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Neural regulation of acetylcholine sensitivity in embryonic sympathetic neurons

(synaptogenesis/neuronal acetylcholine receptor/development)

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ABSTRACT **The development of transmitter sensitivity is an important component of synaptic differentiation. Despite a wealth of information about the appearance of acetylcholine (AcCho) sensitivity at the neuromuscular junction, the onset and regulation of this critical aspect of synaptogenesis has not previously been examined for synapse formation between neurons. To determine whether there is a role of presynaptic input in the induction of AcCho sensitivity at interneuronal synapses, AcCho-induced currents were measured in embryonic sympathetic neurons before and after synapse formation *in vitro*. The total AcCho sensitivity of postsynaptic neurons was increased nearly 10-fold after innervation. The effects of innervation are mimicked by medium conditioned by preganglionic neurons, suggesting that presynaptic neurons regulate postsynaptic AcCho sensitivity by release of a soluble factor. These observations provide evidence that presynaptic input regulates neuronal sensitivity to an identified synaptic transmitter.**

Synaptogenesis between motoneurons and muscle is accompanied by both pre- and postsynaptic cell differentiation (see ref. 1 for review). A well-characterized aspect of muscle cell development with innervation is the enhanced sensitivity to transmitter at the site of nerve–muscle contact (refs. 2–4 and see refs. 5–7 for review). Arrival of the motoneuron is followed by a rapid increase in acetylcholine (AcCho) sensitivity colocalized with sites of presynaptic transmitter release (3). This is due, in part, to an increase in the number of AcCho receptors (AcChoR; see ref. 6 for review) and may depend upon a factor released from the motor nerve (see refs. 5–7 for review).

In contrast to the wealth of information available on the development of AcCho sensitivity at the neuromuscular junction, this important aspect of synaptogenesis has not been studied at synapses formed between neurons. *In vivo* studies have been hampered by the difficulties of recording from neurons before and shortly after the arrival of presynaptic input and establishment of synapses. Although this is technically straightforward using an *in vitro* approach, there are few culture preparations for which synapses have been described between identified pre-and postsynaptic neurons (8–10).

In the current study the developmental regulation of neuronal AcCho sensitivity with synaptogenesis was studied by using an *in vitro* preparation to innervate sympathetic neurons with their preganglionic partners and assay transmitter-evoked responses before and after synapse formation. This study indicates that innervation of sympathetic neurons is accompanied by a 10-fold increase in the total AcCho sensitivity. Since this enhancement of AcCho sensitivity is mimicked by medium conditioned by the preganglionic neurons, these findings suggest that neural regulation of transmitter sensitivity may play an important role in synapse formation between neurons. A preliminary account of some of these findings has been reported in abstract form (11).

MATERIALS AND METHODS

Cell Culture. Cultured lumbar sympathetic neurons were prepared with minor modification of previously described techniques (12, 13). Briefly, lumbar ganglia (L1–5) were removed from embryonic day 9–10 chickens and incubated for 30 min in a Ca^{2+}- and Mg^{2+}-free phosphate-buffered medium containing 0.01% trypsin (Sigma, type IIIs). The ganglia were then mechanically dispersed to single cells by repeated passage through a fire-polished Pasteur pipette and plated at $\approx 2 \times 10^3$ neurons per 35-mm dish on a 0.1% poly-(L-ornithine) substrate. The neurons were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with horse serum (10%), penicillin (50 units/ml), streptomycin (50 μg/ml), glutamine (2 mM; GIBCO), nerve growth factor (0.1 μg/ml; 2.5S NGF, kindly provided by P. Osborne, Washington University Medical School, St. Louis), and embryonic day 11 chicken embryo extract (CEE; 2% or 5%, vol/vol). Under these growth conditions the proliferation of nonneuronal cells is suppressed, resulting in cultures composed of ≈90% cells with neuronal morphology that are sensitive to AcCho (see text) and contain catecholamine as assessed by formaldehyde histofluorescence (not shown). Neurons maintained in 5% CEE media are ≈2-fold more sensitive to AcCho than those in 2% CEE media (e.g., see refs. 12 and 13), so that the latter growth condition was used in this study, unless otherwise noted.

Sympathetic neurons were innervated *in vitro* by coculture with explants of the *dorsal* portion of spinal cord from mid to low thoracic regions, which, in the chicken, contains the preganglionic neurons of the column of Terni (14). Dorsal spinal cord was dissected free of ventral portions by making a midline longitudinal incision to the approximate depth of the central canal, resecting the sides, and removing the most lateral portions that contain the ventral cord. The remaining (dorsal) portion of the cord was then cut to 0.5-mm-diameter pieces and placed *in vitro* with sympathetic neurons in a small volume of medium to allow rapid attachment of the explants to the substratum (e.g., see refs. 4 and 15). After 12 hr, when the explants had attached and process outgrowth had initiated, the culture volume was brought back to 1.5 ml and the cultures were maintained in a humidified chamber.

Physiological Recording. Membrane potential and synaptic and AcCho-activated currents were recorded with the whole cell configuration of the patch clamp technique (16). Patch

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Abbreviations: AcCho, acetylcholine; AcChoR, acetylcholine receptor; CEE, chicken embryo extract.

electrodes (8–12 MΩ) were prepared from borosilicate glass (1.2 mm outside diameter, Glass Company of America, Bargaintown, NJ) by a two-stage pull on a vertical electrode puller (Kopf) and filled with 140 mM KCl/2 mM $MgCl_2$/11 mM K_2EGTA/1 mM $CaCl_2$/5 mM MgATP/10 mM Hepes, pH 7.2. The external recording medium contained 120 mM NaCl/5.4 mM KCl/1.2 mM NaH_2PO_4/0.8 mM $MgSO_4$/10 mM $CaCl_2$/6 mM glucose/12.5 mM Hepes, pH 7.4. Neurons were voltage clamped to −60 mV unless otherwise noted. Macroscopic currents were recorded with a Dagan 8900 patch clamp amplifier with 2-kHz filtering and stored on video tape with a PCM digitizer (Neurodata DR384) for subsequent analysis. Peak current amplitude was measured with a Nicolet digital oscilliscope and expressed as mean ± SEM (n = number of cells tested).

Drug Application. Drugs were applied by addition to the recording medium or by pressure ejection at 5–8 pounds/inch^2 for 10 sec (34–48 kPa) from a 2- to 5-μm-tipped pipette placed ≈20–50 μm from the soma. Drugs applied by pressure in this manner are diluted by <10% (17). AcCho solutions were prepared fresh daily and applied at 1 μM to 1 mM in recording medium as indicated in the text.

RESULTS

AcCho Sensitivity: Dose–Response Characteristics. The sensitivity of sympathetic neurons to cholinergic activation was measured by pressure ejection of AcCho, at known concentrations, onto cells voltage clamped to rest potential (−60 mV; Fig. 1). The amplitude of the peak AcCho-induced current and the rate of current decay were clearly dependent on agonist concentration. The response to AcCho exhibited steep dose dependence, with half-maximal activation at ≈30 μM. Maximal responses were consistently evoked by 100 μM AcCho and were equal to currents evoked by 1 mM AcCho. Therefore, 100 μM was used to assay changes in total AcCho sensitivity of the neurons as a function of innervation. At these saturating concentrations of AcCho,

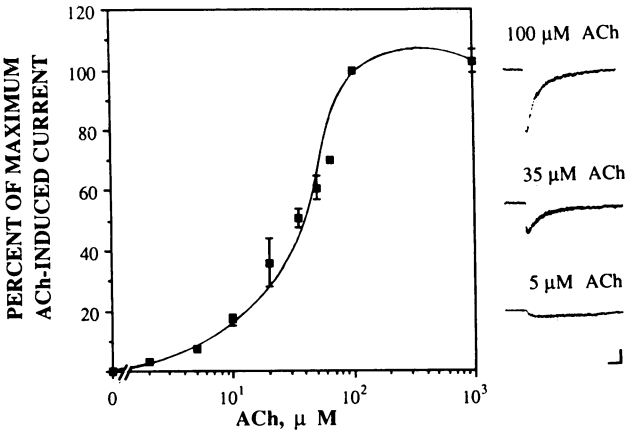

FIG. 1. AcCho (ACh) dose–response curve. The concentration dependence of AcCho-induced current was determined for 46 sympathetic neurons voltage clamped to −60 mV. The maximal peak current is evoked by concentrations of AcCho ≥100 μM. All recordings were obtained from sympathetic neurons prepared and maintained *in vitro* for 4–6 days in 5% CEE medium and voltage clamped to −60 mV as described in the text. AcCho was prepared in recording medium at the indicated concentrations and applied by pressure ejection for 10 sec at 5–8 pounds/inch^2. Each point represents the mean ± SEM of the AcCho-evoked current response of 3–10 neurons from seven separate platings (two or three determinations per neuron; two concentrations tested per neuron). All data are normalized to the response obtained at 100 μM to allow comparison between platings. Typical current responses to AcCho are shown on the right. Calibration: 2 sec × 50 pA.

determinations of relative AcCho sensitivity are not influenced by changes in receptor affinity. It should be noted, however, that due to the rapid desensitization of the AcCho response, the measured responses at concentrations of 100 μM and above most likely underestimate the absolute peak amplitude.

***In Vitro* Innervation of Sympathetic Neurons.** Lumbar sympathetic neurons were innervated after 3–4 days *in vitro* by coculture with explants of the dorsal portion of the spinal cord as described in *Materials and Methods*. In the chicken, the preganglionic neurons of the column of Terni are located dorsal to the central canal so that these explants are greatly enriched in the appropriate presynaptic neuronal population. Thus, the sympathetic neurons can be reliably innervated *in vitro* by preganglionic input without contamination by ventral motoneurons, the other major source of cholinergic spinal neurons. Within 24 hr of coculture, outgrowing neurites, visualized with phase-contrast optics, contact ≈80% of the sympathetic neurons within a 200-μm radius (Fig. 2*A*). Spontaneous synaptic potentials (or currents) were detected in ≈70% of the contacted neurons with whole cell clamp recording (Fig. 2 *B* and *C*). The synaptic current–voltage characteristics indicate that the extrapolated reversal potential is similar to values reported for AcCho-induced currents in these neurons (12). Furthermore, the addition of the nicotinic receptor antagonists *d*-tubocurarine (Fig. 2*E*) or hexamethonium (not shown) abolished these currents reversibly.

These data indicate that nicotinic cholinergic transmission can be established between dorsal spinal cord and sympathetic neurons in coculture. To identify the source of innervation the dorsal spinal cord explants and individual sympathetic neurons were activated by extracellular stimulation or by focal application of 56 mM K^+. Depolarization of the spinal cord explants, but not of other sympathetic neurons in the area, increased the frequency of synaptic currents, suggesting that the cholinergic synaptic input arises solely from the explants (data not shown). A neuron was considered uninnervated if no synaptic currents were detected with 5 min of recording in this configuration.

Assay of AcCho Sensitivity in Innervated Neurons. Fig. 3 summarizes data from 187 neurons assayed in 16 separate experiments to determine the AcCho sensitivity of cells grown for 4 days alone and then for 4 days in the presence or absence of preganglionic input. In 14 of 16 experiments, neurons receiving functional synaptic input were 2- to 20-fold more sensitive to AcCho than sibling neurons grown without dorsal spinal cord explants. The average sensitivity of innervated cells was 9.9 ± 1.8 times greater than that of uninnervated controls (Fig. 3 *A* and *B*). In control cultures (i.e., without explants) the sensitivity of sympathetic neurons to AcCho increased by less than 20% from 4 to 8 days *in vitro*, the same period of time during which innervation enhanced sensitivity of sibling neurons by nearly 10-fold.

Dorsal spinal cord explants selectively enhance the sensitivity of sympathetic neurons to AcCho. Thus, in contrast to AcCho, 100 μM γ-aminobutyric acid evoked comparable responses in both innervated and uninnervated sympathetic neurons (the mean peak current in innervated cells was 279 ± 25 pA, n = 5, vs. 293 ± 43 pA, n = 7, in uninnervated neurons). In addition, the enhanced sensitivity to AcCho is not accompanied by alterations in acetylcholinesterase activity, as had been seen with denervation of sympathetic neurons *in vivo* (18, 19), since currents evoked by AcCho and the nonhydrolyzable cholinergic agonist carbamoylcholine were comparably increased in innervated neurons compared with control. Finally, culture of sympathetic neurons with noncholinergic neurons (e.g., sympathetic ganglion explants) had no effect on AcCho sensitivity (data not shown).

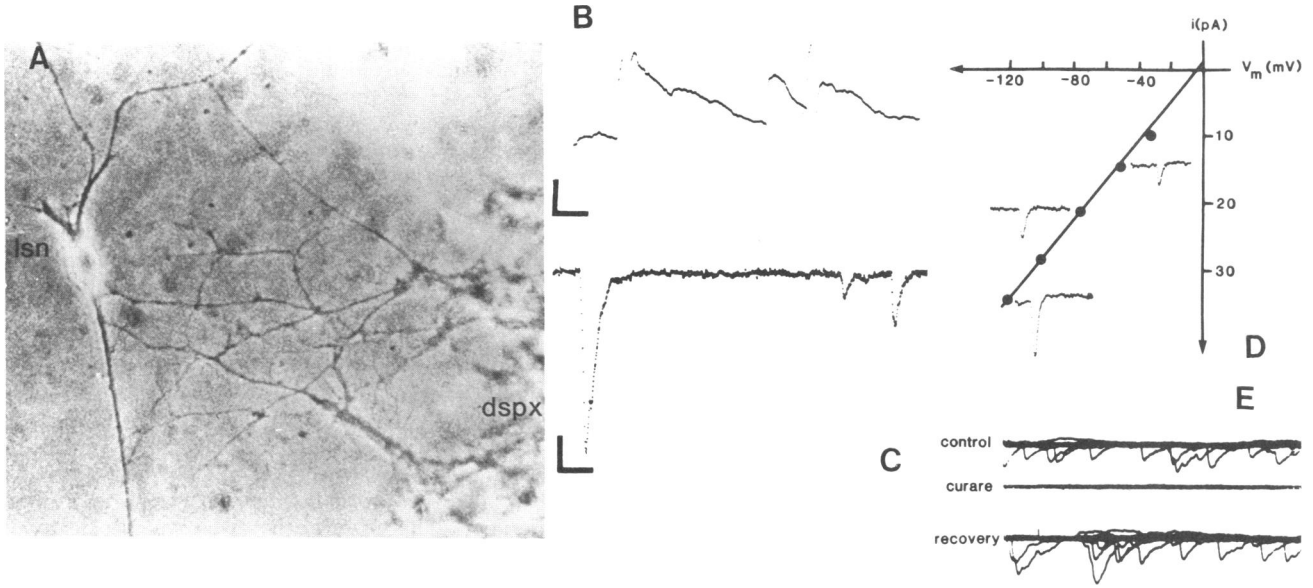

FIG. 2. Innervation of sympathetic neurons *in vitro*. Lumbar sympathetic neurons (lsn) from day 9–10 embryonic chickens were dispersed and maintained *in vitro* for 3–4 days before being cultured with explants of dorsal spinal cord (dspx). Light microscopic examination of a 200-μm region around the explant reveals extensive neuritic outgrowth and contact of ≈80% of the adjacent sympathetic neurons, as shown in the phase-contrast micrograph (*A*). Whole cell current (*B*) and voltage (*C*) clamp recording reveals spontaneous synaptic input in the cell pictured in *A*. Calibrations: *B*, 15 mV × 50 msec; *C*, 25 pA × 20 msec. The current–voltage characteristics of the synaptic currents were examined in three neurons, one of which is shown in panel *D*. Data shown include 277 synaptic currents, with 20–73 events per point. The SEM was <10% of the mean at each of the holding potentials plotted. (*E*) Block of synaptic currents by 10 μM *d*-tubocurarine (curare) was demonstrated in four experiments, one of which is shown here. *d*-Tubocurarine was applied by pressure ejection from a 2-μm-tip-diameter pipette placed ≈50 μm from the cell soma. Holding potential was −60 mV throughout.

AcCho Sensitivity of Uninnervated Neurons in Culture with Spinal Cord Explants. In 13 experiments that examined the sensitivity of the uninnervated neurons in cultures with dorsal spinal cord explants, nearly 80% of the neurons lacking detectable synaptic input were more sensitive than sibling neurons grown alone (Fig. 3*C*). Thus, culture with the spinal cord explants apparently increased the AcCho sensitivity of sympathetic neurons in the absence of functional innervation. This result is reminiscent of early observations that the AcCho sensitivity of developing muscle *in vitro* increases with proximity to cocultured spinal cord explants (15, 20). The enhanced AcCho sensitivity of sympathetic neurons cultured with dorsal spinal cord explants is not due to mere contact by neurites from the explant, however. Neurons that by visual examination were physically contacted by outgrowing neurites but not functionally innervated were equivalent in sensitivity to cells that were not contacted by neurites from the explant.

Effect of Dorsal Spinal Cord Conditioned Medium on AcCho Sensitivity. Many investigators have suggested that the localized increase in AcChoR sensitivity at developing neuromuscular junctions might be induced by neurotrophic factors (see refs. 5–7 for review). To determine if soluble factors from the spinal cord explants regulate neuronal AcCho sensitivity, the AcCho-evoked currents of sympathetic neurons grown in medium conditioned by high density dorsal spinal cord cultures was compared to that of sympathetic neurons grown alone. This neuronal conditioned medium was obtained by plating either spinal cord explants or dissociated spinal cord neurons (material from two thoracic plus upper lumbar cords per 35-mm dish), and arresting the growth of nonneuronal cells by irradiation [5000 rads (50 Gy) of γ irradiation]. Medium conditioned by the neuronal cell cultures was collected (undiluted) after 1 week. In three experiments I found that conditioned medium from dorsal spinal cord cultures increased AcCho sensitivity of sympathetic neurons by an average of 4.5-fold, suggesting that

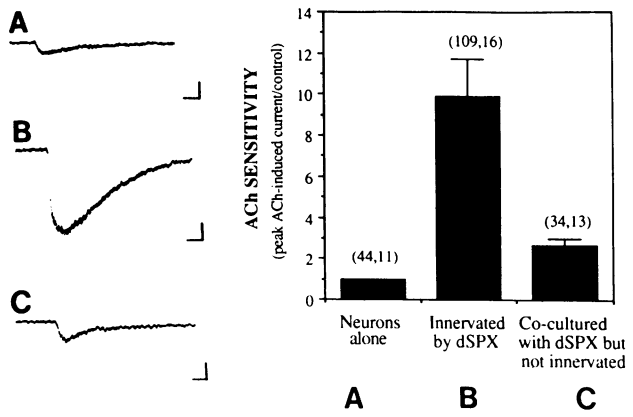

FIG. 3. AcCho (ACh) sensitivity of uninnervated vs. innervated sympathetic neurons. The peak AcCho sensitivity of 187 neurons from 16 separate platings was determined for the indicated conditions. (*A*) Sympathetic neurons were grown alone (i.e., in the absence of presynaptic input) for 8 days *in vitro*. A typical inward current response to 100 μM AcCho in these control cells is shown on the left. AcCho was applied by pressure ejection. Calibration: 50 pA × 2 sec. The histogram on the right shows the magnitude of the peak current, set as 1 for the control cells. Numbers in parentheses indicate number of neurons and number of platings tested. (*B*) Sympathetic neurons were grown alone (4 days) and then in the presence of dorsal spinal cord explants (dSPX; 4 days). The sympathetic neurons were functionally innervated, as judged by recording of spontaneous synaptic currents in whole cell voltage clamp. A typical inward current response to 100 μM AcCho and the mean peak response compared with the mean control responses ± SEM are shown. Calibration: 125 pA × 2 sec. (*C*) Sympathetic neurons were grown in the presence of dorsal spinal cord explants (as in *B*) but were not functionally innervated. Lack of synaptic input was determined by the absence of recorded synaptic currents in the neurons with depolarization of adjacent explants. A typical inward current response to 100 μM AcCho and the mean peak response ± SEM compared with control are shown. Calibration: 50 pA × 2 sec. The holding potential was −60 mV throughout.

a secreted factor may control neuronal AcCho sensitivity (Fig. 4).

To determine whether the soluble factor was derived from neuronal or nonneuronal cells present in the spinal cord explants, sympathetic neurons were also grown in medium conditioned by nonneuronal cell cultures. Nonneuronal conditioned medium was produced by plating dispersed spinal cord cells and passaging the cells three times. This procedure resulted in a confluent layer of flat cells free of neurons. Medium conditioned by such nonneuronal cell cultures was collected after 1 week. The nonneuronal conditioned medium was then transferred (undiluted) to sympathetic neurons grown alone. In two experiments nonneuronal conditioned medium had no significant effect on sympathetic neuron AcCho sensitivity (Fig. 4). In these same experiments innervated neurons in sibling cultures were 11-fold more sensitive to AcCho (515 ± 45 pA vs. 47 ± 10 pA for control, $n = 10$). A role of nonneuronal cells in regulating neural AcCho sensitivity is not entirely excluded by these experiments, however. The nonneuronal cells obtained after repeated passage of dispersed spinal cord cells may not be equivalent to those in the explants.

Although neuronal conditioned medium increased the AcCho sensitivity of most neurons tested, the increase, in general, was smaller than that seen in innervated neurons (Fig. 4*B*). It is possible that at sites of contact by presynaptic neurons a secreted factor could reach a higher local concentration than that achieved in the conditioned medium. Alternatively, other factors not present in the conditioned medium may be involved in the regulation of neuronal AcCho sensitivity.

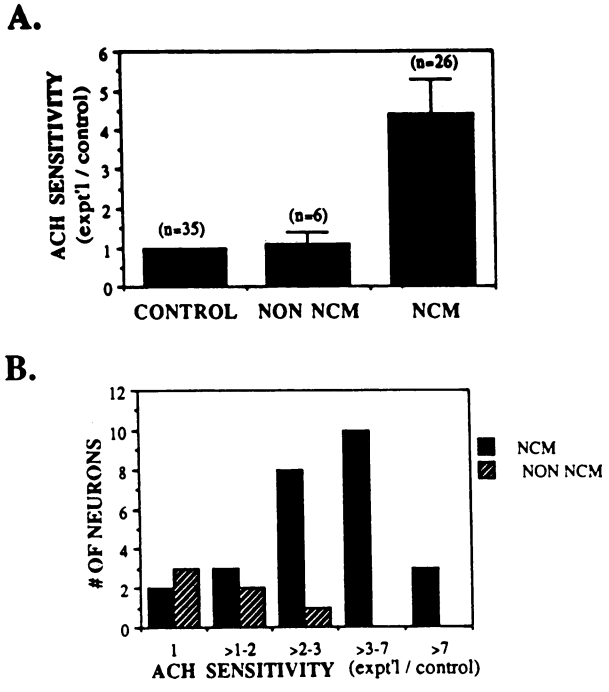

FIG. 4. AcCho (ACh) sensitivity of sympathetic neurons treated with conditioned medium. (*A*) Control sympathetic neurons were maintained *in vitro* for 8 days under standard conditions (2% CEE medium). After 4 days in control medium, experimental sympathetic neurons were treated for 4 days with medium conditioned by nonneuronal cells (NON NCM) or with medium conditioned by neurons (NCM). AcCho sensitivity was measured as the peak inward current in response to maximal (100 μM) AcCho in neurons voltage clamped to −60 mV. The data are mean ± SEM normalized to the mean peak inward current in control (untreated) cells from sibling cultures. (*B*) Data from the same set of experiments displayed to show the range of AcCho sensitivities obtained with conditioned medium treatments. Conditioned media were prepared as described in the text and applied undiluted.

Activity of muscle cells clearly influences the distribution of AcCho sensitivity during development (5–7). Furthermore, some investigators have proposed that activation of AcChoRs might be important in synaptogenesis and in the regulation of AcChoR distribution at the neuromuscular junction (see refs. 1, 5–7, and 21 for review). Preliminary experiments have examined the possibility that AcCho itself might be responsible for the observed increase in neuronal AcCho sensitivity induced by innervation. Sympathetic neurons were grown with and without dorsal spinal cord explants and in the continuous presence of 10 μM *d*-tubocurarine. The average increase in AcCho sensitivity in these *d*-tubocurarine-treated cells was equivalent to that of untreated cultures (Control; 28 ± 8 pA, $n = 7$, vs. neurons with dorsal spinal cord explants, 305 ± 58, $n = 4$). The data indicate that *d*-tubocurarine does not block the effect of presynaptic input on postsynaptic cell AcCho sensitivity. This experiment demonstrates that synaptogenesis between neurons as well as the AcCho sensitivity of the postsynaptic cell is unaffected by pharmacological block of transmission.

DISCUSSION

This study demonstrates that embryonic chicken sympathetic neurons exhibit low sensitivity to AcCho when grown *in vitro* in the absence of preganglionic input. Innervation of the neurons is accompanied by a dramatic increase in their transmitter sensitivity. Innervated neurons did not differ in either their average rest potential (V_m) or their input resistance (R_{in}) from neurons grown in the absence of spinal cord explants ($V_m = -45$ mV ± 1.5 mV, $n = 36$, for control vs. −45 mV ± 2.2 mV, $n = 16$, for innervated; $R_{in} = 0.8 \pm 0.02$ GΩ, $n = 12$, for control vs. 0.8 ± 0.08 GΩ, $n = 12$, for innervated). Therefore, it is unlikely that the differences in AcCho sensitivity were simply a function of differences in cell viability or membrane area.

Although the current study indicates that innervation increases transmitter sensitivity, it does not distinguish between several possible mechanisms. Innervation may increase the number of AcChoR channels available for activation, enhance the net flux of ions through the existing AcChoR channels, or both. Developmental increases in AcChoR single-channel conductance are seen after innervation in muscle (see ref. 6 for review), but are accompanied by concurrent decreases in channel open time.

Berg and collaborators (22–25) have studied the role of soluble factors in the regulation of neuronal transmitter sensitivity in some detail. The AcCho sensitivity of ciliary ganglion neurons grown in eye extract is much greater than that of those grown in elevated K^+ or K^+ plus eye extract. The observed change in AcCho sensitivity apparently reflects changes in the number of channels available for activation by transmitter rather than alterations in the AcChoR channel properties (25).

Antibodies (26) and toxins that bind to the neural nicotinic AcChoR (27–29) as well as cDNA probes (30) to the neural AcChoR mRNA can be used to determine whether significant changes in the number of functional AcChoRs are involved in the dramatic induction of AcCho sensitivity after innervation described in the current study. Recent work has indicated that sympathetic neurons innervated *in vitro* by spinal cord explants have greater numbers of α-bungarotoxin-binding sites present on the cell surface (31, 32). Although α-bungarotoxin does not bind to the functional AcChoRs of these neurons (see ref. 6 for review), it is nevertheless intriguing that the number of toxin-binding sites is apparently coordinately regulated with AcCho sensitivity after synaptogenesis *in vitro*, as demonstrated in this study.

Amphibian sympathetic neurons *in vivo* also exhibit an increase in AcCho sensitivity at about the time of ganglionic synaptogenesis (L. Marshall and P. Dunn, personal communication), suggesting that in this system an inductive effect of preganglionic input may also be involved. In the present study the sympathetic neurons were removed from the animal at a time of low AcCho sensitivity (31), before functional transmission has been reported to occur. Thus, the increase observed after innervation *in vitro* may be comparable to the observations of Marshall and Dunn *in vivo*.

O'Brien and Fischbach (8) have recently demonstrated that chicken spinal motoneurons grown *in vitro* exhibit enhanced glutamate sensitivity when cultured with other spinal cord cells. Culture of motoneurons with spinal cord cells may also result in an altered distribution of glutamate receptor channels, since discrete areas of high sensitivity are detected by glutamate iontophoresis along motoneuron processes. Glutamate itself does not enhance motoneuron sensitivity, but no information is available in this system for effects of spinal cord neuron conditioned media. Simple redistribution of receptors cannot account for the increased response to glutamate in motoneurons (8) or the increased response of sympathetic neurons to AcCho (present study), since both groups measured the current evoked by agonist applied over an $\approx$300 μm radius around the neuronal soma. If glutamate is the transmitter at interneuron–motoneuron synapses, the studies on motoneurons, along with these observations on sympathetic neurons, suggest that presynaptic input plays a general role in the regulation of transmitter sensitivity at synapses formed between neurons.

Finally, preliminary examination of amplitude histograms of synaptic currents in innervated sympathetic neurons indicates that within the first 24 hr of innervation the average amplitude of the synaptic currents is small (unit synaptic current amplitudes of 22–30 pA). These results suggest that enhancement of AcCho sensitivity observed in the present study after 4 days of innervation could profoundly affect the efficacy of synaptic transmission at this stage of development.

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