Choline Acetyltransferase Activity Is Increased in Combined Cultures of Spinal Cord and Muscle Cells from Mice

Abstract. *The activity of choline acetyltransferase was more than tenfold greater in combined cultures of spinal cord and muscle cells than in cultures of spinal cord cells alone. This increase was associated with the formation of functional neuromuscular junctions in culture. Counts of silver-stained cells and determinations of other enzyme activities indicated that the increased choline acetyltransferase activity was not due to nonspecific neuronal survival but reflected greater activity in the surviving neurons. Hence, muscle had a marked, highly specific trophic effect on the cholinergic neurons that innervated it.*

Vertebrate skeletal muscle is controlled, via neuromuscular junctions, by cholinergic neurons in the anterior horns of the spinal cord. Considerable information concerning the anatomy, physiology, and biochemistry of these junctions has been obtained with intact animal preparations. In addition, explants of spinal cord and muscle cultured together form functional neuromuscular junctions (*1*). Also, cells dissociated from embryonic chick spinal cord form morphologically (*2, 3*) and electrophysiologically (*3*) characterized neuromuscular junctions with embryonic chick muscle cells (myotubes) in culture. Recently, the techniques for culture of mouse spinal cord cells (*4*) and myotubes (*5*), alone and combined (*6*), have been developed, and studies of the biochemical events associated with the establishment of neuromuscular junctions have begun to appear (*7*).

We assayed combined cell cultures of spinal cord and muscle for the appearance of neuromuscular junctions and for the activities of enzymes of acetylcholine metabolism which are crucial to the function of these junctions. The activity of the neuronal marker enzyme choline acetyltransferase (CAT) (E.C. 2.3.1.6) was more than tenfold greater in combined cultures of spinal cord cells and myotubes (SC-M) than the sum of CAT activities in spinal cord (SC) cultures and myotube (M) cultures grown separately. In contrast, cholinesterases, as well as several enzymes involved in energy metabolism, were not markedly changed in SC-M cultures compared to SC and M cultures alone.

Collagen-covered Falcon plastic 60-mm culture dishes were inoculated with 0.7×10^6 viable cells dissociated from a hind-limb muscle of mouse embryos (19 to 21 days of gestation). Myotubes were formed by day 4 of culture, and spontaneous twitching was first noted by day 8. On day 14 of myotube culture, a cell suspension was prepared by trypsin dissociation (*4*) of spinal cords (including meninges and some of the dorsal root ganglia) from mouse embryos 15 days of gestation, and 3×10^6 viable cells were added to either the muscle cultures or to collagen-covered culture dishes without myotubes. Dishes from each series of SC, M, and SC-M cultures were washed (*8*), drained, and stored at -100°C on days, 2, 7, 14, and 21 after addition of the spinal cord cells. The cells were harvested, homogenized, and assayed for enzyme activities, protein, and DNA as described (*8, 9*). The presence of neuromuscular connections in replicate SC-M cultures was determined by making intracellular recordings from myotubes while neurites that appeared to contact these muscle cells were being stimulated with an extracellular pipette (*4*).

The morphology and electrophysiology of the cultures were similar to descriptions for chick cultures (*2, 3, 7*). The cells from spinal cord and dorsal root ganglia, cultured alone or in SC-M cultures, showed significant neurite formation by day 2. A silver stain on day 34 (*10*) showed that nerve processes ramified throughout the SC or SC-M cultures, frequently reaching several hundred micrometers in length. There was a progressive increase in functional junctions, as defined by the presence of evoked endplate potentials in the myotubes, from about 3 percent of tested nerve-muscle pairs on day 7 to 22 percent on day 21. These results are consistent with the percentage of connections found in combined cultures of dissociated chick spinal cord and muscle cells (*3*).

Total and specific activities of CAT (Fig. 1) were higher in SC-M than SC cultures at all times. This result has been replicated several times without failure. Total activity of the enzyme (Fig. 1A) in SC dishes had reached a plateau by day 14, while enzyme activity in SC-M cultures was still increasing linearly on day 21. The ratio of

Table 1. Size distributions of silver-impregnated cells in SC and SC-M cultures. Forty-six randomly chosen microscopic fields, each approximately 1 mm², were examined for each culture.

Culture type	Stained cells (No.)			
	Greatest diameter > 30 μm		Diameter < 30 μm; process length > 150 μm	Total
	Process length > 150 μm	Process length < 150 μm		
SC	37	14	97	148
SC-M	59	12	28	99

Table 2. Activities of cholinesterase (ChE), creatine phosphokinase (CPK), myokinase (MK), and phosphorylase (PH) in nerve, muscle, and nerve-muscle cultures. Values from SC-M cultures on days 7, 14, and 21 are means for two homogenates from replicate cultures; all others are from homogenates of two or three pooled cultures. Values are given per 60-mm culture dish. Units for ChE are nanomoles per minute; other enzyme units are micromoles per minute. The number of days after spinal cord cell addition is indicated after culture type in the first column.

Culture type and day	Protein (mg)	DNA (μg)	Enzyme activities (units)			
			ChE	CPK	MK	PH
SC 2	0.2	11	1	190	20	1
M	1.4	29	13	680	820	50
SC-M 2	1.6	36	22	1100	1100	61
SC 7	0.7	48	8	1200	110	8
M	1.7	29	18	600	620	45
SC-M 7	1.8	47	22	1700	1500	68
SC 14	0.7	41	14	1500	150	15
M	1.4	18	16	800	1100	68
SC-M 14	2.1	34	7	2100	1800	86
SC 21	0.8	29	20	1400	170	18
M	1.6	13	13	1000	1000	66
SC-M 21	2.6	26	29	2400	2000	78

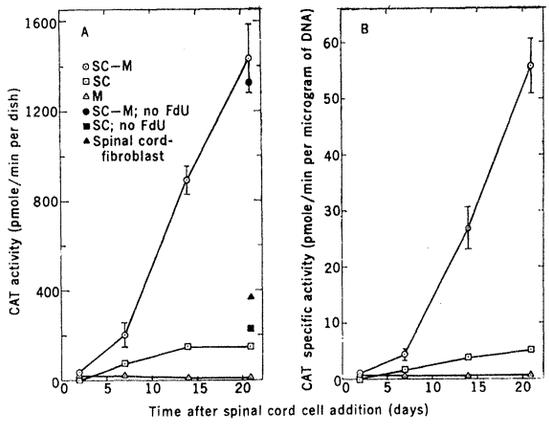

Fig. 1. Development of choline acetyltransferase activity in cultures of spinal cord cells (SC), muscle cells (M), and spinal cord and muscle cells combined (SC-M). Activity is given as picomoles of acetylcholine produced per minute in (A), and is normalized to DNA in (B). The mean and range (brackets) from duplicate SC-M homogenates are shown for days 7, 14, and 21 after addition of nerve cells. Other values are from homogenates of two or three cultures. Activities from spinal cord cell suspensions grown on fibroblasts or cultured without FdU treatment are shown for day 21 (closed symbols).

SC-M to SC total activity was 12.5 on day 2, decreased to 2.8 on day 7, and then increased to 7.5 and 11.4 on days 14 and 21, respectively. Since the apparent CAT activity was low in muscle cultures, this activity in SC-M cultures was presumably due only to spinal cord cells (*11*). Deletion of fluorodeoxyuridine (FdU), used to inhibit multiplication of flat cells that make up the background, did not change the total enzyme activity in either SC or SC-M dishes on day 21. Data for CAT specific activity relative to DNA content (Fig. 1B) show similar relationships. Estimates of specific activity relative to DNA of spinal cord cells alone in SC-M cultures resulted in similar curves. Hence, the data suggested that the increased CAT activity was due to a trophic effect of muscle on spinal cord neurons.

Alternatively, the higher activities in the SC-M cultures might be due to a nonspecific increase in plating efficiency of the spinal cord inoculum on the muscle cultures. We feel that this is not the explanation for the higher CAT activities in the SC-M plates and that a more specific inductive or trophic effect must have been involved for the following reasons. (i) When the spinal cord cells were inoculated onto fibroblasts from muscle cultures (*12*), the CAT activity on day 21 was 2.5 times higher (Fig. 1A) than that in SC cultures, although DNA and protein contents were similar in the two conditions. Thus, spinal cord cells plated on muscle had four times more CAT activity on day 21 than did spinal cord cells plated on fibroblasts. (ii) Silver-impregnated cells (*10*), presumably neurons, were counted in replicate SC and SC-M dishes on day 34 (Table 1). Despite uncertainties in any histologic assessment of the total neuronal population in a culture, the difference between neuron counts in SC-M and SC cultures was less than an order of magnitude. (iii) The DNA and protein contents and the activities of four other enzymes (cholinesterase, creatine phosphokinase, myokinase, and phosphorylase were examined in the same cultures (Table 2). The values for SC-M cultures were similar to the sums of values for SC and M cultures except that DNA tended to be less than additive in combined cultures and myokinase showed a consistent small increment. (iv) The time course of the rise in CAT activity was different in the SC and SC-M cultures, a result not compatible with a simple proportional development after an initially dissimilar plating efficiency.

Thus, the marked increase in activity of CAT in SC-M cultures was specific for that enzyme among those examined, was not due to a nonspecific increase in plating efficiency, and was not dependent on FdU treatment. The influence of the dorsal root ganglion neurons in these cultures (*4*) may also be significant (*13*). This increase was associated with, and may be crucial to, the formation or function (or both) of neuromuscular junctions in culture. Although trophic influences of nerve on muscle are well known, feedback effects of muscle on the development of innervating neurons has been examined only in morphogenetic terms [for review see (*14*)]. We believe that our study is the first demonstration that postsynaptic cells may specifically increase the presynaptic cell enzyme activity responsible for the neurotransmitter used at that junction. Although tissue culture conditions differ from those in vivo, similar specific interactions may occur during development or even in the adult nervous system. Tyrosine hydroxylase, the rate-limiting enzyme in catecholamine synthesis, is apparently induced when synaptic input activity to the adrenergic neuron involved is increased (*15*). Whether the type of direct feedback reported here occurs at other synaptic connections remains to be determined.

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12. Most myotubes were removed from companion muscle cultures with collagenase, and the background cells (presumably fibroblasts) were suspended by trypsin treatment. These cells were cultured and passed four times to remove remaining myotubes, and 2×10^5 cells were plated per 60-mm dish.
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16. Fluorodeoxyuridine was a gift of Hoffmann-LaRoche, Nutley, New Jersey.

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