The Journal of Neuroscience, July 1991, 11(7): 2177–2186

Regulation of Acetylcholine Receptors on Chick Ciliary Ganglion Neurons by Components from the Synaptic Target Tissue

Stanley W. Halvorsen,[a] Herbert A. Schmid,[b,c] Adrienne E. McEachern,[c,d] and Darwin K. Berg

Department of Biology, University of California, San Diego, La Jolla, California 92093

Chick ciliary ganglion neurons have nicotinic acetylcholine receptors (AChRs) that mediate synaptic transmission through the ganglion. A soluble component of about 50 kDa from embryonic eye tissue, the synaptic target of the ganglion, increases the development of ACh sensitivity by the neurons 10-fold over a 1-week period in culture. The increased sensitivity does not arise from a change in agonist affinity or esterase activity. Both the basal ACh response obtained in the absence of the 50-kDa component and the elevated responses obtained with it can be inhibited by neuronal bungarotoxin (nBgt) but not by α-bungarotoxin (αBgt). Increases of less than twofold are observed for the binding of anti-AChR monoclonal antibody 35 (mAb 35), nBgt, and αBgt to the neurons under these conditions. Extract fractions containing the 50-kDa component also enable the neurons to enhance their ACh responses through a cAMP-dependent mechanism. Either the 50-kDa fraction induces the appearance of a new type of AChR regulated by cAMP, or it alters the function of existing AChRs.

The 50-kDa fraction produces no change in neuronal growth but can increase GABA responses sixfold, indicating that its effects are not confined to AChRs. It is not clear whether a single molecular species is responsible for the diverse regulatory effects or whether several types of active components are present in the fraction. The component which enhances ACh sensitivity is trypsin-sensitive and heat-labile, as expected for a protein. The component may be widely distributed since the 50-kDa fraction from a number of tissues can increase the ACh response. The fraction from eye tissue, however, has a specific activity 5–10 times greater than that of the liver fraction. A wide distribution would suggest multiple targets and roles for the component during development.

Received Nov. 9, 1990; revised Feb. 19, 1991; accepted Feb. 20, 1991.

We thank Susan Tsunoda and Amy Lovejoy for preparation of the cell cultures and Lynn Ogden for expert technical assistance. Grant support was provided by the National Institutes of Health (Grants RO1 NS12601 and PO1 NS25916), the Muscular Dystrophy Association, and the American Heart Association with funds contributed in part by the California Heart Association.

Correspondence should be addressed to Darwin K. Berg, Department of Biology, B-022, University of California, San Diego, La Jolla, CA 92093.

[a] Present address: Department of Biochemical Pharmacology, School of Pharmacy, SUNY at Buffalo, Buffalo, NY 14260.

[b] Present address: Max-Planck-Institute fur physiologische und klinische Forschung, W.G. Kerckhoff Institut, 6350 Bad Nauheim, Germany.

[c] These two authors contributed equally.

[d] Present address: Department of Molecular and Cellular Physiology, Stanford University School of Medicine, Stanford, CA 94305.

Controlling the number and distribution of neurotransmitter receptors on the postsynaptic cell is an essential part of synapse formation. Cell–cell interactions are known to regulate this process at the vertebrate neuromuscular junction where presynaptic input induces a high concentration of relatively stable nicotinic acetylcholine receptors (AChRs) in the postsynaptic membrane (for review, see Schuetze and Role, 1987). Two molecules likely to influence the distribution of muscle AChRs have been purified (Usdin and Fischbach, 1986; Nitkin et al., 1987).

Neurotransmitter receptors on neurons may be subject to more complex regulation than that observed for muscle AChRs. Both anterograde signals from the presynaptic input and retrograde signals from the synaptic target tissue are likely to act on neurons to regulate transmitter receptors. Best studied are the AChRs of autonomic neurons. Preganglionic denervation has been reported to increase the ACh response of frog and mudpuppy parasympathetic neurons (Kuffler et al., 1971; Roper, 1976; Dennis and Sargent, 1979) while decreasing the total number of AChRs revealed by immunocytochemistry on the cells (Sargent and Pang, 1988). Denervation of frog sympathetic neurons and chick ciliary ganglion neurons has little if any effect on the neuronal ACh response (Dunn and Marshall, 1985; McEachern et al., 1989), though denervation does decrease substantially the size of the intracellular AChR pool in ciliary ganglion neurons (Jacob and Berg, 1987, 1988).

Retrograde signals appear to play a more dramatic role. Postganglionic axotomy, which isolates neurons from their synaptic targets, reduces the ACh response of ciliary ganglion neurons in newly hatched chicks by about 10-fold in 5 days (Brenner and Martin, 1976; McEachern et al., 1989) and reduces the total number of AChRs associated with the neurons to a similar extent (Jacob and Berg, 1987, 1988). The effect is specific in that surviving neurons are similar to unoperated neurons in size and ultrastructural features and show no unilateral decrease in their GABA responses (Jacob and Berg, 1988; McEachern et al., 1989).

The axotomy studies suggest that information from the synaptic target tissue may be necessary to maintain neurotransmitter receptors on neurons. The information could be conveyed by a component of target tissue origin acting transynaptically. Nerve growth factor is an example of a target-derived component necessary for the survival and development of sensory and sympathetic neuron populations (for review, see Barde, 1989).

Chick ciliary ganglion neurons provide a system for identifying target-derived components that regulate neuronal AChRs. The neurons can be maintained in cell culture where they express AChRs and form functional synapses. Embryonic eye tissue, which contains all the normal synaptic targets of the neurons, has several components that influence the development of the

neurons in cell culture. One is ciliary neurotrophic factor (CNTF), a protein that can increase neuronal survival in culture (Barbin et al., 1984; Rudge et al., 1987) while specifically downregulating the number of α-bungarotoxin binding components (αBgtBCs) on the neurons (Halvorsen and Berg, 1989). The mammalian homolog of the chicken CNTF gene has recently been cloned from rabbit and rat and found to encode a protein of 23 kDa (Lin et al., 1989; Stöckli et al., 1989). Eye tissue also contains a growth-promoting activity (GPA) that enhances neuronal growth in culture, and a choline acetyltransferase-stimulating activity (CSA) that increases the amount of choline acetyltransferase activity in culture without changing neuronal growth (Nishi and Berg, 1981). GPA includes CNTF (Halvorsen and Berg, 1989), but CSA is clearly a separate component, having a nominal size by gel filtration of 40–50 kDa (Nishi and Berg, 1981).

We report here that a component(s) of about 50 kDa in embryonic eye extract causes a 10-fold increase in the ACh response of chick ciliary ganglion neurons while producing a 1.5-fold increase in the total number of AChRs detectable on the cells with an anti-AChR monoclonal antibody (mAb). Moreover, the component(s) enhances the ability of the neurons to increase their ACh responses in a cAMP-dependent manner. The component(s) is a candidate for a target-derived molecule that regulates the development of neurotransmitter sensitivity in neurons.

Materials and Methods

Cell cultures. Freshly isolated ciliary ganglia from 8-d chick embryos were dissociated with trypsin and grown at 37°C in a 5% CO_2/95% air atmosphere on a substratum of collagen and lysed fibroblasts as previously described (Nishi and Berg, 1981). Cultures were prepared with 10^4 neurons (1½ ganglion equivalents) in 35-mm dishes. The basal culture medium consisted of Eagle's minimal essential medium containing 10% (v/v) heat-inactivated horse serum, 50 U/ml penicillin, and 50 μg/ml streptomycin. The medium was supplemented with embryonic eye extract or extract fractions as indicated below, all at 3% (v/v; about 9 μg of protein/ml) unless otherwise indicated. The medium was replaced at 2-d intervals. These conditions provide for the long term survival of essentially all chick ciliary ganglion neurons in culture (Nishi and Berg, 1981).

Extract fractions. Eye extract was prepared from 17–19-d chick embryos in a modified Puck's saline containing glucose, as previously described (Nishi and Berg, 1981). The extract (ca. 2 mg of protein/ml) was concentrated 10-fold using an Amicon concentrator with a PM-10 filter (10,000 MW cutoff). The concentrated extract (1.5 ml) was loaded onto a 2.1 × 42-cm column of LKB ACA 44 Ultragel equilibrated in PBS (0.1 M NaCl and 0.01 M $NaPO_4$, pH 7.4). Fractions (3 ml) were collected, filtered for sterility, and assayed within 2 weeks. Fractions containing material of 20–30 kDa and 0.1–0.3 mg/ml protein were pooled and used as a source of CNTF, either fresh or stored frozen at −70°C.

Extracts from other tissues were prepared as described above and fractionated by passage over the same column. Fractions were assayed for protein by the method of Lowry et al. (1951) and then diluted to yield concentrations equivalent to those obtained by fractionating eye extract.

Binding assays. For mAb 35 and αBgt, binding assays on cells in culture were initiated by replacing the culture medium with 1.0 ml fresh medium (without extract supplements) containing either 10^{-8} M ^{125}I-αBgt or 2.5×10^{-9} M ^{125}I-mAb 35. After 60 min at 37°C, the reaction was terminated by removing the medium and rinsing the cells four times with 2-ml aliquots of HEPES solution (137 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 0.9 mM Na_2PO_4, 0.4 mM KH_2PO_4, 1.8 mM $CaCl_2$, 2 mg/ml BSA, and 10 mM HEPES, pH 7.4). The cells were solubilized in 0.5 ml of 5 mM $NaPO_4$, pH 7.4, containing 0.5% Triton X-100 and 50 mM NaCl, and measured for radioactivity with a model 1191 Tracor γ-counter. Specific binding was calculated as the difference between total binding and nonspecific binding, where nonspecific binding was taken to be that occurring in the presence of 10^{-6} M unlabeled αBgt for ^{125}I-αBgt and in the presence of 0.5×10^{-6} M unlabeled mAb 35 for ^{125}I-mAb 35.

For neuronal Bgt (nBgt) binding assays, cultures were first incubated in 10^{-8} M αBgt for 20 min at 37°C to prevent nBgt from binding to αBgt sites (Halvorsen and Berg, 1986). ^{125}I-nBgt was then added to a final concentration of 10^{-8} M in 0.7 ml, and the assay was continued as described above. Nonspecific binding was determined by including 10^{-7} M nBgt in the 20-min preincubation with αBgt.

The amount of specific binding per culture was corrected for variations in cell number and/or cell growth among cultures by normalizing the binding for the relative amount of cytoplasm in the cultures. Lactic acid dehydrogenase (LDH), a constitutive cytoplasmic enzyme, was assayed as a means of comparing the relative amounts of cytoplasm present as previously described (Nishi and Berg, 1981). LDH activity was determined in aliquots of cell extracts by following spectrophotometrically the conversion of NADH to NAD in the presence of sodium pyruvate. One unit of LDH activity was defined as a decrease of 1 OD unit/min in absorption at 340 nm using a pathlength of 1 cm.

Electrophysiology. In most cases, intracellular recordings were used to examine the electrophysiological properties of neurons. Ciliary ganglion cultures were prepared from 8-d chick embryos and examined after 6–7 d in cell culture unless otherwise indicated. The procedures have been described in detail elsewhere (Smith et al., 1983; McEachern et al., 1985, 1989). Cultures were maintained at 37°C on the stage of an inverted microscope and continuously perfused with recording solution [137 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 0.9 mM Na_2PO_4, 0.4 mM KH_2PO_4, 5.4 mM $CaCl_2$, 5.6 mM glucose, 2.5 mM HEPES, pH 7.4, and the essential amino acids and vitamins present in Eagle's minimum essential medium (MEM)]. Neurotransmitter sensitivities were determined by measuring changes in membrane conductance caused by transmitter being pressure-ejected (1–3 psi for 1–3 sec) from a pipette (4–6 μm tip diameter) positioned 10–20 μm from the soma (Choi and Fischbach, 1981; McEachern et al., 1985; McEachern and Berg, 1988). Transmitter sensitivities determined in this way are likely to represent the summed response of receptors on the soma since the procedure immerses the soma in the test compound. Sensitivities were calculated as the difference between the maximum conductance measured in the presence of transmitter and the resting conductance prior to application of transmitter. Unless otherwise indicated, the results have been corrected for differences in cell size by expressing them as conductance per soma surface area (pS/μm²) for individual neurons, where the surface area was calculated from the cell diameter measured in the microscope. Data were accepted only when the neuron had a resting potential more negative than −45 mV and could fire overshooting action potentials in response to intracellular stimulation, and when the recording electrode was no more than 3 mV out of balance when withdrawn from the neuron. Unless otherwise indicated, ACh was applied at 10 or 30 μM to obtain accurate, quantifiable responses with little desensitization. The EC_{50} for ACh is about 100 μM (Smith et al., 1983). Since GABA responses were small, the compound was routinely tested at 50 μM, a concentration near the EC_{50} for GABA (McEachern and Berg, 1988). Voltage–current relationships for neurons were linear over the range of −80 to −48 mV. In some cases, ACh depolarized the membrane beyond −48 mV, but little distortion of the response would have resulted from the small incursion into the nonlinear range of the current–voltage relationship (Margiotta and Berg, 1986).

In some cases, whole-cell voltage clamp recordings were used to examine ACh responses. Such experiments included most of those testing the heat sensitivity of extract components and the αBgt sensitivity of ACh responses. The experiments used procedures previously described (Hamill et al., 1981; Margiotta et al., 1987a,b) and were performed on ciliary ganglion cultures prepared as described above or on ciliary ganglion neurons freshly dissociated from 16–18-d chick embryos (Margiotta and Gurantz, 1989). During the recordings, the neurons were perfused with (in mM) 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 10 HEPES (pH 7.2). The patch pipette contained (in mM) 140 CsCl, 2 EGTA, 10 glucose, 2 Mg/ATP, and 5 HEPES (pH 7.2). In some cases, the EGTA/Ca^{2+} ratio in the pipette was adjusted to generate a free Ca^{2+} concentration between 10^{-8} and 10^{-6} M according to the program of Fabiato (1988). ACh at 10–500 μM was applied to neuron somata from a perfusion pipette by pressure at 6–12 psi for 2–4 sec. Membrane currents were collected using an Axopatch 1C patch clamp amplifier equipped with capacity compensation (Axon Instruments, Burlingham, CA). The currents induced by ACh were filtered at 10 kHz and digitized at sample intervals of 2 msec using an Axolab 1100 in connection with the PCLAMP data acquisition and stimulation program (Axon Instru-

ments) on an IBM-compatible computer (Descpro 286) with math coprocessor 80387-25. Whole-cell currents elicited by ACh microperfusion were usually measured at an applied holding potential of −70 mV and were not electronically corrected for series resistance errors. Series resistances, estimated as previously described (Margiotta and Gurantz, 1989), were typically less than 3.5 MΩ. This would produce an error of less than 7 mV in the actual holding potential at the peak whole-cell current induced by 30 μM ACh (maximum response of 2000 pA).

Sensitivity data were routinely compared by constructing log-normal distributions and calculating the geometric means in each case. This was done because the distribution of response magnitudes was skewed in each case rather than normal (Dennis and Sargent, 1979; Hancock et al., 1988). For calculation of geometric means, null ACh responses were usually assigned the value of 0.1 pS/μm² since this was estimated to be the maximum response that would have been too small for detection in the present recordings. Unless otherwise indicated, the data are presented as geometric means with SEMs calculated as averages of the differences between the upper and lower geometric error limits. Results were compared statistically by Student's *t* test.

Materials. White leghorn chick embryos were obtained locally and maintained at 37°C in a humidified incubator. Tissue extracts and culture media were prepared as previously described (Nishi and Berg, 1981). αBgt and nBgt (also known as Bgt 3.1) were purified from *Bungarus multicinctus* venom as previously described (Ravdin and Berg, 1979; Ravdin et al., 1981). αBgt was radioiodinated to a specific activity of 0.5–0.7 × 10^{18} cpm/mol using a modified chloramine T method previously described for antibodies (Lindstrom et al., 1981). mAb 35 was purified and radioiodinated to a specific activity of 2–3 × 10^{18} cpm/mol as previously described (Smith et al., 1985). The hybridoma cell line secreting mAb 35 was generously provided by Dr. J. Lindstrom (Salk Institute). Other materials were obtained as previously indicated (Halvorsen and Berg, 1986, 1987). 8-Bromo-cyclic AMP (8-Br-cAMP) and 3-iso-butyl-l-methylxanthine (IBMX) were purchased from Sigma.

Results

Increased ACh sensitivity. Extracts prepared from embryonic chick eye tissue were examined for components that regulate the ACh response of ciliary ganglion neurons in cell culture. Gel filtration was used to fractionate the extracts, and neurons were grown for 6 d in media supplemented with CNTF and individual column fractions. Neurons receiving only CNTF developed ACh responses that were about a tenth of that obtained with complete extract. Neurons receiving column fractions that contained material of about 50 kDa developed large ACh responses, similar to those obtained from neurons grown with complete eye extract (Fig. 1). No other column fractions had such a pronounced and reproducible effect, though sometimes a small enhancement of the ACh response was observed for cells grown with fractions containing smaller material. Normalizing the responses for cell size (pS/μm²) indicated that the 50-kDa fraction caused a specific 9.9 ± 2.0-fold increase in the ACh response (mean of 11 separate experiments).

The increased ACh response brought on by growth in the 50-kDa fraction could not be accounted for by a change in the affinity of the receptor for agonist. Even high concentrations of ACh (0.5–1.0 mM) that elicited near-maximal responses and partially desensitized the neurons revealed a substantial enhancement of the ACh response in cells grown with the 50-kDa fraction (4.6 ± 0.7-fold, 14–15 neurons for each condition, combined from two separate experiments). The increased sensitivity also could not be accounted for by a change in acetylcholinesterase activity. Testing neuronal responses to carbachol, an agonist resistant to hydrolysis by esterases, showed a difference of 5.4 ± 1.9-fold (16–19 neurons for each condition, combined from three separate experiments) in the sensitivities of neurons grown with and without the 50-kDa fraction.

Developmental increase. The change in ACh sensitivity caused by the 50-kDa fraction occurred slowly in cell culture and represented a developmental increase. When normalized for cell size (pS/μm²), neurons receiving only CNTF showed no specific increase in ACh response over the 7-d test period (Fig. 2). In contrast, neurons receiving the 50-kDa fraction showed a specific increase in the ACh response that was proportional to the time of growth with the fraction. The increase did not require CNTF. Omitting CNTF from cultures grown with the 50-kDa fraction did not reduce the ACh response of the neurons (data not shown), though it did reduce neuronal survival in some cases.

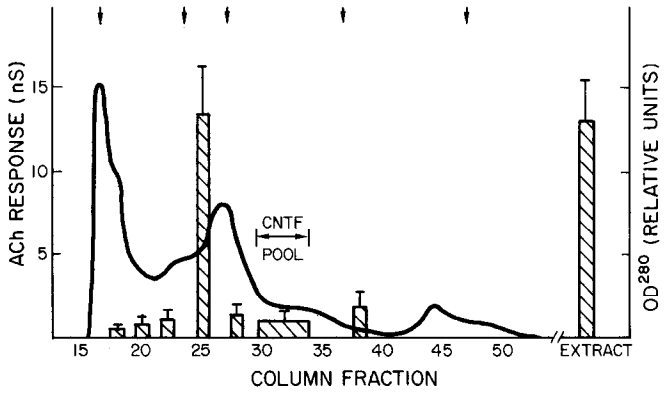

Figure 1. Effects of eye extract fractions on ACh responses from ciliary ganglion neurons. Eye extract was fractionated by gel filtration, and the fractions were tested for effects on neuronal ACh sensitivity. Neurons were grown for 6–7 d in basal media supplemented with complete eye extract (*Extract*), or CNTF (*pooled fractions 30–33*), or CNTF plus an additional fraction (*fraction 18, 20, 22, 25, 28,* or *38*), all at 3% (v/v). ACh responses were then measured with intracellular recording, and the geometric mean response (nS) ± SEM for 5–12 neurons was calculated in each case (*hatched bars*). The *solid curve* indicates the absorption at 280 nm for the column eluate, measured with a Uvicord. The *arrows,* from left to right, indicate the elution positions of blue dextran (2000 kDa), BSA (68 kDa), ovalbumin (43 kDa), soybean trypsin inhibitor (21.5 kDa), and cytochrome C (12.5 kDa). *Fraction 25,* containing material of about 50 kDa, elevated the neuronal ACh response to the level obtained for neurons grown with complete extract. Similar results were obtained in two other complete fractionation experiments (though we were not always so lucky as to select the peak tube for assay). In each case, a maximum effect was obtained for fractions containing material of about 50 kDa, and the extent of the effect was comparable to that obtained with the complete extract. Normalizing the results for cell size as described in Figure 2 did not alter the pattern. Fifteen additional preparations of eye extract were fractionated by gel filtration, and the resulting 50-kDa fraction in each case was found to promote a similar increase in ACh sensitivity.

The active component(s) in the 50-kDa fraction was trypsin-sensitive and heat-labile. Treating the material with 22 μg/ml trypsin for 1 hr at 37°C substantially reduced its ability to influence the development of ACh sensitivity. At 3% (v/v), the trypsinized 50-kDa material supported only 20 ± 10% of the increase caused by untreated material (mean ± SEM of two experiments; 22 neurons). If the trypsin was first inactivated by incubation with 5 μg trypsin inhibitor per μg trypsin, no reduction in potency was observed for the material. Heating the 50-kDa fraction to 60°C for 1 hr partially blocked its effects. At 3% (v/v), the heated fraction achieved 54 ± 27% of the increase induced by untreated material (mean ± SEM of three experiments; 16 neurons). Heating the fraction to 100°C for 1 hr blocked nearly all of the stimulatory effect (Fig. 3).

The ACh response of chick ciliary ganglion neurons is subject to agonist-induced desensitization (Margiotta et al., 1987a,b). Whole-cell voltage clamp recordings such as those shown in

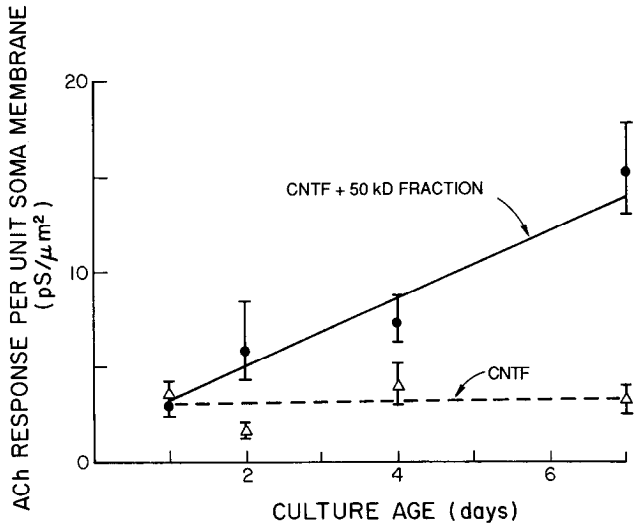

Figure 2. Developmental increase in the ACh response caused by the 50-kDa fraction. Neurons were grown for the indicated times in basal medium supplemented with CNTF (*open triangles*) or CNTF plus the 50-kDa fraction (*filled circles*) and then assayed for sensitivity to 100 μM ACh. Results are expressed as pS/μm² of soma membrane to normalize for cell size. Values represent the geometric mean ± SEM of 12–22 neurons per condition at each time point and are combined from two separate experiments. No specific increase in ACh response is observed for neurons receiving only CNTF. In contrast, neurons receiving both CNTF and the 50-kDa fraction undergo a specific increase in the ACh response over the 7-d period.

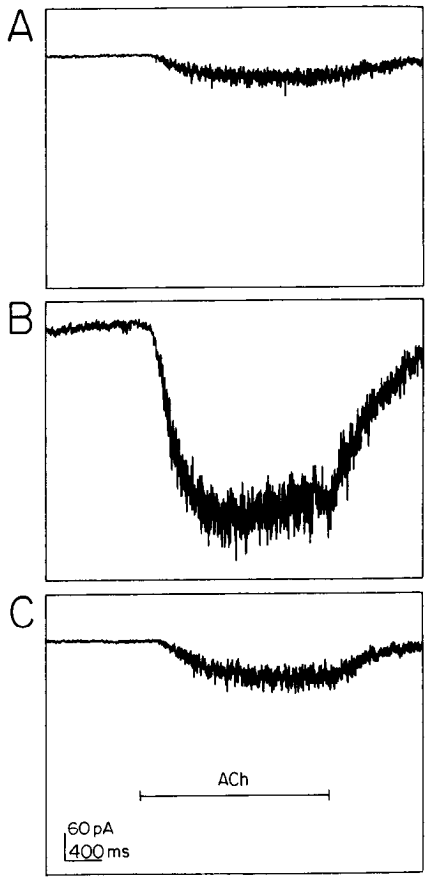

Figure 3. Whole-cell voltage clamp recordings of ACh responses from neurons grown with heat-inactivated 50-kDa fraction. Neurons were grown for 6–7 d in basal medium supplemented with (*A*) CNTF, (*B*) CNTF plus 50-kDa fraction from eye extract, or (*C*) CNTF plus 50-kDa fraction that had been heat-inactivated at 100°C for 1 hr. The traces show whole-cell voltage clamp recordings from cells while 30 μM ACh was applied by pressure to the soma from a nearby pipette. *Calibration bars*: *vertical,* 60 pA; *short horizontal,* 400 msec; *long horizontal,* ACh application (2 sec). The heat treatment largely eliminated the ability of the 50-kDa fraction to enhance ACh sensitivity. The example shown in *panel C* is the largest response observed for neurons grown with heat-inactivated 50-kDa component. No dramatic differences were observed in the time course of the responses.

Figure 3 and intracellular recordings (not shown) illustrate that the time course of the ACh response is not substantially altered by growing the neurons with the 50-kDa fraction. This makes it unlikely that the 10-fold increment in ACh sensitivity caused by the 50-kDa fraction derives predominantly from a change in the known forms of desensitization. It remains possible, however, that a very rapid form of desensitization exists and is modified by the 50-kDa fraction in a way that accounts for the observed changes in ACh sensitivity.

Tissue distribution. Eye tissue was selected initially for preparation of the extracts because the tissue contains all of the structures innervated by ciliary ganglion neurons *in vivo*. Extracts were also prepared from other embryonic tissues and fractionated by gel filtration to determine if they contained material of 50 kDa that could promote the development of ACh sensitivity by ciliary ganglion neurons in cell culture in a similar fashion. Indeed, all tissue extracts examined had some 50-kDa stimulatory activity when assayed at high concentration (Table 1). A quantitative comparison was carried out to determine the relative amounts of 50-kDa stimulatory activity in embryonic eye and liver tissue. Dose–response curves were constructed using 50-kDa fractions diluted to equivalent protein concentrations. The 50-kDa fraction from eye extract achieved a half-maximal effect at 0.4–0.5% (v/v), while the 50-kDa fraction from liver had little effect at concentrations below 3% (v/v) (Fig. 4). This suggests a difference in specific activity of at least 5- to 10-fold for the 50-kDa fractions from the two tissues.

mAb 35 binding sites. One mechanism by which the 50-kDa fraction might produce a developmental increase in the ACh response is by increasing the total number of AChRs on the neurons. We examined this possibility by using the anti-AChR monoclonal antibody mAb 35 to quantitate the number of

Table 1. Relative ACh responses for neurons grown with 50-kDa fractions from various sources

Source of 50-kDa fraction	Relative ACh response	
None	1.0 ± 0.2	(47/6)
Eye	12.2 ± 2.2	(56/6)
Heart	11.4 ± 2.5	(16/2)
Muscle	7.9 ± 1.7	(16/2)
Brain	4.5 ± 1.7	(9/2)
Lung	7.6 ± 0.9	(11/2)
Liver	3.1 ± 0.8	(33/4)

ACh responses were recorded from neurons grown for 6 d in media containing CNTF or CNTF supplemented with 50-kDa fractions from the indicated sources at 3% (v/v) from 0.3 mg/ml stock solutions. Values represent the geometric mean response ± SEM, normalized for the mean response obtained from neurons in the same set of experiments grown without the 50-kDa fraction. The numbers in parentheses indicate the number of neurons tested in each case followed by the number of separate experiments from which data were compiled.

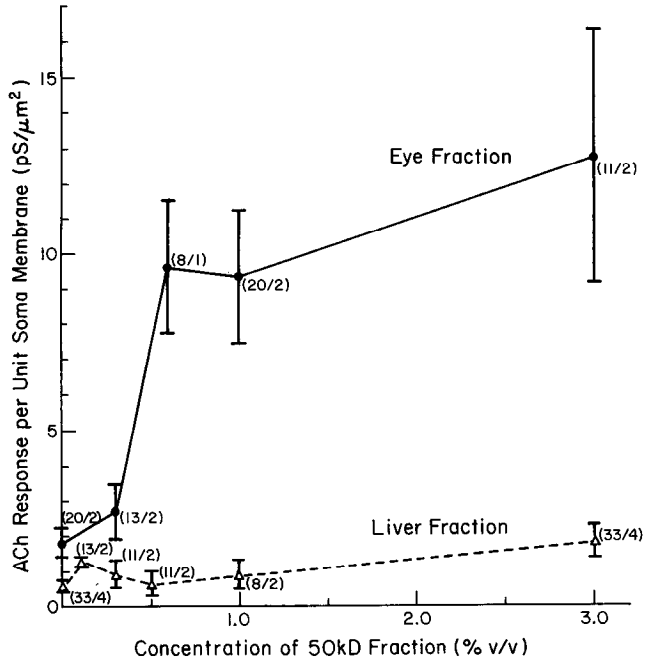

Figure 4. Comparative dose–response curves for the 50-kDa fractions from eye and liver extracts. Neurons were grown for 6–7 d in basal medium with CNTF and the indicated concentration of 50-kDa fraction isolated from eye (*filled circles*) or liver (*open triangles*) extract. The fractions were diluted initially to contain equivalent amounts of protein, i.e., 100% (v/v) = 0.3 mg/ml protein. The results have been normalized for soma membrane (pS/μm^2) to correct for cell size. Values represent the geometric mean ± SEM; the *numbers* in *parentheses* indicate the number of neurons for each point followed by the number of experiments from which data were compiled. With respect to absolute ACh responses, the liver fractions show little effect. The "0%" determinations, however, differ for the two sets of data, reflecting the variation among experiments. If the data are normalized to the corresponding "0%" value in each case, a threefold enhancement of ACh sensitivity is obtained with the liver fraction at 3% (v/v). A similar increase is obtained with the eye fraction at about 0.4% (v/v), suggesting a difference in activity of about eightfold for fractions from the two sources. The same conclusion is reached if the data are not corrected for cell size, i.e., compared as nS/cell.

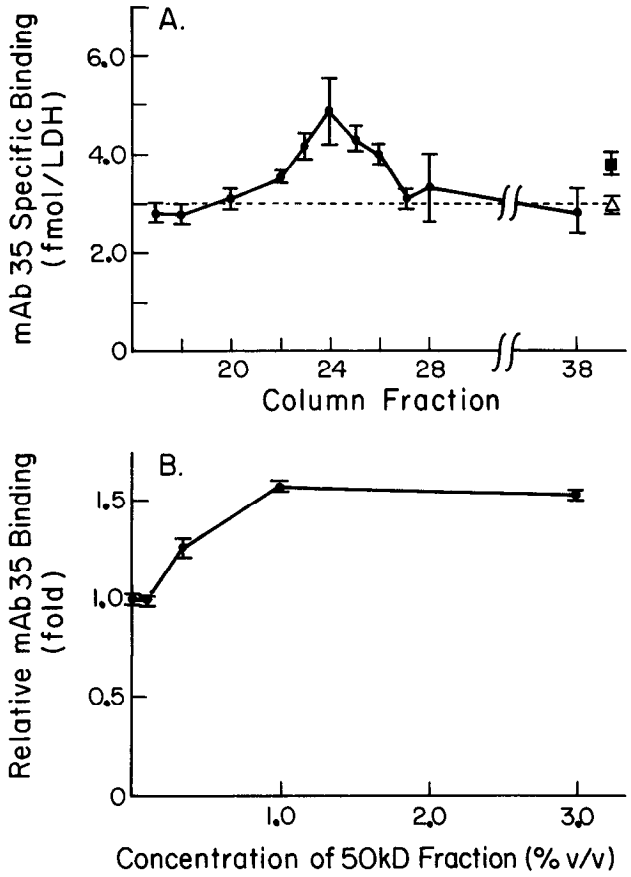

Figure 5. Effects of the 50-kDa eye extract fractions on mAb 35 binding. Neurons were grown for 6–7 d in basal medium supplemented in *panel A* with eye extract (*filled square*), CNTF (*open triangle, dashed line*), or CNTF and the indicated fractions (*filled circles, solid line*) and in *panel B* with the indicated dilutions of a 0.3 mg/ml 50-kDa eye extract fraction, and then assayed for ^{125}I-mAb 35 binding and LDH. Values represent the mean ± SEM of three cultures per point from a single experiment in *panel A* (a second experiment gave similar results) or seven cultures per point combined from two separate experiments in *panel B*. Only fractions containing material in the range of 50 kDa produced an increase in the specific binding of mAb 35.

AChRs. Several lines of evidence indicate that mAb 35 recognizes the functional, synaptic AChR on chick ciliary ganglion neurons (Jacob et al., 1984; Smith et al., 1985, 1986; Halvorsen and Berg, 1986, 1987, 1990). Neurons were grown with CNTF and column fractions for 6 d as described in Figure 1 and then assayed for ^{125}I-mAb 35 binding. The results were normalized to LDH activity in the cultures to control for differences in cell growth caused by the fractions. Expressed in this way, the data show that only the region of the column eluate containing material of about 50 kDa altered the relative level of AChRs on the neurons (Fig. 5*A*). Combining the results obtained with 50-kDa fractions from a number of such columns yielded an average increase of 1.5 ± 0.1-fold in the number of AChRs (mean ± SEM, 17 separate determinations). Scatchard analysis confirmed that the increase represents a change in the number of sites (1.6 ± 0.2-fold, $n = 2$) rather than a change in the affinity of the sites for mAb 35. The concentration dependence of the effect was similar to that observed for the enhancement of ACh sensitivity by the 50-kDa fraction. A half-maximal increase in the number of mAb 35 binding sites was achieved with growth in the 0.4–0.5% (v/v) 50-kDa fraction (Fig. 5*B*).

αBgt binding sites. Though the 50-kDa fraction elevated the level of mAb 35 binding on the neurons to that obtained with complete extract, the relative increase was much smaller than that observed for the ACh response. This raised the possibility that a second class of AChRs, a class not recognized by mAb 35, might be responsible for the altered sensitivity. Ciliary ganglion neurons are known to have another class of membrane components that bind cholinergic ligands. These are the αBgtBCs that bind αBgt and cholinergic ligands, but not mAb 35, and are located predominantly in nonsynaptic regions on the neurons (Jacob and Berg, 1983; Loring et al., 1985; Smith et al., 1985). No physiological function has yet been identified for the αBgtBCs. Recently, however, two genes have been cloned encoding putative subunits of αBgtBCs in chicken brain (Schoepfer et al., 1990). Sequence analysis of the genes suggests that they encode ligand-gated ion channels. A mAb to one of the αBgtBC gene products immunoprecipitates a large fraction of the αBgtBCs from chick ciliary ganglia (S. Halvorsen and D. Berg, unpublished observations). Expression of that same gene product in

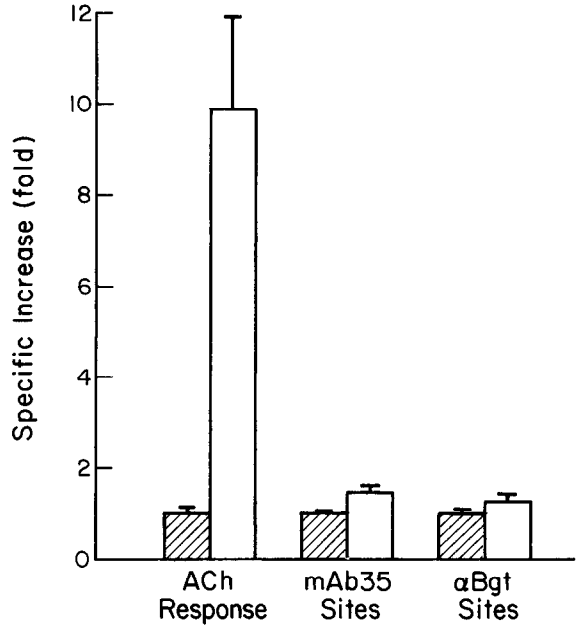

Figure 6. Relative increases in ACh response, mAb 35 binding, and αBgt binding caused by the 50-kDa fraction. Neurons were grown for 6–7 d in basal medium with CNTF (*hatched bars*) or with CNTF and 50-kDa fraction (*open bars*) and then assayed for ACh responses (pS/μm², *ACh response*), ^{125}I-mAb 35 binding (fmol/LDH; *mAb 35 sites*), and ^{125}I-αBgt binding (fmol/LDH; *αBgt sites*). Results have been compiled from 11, 17, and 4 separate experiments, respectively. Geometric means were calculated for the ACh responses and arithmetic means for the binding data. The means were normalized to the values obtained for neurons grown in basal medium with CNTF only.

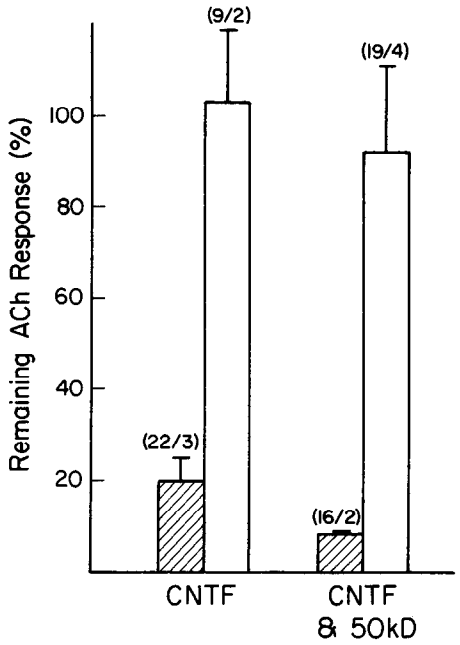

Figure 7. Toxin sensitivities of ACh responses from neurons grown with and without the 50-kDa fraction. Neurons were grown for 5–7 d in basal medium with CNTF or with CNTF and the 50-kDa fraction and then tested for ACh responses either with intracellular recording or with whole-cell voltage clamp in the presence of 10 nM nBgt (*stippled bars*) or 100 nM αBgt (*open bars*) for at least 20 min. Results were normalized for cell size as pS/μm² or pA/μm² and then expressed as a percentage of the mean response obtained from control neurons in the absence of toxin. The geometric mean ± SEM was calculated for each condition. The *numbers* in *parentheses* indicate the number of neurons tested followed by the number of experiments; comparable numbers of control neurons were tested. nBgt blocks the ACh response from cells grown with and without the 50-kDa fraction; αBgt has no significant effect in either case.

Xenopus oocytes produces functional AChRs that are blocked by αBgt (Couturier et al., 1990). Accordingly, we tested the ability of the 50-kDa fraction to alter the number of ^{125}I-αBgt binding sites on ciliary ganglion neurons. A specific increase of 1.3 ± 0.1-fold (mean ± SEM, 15 cultures per condition combined from four experiments) in the number of αBgt binding sites was observed when neurons were grown for 6 d with the 50-kDa fraction. As with mAb 35 binding sites, the increase was specific but was much smaller than the increase observed in the ACh response (Fig. 6).

Toxin sensitivities. Another approach to characterizing the AChRs responsible for the increased response is to examine their sensitivities to neurotoxins. It has recently been shown that neuronal AChR subtypes can differ in their responses to nBgt (Duvoisin et al., 1989; Luetje et al., 1990; Sorenson and Chiappinelli, 1990). In previous studies with chick ciliary ganglion neurons, nBgt was found to block the ACh response while αBgt failed to do so (Ravdin and Berg, 1979). Neurons grown with CNTF or with CNTF plus the 50-kDa fraction have ACh responses that are largely blocked by incubating the cells for 20 min at 37°C with 10 nM nBgt (Fig. 7). In contrast, αBgt at 100 nM was unable to inhibit significantly the ACh response of neurons grown either with CNTF alone or with CNTF in combination with the 50-kDa fraction. The concentration of αBgt used was more than adequate to saturate the known αBgt binding sites on the neurons (Ravdin et al., 1981).

If the 50-kDa fraction induces the appearance of a new type of AChR on the neurons, it is a class of receptor that does not appear to be recognized very well by αBgt or mAb 35, and yet is sensitive to nBgt. To identify such receptors, we measured ^{125}I-nBgt binding. Neurons grown with the 50-kDa fraction for 7 d bound 1.2 ± 0.4-fold (n = 3 experiments) as much ^{125}I-nBgt as did control neurons. This is within the range of values obtained for the relative increments both in ^{125}I-mAb 35 binding and in ^{125}I-αBgt binding to neurons grown with the 50-kDa fraction (Fig. 6) and is consistent with previous studies suggesting that nBgt and mAb 35 recognize a common class of AChRs on the neurons (Halvorsen and Berg, 1986, 1987). No evidence was obtained for a new class of AChRs that bound nBgt but not mAb 35 or αBgt.

cAMP-dependent regulation. A different mechanism by which the 50-kDa fraction may increase the ACh sensitivity of neurons is by enhancing the response of existing AChRs. A change in AChR function has previously been invoked to explain a cAMP-dependent increase in ACh responses observed for ciliary ganglion neurons in the absence of protein synthesis (Margiotta et al., 1987a). Typically, incubating the neurons in 8-Br-cAMP and IBMX for 6 hr produces a 2- to 3-fold increase in the ACh response (Margiotta et al., 1987a; Margiotta and Gurantz, 1989; Vijayaraghavan et al., 1990). A similar increment in the response was observed in the present experiments when neurons were grown with CNTF plus the 50-kDa fraction and then incubated for 6 hr with 8-Br-cAMP plus IBMX (Fig. 8). In contrast, neurons grown only with CNTF had a much lower ACh response, and the response was not increased by incubation with the 8-Br-cAMP and IBMX (Fig. 8). To ensure that activation

of voltage-gated channels did not contribute to the measurements, data were excluded in the analysis if the cell depolarized to more than −48 mV during the response. The results indicate that the 50-kDa fraction enhances the ability of ciliary ganglion neurons to regulate their ACh responses in a cAMP-dependent manner and, in fact, may be required for the effect.

GABA responses. A noncholinergic neurotransmitter receptor found on ciliary ganglion neurons is the $GABA_A$ receptor that binds benzodiazepines and activates a chloride conductance (McEachern et al., 1985; McEachern and Berg, 1988). We tested the GABA responses of neurons grown in the 50-kDa fraction to determine whether noncholinergic receptors were also affected. An enhancement of 6.1 ± 1.2-fold (mean ± SE, 58–68 neurons for each condition, combined from eight experiments) was observed for neurons grown for 6–7 d with the 50-kDa fraction. We conclude that the stimulatory effect of the 50-kDa fraction is not confined to cholinergic receptors though it produces little enhancement of neuronal growth and little increase in the size of several classes of cholinergic binding sites on the cell surface.

Discussion

The results indicate that a soluble component(s) of about 50 kDa from the synaptic target tissue permits the development of a substantial ACh response from chick ciliary ganglion neurons in culture. The increased ACh sensitivity is accompanied by only a small increase in the number of known AChRs on the neurons. In addition, fractions containing the component enable the neurons to increase their ACh responses in a cAMP-dependent manner. Either a new population of receptors is being induced and their function is enhanced by a cAMP-dependent process, or the function of existing receptors is being altered by the component.

The 10-fold increase in ACh sensitivity observed for neurons grown with the 50-kDa component does not derive from a change in receptor affinity for agonist. Even at ACh concentrations sufficient to elicit near-maximal responses, an enhancement of about fivefold was observed. High agonist concentrations were not routinely used for assaying ACh responses because they partially desensitize the receptors and produce an underestimate of the enhancement. Changes in extracellular esterases also fail to account for the different levels of ACh sensitivity since qualitatively similar results were obtained with carbachol as an agonist. Though the ACh response is known to undergo agonist-induced desensitization (Margiotta et al., 1987b), it is unlikely that the effect of the 50-kDa component is simply to reduce desensitization since growth in the component has little effect on the time course of the ACh response. It remains possible, of course, that the effect derives from changes in a more drastic and rapid rate of desensitization that has yet to be described for neuronal AChRs.

Is a new type of AChR species being induced by the 50-kDa component? A number of neuronal AChR genes have been identified both in rat and in chick (Boulter et al., 1986, 1987, 1990; Goldman et al., 1987; Ballivet et al., 1988; Deneris et al., 1988, 1989; Nef et al., 1988; Schoepfer et al., 1988, 1990; Wada et al., 1988; Duvoisin et al., 1989; Couturier et al., 1990). The gene combinations that produce functional AChRs *in vivo* have yet to be determined. Affinity labeling studies have shown that mAb 35 and nBgt can recognize the same class of receptors on chick ciliary ganglion neurons (Halvorsen and Berg, 1987) and that the receptors appear to include functional, synaptic AChRs

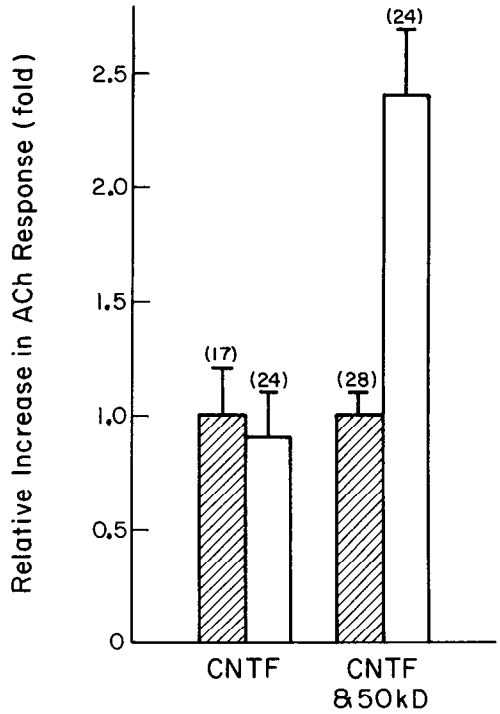

Figure 8. Dependence of cAMP-dependent regulation of AChRs on the 50-kDa fraction. Neurons were grown for 6–7 d in basal medium with *CNTF* or *CNTF* plus the 50-kDa fraction (*CNTF + 50 kD*) and then incubated with (*open bars*) or without (*hatched bars*) 2 mM 8-Br-cAMP and 1 mM IBMX for 6 hr. Afterward, the neurons were tested for ACh sensitivity with intracellular recording, and the results were corrected for cell size (pS/μm^2 of soma membrane). To combine data from several experiments, individual values were normalized to the geometric mean response of control neurons (neurons without the 8-Br-cAMP + IBMX) in that experiment. The values shown represent the geometric mean ± SEM of the normalized responses from the number of neurons indicated in *parentheses,* compiled from four to seven separate experiments. The cyclic nucleotide treatment failed to enhance ACh responses from neurons grown only in *CNTF* but did enhance the responses of neurons grown with *CNTF* plus the 50-kDa fraction. Null responses were excluded from the calculations to avoid overweighting them in the normalization. Qualitatively similar conclusions are reached, however, if the null responses are included (and assigned the nominal value of 0.1 pS/μm^2 as described in Materials and Methods). Similar conclusions are also reached if the data are normalized and averaged as arithmetic means rather than as geometric means.

(Ravdin and Berg, 1979; Jacob et al., 1984; Smith et al., 1985, 1986; Halvorsen and Berg, 1990). Ciliary ganglion neurons also have αBgtBCs with nicotinic pharmacology (Chiappinelli and Giacobini, 1978; Ravdin et al., 1981); they are located predominantly in extrasynaptic regions (Jacob and Berg, 1983; Loring et al., 1985). Though no function has yet been demonstrated for αBgtBCs, recent studies suggest that they are likely to represent AChRs (Couturier et al., 1990; Schoepfer et al., 1990). Neither αBgtBCs nor the AChRs recognized by mAb 35 and nBgt are increased more than 50% by the 50-kDa component. If a new AChR species is being induced, however, it should bind nBgt. This follows from the observation that nBgt blocks the ACh response of neurons grown in extracts containing the 50-kDa component. To reconcile these observations, one would have to argue either that the new receptor species binds nBgt in a rapidly reversible manner or that the species generates a relatively large ACh response per nBgt binding site.

An alternative possibility is that the 50-kDa component changes the functional properties of known receptor classes. This might occur by subunit substitution as seen for muscle AChRs during development (Mishina et al., 1986; Gu and Hall, 1988), by a posttranslational modification of the receptor, or by a change in some component that interacts with the receptor.

Evidence that the functional properties of neuronal AChRs can be changed comes from the demonstration that the ACh response of chick ciliary ganglion neurons can be enhanced in a cAMP-dependent manner (Margiotta et al., 1987a; Margiotta and Gurantz, 1989; Vijayaraghavan et al., 1990), and the enhancement appears to involve a conversion of existing AChRs from a "silent" state to a functionally available one (Margiotta et al., 1987a). Here, we show that acquisition of the cAMP-dependent regulation in culture depends at least in part on the 50-kDa component. No cAMP-dependent enhancement was observed for ACh responses from neurons grown in the absence of the component. (It is possible that a small enhancement occurred under the basal conditions but went undetected because of the low sensitivities obtained in the absence of the 50-kDa component.) As a result, neurons grown with and without the 50-kDa component differed by more than 20-fold in their ACh responses when treated with a cAMP analog. If ciliary ganglion neurons in culture have significant resting levels of cAMP, one might expect that converting the receptors to a cAMP-regulated mode would in itself substantially increase the ACh sensitivity. This could account for the stimulatory effects of the 50-kDa component on the ACh response.

In vivo, the cAMP-dependent regulation of ACh sensitivity is acquired during development (Margiotta and Gurantz, 1989). Prior to embryonic day 9, cAMP analogs have no effect on the ACh response of freshly dissociated ciliary ganglion neurons, whereas after embryonic day 10 the effects appear maximal. This is the same time at which the neurons first establish functional synapses on the target tissue and become dependent on contact with the target tissue for survival (Landmesser and Pilar, 1974). Both the timing of the effect *in vivo* and its dependence in cell culture on a component from the target tissue are consistent with a model in which the component is dispatched from the target tissue to guide certain aspects of neuronal development in the ganglion.

The dependence of ACh sensitivity on target-derived components has also been examined recently with embryonic ciliary ganglion neurons *in vivo* (Engisch and Fischbach, 1990). Early removal of the eye cup prevented the neurons from contacting their normal synaptic targets but did not prevent them from acquiring substantial ACh sensitivities. The stages examined (embryonic days 6.5–18) included those studied here (embryonic day 8 plus 1–7 d in culture). While further work will be required to reconcile these observations, one possibility is suggested by Engisch and Fischback who point out that less than 20% of the neurons survive eye cup removal. The remaining neurons may utilize other sources for obtaining regulatory factors normally provided by the eye. Certainly, other tissues have components that can mimic the 50-kDa component from eye tissue in supporting the development of neuronal ACh sensitivity in cell culture.

The effects of the 50-kDa fraction are not confined to cholinergic receptors on the neurons. A sixfold enhancement of the GABA response was observed for neurons grown with the component. It would be of interest to know whether the same disparity in receptor number versus function occurs for changes in $GABA_A$ receptors as observed for cholinergic receptors, but existing methods do not permit quantitation of $GABA_A$ receptors on the neurons in culture (McEachern and Berg, 1988). The fact that the 50-kDa fraction produced no change in LDH levels indicates that the effects observed on transmitter responses did not arise as a consequence of increasing cell growth in general.

It is possible that the 50-kDa fraction achieves multiple regulatory effects in the neurons by acting on a single common mechanism, e.g., changing the efficacy of a second messenger system. Alternatively, the 50-kDa fraction may include a number of active components responsible for the diverse effects. One component, for example, may cause the observed increases in binding sites while another induces the cAMP-dependent regulation of ACh responses and a third influences GABA responses. The 50-kDa material is likely to contain CSA, a factor of 40–50 kDa in eye extract that enhances the levels of choline acetyltransferase activity in the neurons (Nishi and Berg, 1981). Further purification will be required to identify specific molecules responsible for the individual regulatory effects.

The soluble 50-kDa eye component responsible for enhancing ACh sensitivity is trypsin-sensitive and heat-labile, as expected for a protein. Heating the material to 60°C for 1 hr reduced the activity to that expected for a sample only one-sixth as concentrated. More was lost after heating to 100°C. The residual activity may represent a different component, a high resistance to heat inactivation, or a partial renaturation upon cooling.

Previous studies have shown that a component present in myotube membrane fragments supports the maintenance of ACh sensitivity by chick ciliary ganglion neurons in culture (Tuttle, 1983). Direct contact between the neuron and the membrane fragment may be necessary for the effect since the component was difficult to solubilize. It differs in this respect from the 50-kDa species described here. The substratum of fibroblast remnants used in the present experiments may have functionally substituted for the muscle component. Other studies have described a soluble component from spinal cord cells that increases the development of ACh sensitivity by chick sympathetic neurons in culture (Role, 1988). The relationship between the spinal cord component and the 50-kDa species obtained from eye tissue remains to be determined.

The active component(s) described here may be present in many tissues, since the 50-kDa fraction of all extracts tested had some stimulatory activity on ACh responses. Not all sources had equivalent activity. If the same molecular species are involved, their distribution suggests that they have more than one type of cellular target and more than one kind of regulatory effect. Precedent for multiplicity of action comes from various protein regulatory factors such as cytokines where, for example, the same molecule can influence the proliferation and differentiation of macrophages on the one hand and expression of neurotransmitter phenotype by neurons on the other (Yamamori et al., 1989). The importance of the present findings is the implication that information in the form of soluble components from the synaptic target tissue can influence the development and regulation of neurotransmitter sensitivity on neurons innervating the tissue.

References

Ballivet M, Nef P, Couturier S, Rungger D, Bader CR, Bertrand D, Cooper E (1988) Electrophysiology of a chick neuronal nicotinic acetylcholine receptor expressed in *Xenopus* oocytes after cDNA injection. Neuron 1:847–852.

Barbin G, Manthorpe M, Varon S (1984) Purification of the chick eye ciliary neuronotrophic factor. J Neurochem 43:1468–1478.

Barde YA (1989) Trophic factors and neuronal survival. Neuron 2: 1525–1534.

Boulter J, Evans K, Goldman D, Martin G, Treco D, Heinemann S, Patrick J (1986) Isolation of a cDNA clone coding for a possible neural nicotinic acetylcholine receptor α-subunit. Nature 319:368–374.

Boulter J, Connolly J, Deneris E, Goldman D, Heinemann S, Patrick J (1987) Functional expression of two neuronal nicotinic acetylcholine receptors from cDNA clones identifies a gene family. Proc Natl Acad Sci USA 84:7763–7767.

Boulter J, O'Shea-Greenfield A, Duvoisin RM, Connolly JG, Wada E, Jensen A, Gardner PD, Ballivet M, Deneris ES, McKinnon D, Heinemann, Patrick J (1990) α3, α5, and β4: three members of the rat neuronal nicotinic acetylcholine receptor-related gene family form a gene cluster. J Biol Chem 265:4472–4482.

Brenner HR, Martin AR (1976) Reduction in acetylcholine sensitivity of axotomized ciliary ganglion cells. J Physiol 260:159–175.

Chiappinelli VA, Giacobini E (1978) Time course of appearance of α-bungarotoxin binding sites during development of chick ciliary ganglion and iris. Neurochem Res 3:465–478.

Choi DW, Fischbach GD (1981) GABA conductance of chick spinal cord and dorsal root ganglion neurons in cell culture. J Neurophysiol 45:605–620.

Couturier S, Bertrand D, Matter J-M, Hernandez M-C, Bertrand S, Millar N, Valera S, Barkas T, Ballivet M (1990) A neuronal nicotinic acetylcholine receptor subunit (α7) is developmentally regulated and forms a homo-oligomeric channel blocked by α-BTX. Neuron 5:847–856.

Deneris ES, Connolly J, Boulter J, Wada E, Wada K, Swanson LW, Patrick J, Heinemann S (1988) Primary structure and expression of β2: a novel subunit of neuronal nicotinic acetylcholine receptors. Neuron 1:45–54.

Deneris ES, Boulter J, Swanson LW, Patrick J, Heinemann S (1989) β3: a new member of nicotinic acetylcholine receptor gene family is expressed in brain. J Biol Chem 264:6268–6272.

Dennis MJ, Sargent PB (1979) Loss of extrasynaptic acetylcholine sensitivity upon reinnervation of parasympathetic ganglion cells. J Physiol (Lond) 289:263–275.

Dunn PM, Marshall LM (1985) Lack of nicotinic supersensitivity in frog sympathetic neurones following denervation. J Physiol (Lond) 363:211–225.

Duvoisin RM, Deneris ES, Patrick J, Heinemann S (1989) The functional diversity of the neuronal nicotinic acetylcholine receptors is increased by a novel subunit: β4. Neuron 3:487–496.

Engisch KL, Fischbach GD (1990) The development of ACH- and GABA-activated currents in normal and target-deprived embryonic chick ciliary ganglia. Dev Biol 139:417–426.

Fabiato A (1988) Computer programs for calculating total from specified free or free from specified total ionic concentrations in aqueous solutions containing multiple metals and ligands. Meth Enzymol 157: 378–417.

Goldman D, Deneris E, Luyten W, Kochhar A, Patrick J, Heinemann S (1987) Members of a nicotinic acetylcholine receptor gene family are expressed in different regions of the mammalian central nervous system. Cell 48:965–973.

Gu Y, Hall ZW (1988) Immunological evidence for a change in subunits of the acetylcholine receptor in developing and denervated rat muscle. Neuron 1:117–125.

Halvorsen SW, Berg DK (1986) Identification of a nicotinic acetylcholine receptor on neurons using an α-neurotoxin that blocks receptor function. J Neurosci 6:3405–3412.

Halvorsen SW, Berg DK (1987) Affinity labeling of neuronal acetylcholine receptor subunits with an α-neurotoxin that blocks receptor function. J Neurosci 7:2547–2555.

Halvorsen SW, Berg DK (1989) Specific down-regulation of the α-bungarotoxin binding component on chick autonomic neurons by ciliary neuronotrophic factor. J Neurosci 9:3673–3680.

Halvorsen SW, Berg DK (1990) Subunit composition of nicotinic acetylcholine receptors from chick ciliary ganglia. J Neurosci 10:1711–1718.

Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pfluegers Arch 391:85–100.

Hancock AA, Bush EN, Stanisic D, Kyncl JJ, Lin CT (1988) Data normalization before statistical analysis: keeping the horse before the cart. Trends Pharmacol Sci 9:29–33.

Jacob MH, Berg DK (1983) The ultrastructural localization of α-bungarotoxin binding sites in relation to synapses on chick ciliary ganglion neurons. J Neurosci 3:260–271.

Jacob MH, Berg DK (1987) Effects of preganglionic denervation and postganglionic axotomy on acetylcholine receptors in the chick ciliary ganglion. J Cell Biol 105:1847–1854.

Jacob MH, Berg DK (1988) The acetylcholine receptor distribution of chick ciliary ganglion neurons following disruption of ganglionic connections. J Neurosci 8:3838–3849.

Jacob MH, Berg DK, Lindstrom JM (1984) A shared antigenic determinant between the *Electrophorus* acetylcholine receptor and a synaptic component on chicken ciliary ganglion neurons. Proc Natl Acad Sci USA 81:3223–3227.

Kuffler SW, Dennis MJ, Harris AJ (1971) The development of chemosensitivity in extrasynaptic areas of the neuronal surface after denervation of parasympathetic ganglion cells in the heart of the frog. Proc R Soc (Lond) 177:555–563.

Landmesser L, Pilar G (1974) Synaptic transmission and cell death during normal ganglionic development. J Physiol 241:737–749.

Lin L-FH, Mismer D, Lile JD, Armes LG, Butler ET III, Vannice JL, Collins F (1989) Purification, cloning, and expression of ciliary neurotrophic factor (CNTF). Science 246:1023–1025.

Lindstrom J, Einarson B, Tzartos S (1981) Production and assay of antibodies to acetylcholine receptors. Meth Enzymol 74:432–459.

Loring RH, Dahm LM, Zigmond RE (1985) Localization of α-bungarotoxin binding sites in the ciliary ganglion of the embryonic chick: an autoradiographic study at the light and electron microscopic level. Neuroscience 14:645–660.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275.

Luetje CW, Wada K, Rogers S, Abramson SN, Tsuji K, Heinemann S, Patrick J (1990) Neurotoxins distinguish between different neuronal nicotinic acetylcholine receptor subunit combinations. J Neurochem 55:632–640.

Margiotta JF, Berg DK (1986) Enkephalin and substance P modulate synaptic properties of chick ciliary ganglion neurons. Neuroscience 18:175–182.

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

Margiotta JF, Berg DK, Dionne VE (1987a) Cyclic AMP regulates the proportion of functional acetylcholine receptors on chick ciliary ganglion neurons. Proc Natl Acad Sci USA 84:8155–8159.

Margiotta JF, Berg DK, Dionne VE (1987b) Properties and regulation of functional acetylcholine receptors on chick ciliary ganglion neurons. J Neurosci 7:3612–3622.

McEachern AE, Berg DK (1988) Benzodiazepine interactions with $GABA_A$ receptors on chick ciliary ganglion neurons. Mol Pharmacol 34:129–135.

McEachern AE, Margiotta JF, Berg DK (1985) γ-Aminobutyric acid receptors on chick ciliary ganglion neurons *in vivo* and in cell culture. J Neurosci 5:2690–2695.

McEachern AE, Jacob MH, Berg DK (1989) Differential effects of nerve transection on the ACh and GABA receptors of chick ciliary ganglion neurons. J Neurosci 9:3899–3907.

Mishina M, Takai T, Imoto K, Noda M, Takahashi T, Numa S, Methfessel C, Sakmann B (1986) Molecular distinction between fetal and adult forms of muscle acetylcholine receptor. Nature 321:406–411.

Nef P, Oneyser D, Alliod C, Couturier S, Ballivet M (1988) Genes expressed in the brain define three distinct neuronal nicotinic acetylcholine receptors. EMBO J 7:595–601.

Nishi R, Berg DK (1981) Two components from eye tissue that differentially stimulate the growth and development of ciliary ganglion neurons in cell culture. J Neurosci 1:505–513.

Nitkin RM, Smith MA, Magill C, Fallon JR, Yao Y-MM, Wallace BG, McMahan UJ (1987) Identification of agrin, a synaptic organizing protein from *Torpedo* electric organ. J Cell Biol 105:2471–2478.

Ravdin PM, Berg DK (1979) Inhibition of neuronal acetylcholine sensitivity by α-toxins from *Bungarus multicinctus* venom. Proc Natl Acad Sci USA 76:2072–2076.

Ravdin PM, Nitkin RM, Berg DK (1981) Internalization of α-bun-

garotoxin on neurons induced by a neurotoxin that blocks neuronal acetylcholine sensitivity. J Neurosci 1:849–861.

Role LW (1988) Neural regulation of acetylcholine sensitivity in embryonic sympathetic neurons. Proc Natl Acad Sci USA 85:2825–2829.

Roper S (1976) The acetylcholine sensitivity of the surface membrane of multiply-innervated parasympathetic ganglion cells in the mudpuppy before and after partial denervation. J Physiol (Lond) 254: 455–473.

Rudge JS, Davis GE, Manthorpe M, Varon S (1987) An examination of ciliary neuronotrophic factors from avian and rodent tissue extracts using a blot and culture technique. Dev Brain Res 32:103–110.

Sargent PB, Pang DZ (1988) Denervation alters the size, number, and distribution of clusters of acetylcholine receptor-like molecules on frog cardiac ganglion neurons. Neuron 1:877–886.

Schoepfer R, Whiting P, Esch F, Blacher R, Shimasaki S, Lindstrom J (1988) cDNA clones coding for the structural subunit of a chicken brain nicotinic acetylcholine receptor. Neuron 1:241–248.

Schoepfer R, Conroy WG, Whiting P, Gore M, Lindstrom J (1990) Brain α-bungarotoxin binding protein cDNAs and mAbs reveal subtypes of this branch of the ligand-gated ion channel gene family. Neuron 5:35–48.

Schuetze SM, Role LW (1987) Developmental regulation of the nicotinic acetylcholine receptor. Annu Rev Neurosci 10:403–457.

Smith MA, Margiotta JF, Berg DK (1983) Differential regulation of acetylcholine sensitivity and α-bungarotoxin–binding sites on ciliary ganglion neurons in cell culture. J Neurosci 3:2395–2402.

Smith MA, Stollberg J, Lindstrom JM, Berg DK (1985) Characterization of a component in chick ciliary ganglia that cross reacts with monoclonal antibodies in muscle and electric organ acetylcholine receptors. J Neurosci 5:2726–2731.

Smith MA, Margiotta JF, Franco A Jr, Lindstrom JM, Berg DK (1986) Cholinergic modulation of an acetylcholine receptor-like antigen on the surface of chick ciliary ganglion neurons in cell culture. J Neurosci 6:946–953.

Sorenson EM, Chiappinelli VA (1990) Intracellular recording in avian brain of a nicotinic response that is insensitive to κ-bungarotoxin. Neuron 5:307–315.

Stöckli KA, Lottspeich F, Sendtner M, Masiakowski P, Carroll P, Glötz R, Lindholm D, Thoenen H (1989) Molecular cloning, expression and regional distribution of rat ciliary neurotrophic factor. Nature 342:920–923.

Tuttle JB (1983) Interaction with membrane remnants of target myotubes maintains transmitter sensitivity of cultured neurons. Science 220:977–979.

Usdin T, Fischbach GD (1986) Purification and characterization of a polypeptide from chick brain that promotes the accumulation of acetylcholine receptors in chick myotubes. J Cell Biol 103:493–507.

Vijayaraghavan S, Schmid HA, Halvorsen SW, Berg DK (1990) Cyclic AMP-dependent phosphorylation of a neuronal acetylcholine receptor α-type subunit. J Neurosci 10:3255–3262.

Wada K, Ballivet M, Boulter J, Connolly J, Wada E, Deneris ES, Swanson LW, Heinemann S, Patrick J (1988) Functional expression of a new pharmacological subtype of brain nicotinic acetylcholine receptor. Science 240:330–334.

Yamamori T, Fukada K, Aebersold R, Korsching S, Fann M-J, Patterson PH (1989) The cholinergic neuronal differentiation factor from heart cells is identical to leukemia inhibitory factor. Science 246: 1412–1416.