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Synapse turnover: The formation and termination of transient synapses

(adhesion of cells/synapse selection/retina cells/myotubes/cell culture)

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ABSTRACT **Neurons dissociated from chick embryo retina form synapses with cultured rat striated muscle cells in 35–90 min when neurite extension is uncoupled from later steps in synapse formation. The results suggest that a reaction is required for synapse formation after neurons adhere to muscle cells. All synapses between retina neurons and muscle cells are terminated in 3–10 days depending on the developmental age of the neurons. The half-lives of synapses between muscle cells and retina neurons from 8-, 12-, or 13-day embryos are 36, 26, and 5 hr and mean synapse life-times are 53, 37, and 7.1 hr, respectively. The results show that synapses turn over and that the rate of turnover increases during development. The results suggest that both synapse formation and termination rates are regulated and that the specificity of synaptic connections can be increased by selective termination of synapses.**

Neurons dissociated from chick embryo retina form $\sim 1 \times 10^9$ synapses per mg of protein *in vitro* that resemble those of the intact retina (1, 2). Biochemical and electrophysiological evidence suggest that acetylcholine (ACh) functions as a neurotransmitter in the retina and that nicotinic ACh receptors mediate transynaptic communication at some retina synapses (3–8).

In this report we examine the specificity of synapse formation by retina neurons. The neurons were cultured with striated muscle cells which also possess abundant nicotinic ACh receptors, to determine whether retina neurons form synapses with muscle cells and whether muscle cells and retina neurons displace one another or are competitive targets for synapse formation by retina neurons that synthesize ACh.

Striated muscle cells can be innervated by inappropriate motorneurons (9), parasympathetic neurons (10), sympathetic ganglion neurons (11), cerebral cortex neurons (12), and clonal neuroblastoma × glioma hybrid cells (13–15). If the formation of synaptic connections between neurons and muscle cells were coded by specific cell recognition molecules, discrete classes of muscle cells of different specificities for synapse formation might be expected. However, only one class of muscle cells was detected with regard to synapse formation, suggesting that much of the specificity of the normal neuromuscular synapse may be acquired after synapses form, by selective termination of synapses (15). A similar hypothesis has been proposed by Changeux *et al.* (16) and the evidence has been the topic of a comprehensive review (17).

In this report we show that retina neurons form synapses with striated muscle cells, that synapses turn over, and that all mismatched synapses between retina neurons and muscle cells eventually are terminated.

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METHODS AND MATERIALS

The following conditions were used except where stated otherwise. Myotube cultures were obtained by fusion from hindlimb muscle myoblasts of newborn Lewis rats as described for rat muscle (15) except that the medium used on the third day and thereafter contained 10% horse serum, not fetal bovine serum. Retina cells (2×10^7) dissociated from 8-, 12-, or 13-day chick embryos (White Leghorn) with 0.05% trypsin (crystallized 3×, Worthington) (1, 5) were suspended in 1.5 ml of medium A (90% Eagle's basal medium with Earle's salts and 10% fetal bovine serum) and added in 20 min to each 35-mm petri dish containing $\sim 2 \times 10^4$ myotubes that had been cultured for 5–10 days. Cultures were incubated without movement at 37° in a humidified atmosphere of 5% CO_2/95% air. Half of the medium was replaced with fresh medium on the second and fourth days and each day thereafter.

Neuron–myotube synapses were detected by the presence of spontaneous depolarizing responses of myotubes, usually 0.5 mV and 15–60 responses per min. Intracellular recordings were with micropipettes filled with 3 M K acetate. Only recordings of muscle cells with stable resting membrane potentials of −45 to −90 mV without artifacts were used. Usually muscle cell was assayed every 3–5 min. Cultures assayed for synapses <1 hr after retina cells were added to muscle cells were tested in medium A. Those assayed at later times were in medium B (Eagle's basal medium adjusted to 3.8 mM $CaCl_2$ and 106 μM choline·HCl, without serum); cells were incubated for 30 min in medium B prior to use.

RESULTS

Neuron Adhesion to Myotubes vs. Synapse Formation. Early steps in synapse formation, such as neurite growth, were uncoupled to a large extent from later steps by covering 80–90% of the surface area of petri dishes with retina cells (2×10^7 cells per 35-mm dish). Fifty percent of the retina cells dissociated with ethylene bis(oxyethylenenitrilo) tetraacetic acid (EGTA) or trypsin adhered to the myotube monolayer in 10 min (Fig. 1) and were not dislodged by shaking or removing the medium. Synapses between neurons and myotubes were found in abundance only after 37 min of incubation with neurons dissociated with EGTA or 75 min with neurons dissociated with trypsin. Such rapid rates of synapse formation have not been

Abbreviations: ACh, acetylcholine; EGTA; ethylene bis(oxyethylenenitrilo)tetraacetic acid.

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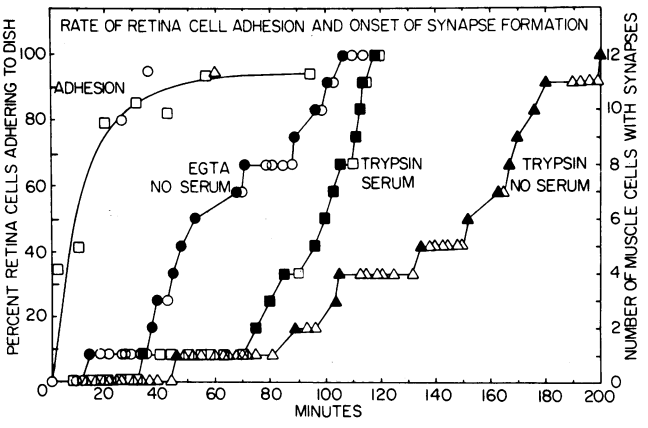

FIG. 1. Rate of adhesion of cells dissociated from 8-day chick embryo retina to a monolayer of rat myotubes (line nearest left ordinate; 100% = 2×10^7 cells), compared with the cumulative number of neuron–myotube synapses detected (other lines). At 0 time, 2×10^7 cells dissociated from retina with 0.02% EGTA or trypsin in 1.5 ml of medium A or medium B (see *Methods and Materials*) were added within 5 or 20 min, respectively, to each 35 mm Petri dish containing rat myotubes. Each solid or open symbol pertaining to synapse formation represents a muscle cell with or without a synapse, respectively. Muscle cells with (●) or without (○) synapses from retina neurons dissociated with EGTA were tested in medium B (32 cells assayed). Muscle cells (32 cells assayed) with (■) or without (□) synapses from retina neurons dissociated with typsin were tested in medium A for the first 60 min of incubation and in medium B thereafter. Muscle cells (53 cells assayed) with (▲) or without (△) synapses from retina neurons dissociated with trypsin were tested in medium B. Adhesion of retina cells dissociated with trypsin and incubated in medium A was determined by counting floating cells with a Coulter counter; adhesion of retina cells dissociated with EGTA, which formed small aggregates within minutes, and of cells dissociated with trypsin and plated in medium B was estimated by microscopic examination. Symbols as above.

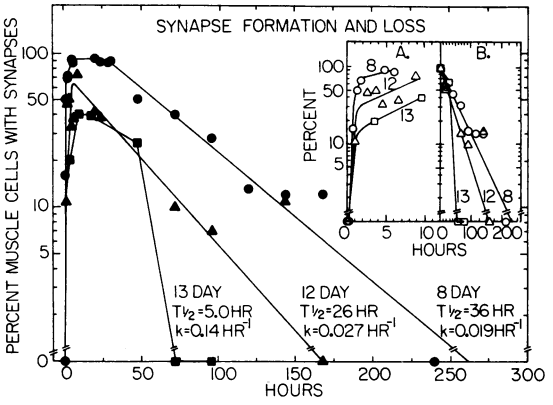

FIG. 2. Rate of formation of synapses between rat muscle cells and retina neurons dissociated with trypsin from 8-day (●), 12-day (▲), and 13-day (■) chick embryos (414, 218, and 129 muscle cells, respectively, were assayed for synapses). The average point represents 21 muscle cells (range, 16–60) assayed for synapses during 30-min periods for the first 2 hr of incubation and 60-min periods thereafter; points are placed at the midpoint of the sampling period. In some cases, the data from two or three experiments were pooled (values from different experiments usually differed by >10%). The 100% level corresponds to ~2×10^4 innervated muscle cells. Each 35-mm dish contained 2×10^7 dissociated retina cells, 2×10^4 rat myotubes, and 1.5 ml of medium B added 30 min before use; however, muscle cells assayed during the first hr of incubation were in medium A. (*Inset A*) Rates of synapse formation shown on an expanded timescale. Percent corresponds to the percent of muscle cells tested with synapses; symbols as above. (*Inset B*) Synapse termination; data normalized so that 100% corresponds to the maximum percent of examined muscle cells with synapses (88, 73, and 39% of muscle cells tested innervated by retina neurons from 8-, 12-, and 13-day chick embryos, respectively) and 0 time corresponds to the time when synapse termination was first detected (30, 8.8, and 20 hr, respectively). $T_{1/2}$, synapse half-life; k, rate constant for loss of synapses.

reported previously; neurite growth probably is the rate-limiting process in the formation of many synapses during embryonic development. The minimum time for synapse formation, found with a neuron dissociated with EGTA, was 14 min; however, this was the only synapse found during the first 33 min of incubation. Fetal bovine serum increased the rate of synapse formation by neurons dissociated with trypsin but had little or no effect on the time required for synapse formation after neurons adhered to myotubes. We conclude that synapses form rapidly and that one or more reactions are required for synapse formation *after* neurons adhere to muscle cells. The results suggest that trypsin either destroys neural molecules that are required for synapse formation, or alters their activity or distribution.

Cycloheximide (4 hr, 20 μg/ml) had no effect on synapse formation; however, the possibility is not excluded that protein molecules synthesized before cycloheximide was added may have been present in sufficient quantity for synaptogenesis.

Synapse Turnover. Rates of synapse formation between neurons from 8-, 12-, or 13-day embryos and myotubes and subsequent synapse termination are shown in Fig. 2. Three phases of synapse formation were found with retina neurons from 8-day embryos (Fig. 2 and *inset A*). During the first phase (0–70 min), neurons adhered to myotubes but only 1 of 14 myotubes examined formed a synapse. In the second phase, synapses appeared abruptly at 75 min and accumulated rapidly until 90 min; at 90 min, 70, 30, and 15% of the examined muscle cells were innervated by 8-, 12-, or 13-day embryo neurons, respectively. These synapses probably were formed by neurons that landed on or near endplate-like sites on the myotube membranes with high concentrations of nicotinic ACh receptors (receptor hot-spots) and thus required little or no neurite extension to form synapses. The rate of synapse formation was slower between 1.5 and 10 hr during the third phase. Maximum innervation was achieved at 4.6, 8.8, and 9.6 hr; 90, 75, and 40% of the myotubes examined then were innervated by neurons from 8-, 12-, or 13-day embryos, respectively. The slow rate of synapse formation may be due to neurons that must extend neurites and/or migrate to contact a receptor hot-spot.

All synapses were terminated over a period of 3–10 days, depending on the developmental age of the neurons, and synapses did not reappear under the conditions used. Synapse termination rates are shown in Fig. 2 and *inset B*. Synapses between muscle cells and retina neurons from 8- or 12-day embryos decreased exponentially after 30 and 8.8 hr of incubation, respectively, which suggests that synapse termination is a first-order process. The half-lives of synapses between muscle cells and retina neurons from 8-, 12-, or 13-day embryos were approximately 36, 26, and 5 hr; the mean synapse lifetimes were 53, 37, and 7.1 hr; and the first-order rate constants (k) for loss of synapses were 0.019, 0.027, and 0.14 hr^{-1}, respectively. Cells seemed healthy and were well-differentiated during the entire period studied. These results show that retina neurons form transient synapses that turn over and that the rate of turnover increases with time. The results suggest that the rate of synapse termination increases during development and that both factors regulate synapse turnover.

Neuron Concentration vs. Number of Synapses Formed. The effect of varying the concentration of retina cells on the number of synapses formed between neurons and muscle after 24 hr of coculture is shown in Fig. 3. Each 35-mm dish (9.6 ×

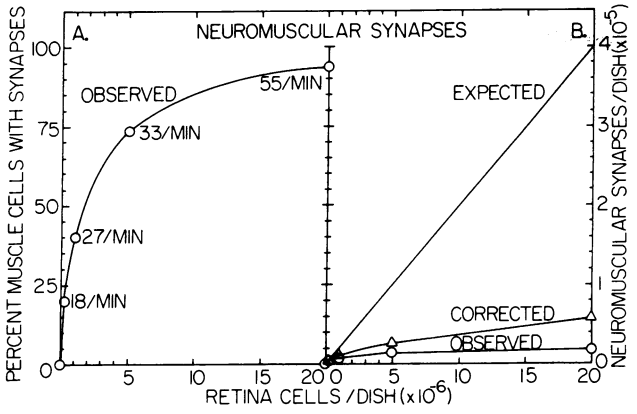

FIG. 3. Relationship between concentration of retina cells dissociated from 8-day embryo with trypsin and percentage of muscle cells with synapses after 1 day of coculture. (*A*) Each point represents 15 muscle cells assayed for synapses; each 35-mm dish (9.6×10^8 μm^2) contained 2×10^4 myotubes and the number of retina cells shown. Number adjacent to each symbol represents mean number of spontaneous muscle responses per min. (*B*) ○, Observed number of innervated muscle cells per dish; 2×10^4 innervated muscle cells per dish corresponds to 100% of the muscle cells with synapses; △, number of synapses per dish corrected for multiple innervation of muscle cells, assuming that 18, 36, and 54 spontaneous muscle responses per min correspond to 1, 2, and 3 synapses per myotube, respectively. The line labeled "expected" represents the number of synapses expected if 2% of the retina cells added had formed synapses with myotubes at each cell concentration tested.

10^8 μm^2 surface area) contained $\sim 2 \times 10^4$ myotubes which occupied 25% of the dish surface area (the average myotube is 850 μm long and 15 μm in diameter) and 0.05 to 20×10^6 retina neurons (6 μm mean cell body diameter) occupying 0.25–90% of the dish. With 2×10^5 retina neurons per dish, 20% of the muscle cells tested were innervated (4000 neuron–muscle synapses per dish). Thus, 2% of the retina neurons added formed synapses with muscle cells, assuming that each myotube was innervated by a different neuron. More muscle cells were innervated at higher concentrations of neurons, but synapses were not proportional to neuron concentration in the range tested. With 2×10^7 retina cells per dish, only 0.1% of the neurons synapsed with myotubes. However, the average rate of spontaneous muscle responses increased from 18 to 55 per min as the concentration of neurons was increased, which suggests that a single muscle cell can be innervated by multiple neurons. At low concentrations of neurons, most muscle cells with synapses probably are innervated by only one neuron. Thus, we assume that 18 muscle responses per min corresponds to a synapse between one neuron and one myotube, and 36 and 54 responses per min correspond to two and three neurons synapsing with one myotube, respectively. The number of synapses per dish observed and the values corrected for multiple innervation are compared with the number of synapses that would be expected if synapses were proportional to neuron concentration and 2% of the neurons synapsed with myotubes (Fig. 3*B*). The average myotube was innervated by three neurons in the presence of 2×10^7 retina cells per dish but only 0.3% of the added cells synapsed with myotubes. Thus, the efficiency of synapse formation decreases 7-fold when the concentration of retina cells is increased from 0.2 to 2×10^6 per dish. These results suggest that a myotube with three synapses is almost saturated by synapses. Similarly, myotubes are innervated simultaneously by less than six motorneurons during normal embryonic development (18) but by only one motorneuron in the adult. The size and number of ACh receptor hot-spots of myotubes probably limit the number of synapses. The average myotube examined by a histochemical method (8) by M. Daniels had one receptor hot-spot, about 320 μm^2, which was <1% of the surface area of the myotube.

Could random interactions between neurons and myotubes account for the observed number of synapses? Approximately 5% of the cells in the posthatched chicken retina possess a high-affinity choline uptake mechanism (19). Assuming that 5% of the cells in the 8-day embryo retina synthesize ACh, we estimate that random contacts between myotube receptor hot-spots and 1×10^4 neurons with ACh per dish (5% of 2×10^5 cells per dish), each neuron with neurites 2000 μm long and 0.5 μm in diameter, could account for 200 to 800 synapses with myotubes, but probably not the 4000 synapses per dish observed. The results suggest, but do not prove, that neuron–muscle synapses are 5- to 20-fold more abundant than would be expected on the basis of random contacts between cells.

Some properties of the synapse target area of muscle cells also can be calculated from the initial rapid rate of synapse formation at 75–90 min after addition of 2×10^7 8-day embryo retina cells per dish (see Fig. 2). If 5% of the retina cells synthesize ACh, then 16,700 of the 1×10^6 neurons with ACh per dish would be expected randomly to land on or near receptor hot-spots of myotubes and form synapses rapidly. Seventy percent of the muscle cells tested were innervated in 90 min; i.e., 14,000 myotubes were innervated per dish, and most of the myotubes were innervated by only one neuron (17 responses per min). These results suggest that the synapse target area of the average myotube is 1–2% of the myotube surface area and agree well with the predicted value. After 24 hr only 3 of the 12–13 neurons with ACh initially landing on the average myotube formed synapses with the myotube, and no change in the number of hot-spots per myotube was detected (M. Daniels, D. Puro, and M. Nirenberg, unpublished data). Thus, retina neurons probably do not induce the formation of hot-spots on myotubes. The demonstration that synapses are not proportional to neuron concentration in the range tested also indicates that the target sites for synapses on myotubes limits synapse formation and can be saturated by synapses.

Properties of Muscle Responses. The effect of 10 μM *d*-tubocurarine on the spontaneous synaptic responses of a muscle cell cultured for 1 day with retina cells from the 8-day chick embryo is shown in Fig. 4*A*. Approximately 40 muscle responses per min were found; such responses were not observed with muscle cells in the absence of retina neurons. Perfusion of the dish with medium containing 10 μM *d*-tubocurarine inhibited muscle responses reversibly. These results suggest that muscle responses are mediated by nicotinic ACh receptors activated by ACh released from retina neurons.

Iontophoretic application of glutamate to retina neurons attached to muscle cells increased the rates and amplitudes of muscle responses (Fig. 4*B*). Iontophoresis of glutamate (−100 nA) onto retina neurons usually increased the rate of muscle response 10- to 30-fold and the response amplitudes 2.5-fold. Muscle cells not in contact with retina cells or without spontaneous responses were not affected by glutamate.

The modal amplitude of spontaneous synaptic responses of a muscle cell cultured with retina neurons from an 8-day embryo (Fig. 4*C*) was 0.4–0.6 mV; responses >2 mV are given in the legend. The durations of muscle responses shown in Fig. 4*C inset* were 25–35 msec; response durations of other muscle cells usually were 15–20 msec (range, 10–40 msec). Spontaneous responses with amplitudes >0.7 mV and of relatively long duration also are common at immature motoneuron–muscle synapses (20).

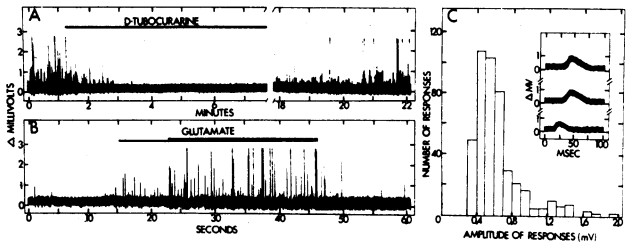

FIG. 4. (*A*) Spontaneous synaptic responses of rat myotube cocultured with 8-day embryo retina neurons for 1 day in the presence or absence of *d*-tubocurarine. Transient shifts in the membrane potential of the muscle cell from a fixed baseline are shown in the penwriter recording. The culture was perfused with medium B (0.5 ml/min) or, as indicated by the bar, with medium B supplemented with 10 μM *d*-tubocurarine. The membrane potential of the muscle cell was −70 mV. (*B*) Muscle cell responses evoked by iontophoresis of glutamate at −40 and −100 nA (thin and thick bars, respectively). Glutamate diffusion from the micropipette was decreased by applying 10 nA constant current. The muscle membrane potential was −60 mV. (*C*) Amplitudes of spontaneous synaptic responses of a muscle cell cultured for 1 day with 2×10^7 retina neurons as a function of the number of reponses observed. Stippled area represents the noise level (0.2 mV). Each bin of the histogram represents 0.1 mV; the first bin is 0.3–0.39 mV. The amplitudes of 23 muscle responses recorded of the 476 which were >2.0 mV (not shown) were: 8 muscle cells, 2.0–3.9 mV: 6 cells, 4.0–5.9 mV; 4 cells, 6.0–7.9 mV; 4 cells, 8.0–9.9 mV; and 1 cell, 15.8 mV. The muscle membrane potential was −75 mV. Oscilloscope traces of responses of this cell are shown in the *Inset*.

Termination of Synapses. Retina cells intially attach to myotubes and other cells without obvious preference (Fig. 5*A*). Neurites extend, adhering to neighboring retina and monolayer cells, and within 1 day (Fig. 5*B*), clusters of retina cells and neighboring areas of the dish devoid of retina cells can be seen. By the sixth day (Fig. 5*C*), >95% of retina cells were in aggregates, 20–100 μm in diameter, composed only of retina cells. Thus, retina cells sort out from other cells in stationary cultures.

The rate of sorting out of retina cells from other cells, determined by measuring the area of the dish occupied by retina cell bodies, is compared with the rate of termination of neuron–muscle synapses in Fig. 6. The surface area of the dish occupied by retina cells from the 8-day embryo decreased from 90% to 15% at the same rate as neuron–myotube synapses were terminated. Retina cells from the 12-day embryo also aggregated and terminated synapses with muscle cells at similar rates initially but the rates were faster than those of neurons from the 8-day embryo. Cell aggregation rates decreased when >90% of the retina cells were in aggregates; aggregates then occupied 15–20% of the surface area of the dish. Most of the myotube

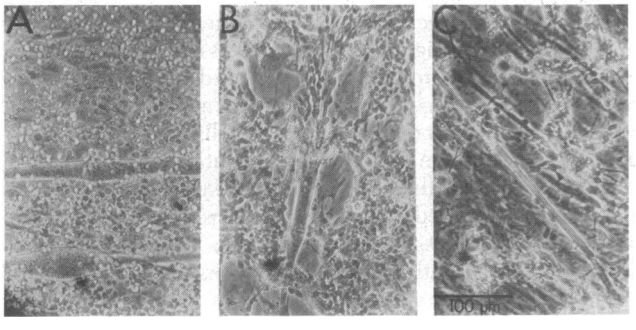

FIG. 5. Phase-contrast photomicrographs at 20 min (*A*), (*B*), and 6 days (*C*) after retina cells dissociated from 8-day embryos with trypsin were added to myotube monolayers. Note retina cell aggregation during the course of incubation.

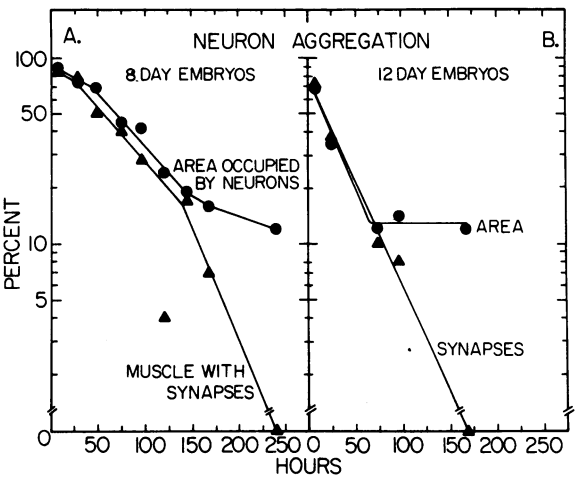

FIG. 6. Rates of aggregation of retina cells dissociated with trypsin from 8- (*A*) or 12-day (*B*) embryos compared with the rates of termination of neuron–muscle synapses. Each 35-mm dish contained 2×10^7 retina cells and approximately 2×10^4 myotubes. ●, Retina cell aggregation determined by measuring area of dish occupied by retina cell bodies. For each symbol, 4800 2.5 × 2.5 μm segments of a grid placed over photomicrographs of random areas of the dish were scored for the presence or absence of a retina cell body; neurites were not scored; 100% corresponds to 9.6 cm^2 occupied by retina cell bodies. ▲, Muscle cells from the same dishes assayed for synapses. Each point corresponds to 10 to 25 muscle cells assayed for synapses.

synapses found then were with neurons in aggregates. However, the rate of synapse termination did not decrease because neurites continue to sort out within the aggregates as synapses form between neurons both in rotating (1, 2) and in stationary cultures (M. Daniels, D. Puro, and M. Nirenberg, unpublished data). Thus, the rate of synapse termination probably is a function of both the rate of neuron aggregation and the rate of sorting out of neurites within aggregates.

Neurons dissociated from 7-day embryo retina with trypsin were incubated for 16 hr in rotating cultures to promote cell aggregation and recovery from trypsin, and the aggregates then were cultured with muscle cells in stationary dishes for 1 day. Sixty percent of the tested muscle cells that were underneath aggregates were innervated. Thus, neurons in aggregates that have recovered at least partially from trypsin treatment synapse with myotubes.

The specific activity of choline acetyltransferase of 8-day embryo retina neurons cultured with muscle cells increased 3.8-fold between culture days 1 and 3 and remained constant thereafter (Fig. 7*A*). Thus, neurons are able to synthesize ACh while synapses with myotubes are terminated. Choline acetyltransferase activity was present in the intact 7-day embryo retina *in ovo* but the specific activity was low (Fig. 7*B*). Enzyme activity was increased markedly in the 11-day embryo retina and almost maximum activity was found in 14-day embryo retina.

Retina neurons were added twice to myotubes, at 0 and at 5 days of culture. On day 6, 80% of the muscle cells tested had synapses whereas, only 11% of the muscle cells cultured with retina neurons added only at 0 time were innervated. Thus, muscle cells do not lose the ability to form synapses while synapses are terminated.

DISCUSSION

The results show that neurons dissociated from chick embryo retina form synapses with cultured rat striated muscle cells in

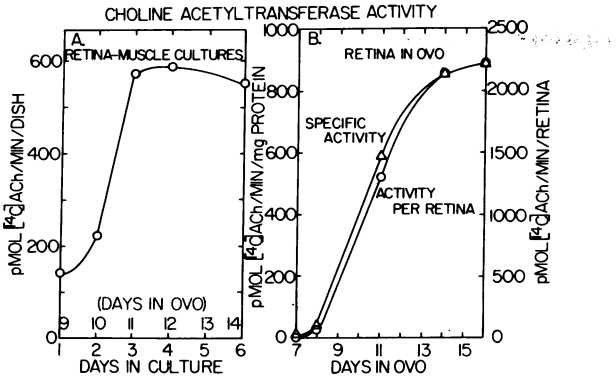

FIG. 7. (*A*) Choline acetyltransferase activity of retina neurons dissociated from 8-day embryos and cultured with rat muscle cells. Each 60-mm dish inoculated with 3×10^6 myoblasts was cultured for 5 days; then, 6×10^7 dissociated retina neurons were added. Cells were cocultured for 1–6 days. At the times indicated, cells were washed, harvested, homogenized, and assayed at four protein concentrations for choline acetyltransferase activity (21) and for protein concentration (22). Enzyme activity was proportional to protein concentration. Mean values are shown. Dishes incubated for for 1, 2, 3, 4, or 6 days contained 2.1, 2.4, 3.3, 2.9, or 1.8 mg of protein, respectively. (*B*) Choline acetyltransferase activity of chick embryo retina *in ovo* as a function of developmental age. Δ, Specific activity, left ordinate; O, pmol [^{14}C]ACh formed/min per retina, right ordinate.

35–90 min when neurite extension is largely uncoupled from later steps in synapse formation and suggest that a reaction of unknown function is required for synapse formation after neurons adhere to muscle cells. Rates of synapse formation with myotubes decrease with time, and all synapses between retina neurons and muscle cells are terminated over a period of 3–10 days depending upon the developmental age of the neuron. Retina neurons aggregate preferentially with one another and sort out from other cells in the dish. Synapse termination rates seem to be coupled to rates of retina cell aggregation and neurite sorting out. Ultrastructural studies (M. Daniels, D. Puro, and M. Nirenberg, to be reported elsewhere) show that synapses between retina neurons become more abundant while neuron–muscle synapses are lost. ***These results suggest that synapses between neurons and muscle cells are terminated whereas some synapses between neurons are retained by a process of selection based on the preferential adhesiveness of retina neurons for one another.*** We suggest that synapse turnover may be required for the assembly of certain neural circuits during embryonic development and perhaps also in the adult at some synapses with memory function.

Neuroblastoma × glioma NG108-15 hybrid cells (13) and sympathetic ganglion neurons (11) also form synapses with cultured striated muscle cells, but synapses were found throughout the period of coculture, 6 weeks and 19 days, respectively, the longest times tested. Synapse turnover was not detected. Either a steady state is attained with respect to synapse formation and termination or stable synapses are formed.

Proteins that specifically increase the aggregation of retina cells have been described (23–26); but developmental changes in aggregation factor concentrations or properties and rates of synapse termination are not correlated. However, aggregation factors that stimulate the aggregation of specific families of neurons, such as those from retina, segregate neurons into separate populations and should thereby increase both the rate of synapse formation between neurons within that cell population and the rate of termination of synapses with neurons outside that cell population.

The experimental strategy of using large cells with defined receptors as synapse formation targets enables one to study rapidly many aspects of synapse turnover, to define the properties of synapses, and the transmitter and receptor phenotypes of small neurons. Synapse target cells with different species of receptors also may be used to explore the specificity of other kinds of synapses.

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