Proc. Natl. Acad. Sci. USA
Vol. 75, No. 5, pp. 2281–2285, May 1978
Biochemistry

Synapse turnover: A mechanism for acquiring synaptic specificity

(retina/muscle cell/neurons/development/cell culture)

ROBERT R. RUFFOLO, JR., GEORGE S. EISENBARTH, JEFFREY M. THOMPSON, AND MARSHALL NIRENBERG

Laboratory of Biochemical Genetics, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20014

Contributed by Marshall Nirenberg, March 10, 1978

ABSTRACT **Neurons are generated in chick retina that are able to form synapses with striated muscle cells for only a brief period during embryonic development. The ability to form synapses is lost with a half-life of 21 hr. Retina neuron–myotube synapses form rapidly but soon are terminated. Chick embryo spinal cord neurons also form synapses with muscle cells for only a limited time during development, but these synapses are long lived. These results show that different classes of synapses turn over at different rates and suggest that part of the specificity of synaptic circuits may be acquired during development by a process of selection based on synapse termination rates.**

Cultured cells dissociated from chick embryo retina have been shown to form approximately 1×10^9 synapses per mg of protein (1), and ultrastructural studies reveal different types of synapses, which closely resemble those formed *in ovo* (1–3). Because relatively few classes of neurons have been identified in the retina and retina synaptic circuits have been studied extensively (4), the developing retina would appear to be a favorable model system for studies on the mechanism of assembly of synaptic circuits.

Acetylcholine probably is a neurotransmitter at some synapses in chick retina. High choline acetyltransferase (EC 2.3.1.6) (5) and acetylcholinesterase (EC 3.1.1.7) (6) activities, acetylcholine (6), a high-affinity choline uptake mechanism (7), and nicotinic (8, 9) and muscarinic (10) acetylcholine receptors are present in chick retina.

In previous studies on the mechanism of synapse formation, chick embryo retina neurons were shown to form synapses rapidly with striated muscle cells; such synapses were transient and eventually disappeared (5). In this report we have used striated muscle cells as synaptic targets to define further the process of synapse formation and to determine the number of neurons that are able to form synapses at different stages of development. We now show that retina neurons are able to form synapses for only a short time during development, that synapses of different types are terminated at different rates, and that populations of synapses can be selected on the basis of differences in synapse termination rates.

METHODS AND MATERIALS

Primary muscle cultures were prepared by fusion of myoblasts obtained from fetal or newborn Fisher rats as described previously (5, 11); however, to select for nondividing cells, 10 μM 5-fluorodeoxyuridine and 100 μM uridine were added on the 3rd or 4th day of culture and removed on the 6th to 9th day. Except where indicated, chick neural retina and spinal cord cells were dissociated with trypsin crystallized three times (Worthington Biochemical Corp.) and cultured with myotubes previously cultured for 8–20 days.

Neuron–myotube synapses were detected by recording spontaneous depolarizing synaptic muscle responses, usually 0.5 mV in amplitude and 10 msec in duration, with intracellular microelectrodes filled with 3 M KCl solution as described by Nelson *et al.* (12). Only those myotubes with stable membrane potentials of at least −40 mV were used. A myotube was considered to be innervated if at least three spontaneous synaptic responses of the muscle cell, judged free of artifacts, were identified during a 2-min recording period.

RESULTS

Neurons dissociated from chick embryo retina at different stages of development were cocultured with rat striated muscle cells. The percent of muscle cells tested that were innervated by retina neurons after 2 or 24 hr of coculture is shown in Fig. 1*A*, and the average frequency of spontaneous synaptic responses of innervated muscle cells, in Fig. 1*B*. Neurons that are able to form synapses with muscle cells appear in chick embryo retina on the 6th or 7th day, are most abundant on the 8th day, and disappear by the 16th day of embryo development. Almost as many synapses were found at 2 hr of coculture as at 24 hr.

The frequency of spontaneous muscle responses of innervated myotubes follows a similar developmental sequence (Fig. 1*B*); however, the frequency of muscle responses was maximum after 24 hr of coculture with neurons from 8-day chick embryo retina, whereas, with more mature neurons, the maximum frequency of muscle responses was observed after 2 hr of coculture. Glutamate (5 mM) applied locally by diffusion from a micropipette increased the frequency of muscle responses at synapses between neurons from 8-day embryo retina and muscle cells, but failed to evoke responses from muscle cells cocultured with neurons from 16-day embryo retina (not shown). The frequency of muscle responses after 24 hr of coculture decreased exponentially as a function of retina development with a half-time of 31 hr and a first-order rate constant of 0.022 hr^{-1} (Fig. 1*B inset*).

To rule out the possibility that cell damage resulting from dissociation with trypsin was responsible for the inability of older neurons to synapse with muscle, 8- and 16-day embryo retinas were dissociated with [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA) (0.02%), and the resulting cells were cocultured with myotubes. Under these conditions, functional synapses were detected with retina neurons from 8-day but not 16-day embryos. Neurons in retina explants (nondissociated tissue fragments) from 8-day embryos formed synapses with striated muscle after 24 hr of coculture, but not neurons in explants from 16- or 18-day embryos (data not shown). These results indicate that the loss of ability of neurons from 16-day embryo or older retinas to form synapses with muscle cells is not due to cell dissociation.

The relation between the percent of muscle cells tested with synapses and the number of retina cells per dish, dissociated

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; BME, basal medium (Eagle's).

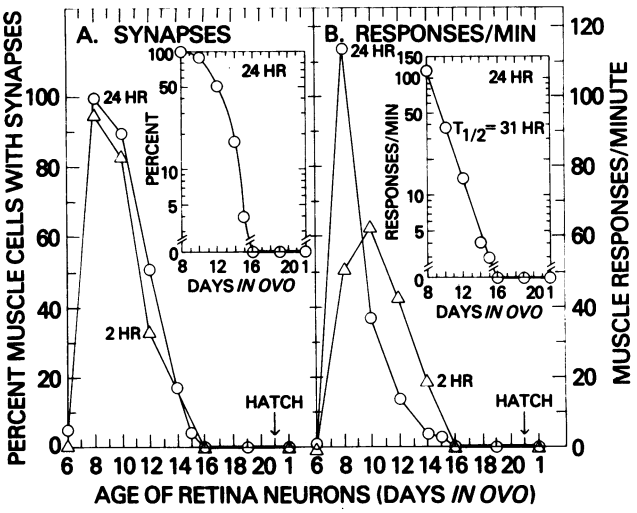

FIG. 1. Synapse formation between neurons dissociated from chick embryo retina at various stages of development and rat striated muscle cells. Thirty million cells dissociated from chick embryo retina with trypsin were cocultured for 2 or 24 hr with myotube monolayers in collagen-coated petri dishes 35 mm in diameter (9.6-cm^2 surface area). The percent of muscle cells tested with synapses is shown in *A* and the mean frequency of spontaneous synaptic responses of innervated muscle cells is shown in *B*. △, Retina cells and muscle cells cocultured for 2 hr; the assay medium was 90% basal medium (Eagle's) (BME) and 10% fetal bovine serum; ○, retina cells and muscle cells cocultured for 24 hr; 1 hr before testing for synapses the medium was changed to BME with the $CaCl_2$ concentration increased from 1.8 to 3.8 mM and the choline chloride concentration adjusted from 6 to 106 μM. The data shown in the *Insets* are from cells cocultured for 24 hr and are plotted with a logarithmic ordinate. Each point represents an average of 30 muscle cells (range 10–70) tested for synapses. $T_{1/2}$ represents the half-time for decrease in muscle response frequency.

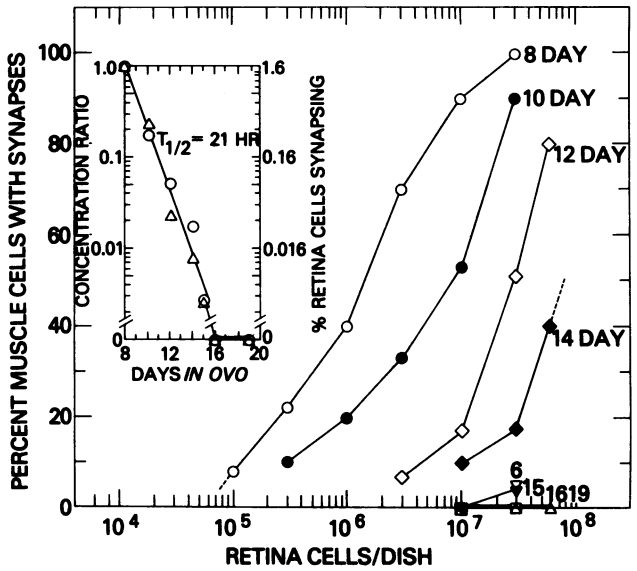

FIG. 2. Loss of neuronal ability to synapse. The percent of muscle cells tested with synapses is shown as a function of the number of dissociated retina cells per dish and of embryo age. Cells were dissociated with trypsin from chick embryo retina of the following ages: ▽, 6 days; ○, 8 days; ●, 10 days; ◇, 12 days; ◆, 14 days; ▼, 15 days; □, 16 days; and △, 19 days. One hour before assay for synapses the medium was changed to BME adjusted to 3.8 mM calcium chloride and 106 μM choline chloride. Each point represents an average of 40 muscle cells (range 10–70). One-hundred percent corresponds to 20,000 myotubes innervated per dish. (*Inset*) Retina cells and myotubes were cocultured for 24 hr prior to assay for synapses; △, the percent of retina cells that form synapses with muscle cells is shown on the right ordinate; and ○, on the left ordinate the concentration ratio (the number of 8-day embryo retina cells needed to innervate 50% of the muscle cells divided by the concentration of retina cells from 8-day or older embryos needed to innervate 50% of the muscle cells). $T_{1/2}$ corresponds to the half-life for loss of ability of retina neurons to form synapses with striated muscle cells as a function of neuron maturation.

from embryos 6–19 days after fertilization, is shown in Fig. 2. As retina cells aged *in ovo*, the number of neurons that formed synapses with myotubes increased between the 6th and 8th day of embryonic development and decreased thereafter. With 20% of the myotubes innervated, most muscle cells that had synapses were innervated by only a single neuron. Because each dish contained approximately 20,000 myotubes, the percent of retina cells synapsing with muscle (Fig. 2 *inset*) is easily calculated (5). At least 1.6% of the retina cells from 8-day chick embryos formed synapses with muscle cells, and this fraction declined logarithmically with embryo age with a half-life of 21 hr. This suggests that the loss of ability of retina neurons to synapse with muscle is an apparent first-order process with a rate constant of 0.035 hr^{-1}. Because the retina cell concentration–percent myotube innervation curves are parallel, concentration ratios (concentration of 8-day embryo retina cells needed to form synapses with 50% of the muscle cells, divided by the concentration of retina cells from 8-day or older embryos needed to innervate 50% of the muscle cells) may be calculated in order to determine the ability of retina neurons at different embryo ages to form synapses relative to retina cells from 8-day embryos. The decline in concentration ratio with embryo age also is exponential and is described by the same regression line as the percent of retina cells synapsing with muscle cells.

The developmental age of the retina cells and the duration of coculture of neurons with muscle were varied and the concentration of retina cells was kept constant (3×10^7 retina cells per dish). After 1 day of coculture, 100% of the myotubes tested were innervated by neurons dissociated from 8-day embryo retina (Fig. 3*A*); however, the maximum percent of muscle cells innervated by neurons from older retina declined progressively as a function of embryo age. No synapses were detected between neurons from 16-day embryo retina and muscle cells cocultured for 20 min to 48 hr. Increasing the concentration of $CaCl_2$ from 1.8 to 3.8 mM and the choline concentration from 6 to 106 μM had little or no effect on the percent of myotubes innervated by retina neurons from 8- to 16-day embryos cocultured 24 hr. The percent of innervated muscle cells was maximum at 24 hr with retina neurons at all developmental ages tested and decreased thereafter. The period of culture required for loss of all synapses depended upon the developmental age of the retina neurons cultured. Retina neurons from 10-day chick embryos required 5 days in culture to terminate all synapses with muscle cells, whereas only 2 days of culture were required to terminate all synapses between neurons from 14-day embryo retina and muscle cells. The time required for termination of synapses appears to be related to the total age of the retina neurons (days *in ovo* plus days of coculture) as shown in Fig. 3*A inset*. Retina neurons terminated all synapses with muscle cells 15–16 days after fertilization. Because synapses are terminated at approximately the same rate that neurons lose the ability to form synapses between the 11th and 16th embryo day, the results suggest that the half-life of synapses between retina neurons and muscle cells is relatively short (less than 21 hr).

To eliminate the possibility that synapse termination is a consequence of cell dissociation, fragments of tissue from 8-day chick embryo retina were explanted and cultured with muscle cells for 5 days. Neurons in explants from 8-day chick embryo

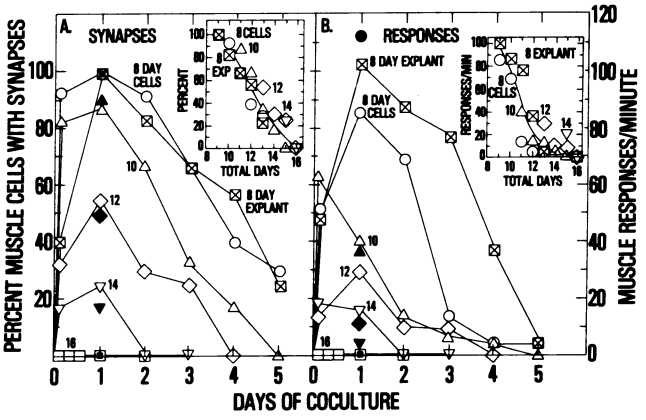

FIG. 3. Percent of muscle cells tested with synapses (*A*) and mean number of spontaneous synaptic responses of innervated muscle cells (*B*). The developmental age of retina cells and duration of coculture of retina cells with muscle cells were varied and the concentration of retina cells was kept constant (3×10^7 retina cells per dish). Cells were dissociated with trypsin from chick embryo retina at the following ages: ○, 8 days; △, 10 days; ◇, 12 days; ▽, 14 days; □, 16 days. The medium was changed 1 hr before assay; open symbols represent an assay medium of 90% BME and 10% fetal bovine serum. Filled-in symbols correspond to an assay medium of BME adjusted from 1.8 to 3.8 mM calcium chloride and from 6 to 106 μM choline chloride. ⊠, Nondissociated explants of retina tissue from 8-day chick embryo retina. Only those myotubes in contact with retina explants were assayed for synapses. Each point represents an average of approximately 20 muscle cells (range 10–70) tested. (*Insets*) Total days shown on the abscissa represents days *in ovo* plus days of coculture and thus corresponds to the age of the retina cells since fertilization. Symbols are as above.

retina formed fewer synapses than dissociated retina cells after coculture with muscle cells for 2 hr, but 100% of the muscle cells were innervated at 24 hr and the rate of termination of synapses by neurons in explants thereafter was identical to that found with dissociated cells from 8-day embryo retina.

The average frequency of spontaneous muscle responses in innervated myotubes is shown in Fig. 3*B* as a function of age of retina neurons and duration of culture. In general, the developmental pattern was similar to that shown in Fig. 3*A* except that the maximum muscle response frequencies found with 10- and 14-day embryo retina neurons were obtained after 2 rather than 24 hr of culture, and the muscle response frequency observed with explants from 8-day embryo retina declined approximately 1 day later than that found with cells dissociated from 8-day embryo retina. Increasing the concentrations of calcium ions and choline had little effect on the frequency of muscle responses in innervated myotubes cocultured for 1 day. In general, the frequency of spontaneous muscle responses decreased faster than the loss of myotubes with synapses (Fig. 3 *insets*). These results show that synapses are transient, and that neurons lose the ability to form synapses at approximately the same rates *in vitro* and *in ovo*.

Cells from 8-day chick embryo retina were dissociated and cocultured with myotubes for 1–6 days and then again dissociated with trypsin and added to fresh myotube monolayers and cultured for an additional 24 hr to determine the effects of *in vitro* aging and denervation on the ability of neurons to form synapses (Fig. 4). The results show that neurons progressively lose the ability to form synapses with muscle cells as the neurons mature *in vitro*. Neurons dissociated from 8-day chick embryo retina, cocultured with muscle cells for 6 days, formed few synapses with myotubes. The number of muscle cells with synapses and the frequency of spontaneous muscle responses were slightly higher than expected for cultures with 1×10^6

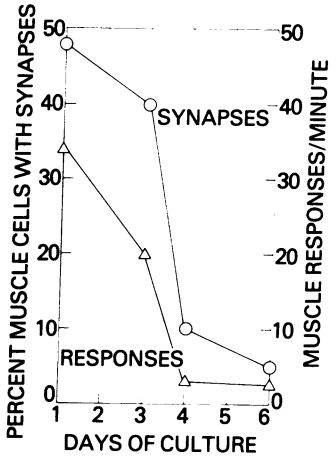

FIG. 4. Effects of *in vitro* aging and denervation on the ability of neurons to form synapses. Cells from 8-day chick embryo retina were dissociated with trypsin and cocultured (1×10^7 retina cells per dish) with myotubes for 1–6 days as shown on the abscissa. Retina cells were again dissociated with 0.05% trypsin and retina cells were added to fresh muscle monolayers (1×10^6 retina cells per dish) and cultured for an additional 1 day. ○, Percent of muscle cells tested with synapses; △, mean number of spontaneous synaptic responses of innervated myotubes per min. Muscle cells were tested for synapses in growth medium (90% BME and 10% fetal bovine serum). Each point represents 10–21 muscle cells tested for synapses.

retina cells per dish compared to retina neurons that matured *in ovo* (see Fig. 2).

Elsewhere we will show that neurons from chick embryo spinal cord gain the ability to form synapses with striated muscle cells at approximately 2 days of embryonic development and lose the ability by the 16th or 17th day of embryogenesis;* this is similar to the pattern of synaptogenesis found with retina neurons. Thus, the question was asked, "Are synapses between spinal cord motor neurons and striated muscle cells, which normally form during development, longer lived than mismatched synapses between retina neurons and muscle cells?" Dissociated cells from 8-day chick embryo retina and spinal cord both formed synapses with striated muscle cells rapidly, but synapse termination was only observed with mismatched retina–muscle cell synapses (Fig. 5). No loss of synapses between spinal cord neurons and muscle cells was detected after 14 days of culture. These results show that retina neurons form transient synapses with striated muscle cells, whereas spinal cord neurons form stable, long-lived synapses; the results strongly suggest that at least part of the specificity of synaptic connections is acquired after synapses form by selection for synapses with slow termination rates.

DISCUSSION

The results show that neurons are generated in chick embryo retina that are able to form synapses with striated muscle cells but then lose the ability to form synapses with a half-life of 21 hr. These neurons appear in retina on the 6th and 7th day of chick embryo development, are most abundant on the 8th day, and lose the ability to form synapses by the 16th day. Synapses between retina neurons and muscle cells form rapidly, but are transient and turn over with a half-life of 21 hr or less. Chick embryo spinal cord neurons also appear by the 2nd day of embryo development and disappear by the 16th or 17th day* but form stable, long-lived synapses with striated muscle cells.

* J. Thompson, G. Eisenbarth, R. Ruffolo, and M. Nirenberg, unpublished observations.

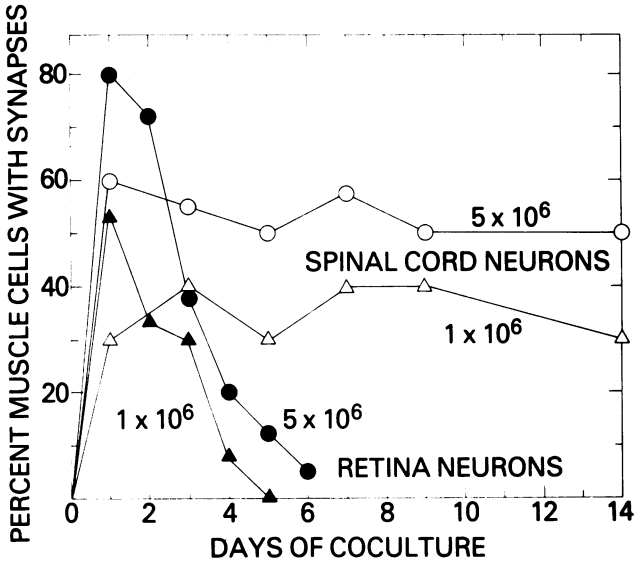

FIG. 5. Synapse turnover by cells dissociated from 8-day chick embryo retina or spinal cord cocultured with muscle for various times. Filled-in symbols correspond to retina cells; open symbols, spinal cord cells. Circles represent 5×10^6 and triangles 1×10^6 retina or spinal cord cells per dish cocultured with muscle cells. The growth medium and assay medium consisted of Eagle's minimum essential medium supplemented with 10% fetal bovine serum, and 10 μM 5-fluorodeoxyuridine and 100 μM uridine (added 16 hr after retina or spinal cord cells were added to myotube monolayers to prevent rapidly dividing cells from overgrowing cultures). Each point represents 10 or 20 muscle cells assayed for synapses.

These results strongly suggest that at least part of the specificity of synaptic connections can be acquired after synapses form by a process of selection based on the rate of synapse termination.

The mechanism of conversion of retina neurons from a synapse-competent to an incompetent state is unknown. The specific activity of choline acetyltransferase is high *in ovo* and *in vitro* and does not decrease while neurons lose the ability to form synapses (5). Thus, the loss of ability of retina neurons to form synapses is not due to a decrease in the number of neurons that are able to synthesize acetylcholine.

Nicotinic acetylcholine receptors are present in 6-day chick embryo retina in low concentration and increase in concentration throughout embryo development (8, 9). Thus, synapses mediated by nicotinic acetylcholine receptors probably form in chick retina between the 6th and 16th day of embryonic development. Synapses recognized by ultrastructural specializations first appear in chick embryo retina on the 13th day of development (13–15). Synapses are formed in abundance between cultured retina neurons (1×10^9 synapses per mg of protein) also starting approximately 13 days after fertilization (1), during the same period that retina neuron–myotube synapses decrease in number. Many ultrastructural specializations that are used to identify synapses were not found with retina neuron–muscle cultures at a time when many synapses were detected by electrophysiological methods.† Thus, early stages of synaptogenesis may be detected by electrophysiologic methods although they are not detected by electron microscopy.

Dissociated neurons and neurons in explants of 8-day chick embryo retina extended long neurites *in vitro*, whereas few neurites were extended by neurons from 16- to 19-day embryo retina or chick retina after hatching. Retina neurons have been shown to become less adhesive as they mature; thus, the diameter of cultured retina cell aggregates decreases during the course of embryonic development (16). However, in stationary cultures, cells dissociated from 16- to 19-day embryo retina formed large, loose aggregates of retina cells when cocultured with muscle. Further work is needed to determine whether the loss of ability of neurons from older chick embryo retina to form synapses with muscle cells is related to the change in adhesive properties of the neurons and/or to the decrease in ability to extend neurites.

A summary of the steps in synapse formation that have been detected is shown in Fig. 6. Neurons that synthesize acetylcholine and are able to form synapses (N^+) are generated in chick retina on the 6th to 7th day of embryonic development (reaction 1). The ability to form synapses is lost (N^-) via an apparent first-order process with a half-life of 21 hr (reaction 2). The apparent rates of synapse termination that were reported previously (5) thus reflect both the rate of loss of ability of neurons to form synapses and the rate of synapse termination. Ninety-four percent of the synapses formed by these neurons therefore are made within 84 hr (4 half-lives) of the time the neurons acquire the ability to form synapses. ***Thus, the sequence of genesis of neurons, their relative positions, and the duration of the synapse-competent state may restrict synaptic targets to cells of approximately the same or older age in neighboring areas.***

As shown in reaction sequence *B*, neurons dissociated from 8-day chick embryo retina adhere to muscle cells (M) and neurons (N) (reaction 3) with a half-time of 10 min (5) and form synapses with muscle cells and probably also with neurons (reaction 5) with a half-time of 25 or 60 min for retina neurons dissociated with EGTA or trypsin, respectively (5). Neurons lose the ability to form synapses (reaction 2), but not the ability to transmit information across synapses formed previously. Synapses may be terminated by reversal of synapse formation (reaction 6) or by cell dissociation as indicated by reaction 7.

In summary, we find that synapses formed by retina neurons and spinal cord neurons with muscle turn over at different rates and that synapses between spinal cord neurons, which presumably include developmentally correct synapses, are retained while synapses between retina neurons and muscle cells are terminated, perhaps due to differences in cell adhesiveness.

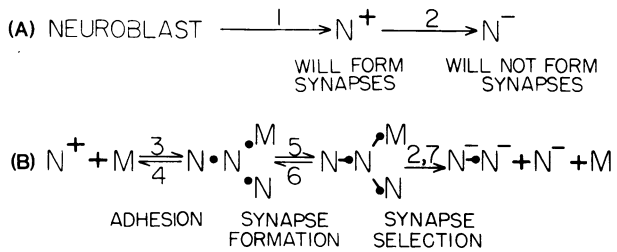

FIG. 6. Summary of proposed steps in synapse formation. In reaction sequence *A*, neurons with choline acetyltransferase activity (N^+) are generated which are able to form synapses (reaction 1). During embryonic development the ability of the neurons to form synapses is lost (N^-) (reaction 2). In reaction sequence *B*, dissociated retina neurons that are able to form synapses (N^+) attach to striated muscle cells (M) and other neurons (N) via reaction 3; • represents cell adhesion. Synapses form via reaction 5; –• represents synapse formation. Synapses may be terminated by reversal of synapse formation (reaction 6) or by dissociation of cells (reaction 7). The loss of the ability of neurons to form synapses (reaction 2) does not affect communication across synapses formed previously. Incorrect, mismatched synapses are terminated at markedly faster rates than those formed by correctly paired synaptic partners. Thus, populations of synapses may be selected on the basis of rate of synapse termination.

† M. Daniels, D. Puro, and M. Nirenberg, unpublished observations.

Similarly, synapses *between* cultured retina neurons are retained after nearly all synapses between retina neurons and muscle cells have been terminated.† These results strongly suggest that both correct and incorrect synapses are formed during embryonic development and that correctly matched synapses are selectively retained, as suggested by Changeux and Danchin (17). The demonstration that neurons acquire the ability to form synapses for a brief period during embryonic development and that some neurons form stable, long-lived synapses whereas other neurons form transient synapses suggests that selection for stable synapses or synapses with slow rates of termination may be a general mechanism for the assembly of some synaptic circuits during development.

R.R.R. is a Pharmacology Research Associate of the National Institute of General Medical Sciences.

1. Vogel, Z., Daniels, M. P. & Nirenberg, M. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 2370–2374.
2. Stefanelli, A., Zacchei, A. M., Caravita, S., Cataldi, A. & Ieradi, L. A. (1967) *Experientia* **23**, 199–200.
3. Sheffield, J. B. & Moscona, A. A. (1970) *Dev. Biol.* **23**, 36–61.
4. Stell, W. K. (1972) in *Handbook of Sensory Physiology*, ed. Fourtes, M. G. F. (Springer-Verlag, Berlin, Heidelberg, W. Germany), Vol. 7, part 2, pp. 111–213.
5. Puro, D. G., DeMello, F. G. & Nirenberg, M. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 4977–4981.
6. Lindeman, V. F. (1947) *Am. J. Physiol.* **148**, 40–44.
7. Baughman, R. W. & Bader, C. R. (1977) *Brain Res.* **138**, 469–485.
8. Vogel, Z. & Nirenberg, M. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 1806–1810.
9. Wang, G.-K. & Schmidt, J. (1976) *Brain Res.* **114**, 524–529.
10. Sugiyama, H., Daniels, M. P. & Nirenberg, M. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5524–5528.
11. Puro, D. G. & Nirenberg, M. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 3544–3548.
12. Nelson, P. G., Peacock, J. H., Amano, T. & Minna, J. (1971) *J. Cell. Physiol.* **77**, 337–352.
13. Meller, K. (1968) in *Veroffentlichungen aus der morphologtischen Pathologie*, eds. Buchner, F. & Giese, W. (Gustav Fischer-Verlag, Stuttgart, W. Germany), Vol. 77, pp. 1–77.
14. Sheffield, J. B. & Fischman, D. A. (1970) *Z. Zellforsch. Mikrosk. Anat.* **104**, 405–418.
15. Hughes, W. F. & LaVelle, A. (1974) *Anat. Rec.* **179**, 297–302.
16. Moscona, A. A. (1962) *J. Cell. Comp. Physiol.* **60**, 65–80.
17. Changeux, J.-P. & Danchin, A. (1976) *Nature* **264**, 705–712.