Activin A and Follistatin Influence Expression of Somatostatin in the Ciliary Ganglion in Vivo

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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, that differentially express the peptide cotransmitter, somatostatin. The present study tests the hypothesis that differences in the level of expression of activin A and its endogenous inhibitor follistatin in CG neuron target tissues are responsible for selective expression of somatostatin in choroid neurons. Intraocular injection of activin A or follistatin (300 ng injected at E10/E11) in cultured embryos 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 to E13) reduced somatostatin positive neurons by 54%. Neuron number, size, and target tissue morphology were unaffected by these treatments. Together with our previous studies, these data suggest that activin A and follistatin are target-derived molecules that regulate neuropeptide phenotype in the ciliary ganglion.

INTRODUCTION

Retrograde signaling 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 signaling that is influenced by the target is neurotransmitter phenotype. The primary neurotransmitter and any peptide cotransmitters 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 co-workers have shown that sympathetic neurons innervating sweat glands in the rat footpad undergo a phenotypic transition from noradrenergic to cholinergic under the influence of a target-derived factor (Schotzinger et al., 1994; Landis, 1996).

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 cotransmitter (Epstein et al., 1988; DeStefano 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.

Previous studies from our laboratory have suggested that ciliary ganglion targets 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 cocultured with choroid-derived smooth muscle or with conditioned medium from the smooth muscle (Coullome 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 regulates neuronal somatostatin expression (Fig. 1). Specifically, excess activin A from the choroid layer induces somatostatin in the choroid neurons, but the presence of follistatin from the iris/ciliary body prevents expression of somatostatin in the ciliary neurons by blocking activin A. Although activin A and follistatin can control somatostatin expression in culture, it is essential to establish their role as regulators of neurotransmitter phenotype in a normal developmental context in vivo. In the current study we address these issues by manipulating activin A and follistatin levels in developing chicken embryos to test their effect on neuropeptide expression in the ciliary ganglion.

**MATERIALS AND METHODS**

**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 shellless 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 No. 15365-36, National Hormone and Pituitary Program, distributor, McKesson BioServices, Rockville, MD) or follistatin (lot No. B3904, National Hormone and Pituitary Program) in L15 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 pipet, one eye was injected through Schlemm's canal behind the lens and onto the vitreous body with the contralateral eye serving as uninjected control. The ciliary ganglia and eyes were collected after 4 days further development. In some embryos, follistatin was applied to the cho-
were collected. 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 3 μl. Daily deliveries were repeated until E14 when ciliary ganglia and eyes were collected.

**Immunohistochemistry**

Ganglia from a single experiment were collected and processed simultaneously. Tissue was fixed in Zamboni’s solution (4% paraformaldehyde, 15% picric acid in 0.1 M sodium phosphate buffer, pH 7.2) for 30 min at 23°C, washed with phosphate-buffered saline solution (PBS, pH 7.2) and saturated with sucrose to 30%. The tissue was embedded in Tissue Tek O.C.T. compound (Sakura Finetek U.S.A., Inc., Torrance, CA) and frozen and 10-μm sections were cut. All sections were collected in order on five different slides; thus each slide represented every fifth section through the serially cut ganglion. 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 Corp., 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 a monoclonal rat anti-peroxidase-peroxidase (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 diaminobenzidine as a substrate with 0.03% NiCl for signal enhancement. A blue filter was used to increase the contrast between the brown PAP reaction product and the pink eosin counterstain.

**Counting Methods and Statistical Analyses**

Slides of stained and mounted sections were masked by another member of the laboratory and mixed prior to counting. This ensured that the counts were performed with the investigator blinded to the conditions of treatment. A profile was scored as a neuron if it had a smooth round or oval outline that exceeded 8 μm in diameter and was distinctly more strongly stained (eosinophilic) than the surrounding nonneuronal tissue, which was amorphous. A nuclear counter stain could not be used because the considerably more intensely staining nonneuronal cell nuclei that surround each neuron obscured the view of the neuronal nuclei. Cytoplasmic counterstains of blue and purple shades were unacceptable because they interfered with detection of the brown peroxidase reaction product that marked the somatostatin-immunoreactive neurons. Nonneuronal cells surrounding the neurons in the ganglion were distinguishable by their flat, crescent shape and were not counted. A blue filter was used to increase the contrast between the brown PAP reaction product and the pink eosin counterstain.

The number of positive neurons and the total number of neurons was determined for every fifth section to obtain a representative count through the ganglia. Most neuron counting was carried out at 250× magnification on a Nikon microscope. Punctate, perinuclear PAP reaction product was distinguished from background staining by viewing through several planes of focus on a single neuron. In some instances, magnification was increased to 400× to confirm the presence of reaction product. A neuron was scored as immunoreactive for somatostatin if punctate staining could be detected in the soma. A neuron was scored as negative if reaction product was not detected and only the evenly distributed pink eosin counterstain was observed. The number of positive neurons and total neurons observed were multiplied by 5 to obtain the values listed in the text and Tables 1 and 2. This method of obtaining total neuron number gave an overrepresentation of the value for each ganglion, but did not affect the outcome of the differences observed under test conditions because all values were compared to controls measured in the same fashion. The number of positive neurons divided by the total yielded the percentage somatostatin-positive neurons determined for each ganglion; this gave a normalized value for each ganglion to allow for direct comparison of control versus treated conditions. Values obtained for total neuron number and the percentage somatostatin-positive neurons showed a normal distribution around the mean. Comparisons of control versus test conditions were done with Statistica (Statsoft, Inc., Tulsa, OK) or with a T155 III statistics calculator and standard statistical tables.

Diameters of cells under all four treatment conditions (control, activin-injected, follistatin-injected, follistatin applied to chorioallantoic membrane) were measured in the same slides that had been scored for reaction to antibodies against somatostatin. Images of ganglia were captured at 400× with a Sony Catseye digital camera and displayed on a computer monitor. Cell body diameters were measured directly on the monitor after calibration of the ruler with a slide micrometer. Measurements were compared with sections whose nuclei were stained with hematoxylin solution (0.75%, Sigma) and dehydrated by standard methods prior to mounting in Pro-Texx.

**RNase Protection Assay**

The RNase protection assay (RPA) was performed as previously described (Darland et al., 1995). In brief, total RNA was isolated using TriReagent (Molecular Research Center, Inc., Cincinnati, OH) acid/phenol extraction (Chomczynski and Sacchi, 1987). Total RNA was obtained from whole eye tissue from the CAM application experiments. Riboprobes were generated in the reaction mix of 12.5 μM cold rCTP (250 μM cold rCTP for CHRPS), 1× transcription buffer, 10 mM dithiothreitol, 10 units RNasin, 500 μM each rUTP, rATP, rGTP (Promega, Madison, WI), 750 ng of template, 25 μCi of [32P]rCTP (3000 Ci/mmol; New England Nuclear, Boston, MA), and 10 units of bacterial RNA polymerase. All mix reagents were from Gibco-BRL (Grand Island, NY), except where indicated. Probes were gel purified and incubated with 15 μg total RNA in a formamide-based hybridization buffer for a 12-h incubation at 45°C. Protection and processing were carried out essentially as described (Gilman, 1991). Protected fragments were run on a 6% acrylamide, 8 M urea, 0.5× TBE gel. Protected fragments were 410 bp (activin A), 223 bp (follistatin), and 100 bp (chick ribosomal protein S17, CHRPS). Dried gels were exposed to phosphorimager ( Molecular Dynamics, Sunnyvale, CA) and the pixel density of the resulting bands was analyzed with IPlab Gel software (Signal Analytics, Vienna, VA).
RESULTS

Detection of Somatostatin in Developing Ciliary Ganglia

Somatostatin expression was assayed by immunohistochemistry using a highly sensitive double peroxidase/antiperoxidase (PAP) detection method. By E14 unstained small and large neurons could be identified by their eosinophilic profiles which could be readily distinguished from the lighter, more amorphous background (Figs. 2A and 2B). The somatostatin-immunoreactive neurons were generally 8–12 μm in diameter and predominantly located in the perimeter of the ganglion. The brown, diaminobenzidine reaction product was punctate, perinuclear, and distinct from the uniformly pink eosin staining of the neuronal profiles (arrowheads in Fig. 2B). Occasional large (20–30 μm) neurons contained somatostatin immunoreactivity. These have been observed by others, but when examined with the electron microscope, they prove to be choroid neurons in that the presynaptic endings are boutons rather than the characteristic calyces found on ciliary neurons (DeStephano et al., 1993). Most of the large neurons (15-30 μm) were unstained and located predominantly, but not exclusively in the core of the ganglion (arrows, Fig. 2B), as previously described for ciliary neurons (DeStephano et al., 1993).

To ensure that somatostatin expression was unaltered during growth under the shellless culture conditions used to perform our experiments, we determined the developmental time course of somatostatin expression. This ex ovo paradigm allows for direct manipulation and observation of chick embryos as they grow (Dunn, 1974; Finn et al., 1998). Embryos are cracked into culture tripods at E3 and develop at a rate similar to that of intact embryos. Ganglia from the shellless 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 (Fig. 3). We observed an increase in somatostatin expression correlated with developmental age. At E8, less than 10% of the neurons are immunoreactive for somatostatin; all of the neuronal cell bodies appear to be similar in size. This corresponds to the beginning of the cell death period in the ganglion (Landmesser and Pilar, 1974) which overlaps with the induction of somatostatin expression (Smet and Rush, 1993). Cell death and somatostatin expression is essentially complete at E14. By this period of development the ciliary and choroid neurons were distinguishable by the collective criteria of soma size, location within the ganglia, and the presence or absence of somatostatin immunoreactivity. Previous studies have established that all somatostatin-immunoreactive cells at E14 are choroid neurons and that this characteristic is 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 by preventing activin from binding to its receptor, then altering the balance

FIG. 2. Immunohistochemical staining for somatostatin in E14 ciliary ganglia. Cryostat sections of E14 ciliary ganglia were incubated with normal rat serum (A) or a rat monoclonal antibody against somatostatin (B). Binding of the primary antibody was visualized with a double PAP procedure, using diaminobenzidine as a substrate. Sections were then counterstained with eosin. Neuronal profiles lacking somatostatin immunoreactivity could be readily observed as eosinophilic, round or oval profiles (open arrowheads). Small, somatostatin-positive choroid neurons are located predominantly in the perimeter or clustered in one quadrant of the ganglion (filled arrowheads), while the larger, somatostatin-negative ciliary neurons are located predominantly in the central region of the ganglion (arrows, A). Calibration bar, 50 μm.
of these factors during development should result directly in changes in the number of somatostatin expressing 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 h postinjection (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. 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 saline-injected eyes had 4929 ± 611 neurons with 42.9 ± 3% somatostatin-positive, while ganglia from uninjected eyes had 5303 ± 722 neurons with 49.3 ± 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 contralateral uninjected eye. Table 1 shows the results of this injection series. The percentage of somatostatin-positive neurons observed in saline-injected ganglia was 47.7 ± 6%. In contrast, the activin A-treated ganglia had 66.4 ± 7.5% somatostatin-positive cells, an increase of 39% relative to control levels (Fig. 5). Only choroid neurons, which comprise approximately 50% of the neuronal population in the ganglia, express somatostatin (Epstein et al., 1988; DeStefano et al., 1993). Therefore, 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. Vehicle-injected ganglia have somatostatin staining patterns similar to untreated ganglia (compare Figs. 4A and 4B with Fig. 2). 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, Figs. 4C and 4D). Higher magnification showed diffuse reaction product within the soma (Fig. 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 ob-

![FIG. 3. 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, which is representative of the adult phenotype. The percentage of neurons in each ganglion that are somatostatin immunoreactive was determined by counting the number of neurons containing punctate, DAB reaction product and dividing by the total number of neuronal profiles observed. Each point is the mean value of percentage somatostatin-positive neurons of at least five ganglia. Error bars represent the standard deviation from the mean.]
FIG. 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 (C), and follistatin CAM application (E)-treated ganglia to indicate relative location. Higher magnification shows small immunoreactive choroid neurons (arrowheads, B) and unstained 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 reaction product (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: 100 μm in A, C, and E and 15 μm in B, D, and F.
tain the presence of follistatin, only $36.8 \pm 10.7\%$ somatostatin-positive neurons were detected. This is a decrement of 23% relative to controls (Fig. 5). In these ganglia, many of the small perimeter neurons, which are normally positive for somatostatin, showed no reaction product (data not shown). Measurements of soma diameter from all three conditions were obtained and no significant differences were observed between treatment conditions (Table 2).

**Chorioallantoic Membrane Delivery of Follistatin**

To increase the 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 choroid 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. The CAM application 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., 1998). From E7 to E13, 1 μg of recombinant follistatin was applied daily onto the CAM. With a standard egg volume of 50 ml, this constitutes approximately 20 ng/ml of follistatin per day. Even if diffusion is only 50% efficient, this level is within the range of effective dose for follistatin to block activin A effects in vitro (Darland et al., 1995). 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 unmanipulated and saline-treated CAM embryos (treated, 6688 ± 1817; untreated, 7138 ± 1435; P = 0.64, two-tailed test). Similarly, no difference between unmanipulated and saline-treated CAM embryos in the percentage of somatostatin-immunoreactive neurons was observed (treated, 52.2 ± 4%; untreated, 49.1 ± 2%; P = 0.11, two-tailed test), 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 (Table 2). Follistatin-treated ganglia were collected at E14, sectioned, and examined for somatostatin immunostaining.

**TABLE 2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>&quot;Choroid&quot; neurons mean diameter $\pm$ SD (n)</th>
<th>&quot;Ciliary&quot; neurons mean diameter $\pm$ SD (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline injection</td>
<td>10.6 ± 1.2 (96)</td>
<td>19.7 ± 2.7 (89)</td>
</tr>
<tr>
<td>Activin injection</td>
<td>10.5 ± 1.3 (90)</td>
<td>20.3 ± 2.5 (81)</td>
</tr>
<tr>
<td>Follistatin injection</td>
<td>10.9 ± 1.2 (112)</td>
<td>20.6 ± 2.6 (110)</td>
</tr>
<tr>
<td>Saline on CAM</td>
<td>11.2 ± 1.1 (93)</td>
<td>20.5 ± 1.6 (87)</td>
</tr>
<tr>
<td>Follistatin on CAM</td>
<td>11.1 ± 1.2 (80)</td>
<td>20.5 ± 1.8 (65)</td>
</tr>
</tbody>
</table>

Note. Mean cell body diameters ($\mu$m) were measured with the investigator blinded as to the treatment conditions. The longest diameter of neurons from fixed and stained ganglia used to quantify the number of somatostatin-positive neurons were measured. Sections from at least three different ganglia were used for each group. Since E14 ciliary ganglia contain two distinct size classes of neurons, measurements were made independently of small, peripheral neurons that are characteristic of choroid neurons versus large, centrally located neurons that are typical of ciliary neurons. The actual variance in size for each group is probably larger because some "large" neurons are likely to be choroid on the basis of their somatostatin immunoreactivity. However, somatostatin immunoreactivity could not be used in these experiments to identify choroid versus ciliary, since this is the variable that is being altered by the treatments. No other independent criteria are available at the light microscopic level to distinguish choroid from ciliary neurons. Cell body diameters of the three injections groups do not differ significantly (one-way ANOVA). Similarly, the cell body diameters of the two CAM application groups do not differ significantly (two-tailed t test).

**FIG. 5.** Change in mean value of percentage somatostatin-positive neurons relative to treatment controls. In the histogram the zero value represents the mean percentage somatostatin-positive neurons for saline-injected or chorioallantoic membrane application controls. Percentage 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 chorioallantoic membrane application control versus follistatin (CAM C vs F). These data are taken from Table 1.
percentage of somatostatin-positive neurons relative to control (see Fig. 5). Clusters of small choroid neurons in the perimeter showed little to no reaction product (Figs. 4E and 4F). Neurons with detectable perinuclear staining remained, but they were often among a cluster of small, negative neurons in the perimeter (arrowheads, Fig. 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

To determine if ciliary ganglion targets were affected by CAM follistatin treatment, we examined sections of iris/ciliary body and choroid layer stained for smooth muscle specific actin (SMSA; Fig. 6). 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. At E14 the iris is a mixture of smooth and striated muscle reflecting the progressive transition to a predominantly striated tissue later in development (Volpe et al., 1993; Link and Nishi, 1998). No change in pigmented epithelium (PE) or stromal (ST) smooth muscle staining pattern in follistatin-treated embryos (Fig. 6B) was detectable relative to controls (Fig. 6A). 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 (Fig. 6D) and follistatin (Fig. 6E) CAM application embryos. 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 (Figs. 6C/6F). 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

We have 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 intracocular 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 support a function in vivo for activin and follistatin in the target-dependent regulation of neuronal differentiation and 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.

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; de Winter 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-stimulating 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. 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 (Darland et al., 1995). 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 results confirm this balance hypothesis. 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 cotransmitter. 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). Activin A does not promote 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 responded to the 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.

Importantly, follistatin, which has no biological activity on its own, but rather prevents activin from binding to its receptor, also affected neuropeptide expression in the ciliary ganglion by reducing the percentage of somatostatin-positive neurons. This reduction was observed with two different application methods, although 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 intracocular injection. One explanation is that the follistatin injected behind the lens had limited accessibility to terminals of the choroid neurons that are throughout the choroid layer. In addition, the injections could only be effectively 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 was 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 cotransmitter phenotype is not completely established and the neurons are still more responsive to exogenous cues. In contrast, with the CAM application method follistatin could be delivered as early as 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 striking reduction in somatostatin expression observed with the CAM application method reflects the ability of follistatin to block the activity of an endogenous factor that regulates somatostatin expression at the choroid neuron target. The endogenous inducer of somatostatin expression is most likely to be activin A. Both ciliary and choroid neurons express the Type IIA serine/threonine kinase receptor for activin A (Kos and Coulombe, 1997) and are able to respond in
In addition, we showed that choroid tissue completely accounted for by activin A (Coulombe and Nishi, 1991). Follistatin binds most strongly with activin A (Nakamura et al., 1990; Sumitomo et al., 1995), while exhibiting a lower affinity for some members of the bone morphogenetic protein (BMP) family (Yamashita et al., 1995; Hogan, 1996; Fainsod et al., 1997). In fact, activin A and BMPs induce differing patterns of message levels for neuropeptides expressed by sympathetic neurons in culture (Fann and Patterson, 1994). Definitive proof that follistatin and activin are involved in determining somatostatin expression in the ciliary ganglion awaits experiments blocking the expression of endogenous molecules; however, such knock-out or knock-down technology remains elusive, although LIF, CNTF, and cardiotrophin-1 were unable to express somatostatin (Coulombe and Nishi, 1991). In vivo induction of ciliary neurons to express somatostatin, a previously established that CG neurons expressing somatostatin. Our previous studies established activin A, while the iris and ciliary body express activin A together with follistatin (Darland et al., 1995). Thus, the sum of these results strongly implicates activin A and follistatin as the target-derived molecules in vivo that regulate neuropeptide expression during development in the ciliary ganglion.

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