neuropeptide phenotype in developing ciliary ganglion neurons is controlled by target-derived factors, consistent with the notion that environmental factors, rather than autonomous cues, regulate this aspect of neuronal development.

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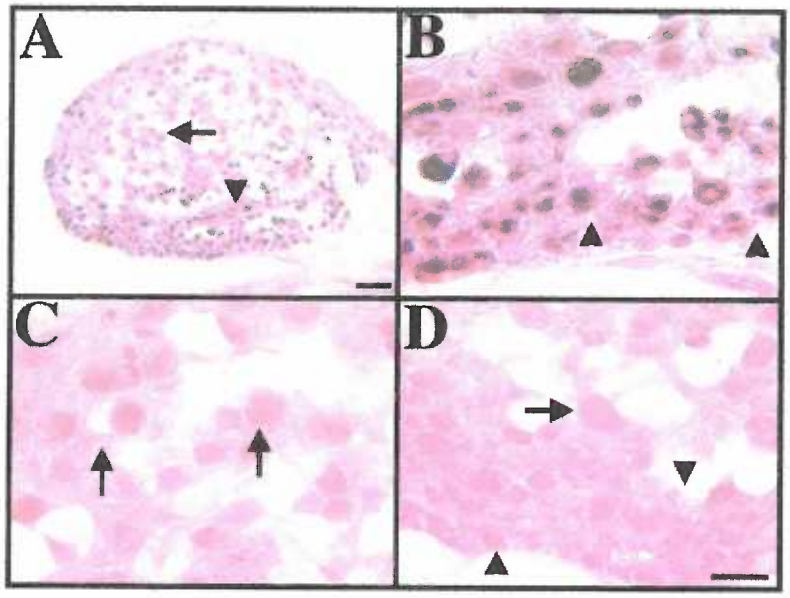

Figure 1. Immunohistochemical staining for somatostatin in E14 ciliary ganglia.

Small, somatostatin positive choroid neurons are located predominantly in the perimeter or clustered in one quadrant of the ganglion (arrowheads, A), while the larger, somatostatin negative ciliary neurons are located predominantly in the central region of the ganglion (arrows, B). Higher magnification shows the perinuclear staining of the DAB reaction product in choroid neurons (arrowheads, B) and the absence of reaction product in ciliary neurons (arrows, C). Staining with normal rat serum shows no immunoreactivity (D). Bar equals 50 μm in A and 20 μm in B-D.

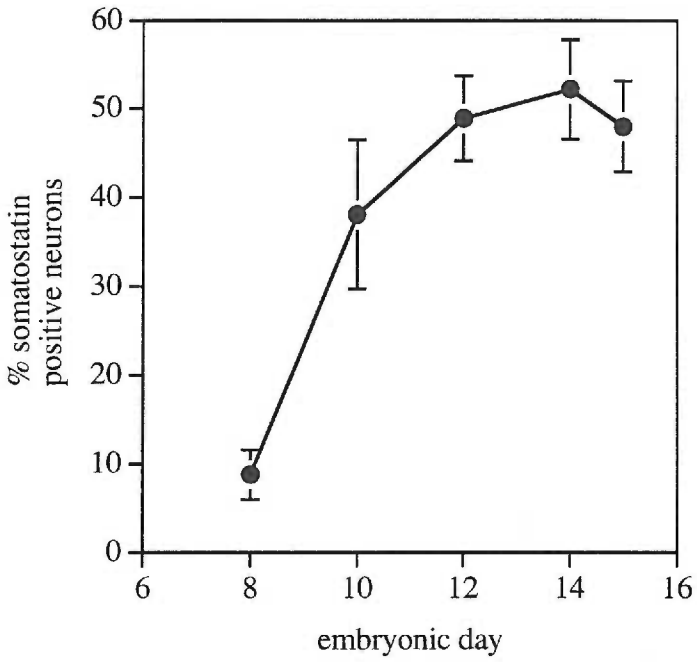

Figure 2. Developmental expression of somatostatin immunoreactivity in ciliary ganglion neurons from shell-less embryos.
At E8 less than 10% of the neurons in the ganglion are positive for somatostatin. By E14 50% of the neurons express somatostatin. Each point respresents the mean value of percent somatostatin positive neurons of at least 5 ganglia. Error bars represent the standard deviation from the mean.

Table 1. Intraocular injection of activin A and follistatin into developing chick eyes.

positive neurons	total neurons	% positive
control injected		
1. 2050	4655	44%
2. 2230	4775	46.7%
3. 2360	5825	41%
4. 2155	4445	48.5%
5. 3005	5395	55.7%
6. 2185	4360	50.1%
7. 1775	4460	39.7%
8. 4045	7245	55.8%
mean ± s.d.	5145 ± 992	47.7 ± 6%
activin A injected		
1. 3615	5880	61.5%
2. 3875	5875	65.9%
3. 2650	5220	50.7%
4. 3290	4345	75.7%
5. 3025	4245	71.3%
6. 2485	3870	64.2%
7. 2365	3450	68.6%
8. 2815	4300	65.5%
9. 4320	5855	73.8%
mean ± s.d.	4782 ± 940	66.4 ± 7.5%
p value	0.45	<0.001
follistatin injected		
1. 920	4320	21.3%
2. 3035	6415	47.3%
3. 1960	5190	37.7%
4. 2165	5435	39.8%
5. 1365	5365	25.4%
6. 2125	4765	44.5%
7. 1670	3970	42.1%
8. 1335	3595	37.1%
9. 2110	4415	50%
10. 1240	4370	28.4%
mean ± s.d.	4784 ± 829	36.8 ± 10.7%
p value	0.41	0.017

E10 or E11 embryos were injected with 300 ng recombinant human activin A, follistatin or vehicle. Ganglia were collected 4 days later. The number of somatostatin immunoreactive neurons, total neurons counted, and percent positive neurons were determined for each. P values are statistical difference from control.

Table 2. Chorioallantoic membrance delivery of activin A and follistatin in vivo.

positive neurons	total neurons	% positive
control CAM		
1. 4840	8215	58.9%
2. 3925	7295	53.8%
3. 3065	6120	50.1%
4. 2610	4985	52.4%
5. 3745	7625	49.1%
6. 3835	6455	59.4%
7. 4840	8215	58.9%
8. 3925	7295	53.8%
9. 3065	6120	50.1%
10. 2610	4985	52.4%
11. 2380	4385	54.3%
12. 2445	4875	50.2%
mean ± s.d.	6381 ± 1356	53.6 ± 3.7%
follistatin CAM		
1. 525	3255	16.3%
2. 1550	6380	24.3%
3. 3365	8045	41.8%
4. 345	4490	7.7%
5. 2505	6510	38.4%
6. 2055	6540	31.4%
7. 1040	5065	20.5%
8. 1880	7220	26%
9. 1370	7375	18.6%
10. 1190	5235	22.7%
11. 1490	7480	19.9%
12. 1550	6075	25.5%
mean ± s.d.	6139 ± 1398	24.4 ± 9.4%
p value	0.67	<0.001

E7 embryos received daily injections of 1μg of follistatin or vehicle alone onto the chorioallantoic membrane (CAM). Ganglia were collected at E14. The number of somatostatin immunoreactive neurons, total number of neurons counted and the percent positive neurons were determined for each. P values indicate statistical difference from control.

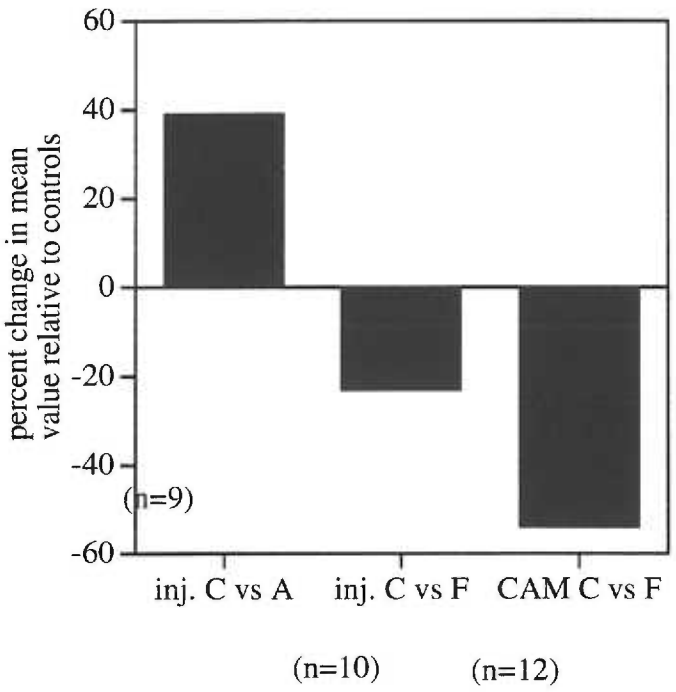

Figure 3. Change in mean value of percent somatostatin positive neurons relative to treatment controls.

In the histogram the zero value represents the mean percent somatostatin positive neurons for injected or choroioallantoic membrane (CAM) application controls. Percent changes in mean value for somatostatin positive neurons are graphed for injected control versus activin A (inj. C vs A), injected control versus follistatin (inj. C vs F) or CAM application control versus follistatin (CAM C vs F). The number of ganglia scored for each test comparison is taken from raw values listed in Tables I and II.

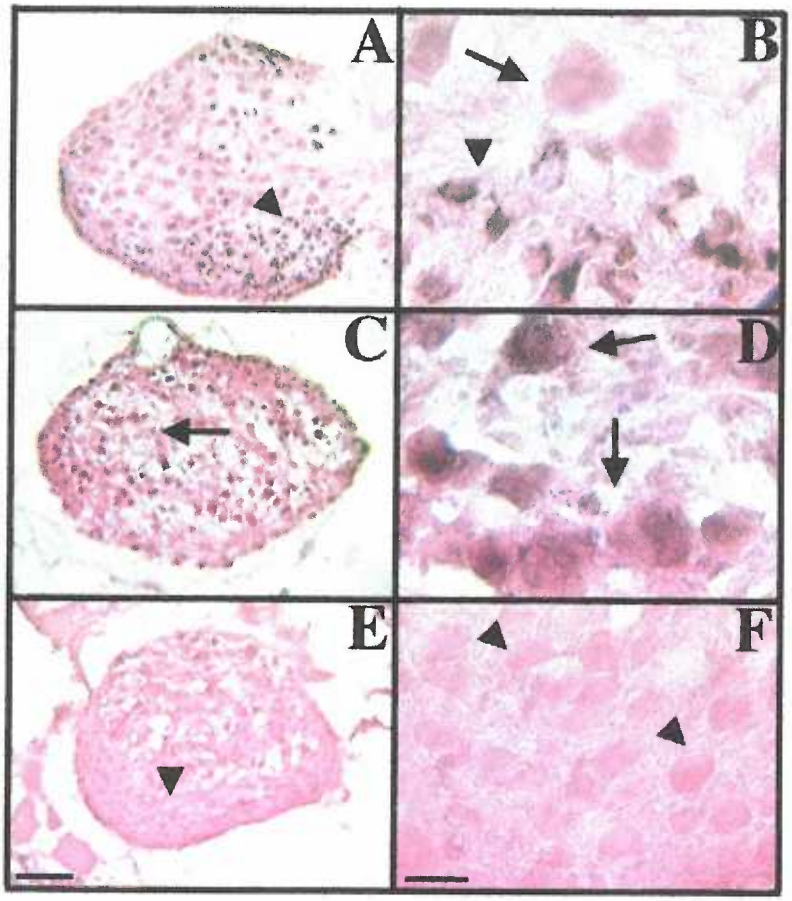

Figure 4. Somatostatin immunoreactive neurons in control, activin A injected and follistatin chorioallantoic membrane (CAM) applied ganglia. Whole ganglia are shown for control (A), activin A injected (B) and follistatin CAM application (C) treated ganglia to indicate relative location. Higher magnification shows small immunoreactive choroid neurons (arrowheads,B) and immunonegative ciliary neurons (arrows, B) in control ganglia. Activin A injection results in an increase in somatostatin immunoreactive neurons in the whole ganglion (C) and higher magnification shows large, centrally located ciliary neurons with diffuse reaction product for somatostatin (arrows, D). Follistatin CAM application results in a reduction of somatostatin positive neurons in the whole ganglion (E) with a notable lack of immunoreactivity in the small choroid neurons in the perimeter of the ganglion (arrowheads, F). Bar equals 50 μm in A/C/E and 15 μm in B/D/F.

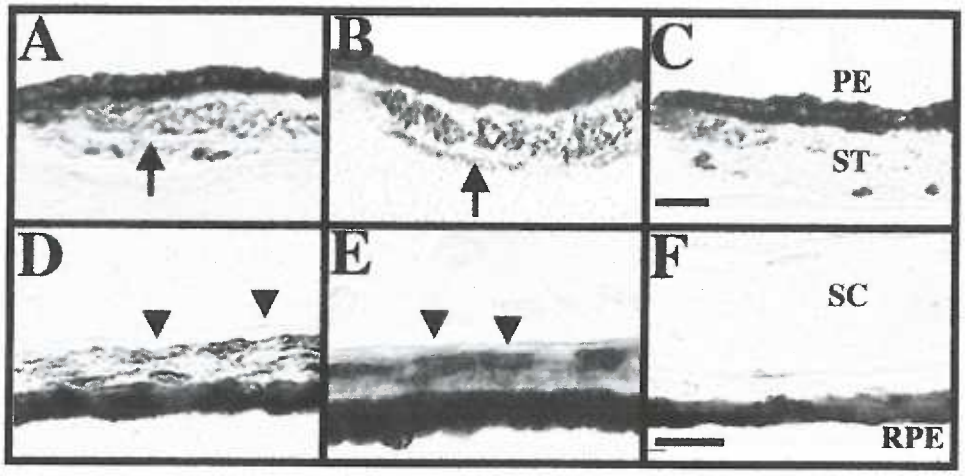

Figure 5. Normal development of ciliary ganglion target tissues with excess follistatin from E7 to E14.

E14 iris and choroid target tissues were examined for immunohistochemical expression of SMSA as an indicator of smooth muscle. Reaction product is detected in the iris stroma (arrows, A/B) and choroid vascular smooth muscle (arrowheads, D/E) in control (A/D) and follistatin (B/E) treated embryos. Normal mouse serum staining of iris (C) and choroid (F) in control embryos is shown for comparison, where stroma (ST), pigmented epithelium (PE), sclera (SC) and retinal pigmented epithelium (RPE) are labelled as indicated. Bar equals 20 μm in A/B/C and 10 μm in D/E/F.

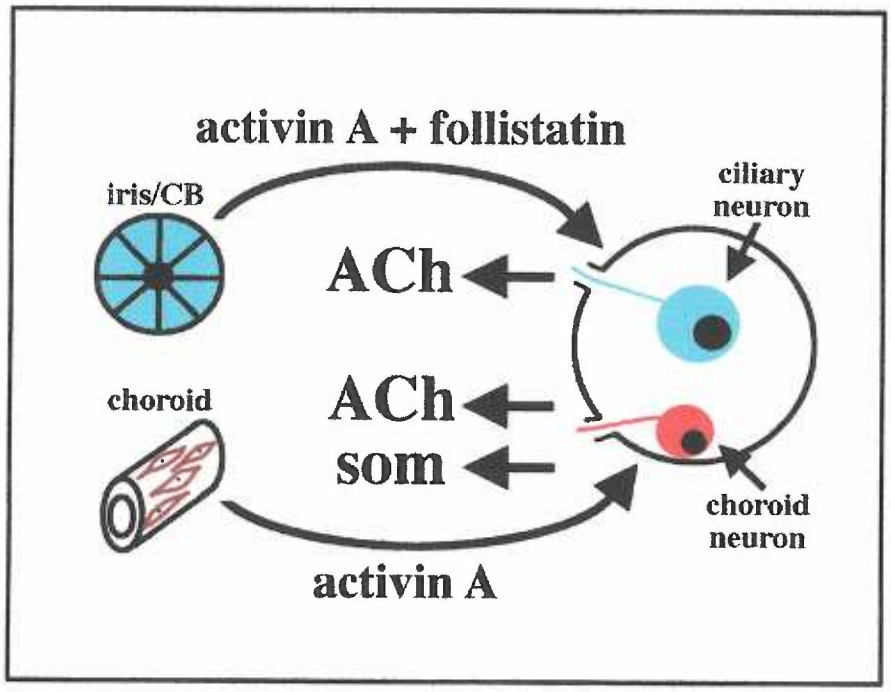

Figure 6. Model for activin A and follistatin control of somatostatin expression in developing ciliary ganglion neurons.

Activin A from the choroid target is in excess relative to follistatin levels. The excess of inducer results in somatostatin expression in choroid neurons that innervate this target. In the iris/ciliary body target there is sufficient follistatin present to inhibit the induction of somatostatin in the innervating ciliary neurons, so this population does not produce the neuropeptide.

Discussion

I. Summary of Results

The data presented here address the role of activin A and follistatin in establishment of neurotransmitter phenotype, specifically somatostatin expression, in the developing ciliary ganglion in vivo. The temporal and spatial pattern of expression of these factors during development coincides with the onset of somatostatin induction in the neurons. Activin A and follistatin are expressed in the target cells in vitro and follistatin in iris cultures can block activin A from inducing somatostatin in cultured ciliary ganglion neurons. Cells derived from the choroid layer target differentiate into smooth muscle in culture and express activin A and low levels of follistatin in a differentiation dependent fashion that is independent of innervation. In vivo function of activin A and follistatin was tested by direct application of these factors to developing embryos. Intraocular injection of activin A induces somatostatin in ciliary ganglion neurons. More importantly, follistatin strongly inhibits expression of somatostatin in the ciliary ganglion neurons with two different delivery methods, intraocular injection and CAM application. These data suggest the model that differential expression of the inhibitor, follistatin, in ciliary ganglion target tissues controls availability of activin A for induction of somatostatin expression in the ciliary ganglion neurons in vivo.

II. Neurotransmitter phenotype: plasticity versus determination

Two points that are worth consideration in neuronal development are the distinct issues of plasticity and determination with regard to neurotransmitter phenotype. Plasticity encompasses the capacity of a neuron to express a range of neurotransmitters and peptides, in essence the potential of the neuron. Determination on the other hand reflects the phenotype that a neuron expresses under normal developmental conditions. Both issues are addressed in the current study. Choroid neurons express somatostatin and ciliary neurons do not; this describes their determined peptide phenotype. The exogenous application of activin A to induce somatostatin reflects the plasticity of ciliary neurons. In contrast, the exogenous application of follistatin tests the idea that activin A, which follistatin binds to and inhibits, is a component of the determination of peptide phenotype in choroid neurons.

This issue of plasticity versus determination has been described in neural crest transplantation studies (Le Douarin, 1980) and in detail in sympathetic cultures (Patterson, 1978). In the majority of cases the studies describe a noradrenergic/adrenergic population adopting a cholinergic phenotype at the behest of environmental influences indicating phenotypic plasticity. The reverse, however, has been described for the ciliary ganglion as well. Quail ciliary ganglion were transplanted into chick trunk neural crest, which normally gives rise to adrenergic neurons, and the resulting chimaeras were examined for incorporation of quail cells into neural crest derivatives (Ziller et al., 1978). Catecholamine histofluorescence was detected in incorporated quail cells, however subsequent studies have demonstrated that this was likely due to non-neuronal cells derived from the ganglion (Dupin, 1984). In a study that clarified this issue, ciliary ganglion neurons from E6.5 were labelled with fluorescent microspheres and transplanted into the ventral pathway of migrating trunk neural crest (Coulombe and Bronner-Fraser, 1986). Labelled neurons were detected in a number of crest derivatives and displayed catecholamine histofluorescence. This is particularly intriguing in light of the fact that there are reports of small numbers of ciliary ganglion neurons (0.5% of neurons in ganglion) that are immunoreactive for tyrosine hydroxylase (TH) in vivo (Iacovitti et al., 1985). Cultured neurons also have TH immunoreactivty, but no enzyme activity, and cannot convert L-tyrosine to levodopa. The neurons also express aromatic L-amino acid decarboxylase (AADC) and if exogenous levodopa is applied, the neurons display dopamine histofluorescence, indicating that some but not all of the the components required for an adrenergic fate are present (Teitelman et al., 1985). The neurons still had ChAT activity, despite the presence of TH. Taken together, these data indicate that the parasympathetic ciliary ganglion neurons, not just sympathetic neurons can display phenotype reversal with regard to their primary neurotransmitter, thus demonstrating plasticity in response to environmental cues.

Apart from this issue, the TH expression observed in vitro and in vivo is certainly worth considering. The in vitro expression could be a result of non-neuronal cell influence in the culture paradigm or a secondary effect of placing the neurons in culture, potentially due to loss of an inhibitory influence once the neurons are removed from contact with the target. Acetylcholine is the primary transmitter associated with somatostatin expression in choroid neurons, but is unaffected by activin A treatment. It would be interesting to determine if activin A had an inhibitory effect on expression of catecholaminergic enzymes, in opposition to its inductive effect on somatostatin

expression. As for the TH expression observed in vivo this presents an additonal puzzle. The TH positive neurons may have an embryonic derivation distinct from the cholinergic neurons. These neurons represent a small proportion of total neurons in the ganglion and as such may not have been represented in the study describing the embryonic derivation of the ciliary ganglion (Narayanan and Narayanan, 1978). Another more interesting possibility is that they represent a subpopulation that innervates an as yet undescribed target in the eye that induces a catecholaminergic phenotype in these neurons.

III. Establishment of neurotransmitter phenotype in ciliary ganglion neurons

The establishment of neurotransmitter phenotype is a critical component of neuronal differentiation. The neurotransmitters and peptides that a neuron expresses are the signals that allow communication with a responding cell. George Bishop stated on this issue, "To wit, the prime function of a neuron is to produce and apply to other tissues a chemical activator" (Bishop, 1965). The outcome of the signal is dependent upon the receptors on the receiving cell and what intracellular machinery is activated or repressed. This is the essence of chemical transmission in the nervous system. In that regard, the neurotransmitter phenotype of a given neuron must be matched with that of the target cell that it innervates. One mechanism for this matching to occur involves target-derived factors that direct this aspect of neuronal differentiation. This allows for signal/receptor matching in conjunction with onset of innervation and neuronal maturation. In the avian ciliary ganglion this event is likely mediated by target-derived activin A and follistatin, to ensure that somatostatin is expressed only in the choroid neurons.

Somatostatin has been proposed to play a key role in modifying acetylcholine release from the synapse (Guillemin, 1976) and in choroid neurons, specifically, is thought to act as part of a negative feedback loop to autoregulate acetylcholine release (Gray et al., 1989; Gray et al., 1990). Somatostatin is not utilized in the cholinergic synapses at the iris/ciliary body target and target-derived expression of follistatin appears to prevent expression of somatostatin in the ciliary neurons. This becomes the proverbial "which came first..." conundrum as to whether the iris/ciliary body and choroid targets expressed differential levels of activin A and follistatin to regulate somatostatin expression or whether the choroid neurons capitalized on the the availability of activin A . The corollary to this being that the somatostatin-inducing activity from the choroid

was subsequently recruited by the choroid neurons as a mechanism to induce expression of an additional signalling molecule to regulate vascular smooth muscle contraction in the choroid layer. Somatostatin as a co-transmitter is more often expressed in conjunction with noradrenergic not cholinergic neurons (Hökfelt et al., 1980; Reichlin, 1983). This is interesting, in particular, when one considers that choroidal vascular smooth muscle in other species receives adrenergic sympathetic input to mediate contraction (Bill, 1962). This does not appear to be the case with the innervation of the smooth muscle in the choroid layer of the chicken.

The plasticity of neurons with regard to their neurotransmitter phenotype has been established in a number of systems, however there is evidence to suggest a period in development after which neurons are refractory to change. This has been clearly described for sympathetic neurons in culture. Neonatal SCG neurons can express a dual adrenergic/cholinergic phenotype in response to conditioned medium (Patterson and Chun, 1974; Patterson and Chun, 1977a; Patterson and Chun, 1977b). These neurons grown in microculture with cardiac myocytes have similar properties however, SCG neurons from adult rats had a much lower incidence of dual phenotype than was observed with the younger animals. In addition SCG explants from various developmental ages were examined after one month in culture for their ChAT and DOPA-decarboxylase (DDC) activities (Ross et al., 1977). There was a notable reduction in ChAT activity in explants derived from older animals. There is thus likely to be a window of time in neuronal differentiation when the neurons are receptive to environmental signals that specify signalling properties, but this is lost as the neurons progress through further differentiation toward a determined phenotype.

We see a similar pattern of age-dependent sensitivity in the plasticity of peptide expression in ciliary ganglion neurons. Intraocular injections of activin A and follistatin were more effective at altering somatostatin expression when given earlier rather than later, during the period of peptide induction. In addition, CAM application of follistatin early in the induction period had a profound inhibitory effect on somatostatin expression, emphasizing that the ciliary ganglion neurons are more receptive to environmentally induced changes in neurotransmitter phenotype early in their development. It is interesting to note that both E8 and E14 ciliary ganglion neurons are dependent upon the continual presence of activin A in the choroid conditioned medium in order to express somatostatin (Coulombe and Nishi, 1991). Activin A levels in the choroid are high from E9 until E14 at which point, message levels drop and follistatin

levels increase (Darland et al., 1995). This drop in activin A continues after E16, suggesting that the choroid neurons no longer require activin A to maintain somatostatin expression. Although we have not tested E16 or E18 ciliary ganglion neurons for somatostatin expression in culture, the target expression of activin A would predict that the neurons are past the period of development where they are dependent on the target for somatostatin inducing cues. Indeed, it would be interesting to determine if an independent period was established after E14 as well as whether or not sensitivity to activin A was maintained in the ciliary neurons.

IV. Activin A expression in the choroid layer

Important components of the activin A induction of somatostatin expression in the choroid neurons are the differentiation dependent control of activin A expression itself in the choroid layer as well as the regulation of this expression by TGFβ. Based upon the expression of activin A in the choroid in vivo and the cells in culture, it is apparent that activin increases with the differentiation state of the vascular smooth muscle and then drops after differentiation is complete. From the in vitro studies comes the indication that TGFβ not only enhances the differentiation state of the choroid cells and subsequent activin A expression, but also is a normal component of the vascular smooth muscle differentiation based upon the blocking analysis. Activin A has been previously described in vascular smooth muscle, but not correlated with differentiation (Kanzaki et al., 1995). TGFβ has been implicated in differentiation of several neural crest derivatives, and specifically promotes a smooth muscle-like lineage (Moses and Serra, 1996; Shah et al., 1996).

The implications for choroid layer development would be that expression of activin A is a component of the normal differentiation program initiated by TGFβ. TGFβ knockout mice have been generated which have extensive vascular abnormalities (reviewed in Matzuk, 1995). These mice also display a "crusty eye" phenotype which may be associated with abnormal aqueous flow and lymphocytic lacrimal gland lesions (McCartney-Francis et al., 1997). The retina and optic nerve of these mice appears normal, but the choroid layer was not examined in detail. It would be interesting to determine if choroid vascular smooth muscle in mice expresses activin A and follistatin. If this is the case then a parallel could be drawn from the data with the avian choroid smooth muscle cells. The prediction would be that in mice lacking TGFβ, vascular smooth muscle differentiation in the choroid layer would be delayed or reduced and

there would be a corresponding loss of activin A expression This prediction is based on the assumptions that other members of the TGFβ superfamily could not compensate for TGFβ in this paradigm and that other factors are unavailable to initiate a differentiation program.

V. Mechanism of activin A and follistatin action in ciliary ganglion neurons

The actual mechanism that is involved in somatostatin induction by activin A is unclear, but generalizations can be made by putting together what is known about activin A signalling and somatostatin gene regulation. Association of activin A from the choroid with the serine/threonine receptor complex expressed in the neurons would result in phosphorylation of SMAD 2 followed by recruitment and phosphorylation of SMAD 4 (Massague, 1996). Based upon TGFβ signalling mechanisms and the downstream effectors of dpp, the drosophila TGFβ homologue, the next series of events would include localization of SMAD 4 to the nucleus and subsequent association with nuclear proteins to form a transcriptional regulatory complex. Transcriptional regulation of the somatostatin gene through the cAMP Response Element (CRE) via association of the CRE binding protein (CREB; Goodman et al., 1990; Andrisani and Dixon, 1990) has been well studied. How SMAD activation and somatostatin gene transcription can be integrated to produce an activin A dependent increase in somatostatin is unclear. Regulation of somatostatin could occur at the level of transcription with a downstream effector of activin A associating with CREB to enhance binding to the CRE or recruitment of transcriptional machinery. The translation efficiency of somatostatin message or its stability are also points of potential regulation. Alternatively, the effects of activin A on somatostatin expression may manifest as an increase in processing of preprosomatostatin or incorporation of peptide into vesicles (Benoit et al., 1990). The pursuit of these questions will yield information on the specifics of how activin A controls somatostatin in choroid neurons, but could also lead to additional information on control of neuropeptide expression in general that may have broader implications in other neuron and neuroendocrine populations.

The regulation of somatostatin expression in ciliary ganglion neurons by activin A and follistatin is proposed to be a reflection of their antagonistic interactions. The current model is that activin A is associated with cell surface heparin sulfate proteoglycans which may be involved in presentation of ligand to the receptor (Willis et al., 1996; de Winter et al., 1996). When follistatin is present it is likely bound to the extracellular

matrix around the cell, to the heparin sulfate proteoglycans or both (Nakamura et al., 1990; Sumitomo et al., 1995). It is through this close association with the cell that it binds to activin A and prevents it from transducing signal through the serine threonine receptor. In the current study, where activin A and follistatin were injected into the eye, it is likely that the proteins were rapidly associated with heparin sulfate proteoglycans in the vitreous body. From this vantage they would be available for detection by ciliary and choroid neuron distal processes. In a study examining neurotransmitter phenotype in sympathetic neurons cultured in Campenot chambers, the authors determined that addition of factors to the distal neurites alone could induce changes in transmitter characteristics (Ure et al., 1992). Extrapolating from this observation, the model for injected activin A and follistatin would then be that exogenous activin A at the distal processes of the ciliary neurons would be at a high enough concentration to overcome the inhibitory effects of local follistatin produced by the iris/ciliary body targets. On the other hand, exogenous follistatin from injections or distributed through the vasculature via the CAM injections, would associate with surface activin A and prevent ligand/receptor binding. In both cases the prediction would be that the activin A and follistatin would be bound either to the target cell surface and triggering/inhibiting receptor activation on the distal tips of the neurons or that the factors would associate with the neuronal processes and mediate their signal/inhibition from the neuron itself. It would be interesting to determine the precise localization of activin A and follistatin in ocular tissues. Both proteins have been localized to the striated muscle of the iris/ciliary body target tissues and activin A has been detected in the smooth muscle cells of the choroid layer around the blood vessels (Darland et al., 1995), but whether these proteins are associated with the neuronal processes as well remains to be seen.

Additional evidence for the importance of activin A and follistatin in developing systems has been obtained with the help of targeted gene disruption. Mice lacking the genes for activin A and B, the Type II receptor and follistatin have been generated and demonstrate varying degrees of severity in their phenotypes (Matzuk et al., 1995;Matzuk et al., 1995;Matzuk et al., 1995). Mice deficient for activin A have craniofacial abnormalities and die soon after birth. In contrast the activin B deficient mice are viable, but have reproductive deficiencies. In the absence of both activin A and B, the mice have characteristics of the individual knockouts, but no combined effects suggesting that the two factors do not have overlapping functions. It is interesting to note in our system that western analysis of choroid conditioned medium with antisera specific for activins A and B demonstrated that only activin A could be

detected (Coulombe et al., 1993), again indicating that they do not share overlapping expression despite their similar functions and receptor interactions (Vale et al., 1991). The Type II serine/threonine kinase receptor knockout has additional abnormalities to those observed in the combined activin A/B knockout, indicating that this receptor may signal for other members of the TGFβ superfamily other than activins. Likely candidates in this case are members of the BMP family (Mehler et al., 1997; Josso and di Clemente, 1997). In the avian ciliary ganglion, the Type IIA, but not the Type IIB receptor has been detected on both ciliary and choroid neurons (Kos and Coulombe, 1997). The Type I receptors are thought to provide signalling specificity by association with the intracellular components (ten Dijke et al., 1996; Massague, 1996), but no information is currently available as to which Type I receptors are expressed on ciliary ganglion neurons. The follistatin knockout mice have widespread defects, retarded growth and die soon after birth. Their phenotype includes the abnormalities observed in the activin A/B knockout mice. The severity of their phenotype indicates the importance of this factor in a number of developmental processes and also implicates follistatin in the regulation of other proteins in addition to the activins.

Our results with the in vivo manipulation of somatostatin levels are in keeping with the importance of follistatin in regulating activin A. Somatostatin levels in ciliary ganglion neurons were significantly inhibited in the presence of exogenous follistatin (see Results, chapter 3). These results point not only to the importance of activin A on somatostatin induction, based upon the presence of the inhibitor, but also support a mechanism for control of somatostatin expression via selective and localized production of follistatin. The role of target-derived activin and follistatin in ciliary ganglion development cannot be directly addressed with the use of the mouse knockouts described above, however. In mammals, the iris/ciliary body is innervated by the parasympathetic ciliary ganglion, but also by a sympathetic contribution (Westheimer and Blair, 1973; Leblanc and Landis, 1988). The choroid layer is innervated by choroid neurons that form a plexus over the vascular system and do not consolidate into a discrete ganglion (Ruskell, 1970;Ruskell, 1971; Flugel et al., 1994). In addition, the peptides NPY and VIP are used as co-transmitters at these targets, respectively. These distinctions emphasize the importance of exploring neuron/target interactions in a simple system such as the avian ciliary ganglion, particularly considering the accessibility of the components during development.

VI. Transmitter phenotype control: systems comparison

Some comparisons can be made between the target-dependent control of neurotransmitter phenotype in the avian ciliary ganglion and that of the sympathetic innervation of the rat footpad sweat gland. A large body of literature exists to describe the action of SGF, produced in the target cells, which induces a noradrenergic to cholinergic switch in phenotype in the innervating sympathetic neurons (Schotzinger et al., 1994; Habecker et al., 1997). Although the exact nature of the molecule is not known, a great deal about its characteristics, mechanism of action and onset of expression has been determined. For instance 6-hydroxydopamine treatment of developing rats results in a loss of sympathetic innervation and a subsequent loss in acetycholine esterase and VIP staining (Yodlowski et al., 1984). Interestingly, sweat gland cells from sympathectomized rats do not produce SGF and this factor is induced in the cultured cells in the presence of sympathetic but not sensory innervation (Habecker and Landis, 1994; Habecker et al., 1995). These results indicate that sympathetic innervation of the target is required for SGF production, which is in turn required for specification of the cholinergic phenotype.

In the avian ciliary ganglion system, in contrast, there are candidate molecules for which a great deal of information has accumulated that is not connected with their possible role as target-derived factors controlling neurotransmitter phenotype. There is evidence to indicate that these factors, activin A and follistatin, are expressed independent of innervation, at least in the choroid target. Choroid cells cultured alone express these factors as they differentiate, however they are taken from choroid layer in vivo that has been innervated. One experiment to address this issue directly would be ablation of the mesencephalic neural crest to prevent formation of the ciliary ganglion. Subsequent analysis of the ciliary ganglion target tissue for activin A and follistatin expression should give an indication of whether this parasympathetic innervation is required for their expression. Another interesting point concerns specificity of response to target-derived factors in different neuronal populations. The post-innervation footpad sweat glands can induce a noradrenergic to cholinergic switch in sympathetic neurons that are normally noradrenergic (Schotzinger and Landis, 1988; Schotzinger and Landis, 1990; Schotzinger and Landis, 1990). There is evidence to indicate that activin A can induce message for some peptides, including somatostatin, in cultured sympathetic neurons (Fann and Patterson, 1994; Fann and Patterson, 1994), but production of protein was not measured in these studies. Upregulation in

somatostatin message in sympathetic neurons does not necessarily correlate with an increase in neuropeptide (Spiegel et al., 1990). It is still not clear how activin A and follistatin may affect neuropeptide expression in other neuronal populations. The data presented in the current study address some of the mechanistic issues involved in activin A and follistatin control of ciliary ganglion peptide expression, but further examination of the issue is needed.

VII. Considerations

It is conceivable that there are other aspects of ciliary ganglion development, in addition to somatostatin expression, that are influenced by the target. One of these is the ion channel repertoire expressed in ciliary ganglion neurons. The delayed rectifier K^+ current has been described in ciliary ganglion neurons in vitro as well as throughout development and does not appear to be influenced by the target (Dourado and Dryer, 1992). In contrast, the Ca^{++} activated K^+ current ($I_{K(Ca)}$) and the A-current (I_A) were not detectable in ciliary ganglion neurons until after contact with the target, nor did these currents develop in cutured neurons in the absence of target cells. Whether this is contact-mediated or the result of a target-derived soluble factor is unclear at this time, however it is apparent that target influence is a likely component in the acquisition of appropriate channels in the neurons (Dourado and Dryer, 1994).

An additional characteristic of the ciliary ganglion neurons that may be target-influenced is myelination. The ciliary neurons innervating the iris/ciliary body target are myelinated but the choroid neurons are not (Marwitt et al., 1971; DeStefano et al., 1993). There are three basic phases in Schwann cell maturation: precursor genesis from migrating neural crest cells, precursor transition to immature Schwann cell and initiation of the myelination program in the Schwann cell. This process has a soluble-factor induced component and an axon-contact dependent component. The transcription factor Krox-20 is required for initiation of the myelination program (Topilko et al., 1994) and can be induced in precursor cells by the combination of CNTF and FGF2 (Murphy et al., 1996). Both CNTF and FGF2 are produced in ciliary ganglion targets and could be responsible for the soluble factor component of induction. The contact-mediated portion of Schwann cell maturation has been proposed to be due to neuregulin (Meyer and Birchmeier, 1995). If this is the case then it is possible to speculate that an inhibitor of neuregulin expression in choroid neurons may be produced in the choroid layer, indirectly preventing axon-induced myelination in the

choroid neurons. This issue of myelination has yet to be pursued much beyond the basic description of the distinction between the neuron populations, but it certainly merits further pursuit.

A final aspect of ciliary ganglion development that is likely to be target mediated is the development of the cholinergic phenotype itself. Functional cholinergic transmission through the ciliary ganglion neurons is not detected until after the target has been contacted (Pilar et al., 1987; Meriney and Pilar, 1987). In addition, there are two distinct periods in ciliary ganglion development where ChAT immunoreactivity is detected in the quail. The earliest immunolocalization for ChAT occurs between E5 and E6, the time at which ciliary neurons form synapses with their target (Coulombe and Bronner-Fraser, 1990). A second wave of immunoreactive neurons is detected, peaking at E12, which likely corresponds to the choroid neurons whose target contact occurs later than that of ciliary neurons. A Cholinergic Stimulating Activity (CSA) from eye extracts has been described that induces high levels of ChAT activity in ciliary ganglion neurons (Nishi and Berg, 1981). The CSA factor has not been described in detail or purified, but it would be interesting to pursue as a possible addition to target-derived factors that influence neurotransmitter phenotype in ciliary ganglion neurons.

At the end of this discourse, several questions remain, particularly with regard to the manipulations of somatostatin expression in vivo. For instance, what are the physiological effects, if any, in changing the neurotransmitter phenotype in the ciliary and choroid neurons? If the ciliary neurons actually express the somatostatin receptor, is it functional when the neurons are forced to express somatostatin and what does this do to the functions of pupillary constriction, accomodation and to a certain degree aqueous outflow that are regulated by these neurons? What is the outcome of inhibiting normal somatostatin expression in choroid neurons? Is there an increase in intraocular pressure as a result of the reduction of inhibition normally subserved by somatostatin's regulatory effects on acetylcholine? In addition, it would be interesting to determine if any of the changes observed by immunohistochemical analysis of somatostatin levels are reflective of changes in somatostatin message stability, translation of message, peptide processing or incorporation into vesicles. The processing aspect of activin A effects on somatostatin could first be addressed in culture and would yield interesting information as to the action of activin A on somatostatin as well as regulation of the peptide in ciliary ganglion neurons. One final consideration remains as to whether or not the dynamic duo of activin A and follistatin may act as target-derived regulators of

neurotransmitter phentoype in other autonomic populations. It would be interesting to determine if this mechanism is replicated, particularly in those neuronal populations that innervate parenchymal, smooth muscle or vascular smooth muscle tissue.

VIII. Conclusion

Cumulatively, the data presented in this study contribute to the understanding of target-derived influences on the establishment of neurotransmitter phenotype in ciliary ganglion neurons in vivo. Previous data indicated that activin A was able to induce somatostatin in the neurons in vitro. The current study extends this to in vivo control of the neuropeptide and suggests a revision to this hypothesis that takes into consideration the differential expression of activin A in the iris/ciliary body versus the choroid layer, which express moderate and high levels, respectively. In addition, the major contribution to the difference in somatostatin expression seen in choroid versus ciliary neurons appears to come from the selective and localized expression of the activin A inhibitor, follistatin, which is at higher levels in the iris/ciliary body relative to the choroid. The mechanism for activin A and follistatin interaction outlined here parallels the antagonistic interactions of these factors observed in other systems. It describes a novel function for these proteins in vivo, specifically in determination of neurotransmitter phenotype.

Summary and Conclusions

The data presented here address the basic biological questions of how neurotransmitter phenotype is established in the developing nervous system and what is the role of target-derived factors in mediating this component of neuronal differentiation. These issues are examined in the parasympathetic avian ciliary ganglion which contains two populations of neurons that differ in their expression of the co-transmitter, somatostatin. Previously published data indicates that activin A can induce somatostatin in ciliary ganglion neurons. The current study presents evidence that differential expression of activin A and its inhibitor, follistatin, in the ocular targets of the ganglion controls this distinction between the neurons in vivo. Activin A and follistatin demonstrate a temporal and spatial pattern of expression that is in accordance with their role as target-derived factors that mediate somatostatin expression. Both targets produce activin A during the window of development when somatostatin induction occurs in vivo. The important difference in expression is seen with follistatin which is much higher in the iris/ciliary body than in the choroid layer, indicating that the presence of the activin A inhibitor prevents expression of somatostatin in the ciliary but not the choroid neurons. In addition, conditioned medium from iris-derived striated muscle cultures contains follistatin and can inhibit somatostatin induction in ciliary ganglion neurons. Expression of activin A in choroid cells is closely associated with the differentiation state of the vascular smooth muscle and is independent of innervation in culture. Testing the hypothesis in vivo involved altering somatostatin levels with exogenous application of activin A and follistatin to change the balance in these factors. The hypothesis would predict that additional activin A available to the ciliary neurons would overcome the follistatin inhibition and result in an increase in somatostatin expression. The opposite would be predicted for follistatin which would inhibit the activin A from the choroid and inhibit expression of somatostatin in choroid neurons. Intraocular injection of activin A and CAM application of follistatin changed somatostatin expression in line with the predicted results.

A model that describes the normal establishment of somatostatin expression in ciliary ganglion neurons can be derived from these results (see Chapter 3, Figure 6). Choroid neurons express the neuromodulatory peptide somatostatin in response to activin A derived from the smooth muscle target cells that these neurons innervate. Expression of somatostatin in ciliary neurons is prohibited due to the production of follistatin in the striated muscle targets, despite the fact that these cells also produce activin A. In

conclusion, these results suggest a novel role for activin A and follistatin in regulating neurotransmitter phenotype during neuronal development. Moreover, the results support a novel mechanism for target-dependent regulation of peptide expression that involves not only differential levels of inducer, in this case activin A, but more importantly involves localized expression of an inhibitor, follistatin, in controlling determination of transmitter phenotype.

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The day shall not be up so soon as I,
To try the fair adventure of tomorrow.

William Shakespeare, King John, *V*, *iv*, 11