

SYNAPSE SELECTION BASED ON DIFFERENCES IN SYNAPSE TURNOVER

JEFFREY M. THOMPSON,* GEORGE S. EISENBARTH,† ROBERT R. RUFFOLO, JR.‡ and MARSHALL NIRENBERG§

Laboratory of Biochemical Genetics, National Heart, Lung, and Blood Institute,
National Institutes of Health, Bethesda, MD 20205, U.S.A.

Abstract — Rat retinal neurons formed transient synapses with rat muscle cells in culture only during a discrete period in development, from the 20th day of embryonic development to the 7th neonatal day. In contrast, chick embryo spinal cord neurons formed synapses at all developmental stages tested, from the 2nd to the 18th day of embryonic development. The percentage of cells from the spinal cord that formed synapses with muscle cells was maximum at 4 days of embryonic development and decreased thereafter. However, the number of synapses with muscle formed by cells from 8-day embryonic spinal cord did not decrease during 14 days of culture. Under identical conditions, all synapses formed between rat retinal neurons and muscle cells were terminated during 7 days of culture. These results show that differences in the rates of turnover of two populations of cholinergic synapses can result in the selective retention of one population of synapses and the loss of the other, and thereby alter the specificity of synaptic connections.

Key words: Synaptogenesis, Retinal neurons, Spinal cord neurons, Tissue culture, Synapse specificity, Development.

Neurons dissociated from chick embryo retina rapidly form synapses with striated muscle cells in culture only between the 6th and 15th day of embryonic development.^{16,18} On the other hand, evidence from other systems has been reported which suggests that during development some neurons retain the ability to form synapses.^{1,9} In adult vertebrates, denervation of striated muscle often leads to the formation of many new motorneuron-muscle synapses, but the specificity of the original synaptic circuits in most cases is not restored.¹²

Chick retinal neuron-muscle synapses are transient and terminate with a half-time of 21 h, as opposed to synapses with neurons from chick embryo spinal cord which are retained for 14 days or more.¹⁸ Evidence for transient synapses in other systems has also been reported.¹⁷ For example, during development of the retinotectal projection in *Xenopus*, synaptic contacts between retinal ganglion neurons and tectal cells progressively shift.^{10,19} Neurons in the adult snail *Helisoma* form transient inappropriate electrical synapses which are broken upon establishment of stable, appropriate synapses.⁵ In the lateral vestibular nucleus of the rat,²⁰ evidence has been reported for degeneration of neuronal connections. Some rat and chick striated muscle cells have been shown to be innervated by multiple neurons initially, but by only single neurons later in development.^{2,3}

In this report, we show that rat retinal neurons form transient synapses with rat muscle cells only during a discrete period in development, whereas chick embryo spinal cord neurons form synapses with rat muscle cells at all developmental stages tested. Spinal cord neurons form synapses with muscle which are selectively retained while retinal synapses are lost.

METHODS

Cultures of striated muscle cells from hindlimbs of newborn rats or 20-day embryos and neurons from rat retina or chick spinal cord at different stages of development were prepared as described previously.¹⁸ Neuron-myotube synapses were detected by recording spontaneous depolarizing synaptic muscle responses, usually 0.5 mV in amplitude and 10 ms in duration, with intracellular

Present addresses: * Department of Anatomical Sciences, Schools of Basic Medical Sciences and Life Sciences, University of Illinois — Urbana/Champaign, Urbana, IL 61801; † Joslin Diabetes Center, 1 Joslin Place, Boston, MA 02215; ‡ Department of Cell Biology, Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, IN 46206, U.S.A.

§ Correspondence to: Marshall Nirenberg, Bld. 36, Rm. 1C06, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20205, U.S.A.

microelectrodes filled with 3 M KCl solution as described by Nelson *et al.*¹³ Only myotubes with stable membrane potentials of -40 mV or more 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 recording period of 2 min. The length of the recording period was extended when the frequency of spontaneous muscle responses was low.

RESULTS AND DISCUSSION

Neurons dissociated with trypsin from rat retina at different stages of development were added to monolayers of differentiated rat striated muscle cells that had been cultured for approximately 9 days. After 24 h of incubation, myotubes were assayed for synapses. Synapses were first detected with neurons from retina of 20-day rat embryos (Fig. 1); the proportion of retinal cells which formed synapses with muscle was maximal 1 day after birth, and then declined. No synapses were found between neurons from 7- to 18-day postnatal rat retina and muscle cells. The frequency of spontaneous synaptic muscle responses followed a similar developmental sequence. Activation of glutamate receptors of rat retinal neurons by local application of 5 mM sodium glutamate in culture medium by diffusion from a micropipette resulted in an increase of acetylcholine secretion from the neurons and as much as a 30-fold increase in the frequency of synaptic muscle responses (not shown). Application of glutamate to muscle cells in the absence of neurons did not evoke depolarizing muscle responses.

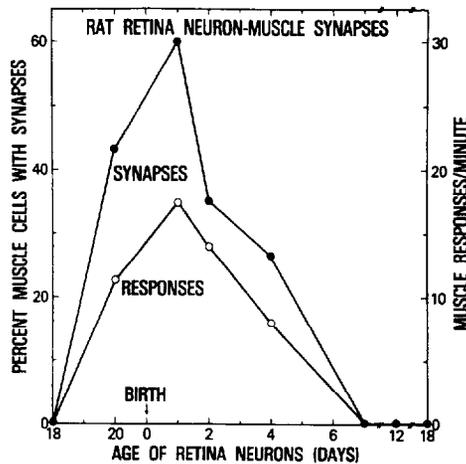


Fig. 1. Synapse formation between neurons dissociated from rat retina at various stages of development and rat striated muscle cells. Twenty million cells dissociated from rat retina with trypsin were cocultured for 24 h with a monolayer of rat striated muscle cells in a 35 mm Petri dish. For all experiments described in this report, the growth medium consisted of Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum. The medium was changed 1 h prior to assay. Symbols represent the following: (●), % of muscle cells tested with synapses; (○), mean number of spontaneous synaptic responses/min of innervated muscle cells. Each point represents 20–30 muscle cells (2–3 dishes) assayed for synapses; a total of 190 cells were tested.

We estimate^{16,18} that each culture dish contained 20,000 myotubes which occupied 20% of the surface area of the dish (9.6 cm²). Since 60% of the muscle cells were innervated (12,000 synapses per dish), we calculate that 0.06% of the cells plated from 1-day neonatal rat retina formed synapses with muscle cells. Probably 0.3–0.6% of retinal neurons may be able to form synapses with muscle cells, since only a fraction of the retinal cells was in contact with muscle cells and some muscle cells probably were innervated by 2 or more neurons.

The ability of neurons dissociated from chick embryo spinal cord to form synapses with rat striated muscle is shown in Fig. 2 as a function of developmental age. Spinal cord neurons dissociated on the 2nd day of chick embryo development formed synapses with muscle cells. The maximum number of synapses with muscle cells was found with neurons from 4-day embryos which agrees well and extends the findings reported by Berg & Fischbach.⁴ We estimate that 0.29% of the spinal cord cells from 4-day embryos formed synapses with muscle cells. If we allow for multiple synapses and for a fraction of neurons which are not in contact with muscle, then 1.5–3% of the cells from spinal cord may be able to form synapses with muscle cells. The number of synapses with muscle formed by cells from 4- to 14-day chick embryo spinal cord decreased via an apparent first-order process with a half-time of 3.8 days (Fig. 2, inset). Synapses between neurons dissociated from 18-day embryo spinal cord and muscle cells were detected after 51 h of coculture, but not after 24 h of coculture. The maximum frequency of spontaneous synaptic responses of muscle cells also was obtained with neurons from 4-day chick embryo spinal cord; the frequency of responses decreased with cells from 4- to 10-day chick embryo spinal cord and remained constant thereafter. Results of Obata¹⁴ suggest that the decrease in frequency of muscle responses may be due to the formation of inhibitory synapses. In other experiments (not shown), local application of 5 mM glutamate to neurons from 6-day chick embryo spinal cord cultured with muscle cells resulted in 2-fold increases in the rate and amplitude of muscle responses.

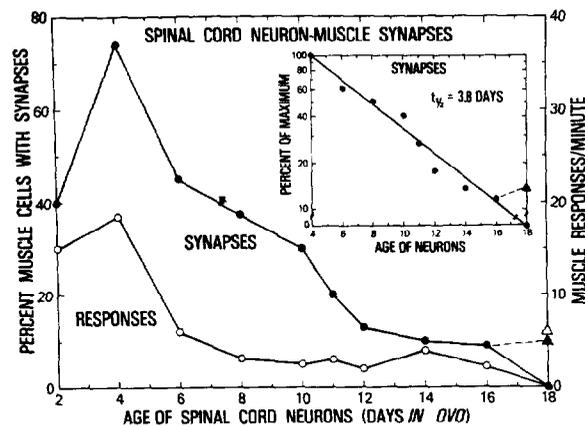


Fig. 2. Synapse formation between neurons dissociated from chick embryo spinal cord at various stages of development and rat striated muscle cells. Five million cells dissociated from chick embryo spinal cord with trypsin crystallized 3 times were cocultured for 24 h, (●), or 51 h, (▲), with a monolayer of rat striated muscle cells in collagen-coated Petri dishes 35 mm in diameter (9.6-cm² surface area). Symbols represent the following: (●, ▲), % of muscle cells tested with synapses; (○, △), mean number of spontaneous synaptic responses/min of innervated muscle cells. The data shown in the inset are the % of maximum innervation of muscle cells plotted with a logarithmic ordinate. $t_{1/2}$ represents the half-time for decrease in the % of muscle cells innervated. Each point represents approximately 30 (range 10–60) muscle cells assayed for synapses; a total of 311 cells were tested.

Choline acetyltransferase (EC 2.3.1.6) specific activity from 6- and 18-day chick embryo spinal cord before and after dissociation with trypsin and after coculture of dissociated spinal cord cells with striated muscle for 1 day is shown in Table 1. Relatively high specific activities of choline acetyltransferase were present in 6- and 18-day embryo spinal cord and were reduced by dissociation with trypsin. However, cells dissociated from 6- or 18-day chick embryo spinal cord which were cocultured for 1 day with muscle cells still possessed relatively high choline acetyltransferase activities. During 1 day of coculture with muscle cells, the enzyme activity of 6-day embryo spinal cord neurons increased, whereas the activity of neurons from the 18-day embryo decreased. These results suggest that cholinergic neurons were abundant in cocultures of both 6- and 18-day embryo spinal cord and muscle cells, and that neurons from 18-day embryos may have been injured more easily by the process of dissociation than neurons from 6-day embryos.

Table 1. Choline acetyltransferase activity of spinal cord neurons

Age of chick embryo spinal cord (days from fertilization)	Treatment	Total activity (pmol ¹⁴ C-acetylcholine formed/min/dish)	Specific activity (pmol ¹⁴ C-acetylcholine formed/min/mg protein)	Protein (mg/dish)
6	Before trypsinization	—	437	—
6	After trypsinization	234*	124	1.95*
6+	1 Day <i>in vitro</i> with muscle	401	176 (393)†	2.28
18	Before trypsinization	—	695	—
18	After trypsinization	1010*	310	3.30*
18+	1 Day <i>in vitro</i> with muscle	501	171 (300)†	2.93
	Muscle <i>in vitro</i> (no neurons)	—	—	1.26

Spinal cords were dissected from 6- or 18-day chick embryos, trypsinized to obtain dissociated cells or minced and sonicated to prepare homogenates. Fifteen million spinal cord cells were added to rat myotubes in a 60 mm plastic tissue culture dish. After 1 day, cells were removed from the dish by scraping and then were sonicated. Freshly trypsinized spinal cord cells were also sonicated to determine choline acetyltransferase activity prior to culture. There were 3.6×10^6 cells recovered from a 6-day embryo spinal cord (0.96 mg protein/cord) and 7×10^6 cells recovered from an 18-day embryo spinal cord (5.05 mg protein/cord). Choline acetyltransferase activity of homogenates was determined as described by Wilson *et al.*²¹

* Amount of choline acetyltransferase activity or protein, per 15×10^6 spinal cord cells, added to each dish.

† Specific activity based on neural protein only (calculated by subtracting the protein determined from muscle cells cultured without neurons from protein in dishes with cocultured neural and muscle cells).

In other experiments (not shown), cells were dissociated from 6-day chick embryo spinal cord and cultured with muscle cells for 1, 3 and 6 days (first stage). Then the cultured cells were dissociated again with trypsin and the number of cells plated initially were plated on fresh muscle cells. After coculture for one additional day (second stage), muscle cells were assayed for synapses to determine whether neurons previously cultured and dissociated could form new synapses. The percentage of muscle cells with synapses found in the second stage was 83, 25 or 10% with neurons cocultured in the first stage for 1, 3 or 6 days, respectively. Thus, the proportion of myotubes innervated by spinal cord neurons decreased with age both *in ovo* (Fig. 2) and *in vitro*. Whether the decrease in the number of myotubes with synapses detected is due to a decrease in the ability of neurons to form synapses, to an increase in fragility of older neurons to dissociation by trypsin, or to programmed cell death^{7,8,11,15} is unknown.

Neurons from chick embryo retina form synapses with muscle cells between the 6th and 15th day of embryonic development,¹⁸ whereas neurons from rat retina form synapses between the 20th embryonic and 7th neonatal day. At later developmental stages, both chick and rat retinal neurons lose the ability to form synapses with muscle cells. In contrast, chick embryo spinal cord neurons acquire the ability to form synapses with muscle cells by the second day of embryonic development, and continue to form some synapses throughout the developmental period tested (2–18-day embryos). The sequence of neuron genesis, the availability of target cells, their relative positions and the duration of the synapse-competent state may determine, in part, the kinds of synaptic connections that form.

Retinal neurons from 1-day neonatal rats formed transient synapses with striated muscle cells (Fig. 3). Synapses were detected 2 h after retinal neurons were added to muscle cells (not shown), reached maximum abundance by 24 h, and then declined progressively as a function of time of coculture. However, after 7 days of coculture, no synapses between rat retinal neurons and muscle cells were detected. The transient nature of the rat retinal neuron synapses with muscle cells is similar to that reported for synapses between 8-day chick embryo retinal neurons and muscle cells which disappear after 8–10 days of coculture with a half-time for loss of synapses of 21 h.^{16,18} The decrease in the number of retinal neuron–muscle synapses during 7 days of coculture is due to the loss during development of the ability of neurons to form synapses with muscle cells and to the termination of synapses with myotubes which have formed.

Neurons from spinal cord, which presumably include motoneurons that normally synapse with striated muscle cells, also formed synapses within 2 h with cultured muscle cells; the maximum percentage of muscle cells innervated (60%) was achieved by 1 day of coculture. However, the number

of synapses between spinal cord neurons and muscle cells did not decrease appreciably during 14 days of coculture (Fig. 3). These results show that cholinergic spinal cord neurons and retina neurons form synaptic connections with striated muscle cells, and that synapses formed by spinal cord neurons are retained whereas those formed by retina neurons disappear by a process of selection based on differences in turnover. Whether synapses between spinal cord neurons and muscle

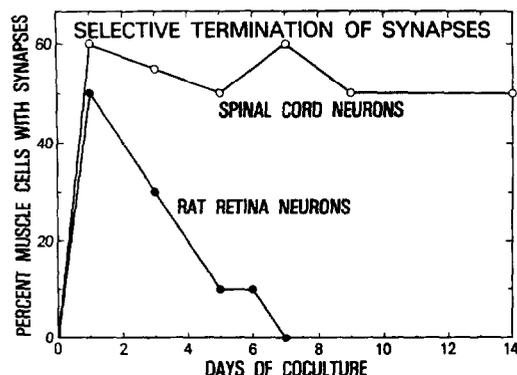


Fig. 3. Synapse turnover by cells dissociated from 8-day chick embryo spinal cord or 1-day neonatal rat retina. (●), 5×10^6 rat retinal neurons per dish cocultured with muscle cells; (○), 5×10^6 chick spinal cord neurons per dish cocultured with muscle cells. Ten mM 5-fluorodeoxyuridine and 100 μ M uridine was added to prevent rapidly dividing cells from overgrowing cultures. Each point represents 20 muscle cells assayed for synapses, a total of 170 cells. Data for spinal cord-muscle synapses are from Ruffolo *et al.*¹⁸

cells continue to turn over, and have attained a steady-state wherein the rate of synapse formation is equal to the rate of synapse termination, or whether spinal cord neurons form stable, long-lived synapses with muscle cells as suggested by Changeux & Danchin⁶ remains to be determined. Both possibilities seem probable since spinal cord neurons were shown to form synapses with muscle cells throughout the developmental period studied (2–18-day embryos). However, the results also suggest that the synapses between chick embryo spinal cord neurons and muscle cells may be either longer-lived or more stable than those between rat retinal neurons and muscle cells, and thus turn over at a slower rate. Although the mechanism has not been determined, the results demonstrate that differences in the rates of turnover of two populations of cholinergic synapses can result in the selective retention of one population of synapses and the loss of the other, and thereby alter the specificity of synaptic connections.

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